Modulation of interleukin-18 release produced positive outcomes on parasitaemia development and cytokines production during malaria in mice

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Abstract. The involvement of interleukin-18 (IL-18) and the effects of modulating its release on the course of malaria infection were investigated using *Plasmodium berghei* ANKA infection in ICR mice as a model. Results demonstrated that plasma IL-18 concentrations in malarial mice were significantly elevated and positively correlated with the percentage parasitaemia development. Significant expressions of IL-18 were also observed in the brain, spleen and liver tissues. Slower development of parasitaemia was observed significantly upon inhibition and neutralization of IL-18, whereas faster development of parasitaemia was recorded when the circulating levels of IL-18 were further augmented during the infection. Inhibition and neutralization of IL-18 production also resulted in a significant decrease of plasma concentrations of pro-inflammatory cytokines (TNFα, IFNγ, IL-1α and IL-6), whereas the anti-inflammatory cytokine, IL-10, was significantly increased. Augmenting the release of IL-18 during the infection on the other hand resulted in the opposite. Early mortality in malarial mice was also observed when the circulating levels of IL-18 were further augmented. Results proved the important role of IL-18 in immune response against malaria and suggest that IL-8 is pro-inflammatory in nature and may involve in mediating the severity of the infection through a pathway of elevating the pro-inflammatory cytokine and limiting the release of anti-inflammatory cytokine.

INTRODUCTION

Interleukin-18 (IL-18) is a pleiotropic cytokine (Dao *et al.*, 1996, Tsutsui *et al.*, 1996, Ushio *et al.*, 1996) that shares many similarities structurally and functionally to the IL-1 family (Okamura *et al.*, 1995; Bazan *et al.*, 1996; Parnet *et al.*, 1996). It was formerly introduced as an IFNγ-inducing factor and was able to induce the production of IFNγ from the activated macrophages, natural killer cells and T cells (Okamura *et al.*, 1995). It has also been reported to be able to induce the production of tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β) in mononuclear cells (Puren *et al.*, 1998). IL-18 is regarded as a pivotal mediator of Th1 cytokines response with its ability to initiate a cytokines cascade with concomitant release of pro-inflammatory mediators such as adhesion molecules (Kohka *et al.*, 1998), nitric oxide (Zhang *et al.*, 1997) and matrix metalloproteinase-9 (Nold *et al.*, 2003a, 2003b). It's crucial roles in the regulation

of the immune responses have been reported in many studies and its diverse involvement in disease conditions has been described in AIDS (Iannello *et al*., 2009), acute pancreatitis (Martin *et al*., 2008), chronic obstructive pulmonary disease (Rovina *et al*., 2009), tumour growth (Hashimoto *et al*., 1999; Zheng *et al*., 2009) etc. Microbial infections such as *Leishmania major* (Ohkusu *et al*., 2000), *Cryptococcus neoformans* (Kawakami *et al*., 1997), and *Plasmodium falciparum* (Kojima *et al*., 2004) have also shown the involvement of IL-18 in the host immune response towards the invading organisms.

Malaria infection has always been associated with the elevation of many inflammatory cytokines such as TNFα, IL-1, IL-6, IL-2, IFNγ, IL-10, TGFβ and IL-18 (Grau *et al*., 1987; Plebunski & Hill, 2000; Singh *et al.*, 2002), which have been proven to play crucial roles in mediating the severe immunopathological reactions associated with the disease (Plebunski & Hill, 2000). The role of IL-18 in malaria infection has not been widely studied and documented. Only a handful studies have described the possible role and involvement of IL-18 during malaria infection in mice and human. In severe falciparum malaria in human, IL-18 was associated with the disease severity (Nagamine *et al*., 2003), suggesting its role in mediating the severe pathology of the disease. Contrary to the human study, IL-18 was reported to play protective role in murine malaria by enhancing IFNγ production (Singh *et al*., 2002) and anti-IL-18 treatment clearly shortened the survival times of malaria-infected mice (Okamura *et al*., 1998).

With many studies demonstrating the promising therapeutic potential of modulating IL-18 bioactivity for the treatment of inflammation-related diseases, the present study was therefore designed to clarify further the role and involvement of IL-18 during malaria infection. Here, we proposed to investigate the effects of modulating IL-18 release and production on the course of malaria infection in mice and the systemic release of pro-inflammatory cytokines TNFα, IL-1α, IFNγ and IL-6 and the anti-inflammatory cytokine IL-10, with the ultimate aim of evaluating whether IL-18 plays a key role in protective immunity against the infection or it mediates the severe pathology of the infection through a pathway of elevating the above said cytokines.

MATERIALS AND METHODS

Animals
Inbred male ICR mice were used as animal model for malaria infection. They were obtained from The Animal Housing Unit, Universiti Sains Malaysia. The mice weighing initially between 17-20g were used throughout the experiments. They were handled as gently as possible and transferred from the animal house to the laboratory at least 30-60 min prior to experiment in order to minimize the effect of stress. All experimental protocols involving the animals were conducted according to the rules and regulation approved by The Animals Ethics Committee, Universiti Sains Malaysia.

Parasites
Plasmodium berghei (*P. berghei*) ANKA strain was used to initiate malaria infection in the mice. The parasite was originally obtained from the Institute of Medical Research, Kuala Lumpur, Malaysia and was maintained in a laboratory at The School of Pharmaceutical Sciences, Universiti Sains Malaysia by a combination of passage in male ICR mice and cryoscopic storage.

Malarial infection procedure
Malaria infection in the mice was initiated by intravenous (i.v) inoculation into the tail vein of normal mouse with 0.2 ml blood, diluted to contain 2×10^7 parasitized red blood cells (PRBC) from a donor mouse infected with *P. berghei*. Controls to malaria-infected mice received an equivalent volume and dilution of normal uninfected mouse red blood cells.

Parasitaemia measurement
Measurement of the parasitaemia levels in the animals was carried out by collecting a drop of blood through venesection of the tail from each malarial animal, onto the edge of

a microscope slide. The blood was drawn evenly across the slide to make a thin blood film before staining with Leishman stain and then viewed under light microscopy with oil immersion (1000x magnification). Leishman positive cells were counted with the aid of a graticule and hand counter. Five fields of approximately 200 cells each were counted and the parasitaemia was calculated as the percentage of the total red blood cells counted containing Leishman positive bodies. The average results from the five different fields were then taken as the final percentage of parasitaemia.

