

Full Length Research Paper

# Cytokine levels connected with experimental trial of malaria pathology during *Plasmodium berghei* ANKA disease in a mouse

Jomo V. Awori

Department of Anatomy and Cell Biology, School of Medicine, University of Nairobi, Nairobi, Kenya.  
Email: awori.jomo3@yahoo.co.uk

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Successful recovery from malaria involves striking a balance between counteracting cytokines. The cytokine imbalance contributes to pathological features, but their exact levels have not been elucidated. This study aimed at investigating the role played by circulating cytokines in pathophysiology of cerebral malaria using experimental cerebral malaria (ECM) model by profiling four serum cytokines using cytometric bead array. 72 BALB/c mice were intraperitoneally inoculated with 0.2 ml  $1 \times 10^4$  parasitized red blood cells at day 0 and randomized into six groups (six mice/group). The mice were sacrificed at day 4, 6, 8, 11 and 20 post-infection. Significantly higher systemic levels ( $P < 0.05$ ) of interferon-gamma (IFN- $\gamma$ ) were observed between day 8 and 20 post inoculation (p.i), while tumor necrosis factor-alpha (TNF- $\alpha$ ) levels were significant at days 4, 8, 11, 14 and 20, respectively in BALB/c mice that survived until day 20 post-infection with a higher parasitemia (up to  $52.6\% \pm 0.8$ ). Significant concentrations ( $P < 0.05$ ) of interleukin-4 (IL-4) were observed between day 4 and 8. IL-5 levels had significant differences at days 11 and 20 p.i. T-cell pathology was revealed by fragmentation of whole genomic DNA during the infection which coincided with elevated IFN- $\gamma$  and TNF- $\alpha$  responses further accelerating the severity of cerebral malaria (CM). This study has demonstrated the correlation between T-lymphocyte pathology and elevated levels of T-helper 1 (Th1) cytokines concentrations in the brain and spleen.

**Key words:** Experimental malaria, *Plasmodium berghei*, pathology, cytokine levels, fragmentation.

## INTRODUCTION

The murine model of malaria is an immunophysiological process caused by *Plasmodium berghei* ANKA in rodents. Experimental malaria models have been developed to investigate the pathogenesis of malaria, because they exhibit similar characteristics to malaria (Desruisseaux et al., 2008), nevertheless, the fundamental and obvious pathological difference with the human cerebral malaria is the marked accumulation of leukocytes and platelets in the

venules and capillaries of the brain in the murine model instead of parasitized red blood cell (RBC) sequestration (White et al., 2010). Consequently, the hallmark of the murine model is the cytopathological evidence of inflammation which is conspicuously absent in the human cerebral malaria (CM) (White et al., 2010). CM seen in about 7% of *Plasmodium falciparum* malaria cases is characterized by neurological features, especially impaired

consciousness (Maitland and Newton, 2005). CM is associated with sequestration of parasitized red blood cells (pRBCs) in the brain microvasculature and secretion of toxic molecules by parasites, as well as inflammatory components of the host immune response, including secretion of cytokines and recruitment of activated leukocytes to the brain (Amante et al., 2010). During malaria, the successful resolution of infection is mediated by the early secretion of pro-inflammatory T-helper 1 (Th1) cytokines that promote parasite killing by macrophages and thereafter to prevent immune-mediated damage (Desruisseaux et al., 2008). Cytokine imbalance contributes to pathological features such as activation of endothelial adhesion molecules and cellular activation (Takahiko et al., 2011). Despite extensive research, the precise mechanisms leading to pathogenesis during cerebral malaria are not fully understood. Laboratory models are important to elucidate the immunological mechanisms involved in CM and the way of alleviating this serious condition, because of the difficulty in following up human cases with CM and the limited possibility to examine its pathological process.

The study aims at establishing the association between specific cytokines and apoptosis of brain migrating lymphocyte in the induction pathology of CM.

