# CYTOKINE-CpG MOTIF OLIGODEOXYNUCLEOTIDE CO-INOCULATION IN THE MURINE MALARIA MODEL

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# Cytokine-CpG Motif Oligodeoxynucleotide Co-inoculation in the Murine Malaria Model

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other university:

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This thesis has been submitted for examination with my approval as university supervisor:

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## ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| **ACT** | Artemisinin Combinational Therapy |
| **ACUC** | Animal Care and Use Committee |
| **AHR** | Ahryl Hydrocarbon Receptor |
| **ALT** | Alanine transaminase |
| **ALP** | Alkaline phosphatase |
| **Ang:** | Angiogenin |
| **ANOVA** | Analysis of Variance |
| **AST** | Aspartate aminotransferase |
| **BSA** | Bovine Serum Albumen |
| **CBRD** | Center for Biotechnology Research and Development |
| **CDC** | Center for Disease Control and Prevention |
| **COX – 2** | Cyclooxygenase - 2 |
| **DBD** | DNA-binding Domain |
| **dL** | Deciliter |
| **DMEM – 10** | Dulbecco's Modified Eagles Medium - 10 |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **ERC** | Ethical Revieww Comittee |
| **FBS** | Foetal Bovine Serum |

| | |
|---|---|
| **Fl** | Femto-Liter |
| **FOX – P3 / FOXp3** | Forkhead Box Protein - P3 |
| **GM-CSF** | Granulocyte Monocyte Colony Stimulating Factor |
| **Hgb** | Haemoglobin |
| **HRP** | Horse-radish Peroxidase |
| **ICAM - 1** | Intercellular Adhesion Molecule - 1 |
| **IgE and IgM** | Immunoglobulin E and Immunoglobulin M |
| **IL - 18** | Interleukin - 18 |
| **IFN γ** | Interferon Gamma |
| **IRF – 5** | Interferon Regulatory Factor - 5 |
| **IPR** | Institute of Primate Research |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **KEMRI** | Kenya Medical Research Institute |
| **KLF** | Kruppel-Like Factor |
| **Mg** | Microgram |
| **NO** | Nitric Oxide |
| **ODNs** | Oligodeoxynucleotides |
| **µL** | Microlitre |
| **KLF – 1** | Kruppel-like factor - 1 |
| **MCH** | Mean corpuscular hemoglobin |
| **MCHC** | Mean corpuscular hemoglobin concentration |

| | |
|---|---|
| **MCV** | Mean corpuscular volume |
| **mM** | Milli Molar |
| **MMPs** | Matrix Metalloproteinases |
| **MMUST** | Masinde Muliro University of Sciences and Technology |
| **NFATc -1** | Nuclear Factor of Activated T-Cells - 1 |
| **NF-kB** | Nuclear Factor – Kappa Light Chain Enhancer of Activated B Cells |
| **NRP – 1** | Neuropilin - 1 |
| **OD** | Optical Density |
| **ODN** | Oligodeoxynucleotides |
| **PBMC** | Peripheral Blood Mononuclear Cells |
| **PBS** | Phosphate Buffered Saline |
| **PCV** | Packed Cell Volume |
| **Pg** | Picograms |
| **RBC** | Red Blood Cells |
| **RPMI 1640** | Roswell Park Memorial Institute medium |
| **SI** | Stimulation Index |
| **SSC** | Scientific Steering Committee |
| **SOP** | Standard Operating Procedures |
| **STAT – 6** | Signal Transducer and Activator of Transcription-6 |
| **TCR** | T-Cell Receptor |

| | |
|---|---|
| **TF** | Transcription Factor |
| **TH1** | T helper 1 |
| **TLR** | Toll-Like Receptor |
| **TMB** | Tetramethylbenzidene |
| **TNF α** | Tumour Necrosis Factor Alpha |
| **VCAM – 1** | Vascular Cell Adhesion Molecule - 1 |
| **WHO** | World Health Organisation |

## ABSTRACT

Malaria is a global problem that affects up to 500 million people and kills hundreds of thousands every year making it a serious global health predicament. Currently, there is only a partially efficacious vaccine licensed for use against malaria and available therapeutic control measures continue to be impeded by the emergence of drug-resistant strains of *Plasmodia* parasites which cause high morbidity and mortality. Cutting-edge research on anti-malarial mechanisms is intensively focused on biochemical and molecular actors with the potential of improving vaccination and therapy against malaria. The outcome of host-pathogen interactions, with respect to *Plasmodia* parasites, is determined by an extremely delicate balance of various biomolecules, cytokines and host-specific factors. *Plasmodia* parasites evade immune mechanisms and it is still unclear what exact modulations of the immune system are required in their elimination. Proper understanding of the intricate mechanisms underlying the immunopathogenesis of malaria is an essential component in the development of vaccination and therapeutic interventions. A vast array of immunopotentiating molecules like unmethylated CpG motif oligodeoxynucleotides (ODNs) operate in concert with cytokines to mediate resistance to 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, plasmacytoid dendritic cells (pDCs) and NK cells. The current project investigated cytokine-CpG motif ODN co-inoculation in BALB/c mice infected with *P. berghei* ANKA strain. Two BALB/c mice groups were infected with virulent *P. berghei* ANKA strain parasites, followed by five consecutive days of cytokine-involving CpG ODN-based gene therapies. One of the *P. berghei*-infected mice groups received IL-18-CpG ODN, and another one received IL-12-CpG ODN co-inoculation. Six other control groups with various therapeutic regimens were involved in the study. At ten days postinfection, all mice groups were humanely sacrificed for the extraction of EDTA-treated blood, plasma and splenocyte samples which were used to quantify a plethora of transcription factors (TFs), physiologic biomolecules, haematological and clinical chemistry parameters, chemokines, cytokines, immunoglobulin M (IgM) and splenocyte recall proliferation in a multiplicity of bioassays. Analysis using one-factor ANOVA unraveled cytokine-CpG co-immunotherapy as a strong trigger of antimalarial mechanisms that lead to overall parasitaemia reduction, less dramatic parasitaemia trends, milder clinico-haematological outcomes, and protective patterns in expression of TFs, physiologic biomolecules, chemokines, cytokines, IgM, and antigen-specific splenocyte recall proliferation. Murine recipients of the cytokine-CpG ODN co-inoculation ditherapy manifested with increased levels of NF-kB, NFATc, IRF-5, AhR, KLF and reduced levels of FOX-P3 and STAT-6 TFs. They also had enhanced of anti-*Plasmodial* activities accompanied by elevations in adiponectin, ANGPT1, NRP-1 and Cox-2 and delevations in Angiogenin, ANGPT2, MMP-8 and MMP-9 physiologic biomolecules. Cytokine-CpG ODN co-injection triggered upregulated concentrations of CCL-2, CCL-5, CXCL-12, CXCL-1, CX3CL-1, recall proliferation SI and downregulated concentrations of CCL-3, CXCL-5, CXCL-10 and CXCL-16. Augmented quantities of IFN-γ, TNF-α, IL-17, IL-23a, and IgM repressed measurements of IL-4, and IL-10 were detected with widespread ramifications in the potential of cytokine-CpG-based DNA therapy in counteracting malaria, other infectious diseases and medical conditions.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

Malaria is a vector-borne infectious disease caused by protozoan *Plasmodium* parasites. It is a parasitic disease affecting red blood cells (RBC) and hepatocytes and is transmitted by female mosquitoes of the genus *Anopheles*. *Anopheles gambiae* and *A. funestus* are the main vectors of the disease in Africa. Malaria is widespread in tropical and subtropical regions including parts of the Americas, Asia and Africa. Each year there are approximately 250-500 million cases of malaria (WHO, 2012), killing between 1-3 million people, the majority of whom are young children in Sub-Saharan Africa (Snow, Guerra, Noor, Myint, & Hay 2005). Infection of the red blood cells by *Plasmodium* causes recurring fever of sudden onset. *Plasmodium vivax* and *P. ovale* can exist as dormant forms that remain in the liver for months or years before producing the disease. With *P. falciparum*, the disease can reoccur after apparent recovery, due to either inadequate treatment or infection with a drug resistant strain. *P. malariae* can rarely persist with very low levels of parasite in the peripheral blood for decades. *Plasmodium knowlesi* produces acute illness but does not cause relapse. Malaria caused by *P. falciparum* is life threatening and can cause multiple organ damage, coma and death. Additionally, severe malaria complications can result in anaemia, cerebral malaria, and in pregnant women malaria parasites may infiltrate the placenta, a condition called placental malaria (WHO, 2012). Socioeconomically, malaria imposes substantial costs to both individuals and governments. Costs to individuals and their families include purchase of drugs for treating malaria at home; expenses in prevention, lost days of work; expenses for burial in case of deaths. Available therapeutic agents are repeatedly being challenged by the emergence of drug resistant *Plasmodia* strains and the recently licenced GSK-formulated malaria vaccine, RTS,S/AS01-Mosquirix$^{TM}$ is only partially efficatious in children. The global scientific community is making concerted efforts to develop new vaccine and pharmacological agents against malaria (WHO Malaria Facts Sheet, 2015). This project report details some experimental contributions towards that direction.

## 1.2 Statement of the Problem

Malaria is a preventable and treatable mosquito-borne disease, whose main victims are children under five years of age in Africa. The *World Malaria Report 2012* summarized data received from 104 malaria-endemic countries and territories for 2011. Ninety-nine of these countries had on-going malaria transmission. There were about 219 million cases of malaria in 2010 and an estimated 660 000 deaths. Africa is the most affected continent: about 90% of all malaria deaths occur there. Between 2000 and 2010, malaria mortality rates fell by 26% around the world. In the WHO African region the decrease was 33% (Keating, 2012). During this period, an estimated 1.1 million malaria deaths were averted globally, primarily as a result of a scale-up of interventions. Malaria remains inextricably linked with poverty. The highest malaria mortality rates are being seen in countries that have the highest rates of extreme poverty (proportion of population living on less than US$ 1.25 per day). Malaria can kill within 24 hours of symptom onset. Approximately half of the world's population is at risk of malaria. Most malaria cases and deaths occur in sub-Saharan Africa. However, Asia, Latin America, and to a lesser extent the Middle East and parts of Europe are also affected. In 2014, 97 countries and territories had ongoing malaria transmission.