Determination of plasma IL-18 concentration

To determine the circulating plasma concentrations of IL-18 during malaria infection, the mice were inoculated with the parasite as described above. Blood samples for plasma from both the control and infected mice were collected on day 1, 2, 3, 4 and 5 following inoculation with the parasites. Blood was withdrawn from the animals by cardiac puncture while they were under terminal general anaesthesia by inhalation of diethyl ether. The animals were finally sacrificed through exsanguination. Plasma was prepared by centrifugation of the blood at 2500 rpm using a bench top centrifuge. IL-18 concentrations in the plasma of control and malaria-infected mice were determined by means of Enzyme-Linked Immunosorbent Assay (ELISA) method using commercially available kit purchased from The Medical & Biological Laboratory Co. Ltd., Nagoya, Japan. The assays were conducted according to the procedures recommended by the manufacturer. Plasma samples were run in duplicate manner in a 96-well microplate coated with anti-mouse IL-18 monoclonal antibody and incubated for 1 hr at room temperature (20–27ºC). Peroxidase conjugate anti-mouse IL-18 monoclonal antibody solutions were then added to the wells and incubated for a further 1 hr at room temperature, followed by the addition of substrate solution (tetramethylbenzidine/ hydrogen peroxide). Finally stop solution (0.5 mol/L sulfuric acid) were added to the wells before reading the absorbance at 450nm using a microplate reader (Multiskan Ascent, Labsystem, Finland). IL-18 concentrations in the samples were determined using the plotted calibration curve from the standard.

Determination of IL-18 expression in local organ tissues

To determine the expression of IL-18 in organ tissues, mice were inoculated with either normal RBC or PRBC as described above. The mice were killed on day 1, 2, 3, 4, 5 and 6 following inoculation and the spleen, liver, brain, kidneys and lungs were removed from the animals and cleaned with phosphate buffered saline solution. The organs were homogenized separately in Tris buffer solution (40mM) in 1:2 ratios and then centrifuged at 12,000 rpm for 20 min. The resultant supernatants were then subjected to electrophoresis on SDS-PAGE (15% separating gel and 4% stacking gel) to separate the protein mixtures in the supernatant. Gel was stained with Coomassie blue after electrophoresis and immuno-blots were applied to transfer the proteins separated on SDS-PAGE gels to nitro-cellulose membranes. The membranes were incubated with antibody against IL-18 and reacted proteins were detected by substrate containing nitroblue-tetrazolium chloride and 5-bromo-4 chloro-3-indolylphosphate in dimethylformamine. Image on the membrane was captured using VersaDoc Imaging System and Quantity One 1-D analysis software (BIO-RAD, USA).

Treatment of malaria-infected mice with recombinant IL-18 (rmIL-8), anti-IL-18 monoclonal antibody (AmIL-18) and recombinant IL-18 binding protein (rmIL-18BP)

In this part of the study, mice were divided into five different groups (N=8 in each group). **Group 1:** Control mice receiving 0.9% normal saline (C+NS); **Group II:** Malarial mice treated with 0.9% normal saline (M+NS); **Group III:** Malarial mice treated with rmIL-18 (M+rmIL-18); **Group IV:** Malarial mice treated with AmIL-18 (M+AmIL-18); **Group V:** Malarial mice treated with rmIL-18BP (M+rmIL-18BP). In the malarial groups, the mice were inoculated with *P. berghei* as

described in previous section. The controls received an equivalent volume and dilution of normal RBC. Treatments were carried out once daily starting on mid day of day 0 following inoculation of the parasites in the morning, and continued until day 4 post infection. All drugs were dissolved in 0.9% normal saline. In group I and II, the control and malarial mice were injected with 0.2 ml normal saline (0.9%) intravenously. In group III, IV and V the mice received 1 µg (0.2 ml, i.v) of either rmIL-18, AmIL18 or rmIL-18BP respectively.

Group I served as the baseline control for the rest of the groups. Group II served as the control for malaria group treated with the IL-18-related drugs. In group III, treatment of malarial mice with rmIL-18 will determine whether augmenting the release of IL-18 in the systemic circulation during the infection will cause any significant changes on the course of malaria infection and also in the release of the pro- and anti-inflammatory cytokines. Similar parameters will also be observed in group IV and V in which the production of IL-18 was either neutralized by AmIL-18 or the action was antagonized by rmIL-18BP (receptor binding protein). All the drugs used in this experiment were purchased from R&D Systems, USA.

Determining the effects of treatment with IL-18-related drugs on parasitaemia development and mortality rate in malarial animals

To determine the effects of treatment of malarial mice with IL-18-related drugs on the course of malaria infection, i.e the parasitaemia development and mortality rate, mice in group I-V as described above will be monitored throughout the infection. Parasitaemia will be measured daily and mortality will be recorded.

Determining the effects of treatment of malarial mice with IL-18-related drugs on the release of pro- and anti-inflammatory cytokines

To determine the effects on the release of circulating pro- and anti-inflammatory cytokines when malarial mice were treated with IL-18-related drugs, blood samples for plasma were collected from the mice in group I-V on day 1, 3 and 5 following inoculation and treatment. The blood was collected from the animals through cardiac puncture while they were under terminal general anaesthesia by inhalation of diethyl ether. Plasma was prepared from the blood through centrifugation and the concentrations of TNFα, IFNγ, IL-1α, IL-6 and IL-10 were measured using commercially available ELISA kit purchased from either The Medical & Biological Laboratory Co. Ltd (MBL), Japan or R&D Systems, USA.

Statistical analysis

Statistical analysis of the data obtained in this study was performed by one-way ANOVA followed by Tukey as a single post hoc test. P value <0.05 was taken as statistically significant. Correlation between plasma IL-18 concentrations and percentage parasitaemia development were determined using linear regression analysis of Spearman's rank-order correlation coefficient.