## MATERIALS AND METHODS

### Animals, parasite and infection

Male BALB/c mice (8±10 weeks old) were purchased from Institute of Livestock Research (ILRI) Kenya. The mice were maintained in the Kenya Medical Research Institute (KEMRI) Animal Facility and supplied with food and water *ad libitum*. Mice were infected intraperitoneally (i.p.) with 0.2 ml 10<sup>4</sup> parasitized RBC obtained from a homologous passage mouse that had been infected with *P. berghei* ANKA (reference line 676m1c11). Control mice were injected i.p. with the same volume of sterile normal saline. The infected mice were randomized into six groups of 6 (in separate cages), each group corresponding to selected day post-infection when cytokine levels were determined. Each of the six groups received two control mice from each set of the control groups. Two parallel experiments (n=2) were modeled on this setup. For parasitemia monitoring, a Giemsa-stained thin smear was made daily from tail blood of each mouse. Mice were judged to have CM only if they displayed behavioral (using SHIRPA box plot [unpublished data]) neurological signs (convulsion, seizures, reduced locomotion, and/or paralysis and coma) and became moribund within the first 2 weeks of infection (6 to 14 days post inoculation [p.i.]). Only mice with experimental cerebral malaria (ECM) features were used for cytokine assays.

### Serum cytokine assays

Infected BALB/c mice were monitored daily until parasitemia exceeded 50%. Blood was collected by heart puncture and non-heparinized blood was pooled from three to five mice at once. Serum was obtained by centrifuging blood at 1,000 g for 10 min. Cytokine profiles were analyzed from the serum samples by cytometric bead array (CBA) according to the manufacturer's

instruction (BD Pharmingen®, 2010). Mouse cytokine kit bearing sensitivities for interferon-gamma (IFN-γ), interleukin (IL-4), IL-5 and tumor necrosis factor-alpha (TNF-α) representing the Th1 and T-helper 2 (Th2) cytokines was used. Reconstituting the standards for the mouse, Th1/Th2 cytokine assay was done according to the manufacturer's instructions (BD Pharmingen®, 2010).

### Analysis of cytokine levels

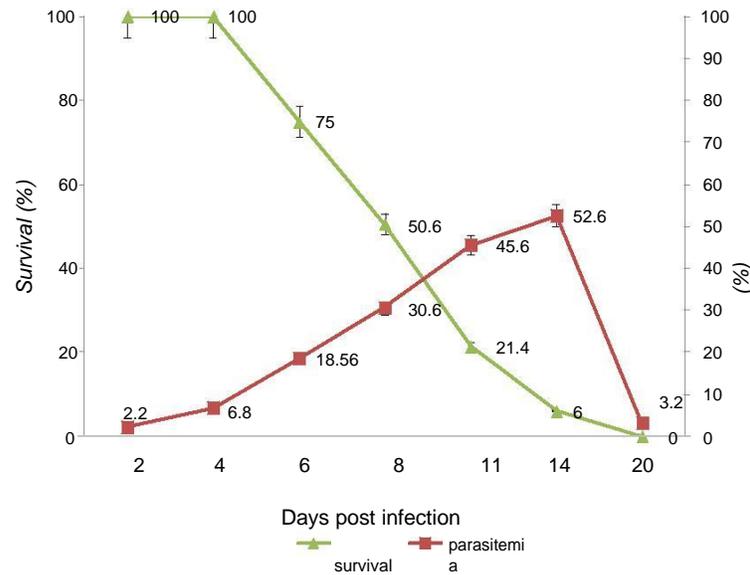
Analysis was done using CELLQUEST (3.3) software (BD Pharmingen®, 2010). The FACSCalibur flow cytometer (BD Pharmingen®) was calibrated with setup beads and the samples acquired. Cytokine concentration was indicated by its fluorescent intensities and then computed using the standard reference curve of CELLQUEST software. Cytokine levels for IFN-γ, TNF-α, IL-4 and IL-5 were analyzed. FCAP array software (BD Pharmingen®, 2010) was used for the analysis.

### Isolation of T-cells from mouse brain and spleen tissues

Briefly, whole brain and spleen tissues from individual infected mice obtained on days 4, 6, 8, 11, 14 and 20 post infection were perfused in 15 ml of 2% Fetal Calf Serum-Hanks Buffered Saline Solution (FCS-HBSS) in sterile petri dish by teasing them until they turned pale in colour according to the methods of Sébastien et al. (2004). Briefly, the pieces were aspirated using a syringe to break up the chunks. Each tissue suspension was transferred into a 50 ml Falcon tube (BD) and was centrifuged at 1500 g for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in 10 ml of collagenase diluted to 1 µg/µl in 2% FCS-HBSS. The tissue suspensions were incubated for 45 at 25°C min, and then centrifuged at 1500 g for 5 min at 25°C and the pellet was resuspended in 6 ml of 2% FCS-HBSS. To separate leukocytes from tissue debris, the cell suspensions were layered onto 10 ml of 30% (v/v) iso-osmotic Percoll gradient solution (Pharmacia®) diluted in 2% FCS-HBSS in 15 ml Falcon tubes and was centrifuged at 1500 g for 10 min at room temperature without brake. To confirm the presence of T cells, the upper layer cells were separated and stained with mouse anti-CD3 (Clone 145-2C 11) (BD, Biosciences) T-cell lineage marker, and then were washed with 2% FCS-HBSS and finally washed with LymphoPrep™ and centrifuged at 1500 g for 10 min. Using a sterile pipette, the supernatant was carefully removed without disturbing the pellet and was preserved at -80°C (Mallone et al., 2011).