According to the latest estimates, released in December 2014, were 198 million cases of malaria worldwide in 2013 (with an uncertainty range of 124 million to 283 million), with 82% of these cases occurring in Africa. There were an estimated 584,000 malaria deaths worldwide in 2013 (with an uncertainty range of 367 000 to 755 000). Although malaria mortality rates have fallen by 47% globally since 2000, and by 54% in the WHO African region, 78% of global malaria deaths were estimated to occur in children under 5 years of age children. Sixteen countries account for 80% of malaria deaths: Nigeria, Democratic Republic of the Congo, India, Angola, United Republic of Tanzania, Uganda, Ghana, Niger, Chad, Mozambique, Burkina Faso, Ethiopia, Côte d'Ivoire, Mali, Guinea, and Cameroon. Two African countries account for nearly 40% of global malaria deaths. Malaria accounts for 40% of total government spending on public health in Africa, and costs Africa US$ 12 billion in direct costs every year and much more in lost productivity (WHO Malaria Facts Sheet, 2015; WHO 2014 World Malaria Report).

## 1.2 Justification of the Study

Investigations on biochemical and molecular agents capable of improving vaccination and therapy, encompass an active part of current malaria research (Pattaradilokrat *et al.,* 2014). The immunotherapeutic combination of CpG ODNs with cytokines like IL-18 and IL-12 is likely to generate more upregulated immunopotentiation compared to independent administration of these components. The biotherapetic co-administration has the potential of combining the advantages of both CpG ODNs gene treatment and the cytokines' effects *in vivo*. Recent studies have indicated that sysnergistic mechanisms are initiated through TLR9 (Toll Like Receptor 9) pathways *in vivo* after cytokines and immunostimulatory CpG oligodeoxynucleotides (ODN) motifs are co-inoculated leading to strong and full protection against some parasitic infections (Li *et al.,* 2004). However, prior to this current research, the effects of such internal interactions and the accompanying improved immunocompetence had not been evaluated in any host in the context of malaria infections.

Cytokines like IL-12 and IL-18 have multiple proven anti-*Plasmodial* properties (Angulo & Fresno, 2002). IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T cells. It stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ. T cells that produce IL-12 have a coreceptor, CD30, which is associated with IL-12 activity. IL-12 plays an important role in the activities of natural killer cells and T lymphocytes. Interleukin-18 is one of the pro-inflammatory cytokines. The activities of IL18 appear to be species specific. An important function of IL18 is the regulation of functionally distinct subsets of T-helper cells required for cell mediated immune responses (Nakanishi, Yoshimoto, Tsutsui, & Okamura 2001). IL18 functions as a growth and differentiation factor for Th1 cells. IL18 upregulates FAS ligand mediated cytotoxic activitiy of murine natural killer cells. IL18 is part of a complex regulatory circuit involved in causing cell death by apoptosis. IL18 induces activated B-cells to produce IFN-gamma that inhibits IgE production (Yoshimoto, Okamura, Tagawa, Iwakura, & Najanishi 1997). Interleukin-18 has been shown to strongly augment the

production of IFN-gamma by T-cells and NK-cells (Micallef *et al*, 1996). The ability of IL18 to enhance IFN-gamma production by NK-cells is dependent on the presence of IL12 (Walker, Aste-Amezaga, Kastelein, Trinchieri, & Hunter, 1999). IGIF enhances T-cell proliferation apparently through an IL2 dependent pathway (Micallef *et al*, 1996). The inflammasome multimeric oligomeric protein complexes that are crucial for host defense to infection and endogenous danger signals promote the secretion of the pro-inflammatory cytokines like interleukin (IL)-1b and IL-18 and cause a rapid and pro-inflammatory form of cell death called pyroptosis. Inflammasomes assemble in the cytoplasm of innate immune cells, such as macrophages and dendritic cells, in response to cytosolic pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs [Latz *et al.,* 2013]) and are critical agents in ati-pathogenic reactions.

The immunopotentiating effects of CpG ODNs include 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, which drive immune responses, and secretion of a variety of cytokines, including high levels of IL-12 (Weeratna, Krieg, & Davis 1999). Additionally, synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA, and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes. Immunostimulatory activities of CpG-ODNs have gained attention as potentially useful therapeutics for immune adjuvant, inflammatory and allergic disease, and for immunoprotective agent. Utilising cutting-edge nanotechnological mechanisms, nanoparticle-bound CpG ODNs have been successfully used to deliver drugs to targeted cells leading to pinpoint delivery and increased immunopotentiating capabilities (Titta *et al.,* 2013), thereby increasing potential applications of such CpG ODN-based immunotherapeutic nanoscale-complexes in dealing with infectious diseases.

Combination of the above described properties of IL-12 and IL-18 in a host, coupled with their inflammasomal functions (Latz, Xiao, & Stutz, 2013), with immunostimulatory CpG ODN motif capabilities can therefore be expected to operate in concert against

microbial agents such as *Leishmania* and *Plasmodia* in a synergistic fashion. However, before this report, it was still unknown whether or not the potential cytokine-CpG synergistic effects could reduce host parasitisation by *Plasmodium.* The potential system-wide effects of cytokine-CpG motif co-inoculations, including effects on transcription factors, physiologic biomolecules, chemokine expression, haematological, humoral and cell mediated immune mechanisms were still yet to be elucidated. It was unknown whether the combined presence cytokine-CpG molecules in host organisms could still initiate the traditional outcomes such as the ones achieved when they are co-administered in other biological experimental situations. By utilising the murine malaria model, this project yielded results that furnished greater insights into the protective effects of cytokine-CpG motif ODN co-inoculations following malaria infection. Current findings indicate that cytokine-CpG ODN biothepeutic immunomodulations are accompanied by a wide spectrum of protective clinico-haematological, immunoparasitological, TF and physiological biomolecular profiles. This research's findings now support the continued investigation and utilisation of cytokine-involving gene therapy, paving the way towards a new CpG-DNA based immune system manipulation with immense ramifications in the way infectious diseases will be combated both at preventative and therapeutic scales.

### 1.3 Research Questions

i) How does cytokine-CpG motif ODN co-inoculation modulate clinical, haematological, parasitological parameters in BALB/c mice infected with *P. berghei* ANKA strain?

ii) How does cytokine-CpG motif ODN co-inoculation modulate transcription and physiologic biomolecular factors in BALB/c mice infected with *P. berghei* ANKA strain?

iii) How does cytokine-CpG motif ODN co-inoculation modulate chemokine expression and recall proliferation responses in BALB/c mice infected with *P. berghei* ANKA strain?

iv) How does cytokine-CpG motif ODN co-inoculation modulate antibody and cytokine responses in BALB/c mice infected with *P. berghei* ANKA strain?

## 1.4 Null Hypothesis

There are no differences in clinical, haematological, parasitological, transcription factor, physiologic biomolecular, and immunological manifestations between BALB/c mice with cytokine-CpG motif ODN co-inoculation and BALB/c mice without cytokine-CpG motif ODN co-inoculation in the context of *P. berghei* ANKA strain malaria infection.

## 1.5 Objectives

### 1.5.1 General Objective

To characterise the diverse effects of cytokine-CpG motif ODN co-inoculation in BALB/c mice infected with *P. berghei* ANKA strain.

### 1.5.2 Specific Objectives

i) To determine effects of cytokine-CpG motif ODN co-inoculation on clinical, haematological, and parasitological profiles in BALB/c mice infected with *P. berghei* ANKA strain.

ii) To determine effects of cytokine-CpG motif ODN co-inoculation on transcription factor and physiologic biomolecular responses in BALB/c mice infected with *P. berghei* ANKA strain.

iii) To determine effects of cytokine-CpG motif ODN co-inoculation on chemokine expression and recall proliferation responses in BALB/c mice infected with *P. berghei* ANKA strain.

iv) To determine effects of cytokine-CpG motif ODN co-inoculation on antibody and cytokine responses in BALB/c mice infected with *P. berghei* ANKA strain.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Malaria

### 2.1.1 Introduction to Malaria

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans (a type of unicellular microorganism) of the genus *Plasmodium* (Bartoloni *et al.,* 2012). Commonly, the disease is transmitted via a bite from an infected female *Anopheles* mosquito, which introduces the organisms from its saliva into the person's circulatory system. In the blood, the protists travel to the liver to mature and reproduce. Malaria causes symptoms that typically include fever and headache, which in severe cases can progress to coma or death. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas (Bartoloni *et al.,* 2012). Five species of *Plasmodium*can infect and be transmitted by humans. The vast majority of deaths are caused by *P. falciparum* and *P. vivax*, while *P. ovale*, and *P. malariae* cause a generally milder form of malaria that is rarely fatal. The zoonotic species *P. knowlesi*, prevalent in Southeast Asia, causes malaria in macaques but can also cause severe infections in humans. Malaria is prevalent in tropical and subtropical regions because rainfall, warm temperatures, and stagnant waters provide habitats ideal for mosquito larvae. Disease transmission can be reduced by preventing mosquito bites by using mosquito nets and insect repellents, or with mosquito-control measures such as spraying insecticides and draining standing water (Keating, 2012).

Malaria is typically diagnosed by the microscopic examination of blood using blood films, or with antigen-based rapid diagnostic tests. Modern techniques that use the polymerase chain reaction to detect the parasite's DNA have also been developed, but these are not widely used in malaria-endemic areas due to their cost and complexity. The World Health Organization has estimated that in 2010, there were 219 million documented cases of malaria. That year, the disease killed between 660,000 and 1.2

million people (WHO, 2012), many of whom were children in Africa (Appendix III and IV). The actual number of deaths is not known with certainty, as accurate data is unavailable in many rural areas, and many cases are undocumented. Malaria is commonly associated with poverty and may also be a major hindrance to economic development.

