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RESEARCH ARTICLE

Inhibition of Activin A suppressed tumor necrosis factor-α secretion and improved histopathological conditions in malarial mice

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ABSTRACT

Malaria infection still remains as one of the most prominent parasitic diseases afflicting mankind in tropical and subtropical regions. The severity of malaria infection has often been associated to exuberant host immune inflammatory responses that could possibly lead to severe immunopathological conditions and subsequent death of host tissues. Activin A is a protein belonging to the transforming growth factor-beta (TGF-β) family that regulates multiple physiological processes and pathological-associated diseases. The biological roles of activin A have been associated with manipulation of inflammation-related processes and modulation of host immune responses. This implies that activin A protein could play a role in malaria pathogenesis since malaria infection has been closely linked to severe immune responses leading to death, However, the actual *in vivo* role of activin A in malaria infection remains elusive. Hence, this study was undertaken to investigate the involvement of activin A in malaria infection as well as to assess the modulating effects of activin A on the cytokine releases (TNF-α, IFN-γ and IL-10) and histopathological changes in major affected organs (kidney, liver, lung, brain and spleen) in malarial mice infected with *Plasmodium berghei* ANKA. Our results showed that the concentrations of plasma activin A were significantly increased in malarial mice throughout the study periods. Also. the systemic activin A level was positively correlated with malaria parasitemia. This indicates that activin A could play a role in malaria pathogenesis and malaria parasitemia development. Plasma TNF-α, IFN-γ and IL-10 cytokine levels were significantly increased in malarial mice at day-5 post infection, suggesting that these cytokines attributed to severe malaria pathogenesis. Histopathological features such as sequestration of parasitized red blood cells (pRBCs) and hemozoin formation were amongst the most common pathological conditions observed in tissues of major affected organs (kidney, liver, lung, brain and spleen) in malarial mice. Neutralization of activin A production via recombinant mouse activin RIIA Fc chimera (rmActivin RIIA Fc chimera) had significantly reduced the parasitemia levels in malarial mice. The release of TNF-α cytokine was significantly reduced as well as the sequestration of parasitized pRBCs and hemozoin formation in major affected organs in malarial mice were also alleviated following inhibition of activin A production. Overall, this preliminary study suggests that activin A could play an immune modulation role in malaria pathogenesis through modulation of TNF-α release that benefits host from severe pathological destructions provoked by intensified inflammatory responses. Further studies are warranted to elucidate the precise mechanism of immune modulation mediated by activin A and its associated immune-modulation mediators in regulating the inflammatory responses elicited during the course of malaria infection.

Keywords: Activin A; malaria pathogenesis; tumor necrosis factor-α (TNF-α); histopathological.

INTRODUCTION

Malaria caused by *Plasmodium* species remains as one of the serious public health threats in tropical and subtropical regions. There are five *Plasmodium* parasites infecting humans which include *Plasmodium falciparum (P. falciparum)*, *Plasmodium vivax (P. vivax)*, *Plasmodium ovale (P. ovale)*, *Plasmodium malariae (P. malariae)* and *Plasmodium knowlesi* (*P. knowlesi*) (Lee *et al*., 2011; WHO, 2018). The pathogenesis of malaria infection in humans begins prior to intrusion of host cells by *Plasmodium* parasites through the bites of *Anopheles* mosquitoes. Malaria parasites continue to replicate in the liver and red blood cells as well as remain sequestered in the erythrocytes and within the microvasculature compartment in host tissues to evade from host immune attack by circulating and tissue-resident immune cells. This will eventually lead to the development of clinically prominent manifestations such as severe anemia and cerebral malaria in humans (Miller *et al*., 1994). Effective control and complete eradication of malaria are still challenging tasks. Increase in the resistance of *Anopheles* mosquito to mosquito insecticides such as dichlorodiphenyltrichloroethane (DDT) and pyrethroids have been demonstrated in malaria endemic regions (Sumarnrote *et al*., 2017). Emergence of drug-resistant malaria parasite strains to the current and available anti-malarial drugs further exacerbated the severity of malaria infection and hindered the efficacy of malaria therapeutic regimen (Menard & Dondorp, 2017). It is undoubtedly raising the needs to unravel more promising therapeutic agents in resolving malaria infection.

Rodent model of malaria has long been used as a tool to investigate and understand the pathogenesis of malaria, host immune response against malaria parasites, as well as basic biology of the *Plasmodium* parasites. To date, *Plasmodium berghei* (*P. berghei*), *Plasmodium chabaudi* (*P. chabaudi*), *Plasmodium yoelii* (*P. yoelii*), and *Plasmodium vinckei* (*P. vinckei*), are the most common species used to establish malaria infection in rodent model (Wykes *et al*., 2009; Zuzarte-Luis *et al*., 2014). Amongst all, *P. berghei* is the only species that develops almost all severe malaria syndromes, including cerebral malaria, placental malaria, acute lung injury / acute respiratory distress syndrome, acute kidney injury, and severe malarial anemia in the rodent model (Craig *et al*., 2012; Zuzarte-Luis *et al*., 2014). *P. berghei* can readily infect mice, young rats and hamsters but seems to be refractory in adult rats. The outcome of *P. berghei* infection in mice and young rats are almost always fatal (Sanni *et al*., 2002). As *P. berghei* resembles the clinically most important human *P. falciparum*, this parasite is commonly used as an infection model for malarial studies (Sherman, 2009). Although phylogenetically different with human malaria parasites, *P. berghei* serves well as a valuable model parasite for disease investigation. Lethal *P. berghei* infection in mice is usually used in chemotherapeutic drug testing as well as the assessment of potential vaccine candidate molecules, and sometimes in the investigation of parasite-host interactions and developmental biology of malaria parasites (Sanni *et al*., 2002; Sherman, 2009).

The severity of malaria infection is highly depending on the intensity of inflammation in which uncontrolled and exuberant host immune inflammatory responses towards parasite invasion could increase susceptibility to malaria infection, septic shock, severe immunopathological conditions and multiple organ damages/failures (Plebanski *et al*., 2000; Francischetti *et al*., 2008; Lopera-Mesa *et al*., 2012). Excessive production of pro-inflammatory cytokines, for instance, TNF-α, IL-1α, and IFN-γ are associated with tissue damage and high mortality rate in humans whilst the release of anti-inflammatory cytokines, for example, IL-4, IL-10 and IL-13 alleviated the intensified host inflammatory responses and facilitated the parasitic clearance from humans (Malaguarnera & Musumeci, 2002; Perkins *et al*., 2011). Overt production of pro-inflammatory cytokines along with the hyper-inflammation occurs in multiple affected organs such as kidneys, lungs, brain, spleen and liver attributed to the severe malaria development (Artavanis-tsakonas *et al*., 2003; Lyke *et al*., 2004). It is critical to understand the interaction between host immune inflammation responses with malaria parasite during malaria infection which could be helpful in identifying suitable immune modulators that target the severe immunopathological conditions in malaria (Dondorp *et al*., 2009, Achtman *et al*., 2012).

Activins are proteins of transforming growth factor-beta (TGF-β) family (Hedger *et al.,* 2011; Sideras *et al*., 2013). Activin proteins contain four subunits (activin A, activin B, activin C, and activin E) and have initially been known as reproductive factors in humans. However, growing evidences showed that activin proteins are essential regulators for multiple biological events such as inflammation, apoptosis, wound repair, cell differentiation, and neural cell survival in various tissues and cells in humans (Hedger *et al*., 2011). Amongst all subunits, activin A gathered the most attention due to its undisputable role in a plethora of physiological and pathological events (Sideras *et al*., 2013). Activin A can be found in various tissues of immune and non-immune cell origins including granulocyte-macrophage colony stimulating factor (GM-CSF) (Shao *et al.,* 1992), triggering receptor expressed on myeloid cells 1 (TREM-1) (Dower *et al*., 2008), bone marrow (Shao *et al*., 1992), monocytes (PBMCs) (Eramaa *et al*., 1992) and macrophage (Wang *et al*., 2008). Increased in the production of activin A based on the reports from different experimental and/or clinical studies highlighted that activin A is involved in the pathogenesis of numerous diseases including acute inflammatory disorders, traumatic injuries, autoimmune disorders and tissue remodeling and injuries (Jones *et al*., 2004; Sideras *et al*., 2013).

Different sources also claimed that activin A has both pro- and anti-inflammation properties, and is capable to modulate cytokine responses and inflammation process, depending on the cell types employed, its activation status and concentration, and the involvement of cytokines (Jones *et al.*, 2000, Phillips *et al*., 2009; Petrakou *et al*., 2013). These studies unraveled the potential role of activin A as a host immune-modulator (Robson *et al*., 2009) based on its significant involvement in the pathophysiology of inflammation-related diseases (Phillips *et al.*, 2009). Since malaria infection has been associated with inflammation, this study attempts to investigate the involvement of activin A in malaria pathogenesis and the modulating effects of activin A in cytokine releases (TNF-α, IFN-γ and IL-10) and histopathological changes in major affected organs (kidney, liver, lung, brain and spleen) in malarial mice through neutralization of activin A production by recombinant mouse activin RIIA Fc chimera in *Plasmodium berghei* ANKA infection murine model. Ultimately, this study aims to shed lights on the potential of activin A as a immunotherapeutic target in reducing inflammation during malaria infection.

