Cytokine-CpG Motif Oligodeoxynucleotide Co-Inoculation in BALB/c Mice Infected With *Plasmodium berghei* ANKA Strain

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ABSTRACT

Approximately 198 million cases of malaria manifested worldwide in 2013, causing 584,000 deaths, further solidifying malaria’s status as a serious global health predicament. A vast array of immunopotentiating molecules like unmethylated CpG motif oligodeoxynucleotides (ODNs) operate in concert with cytokines in rendering hosts resistant to parasitic infections. The CpG ODNs exert potent immunostimulatory effects via nexus with dendritic cell Toll-like receptors (TLRs) like TLR 9 and by activating immune cells like B-cells and NK cells. Investigations were performed to resolve the anti-malarial effects of cytokine-CpG ODN co-inoculation in BALB/c mice infected with *Plasmodium berghei* ANKA strain. Two BALB/c mice groups were infected with virulent *P. berghei* ANKA strain parasites, followed by five consecutive days of cytokine-CpG ODN co-therapies. Six control groups with various regimen were included. Parasitaemia, and clinico-haematological outcomes accompanying the immunotherapies were quantified. Cytokine-CpG ODN interventions elicited antimalarial mechanisms involving lower peak parasitaemia, less dramatic parasitaemia trends and overall suppression of parasitaemia. Cytokine-CpG ODN co-administration also induced milder symptomatic sequelae in which lethargy, appetite distortion, convulsions and adverse clinico-haematological outcomes were repressed with ramifications in the potential of cytokine-CpG-based DNA therapy in counteracting malaria.

Key words: *P. berghei* ANKA, Parasitaemia, Malaria, BALB/c Mice, Cytokines, CpG Motif ODN.

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INTRODUCTION

Malaria is widespread in tropical and subtropical regions including parts of the Americas, Asia and Africa. There were about 219 million cases of malaria in 2010 and an estimated 660 000 deaths and Africa is the most affected continent: about 90% of all malaria deaths occur there (Keating, 2012). Malaria caused by *P. falciparum* is life
threatening and can cause multiple organ damage, coma and death. Furthermore, severe malaria complications can result in anaemia, cerebral malaria (CM), and in pregnant women malaria parasites may infiltrate the placenta, a condition called placental malaria (PM). At present there is only a partially efficacious RTS,S/AS02A (Mosquirix™) antimalarial vaccine licensed for antimarial use and available therapeutic interventions continue to be impeded by emergence of drug-resistant strains of Plasmodia which cause high morbidity and mortality (Walsh, 2015). Plasmodia parasites evade immune mechanisms and up to date it remains unclear what exact immunophysiological modulations are required in their elimination. The outcome of host-pathogen interactions, with respect to Plasmodia parasites is determined by an extremely delicate balance of various biomolecules. Therefore proper understanding of intricate mechanisms underlying the pathogenesis of malaria is essential in controlling Plasmodia. Cutting-edge research programmes on counter-malarial mechanisms are intensively focusing on promising biochemical and molecular actors in advancing vaccination and treatment systems against malaria.

The short single-stranded synthetic CpG oligodeoxynucleotide (or CpG ODN) molecules contain a cytosine triphosphate deoxyxynucleotide (“C”) followed by a guanine triphosphate deoxynucleotide (“G”). The “p” in “CpG” represents the phosphodiester link between consecutive nucleotides, although some ODN have a modified phosphorothioate (PS) backbone instead. Unmethylated CpG motifs are powerful immunostimulants (Weiner et al., 1997; Li et al., 2004) and CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundant presence in microbial genomes and their rarity in vertebrate genomes (Li et al., 2004). The immunotherapeutic combination of CpG motif oligodeoxynucleotides (ODNs) with inflammasome cytokines like IL-18 and IL-12, can be expected to generate more upregulated immunopotentiation compared to independent administration of these components. Such interventions have the potential of combining the advantages of both CpG ODN treatment and cytokine effects thereby activating synergistic antimicrobial effects in vivo. Protective CpG ODN-involving mechanisms are initiated through TLR 9 (Toll Like Receptor 9) pathways and when cytokines and immunostimulatory CpG motif ODNs are therapeutically co-administered against parasitic infections in vivo, strong protection occurs (Li et al., 2004; Barasa et al., 2015). Through interconnection with dendritic cell Toll-like receptors (TLRs) like TLR 9 CpG ODN motifs provoke and up regulate widespread immune functionalities. However, prior to this communication, the parasitological and clinico-haematological effects of in vivo cytokine-CpG synergistic interactions and any potentially accompanying enhanced protection were yet to be evaluated in the context of malaria infections. The variety of immunoactivating effects of CpG ODNs includes direct induction of B cell proliferation and immunoglobulin (Ig) secretion, as well as activation of monocytes, macrophages, and dendritic cells to upregulate their expression of costimulatory molecules, that promote immune responses, and secretion of a multiplicity of cytokines, including high levels of IL-12 (Weeratna et al., 1999; Gramzinski et al., 2001). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs imitate the direct immunostimulatory effects of native bacterial DNA, and activate a spectrum of cell types including macrophages, dendritic cells, NK cells, and B lymphocytes. Immunostimulatory activities of CpG-ODNs have gained attention as potentially useful therapeutics for inflammatory and allergic diseases, and for inoculation as immune adjuvants or immunoprotective agents. Accurately targeted drug delivery to cells was accomplished using CpG ODN-complexed nanoparticles causing increased immunopotentiating capabilities (Kerkmann et al., 2006; Chinnathambi et al., 2012; Alexandre de Titta et al., 2013), and this diversifies the possible applications of such CpG ODN-based co-therapeutic nanoscale-complexes in dealing with infectious diseases. Symptomatic, haematological, clinical chemistry and parasitological effects of cytokine-CpG ODN co-injection in P. berghei-infected BALB/c mice were quantified in order to determine the protective outcomes induced via such co-therapies against malaria. Findings, reported herein indicate that cytokine-CpG co-inoculation reduces parasitaemia progression and leads to less severe clinical and haematological manifestations.

MATERIALS AND METHODS

Study Site

These investigations were done at the Kenya Medical Research Institute’s (KEMRI) Center for Biotechnology Research and Development (CBRD) and the Institute of Primate Research (IPR), Nairobi, Kenya. They were approved by the KEMRI Scientific Steering Committee (SSC) and ethical approval for was granted by the KEMRI Animal Care and Use Committee (ACUC) and the Ethical Review Committee (ERC).