Despite a need, no effective vaccine currently exists, although efforts to develop one are ongoing. Several medications are available to prevent malaria in travellers to malaria-endemic countries (prophylaxis). A variety of antimalarial medications are available. Severe malaria is treated with intravenous or intramuscular quinine or, since the mid-2000s, the artemisinin derivative artesunate, which is superior to quinine in both children and adults and is given in combination with a second anti-malarial such as mefloquine. Resistance has developed to several antimalarial drugs; for example, chloroquine-resistant *P. falciparum* has spread to most malarial areas, and emerging resistance to artemisinin has become a problem in some parts of Southeast Asia (Bartoloni *et al.*, 2012; Nadjm & Behrens, 2012).

### 2.2.1 Vaccine Agents under Development Against Malaria

A completely effective vaccine is not yet available for malaria, although several vaccines are under development. SPf66 a synthetic peptide based vaccine developed by Manuel Elkin Patarroyo team in Colombia was tested extensively in endemic areas in the 1990s, but clinical trials showed it to be insufficiently effective, 28% efficacy in South America and minimal or not efficacy in Africa (Graves, & Gelband 2006). Other vaccine candidates, targeting the blood-stage of the parasite's life cycle, have also been insufficient on their own. Several potential vaccines targeting the pre-erythrocytic stage are being developed, with RTS,S showing the most promising results so far., (RTS,S Clinical Trials Partnership *et al.*, 2012) The CSP (Circum-Sporozoite Protein) was a vaccine developed that initially appeared promising enough to undergo trials. It is also based on the circumsporozoite protein, but additionally has the recombinant (Asn-Ala-Pro15Asn-Val-Asp-Pro) 2-Leu-Arg (R32LR) protein covalently bound to a purified

*Pseudomonas aeruginosa* toxin (A9). However, at an early stage a complete lack of protective immunity was demonstrated in those inoculated. The study group used in Kenya had an 82% incidence of parasitaemia whilst the control group only had an 89% incidence. The vaccine intended to cause an increased T-lymphocyte response in those exposed, this was also not observed. The NYVAC-Pf7 multi-stage vaccine attempted to use different technology, incorporating seven *P. falciparum* antigenic genes. These came from a variety of stages during the life cycle. CSP and sporozoite surface protein 2 (called PfSSP2) were derived from the sporozoite phase. The liver stage antigen 1 (LSA1), three from the erythrocytic stage (merozoite surface protein 1, serine repeat antigen and AMA-1) and one sexual stage antigen (the 25-kDa Pfs25) were included. This was first investigated using Rhesus monkeys and produced encouraging results: 4 out of the 7 antigens produced specific antibody responses (CSP, PfSSP2, MSP1 and PFs25). Later trials in humans, despite demonstrating cellular immune responses in over 90% of the subjects had very poor antibody responses (GSK Press Releases, 2015).

In 1995 a field trial involving [NANP] 19-5.1 proved to be very successful. Out of 194 children vaccinated none developed symptomatic malaria in the 12 week follow up period and only 8 failed to have higher levels of antibody present. The vaccine consists of the schizont export protein (5.1) and 19 repeats of the sporozoite surface protein [NANP]. Limitations of the technology exist as it contains only 20% peptide and has low levels of immunogenicity. It also does not contain any immunodominant T-cell epitopes.

RTS,S is the most recently developed recombinant vaccine. It consists of the *P. falciparum* circumsporozoite protein from the pre-erythrocytic stage. The CSP antigen causes the production of antibodies capable of preventing the invasion of hepatocytes and additionally elicits a cellular response enabling the destruction of infected hepatocytes. The CSP vaccine presented problems in trials due to its poor immunogenicity. The RTS,S attempted to avoid these by fusing the protein with a surface antigen from Hepatitis B, hence creating a more potent and immunogenic vaccine. When tested in trials an emulsion of oil in water and the added adjuvants of monophosphoryl A and QS21 (SBAS2), the vaccine gave protective immunity to 7 out of 8 volunteers when challenged with *P. Falciparum* (RTS,S Clinical Trials Partnership *et al.*, 2012).

RTS,S/AS01 (commercial name: Mosquirix™), was engineered using genes from the outer protein of *Plasmodium falciparum* malaria parasite and a portion of a hepatitis B virus plus a chemical adjuvant to boost the immune system response. Infection is prevented by inducing high antibody titers that block the parasite from infecting the liver. It is being developed by the non-profit PATH Malaria Vaccine Initiative (MVI) and GlaxoSmithKline (GSK) with support from the Bill and Melinda Gates Foundation. In November 2012 a Phase III trial of RTS,S found that it provided modest protection against both clinical and severe malaria in infants (RTS,S Clinical Trials Partnership *et al.,* 2012).

The GlaxoSmithKline (GSK)-formulated malaria vaccine, named RTS,S, is said to have reduced the amount of cases amongst young children by almost 50 percent and among infants by around 25 percent, following the conclusion of an 18-month clinical trial. In a bid to expand the novel vaccine program to accommodate a larger group and guarantee a sustained availability for the general public, the GlaxoSmithKline did submit an application for a marketing license with the European Medicines Agency (EMA) in July 2014 (GSK, 2014). GlaxoSmithKline embarked on this project as a non-profit initiative, with most of its funding coming from the Bill and Melinda Gates Foundation, a major contributor to malaria eradication in Africa. GlaxoSmithKline has been developing the Malaria vaccine for three decades, and now has the backing of the UN's Swiss-based WHO, saying it will recommend the use of RTS,S for use starting in 2015, providing it gets approval (Kelland, 2013). On 24$^{th}$ July 2015, Mosquirix$^{TM}$ received a positive opinion from the EMA to be used to vaccinate children aged 6 weeks to 17 months outside the European Union (EMA, 2015). The WHO formulated a policy recommendation on use of the vaccine in national immunisation programs approved by national regulatory authorities in the sub-Saharan African countries (GSK, 2015). The SANARIA® PfSPZ vaccine is a candidate malaria vaccine developed by Sanaria (Zhang, Chavchich, & Waters 2012). Clinical trials have been promising, but it has been subject to some criticism regarding the ultimate feasibility of large-scale production and delivery in Africa.

### 2.3.0 The Mouse-*P. berghei* Infection as an Animal Model in Malaria Research

Although human malaria parasites are seen as a major priority for research because of the mortality associated with this infection, they are not always the optimal system in which to study the biology and immunology of *Plasmodium*. The host specificity of human malaria parasites represents a major constraint on *in vivo* studies (Ozwara *et al.,* 2003). A good laboratory model should be relevant for human malaria and offer the ability to study the biology of the parasite at the cellular and molecular level. Immunobiological and chemotherapeutic studies have made use of the fact that a number of *Plasmodium*species found naturally in mice like *P. berghei* are very similar biologically and antigenically and have similar host-parasite relationships to the human-infecting *Plasmodia* (Ozwara *et al.,* 2003). Murine malaria parasites like *P. berghei* are used in many research institutes for studies aiming at the development of new drugs or a vaccine against malaria (Carter & Diggs, 1977). *Plasmodium berghei* is a valuable model organism for the investigation of human malaria because it is similar to human malaria parasites in most essential aspects of morphology, physiology and life cycle and the manipulation of the complete lifecycle of the parasite, including mosquito infections, is simple and safe (Carter & Diggs, 1977).

### 2.3.1 Valuable Characteristics of *P. berghei* as a Model in Malaria Studies

*Plasmodium berghei* is an important model parasite for the investigation of the developmental biology of malaria parasites (Figure 2.0), parasite-host interactions, vaccine development and drug testing due to several reasons: the basic biology of rodent and human parasites is similar; the genome organisation and genetics is conserved between rodent and human parasites; housekeeping genes and biochemical processes are conserved between rodent and human parasites; the molecular basis of drug-sensitivity and resistance show similar characteristics in rodent and human parasites; the structure and function of vaccine candidate target antigens are conserved between rodent and human parasites (for example, CTRP, P25 and 5 P28 of ookinetes; AMA1 and MSP1 of merozoites; P45/48, P47 and P230 of gametes); rodent parasites allow *in vivo*

investigations of parasite-host interactions and *in vivo* drug testing; technologies for *in vitro* cultivation and large scale production and purification of the different life cycle stages are available and methodologies for genetic modification are also available (Ozwara *et al.*, 2003).

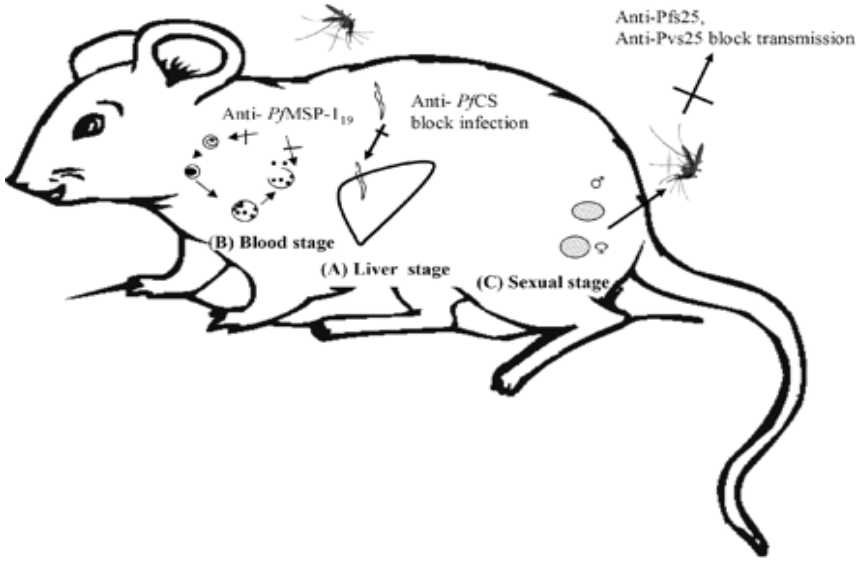



**Figure 2.0: *Plasmodial* parasite life cycle stages for which murine transgenic parasites expressing human malaria antigens are available.**

There currently exist and can be used to assess functional immune responses *in vivo.* (A) Liver stage, CS(Pf); (B) asexual blood stage, Pb-PfM19; and (C) sexual stage, Pv25DR, Pv25DR3, and TrPfs25Pb.