MATERIALS AND METHODS

Animals and malaria model establishment

A total of sixty-four male ICR mice with average weight between 16-20g were used in this study. *Plasmodium berghei* ANKA malaria parasite used in the study was obtained from Institute for Medical Research (IMR), Malaysia. The animals were maintained at room temperature (25°C–27°C) under mosquito-netted cage at animal house of Faculty of Medicine and Health Science, Universiti Putra Malaysia, with food and water supply *ad libitum*. The infection was initiated by intraperitoneal (i.p) inoculation of 2×10^7 parasitized red blood cells (pRBCs) into ICR mice in a volume of 0.2ml while the control uninfected mice were given 0.2ml of uninfected red blood cells (RBCs) (Basir *et al*., 2012). All the procedures abided the rules and regulations stated by Animal Care and Use Committee (ACUC) of Universiti Putra Malaysia (UPM) (Medical and Animal Ethics approval number: UPM/FSPK/PADS/BR-UUH/00365).

Parasitemia measurement

Parasitemia levels in mice were measured by examination of the thin blood film at day 1, 2, 3, 4, 5 and 6 post infection. A drop of blood was taken from the tail of mice. Blood was dropped on a side of slide and smeared evenly to produce thin blood film. The slide was then stained with Leishman's stain and viewed under digital microscope (Nikon Eclipse 80i, US) with 1000× magnification (immersion oil applied). Five fields of view containing 200 red blood cells were taken and parasitemia for each field was counted by dividing total amount of Leishman-positive RBCs by total amount of RBCs and then times 100. Leishman positive cells were counted with the aid of software Image Pro Plus Ver. 5.1. Parasitemia counts were expressed as percentage of RBCs containing Leishman positive cells. The final percentage of parasitemia was based on the average parasitemia for the five fields consisting of Leishman positive cells.

Determination of systemic activin A levels in malarial mice

Malaria-infected mice and control uninfected mice were assigned randomly into two groups of eight mice each. Whole bloods were collected from mice via cardiac puncture. Plasma was prepared by centrifuged the whole bloods at 15,000rpm for 10 minutes. Plasma was stored at -70°C before use. Systemic activin A concentration was determined using Human/Mouse/Rat Activin A Quantikine ELISA Kit (R&D systems, USA) in accordance to manufacturer's instructions at day 1, 2, 3, 4, 5 and 6 post inoculation.

Preparation of Recombinant Mouse Activin RIIA Fc Chimera

The recombinant mouse activin RIIA Fc chimera (rmActivin RIIA Fc chimera) (R&D Systems, USA) was obtained from myeloma cell line with effective dose (ED^{50}) was typically 6–36ng/mL (R&D Systems, USA). Recombinant mouse activin RIIA Fc chimera was supplied in lyophilized form of 50µg per vial and was diluted to 25µg/mL using PBS in this study (R&D Systems, USA). The prepared drug was stored at -70°C prior to use. The therapeutic dosage in this study was 2.5µg/0.1 mL, adapted from previous literatures and also in accordance with manufacturer's descriptions.

Effects of activin A modulation on the parasitemia development of malarial mice

Twenty-four ICR mice with weight between 16–20g were randomly assigned into three groups of eight mice each. The groupings were as below:

Group 1: Control mice (C) + Phosphate Buffer Saline (PBS) (0.1mL, i.v) (C+PBS)
Group 2: Malaria (M) + Phosphate Buffer Saline (PBS) (0.1mL, i.v) (M+PBS)
Group 3: Malaria (M) + rmActivin RIIA Fc chimera (2.5µg/0.1 mL/mouse, i.v) (M+ rmActivin RIIA Fc chimera)

Control and malarial mice were injected intraperitoneal (i.p) with 0.2ml of uninfected RBCS and 2×10^7 PRBCs, respectively. On day 2 to day 5 post-inoculation, each group received one dose of treatment with phosphate buffer saline (PBS) or recombinant mouse activin RIIA Fc chimera (rmActivin RIIA Fc chimera), as indicated in the above grouping, respectively. Parasitemia development in all experimental animal groups were monitored and recorded daily throughout the study periods.

Effects of activin A modulation on cytokines release and histopathological changes during malaria infection

Twenty-four ICR mice of weight between 16–20g were randomly grouped into three groups of eight mice each. The groupings were as below:

Group 1: Control mice (C) + Phosphate Buffer Saline (PBS) (0.1mL, i.v) (C+PBS)
Group 2: Malaria (M) + Phosphate Buffer Saline (PBS) (0.1mL, i.v) (M+PBS)
Group 3: Malaria (M) + rmActivin RIIA Fc chimera (2.5µg/0.1 mL/mouse, i.v) (M+ rmActivin RIIA Fc chimera)

Cytokine analysis

All mice from the above groupings received one dose of treatment with phosphate buffer saline (PBS) or recombinant mouse activin RIIA Fc chimera (rmActivin RIIA Fc chimera), consecutively from day 2 to day 5 post-inoculation. For cytokine analysis, bloods were collected from mice at day 5 post-infection via cardiac puncture while the mice were anaesthetized through inhalation of diethyl ether. Plasma samples were prepared by centrifuged the whole blood at 15,000rpm for 10 minutes using microcentrifuge (Sartorious, Germany) and stored at -70°C prior to analysis. Plasma levels of TNF-α, IFN-γ, and IL-10 at day 5 post infection were measured by commercial Mouse TNF-alpha Quantikine ELISA Kit (R&D systems, USA), Mouse IFN-gamma Quantikine ELISA Kit (R&D systems, USA) and Mouse IL-10 Quantikine ELISA Kit (R&D systems, USA), respectively as per manufacturer's instructions.

Histopathological analysis

All the mice in this study were anesthetized with diethyl ether 1.9% and sacrificed at day 5 post-infection for histopathological analysis. Perfusion was performed using PBS intracardially for 5 minutes before analysis. Five major organs (brain, lungs, spleen, liver, and kidneys) were extracted, fixed in 10% formalin and subjected to tissue processing using tissue processor (Leica, Germany). Processed tissues were transferred to Histo-Embedder (Leica, Germany) for tissues embedding using liquid paraffin wax. Embedded tissues were sectioned into 5.0µm thick via microtome machine and stained with hematoxylin and eosin (H&E) with an auto-stainer (Leica, Germany). The pathological changes of the affected host tissues in disease and treatment stages were observed under digital microscope (Nikon Eclipse 80i, US) with 100x, 200x, 400x, and 1000x magnifications.

Statistical Analysis

All the data in this study were analyzed via Graph Pad Prism ver 5.01 (GraphPad Prism Inc., USA). Results were expressed as mean ± standard error of the means (SEM). Mann-Whitney U Test was performed to compare the systemic level of activin A in malarial mice and control uninfected groups. Kruskal-Wallis test followed by Dunn's post hoc analysis was performed to compare the parasitemia levels and the concentration of TNF-α, IFN-γ and IL-10 between different experimental groups. A p-value less than 0.05 ($p<0.05$) was considered as statistically significant.

RESULTS

Systemic activin A concentrations in malarial mice

The involvement of activin A in malarial mice was assessed by measuring the concentration of activin A in the plasma (Figure 1a). Activin A was detected in malarial mice throughout the study period. Activin A concentration was significantly increased from day 2 to day 6 post-infection as compared to control uninfected mice. Also, Activin A concentration was positively correlated with malaria parasitemia (Figure 1b), suggesting that activin A is involved in the malaria pathogenesis.

Parasitemia development in malarial mice treated with recombinant mouse activin RIIA Fc chimera (rmActivin RIIA Fc chimera)

The parasitemia levels of malarial mice treated with PBS and rmActivin RIIA Fc chimera is depicted in Figure 2. The parasitemia levels in malarial mice were significantly increased consecutively from day-2 until day-6 post infection compared to malarial mice receiving PBS. Conversely, the parasitemia levels in malarial mice treated with rmActivin RIIA Fc chimera showed significant reduction from day 4 to day 6 post-infection compared to malarial mice treated with PBS.

Modulating effects of Activin A on the cytokines release (TNF-α, IFN-γ and IL-10)

The modulating effects of Activin A on the release of TNF-α, IFN-γ and IL-10 cytokines during malaria infection at day 5 post-infection is illustrated (Figure 3a, 3b and 3c). TNF-α, IFN-γ and IL-10 cytokine levels were significantly increased in malarial mice compared to control mice at day 5 post-infection. In malarial mice receiving rmActivin RIIA Fc chimera treatment, TNF-α level was significantly reduced whilst a lack of significant modulating effects of Activin A on the production of IFN-γ and IL-10 cytokines were shown in this study, demonstrated by a slight reduction in the plasma IFN-γ and IL-10 cytokine levels.