RESULTS

Plasma IL-18 concentrations in malarial mice and correlation with parasitaemia development

IL-18 concentrations in the plasma of malarial mice were found to be significantly elevated throughout the infection as compared to the control group (Fig. 1). IL-18 concentrations were significantly increased starting from day 2 (18.0 ± 5.0 ng/ml plasma vs 9.5 ± 1.3 ng/ml plasma, P<0.001). The concentrations were further elevated on day 3 (40.0 ± 6.0 ng/ml plasma vs 8.5 ± 0.9 ng/ml plasma, P<0.0001), day 4 (55.0 ± 8.0 ng/ml plasma vs 9.0 ± 1.0 ng/ml plasma, P<0.0001) and day 5 (57.1 ± 6.0 ng/ml plasma vs 9.0 ± 0.8 ng/ml plasma, P<0.0001). Day 1 of the infection did not show any significant increase in the plasma IL-18 concentration (10.5 ± 2.5 ng/ml plasma vs 10.0 ± 1.5 ng/ml plasma, P>0.05). Day 6 measurement of IL-18 level was unable to be recorded as most of the infected mice died before blood collection.
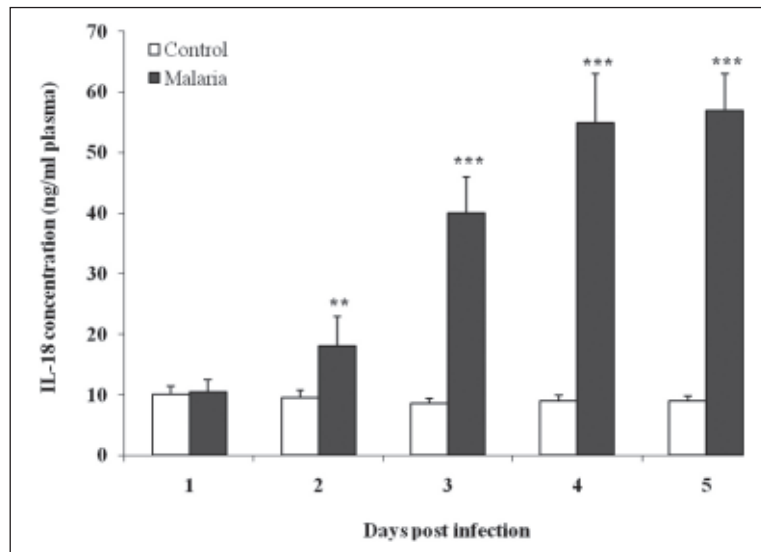
Figure 1. Plasma IL-18 concentrations measured in the control and malaria-infected mice. Results are expressed as the mean ± s.e.m (N=8). Circulating plasma IL-18 concentration were significantly elevated in malaria-infected mice throughout the infection period with a gradual increase in pattern was observed. ** P<0.001, ***P<0.0001: significant differences observed between the control and malaria-infected groups

Linear regression analysis performed on the circulating plasma IL-18 concentrations versus the percentage development of parasitaemia throughout the infection revealed that there was a significant positive correlation (r^2=0.7, P<0.001) observed between the two parameters (Fig. 2), in which the higher the degree of parasitaemia, the higher the concentration of IL-18 being released into the systemic circulation.

IL-18 expression in local organ tissues
Western blot analysis on five major organs tissues showed that IL-18 was significantly expressed in the spleen, liver and brain of the infected mice but not in the lungs and kidneys (Fig. 3). The spleen and brain showed an increasing pattern of IL-18 expression throughout the infection. Expression of IL-18 in the liver was only obvious on day 2 of infection.

Effects of treatment with IL-18-related drugs on parasitaemia development and mortality rate of malaria-infected mice
Observation on the parasitaemia development in malarial mice during treatment with IL-18-related drugs showed that inhibition of IL-18 production using rmIL-18BP in malarial mice significantly decreased the parasitaemia development as compared to malarial mice receiving normal saline (Fig. 4). This significant inhibition was observed starting from day 2 until day 5 following infection and treatment. Similar observations also occurred in the malarial mice treated with AmIL-18 that neutralizes the circulating release of IL-18. On the contrary, treatment of malarial mice with rmIL-18 which augmented the level of circulating IL-18 resulted in a further significant increase in parasitaemia development as compared to saline treated malarial mice (Fig. 4).

Earlier mortality and shorter survival time were recorded in malarial mice treated with rmIL-18, suggesting that augmenting the levels of circulating IL-18 contribute towards faster death in malarial mice (Table 1). In malarial mice treated with rmIL-18, mortality was recorded as early as day 4 post infection with 25% of the mice succumbed to the infection, which is comparable to the malarial mice treated with normal saline that recorded
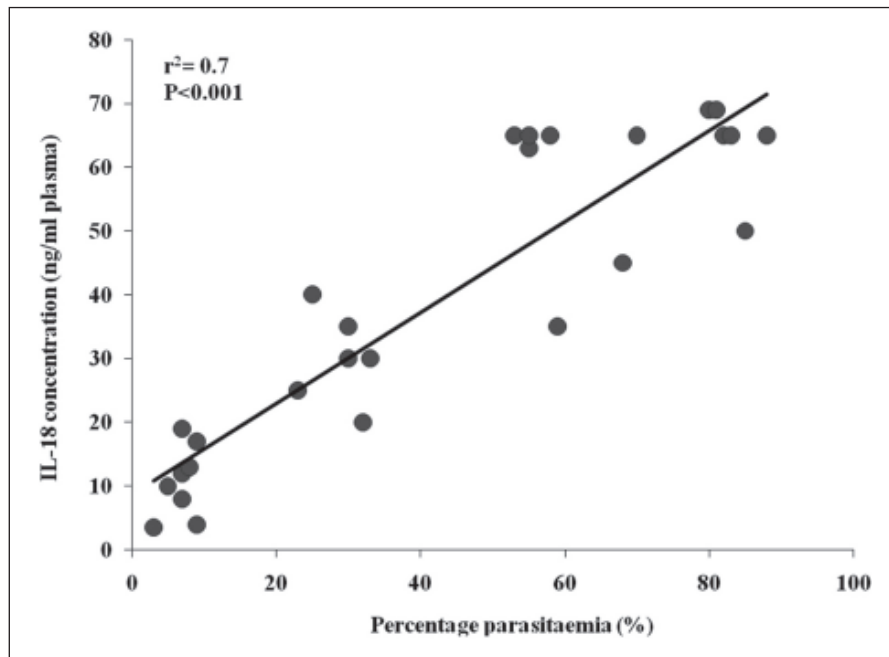
Figure 2. Scatter plot showing positive correlation between IL-18 plasma concentrations and the percentage of parasitaemia development in malaria-infected mice throughout the infection period (r^2 = 0.7, P<0.001). Coefficient correlation was calculated using Spearman's rank test
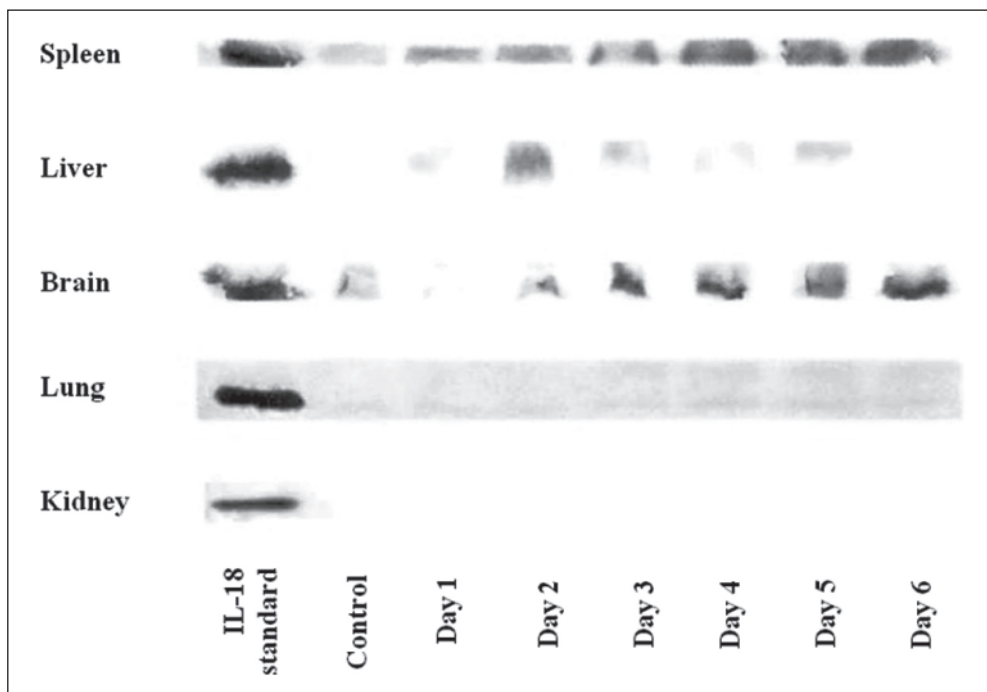