DNA fragmentation was investigated using the whole genomic DNA extracted from spleen and brain lymphocytes since internucleosomal degradation of DNA is the biochemical hallmark of apoptosis. Genomic DNA was extracted from the isolated T-lymphocytes using a modification of the Chelex® DNA extraction method (Abdel-Muhsin et al., 2002). 200 µl cell suspension was placed into sterile 1.5 ml vial and 1 ml of 0.5% saponin in 1X phosphate buffered saline (PBS) was added. The vial was inverted several times and incubated at 4°C for 24 h. The filtrate was resuspended in 1 ml of 1X PBS and then was incubated for further 30 min at 4°C. The tube was centrifuged at 10,000 g for 30 s at room temperature. The filtrate was resuspended in 50 µl of 20% Chelex® and 100 µl of DNase water was added. The vial was heated for 10 min at 95°C in a heating block while vortexing at intervals of 3 min. The vial was centrifuged at 10,000 g for 2 min at room temperature. The filtrate was collected into a sterile vial (Eppendorf®) and centrifuged at 10,000 g for 2 min at room temperature. The supernatant which had the eluted DNA sample was collected into a sterile 1.5 ml vial (Abdel-Muhsin et al., 2002).

To demonstrate DNA cleavage, the eluted DNA samples were run on agarose gel and visualized on a ultraviolet (UV) light box.



**Figure 1.** Comparison of parasitemia levels (% mean $\pm$ SD) and survival incidence curve (% Mean $\pm$ SD) for BALB/c mice showing CM symptoms (n = 6). On day 14, only 6% of the mice were still alive and all the surviving mice died at day 20 post infection, respectively with a parasitemia of  $\leq 3.2\% \pm 0.5$  with few schizonts detectable on blood films. Some animals which were ill by day 6 did not encounter early death but appeared to display CM reversal whereby they seemed to have reversed their symptoms after 24 h and survived to day 20 p.i. Mice with these features died later at day 20 of severe anaemia and hyperparasitemia.

Agarose gel (1.0% w/v) was prepared by dissolving 1.0 g of agarose powder into 100 ml of 1X TBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 min to completely dissolve the powder. The cooled gel solution was poured in a casting tray having combs and was left for 10 min to gel (polymerize). Ethidium bromide (1x [3  $\mu$ l]) was incorporated before setting of the gel to facilitate visualisation of DNA under UV light. The DNA sample (5  $\mu$ l) was mixed with 3  $\mu$ l of loading dye (Bromophenol blue) and was loaded into the wells and subjected to electrophoresis at 80 V for 45 min. The gel was viewed on a UV light box to visualize the DNA ladder pattern (Sambrook and Gething, 1989). DNA samples (5  $\mu$ l) from non-infected healthy mice were run alongside as controls.

#### Ethical and biosafety considerations

Permission to carry out the study was obtained from Kenya Medical Research Institute (KEMRI) Scientific Committee and Ethical Review Committee (KEMRI SSC No 2217). The experiment was carried out in compliance with Animal Care and Use Committee (ACUC) Regulations of KEMRI. Standard Operating Procedures (SOPs) available at the malaria and immunology laboratories were followed in all experiments.

#### Statistical analysis

Readings falling below the detectable threshold ( $\leq 0$ ) were disregarded in the statistical analysis. Data was analyzed using EXCEL statistical software. The test and control groups were tested for homoscedasticity in the variances using the F-test and were

found to have statistically significant differences. Statistical analysis for the geometric means of cytokine concentrations were performed using un-paired student t-test assuming unequal variances to test significance between the means of the groups at 95% confidence level.