Modulating effects of Activin A on the histopathology of major affected organs in malarial mice

i) Brain

Sequestration of parasitized red blood cells (pRBCs) (the hallmark of cerebral malaria) in the small blood vessels was observed in the brain of malarial mice (Figure 4B) (Cox-Singh *et al.*, 2010; Scull *et al.*, 2010). Hemozoin-containing red blood cells were also found in the brain of malarial mice (Figure 4C). In contrast, hemozoin formation and sequestration of pRBCs within the microvasculature compartment of brain tissues were absence in malarial mice treated with rmActRIIA Fc chimera (Figure 4D and Figure 4E).

ii) Lung

Sequestration of pRBCs (Figure 5B) that observed in the lung tissues of malarial mice (Figure 5B) contributes to the severity of malaria infection (Vásquez and Tobon 2012). Hemozoin

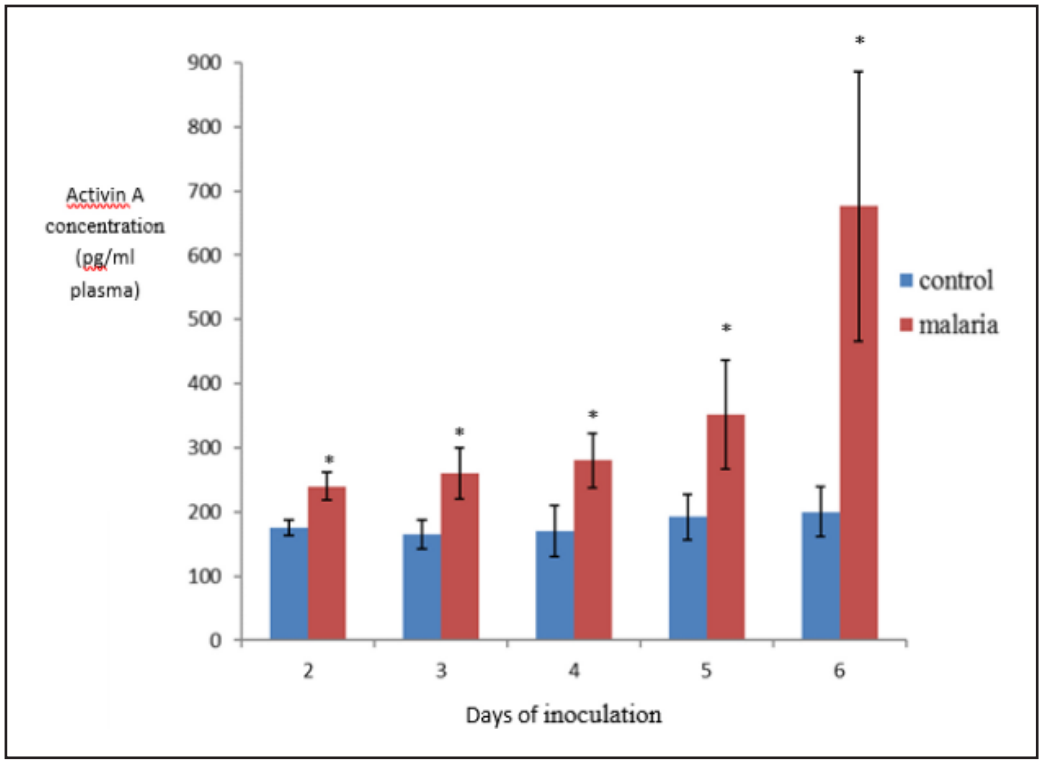

Figure 1. The release of activin A in the plasma of malaria-infected and control mice. The results were presented as mean ± s.e.m. (N=8). * indicates significant differences between control and malaria-infected mice via Mann-Whitney U Test.

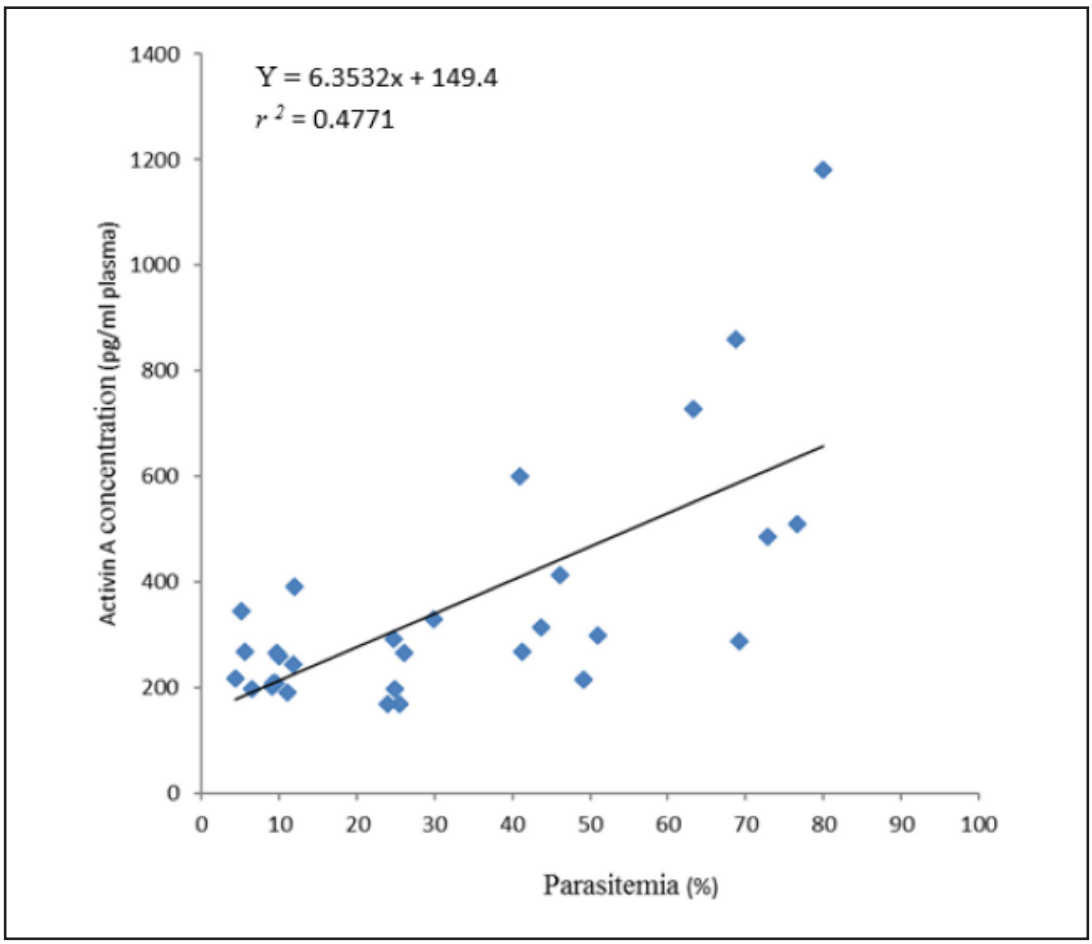

Figure 2. Correlation between activin A concentration (pg/mL) and parasitemia (%) in malaria-infected mice. Spearmen's rank test was used to determine the relationship between these two parameters.

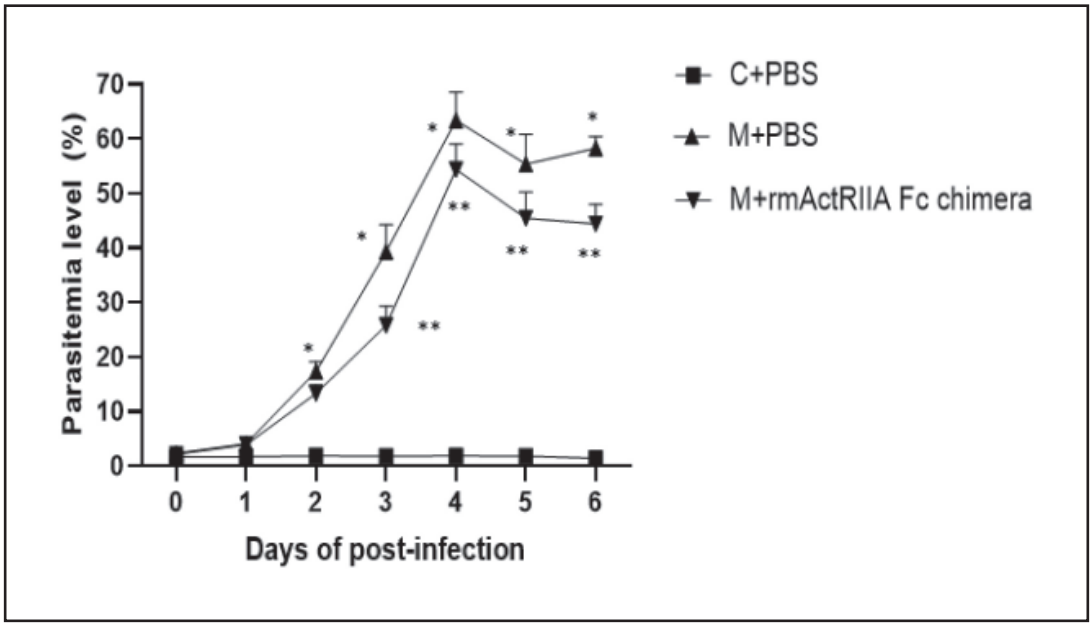

Figure 3. Parasitemia development in control, malarial mice treated with PBS and malarial mice treated with rmActivin RIIA Fc chimera. Results were presented as mean ± s.e.m. of N=8. * denotes the significant differences between malaria-infected and control mice, while * * denotes the significant differences between malarial mice treated with PBS and malarial mice treated with rmActivin RIIA Fc chimera via Kruskal-Wallis test followed by Dunn's post hoc analysis.