Study Design

There were eight groups of mice; two main experimental cytokine-CpG co-inoculation groups and six control groups that were used. The groups were designated as CpG/IL-18/ P. berghei; CpG/IL-12/ P. berghei; IL-18/ P. berghei; IL-12 P. berghei; CpG / P. berghei; P. berghei;
CpG and uninfected mice groups. Each mice group had 18 mice. Generally, the cytokines (IL-12 and IL-18) were chosen due to their protective roles in parasitised murine hosts (Angulo et al., 2002; Li et al., 2004; Gramzinski et al., 2001). The mice groups with names containing ‘P. berghei’ were infected simultaneously with P. berghei parasites. On day one post-infection mice groups were treated as follows: the CpG/IL-12/P. berghei group was treated with both CpG ODNs and IL-12, the CpG/IL-18/P. berghei group was treated with both CpG ODNs and IL-12, the IL-18/P. berghei and IL-12/P. berghei groups were treated with IL-18 and IL-12, respectively, the CpG/P. berghei group was treated with CpG ODNs, the P. berghei group remained untreated, the CpG group (uninfected) received CpG ODNs only, while the uninfected group remained untreated. The CpG/IL-18/P. berghei and CpG/IL-12/P. berghei groups were the main groups under investigation, while the other six groups were used as controls. Treatments were repeated for 5 days. Parasitaemia and clinical characteristics were monitored on a daily basis in all mice (Barasa et al., 2015). After ten days, all mice were anaesthetized and humanely euthanised for extraction of EDTA blood via intracardiac puncture using a disposable 1ml syringe and a 26 x 6 mm needle for haematological and clinical chemistry analysis soon after the animal phase of experimentation.

Experimental Mice, Parasites, and Infections

Twelve week-old female BALB/c mice purchased from KEMRI were intraperitoneally injected, using a needle of size 26 G, with 1x104 virulent wild type P. berghei ANKA-parasitized red blood cells obtained from donor infected BALB/c mice. Blood for daily parasitaemia determination was extracted from all mice (approximately 50 µl per mouse) and used to prepare triplicate Giemsa-stained thin blood smears and parasitaemia were expressed as a percentage of at least 2000 RBCs (Barasa et al., 2015). After ten days, all mice were anaesthetized and humanely euthanised for extraction of EDTA-treated blood via intracardiac puncture using a disposable 1ml syringe and a 26 x 6 mm needle for haematological and clinical chemistry analysis soon after the animal phase of experimentation.

Recombinant Cytokines and CpG Motif Oligodeoxynucleotides (ODNs)

Commercially available recombinant murine cytokines (rIL-18 and rIL-12) were purchased and processed for intradermal inoculation according to manufacturer’s specifications (Becton Dickinson, USA). The recombinant cytokines were reconstituted to final concentrations of 500 ng/mL in total volumes of 50 µl of PBS each (Barasa et al., 2015). Synthetic CpG motif oligodeoxynucleotides (ODN; M362) containing CpG motifs synthesized with a nuclease-resistant phosphorothioate backbone (Invivogen, USA) were used. The CpG ODN M362 sequence 5’-TCGTCGTCGTTCGACGACATTGAT-3’ (25 mer) contained the CpG motifs required for immunostimulation in these experiments. The CpG ODN M362 was shipped at room temperature and stored at −20°C. This type C CpG ODN combines features of both types A and B CpG ODN and contained a complete phosphorothioate backbone and a CpG-containing palindromic motif. Type C CpG ODNs induce strong IFN-α production from pDC and B cell stimulation (Weeratna et al., 1999).

Upon resuspension, aliquots of CpG M362 were prepared and stored at -20°C. The resuspended product was capable of remaining stable for 6 months at -20°C and repeated freeze-thawing cycles were avoided. Each BALB/c mouse was intramuscularly inoculated at the appropriate time with 50 µg of CpG ODN M362 in a 50 µl volume of phosphate buffered saline using a 27.5-gauge needle as previously described (De Rose et al., 2002; Barasa et al., 2015).

Clinical, Haematological and Parasitological Monitoring

Soon after infection the mice were closely monitored on a daily basis for lethargy, hair ruffling, appetite, roll-over movement’s diarrhea, skin turgor reduction, limb paralysis, convulsions (Carvalho et al., 2006). Clinical parameters measured were scored arbitrarily on a scale of one to ten and each one to ten range score was represented on a clinical parameter score table as a single ‘+’. Thus, in the clinical parameter score table, the higher the number of the ‘+’ signs, the greater the symptomatic intensity and vice versa. Body weights of mice in grams were measured on a daily basis. Parasitaemia values were determined by examination of Giemsa-stained blood smears collected from the tail vein. Blood drops from pricked tail veins (5 to 15 µl) were placed onto microscope slides for the preparation of thick and thin Giemsa-stained smears. At least 2000 red blood cells (RBC) were counted in every parasitaemia count session (Ozwara et al., 2003; Barasa et al., 2010; Helegbe et al., 2011). The EDTA-treated whole blood samples were used for clinical chemistry and haematological analysis. A Reflotron® Plus reflectance photometric clinical chemistry analyser was used to quantify bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and glucose. The leukocyte differential count was carried out with immersion with an Olympus® CX 41 light microscope, using 26x76 mm microscopic slides. A Sysmex SF-3000® automated hematology
ana\textit{lyser was used to Measure RBC, total leukocytes, packed cell volume (PCV), corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), platelets.}

**Data Analysis**

The Graphpad Prism-6®, 2015 software was used for data analysis. Group mean values of parasitaemia, clinical chemistry and haematological parameters were compared using one-way Analysis of Variance (ANOVA) and values where \( P < 0.05 \) were considered significant.