Figure 3. IL-18 expression determined in major organs (spleen, liver, brain, lung and kidney) of control and malaria-infected mice using western blot analysis. Recombinant IL-18 was used as standard. Day 1-6 denotes the malaria-infected mice on each day post inoculation with *P. berghei*
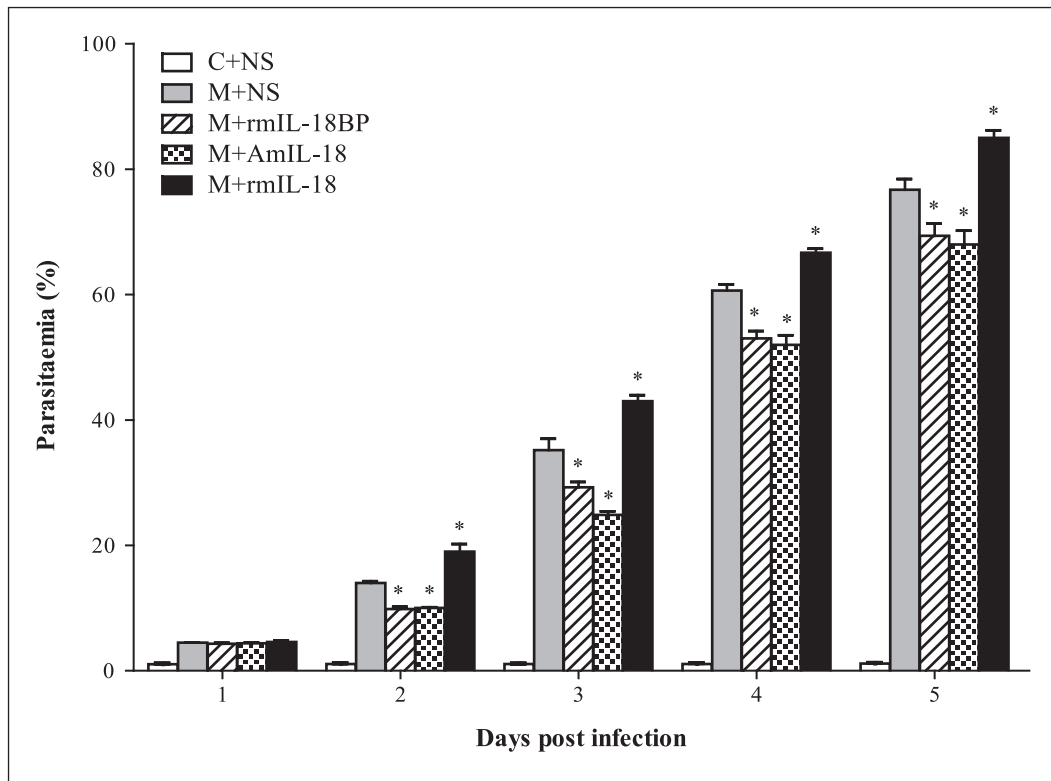
Figure 4. Percentage of parasitaemia development in the control and malarial mice treated with either 0.9% normal saline, rmIL-18BP, AmIL-18 or rmIL-18. Results are mean ± s.e.m of N=8. There was a significant decrease in parasitaemia development when IL-18 production was inhibited or neutralized during the infection. Increasing the circulating concentration of IL-18 resulted in a faster development of parasitaemia. *P<0.05: significant differences measured between malarial group treated with normal saline and malarial group treated with the IL-18-related drugs

Table 1. Percentage mortality recorded in the control and malarial mice treated with either normal saline, rmIL-18BP, AmIL-18 or rmIL-18

Days post infection	Percentage Mortality (%)				
	C+NS	M+NS	M+rmIL-18BP	M+AmIL-18	M+rmIL-18
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	25
5	0	17	17	17	100
6	0	100	100	100	100

Keynotes: C+NS = Control + Normal saline; M+NS = Malaria + Normal saline; M+rmIL-18BP = Malaria + Recombinant mice IL-18 binding protein; M+AmIL-18 = Malaria + Anti-IL-18 monoclonal antibody; M+rmIL-18 = Malaria + recombinant mice IL-18.

zero mortality on day 4. By day 5, 100% mortality was recorded in the rmIL-18-treated malarial group whereas saline-treated malarial group only showed 17% mortality on day 5. A hundred percent mortality in the saline-treated malarial group was only recorded on day 6 post infection. Inhibition and neutralization of IL-18 during the infection however, did not produce any significant effects on the mortality rate of the malarial mice. Both groups showed similar mortality rate with the saline-treated malarial group.