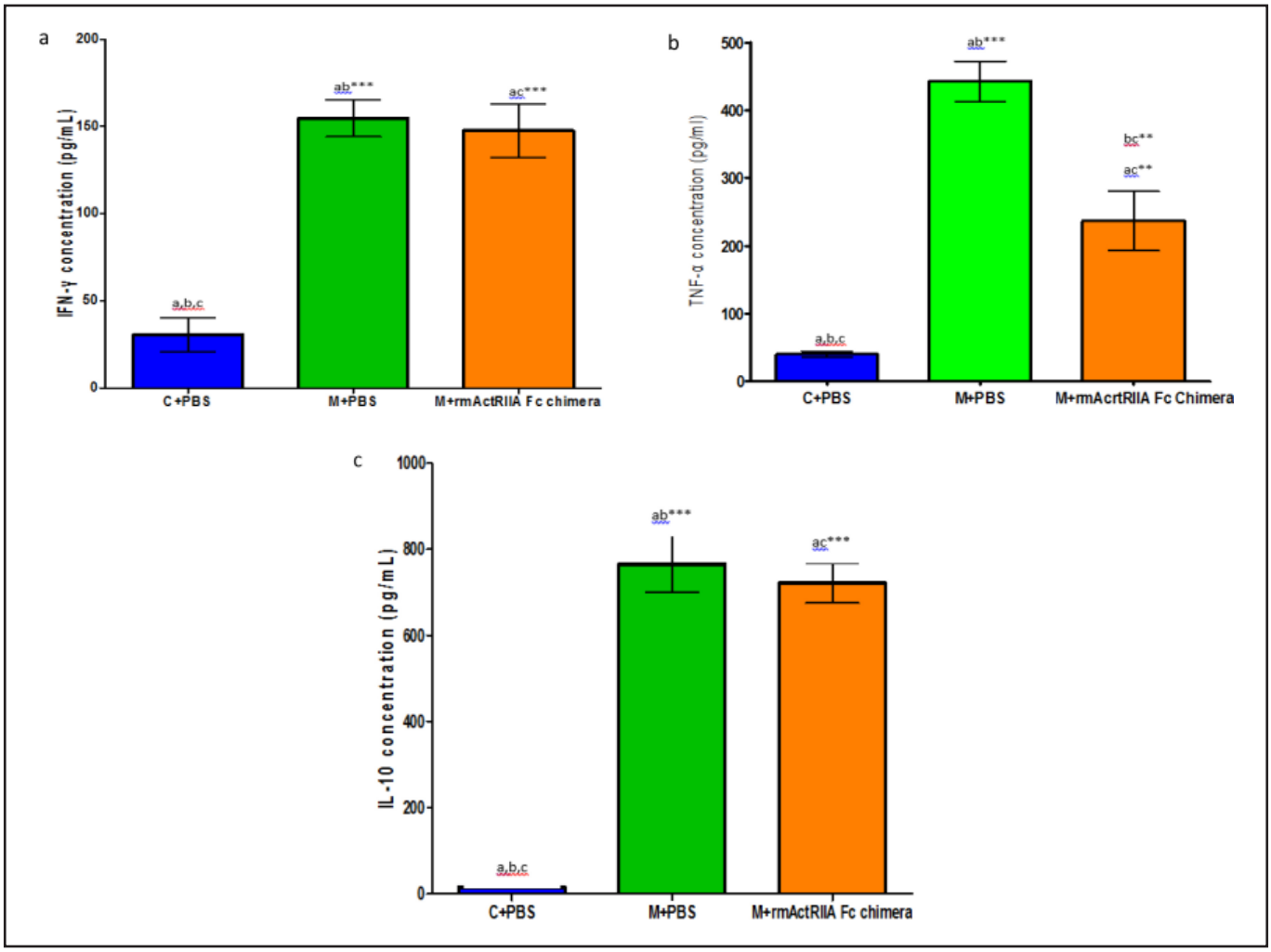

Figure 4. Concentration of TNF-α, IFN-γ and IL-10 in C+PBS, M+PBS, and M+rmActRIIA Fc chimera mice. Results were expressed in mean ± s.e.m. of N=8. **P<0.005 and ***P<0.0005 significant differences between control and malaria-infected group and malaria-treated group on day 5 of post inoculation using Kruskal-Wallis test followed by Dunn's post hoc analysis. Keynote: C+PBS = control + phosphate buffer saline; M+PBS = malaria + phosphate buffer saline; M+rmAcrtRIIA Fc Chimera = Malaria + recombinant mouse activin RIIA Fc chimera; abc = control (a) compared to malaria-infected (b) and malaria-treated group (c); ab*** = p<0.0005 significant difference when b compared to a; ac** = p<0.005 significant difference when c compared to a; bc** = p<0.005 significant difference when b compared to c.

formation, thickening of septal (Figure 5C), and hyalinized membrane formation (Figure 5D) were the histopathological features seen in malaria-infected lung tissues. The hemozoin formation was evident by the presence of brownish spots between interstitium (Figure 5B). Hyalinized membrane seen in malarial mice (Figure 5D) was due to alveolar hemorrhage associated with capillaritis and leakage of red blood cells into interstitium (Iaochimescu and Stoller, 2008). Histopathological conditions of lung tissues in malarial mice receiving rmActivin RIIA Fc chimera were improved, marked by a reduction in pRBCs sequestration and hemozoin formation (Figure 5E and Figure 5F).

iii) Liver

Liver enlargement as well as other histopathological changes such as sequestration of pRBCs in the blood vessels, sinusoids dilation, hemozoin formation in sinusoids, hyperplasia and hypertrophy of Kupffer cells and infiltrations of inflammatory cells were observed in malarial mice (Figure 6B, Figure 6C and Figure 6D). These histopathological pictures were absent in the counterpart of liver tissues in control mice (Figure 6A). Histological analysis also revealed the presence of inflammatory cells in liver portal vein. The infiltrating inflammatory cells including monocytes and macrophages are important for protection against malaria infection (Chua *et al.*, 2013; Ioannidis *et al.*, 2014). A milder hemozoin formation accompanied with less congestion caused by pRBCs were observed in malarial mice treated with rmActRIIA Fc chimera (Figure 6E).

iv) Spleen

Histopathological changes such as hemozoin formation, sequestration of pRBCs (Figure 7B) and the enlargement of white pulp (Figure 7C) were observed in malarial mice. The histopathological conditions of the spleen of malaria mice treated with rmActRIIA Fc Chimera were improved (reduction in pRBCs sequestration, mild enlargement of the white pulp and hemozoin formation was reduced in red pulp when compared to malaria-infected mice (Figure 7E and Figure 7F).

v) Kidney

Sequestration of pRBCs was observed in renal vessels of kidney tissues (Figure 8C). Vascular congestion in the medullary region (Figure 8D), another pathology found in

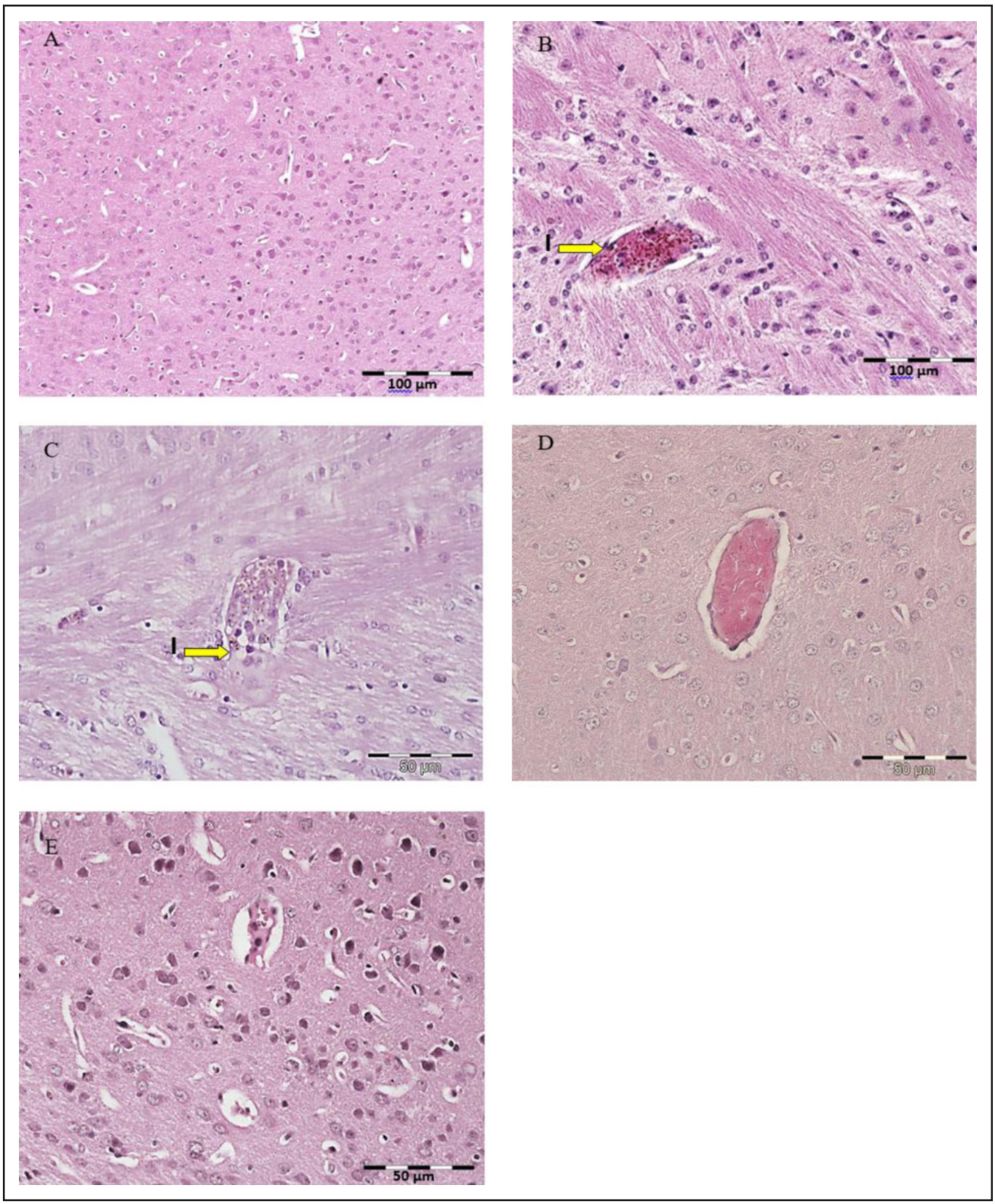

Figure 5. Light microscopy of brain tissue in normal uninfected mice, malarial mice and malarial mice treated with rmActRIIA Fc chimera. A: Light micrograph of brain tissues in control mice. B: Light micrograph of brain tissues in malarial mice; H&E x200. I: Sequestration of pRBCs. C: Light micrograph of brain tissues in malarial mice; H&E x200. I: Hemozoin-containing red blood cells in blood vessel. D: Light micrograph of brain tissues in rmActRIIA Fc chimera treated mice; H&E x200. No sequestration was found in rmActRIIA Fc chimera treated mice. E: Light micrograph of brain tissues in rmActRIIA Fc chimera treated mice; H&E x400. No hemozoin-containing red blood cells were observed.