## 2.4 Immune Responses to Malaria

### 2.4.1 Interaction of *Plasmodia* with the Immune System

Both the cellular and humoral arms (Barasa, Maamun, Kagasi, Ozwara, & Gicheru. 2010a) of the adaptive immune system are pivotal elements in the eradication of *Plasmodium*from the body, and both are critically dependent on α/ß CD4+ lymphocytes. It has been firmly established that CD4+ T cells are comprised of at least two

functionally different subsets, distinguished on the basis of cytokine secretion in Th1 (IFN--producing) and Th2 (interleukin-4 [IL-4]/IL-5-producing) cells (Dong & Flavell 2001). The CD4+ T cells, of either Th1 or Th2 type, also have regulatory functions in human *P. falciparum* malaria. Both Th1 and Th2 responses seem to be required to control the infection, but they need to be adequately tuned in intensity and time. Thus, both Th1 and Th2 are activated during infection with *P. yoelii* 17XL in C57BL/6 mice, but early activation of Th2 cells is deleterious, conferring susceptibility to infection (Kobayashi *et al.,* 1996). In addition, in *P. chabaudi chabaudi* AS infection there is a shift from Th1 to Th2 during peak parasitemia that is important for clearance of parasites (Helmby, Kullberg & Troye-Blomberg 1998). The CD8+ T lymphocytes contribute to the fight against the parasite through secretion of cytokines as IFN-γ.

### 2.4.2 Involvement of Cytokines in Early Protection from Malaria

In immunity to malaria, there is usually a requirement for early production of IFN-γ to give resistance against infection. In support of this view, analysis of IFN-γR$^{-/-}$ mice infected with *P. chabaudi chabaudi* reveals a critical role of IFN-γ in immunity against this pathogen, although the production of parasite-specific immunoglobulins was not affected (Favre, Ryffel, Bordmann, & Rudin 1997). Interestinglyit was reported that IFN responsive factor (IRF-1)$^{-/-}$ mice infected with *P. berghei* showed lower mortality than wild-type mice, although they produced no IFN-γ or NO (Tan, Feng, Asano, & Kara, 1999). These animals suffered a later onset of disease and a reduced peak of parasitemia. These results may therefore indicate that IFN-γ and/or other molecules under the control of IRF-1 can actually have a pathological role. However, a striking lack of correlation between changes in parasitemia and clinical symptoms has been repeatedly observed (Cross, & Langhorne 1998). To illustrate this point, in C57BL/6 mice infected with *P. yoelii* 17XL and treated with anti-IFN-γ (exacerbating effect) or anti-IL-10 (protective effect) there were severe consequences on survival with no apparent changes in parasitemia (Kobayashi, Morii, Matsui, Fujino, Watanabe, Weidanz & Tsuji 2000).

It is likely that there are mechanisms of resistance independent of IFN-γ and NO. In this regard, it is interesting that *in vivo* treatment with anti-IFN-γ exacerbates *P. yoelii* 17XL infection in C57BL/6 because mice treated with antibody die earlier (Kobayashi *et al.,* 2000). In contrast, treatment with aminoguanidine, an irreversible inhibitor of NO production, has no effect (Kobayashi *et al.,* 2000). Consistently, mice lacking inducible nitric oxide synthase (iNOS$^{-/-}$) cleared *P. berghei* XAT (an attenuated variant of *P. berghei* NK65) as effectively as did wild-type animals. In this case, resistance was dependent on IFN-γ, since its *in vivo* blocking provoked progression of parasitemia and death (Yoneto *et al.,* 1997). Again, the evidence supports an absence of strict dependency between IFN-γ and NO.

An important role for IL-12 in early responses against *Plasmodium*has been proposed, and although it may have a role in making mice resistant or susceptible, it is also possibly involved in the pathology itself (Adachi *et al.,* 2001). IL-12 appears to be critically linked to protection through IFN-γ production, thereby allowing an early and sustained Th1 response. Thus, after infection of C57BL/6 GKO (IFN-γ$^{-/-}$) with *P. chabaudi* AS, higher levels of parasitemia during acute infection and severe mortality were found. These phenomena were associated with reduced amounts of IL-12p70, TNF-α, and NO. In contrast, in addition to its role in resolving primary infection, IL-12 has been shown to be required for the production of a protective immunoglobulin G2a (IgG2a) antibody. This finding suggests that the immunoregulatory activity of IL-12 extends to the antibody responses against *Plasmodium*.

Many reports indicate that IL-18 enhances Th1 immune responses depending on IL-12, but it can also potentiate Th2 immune responses when IL-12 is not available (Nakanishi *et al.,* 2001). Concerning the role of IL-18 on early immunity against *Plasmodium*, recent results have suggested that IL-18 plays a protective role by enhancing IFN-γ production *in vivo*. Hence, it has been shown that IL-18 knockout mice were more susceptible to *P. berghei* ANKA than wild-type mice. Besides, administration of neutralizing IL-18 antibody exacerbated infection in wild-type mice C57BL/6. Noteworthy, IL-18 has been found not to be absolutely required for hepatotoxicity produced after infection with *P. berghei*, which is actually dependent on IL-12 production (Adachi *et al.,* 2001).

Therefore, although much of the evidence available suggests that both IL-18 and IL-12 act, at least in part, through induction of IFN-γ, these two proinflammatory cytokines do play different specific roles in responses against *Plasmodium*. A similar picture to that depicted for IFN-γ emerges for the role of TNF-α in early responses against *Plasmodium.* Treatment with anti-TNF-α monoclonal antibody results in a tendency toward longer times for parasite clearance. Interestingly, this effect is associated with reduced levels of IFN-γ. In support of this general view, an association between the ability to produce high levels of TNF-α and an accelerated cure and improved prognosis has been reported in humans (Mordmuller *et al.,* 1997).

### 2.4.3 Cytokines in Malaria Immunopathophysiology

The pathogenesis of malaria is complex and most likely entails immunologic and nonimmunologic mechanisms (Kobayashi *et al.,* 2000). In general, it is now accepted that severe malaria is the consequence of alterations in many tissues and organs. These dysfunctions often lead to metabolic acidosis and localized ischemia. In this section, we focus on the involvement of cytokines in the immunologic mechanisms. It is evident that parasite factors can contribute to the severity of disease, as is clear from their ability to infect a high percentage of erythrocytes or to induce production of proinflammatory cytokines. In particular, much evidence has been accumulated that points to glycosylphosphatidylinositols from *Plasmodium*as important pathogenic factors due to their ability to induce TNF-α and IL-1 (Adachi *et al.,* 2001).This view is strongly supported by the fact that the toxicity of malarial parasite extracts can be neutralized with monoclonal antibodies against this moiety in experimental models It is noteworthy that recent work suggests that the presence of anti-glycosylphosphatidylinositol antibodies in the serum of patients may provide protection against clinical symptoms of malaria Therefore, cytokines, viewed as potential pathogenic elements, can contribute either directly or indirectly to many pathological processes (Kobayashi *et al.,* 2000). Of these, control of cerebral malaria (CM) and severe malarial anaemia (SMA) are critical for

patient care. Although both *P. falciparum* and *P. vivax* can cause SA, only *P. falciparum* causes the many complications associated with CM.

Cerebral malaria (CM) is characterized by a coma situation in patients with *P. falciparum* infection that is often accompanied by metabolic acidosis, seizures, and hypoglycemia. Animal models of malaria have provided convincing evidence of the important role of inflammatory processes in the development of CM. Monkey, rat, and mouse models for CM have been developed, although none of them completely duplicates the situation in humans. The most complete information has been obtained from experiments in mice. And there has been emphasis on the role of adhesion molecules and platelets in immune-mediated damage of vascular endothelium of the brain. Not all proinflammatory cytokines are equally relevant for the development of CM. The best-documented evidence implicates IFN-γ, TNF-α, and IL-12, whereas no evidence has been found for IL-6 (Kobayashi *et al.*, 2000).In the case of IL-12 and/or IFN-γ, strong support for their involvement in the pathogenesis of CM comes from studies with knockout mice infected with *P. berghei* ANKA. Consistently, experiments with IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ have revealed an essential requirement of this cytokine in the development of CM (Yoneto *et al.*, 1999). Overall, an inhibition of TNF-α production and ICAM-1 expression (Yoneto *et al.*, 1999), or IL-12 production was found in experiments with these animals. Interestingly, NO seems not to be involved in the pathogenesis of CM. In addition, IRF-$1^{-/-}$ mice infected with *P. berghei* were resistant to CM (Yoneto *et al.*, 1999), a result consistent with a major pathological role for IL-12 and IFN-γ.

Less clear is the role of anti-inflammatory cytokines in the control of CM. Although data based on knockout mice indicate that IL-4 and IL-10 are not required for development of CM, in models in which IL-12 and IFN-γ display a dominant pathological role a protective effect has been demonstrated for IL-10. It was found that infected wild-type mice mounted an early Th1 response that shifted to a late Th2 response, whereas in infected IRF-$1^{-/-}$ animals an early protective Th2 was found that avoids Th1-dependent pathological damage. Moreover, neutralization of IL-10 *in vivo* has been demonstrated to increase the percentage of mice with CM in a CM-resistant strain (Favre *et al.*, 1997). Interestingly, sex-related determinants might play a role in determining the relative

importance of IL-10. Thus, infection of IL-$10^{-/-}$ mice with *P. chabaudi chabaudi* (AS) led to exacerbated pathology in female mice. In that study hypoglycemia, hypothermia, and loss of body weight were significantly greater in female IL-$10^{-/-}$ mice than in male knockout mice and all wild-type mice during the acute phase of infection (Favre *et al.*, 1997). The role of TNF-α in CM seems to be well established. Thus, transgenic mice expressing high levels of soluble TNFR-1-FcIgG3 under the control of the $\alpha_1$-antitrypsin gene promoter are markedly protected from CM caused by *P. berghei* (Kobayashi *et al.*, 2000). However, apparently contradictory findings were reported (Shear, Marino, Wanidworanun, Berman, & Nagel, 1998), who used TNF-$\alpha^{-/-}$ mice infected with nonlethal *P. yoelii*. These animals had slightly higher levels of infected erythrocytes, but their susceptibility to death from this infection was unaffected. It was therefore concluded that TNF-α was not absolutely required for death. A possible explanation could lie in the time and site of cytokine production. In this regard it has been shown that TNF-α, produced locally in the central nervous system, has an essential role in the development of CM (Angulo *et al.,* 2002). Moreover, TNFR$2^{-/-}$ mice but not TNFR$1^{-/-}$ mice were considerably protected from CM. This protection was associated with a lack of upregulation of ICAM-1. It has been suggested that membrane-TNF-α-TNFR2 interactions are critical for CM because of the requirement for TNF-α in the upregulation of ICAM-1 in cerebral vascular endothelium (Kobayashi *et al.*, 2000).