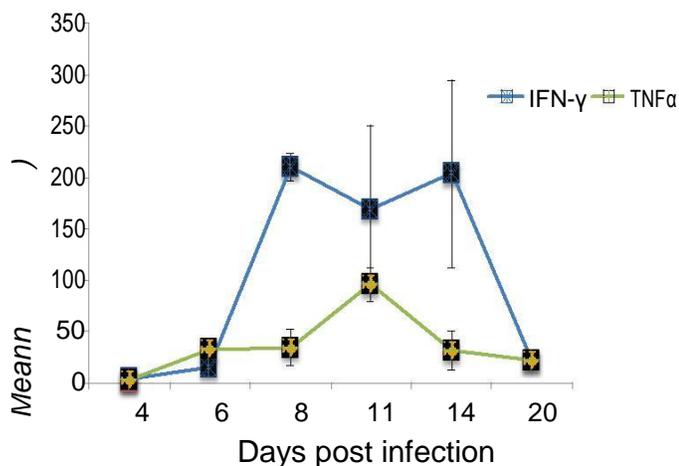
## RESULTS

### Parasitemia levels and survival rates

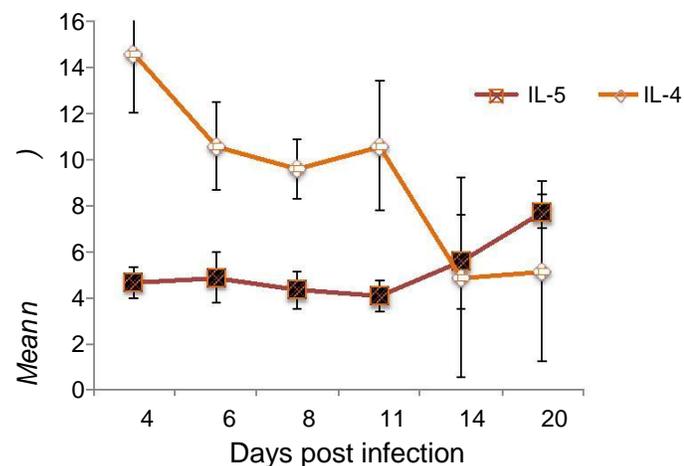
All the experimental mice developed a patent parasitemia by day 4 post-inoculation with gradual increase in parasitemia between day 0 and 4 and thereafter, the parasitemia rose rapidly reaching mean peak of  $52.6 \pm 0.8$  (mean $\pm$ standard deviation (SD)) at day 14 with only 6% of the mice surviving and dying at day 20 post infection due to overwhelming hyperparasitemia (Figure 1).

### IFN- $\gamma$ production

Analysis on IFN- $\gamma$  showed no significant difference ( $P > 0.05$ ) in IFN- $\gamma$  production at day 4 post infection between the test group (Mean $\pm$ SD,  $4.6 \pm 1.7$ ), and that of the control uninfected mice ( $2.3 \pm 0.5$ ) [ $P = 0.2$ ]. Similarly, no significant increase was registered at day 6 p.i ( $15.4 \pm 9.5$ ) as compared to the control uninfected mice ( $2.6 \pm 0.3$ ). Peak IFN- $\gamma$  levels were observed at day 8 post



**Figure 2.** Basal Th1 cytokine production trends during the course of infection with *Plasmodium berghei* ANKA. Error bars represent standard deviation of the mean.



**Figure 3.** Basal Th2 cytokine production trends during the course of infection with *Plasmodium berghei* ANKA. Error bars represent standard deviation of the mean.

infection (210.6±133.0) as compared to the control uninfected mice (2.2±0.5 [P=0.05]) followed by a drop in levels at day 11 post-infection (169.8±80.5) as compared to the control uninfected mice (2.3±0.3 [P=0.02], P<0.05). Conversely, there was a significant (P<0.05) upsurge at day 14 post-infection (203.6±91.6), while control uninfected mice (2.6±0.2 [P=0.02]) registered an insignificant increase (Table 1). There was a significant decline of the levels at day 20 post infection (Table 1) in the experimental group (22.0±3.5) as compared to the control uninfected mice (2.6±0.2 [P=0.01]). IFN-γ levels were the most dominant TH1 cytokine in circulation followed by TNF levels. An exponential upsurge in IFN-γ levels were detected between the 4<sup>th</sup> and 8<sup>th</sup> day and subsequently

decreased on the 11th day, although the concentration rose again on the 14th day, but decreased significantly (P<0.05) on the 20th day for the surviving mice (Figure 2).