**RESULTS**

**Parasitological, Clinical and Haematological Responses**

All mice groups were monitored on a daily basis for parasitaemia development. Beginning from day one post-infection, all mice groups on average became parasitaemic with the \( P. \text{ber}ghei \) group recording the highest parasitaemia levels of all mice groups. The uninfected mice maintained their status quo throughout the study. There were significant (\( P < 0.05 \)) differences in parasitaemia levels between the mice that were treated with cytokine-CpG co-inoculations and the rest of the mice in the study. On average, the parasitaemia (5.591\%) experienced throughout the experiments by the malaria infected mice that were given cytokine-CpG ODN co-inoculations were three times lower than the average parasitaemia (11.826 \%) in the control experiment groups. The highest detected parasitaemia in the CpG/IL-18/\( P. \text{ber}ghei \) group of mice was 8.0\% while in the CpG/IL-12/\( P. \text{ber}ghei \), levels rose to a peak concentration of 9.0\%, significantly (\( P < 0.05 \)) less compared to the \( P. \text{ber}ghei \) group that experienced the highest peak total parasitaemia level of 70.20\%. For a majority of the mice groups, peak parasitaemia were experienced over the last three days just before euthanisation for sample collection. The \( P. \text{ber}ghei \) infected group and the CpG/P. \( \text{ber}ghei \) group experienced stronger upturns in parasitaemia trends beginning from day six postinfection than the rest of the groups. The CpG/IL-18/\( P. \text{ber}ghei \) group and the CpG/IL-12/\( P. \text{ber}ghei \) groups experienced relatively equivalent levels of average total parasitaemia; 5.727 and 5.455\%, respectively. Generally, parasitaemia levels of below 10\% were associated with less severe clinical symptoms compared to levels above 10\%. Trends in the expansion of differential parasitaemia stages were similar to the total parasitaemia trends. Ring stages of malaria parasites were demonstrable from day one post infection and generally levels started increasing more rapidly beginning from day six post infection. Just like for total parasitemia, the experimental groups CpG/IL-18/\( P. \text{ber}ghei \), and CpG/IL-12/\( P. \text{ber}ghei \) both experienced lower differential parasitaemia than the rest of the groups. The CpG/\( P. \text{ber}ghei \) group had the second steepest upward acceleration in parasite count while less steeper intermediate total parasitaemia development trends were witnessed in the IL-18/\( P. \text{ber}ghei \) and IL-12/\( P. \text{ber}ghei \) groups (Figures 1 to 4). More importantly, both total and differential parasitaemia trends in the CpG/IL-18/\( P. \text{ber}ghei \) and CpG/IL-12/\( P. \text{ber}ghei \) groups remained strongly reduced, barely striking the 9.2\% barrier level and sharp day-6 up-turns witnessed in the infected controls were notably absent in these two main experimental groups.

**Total Parasitaemia Levels**

The highest mean total parasitaemia, 25.34\% was recorded in the \( P. \text{ber}ghei \) group. This was over five times higher than the parasitemia measured in the CpG/IL-18/\( P. \text{ber}ghei \) and CpG/IL-12/\( P. \text{ber}ghei \) groups of mice; 5.727 and 5.455, respectively. The total parasitaemia differences amongst the groups were highly significant at \( P < 0.0001, F (7, 70) = 9.342 \). The CpG/\( P. \text{ber}ghei \) group (the second highest total parasitaemia group) had a total parasitaemia level of 15.73, five times higher than parasitaemia in the CpG/IL-18/\( P. \text{ber}ghei \) and CpG/IL-12/\( P. \text{ber}ghei \) groups of mice. The IL-12/\( P. \text{ber}ghei \) group a mean total parasitemia level of 10.64, approximately two times higher than that of the CpG/IL-12/\( P. \text{ber}ghei \) group. The IL-18/\( P. \text{ber}ghei \) group a mean total parasitemia level of 9.455, 0.6 times higher than that of the CpG/IL-18/\( P. \text{ber}ghei \) group.

**Ring Stage Parasitaemia Levels**

The \( P. \text{ber}ghei \) group had highest mean levels of ring stage parasitaemia (9.036\%). This level was over six times higher than the parasitemia measured in the CpG/IL-18/\( P. \text{ber}ghei \) and CpG/IL-12/\( P. \text{ber}ghei \) groups of mice; 2.061 and 1.909 (SD 0.8336, SE 0.2513), respectively. The ring stage parasitaemia differences amongst the groups were highly significant at \( P < 0.0001, F (7, 70) = 9.3429 \). The CpG/\( P. \text{ber}ghei \) group had a mean ring stage parasitaemia level of 5.578, almost 3-fold higher than the two cytokine-CpG co-inoculation groups of mice. The IL-12/\( P. \text{ber}ghei \) group had a mean ring stage parasitaemia level of 3.78, while in the IL-18/\( P. \text{ber}ghei \) levels averaged at 3.4. In the CpG/ODN and uninfected groups ring parasitaemia concentrations remained as expected at 0.0 and 0.0, respectively.

**Trophozoite Stage Parasitaemia Levels**

The \( P. \text{ber}ghei \) group had highest mean levels of
trophozoite stage parasitaemia; 7.864%. These trophozoite levels were over six times higher than the trophozoite parasitaemia measured in the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups of mice; 2.100 and 1.827, respectively. The trophozoite parasitaemia differences were significant at P<0.0001, F (7, 70) =
For the CpG / *P. berghei* group of mice parasitaemia levels were 5.145 (second highest trophozoite levels) while in the CpG ODN and uninfected groups trophozoite parasitaemia levels were both at 0%. 

Figure 3. Trophozoite Stage Parasitaemia Development Trends.

Figure 4. Schizont Parasitaemia Trends.
The trophozoite parasitaemia level in the CpG/ P. berghei group, 5.145, was more than two times higher than the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups of mice. The IL-12/ P. berghei group a mean trophozoite level of 3.245, was also more than two times higher than in the CpG/IL-12/ P. berghei group. Levels in the IL-18/ P. berghei group were almost one and a half times higher than in the CpG/IL-18/ P. berghei group.

**Schizont Stage Parasitaemia Levels**

The overall highest schizont stage parasitaemia concentrations, 8.436% were recorded in the P. berghei group. These schizont levels were approximately four times higher than the parasitaemia measured in the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups of mice; 2.030 and 2.491, respectively. The schizont parasitaemia differences were significant at P < 0.0001, F (7, 70) = 11.15. The schizont parasitaemia levels in the CpG / P. berghei group, 5.700, were more than two times higher than in the cytokine/CpG co-inoculated groups of mice put together. The IL-12/ P. berghei group mean schizont level of 3.736, was about one and a half times higher that of the CpG/IL-12/ P. berghei group. The IL-18/ P. berghei group a mean schizont level, 3.464, was also about one and a half times higher than in the CpG/IL-18/ P. berghei group (2.030). For the IL-18/ P. berghei, CpG ODN and uninfected groups of mice schizont parasitaemia levels were at 3.464, 0 and 0%, respectively (Table 1 and Figure 5[a-h]).