The effects of modulating IL-18 production on the plasma levels of pro- and anti-inflammatory cytokines in malaria-infected mice

Results from this study demonstrated that all the cytokines being investigated (TNFα, IFNγ, IL-1α, IL-6 and IL-10) were significantly elevated in the malarial mice throughout the infection as compared to the control group (Fig. 5 (a-e)). These were recorded in the malarial group treated with 0.9% normal saline (M+NS) as compared to the control group which was inoculated with normal red blood cells and treated with 0.9% normal saline (C+NS). All the cytokines were significantly elevated starting from day 1 post infection until the end of the experimental period on day 5. Increasing patterns of elevation were observed for all the cytokines during the infection.

Results in this study also showed that modulation of IL-18 production with IL-18-related drugs produced a significant impact on the production of TNFα, IFNγ, IL-1α, IL-6 and IL-10. Neutralization and inhibition of IL-18 by treatment with AmIL-18 and rmIL-18BP respectively, resulted in significant reductions in the elevated levels of the pro-inflammatory cytokines TNFα, IFNγ, IL-1α and IL-6 (Fig 5a, 5b, 5c and 5d respectively). Among the pro-inflammatory cytokines, the release of TNFα was the most strongly reduced to almost nearly the baseline levels (more or less similar to the levels recorded in the control mice treated with saline (Fig. 5a). In contrast, treatment of the malarial mice with rmIL-18 resulted in opposite effects. A further significant increase in the production

of the pro-inflammatory cytokines was observed. For IFNγ, the significant elevation in the plasma levels of malarial mice were recorded starting from day 1 through until day 5 post infection (Fig. 3b), whereas for TNFα, IL-1α and IL-6, the further significant elevation occurred on day 3 until day 5, but not on day 1 post infection (Fig. 5a, 5c and 5d respectively).

Observations on the anti-inflammatory cytokines IL-10 however showed contrasting effects from the pro-inflammatory cytokines upon modulation of the IL-18 production (Fig. 5e). Neutralization and inhibition of IL-18 with AmIL-18 and rmIL-18BP treatments respectively, resulted in a significant increase in IL-10 release in the systemic circulation of malarial mice on day 3 and day 5 post infection, whereas treatment with rmIL-18 significantly reduced the elevated levels of IL-10 during the infection.

DISCUSSIONS

The outcomes from the present study demonstrated the significant involvement of IL-18 in malaria infection with its circulating plasma levels significantly elevated among the malaria-infected mice throughout the infection especially towards the later critical stages of the infection. Significant expressions of IL-18 were also detected locally in the brain and spleen tissues with increasing pattern of expression observed from the early until late phase of infection. In the liver tissue, IL-18 expression was only significantly observed on day 2 post infection. Perhaps, the most plausible explanation for the observation on IL-18 expression in the liver was probably after day 2 following infection, the parasite shizonts ruptured and all the parasites left the liver and starts invading the erythrocytes. And that also explain why circulating plasma IL-18 concentrations were only significant starting from day 2 post infection and not on day 1.

A significant positive correlation was also observed between the elevated plasma IL-18 concentration and the percentage parasitaemia development, which at this stage may reflect IL-18 involvement in either
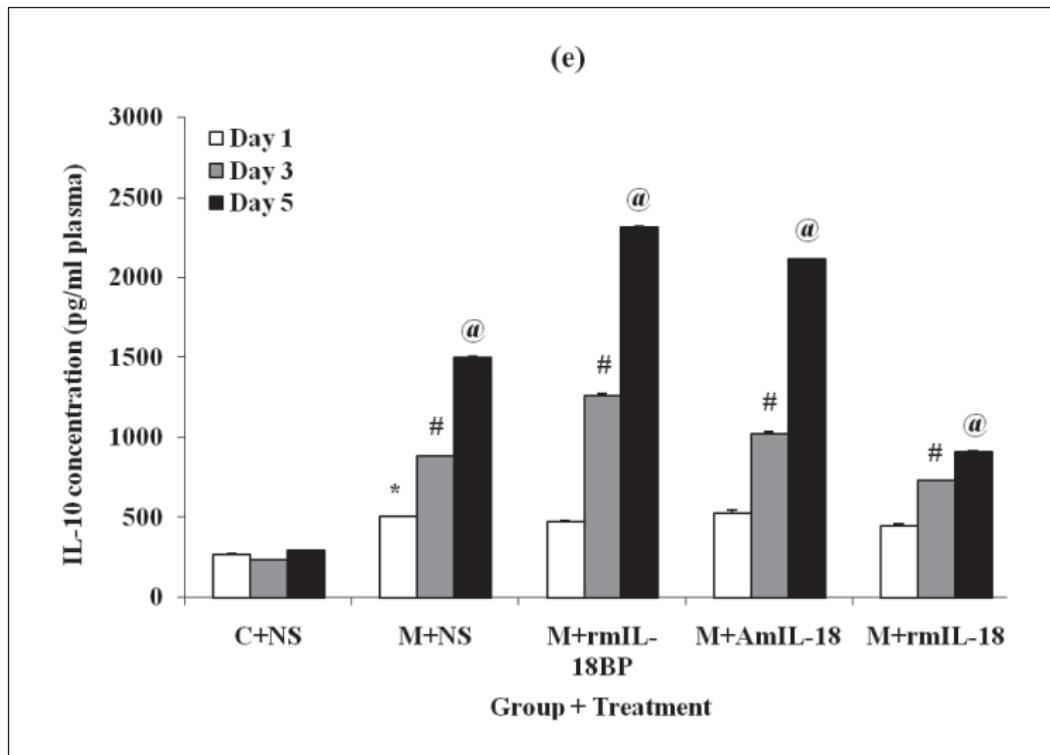
(a)

(b)

(c)