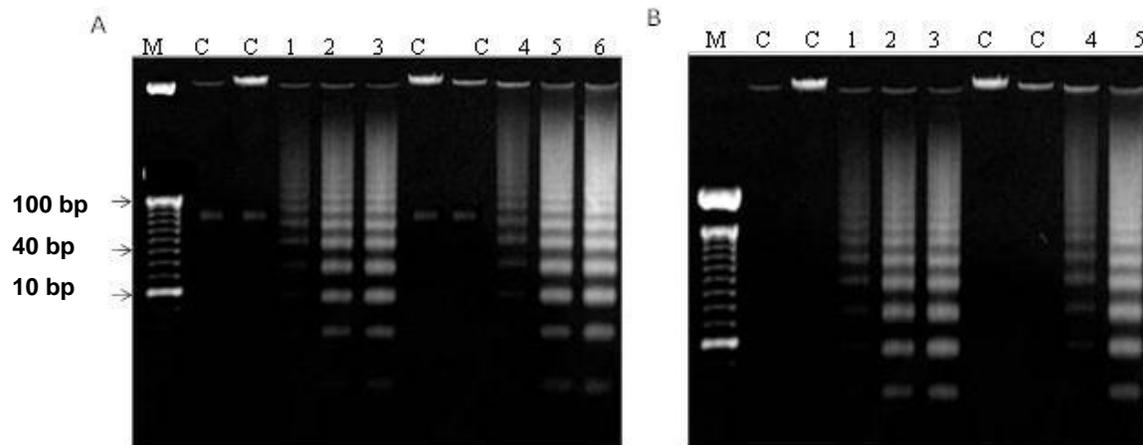
### TNF-α production

TNF-α for the test group at day 4 post infection (2.9±0.2), was significantly different as compared to that of the control uninfected mice (2.5±0.1 [P=0.04], P<0.05) (Table 2). Although TNF-α levels rose exponentially (Table 2) for the test group at day 6 post infection (32.2±7.6) as compared to the control uninfected group (2.5±0.3 [P=0.4]), this change was not statistically significant (P>0.05). The levels for the test group were significantly higher (33.9±17.5) as compared to the control uninfected mice (2.5±0.1 [P=0.04]) at day 8 post infection (P<0.05) (Table 2). The levels remained significantly higher (P<0.05) for the test group (95.5±17.0) as compared to the control uninfected group (2.2±0.2 [P=0.01]) at day 11 post infection. Conversely, although not statistically significant (P>0.05), the levels of the test group declined (31.6±19.1) while that of the control uninfected group was 2.6±0.2 [P=0.06] at day 14 post infection (Table 2). At day 20 post infection, the levels of the experimental group declined significantly (22.1±3.6, Table 2) as compared to the control uninfected group (2.6±0.2 [P=0.01], P<0.05). TNF-α levels were distant second to IFN-γ levels.

Between the 4 and 6th day, an exponential increment in TNF-α levels was detected indicating its early involvement in protection against the parasite and constantly increased until day 11. However, the production increased between the 6 and 8th day with peak production being detected on the 11th day, and subsequently decreased between the 14 and 20th day (Figure 2).

### Interleukin-4 production

IL-4 for the test group was significantly higher (14.6±2.5) as compared to that of the control uninfected mice (3.1±0.6 [P=0.04], P<0.05) at day 4 post infection (Table 3). The levels for the experimental group remained significantly elevated at day 6 p.i (10.6±1.9) as compared to control uninfected mice (2.6±0.2, P<0.05). There was a decline in the IL-4 levels for the test group (9.6±1.3) as compared to control uninfected mice (3.1±0.6, P<0.05) at day 8 post-infection (Table 3). There was an increase in the IL-4 levels in the test group at day 11 post infection (10.6±2.8) as compared to the control uninfected mice (2.9±0.5 [P=0.5]); though, no significant difference was observed (P≥0.5) (Table 3). The levels then declined (4.9±4.3) (Figure 3) while the control uninfected mice increased (2.5±0.2); although; no significant differences were observed (P>0.05), whereas at day 20 post infection, insignificant increment (Table 3) was registered



**Figure 4.** Cytokine induced DNA cleavage during infection with ECM. Agarose gel electrophoretic fraction of DNA was prepared in two independent experiments, A and B. In A, Lanes 1, 2, 3, 4, 5 and 6 represent DNA extracted from the spleen lymphocytes at days 8, 9 and 14 post infection, while Lane C shows splenic cellular DNA from uninfected control mice. Similarly, DNA cleavage was observed from brain tissue (B), lanes 1, 2, 3, 4 and 5 represent cellular DNA cleavage as observed on days 9, 12 and 20 post infection, while Lane C represents negative control from uninfected control mice. Lane 1 represents 100 bp DNA molecular marker in both cases.