**Clinical Manifestations**

The most severe malarial symptoms were detected in the P. berghei group. It is worth noting that the P. berghei group also experienced the highest parasitaemia of all the groups. Overall, less severe clinical symptoms were experienced in the CpG/IL-18/P. berghei, CpG/IL-12/P. berghei, CpG/ODN and uninfected mice groups compared to the rest of the mice groups. With the exception of the CpG ODN and uninfected group, all mice groups experienced lethargy. Ruffling of hair was notable only in the IL-18/P. berghei, IL-12/P. berghei, CpG/P. berghei and the P. berghei groups. The rest of the groups did not experience the ruffling of hair. Appetite distortion occurred in all mice groups except in the CpG/IL-18/P. berghei, and IL-12/P. berghei groups. Urine became very dark coloured in the CpG/IL-12/P. berghei and also in the P. berghei groups beginning from day 5 post infection. Urine also darkened in the CpG/IL-18/P. berghei and IL-18/P. berghei groups. The other groups did not experience this clinical symptom. Turgidity of the skin sharply dropped in the P. berghei and CpG/P. berghei groups, and this characteristic occurred mildly in the rest of the mice groups, except in the CpG ODN and uninfected groups which did not exhibit any skin abnormalities, including turgidity reduction. Extreme limb paralysis was experienced in the P. berghei groups and also in the CpG/P. berghei groups. Mild limb paralysis was noted in the IL-18/P. berghei, IL-12/P. berghei, CpG/P. berghei, and in the CpG/ODN groups. While intense convulsions were noted to occur in the P. berghei group and CpG/P. berghei groups the other groups did not demonstrate this feature. Intense roll-over movements and restlessness were notable in the IL-12/P. berghei, CpG/P. berghei, and in the P. berghei groups. The IL-18/P. berghei group had mild levels of roll-overs movements in their cages. Severe diarrhoea was noted in the P. berghei group and also, albeit with less severity in the IL-12/P. berghei and CpG/P. berghei groups. The CpG/IL-18/P. berghei, CpG/ODN, CpG/IL-12/P. berghei and uninfected group did not have any diarrhoea. A high level of piloerection was observed in the P. berghei group and also mildly in the CpG/IL-18/P. berghei, IL-18/P. berghei, and IL-12/P. berghei groups. However there was no piloerection in the rest of the mice groups. Below is a table showing in summary, the intensity of clinical manifestations measured in all mice groups (Table 2).

**Weight Changes**

The uninfected mice group had a mean body weight level of 22.45. The CpG/IL-18/P. berghei and CpG/IL-12/P. berghei had mean weight levels of 22.57 which significantly (P < 0.0001, F; 7, 70 = 13.98) differed from the P. berghei group mean of (16.89) that experienced drastic body weight decline. The lowest body weight, 13.80, was also recorded in the P. berghei group. The highest body weight of 27.4 was recorded in the CpG/IL-18/P. berghei group. The CpG ODN group had a mean weight of 21.18 while the IL-18/P. berghei, IL-12/P. berghei and CpG/ P. berghei groups had intermediate body weight levels ranging between 17.20 and 19.90 (Figures 6 and 7).

**Haematological and Clinical Chemistry Responses**

With regard to all the RBC indices (PCV, Hgb, MCV, MCHC and RBC), the P. berghei group had the lowest values; 26.9%, 9.2 g/dL, 44.2 fL, 12.6 pg, 34.2 g/dL, 6.08 x 1000/mL, respectively. With the exception of MCV, there were significant (P < 0.05) variations in all measured RBC indices values. The CpG/IL-18/P. berghei group had significantly (P < 0.05) higher values for these indices; 43.2%, 16.3 g/dL, 16.5 pg, 42.3 g/dL, 10.3 x 1000/mL, respectively, with the exception of MCV (42.1 fL) which did not vary significantly among the groups. For the CpG/IL-12/P. berghei groups, the values for these indices were 44.2%, 15.4 g/dL, 16.3 pg, 43.1
WBC cell types with the exception of basophils, and eosinophils compared to the rest of the mice groups. The CpG/IL-18/P. berghei and CpG ODN groups had significantly lower values of AST recorded in the CpG/IL-18/P. berghei and uninfected groups, 170.1, 173.1 and 175.2, respectively, and the P. berghei and CpG ODN groups had the highest mean records of AST concentrations, 190.4 and 195.3, respectively. Intermediate values were recorded from the IL-18/P. berghei, IL-12/P. berghei, IC and CpG ODN groups. Significantly lower levels of glucose, 92.4 g/dL we detected in the P. berghei group, while in the CpG/IL-18/P. berghei, CpG/IL-12/P. berghei, IL-12/P. berghei and uninfected groups levels were higher and they ranged from 117.3 g/dL to 120.4 g/dL. Intermediate concentrations of glucose, 100.9 g/dL, 107.6 g/dL, and 114.3 g/dL were recorded in the CpG/IL-18/P. berghei, IL-12/P. berghei, CpG/ODN groups.

Table 1. Parasitaemia data summary for all the mice groups.

<table>
<thead>
<tr>
<th>Stage</th>
<th>CpG/IL-18/ P. berghei</th>
<th>CpG/IL-12/ P. berghei</th>
<th>IL-18/ P. berghei</th>
<th>IL-12/ P. berghei</th>
<th>CpG / P. berghei</th>
<th>P. berghei</th>
<th>CpG ODN</th>
<th>Uninfected mice</th>
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<tbody>
<tr>
<td>Schizont</td>
<td>Means</td>
<td>5.727</td>
<td>5.455</td>
<td>9.455</td>
<td>10.64</td>
<td>15.73</td>
<td>25.34</td>
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<tr>
<td></td>
<td>SD</td>
<td>2.195</td>
<td>2.583</td>
<td>5.837</td>
<td>5.784</td>
<td>12.76</td>
<td>23.63</td>
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<td>Trophozoite</td>
<td>ANOVA</td>
<td>F (7, 70) = 9.342, P &lt; 0.0001</td>
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</tr>
<tr>
<td></td>
<td>Means</td>
<td>2.100</td>
<td>1.827</td>
<td>2.955</td>
<td>3.245</td>
<td>5.145</td>
<td>7.864</td>
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<td>1.028</td>
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<td>1.739</td>
<td>3.919</td>
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<td>1.965</td>
<td>1.895</td>
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</tbody>
</table>

With levels ranging from 61.3 to 64.2, compared to the rest of the groups. The CpG/ P. berghei and the P. berghei groups had 58.9 and 58.2, respectively. Significantly lower values of AST were recorded in the CpG/IL-18/P. berghei, CpG/IL-12/P. berghei and uninfected groups, 170.1, 173.1 and 175.2, respectively, and the P. berghei and CpG ODN groups had the highest mean records of AST concentrations, 190.4 and 195.3, respectively. Intermediate values were recorded from the IL-18/P. berghei, IL-12/P. berghei, IC and CpG ODN groups. Significantly lower levels of glucose, 92.4 g/dL we detected in the P. berghei group, while in the CpG/IL-18/P. berghei, CpG/IL-12/P. berghei, IL-12/P. berghei and uninfected groups levels were higher and they ranged from 117.3 g/dL to 120.4 g/dL. Intermediate concentrations of glucose, 100.9 g/dL, 107.6 g/dL, and 114.3 g/dL were recorded in the CpG/IL-18/P. berghei, IL-12/P. berghei, CpG/ODN groups.