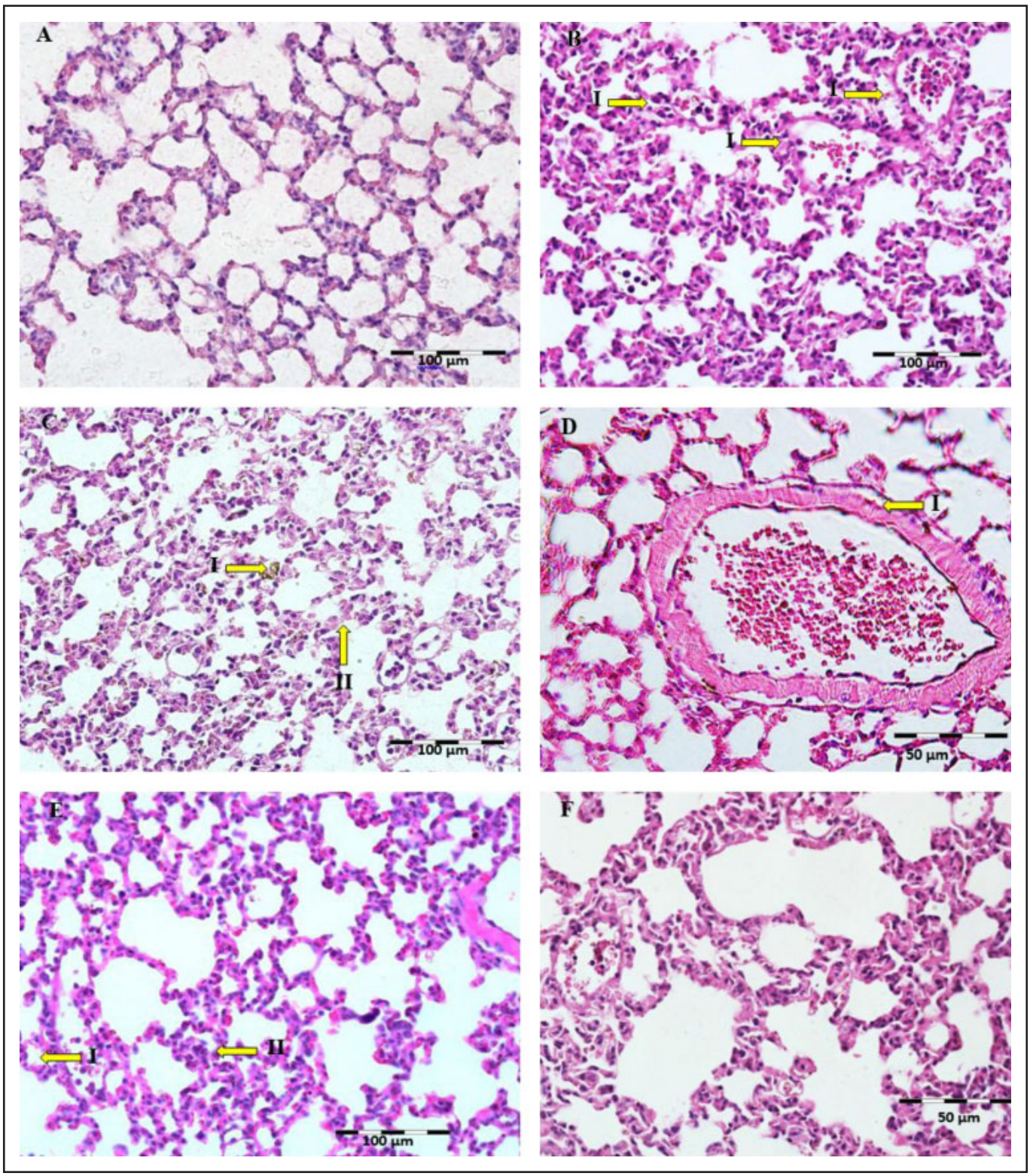

Figure 6. Light microscopy of lung tissue in normal uninfected mice, malarial mice and malarial mice treated with rmActRIIA Fc chimera. A: Light micrograph of lung tissues in control mice, H&E x200. B: Light micrograph of lung tissues in malaria-infected mice, H&E x200. I: Sequestration of pRBCs. C: Light micrograph of lung tissues in malaria-infected mice, H&E x200. I: Hemozoin between intersitium; II: Thickening and disordered of septal. D: Light micrograph of lung tissues in Malaria mice; H&E x400. I: Hyalinized membrane. E: Light micrograph of lung tissues in rmActRIIA Fc Chimera treated mice; H&E x200. I: Less sequestration of pRBCs; II: Less hemozoin. F: Light micrograph of lung tissues in rmActRIIA Fc Chimera treated mice, H&E x400. No hyalinized membrane found.

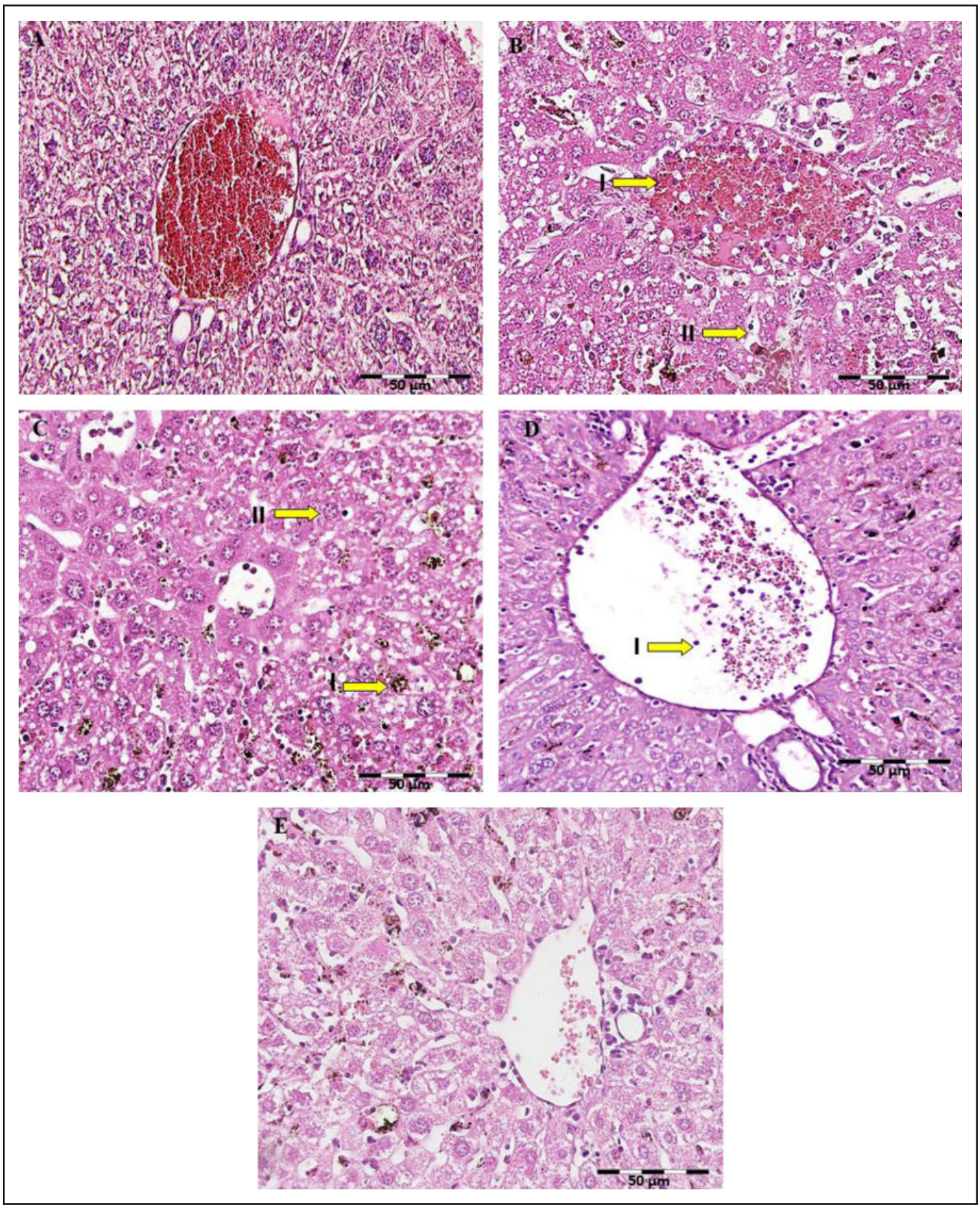

Figure 7. Light microscopy of liver tissue in normal uninfected mice, malarial mice and malarial mice treated with rmActRIIA Fc chimera. A: Light micrograph of liver tissues in control mice. B: Light micrograph of liver tissues in malaria-infected mice; H&E x400. I: PRBCs in blood vessel; II: Sinusoid dilation. C: Light micrograph of liver tissues in malaria-infected mice; H&E x400. I: Hemozoin in sinusoid; II: Hyperplasia and hypertrophy of kupffer cells. D: Light micrograph of liver tissues in malaria-infected mice; H&E x400. I: Inflammatory cells were observed in liver tissues. E: Light micrograph of liver tissues in rmActRIIA Fc Chimera mice; H&E x400. Improved histopathological conditions were observed.