In any case, other factors also seem to play a decisive role in the development of CM. Thus, despite a production of TNF-α similar to that in wild-type animals, CD$40^{-/-}$ and CD$40L^{-/-}$ mice infected with *P. berghei* ANKA were protected from CM and survived. In addition to the unchanged systemic production of TNF-α, the expression of TNF-α in the brains of these mutants which did not correlate with local macrophage sequestration and circulating, but not cerebral TNF-α is sufficient to induce upregulation of CD54 (ICAM-1) in brain endothelial cells (Angulo *et al.,* 2002).

## 2.5 CpG Motif Oligodeoxynucleotides (ODNs)

### 2.5.1 CpG Motif Oligodeoxynucleotides and their Immunostimulatory Roles

Bacterial DNA, but not vertebrate DNA, causes direct stimulation of several components of the vertebrate immune system (Figure 2.1). This activation is due to the presence of unmethylated CpG dinucleotides (Weeratna *et al.,* 1999), which are present at the expected frequency in bacterial DNA, but are underrepresented and methylated in vertebrate DNA. The immunostimulatory effects include 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, which drive immune responses, and secretion of a variety of cytokines, including high levels of IL-12 (Weeratna *et al.,* 1999). These cytokines then, in turn, stimulate natural killer (NK) cells to secrete IFN-γ and to have increased lytic activity. Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-γ. These effects can also be obtained with synthetic oligodeoxynucleotides (ODN) or plasmid DNA vectors containing CpG immunostimulatory motifs (Klinman, Conover, & Coban 1999). However, CpG motif ODN also have the potential of causing polyclonal B cell activation (Appendix VIII) leading to autoimmunity and in the presence of LPS (lipopolysaccharide) they have been reported to facilited septic shock. A recent study involving *Leishmania major* parasites demonstrated that cytokine-CpG motif co-inoculation leads to strong protection against *L. major* (Li *et al.,* 2004).

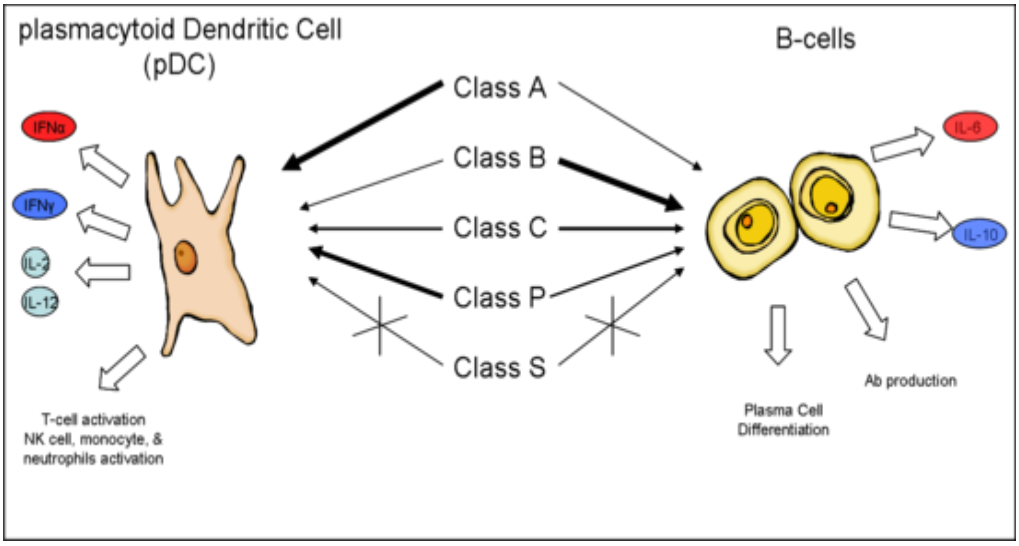



**Figure 2.1: The different classes of ODN elicit different responses in pDC and B cells.** Class A CpG ODNs activate dendritic cells and IFN- γ production, while class B activat B cells. Class C CpG ODNs possess the properties of both class A and B CpG ODNs. Source: (Li *et al.*, 2004).

CpG oligodeoxynucleotides (or CpG ODN) are short single-stranded synthetic DNA molecules that contain a cytosine triphosphate deoxynucleotide ("C)" followed by a guanine triphosphate deoxynucleotide ("G"). The "p" refers to the phosphodiester link between consecutive nucleotides, although some ODN have a modified phosphorothioate (PS) backbone instead. When these CpG motifs are unmethlyated, they act as immunostimulants (Weiner, Liu, Wooldridge, Dahle, & Krieg 1997). CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes but their rarity in vertebrate genomes (Bauer & Wagner, 2002). The CpG PAMP is recognized by the pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9; appendices V and VI), which is constitutively expressed only in B cells and plasmacytoid dendritic cells (pDCs) in humans and other higher primates (Rothenfusser, Tuma, Endres & Hartmann, 2002). Since 1893, it has been recognized that Coley's toxin, a mixture of bacterial cell lysate, has immunostimulatory properties that could reduce the progression of some carcinomas (Coley, 1991), but it was not until 1983 that it was specifically identified bacterial DNA as the underlying component of the lysate that elicited the response (Tokunaga *et al.*, 1984). Then Krieg *et al.*, (1995) demonstrated that

the CpG motif within bacterial DNA was responsible for the immunostimulatory effects and developed synthetic CpG ODN (Krieg *et al.,* 1995). Since then, synthetic CpG ODN have been the focus of intense research due to the Type I pro-inflammatory response they elicit and their successful use as vaccine adjuvants. Innate immune system recognizes bacterial DNA containing unmethylated CpG dinucleotides in the context of particular base sequences (CpG motifs) to secrete various cytokines. Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA, and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes. Immunostimulatory activities of CpG-ODNs have gained attention as potentially useful therapeutics as immune adjuvants, inflammatory and allergic disease control, and for use as immunoprotective agents (Figures 2.2 and 2.3).

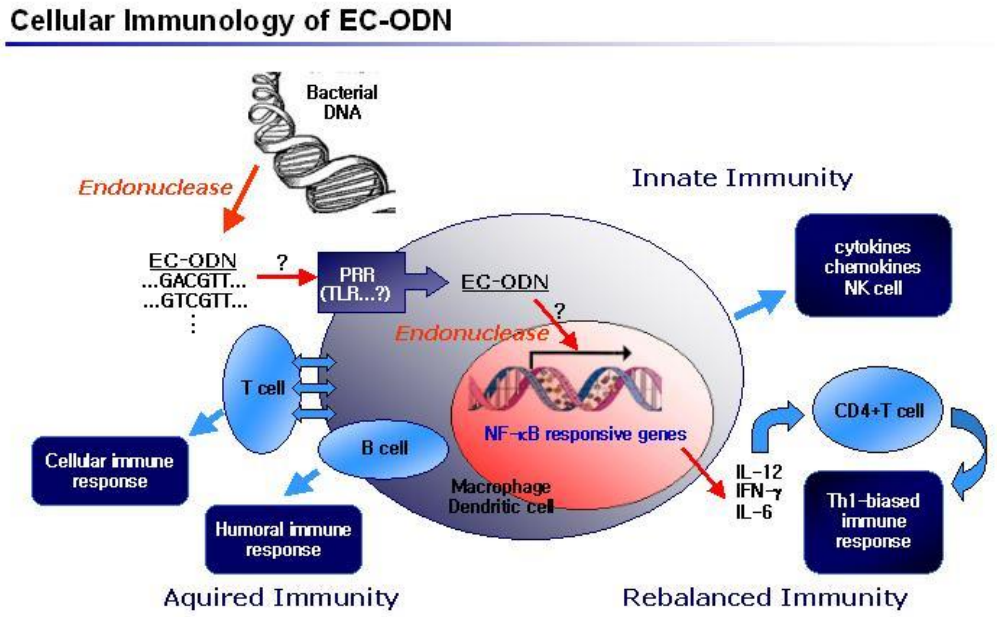



**Figure 2.2: CpG ODNs' involvement in both innate and acquired immune parameters.** Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA. Source: (Li *et al.,* 2004).

### 2.5.2 Synthetic CpG Oligodeoxynucleotide and CpG Oligodeoxynucleotide Classes

Synthetic CpG ODN differ from microbial DNA in that they have a partially or completely phosphorothioated (PS) backbone instead of the typical phosphodiester backbone and a poly G tail at the 3' end, 5' end, or both. PS modification protects the ODN from being degraded by nucleases such as DNase in the body and poly G tail enhances cellular uptake (Dalpke, Zimmermann, Albrecht, & Heeg, 2002). The poly G tails form intermolecular tetrads that result in high molecular weight aggregates. These aggregates are responsible for the increased activity the poly G sequence imparts; not the sequence itself (Wu, Lee, Raz, Corr, & Carson 2004). Numerous sequences have been shown to stimulate TLR9 with variations in the number and location of CpG dimers, as well as the precise base sequences flanking the CpG dimers. This led to the creation of five unofficial classes or categories of CpG ODN based on their sequence, secondary structures, and effect on human peripheral blood mononuclear cells (PBMCs). The five classes are Class A (Type D), Class B (Type K), Class C, Class P, and Class S (Vollmer & Krieg 2009). It is important to note that during the discovery process, the "Classes" were not defined until much later when it became evident that ODN with certain characteristics elicited specific responses. Because of this, most ODN referred to in the literature use numbers (i.e., ODN 2006, ODN 2007, ODN 2216, ODN D35, ODN K3). The numbers are arbitrary and come from testing large numbers of ODN with slight variations in attempts to find the optimal sequence. In addition, some papers will give different names to previously described ODN, complicating the naming convention even more.