g/dL, and 10.1 x 1000/mL, respectively. The MCV value was 43.7 fl. The IL-18/P. berghei, IL-12/P. berghei, and CpG/IL-12/P. berghei, groups had intermediate levels of PCV ranging from 37.7 to 38.9%, while the CpG ODN, and uninfected mice groups had 45.2 and 45.6% respectively. Similar intermediate levels were recorded from the IL-18/P. berghei, IL-12/P. berghei, CpG /P. berghei groups for the rest of the RBC indices (Table 3) and higher values were recorded for these indices from the CpG ODN, and uninfected mice groups. The CpG/IL-18/P. berghei and CpG/IL-12/P. berghei and CpG OD groups had significantly higher levels of WBCs, segmented neutrophils, band neutrophils, lymphocytes, mononuclear cells, and eosinophils compared to the rest of the groups, while the percentage values recorded for basophils exhibited no significant variation. The P. berghei group had much lower concentrations of WBC cell types with the exception of basophils, while the IL-18/P. berghei, IL-12/P. berghei, CpG /P. berghei and uninfected groups registered mid-level concentrations of the WBC cell types (Table 3). There were significant variations in the numbers of platelets measured with the P. berghei group recording the lowest levels, 126 x 1000, and the highest levels being recorded in the IL-18/P. berghei and CpG ODN groups, 302 x 1000 and 315 x 1000, respectively. The uninfected mice had platelet values that averaged to 276 x 1000. The CpG/IL-12/P. berghei, IL-18/P. berghei, IL-12/P. berghei, and CpG / P. berghei groups had levels ranging from 130.1 to 139. Total bilirubin levels in the CpG/IL-18/P. berghei and CpG/IL-12/P. berghei groups were slightly lower than in the rest of the groups except the CpG ODN and uninfected groups which had similar levels. There were no significant variations in creatinine and ALT levels among the mice groups. The CpG/IL-18/P. berghei, CpG/IL-12/P. berghei, CpG ODN and uninfected groups were found to have slightly higher concentrations of alkaline phosphatase.

<table>
<thead>
<tr>
<th>Stage</th>
<th>CpG/IL-18/ P. berghei</th>
<th>CpG/IL-12/ P. berghei</th>
<th>IL-18/ P. berghei</th>
<th>IL-12/ P. berghei</th>
<th>CpG / P. berghei</th>
<th>P. berghei</th>
<th>CpG ODN</th>
<th>Uninfected mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizont</td>
<td>Means</td>
<td>5.727</td>
<td>5.455</td>
<td>9.455</td>
<td>10.64</td>
<td>15.73</td>
<td>25.34</td>
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<tr>
<td></td>
<td>SD</td>
<td>2.195</td>
<td>2.583</td>
<td>5.837</td>
<td>5.784</td>
<td>12.76</td>
<td>23.63</td>
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<tr>
<td>Trophozoite</td>
<td>ANOVA</td>
<td>F (7, 70) = 11.68, P &lt; 0.0001</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>Means</td>
<td>2.491</td>
<td>3.464</td>
<td>3.736</td>
<td>5.700</td>
<td>8.436</td>
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<tr>
<td></td>
<td>SD</td>
<td>0.8894</td>
<td>1.679</td>
<td>1.965</td>
<td>1.895</td>
<td>4.882</td>
<td>7.810</td>
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</tr>
</tbody>
</table>
DISCUSSION

This study was conducted to determine the clinical symptoms, haematological, clinical chemistry and parasitological responses elicited in the *P. berghei* - murine (BALB/c) model of malaria following combinatorial cytokine-CpG ODN biotherapies involving the proinflammatory cytokines IL-12 and IL-18. Generally,
cytokine-CpG ODN co-injection in the causing early IFN-γ release and parasitaemia reduction (Erik et al., 2014). Down regulation of IL-12 functions was found to be positively correlated with severity of *P. falciparum* malaria in African children (Adrian et al., 2000) and mild *P. falciparum* malaria was reportedly associated with high IL-12 and IL-18 levels in plasma (Malaguarnera et al., 2002). The heterodimeric cytokine IL-12 was also described as a component of a mild malaria cytokine cluster that also included IFN-γ, IL-2, IL-5 and IL-6. Reduced levels of IL-12 were also associated with severe malaria (Chaiyaroj et al., 2004) and plasma levels of IL-12 were found to be inversely correlated with *P. falciparum* parasitaemia and PBMC nitric oxide synthase activities (Boutlis et al., 2003). The CpG ODN 1826 was demonstrated to trigger a greater level of protection compared with CpG ODN 1585. The protective outcomes of the two CpG ODNs were found to be dependent on interleukin-12, and IFN-γ. The immune cellular subsets NK cells, CD8+ T cells (but not CD4+ T cells), and nitric oxide were involved in the protection mediated by CpG ODN 1585 (Gramzinski et al., 2001). In this current report the various anti-malaria attributes of IL-12 appeared to synergise with immunostimulatory CpG activities like activation of proinflammatory reactions, B-cells and plasmacytoid dendritic cells activities (Rothenfusser et al., 2002) leading to reduced parasitaemia in mice that received both IL-12 and CpG in coincidence. In the current study, both independent and CpG accompanied rIL-18 therapy in *P. berghei* infected mice significantly reduced parasitaemia development. Interleukin-18, a member of the IL-1 cytokine super-family and an interferon gamma inducing factor, also enhances T and NK cell maturation cytokine production, BALB/c-*P. berghei* ANKA drastically reduced parasitaemia and was associated with milder clinical and haematological outcomes. The two main experimental groups, the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups, experienced reduced parasitaemia. Besides having significantly reduced total and differential parasitaemia levels, they also had lower peak parasitaemia, and relatively stabilised total and differential parasitaemia trends. This indicated that the cytokine-CpG motif combination caused anti-*P. berghei* responses leading to reduced parasitaemia. Previous reports (Nakanishi et al., 2001; Li et al., 2004) have shown that both independent and combined (with CpG ODNs) presence of cytokines elicits anti-parasitic responses and the cytokines IL-18 and IL-12 themselves have anti-malarial effects (Angulo et al., 2002; Normaznah et al., 1999). Treatment of *P. berghei* infected mice with anti-IL-12 was shown to cause increased parasitaemia, fatal results, and lower IFN-γ mRNA expression and secretion activities (Yoshimoto et al., 1998). IL-12 triggers IFN-γ

<table>
<thead>
<tr>
<th></th>
<th>CpG/IL-18/ <em>P. berghei</em></th>
<th>CpG/IL-12/ <em>P. berghei</em></th>
<th>IL-18/ <em>P. berghei</em></th>
<th>IL-12/ <em>P. berghei</em></th>
<th>CpG / <em>P. berghei</em></th>
<th><em>P. berghei</em></th>
<th>CpG ODN</th>
<th>Uninfected mice</th>
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<tr>
<td>Lethargy</td>
<td>++ ++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+ ++</td>
<td>+ ++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hair Ruffling</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Appetite Distortion</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+ ++</td>
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<td>Urine Colour</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ ++</td>
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<tr>
<td>Skin Turgor</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+ ++</td>
<td>+</td>
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<tr>
<td>Limb Paralysis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Convulsions</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
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</tr>
<tr>
<td>Roll-over movements</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
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<td>Diarrhoea</td>
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<td>++</td>
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<td>Piloerection</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
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</tbody>
</table>

Clinical observations were quantified using an arbitrary scale and reported as either absent (-), mild (+), moderate (++) or severe (+++).
Figure 6. Daily Body Weight Measurements in the Mice Groups.