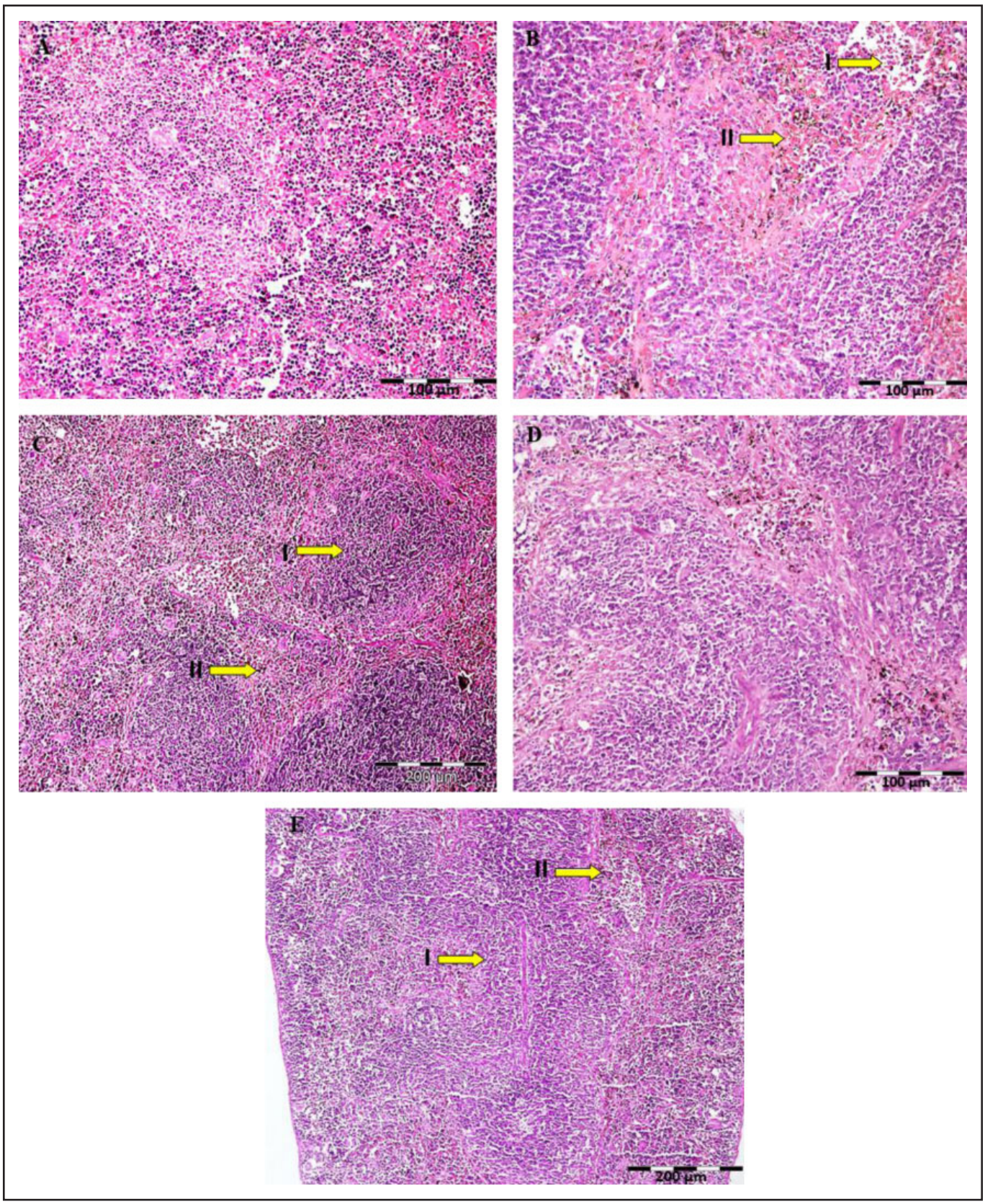

Figure 8. Light microscopy of spleen tissue in normal uninfected mice, malarial mice and malarial mice treated with rmActRIIA Fc chimera. A: Light micrograph of spleen tissues in control mice, H&E x200. B: Light micrograph of spleen tissues in malaria-infected mice, H&E x200. I: Sequestration of parasitized red blood cells in sinus; II: Hemozoin in sinusoid. C: Light micrograph of spleen tissues in malaria-infected mice, H&E x100. I: Enlargement of white pulp; II: dissemination of hemozoin in red pulp. D: Light micrograph of spleen tissues in rmActRIIA Fc Chimera mice, H&E x200: Less parasitized red blood cells were observed. E: Light micrograph of spleen tissues in rmActRIIA Fc Chimera treated mice, H&E x100. I: Only mild enlargement of white pulp when compared with malarial mice; II: Less hemozoin in red pulp.

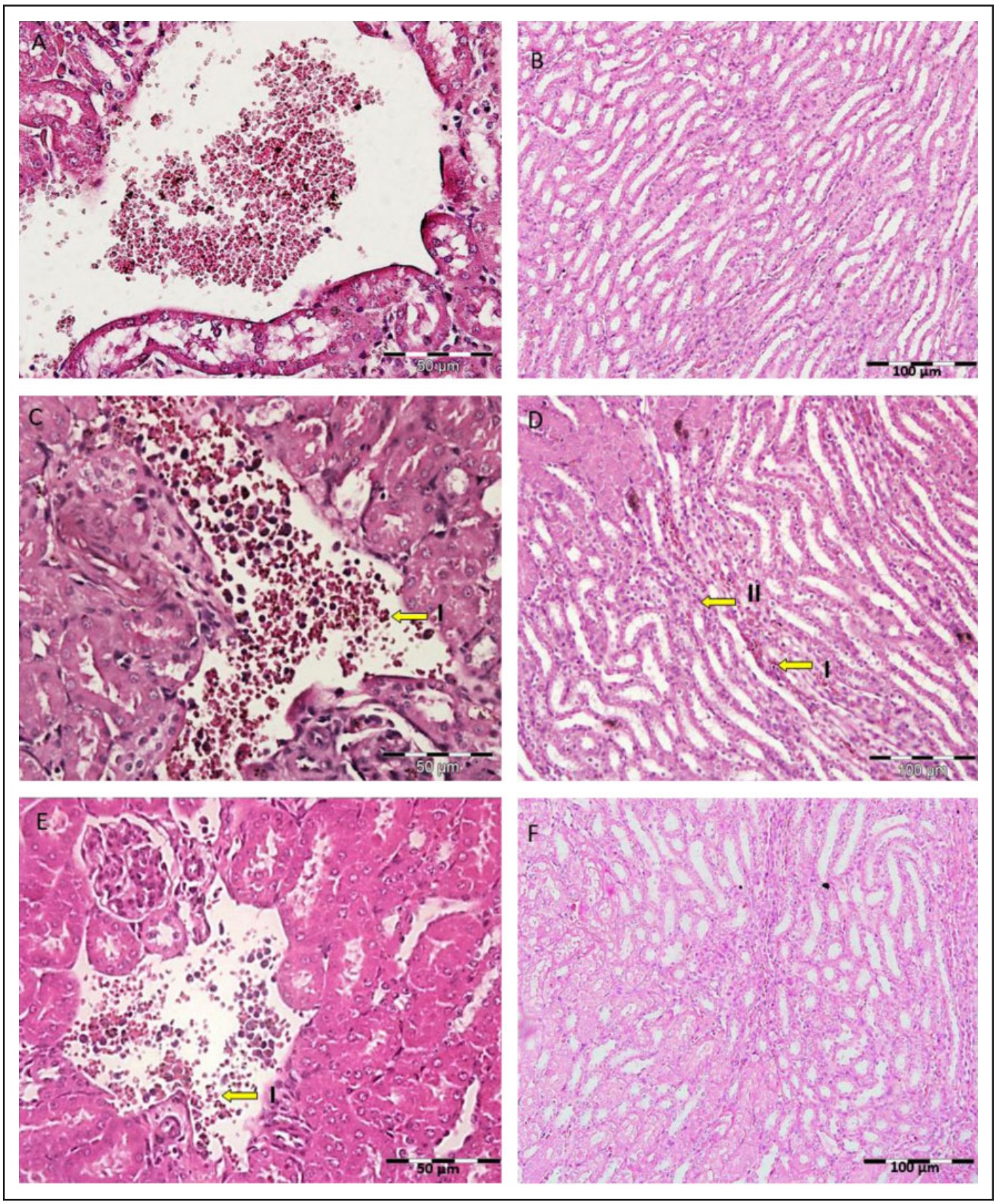

Figure 9. Light microscopy of kidney tissue in normal uninfected mice, malarial mice and malarial mice treated with rmActRIIA Fc chimera. A: Light micrograph of cortex in control mice; H&E x200. B: Light micrograph of medulla in control; H&E x400. No malaria histopathological conditions were observed. C: Light micrograph of cortex in malaria-infected mice; H&E x200. I: sequestration of PRBCs. D: Light micrograph of medulla in malaria-infected mice, H&E x200. I: Hemozoin in medulla; II: Medullary vascular congestion. E: Light micrograph of cortex in rmActRIIA Fc Chimera treated mice; H&E x400. I: Less parasitized red blood cells were seen. F: Light micrograph of medulla in rmActRIIA Fc Chimera treated mice; H&E x200. Improved histopathological conditions in rmActRIIA Fc Chimera treated mice.

malaria-infected mice (Day *et al*., 2005) was also observed in this study. An overall improvement on the sequestration of pRBCs in both cortex and medulla regions were observed in malarial mice treated with rmActRIIA Fc Chimera. (Figure 8E and Figure 8F).