( $5.1 \pm 3.9$ ) as compared to the control uninfected mice ( $2.7 \pm 0.3$ ,  $P > 0.05$ ). On the 4th day post infection, significant levels ( $P < 0.05$ ) of IL-4 were detected, while it tended to decrease latter, although on the 11th day the concentration rose again and subsequently decreased on the 14th day and thereafter it hit a bottom low till the 20<sup>th</sup> day (Figure 3).

### Interleukin-5 production

The difference in IL-5 levels for both the experimental group ( $4.5 \pm 0.7$ ) was compared to that of the control uninfected mice ( $7.8 \pm 0.8$ , [ $P = 0.06$ ],  $P > 0.05$ ) at day 4 post infection. Correspondingly, the levels of the test group ( $4.9 \pm 1.1$ ) and that of the control uninfected mice ( $5.8 \pm 1.1$  [ $P = 0.16$ ]) were not significantly different ( $P > 0.05$ ) at day 6 post infection (Table 4). Similarly, there was no significant difference for IL-5 levels ( $P > 0.05$ ), at day 8 post infection in the test group ( $4.3 \pm 0.8$ ) as compared to uninfected control group ( $5.9 \pm 0.9$  [ $P = 0.2$ ]).

The IL-5 levels in the test group were lower ( $4.1 \pm 0.7$ ) than the control uninfected group ( $5.8 \pm 1.4$  [ $P = 0.05$ ],  $P > 0.05$ ) at day 11 post infection. At day 14 post infection, no significant production levels was observed in the test group ( $5.6 \pm 2.0$ ) as compared to the control uninfected group ( $5.8 \pm 1.4$  [ $P = 0.9$ ],  $P > 0.05$ ). However, the levels rose significantly in the test group ( $3.4 \pm 1.6$ ) as compared to the control uninfected group ( $7.8 \pm 0.8$  [ $P = 0.01$ ],  $P < 0.05$ ) at day 20 post infection. IL-5 levels were the lowest across in all the intervals and there was a corresponding drop in IL-4 during the course of infection.

**Table 1.** IFN- $\gamma$  geometric mean levels (pg/ml) and standard deviations for the experimental and the control groups during the course of ECM infection.

Days post infection	IFN- $\gamma$	IFN- $\gamma$ (control)
4	$4.59 \pm 1.74^a$	$2.26 \pm 0.45^a$
6	$15.44 \pm 9.46^a$	$2.58 \pm 0.29^a$
8	$210.63 \pm 13.01^{a*}$	$2.24 \pm 0.47^b$
11	$169.76 \pm 80.48^{a*}$	$2.32 \pm 0.34^b$
14	$203.62 \pm 91.62^{a*}$	$2.59 \pm 0.17^b$
20	$22.05 \pm 3.51^{a*}$	$2.59 \pm 0.17^u$

\*Means within a row followed by a different letter are significantly different at  $P < 0.05$ .

IL-5 production was constant between the 4 and 11th day, although a rise was detected on the 6th day and subsequently increased between 11 to 20th day post infection (Figure 3).

### Cytokine induced T-cell Apoptosis

Fragmentation of the DNA was shown to be extensively induced in T cells from infected mice and was held to account for severity in pathology of malaria. On day 8 post infection when the levels of Th1 cytokines were at their optimum, concomitant DNA cleavage of T cells was detected with extensive apoptosis (lanes 1 to 6 in Figure 4). Apoptosis patterns of DNA cleavage were almost comparable at all intervals (Figure 4).

**Table 2.** Geometric mean (pg/ml) and standard deviations of TNF-α levels for the experimental and the control groups during the course of ECM infection.

Days post infection	TNF-α	TNF-α (control)
4	2.94±0.16 <sup>a*</sup>	2.40±0.08 <sup>b</sup>
6	32.24±7.59 <sup>a</sup>	2.46±0.32 <sup>a</sup>
8	33.93±17.48 <sup>a*</sup>	2.46±0.08 <sup>b</sup>
11	95.52±17.02 <sup>a*</sup>	2.24±0.18 <sup>b</sup>
14	31.55±19.15 <sup>a</sup>	2.59±0.17 <sup>a</sup>
20	22.05±3.51 <sup>da*</sup>	2.59±0.17 <sup>u</sup>

\*Means within a row followed by a different letter are significantly different at P<0.05.

**Table 3.** Geometric mean (pg/ml) and standard deviations of IL-4 levels for the experimental and the control groups during the course of ECM infection.