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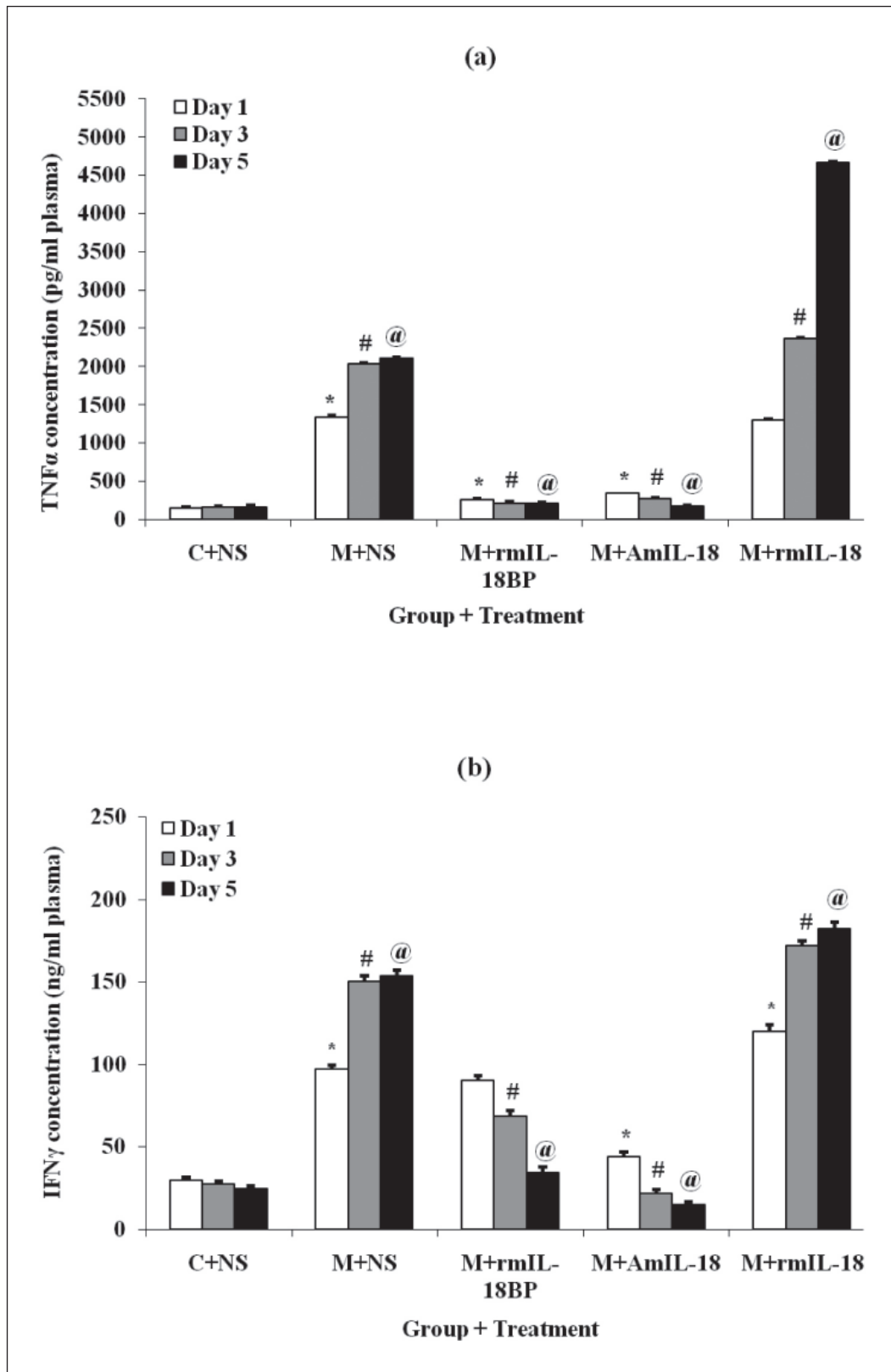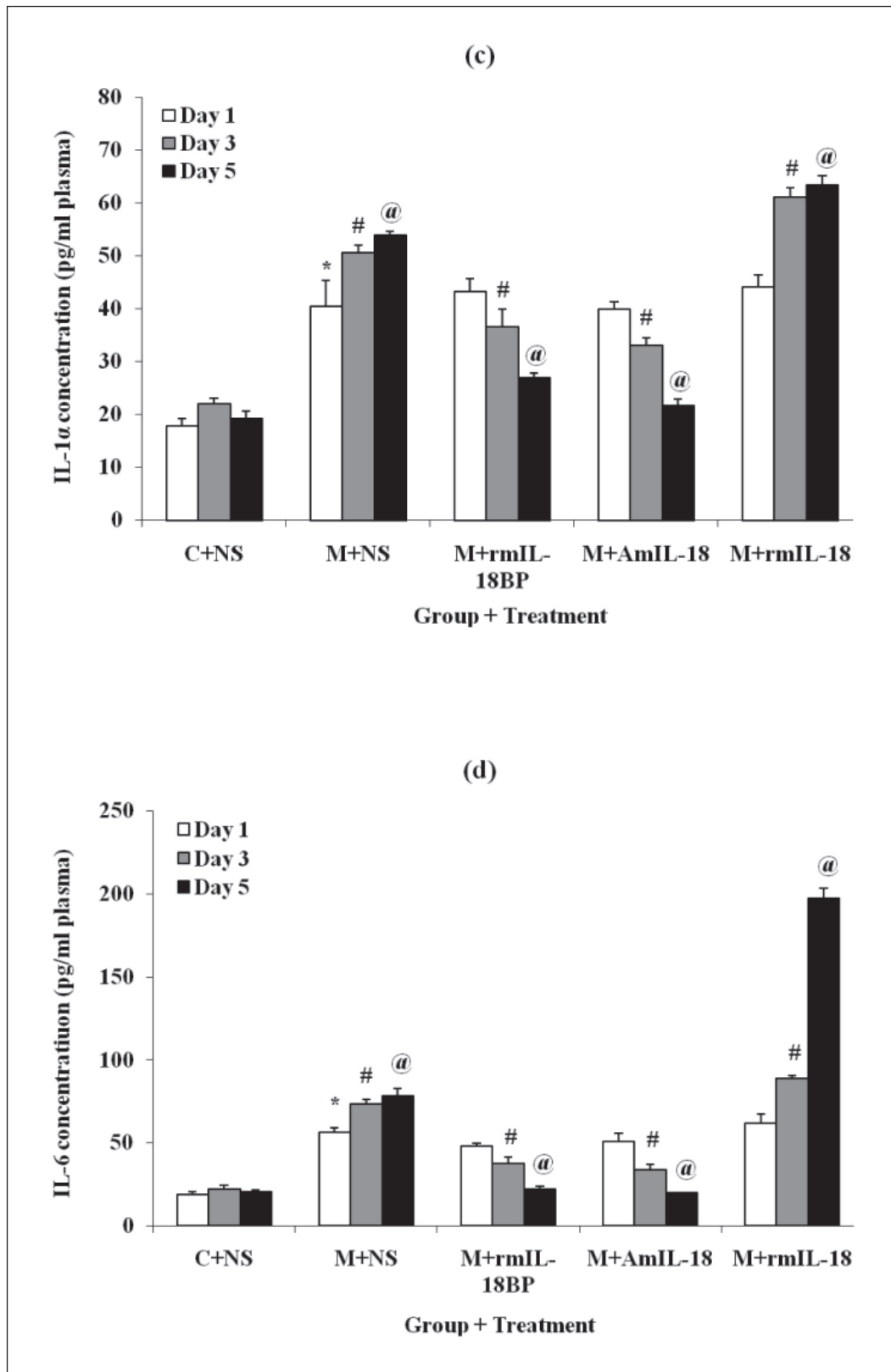
Figure 5 (a-e). Plasma concentrations of TNF (a), IFN (b), IL-1 (c), IL-6 (d) and IL-10 (e) in the control and malarial mice treated with either 0.9% normal saline, rmIL-18BP, AmIL-18 or rmIL-18. Results are expressed as mean ± s.e.m. Inhibition and neutralization of IL-18 resulted in the decrease of pro-inflammatory cytokines (TNFα, IFNγ, IL-α and IL-6) and an increase in anti-inflammatory cytokine (IL-10) plasma concentrations. In the contrary, augmenting the levels of circulating IL-18 caused an increase in pro-inflammatory cytokines and decrease in anti-inflammatory cytokine release. *, #, @ denote the significant differences (P<0.05) between the M+NS group and M + (rmIL-18BP or AmIL-18 or rmIl-18) on day 1, 3 & 5 respectively after the initiation of malaria infection.