DISCUSSION

Severe malaria infection in humans is often a consequence of dysregulated host immune responses, which causes intensified inflammation and subsequently irreversible tissues/organs damages in humans. TNF-α, IFN-γ and IL-10 are amongst the key cytokines involved in the host immune responses during malaria infection (Angulo & Fresno, 2002; Clark *et al*., 2006; Boström *et al*., 2012; Rodrigues-da-Silva *et al*., 2014). Targeting host inflammation besides malaria parasites could be one of the holistic approaches in tackling malaria infection. This preliminary study explored experimental *P. berghei* ANKA infection murine model to study the role of activin A and its modulating effects on the key cytokines release and the histopathological changes during the course of malaria infection.

Numerous studies have proposed that activin A is a crucial mediator of host immune inflammatory responses (Phillips *et al*., 2009; Hedger *et al*., 2011; Hedger *et al*., 2013). Nonetheless, studies on the role of activin A in malaria context are in paucity. A study by Spottiswoode *et al.* (2017) reported that although activin A may not exert crucial role in regulating the expression of hepcidin- a iron regulatory hormone that has been linked with host susceptibility to malaria superinfection (Portugal *et al*., 2011), however, activin A is induced in malaria infection wherein its level is positively correlated to hepcidin in human volunteers experimentally infected with malaria. The study also showed that activin A mRNA is significantly up-regulated in human peripheral blood mononuclear cells co-cultured with *P. falciparum*-infected RBC (Spottiswoode *et al*., 2017). This study provides evidence on the induction of activin A in malaria infection, but its actual role in malaria context remains to investigate. Consistently, our study observed a rapid induction of plasma activin A concentration in malarial mice since day 2 post-infection. Positive correlation between plasma activin A and malaria parasitemia was also recorded. The higher the parasitemia level, the higher the number of matured parasites which subsequently lead to more frequent host invasion as well as decrease in the number of circulating erythrocytes due to the destruction of erythrocytes by malaria parasites (Cantrell *et al*., 1970; Huh *et al*., 2010). Conversely, parasitemia levels in malarial mice had significantly reduced following inhibition of activin A via recombinant mouse Activin RIIA Fc chimera. We speculate that activin A could have a role in malaria pathogenesis and is associated with hyper-parasitemia. Several lines of evidence suggested that high malaria parasitemia are correlated well to disease severity and the course of malaria infection, leading to multiple organ failures and death in the susceptible hosts (Day & Fowkes, 2001; Koh *et al*., 2004; Rajkumar *et al*., 2012). Nonetheless, the linkage between activin A and parasitemia development is poorly understood and the actual mechanism driving this relationship requires more extensive studies.

Our findings revealed that in malarial mice, TNF-α, IFN-γ and IL-10 levels were markedly increased at day 5 post-infection, suggesting the role of these cytokines in contributing to severe malaria pathology. Macrophages and monocytes are predominant immune effector cells in host defense against malaria parasites by phagocytosing free parasites or parasitized erythrocytes in human or murine malaria infection (Celada *et al*., 1983; Couper *et al*., 2007). The early stage of protection against malaria parasite invasion requires a pro-inflammatory response that involves secretion of key cytokines such as TNF-α, IFN-γ, and IL-12 to control the parasitemia level and to maintain the erythropoiesis process (Stevenson *et al.*, 1995; Perkins *et al*., 2011) whilst anti-inflammatory cytokines including IL-10, IL-4 and transforming growth factor (TGF)-β are released to prevent and/or to balance the production of excessive inflammatory responses that cause detrimental effects on host tissues (Clark *et al*., 2006). TNF-α and IFN-γ have been found to exert both beneficial and detrimental effects in malaria infection. The production of TNF-α at the early stage of malaria infection is mediated through the activation of macrophages by IFN-γ. TNF-α also synergizes with IFN-γ in eradicating malaria parasites through the release of nitric oxide (NO) and other toxic radicals (Artavanis-tsakonas *et al*., 2003). In a study, TNF-deficient mice had significantly reduced its capability to control the erythrocytic stage of malaria infection (Hernandez-Valladares *et al*., 2006). Additionally, transient elevation of TNF-α response as depicted in T cell studies protected the host against severe *P. falciparum* infection and reinfection (Ramharter *et al*., 2004). Nevertheless, exaggerated production of TNF-α had been linked to severe malarial pathology including cerebral malaria and fever in both human host and murine models (Kwiatkowski *et al*., 1990; Depinay *et al*., 2011). Highly circulating TNF-α levels also detected in patients with uncomplicated and severe *P. falciparum* infections (Perera *et al*., 2013). Although a recently published systematic review had associated the increase in TNF-α level with cerebral malaria caused by *P. falciparum* in humans, however, the study reported that current evidence is imprecise and inconsistent that requires more observational studies to prove it (Leão *et al*., 2020).

IL-10 is a key immunoregulator that counteracts excessive inflammatory responses caused by Th1 cells (Nyangoto, 2005) and pro-inflammatory cytokines such as TNF-α and IFN-γ, thus confers protective immunity to erythrocytic malaria parasites (Artavanis-tsakonas *et al*., 2003). In experimental cerebral malaria, the inhibitory effect of IL-10 on the release of TNF-α and IFN-γ has been found to benefit the host by counteracting the pathological role of macrophage (Kossodo *et al*., 1997). In different murine models, IL-10-deficient mice exhibited higher plasma levels of pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-12 (Linke *et al*., 1996; Li *et al*., 1999) and showed a higher risk of developing cerebral (Sanni *et al*., 2004; Niikura *et al*., 2010) and hepatic pathology (Couper *et al*., 2008; Niikura *et al*., 2008) despite the parasitemia levels of these mice are much lower compared to wild-type mice. These findings illustrated the importance of IL-10 regulation in controlling the pathology-inducing inflammatory response and preventing severe immunopathology during malaria infection. However, IL-10 could play a negative role during malaria infection. In rodent model infected with lethal *P. yoelli* 17XL, high level of IL-10 had been associated to high parasitemia level inhibition of pro-inflammatory responses and massive destruction of erythrocytes, leading to severe malarial anemia and death (Kobayashi *et al*., 2000; Omer *et al*., 2003). Another study reported that the increased IL-10 level inhibited Th1 cell responses at early phase of malaria infection, which heightened the parasitemia levels and mortality rate (Wu *et al*., 2007). These studies suggested that IL-10 production in wrong timing and magnitude may suppress the inflammatory response to malaria parasites and thus leading to deleterious outcomes.

Histopathological analysis revealed that the sequestration of pRBCs in the microvasculature compartment of different organs/tissues and hemozoin formation in malarial mice representing the most common histological pictures observed in major affected organs (brain, lung, kidneys, spleen and liver) during malaria infection. Enlargement of spleen and liver, architecture losses in spleen, septa thickening and hyaline formation in lungs, dilation of sinusoids, hyperplasia of Kupffer cells in liver were the organs-specific histopathological features observed in malaria mice in this study. Sequestration of parasitized erythrocytes in the microvasculature compartments of various organs followed by its consequences that include inadequate tissue perfusion and hypoxia are the main pathological mechanisms attributed to severe malaria-related pathology including cerebral malaria, hepatic injury, splenomegaly, pulmonary edema and kidney injury (Mackintosh *et al*., 2004; Franke-Fayard *et al.*, 2010; Idro *et al*., 2010; Kim *et al*., 2018). In human malaria infection, sequestration of *P. falciparum* is facilitated by *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) that interacts with the receptors on infected erythrocytes surface such as intercellular adhesion molecule-1 (ICAM-1) and CD36 (Tripathi *et al*., 2006; Smith *et al*., 2013). Sequestration in infected erythrocytes may cause vascular obstruction, metabolic disruptions consisting of acidosis, and activation of local endothelial cells, leading to proinflammatory cytokine releases (Miller *et al*., 2002; Schofield & Grau, 2005; Mishra & Newton, 2009). Accumulation of other cells such as platelets and leucocytes have been found in the microvasculature compartments of patients with cerebral malaria whereby these cells are able to accelerate the obstruction of microvascular by stimulating the production of chemotactic and inflammatory mediators (Grau *et al*., 2003; Hunt & Grau, 2003). In rodent malaria model, sequestration of parasitized erythrocytes in organs such as lungs and adipose tissue is mediated via adherence to CD36 receptor. A small protein, schizont membrane-associated cytoadherence protein (SMAC) is involved in CD36-mediated sequestration in infected erythrocytes in *P. berghei* infection, however, SMAC is not transported to the surface of infected red blood cells but rather is exported into cytoplasm of host erythrocytes. Such orthologs are absence in human malaria parasites as well (Franke-Fayard *et al*., 2005; Franke-Fayard *et al*., 2010; Fonager *et al*., 2012). Indeed, a study reported that *P. berghei* expressed *P. falciparum* orthologues, PfSBP1 and PfMAHRP1 that are involved in trafficking a still unidentified parasite ligand which mediates the adhesion of parasitized erythrocytes to CD36 *in vitro* and sequestration *in vivo*. These *P. falciparum* orthologues, PfSBP1 and PfMAHRP1 are complementary to the respective gene deletions in *P. berghei*, suggesting the existence of evolutionary conservation in the trafficking machinery that underlies the malaria parasite virulence between rodent and human (De Niz *et al*., 2016). The identification of rodent parasite ligands and their associated trafficking machinery involved in the sequestration of pRBCs in rodent malaria models could shed lights on the primary mechanism that mediates sequestration and malaria virulence by *Plasmodium*, wherein these findings can be translated into human malaria context (De Niz *et al*., 2016).