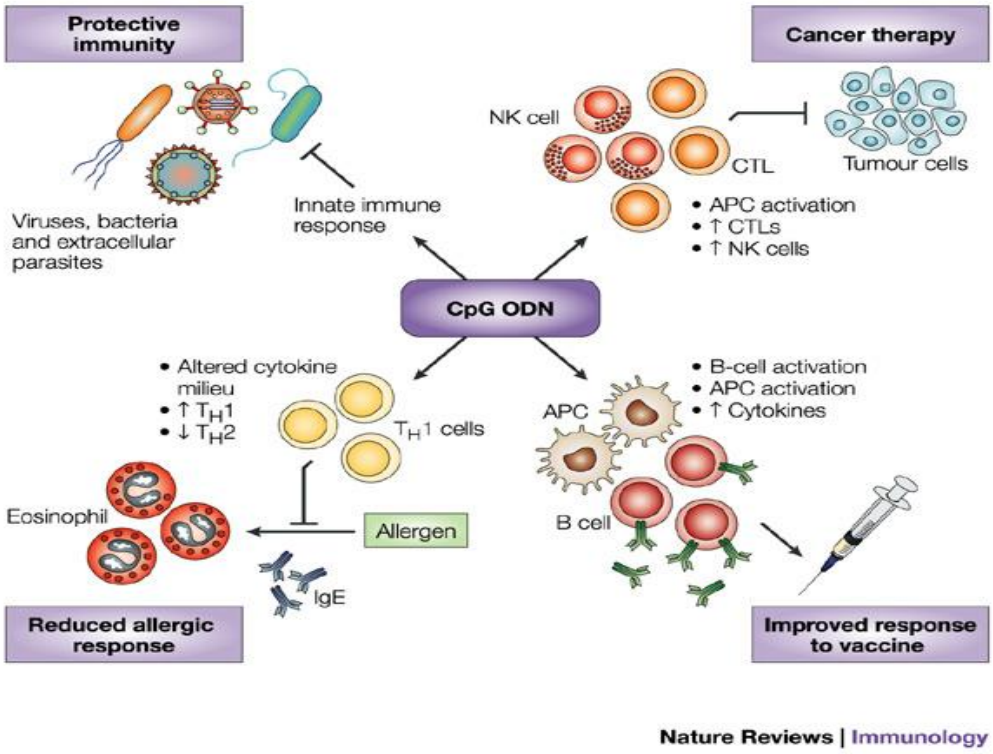



**Figure 2.3: Applications of CpG ODNs**

The CpG ODNs have found immnse applications in protective immunity and in initiatives against cancers and allergy (Source: Krieg *et al.,* 1995).

One of the first Class A ODN, ODN 2216, was described by Krug *et al.*.2001. This class of ODN was distinctly different from the previously described Class B ODN (ODN 2006) in that it stimulated the production of large amounts of Type I interferons (Figure 2.3), the most important one being IFN-α, and induced the maturation of plasmacytoid dendritic cells. Class A ODN are also strong activators of NK cells through indirect cytokine signaling. The structural features defining Class A ODN include: the presences of a poly G sequence at the 5' end, the 3' end, or both; an internal palindrome sequence; GC dinucleotides contained within the internal palindrome; A partially PS-modified backbone (Figure 2.4). Class A ODN typically contain 7 to 10 PS-modified bases at one or both ends that resist degradation by nucleases and increase the longevity of the ODN. The above rules strictly define the class, but variability of the sequence within these

"rules" is possible. It should also be noted that changes to the sequence will affect the magnitude of the response. For example, the internal palindrome sequence can be 4 to 8 base pairs in length and vary in the order of bases, however the pattern, 5'-Pu Pu CG Pu Py CG Py Py-3', was found to be the most active when compared to several other sequences. The poly G tail found at either end of the DNA strand can vary in length and even number (Type D only have a poly G sequence on the 3'end), but its presence is critical to the activity of the molecule.

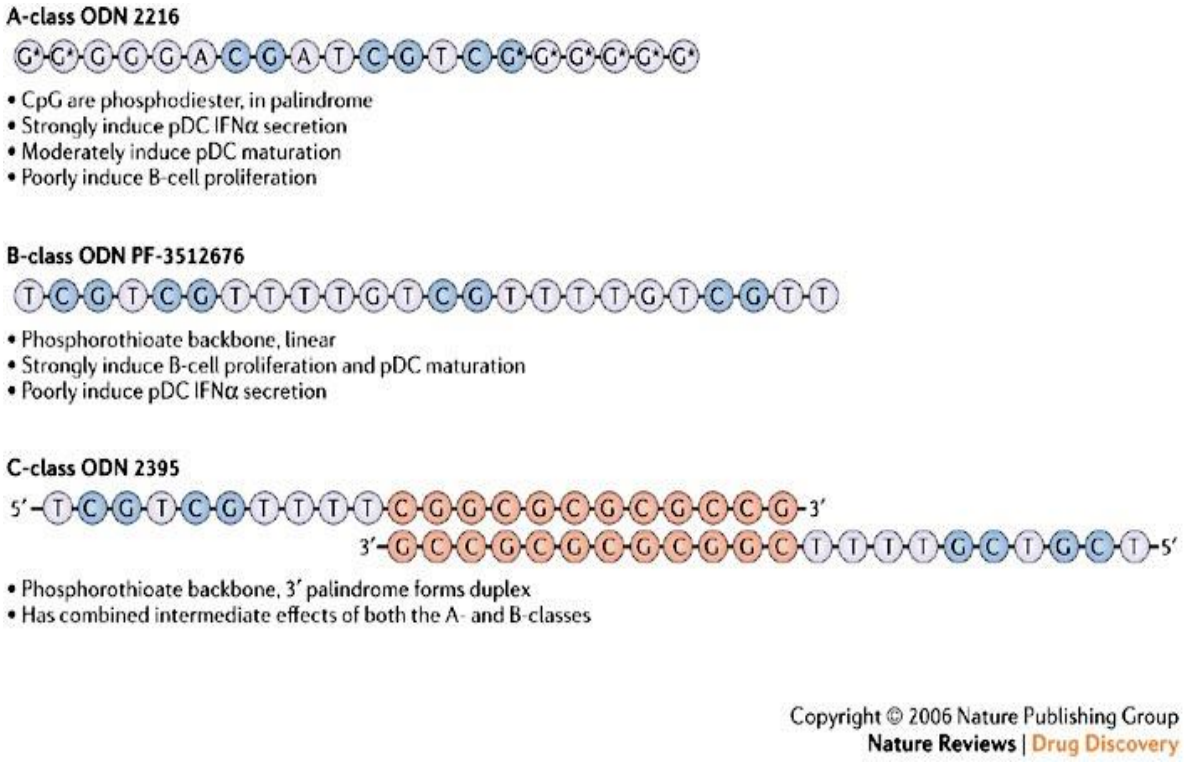



**Figure 2.4: General structures of CpG ODN Classes.**

(Source: Krieg *et al.,* 1995).

Krieg *et al.,* (1995) were the first to describe Class B ODN . Class B ODN (i.e. ODN 2007) are strong stimulators of human B cell and monocyte maturation. They also stimulate the maturation of pDC but to a lesser extent than Class A ODN and very small amounts of IFNα. The structural features defining Class B ODN: one or more 6mer CpG motif 5'-Pu Py C G Py Pu-3'; a fully phosphorothioated (PS-modified) backbone;

Generally 18 to 28 nucleotides in length. The strongest ODN in this class have three 6mer sequences (Hartmann *et al.*, 2000). B ODN have been studied extensively as therapeutic agents because of their ability to induce a strong humoral immune response, making them ideal as a vaccine adjuvant. Type C CpG ODNs combine features of both types A and B. They contain a complete phosphorothioate backbone and a CpG-containing palindromic motif. Type C CpG ODNs induce strong IFN-a production from pDC and B cell stimulation. A cationic polysaccharide bearing a β-1,3-glucan main-chain structure (CUR-N+) forms a complex with a hetero-sequence oligonucleotide, that is, a CpG ODN, and facilitates the transportation of the resultant complex into a murine macrophage-like cell J774.A1, which induces an efficient secretion of a cytokine (IL-12) as compared with that induced by conventional carriers such as poly (ethyleneimine) (PEI) and poly (L-lysine) (PLL) (Ikeda *et al.*, 2007).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Experimental Mice, Parasites, and Infections

Twelve-week-old female BALB/c mice purchased from CBRD, KEMRI and IPR were intraperitoneally injected, using a needle of size 26 G, with $1x10^4$ virulent wild type *P. berghei* ANKA-parasitized red blood cells obtained from donor infected BALB/c mice. Parasitaemia and reticulocyte levels were monitored daily in all mice in the experiments. Blood was extracted from the tail veins of the mice (approximately 50 µl per mouse) for parasitaemia determination and used to prepare triplicate Giemsa-stained thin blood smears and parasitaemia were expressed as a percentage of at least 2000 RBCs. Haemoglobin was measured in a 96-well plate at 570 nm on Bio-Rad Model 3550 Micro plate reader (Biorad, Philadelphia, USA) as previously described (Helegbe *et al.*, 2011). The mice were anaesthetized on day 10 post infection for the extraction of various biological samples for laboratory analysis after euthanasia.