Keynotes: C+NS = Control + Normal saline; M+NS = Malaria + Normal saline; M+rmIL-18BP = Malaria + Recombinant mice IL-18 binding protein; M+AmIL-18 = Malaria + Anti-IL-18 monoclonal antibody; M+rmIL-18 = Malaria + recombinant mice IL-18

the protective immunity against the parasite or it may be involved in mediating the disease severity. Our further investigation on the effect of treatment with IL-18-related drugs in malarial mice then demonstrated that augmenting the circulating levels of IL-18 by treatment with rmIL-18 resulted in an earlier mortality and shorter survival time of the malarial mice. This is in contrast with an earlier finding that suggested a protective role of IL-18 in which malarial mice that received anti-IL-18 treatment exhibited shorter survival times (Okamura *et al.*, 1998). Our result at this stage, clearly indicated that IL-18 may well be involved in mediating

the severity of the infection which is also reflected by the positive relationship between IL-18 levels and parasitaemia development in which higher circulating levels of IL-18 correlated with higher degree of parasitaemia.

We further our investigation by looking into the effects of treatment of malarial mice with IL-18-related drugs on the release of pro-inflammatory cytokines TNFα, IFNγ, IL-1α and IL-6, and the anti-inflammatory cytokine, IL-10 during the infection. The aim was to determine whether modulation of IL-18 production during the infection would produce any significant impact on the release

of these major cytokines which are known to be involved during immune response against malaria. Results from our study demonstrated that malaria infection caused significant elevation of all the pro-inflammatory cytokines (TNFα, IFNγ, IL-1α and IL-6) and also the anti-inflammatory cytokine IL-10 throughout the infection, which was in line with many previous observations (Grau *et al.*, 1989; Kwiatkowski *et al.*, 1990; Clark & Rockett, 1994). Modulation of IL-18 production using IL-18 related drugs during the infection also showed significant effects on the release and production of all the cytokines. Inhibition and neutralization of IL-18 production in the malaria-infected animals significantly decreased the release of all the pro-inflammatory cytokines (TNFα, IFNγ, IL-1α and IL-6) in the systemic circulation, and at the same time the anti-inflammatory cytokine, IL-10 release was significantly increased. When the already elevated level of IL-18 was further augmented by a challenge with rmIL-18 during the infection, a further significant increase in the pro-inflammatory cytokines release was observed whereas the anti-inflammatory cytokine decreased significantly. These results proved that IL-18 was an inducer of pro-inflammatory cytokines (TNFα, IFNγ, IL-1α and IL-6) production, but at the same time it limited the release of anti-inflammatory cytokine, IL-10 during its activity. This modulatory effect of IL-18 on the pattern of cytokines release during the infection may suggest IL-18 crucial role(s) in mediating the severity of the disease.

Cytokines production during malaria infection has well been documented both in human (Grau *et al.*, 1988; Clark *et al.*, 1991) and mouse (Grau *et al.*, 1987). TNFα, IL-1α, IL-6, IL-10 and IFNγ have been found to be elevated during *P. berghei* ANKA infection in CBA/Ca mice (Grau *et al.*, 1987; Kossodo & Grau, 1993). TNFα, IFNγ, IL-1α, and IL-6 are pro-inflammatory cytokines produced by Th1 cells and evidence has indicated that their systemic release were detrimental to the host and has been linked to the severe pathogenesis and immunopathological reactions associated with the infection (Kossodo & Grau, 1993). Induction of these