Hemozoin is a crystalline, brown pigment that is produced during heme detoxification by all *Plasmodium* parasites, including species-infecting mice (*P. berghei* and *P. chabaudi*) during intra-erythrocytic development stage. Hemozoin is sequestered in digestive vacuole of *Plasmodium* as a byproduct after the digestion of host hemoglobin for nutrients and energy supply (Francis *et al*., 1997). Hemozoin have been demonstrated to sequester in different organs (spleen, brain and liver) besides red blood cells, to be actively phagocytosed or being rapidly uptake after its release from ruptured schizont by circulating and tissue-resident immune cells, in particular monocytes and macrophages as well as the capability to modulate host innate and inflammatory responses. Ultimately, these findings highlighting a central role of hemozoin in malaria immunopathogenesis. (Sullivan *et al*., 1996; de Souza *et al*., 2010; Shio *et al*., 2010; Autino *et al*., 2012; Olivier *et al*., 2014). In murine and human malaria, abundance of circulating phagocytes as well as tissue phagocytes in lymphoid organ (spleen) and brain are loaded with hemozoin that correlated well with malaria disease severity (Lyke *et al*., 2003; Franke-Fayard *et al.*, 2010; Mohapatra *et al*., 2014). Hemozoin-monocyte complex had been linked to severe malaria anemia (Novelli *et al*., 2010), cytokine dysregulation (Keller *et al*., 2006) and immunosuppression (Arese & Schwarzer, 1997), reflecting its pathological roles in malaria parasitemia development. Hemozoin also serves as a robust biomarker in diagnosing malaria infection because of its presence in the erythrocytic stages in all clinically relevant *Plasmodium* species (Lukianova-Hleb *et al*., 2014; Rifaie-Graham *et al*., 2019).

Consistently with different reported studies, our study demonstrated that immunopathogenesis of severe malaria infection is caused by high levels of systemic cytokines including TNF-α, IFN-γ and IL-10 (Lyke *et al*., 2004; Clark *et al*., 2006) as well as pronounced histopathological features (sequestration of malaria parasites in the red blood cells and host tissues and hemozoin formation) in different organs/tissues of malarial mice. Insights from *in vivo* studies showed that both TNF-α and IFN-γ are imperative for the development of cerebral malaria by triggering the expression of nitric oxide and ICAM-1 (Rudin *et al*., 1997; Veronique *et al*., 2000; Flori *et al*., 2005; Poovassery *et al*., 2010; Hansen, 2012). Different sources also reported that hemozoin stimulates the gene transcription and expression of TNF-α. It has been observed that hemozoin induced- TNF-α release by human monocytes is suppressed by IgM purified from malaria patients (Biswas *et al*., 2001), which further supported the modulation role of hemozoin in inflammation-related processes. In *P. berghei* ANKA infection, hemozoin activated macrophages to produce pro-inflammatory cytokines, for example, TNF-α that in turn accelerates the activation of hepatic stellate cells to promote liver fibrosis (Kim *et al*., 2018). In this study, we speculate that activin A could have a role in malaria pathogenesis and parasitemia development based on its positive correlation to malaria parasitemia development and sustainable increased in systemic activin A levels during the course of malaria infection. Sustained release of high levels of activin A could probably augment the inflammatory responses through TNF-α and other inflammatory mediators, resulting in tissue/organ damages and even death in malarial mice.

The immune modulation role mediated by activin A in malaria pathogenesis is poorly understood. Production and subsequent release of activin A from cells is triggered by the interaction of activin A ligand with toll-like receptors (TLRs) and signaling pathways through NFkB transcription factor and Jnk/p38 MAP kinase. Increase in the expression of activin A appears to have biphasic response in innate immunity wherein activin A acts as a general amplifier to aggravate the inflammatory responses and exerts suppressive effect on the adaptive immune responses. High levels of activin A induce sustained expression of high mobility group box 1 (HMGB1) and pro-inflammatory cytokines and chemokines such as MCP-1, IL-6, KC, IL-5, IL-1β, IL-4, and TNF-α in which the HMGB/cytokines complex will trigger the activation of TLR

signaling pathway, causing a positive feedback loop that can sustain the production of activin A as long as the signs of tissue damage or infectious agents remain persistent in the affected tissues (Klune *et al*., 2008; Phillips *et al*., 2009; Apostolou *et al*., 2012; Sideras *et al*., 2013). HMGB1, a danger-associated molecular patterns (DAMPs) had been well related to malaria pathogenesis and stimulation of pro-inflammatory cytokines production (Alleva *et al*., 2005; Kumar *et al*., 2008). HMGB1 level also had been reported to increase in *P. falciparum* infection and HMGB1 could serve as a prognostic marker for malaria severity (Angeletti *et al*., 2013; Higgins *et al*., 2013). Furthermore, activin A can be produced by various immune cells including monocytes/macrophages which are the primary effector immune cells in malaria infection and also by stressed/injured tissue-resident cells including epithelial, mesenchymal and endothelial cells (Sideras *et al*., 2013). The secretion of activin A is usually accompanied with the expression of various inflammatory mediators. This strongly suggests that activin A could promote local inflammation and tissue injuries in different major affected organs during malaria infection. Further study interrogating on the gene and protein expression of activin A and its subsequent downstream inflammatory responses at both local and systemic levels are needed to fully address the role of activin A in malaria pathophysiology.

In this, study, we observed that TNF-α level in malarial mice was significantly reduced whilst only a small reduction in IFN-γ and IL-10 levels were recorded after neutralization of activin A with recombinant mouse Activin RIIA Fc chimera. Also, the histological pictures of major affected organs (brain, lung, kidneys, spleen and liver) in malarial mice were mitigated following inhibition of activin A production. These histological improvements in major affected organs include reduction in pRBCS sequestration, decreased in microvascular congestion and lesser hemozoin formation. It is very likely that the improvised histopathology conditions and restricted damage in all affected organs could be due to a reduction in TNF-α concentration. We speculate that activin A-mediated TNF-α release could be the plausible mechanism involved in alleviation of tissue pathology in malarial mice. Other studies had reported that inflammation-related activity exerted by activin A can be modulated by follistatin (Dohi *et al*., 2005; Hardy *et al*., 2006), an inhibitor of activin A that has high binding affinity to activin A (Nakamura *et al*., 1990) *in vivo*. In LPS challenge mouse model, although pre-administration of follistatin is unable to impede the release of activin A in acute phase, but it can modulate the secretion of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1b in a dose-dependent manner (Jones *et al*., 2007). Treatment with activin A neutralizing fusion protein (Apostolou *et al*., 2012) or prophylactic administration of follistatin (Jones *et al*., 2007) had been shown to block the activin A activity and improved the survival in mice challenged with LPS. These aforementioned studies have undoubtedly supported the attribution of activin A in mediating the severity of inflammation-related diseases and modulation of activin A activity by its inhibitors could attenuate the consequences of a plethora inflammatory syndromes.

Overall, this study has its own limitations. This study relies on a single effective dose of recombinant mouse rmActRIIA Fc chimera to assess the modulating effects of activin A in cytokines release and tissue histopathology in malaria infection using an experimental murine malaria infection. The neutralizing effects of recombinant mouse rmActRIIA Fc chimera for activin A activity should be carried out at variable doses in different independent studies to identify the optimal therapeutic dosage that effectively limits the tissue damage caused by excessive inflammatory responses. The modulating effects of activin A should include more inflammatory and non-inflammatory markers, but should not limited to TNF-α, IFN-γ and IL-10 only following neutralization of activin A. The source of activin A production in malarial mice remains elusive since it can be produced by various immune or non-immune acells. Immunohistochemical staining of activin A and its associated inflammatory mediators on affected tissues and also on tissue-resident immune cells should be performed as well as the gene and protein expression at systemic level in malarial mice should be conducted to elucidate the functional role of activin A and also to unravel the activin A signaling pathway responsible for malaria pathogenesis. The modulating effects of activin A on the histopathological features of affected organs should be examined using both qualitative and quantitative measurements that includes histological scoring.

CONCLUSION

This study highlights that activin A could play a role in malaria pathogenesis, demonstrated by significantly and progressively increase in activin A level in malarial mice as well as a positive correlation between activin A and malaria parasitemia level during the course malaria infection. High levels of TNF-α, IFN-γ and IL-10 levels were recorded in malarial mice, suggesting the contribution of these cytokine in severe malaria-associated pathology. Prominent histo-pathological pictures such as sequestration of parasitized erythrocytes and hemozoin formation are observed in major affected organs in malarial mice. The immune-modulation role of activin A in malarial mice was evident by a reduction in TNF-α release following neutralization of activin A by recombinant mouse rmActRIIA Fc chimera. It is speculated that low level expression of TNF-α through inhibition of activin A release attributed to improvised histopathological conditions in major affected organs in malarial mice. Nevertheless, the origins of activin A and its precise immune-modulation mechanism in malaria context are still not clear. The questions on the source of activin A during malaria infection, the activin A signaling pathway involved and its immune-modulation role in malaria pathophysiology remain unanswered. Further investigations are warranted to completely dissect the functional roles/consequences of activin A in the complex network of host-pathogen interactions.

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Competing Interests

The authors declare that they have no competing interests.

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