For anaesthetisation, a Ketamine/Diazepam mixture at ratios of 1:1 v/v were administered at 0.1 ml/kg to all mice for restraint, anesthetic induction or for allowing non-painful cardiac puncture procedures using needle size 26 G. This gave excellent muscle relaxation, and minimal respiratory or cardiovascular depression. When combined, and administered as described, the dose was 5 mg/kg ketamine and 0.25 mg/kg diazepam. All mice were killed “humanely” by placing them on top of the cage and breaking their neck by applying firm pressure at the base of the skull and sharply pinching and twisting between thumb and forefinger while at the same time pulling backward on the tail. These murine model-based investigations were approved and performed in accordance with the KEMRI and IPR institutional guidelines. Each group of mice were housed in the KEMRI animal care facilities in standard 67–75 square inch cages throughout the experiments. The mice were provided with commercial rodent diet, and water was provided *ad libitum.* The mice cage facilities were inspected for proper lighting, ventilation, temperature, foot bath sterilization according to standard operating procedures (SOPs).

### 3.2 Study Design and Usage of Mice

This study characterised cytokine-CpG motif co-inoculation in mice BALB/c infected with *P. berghei* ANKA strain. 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. One day one post-infection mice groups were treated as follows: the CpG/IL-18/ *P. berghei* group was treated with both CpG ODNs and IL-18, the CpG/IL-12/*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. Respective therapies were repeated for 5 conscutive days. Generally, the cytokines (IL-12 and IL-18) were chosen due to their previously illustrated protective roles in parasitised murine hosts (Angulo *et al.*, 2002; Li *et al.*, 2004). Interleukin – 18 was chosen for these investigations due to its previously demonstrated capability to induce protection against other intracellular parasites (Li *et al.*, 2004), while IL-12 was a preferred cytokine due to its anti-parasitic proinflammatory properties (Angulo *et al.*, 2002). Each mice group had 18 mice and consequently a total of 144 (18 × 8) mice were involved in the analysis of each experimental objective. The mice were placed into eight groups and exposed to general experimental plans as outlined below;

**Table 3.0: General Experimental Plan**

| GROUP NAME | STAGE 1 | STAGE 2 (Therapy) | STAGE 3 (Monitoring) | STAGE 4 |
|---|---|---|---|---|
| **CpG/IL-18/ *P. berghei*** | Infection | **CpG/IL-18** | Parasitaemia and Clinical | Lab. analysis |
| **CpG/IL-12/ *P. berghei*** | Infection | **CpG/IL-12** | Parasitaemia and Clinical | Lab. analysis |
| **IL-18/ *P. berghei*** | Infection | **IL-18** | Parasitaemia and Clinical | Lab. analysis |
| **IL-12/ *P. berghei*** | Infection | **IL-12** | Parasitaemia and Clinical | Lab. analysis |
| **CpG/ *P. berghei*** | Infection | **CpG** | Parasitaemia and Clinical | Lab. analysis |
| ***P. berghei*** | Infection | __ | Parasitaemia and Clinical | Lab. analysis |
| **CpG** | __ | **CpG** | Parasitaemia and Clinical | Lab. analysis |
| **Uninfected** | __ | __ | Parasitaemia and Clinical | Lab. analysis |

Sampled mice were infected simultaneously with *P. berghei* as elaborated (section 3.1 above). Parasitaemia (using Giemsa-stained blood films) and clinical characteristics (section 3.3) were monitored on a daily basis in all these mice. On day one post-infection, mice were treated as depicted in the stage two column in the above table (table 3.0); they were injected at separate sites with both respective cytokine and CpG ODN combinations (See section 3.7). Treatments were repeated for 5 consecutive days. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups constituted the main groups under investigation, while the other six groups were the control groups (Ozwara *et al.*, 2003). After ten days, all mice were anaesthetized and humanely euthanised (section 3.2) for the extraction of EDTA-treated blood and splenocytes. Haematology (on blood films and EDTA blood), recall proliferation (on splenocytes), antibody, transcription and physiologic factor, chemokine and cytokine bioassays (using -20°C-preserved plasma samples) were then conducted (stage four; lab. analysis) soon after the animal phase experiments.

### 3.3 Clinical, Haematological and Parasitological Monitoring

Soon after infection with *P. berghei*, the mice were closely monitored on a daily basis for agility, hair ruffling, appetite, and roll-over movements (Carvalho, Ferreira-da-Cruz, Daniel-Ribeiro, Pelajo-Machado & Henrique 2006). An arbitrary scale ranging from level 1 up to level 10 was used for tabulated scoring of the mildness or severity of the clinical manifestations where levels 1, 5 and 10 represented the least, intermediate and most severe clinical condition scores respectively. Body weights of mice in grams were measured on a daily basis. The EDTA-treated whole blood samples were used for differential white blood cell counts, measurement of erythrocyte and reticulocyte populations, and measurement of Packed Cell Volume (PCV), Mean Cell Volume (MCV), MCH, MCHC, using a Symex SF-3000® haematology analyser system (Symex Corporation, Kobe, Japan). Bilirubin, Albumen, ALP, ALT, AST, creatinine and glucose were also quantified using a Reflotron® Plus reflectance photometric clinical chemistry analyser (Hoffmann-La Roche, Basel, Switzerland). Haemoglobin concentration was also ascertained by dividing PCV by three basing on the formular 3Hb=PCV (Barasa *et al.*, 2010a). Parasitaemia scores were determined by examination of Giemsa-stained blood smears collected from the tail vein. Parasitaemia was quantified as the total number of parasitized RBCs per 2000 total counted RBCs (Barasa *et al.*, 2010b; Helegbe *et al.*, 2011). A drop of blood from a pricked tail vein (5-10 µl) was then placed onto a microscope slide close to the frosted end of the slide. The blood film was touched with another slide inclined at 45° and after the blood had spread on its edge it was moved in the forward direction to make a smear. The smear was air dried in for 5 minutes then fixed using 100% methanol.

Fixed slides were stained for 10 min in 10% Giemsa solution (Appendix I). Ten microlitres of erythrocyte pellet was used to prepare a thick smear as follows. The erythrocytes were spread gently on a slide using the edge of another slide then the smear air dried properly. The smear was fixed in acetone for 20 seconds with agitation and stained in the same way as thin smear. Smears were observed microscopically at × 100 magnification. At least 2000 red blood cells (RBC) were counted in every parasitaemia count session. This was done by counting erythrocytes from a quarter of each field and

multiplying by four. Counting was done with the aid of manual lab counters. Parasitaemia was calculated as follows: count the number of infected erythrocytes in a minimum of 2000. Parasitaemia % = (Total counted parasites ÷ Number of erythrocytes counted) × 100. To determine levels of developmental stages of the parasites differential count were performed (for rings, trophozoites and schizonts). All the slides were stored in standard slide storage racks at room temperature (Barasa *et al.*, 2010a).

### 3.4 Recombinant Cytokines and Palindromic CpG Motif ODN Sequences

Commercially available recombinant murine cytokines (rIL-18, and rIL-12) were purchased and processed for intradermal inoculation according to manufacturer's instructions (Becton Dickinson, USA). Recombinant cytokines were reconstituted to final concentrastions of 500 ng/mL in total volumes of 50 µl of PBS each. Synthetic CpG motif oligodeoxynucleotides (ODN) containing CpG motifs synthesized with a nuclease-resistant phosphorothioate backbone (Invivogen, USA) were purchased (Catalog number: CpG ODN M362) and used. The CpG ODN M362 sequence 5'-tcgtcgtcgttc:gaacgacgttgat-3' (25 mer) contains the CpG motifs required for immunostimulation in these experiments. While a few nucleobases are of phosphodiester-form, most of the others in the sequences are phosphorothioated (nuclease resistant). The palindrome is underlined. The CpG ODN M362 was shipped at room temperature and stored at -20˚C for up to 1 year. The type C CpG ODNs (Figure 3.0) combine features of both types A and B. They contain 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. Upon resuspension, aliquots of CpG M362 was prepared and stored at -20˚C. The resuspended product is stable for 6 months at -20˚C if properly stored. Repeated freeze-thawing cycles were avoided. The CpG ODN were processed by methods used by Klinmann *et al.,* 1999. Briefly, they were ethanol-precipitated as $Na^+$ salts and then resuspended in 10 mM Tris, pH 7.0/1 mM EDTA) for storage at −20°C and had undetectable lipopolysaccharide levels by chromogenic Limulus amoebocyte assay (BioWhittaker, Virginia, USA) and undetectable protein contamination by the

bicinchoninic acid protein assay kit (Pierce Chemicals, Dallas, USA). Each BALB/c mouse was intramuscularly inoculated at the appropriate time with a 50µL preparation of the CpG ODN in phosphate buffered saline (PBS; Appendix I) as previously described (De Rose *et al.*, 2002). Briefly, after infection with *P. berghei* parasites on day 0, relevant mice groups were injected with 50 µg CpG ODN M362 by use of a 27.5-gauge needle in a volume of 50 µl. The IL-12/CpG ODN and IL-18/CpG ODN immunotherapeutic combinations were done for 5 consecutive days from day 1.


**Figure 3.0: A schematic representation of the sequential composition of the class C M 362 ODN obtained from Invivogen® for co-inoculation with cytokines. Delivered sequences contained 25 mer palindromic CpG ODN motifs.**

### 3.5 *In Vitro* Propagation of *Plasmodium berghei*

For *in vitro* propagation of *P. berghei*, murine sources with 1-4 % parasitaemia levels were intracardially bled using a 23-G needle attached to a syringe containing 0.1 mL of heparin stock solution (Sigma®, St. Louis [Missouri], USA). Blood from all mice were pooled into a 50 mL tube containing 5-10 mL complete culture medium. The culture medium contained: RPMI1640 (3/4): DMEM-F12 (1/4); bicarbonate 32 mM, HEPES 25 mM; Albumax II 0.5%; glucose 3 g/L; hypoxanthine 200 µM; calcium 2 mM; gelatin 0.1%; choline 1 mM. Cultures were maintained at 37°C and medium was changed daily. Fresh mouse non-parasitised RBCs (NRBCs) were added twice a week (dilution 1:4). Haematocrit was maintained at 2.5% (Jambou, El-Assaad, Combes & Grau 2011). The extracted blood was spun at 1500 rpm for 10 minutes and parasites resuspended in 120 mL of complete culture medium in a 500 mL flask. The flask was gased and incubated overnight at 37 ºC. New gassing needles were heat sterilized before connection to a gas

pipe fitted with a 0.2 µm filter and the cultures were gassed for 25 minutes. The gases in the mixture were (5% $CO_2$, 5% $O_2$, 90% $N_2$). The flasks were tightly capped and transferred to an incubator (37 °C). The incubators and exterior of culture flasks were swabbed with 70 % ethanol to ensure sterility (Waters, & Edstein 2012).