pro-inflammatory cytokines from the macrophages during shizont rupture was thought to be the initial event in malaria pathology (Clark *et al.*, 1981). Toxin products from the parasites provide the essential trigger for the pro-inflammatory cytokines release and initiation of immune response during the infection. The sequestration phenomenon in major organs was one of the pathological conditions closely associated with pro-inflammatory cytokines secretion by host cells after activation by parasite-derived toxins (Mannel & Grau, 1997). Whilst the early release of TNFα and IFNγ were thought to be crucial in the control of parasitaemia development during malaria (Kremsner *et al.*, 1995), and furthermore pro-inflammatory cytokines are appreciated as a key importance in host defence against infection (Dinarello, 1992; Cunnane *et al.*, 2003), overproduction of these cytokines during the late stages of infection may lead to over-reactive immune response that contributed towards the severe pathology of malaria. We have shown in this study that treatment with rmIL-18 which increased further the circulating levels of TNFα and IFNγ was parallel with the higher degree of parasitaemia and earlier mortality of the malarial mice, whereas inhibition of their release through IL-18 inhibition produced the opposite outcome. These findings associating further the pro-inflammatory cytokines such as TNFα and IFNγ with malaria severity. A well-balanced immune response is thought to be critical in the disease outcome with minimal pro-inflammatory cytokines release at the early stages of infection may be beneficial in limiting parasiteamia development, but excessive release towards the later stages of infection can lead to exacerbation of immune response with fatal consequences. Results from this study have also proven that IL-18 plays a pro-inflammatory role during malaria infection through the elevation of the above said cytokines. It may well play a protective role in murine malaria by enhancing IFNγ production as suggested by a previous study (Singh *et al.*, 2002), but excessive release of IFNγ and other pro-inflammatory cytokines towards the later

stages of infection may proved to be detrimental to the host and contributed towards severe pathology.

Anti-inflammatory cytokine like IL-10 is produced by Th2 typed cells, B cells and macrophages and is a powerful regulator of the inflammatory response (Howard & O'Garra, 1992). Its anti-inflammatory action is believed to be mediated via its inhibitory actions on IL-12 (D'Andrea *et al.*, 1993). IL-10 is able to suppress the production of Th1 cytokines (Moore *et al.*, 1993) through indirect inhibition of the antigen presenting function of macrophages (Bogdan *et al.*, 1991). IL-10 production is also thought to be stimulated by TNFα and the resulting IL-10 released in turn exerts a negative feedback on TNFα production (Fiorentino *et al.*, 1991). The involvement of IL-10 in malaria infection in mice has been implicated since IL-10 deficient mice infected with *P. chabaudi chabaudi* showed a high mortality rate and increased IFNγ response, which indicates that IL-10 contributes towards protective responses during the infection (Linke *et al.*, 1996). Another study in murine malaria suggested that parasites clearance requires an early release of IFNγ which is a type 1 immune response, followed by IL-10 response which is a type 2 for complete removal of the parasites (Taylor-Robinson, 1995). Whilst parasites clearance depends on both IL-10 and IFNγ release, severe pathology of malaria was associated with an exaggerated type 1 response (Ho *et al.*, 1995), which implicates that pro-inflammatory cytokine like IFNγ contributes to the ensuing pathology of malaria, while anti-inflammatory cytokine plays protective and counter-regulatory roles, which is a key to survival. In human malaria, IL-10 was able to counter-regulates the pro-inflammatory response induced by *P. falciparum* and in patient with severe *falciparum* malaria, this host-protective mechanism might be deficient (Ho *et al.*, 1998). Studies of IL-10 in severe human malaria particularly cerebral malaria suggested the restraining role of IL-10 in the immunopathological processes leading to severe pathology (Kossodo *et al.*, 1997; Day *et al.*, 1999), possibly through its ability to inhibit the production of pro-inflammatory cytokines and several chemokines (Moore *et al.*, 2001) which plays essential role in many complications of cerebral malaria (Grau *et al.*, 1989; Kwiatkowski *et al.*, 1990; Clark & Rockett, 1994). In the present study, we found that IL-18 release during malaria infection somehow limits the capacity of the malarial mice to produce IL-10 even though the concentration of circulating IL-10 in the plasma was still significantly higher as compared to control uninfected mice. This is proven when IL-18 was inhibited, a further increased in IL-10 levels was observed and the levels were decreased when IL-18 concentrations were augmented in the circulation. This suggests the regulatory role of IL-18 on anti-inflammatory cytokine like IL-10 by limiting their release during the infection.

Since immunopathogenic processes involving pro-inflammatory cytokines release have been widely recognized as having a central role in malaria, main focus and objective should now be directed at modifying these cytokines production. Agents that possess this property could well become a useful adjunctive therapeutic in reducing the mortality and morbidity associated with malaria. IL-18 was chosen to be investigated in this study due to its unique Th1 pathway inducing capacity and could serve as a marker for type 1 immune response in malaria infection. We have demonstrated in our findings that IL-18 modulation produced a positive impact on the mortality, parasitaemia development and cytokines release in malarial mice, in which suppression of IL-18 activity helped controlled the parasites growth and development, reduced the release of pro-inflammatory cytokines and increased the circulating anti-inflammatory cytokine. Augmenting the release of IL-18 during the infection on the other hand, shortened the survival time of infected mice, promoted faster parasitaemia development, further increased the release of pro-inflammatory cytokines and inhibited anti-inflammatory cytokine. These intriguing findings may suggest that IL-18 could be a promising target for pharmacotherapeutic strategy in malaria

treatment in which the host may benefit from IL-18 inhibition. It may also serve as an important additional therapeutic approach in endemic areas of malaria that has shown resistance towards the conventional antimalarial therapy.

In conclusion, this study has provided strong evidence on the crucial role and involvement of IL-18 in mediating the severity of malaria infection possibly through the induction of pro-inflammatory cytokines such as TNFα, IFNγ, IL-1α and IL-6 and at the same time limiting the release of anti-inflammatory cytokine, IL-10. The positive impact on the pattern of cytokines release upon IL-18 modulation during the infection suggests its close association with the severity of the disease and may serve as a potential marker for possible development of immunotherapeutic agent in malaria therapy. Targeting IL-18 in malaria therapy may prove to be beneficial to the host since the outcome from this study has demonstrated that suppressing IL-18 production can increase the concentration of anti-inflammatory cytokine IL-10 which was thought to exert protective effects during the infection. Increasing its circulating concentrations during the infection has also been proven in this study to further increase the release of pro-inflammatory cytokines that can contribute towards severe malaria pathology. Our overall findings demonstrated close relationship of IL-18 with malaria severity through a pathway of increasing parasite development and pro-inflammatory cytokines release. Its release during the infection is therefore proven to be detrimental to the host and immunopharmacological therapy aiming at inhibiting and suppressing IL-18 bioactivity may prove beneficial in malaria treatment.

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