Days post infection	IL-4	IL-4 (control)
4	14.60±2.52 <sup>a*</sup>	3.11±0.60 <sup>b</sup>
6	10.60±1.90 <sup>a*</sup>	2.55±0.20 <sup>b</sup>
8	9.61±1.29 <sup>a*</sup>	3.11±0.60 <sup>b</sup>
11	10.60±2.83 <sup>a</sup>	2.88±0.52 <sup>a</sup>
14	4.88±4.32 <sup>a</sup>	2.55±0.20 <sup>a</sup>
20	5.15±3.92 <sup>da</sup>	2.71±0.25 <sup>da</sup>

\*Means within a row followed by a different letter are significantly different at P<0.05.

**Table 4.** Geometric mean (pg/ml) and standard deviations of IL-5 levels for the experimental and the control groups during the course of ECM infection.

Days post-infection	IL-5	IL-5 (control)
4	4.66±0.66 <sup>a</sup>	7.76±0.75 <sup>a</sup>
6	4.87±1.09 <sup>a</sup>	5.80±1.06 <sup>a</sup>
8	4.34±0.82 <sup>a</sup>	5.94±0.90 <sup>a</sup>
11	4.09±0.68 <sup>a*</sup>	5.95±0.89 <sup>b</sup>
14	5.58±2.05 <sup>a</sup>	5.80±1.06 <sup>a</sup>
20	7.76±0.75 <sup>da*</sup>	3.42±1.58 <sup>b</sup>

\*Means within a row followed by a different letter are significantly different at P<0.05.

**DISCUSSION**

In the present study, the development of experimental cerebral related complications was analyzed in BALB/c mice that are susceptible to ECM (Neill and Hunt, 1992). Although, several mechanisms have been proposed to explain CM immunopathogenesis, the precise mecha-

nisms is still unknown. Dysregulation of homeostasis of pro- and anti-inflammatory factors has been linked to the severity of CM (Sarfo et al., 2011). Upon infection with *P. berghei*, BALB/c mice exhibited varied parasitaemia responses to the infection which formed the basis for monitoring the cytokine concentrations depending on the survival rates. In this study, Th1 set of cytokines showed significantly different patterns across the course of infection as compared to their corresponding concentrations in the control groups. During the present study, parasite load estimations (Figure 1) was associated with high pro-inflammatory cytokines concentrations (IFN-γ and TNF-α) (Figure 2) suggesting that the parasites were critical for inducing dysregulated pro-inflammatory disease pathogenesis among other factors relating to similar studies on monkeys done by Belhoue et al. (2008). An exponential surge of IFN-γ and TNF-α for the test group was observed between day 6 and 14, respectively. IFN-γ levels in the test group were significantly different (P<0.05) between day 8 and 14 post infection, respectively.

The mice displayed an increased trend in IFN-γ and TNF-α (Figure 1, Tables 1 and 2) over the entire infection period which is initially beneficial by reducing the parasite load and later detrimental by decreasing the humoral response. A significant linear elevation for IFN-γ was observed between day 6 and 8 post infection when the parasitemia levels were also on the upward trend. The results presented in this study show that the TNF-α produced at day 4 post infection could probably confer some protection from CM, as T-cell apoptosis was not observed converging with previous studies by Mitchell et al. (2005) showing that a later production of TNF-α is essential for the development of CM. TNF-α up-regulates the expression of adhesion molecules, thereby exacerbating parasite sequestration and correlates with severe disease (Mitchell et al., 2005).

The kinetic pattern of Th2 cytokines during immune responses is to limit the consequences of an exaggerated proinflammatory response and to counterbalance the production of Th1 cytokines (Mitchell et al., 2005). Significant transient increase in concentrations of IL-4 (P<0.05), was noted between day 4 and 8 post infection, but afterwards its levels remained low throughout the course of infection which is critical for biasing the immune reaction toward a Th2 phenotype (Waknine-Grinberg et al., 2010). IL-4 is an anti-inflammatory cytokine and thus increased production during initial parasite infection (days 4 to 8 post infection) was necessary to ameliorate the disease outcome as evidenced in the absence of CM symptoms in those mice (Mitchell et al., 2005). The current study show that *P. berghei* infection caused an immediate significant shift in the IL-4 response of infected mice skewing towards a Th2 shift and later the mice displayed a prevalently Th1 response as it directs polarization of naive T helper cells towards the Th2 phenotype variety of anti-inflammatory effects (Waknine-Grinberg et al., 2010).