Figure 7. Overall Body Weight Measurements.
Table 3. Hematological and clinical chemistry parameter levels analysed in the 8 groups of mice.

<table>
<thead>
<tr>
<th></th>
<th>CpG/IL-18</th>
<th>CpG/IL12</th>
<th>IL-18</th>
<th>IL12</th>
<th>CpG / P.</th>
<th>P.</th>
<th>CpG ODN</th>
<th>Uninfected mice</th>
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<tr>
<td></td>
<td>P. berghei</td>
<td>P. berghei</td>
<td>P. berghei</td>
<td>P. berghei</td>
<td>P. berghei</td>
<td>P. berghei</td>
<td>P. berghei</td>
<td></td>
</tr>
<tr>
<td>RBC (x 1000/ml)</td>
<td>10.3</td>
<td>10.1</td>
<td>8.34</td>
<td>9.6</td>
<td>9.32</td>
<td>6.08</td>
<td>10.2</td>
<td>10.53</td>
</tr>
<tr>
<td>WBC (x 1000)</td>
<td>13.3</td>
<td>13.4</td>
<td>6.4</td>
<td>6.8</td>
<td>6.3</td>
<td>5.8</td>
<td>09.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Segmental Neutrophils (%)</td>
<td>14.2</td>
<td>9.7</td>
<td>10.5</td>
<td>8.5</td>
<td>8.8</td>
<td>7.6</td>
<td>10.3</td>
<td>11.3</td>
</tr>
<tr>
<td>Band Neutrophils (%)</td>
<td>4.2</td>
<td>1.2</td>
<td>2.0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>79.4</td>
<td>69.1</td>
<td>42.2</td>
<td>38.2</td>
<td>37.4</td>
<td>36.3</td>
<td>75.4</td>
<td>67.3</td>
</tr>
<tr>
<td>Mononuclear cells (%)</td>
<td>4.1</td>
<td>2.4</td>
<td>2.3</td>
<td>2</td>
<td>1.2</td>
<td>0.9</td>
<td>3.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.9</td>
<td>1.7</td>
<td>1.9</td>
<td>1.2</td>
<td>1.2</td>
<td>1.1</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>1</td>
<td>1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
<td>1.2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Platelets (x 1000)</td>
<td>302</td>
<td>139</td>
<td>134.2</td>
<td>136.2</td>
<td>130.1</td>
<td>126</td>
<td>315</td>
<td>276</td>
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<tr>
<td>PCV (%)</td>
<td>43.2</td>
<td>44.2</td>
<td>37.7</td>
<td>38.7</td>
<td>38.9</td>
<td>26.9</td>
<td>45.2</td>
<td>45.6</td>
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<tr>
<td>MCV (fl)</td>
<td>42.1</td>
<td>43.7</td>
<td>45.2</td>
<td>40.2</td>
<td>41.7</td>
<td>44.2</td>
<td>44.3</td>
<td>43.3</td>
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<tr>
<td>MCH (pg)</td>
<td>16.5</td>
<td>16.3</td>
<td>38.2</td>
<td>36.9</td>
<td>18.4</td>
<td>12.6</td>
<td>16.9</td>
<td>17.9</td>
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<tr>
<td>MCHC (g/dl)</td>
<td>42.3</td>
<td>43.1</td>
<td>37.9</td>
<td>38.1</td>
<td>38.6</td>
<td>34.2</td>
<td>47.5</td>
<td>46.5</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>1.1</td>
<td>0.82</td>
<td>1.4</td>
<td>1.6</td>
<td>1.2</td>
<td>1.9</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.6</td>
<td>0.8</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/I)</td>
<td>61.3</td>
<td>61.9</td>
<td>45.9</td>
<td>45.1</td>
<td>58.9</td>
<td>58.2</td>
<td>64.2</td>
<td>62.8</td>
</tr>
<tr>
<td>ALT (U/I)</td>
<td>43.7</td>
<td>44.6</td>
<td>42.2</td>
<td>41.1</td>
<td>43.3</td>
<td>43.4</td>
<td>43.3</td>
<td>44.4</td>
</tr>
<tr>
<td>AST (U/I)</td>
<td>170.1</td>
<td>173.1</td>
<td>180.2</td>
<td>176.1</td>
<td>190.4</td>
<td>195.2</td>
<td>164.8</td>
<td>175.2</td>
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<tr>
<td>Glucose (g/dl)</td>
<td>119.8</td>
<td>120.4</td>
<td>107.6</td>
<td>117.4</td>
<td>100.9</td>
<td>92.4</td>
<td>114.3</td>
<td>117.3</td>
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</table>

production in T-cells and NK cells leading to protective T helper 1 (Th1) responses against intracellular microbes. In another study by Romero et al. 2007, it was shown that IL-12 deficient mice fail to generate protective immunity to P. berghei even after immunization with sporozoites. Introduction of IL-12 during P. berghei infection both via soluble T. gondii antigens (STAg)-elicited mechanisms and recombinant cytokine treatment protects from ECM pathology by and cytotoxicity (Gracie et al., 2003). The protective roles of IL-12 against the blood stages of both lethal P. berghei ANKA and the non-lethal P. yoelii strain have been documented previously (Singh et al., 2002). Malaria- infected mice in these experiments were found to have increased IL-18 and IL-12 mRNA expression, inflammatory cell infiltration into splenic and hepatic sites, lower necrosis and hemozoin pigment deposition. Interleukin-18 was shown to mediate immunity to blood stages of murine Plasmodia via induction of IFN-γ production and treatment with IL-18 delayed parasitaemia and increased survival rate. In contrast, injection of anti-IL-18 antibodies exacerbated infection and shortened survival of malaria infected mice (Singh et al., 2002). Early production of IFN-γ, a cytokine induced by IL-18, has been linked to protection from murine cerebral malaria (Mitchell et al., 2005). Similarly, this would also be expected to occur in mice that received both IL-18 and CpG motif co-inoculation. High circulating plasma levels of IL-18 have been associated with mild P. falciparum malaria in and simultaneous increases in both IL-18 and IL-12 have been implicated in defense against P. falciparum by modulating the synthesis of inflammatory cytokines (Malaguarnera et al., 2002). Elevated proinflammatory IL-18 cytokine levels were also associated with uncomplicated P. falciparum malaria limiting progression to life-threatening complications (Torre et al., 2001) and IL-18 responses may be impaired with increased P. falciparum malaria severity (Chaiyaroj et al., 2004). Caspase-1 activation of and IL-18, which is
associated with inflammatory pathways, was previously out ruled as contributor to *P. berghei* ANKA-induced immunopathology. Combination of these IL-18 anti-malarial effects would be expected to operate in concert with coincidental immunostimulatory CpG inoculation thereby limiting parasitaemia development as it was witnessed in the current study.