From the study, the IL-4 concentrations were insignificantly lower as to prevent the mice from getting

an infection. This early regulation of pro-inflammatory response during murine malaria infection is mediated by regulatory T- cells in an IL-4 dependent manner (Chen et al., 2009). The regulation of TNF- $\alpha$  levels by IL-4 and IL-5 appears to contribute to the prevention of severe malarial anemia in humans (May et al., 2000) as observed in this study, however, the role that both IL-4 and -5 plays may depend on their levels, since very high levels of IL-4 have been associated with severe malaria in humans (Lyke et al., 2004) and some animal models (Kobayashi et al., 2000).

The study reveals a relative deficiency in IL-5, albeit not statistically significant in days 6, 8, and 14 post infection, whereas significant differences were observed in days 11 and 20 post infection in mice that died of severe malaria suggesting a loss of down-regulatory function. IL-5 concentrations were remarkably low throughout the infection course, but it was peculiarly significant ( $P < 0.05$ ) at days 11 and 20 post infection, because the transient increase was necessary to ameliorate the disease outcome in the late stages of the infection. IL-4 and IL-5 levels in the test group were significantly higher ( $P < 0.05$ ) as compared to the control group at day 6 post infection which demonstrates that Th2 cytokines were counter balancing the pro-inflammatory cytokines during the initial phase of infection. The inverse relationship between Th1 and Th2 cytokine milieu was striking as revealed by a continued suppression of Th2 responses in the test group during the course of infection which in effect led to the overproduction of the Th1 immune responses. The early production of IL-4 together with IL-5 acts predominantly in an autocrine manner by down-regulating the activation of antigen-presenting cells, which then has the downstream effect of inhibition of T-cell activation and expansion (Mitchell et al., 2005). The Th2 cytokines in the study were marginally up-regulated in and therefore they have played no role in CM pathology as this part of normal fluctuation.

In this study, evidence is provided indicating that IFN- $\gamma$  and TNF- $\alpha$  cytokines are altered in CM pathology. The elevated levels of IFN- $\gamma$  and TNF- $\alpha$  is particularly remarkable since they are involved in parasite clearance and preserve erythropoiesis (Stevenson and Riley, 2004). The possible involvement of cytokines production in disease severity was investigated by DNA fragmentation of T-lymphocytes in the spleen and brain tissues during malaria infection (Figure 4). The study demonstrates an elevated systemic significant increase in pro-inflammatory cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) in the serum coinciding with T-lymphocytes pathology in the spleen and brain. This is because while the Th1/Th2 dichotomy is highly stereotypic (Kelso, 1995), the activation of inappropriate effector responses conceivably could lead to the development of immune-mediated pathology.

Although, the study did not determine the concentrations of cytokines in the brain and spleen, it can

be deduced that up-regulation of TNF- $\alpha$  and IFN- $\gamma$  in these tissues was higher as indicated by the fragmentation of the T-lymphocytes DNA from these tissues (Figure 4). Similar results obtained by Weiss et al. (1990) reported that in malaria-infected mice, reactive lymphocytes disappeared from the blood, but their number increases significantly in the spleen suggesting that elevated levels of IFN- $\gamma$  and TNF- $\alpha$  probably synergistically induces deleterious effects, including killing of T-lymphocytes. High levels of Fas-Ligand in sera of human (Kern et al., 2000), monkey (Matsumoto et al., 2000) and mice (Helmsby et al., 2000) are associated with severity of malaria. This study concurs with previous studies by Tewari et al. (2007) which demonstrated that IFN- $\gamma$  induces apoptosis of activated CD4<sup>+</sup> T cells as apoptosis was largely observed in the brain lymphocytes. Through, a comparison of the patterns of cytokine responses in the mice, the present study was able to exhibit different courses and outcomes of infection that can be used to deduce instances of cytokine excess or deficiency. Elevated cytokine levels for IFN- $\gamma$  and TNF- $\alpha$  coincided with the development of CM, implying that they act as double-edged swords in the pathogenesis of malaria. Therefore, the complications seen in ECM may be related to cytokine-mediated injury required for parasite clearance. The findings have provided new insights on the earlier involvement of serum Th2 cytokine levels important in understanding the pathogenesis of CM. From this study, it can be deduced that elevated levels of serum Th1 cytokines concentrations in the brain and the spleen could probably lead to T-lymphocyte DNA cleavage during experimental malaria. Further investigations are required on the extensive cytokine network during murine malaria to extend knowledge regarding the immune responses.

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