In addition, the absence of cytokine-CpG combinations in the control groups ,especially the *P. berghei* control group, was associated with higher parasitaemia than in the two main groups given such combinations; the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups. Thus, in vivo combination of antimalarial consequences elicited by both cytokines and CpG ODN actors promoted a greater magnitude of protection compared to independent administration of these agents. Cytokine-CpG motif ODN co-therapies done in the current study caused significant reductions in populations of differential *P. berghei* blood stages; ring, trophozoite and schizont stages. This was an expected outcome as it also applied similarly, as reported, to the total parasitaemia results. *Plasmodium berghei* has a preference for infecting reticulocytes but can also invade mature red blood cells. The blood stage development of *P. berghei* in laboratory rodents such as BALB/c mice is usually asynchronous; the different developmental stages, such as rings, trophozoites and schizonts are simultaneously present in the blood during the course of infection (Carter et al., 1977; Landau et al., 1978). During schizogony parasites disappear from the peripheral circulation and sequester in the capillaries of the inner organs, such as lungs, brain and spleen. Adhesion of and sequestration of *Plasmodia* in tissues enables the parasites to evade clearance via splenic mechanisms and *P. berghei* sequestration mechanisms are to a great extent analogous to the mechanisms involved in the sequestration of *P. falciparum* via binding to the CD 36 molecule (Fonager et al., 2012). Overall anti-*Plasmodial* and schizionicidal activities of cytokine-CpG motif co-inoculations could be expected to limit such forms of sequestration by reducing expansion of these differential *P. berghei* forms.

The sequestration of malaria parasites in organ microvasculature has been linked to severe disease and schizonts of both *P. falciparum* and *P. berghei* are known to exhibit clear sequestration capabilities and PfEMP1-mediated sequestration of *P. falciparum* IRBCs is a major feature that has been linked to CM-related pathology (Franke-Fayard et al., 2010). *Plasmodium falciparum* IRBCs become more rigid, more spherical and less deformable as the parasite matures in iRBCs causing difficulties in passage through the microvasculature (Suwanarusk et al., 2004). Although this study did not investigate internal organ damage, it is likely that cytokine CpG motif ODN co-inoculation may reduce organ specific parasite adhesion and sequestration, since it significantly suppressed proliferation of tissue adhering schizont stages (Franke-Fayard et al., 2010). This study has revealed that cytokine-CpG motif ODN co-inoculations in the murine model are associated with less severe clinical features of murine malaria since symptoms like hair ruffling, appetite loss, skin turgor reduction, limb paralysis, convulsions, nervousness in cages and diarrhoea were less experienced in the two groups that received the co-inoculations compared to control groups. Release of merozoites from infected red cells when they rupture causes fever, a hallmark symptom of malaria infection, and the other manifestations of malaria (Svensson et al., 1995). Prodromal symptoms, such as vomiting, nausea, malaise, anorexia, lassitude, dizziness, a desire to stretch limbs and yawn, headache, backache in the lumbar and sacroiliac region, myalgias, and chilliness may occur.

The fever is usually irregular shivering and mild chills although in advanced infections the pattern of fever becomes less regular (Bartoloni et al., 2012). Malarial complications may involve supervening symptoms such as acute renal failure, dysfunctions of hematopoietic systems (for example, severe anaemia) pulmonary oedema, generalized convulsions, circulatory collapse, followed by coma and death. Metabolic acidosis and hypoglycemia are also common systemic complications (Trampuz et al., 2003). Current findings illustrate the ability of cytokine-CpG motif ODN co-inoculations to reduce the severity of various clinical manifestations during *P. berghei* malaria. In consistency with our findings in the control mice groups which had lower body weights, malaria has also been shown to contribute to weight reduction and suboptimal growth especially in children (Sowunmi et al., 2007) and weight reduction effects of *P. berghei* malaria have been published previously (Basir et al., 2012). The current study’s control mice groups had lower appetite which could have resulted in weight loss and lethargy that they also experienced and the *P. berghei* untreated control group particularly experienced the most severe symptoms, in consistency with its high parasitaemia and untreated status.

The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups experienced higher PCV, Hgb, MCH, MCHC and RBC levels compared to *P. berghei* infected control groups. Reductions in these parameters have been linked to untreated malaria in previous communications (Kotepui et al., 2015; Ayodele et al., 2015) and haematological anomalies including anaemia, thrombocytopenia, and leukocytosis or leukopenia and are amongst the major characteristics that accompany malaria infections and they are usually worse in cases of *P. falciparum* infection (Kotepui et al., 2015). In agreement with the current study’s outcomes, the stated haematological parameters have been negatively
correlated with increased parasitaemia and positively correlated with anaemia (Aydeler et al., 2015). The severity and type of anaemia can be determined by the levels of haematological indices such as PCV, Hgb, MCH, MCHC and RBC levels (Dondorp et al., 2000); the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups of mice experienced less severe malarial-related haematological outcomes compared to malaria-infected control groups. This study also demonstrated that the cytokine-CpG combination in the context of murine P. berghei malaria was accompanied by increased levels of total leukocytes, neutrophils, eosinophils, lymphocytes and mononuclear cell counts. Increased WBC counts have been implicated in the control of P. falciparum malaria (McKenzie et al., 2005) and experimental ascaris-elicited eosinophilia has previously been connected to depression of P. berghei infection in mice (Zainal-Abidin et al., 1984) and anti-Plasmodial activities of eosinophils in the human system have also been detailed (Kurtzhals et al., 1998).

The CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups which had the highest WBC and eosinophil and neutrophil counts in comparison to all controls, coincidentally had the lowest parasitaemia levels, and milder clinical manifestations (compared to malarial-infected controls), indicating a possible eosinophilic role in mediation of reduced P. berghei severity. Prolonged neutrophil dysfunction after P. falciparum malaria is related to hemolysis and heme oxygenase-1 induction (Cunnington et al., 2012). Similar to the current cytokine-CpG motif co-inoculation study’s findings, anti-malarial activities of neutrophils were described (Kumaratilake et al., 1992) and neutrophil paralysis was shown to occur in cases of intensive P. vivax malaria (Leoratti et al., 2012). In studies carried out on Thai soldiers, eosinophilia was shown to accompany the healing process following the treatment of P. falciparum malaria (Shanks and Wilairatanaporn, 1992) and a potent eosinophilic response following antimalarial therapy was shown to predict a good recovery from malaria-associated anaemia (Camacho et al., 1999). In agreement with the present study’s findings, it was found that malaria infection significantly associated with reduced lymphocyte counts (Kotepui et al., 2014) and it is known that animals deprived of T lymphocytes suffer severe Plasmodial infections and cannot be immunized against malaria parasites (Allison et al., 1983). CD4+ T cells were shown to expand in P. berghei (NK-65) infected and immunized BALB/c Mice leading to protection (Vineet et al., 2015). Antimalarial immunity can be transferred in mice via adoptive transfer of T lymphocytes of the Ly1+ phenotype, and reduced levels of CD4+, CD8+, B, and CD3+ cells were linked to increased P. falciparum infections (Kassa et al., 2006), graphically illustrating the crucial role of lymphocytes in protection (Allison et al., 1983). Mice lacking CD 4+ and CD 8+ T cells were found to have significantly higher parasitaemias following P. chabaudi infections (Suss et al., 1988). This current study also found that higher P. berghei parasitaemia events were accompanied by both thrombocytopaenia and lower mononuclear cell counts, thus agreeing with previous research findings that indicated lower levels of these cell types being associated with malarial parasitisation (Kotepui et al., 2014). Mononuclear cells are required in elimination of Plasmodia as illustrated by reports of increased nitric oxide production and mononuclear cell nitric oxide synthase activities in malaria-tolerant Papuan adults (Boutilis et al., 2003). The BALB/c mice that were inoculated with both cytokine and CpG motifs displayed significantly higher mononuclear and thrombocyte counts and these were also the same groups with lower parasitisation and more symptomatic. Likewise, platelets have been shown to have significant Plasmodicidal activities as both mouse and human platelets bind malarial-infected red cells and kill the parasite within (McMorran et al., 2009). Preferential binding of platelets to Plasmodium-parasitised cells, triggers the activation and discharge of the Platelet factor 4 (PF4) molecule, which is cytotoxic to the Plasmodia. The PF4 enters into the cell and parasite via the Duffy molecule.

In previous studies (Adeosun et al., 2007; Singh et al., 2015), increased levels of Plasmodium infections were correlated with elevation of bilirubin, creatinine and reduced albumin in plasma samples, concurring with the present outcomes showing that the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups experienced slightly lower creatinine and bilirubin concentrations with simultaneous increases in albumin levels in comparison to the P. berghei-infected control groups. Jaundice with hepatic dysfunction and high levels of serum hyperbilirubinemia have been linked to severe P. falciparum infections (Abro et al., 2009).

Bilirubin is a breakdown product of normal heme catabolism, caused by the body’s clearance of aged or damaged hemoglobin-containing RBC and its concentrations in plasma increase with increased RBC breakdown such as it happens in severe malaria infection. Serum creatinine (a blood measurement) is an important indicator of renal health because it is an easily measured by product of muscle metabolism that is excreted unchanged by the kidneys. Creatinine itself is produced via a biological system involving creatine, phosphocreatine (also known as creatine phosphate), and adenosine triphosphate (ATP, the body’s immediate energy supply). Current findings of slightly lower bilirubin and creatinine values in the CpG/IL-18/ P. berghei and CpG/IL-12/ P. berghei groups therefore reflect, respectively, that these two groups experienced less Plasmodium-driven RBC damage and
less disturbances in kidney functionality. While ALP and ALT levels did not vary significantly among the mice groups. AST levels were higher in *P. berghei*-infected control groups than in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* main experimental groups. Previous experiments showed that with increase in malaria-generated hepatic damage, serum ALT, ALP and AST activities increase, showing positive correlation with liver damage (Abro et al., 2009; Umm-e et al., 2014). Lower concentrations of AST in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* hereby indicate some protection from liver damage was induced by this antimalarial therapy. Alanine aminotransferase (ALT/ALAT) is a transaminase enzyme (EC 2.6.1.2). It was formerly called serum glutamate-pyruvate transaminase (SGPT) or serum glutamic-pyruvic transaminase (SGPT). Alanine aminotransferase (ALT) is found in plasma and in various body tissues, but is most common in the liver. It catalyzes the two parts of the alanine cycle. Alanine aminotransferase (ALT) is commonly measured clinically as a part of a diagnostic evaluation of hepatocellular injury, to determine liver health. When used in diagnostics, it is almost always measured in international units/liter (IU/L). Significantly elevated levels of ALT (SGPT) often suggest the existence of other medical problems such as viral hepatitis, diabetes, congestive heart failure, liver damage, bile duct problems, infectious mononucleosis, or myopathy, so ALT is commonly used as a way of screening for liver problems. Elevated ALT may also be caused by dietary choline deficiency. However, elevated levels of ALT do not automatically mean that medical problems exist. Fluctuation of ALT levels is normal over the course of the day, and they can also increase in response to strenuous physical exercise. Alkaline phosphatase (ALP) is an enzyme in the cells lining the biliary ducts of the liver. Alkaline phosphatase (ALP) levels in plasma rise with large bile duct obstruction, intrahepatic cholestasis, or infiltrative diseases of the liver. Aspartate aminotransferase (AST), also called serum glutamic oxaloacetic transaminase, is similar to ALT in that it is another enzyme associated with liver parenchymal cells. It is raised in acute liver damage, but is also present in red blood cells, and cardiac and skeletal muscle, so is not specific to the liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage. Elevated AST levels are not specific for liver damage, and AST has also been used as a cardiac marker.

The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* mice groups were found to have significantly higher glucose concentrations than *P. berghei*-infected controls including the untreated *P. berghei* group in which extremely low glucose levels were detected. Inhibition of glycogenolysis has been implicated in mediation of hypoglycaemia, which constitutes a major complication of severe malaria (Van Thien et al., 2001). In uncomplicated malaria, insulin resistance may occur, thereby promoting a rise in plasma glucose. Progressive infection increases host/parasite glucose demand and the resulting glucose insufficiency raises the risk of hypoglycaemia (Binh et al., 1997). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* mice groups has similar glucose levels to those detected in normal/uninfected and CpG motif receiving control mice, indicating that cytokine-CpG therapy prevented hypoglycaemic tendencies in this context.

CONCLUSION

One of the major revelations from this project was that cytokine-CpG ODN co-therapy in the BALB/c-*P. berghei* ANKA model drastically reduces parasitaemia progression and such immunotherapeutic interventions mediate milder malarial clinical and haematological outcomes. Not only does the cytokine-CpG ODN combinational DNA therapy suppress total *Plasmodium* parasitaemia but it also causes declines in differential parasitaemia, confers lower peak parasitaemia, and relatively stable total and differential parasitaemia trends. Cytokine-CpG ODN co-injection also strongly impedes haematological damage and symptomatic severity.

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