### 3.6 Preparation of *P. berghei* Antigens for Recall Proliferation and ELISA

*Plasmodium berghei* parasite antigen were required for use in recall proliferation and enzyme linked immunosorbent assays. *Plasmodium berghei* parasites from culture and infected mice were used for the preparation of saponin-lysed and sonicated antigens for recall proliferation and ELISA assays respectively. For the preparation of antigens via sonication, parasites were first washed twice by centrifugation at 1200 rpm, 24ºC for 10 minutes with an equivalent volume of Alsever's solution (Gicheru, Olobo, Kariuki & Adhiambo 1995). The parasites were suspended at a final concentration of $10^9$ parasites/ ml in incomplete RPMI 1640 and sonicated at 14-18 amplitude microns for 5 periods of 45 seconds each in ice. The parasites suspension was centrifuged at 10 000 g for 30 minutes. Aliquots of soluble and crude parasite antigens were stored at -70ºC. For the preparation of saponin-lysed antigen, 50 % PCV of parasite pellets were lysed using 0.15% saponin in RPMI incomplete 1640, added to the parasites in the ratio of 1:4. The mixture was incubated for 10 minutes at 4ºC with mixing after every 3 minutes. Centrifugation was done at 4000 rpm at 24ºC for 10 minutes. The pellet was loosened and resuspended for another round of saponin mediated lysis and spinning as before. The parasite pellets were then resuspended in incomplete RPMI 1640 (Sigma, USA) and spun as before to wash. This washing was done twice. The crude parasites were resuspended to contain $10^5$ parasites per every 20 µl volume ($5.0\times10^6$ parasites/ml). Aliquots of saponin-lysed crude parasite antigen were stored at -70ºC until use (Gicheru *et al.,* 1995; Gicheru *et al.,* 2001).

### 3.7 Isolation of Plasma from EDTA-treated Whole blood

Murine whole blood obtained via cardiac puncture was collected into 1.5-ml EDTA coated Eppendorf tubes containing 8 µl of 0.5 M EDTA and were chilled on ice and gently mixed. Gentle mixing of the blood and anticoagulant was done followed by storage on ice. Blood volume was estimated followed by the addition of an amount of 0.5 M EDTA so the final concentration of EDTA became 5 mM. for example, for to 1 ml of blood, one would need to add additional 2 µl of EDTA. Centrifuging of the sample was then performed for 15 min at 3,000 rpm (1500 × g) at 4° C without breaking to stop centrifuge. The supernatant (plasma) was carefully transferred into a 0.5 ml-Eppendorf tube and spun at 3,000 rpm for 15 sec at 4° C. The plasma that was stored in aliquots at −20° C. until used.

### 3.8 Transcription Factor Bioassays

#### 3.8.1 Sources of Antibodies and Kits for Transcription Factor Bioassays

Bioassay kits for NF-kB, IRF-5 and KLF-1 were purchased from US Biological Life Sciences® (Salem, USA), while those for STAT-6 were purchased from Neo Scientific® (Cambridge, Massachussetts, USA), those for NFATc-1 from Antibodies-online® (Atlanta, USA), FOXP3 bioassay kits from Mybiosource® (San Diego, California, USA), the AHR bioassay kit was from LSBio™ (Seattle, Washington, USA).

#### 3.8.2 NF-kB and STAT-6 Bioassays

In this quantitative competitive enzyme immunoassay technique, standards and samples (100 µL) were added to the appropriate wells in triplicate to the Nuclear Factor kappa B (NF-kB) (mouse) antibody and STAT-6 antibody pre-coated and (pre-blocked) 96-well microtiter plates. One hundred microliters of PBS (pH 7.0-7.2) were then added into duplicate blank control wells. Fifty microliters of protein HRP conjugates were added to

each well (NOT blank control well) and mixed well. Mixing well in this step was critical important. The plates was then covered and incubated for 1 hour at 37°C. Automatic washing of the microtiter plate was then done five times followed by decanting. Fifty microliters of tetramethylbenzedene conjugate (Sigma, USA) were then added including in the blank control well, followed by incubation for 10-15 minutes at 20-25°C while avoiding sunlight. The product of the enzyme-substrate reactions formed blue colored complexes and finally, a sulphuric acid stop solution was added to stop the reactions, and mixed well, turning the solution yellow. The intensity of color was measured spectrophotometrically to determine the Optical Density (O.D.) at 450nm in a microplate reader. The intensity of the color was inversely proportional to the NF-kB and STAT-6 concentrations since protein from samples and protein-HRP conjugate compete for the anti-NF-kB or anti-STAT-6 protein antibody binding site. Since the number of sites is limited, as more sites are occupied by NF-kB or STAT-6 protein from the sample, fewer sites are left to bind the NF-kB protein-HRP or the STAT-6 protein-HRP conjugates. Standard curves were plotted relating the intensity of the color (O.D.) to the concentration of standards. The target protein concentration in each sample was then interpolated from these standard curves.

### 3.8.3 NFATc Bioassay

Ninety-six well mictrotiter plates pre-coated with an antibody specific to Nuclear Factor of Activated T-cells, Cytoplasmic 1 (NFATC1) were used for this NFATc-1 sandwich ELISA procedure. After determining the wells for diluted standard, blank and sample, labeling of 7 wells for standard, and 1 well for blank was done. One hundred microliters each of dilutions of standard, blank and samples were then added into the appropriate wells. The ninety-six well mictrotiter plates were then covered with the plate sealer and incubated for 2 hours at 37 °C. Liquid contents of each well were removed, and without washing, 100 µL of biotin-conjugated antibody specific to NFATC-1 working solution added to each well. Incubation for 1 hour at 37 °C was then done after covering it with the plate sealer. The solution was then aspirated and washed with was solution (0.05 %

tween 20; pH 7.2-7.4) using an autowasher, and allowed to sit for 1~2 minutes. The remaining liquid was removed completely from all wells by snapping the plate onto absorbent paper and then washed again 3 times. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was then inverted and blotted against absorbent paper. One hundred microliters of avidin conjugated to horseradish peroxidase (HRP) working solution was then added to each well and incubated for 30 minutes at 37 °C after covering it with the plate sealer. Repeated aspiration/wash processes were conducted for a total of 5 times as conducted in the fourth step. Ninety microliters of TMB substrate solution were then added to each well and covered with a new plate sealer. Incubation then followed for 15 - 25 minutes at 37 °C (without exceed 30 minutes and with protection from sunlight. The liquid turned blue by the addition of substrate solution. Fifty microliters of sulphuric acid stop solution were then added to each well, thereby converting the solution from blue into yellow colour. Whenever color change did not appear uniform, the plate was gently tapped to ensure thorough mixing. Any drops of water and fingerprints on the bottom of the plate werer removed and it was confirmed there were no bubbles on the surface of the liquid. Then the microplate reader was activated and measurement conducted at 450 nm immediately. The intensity of color was measured spectrophotometrically to determine the Optical Density (O.D.) at 450nm in a microplate reader.

### 3.8.4 FOXP3, IRF-5 and KLF-1 Bioassays

The FOXP3, IRF-5 and KLF-1 molecules were detected via the Sandwich-ELISA method. The 96-well micro ELISA plate provided with the kit had pre-coated antibody specific to mouse FOXP3, IRF-5 and KLF-1. One hundred microliters each of dilutions of standard, blank and samples were added into the appropriate wells. The ninety-six well mictrotiter plates were then covered with the plate sealer and incubated for 2 hours at 37 °C. Liquid contents of each well were removed, and without washing, 100 µL of biotin-conjugated antibody specific to FOXP3, IRF-5 and KLF-1 working solution added to each well. Incubation for 1 hour at 37 °C was then done after covering it with the plate

sealer. The solution was then aspirated and washed with was solution (0.05 % Tween 20; pH 7.2-7.4) using an autowasher, and allowed to sit for 1~2 minutes. The remaining liquid was removed completely from all wells by snapping the plate onto absorbent paper and then washed again 3 times. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was then inverted and blotted against absorbent paper. One hundred microliters of avidin conjugated to horseradish peroxidase (HRP) working solution was then added to each well and incubated for 30 minutes at 37 °C after covering it with the plate sealer. Repeated aspiration/wash processes were conducted for a total of 5 times as conducted in the fourth step. Ninety microliters of TMB substrate solution were then added to each well and covered with a new plate sealer. Incubation then followed for 15 - 25 minutes at 37 °C (without exceed 30 minutes and with protection from sunlight. The liquid turned blue by the addition of substrate solution. Fifty microliters of sulphuric acid stop solution were then added to each well, thereby converting the solution from blue into yellow colour. Whenever color change did not appear uniform, the plate was gently tapped to ensure thorough mixing. Any drops of water and fingerprints on the bottom of the plate werer removed and it was confirmed there were no bubbles on the surface of the liquid. Then the microplate reader was activated and measurement conducted at 450 nm immediately. The intensity of color was measured spectrophotometrically to determine the Optical Density (O.D.) at 450nm in a microplate reader. The OD value was proportional to the concentration of mouse FOXP3, IRF-5 and KLF-1. Calculation of the concentration of mouse FOXP3, IRF-5 and KLF-1 in the samples was accomplished by comparing the OD of the samples to the standard curve.

### 3.8.5 Ahryl Hydrocarbon Receptor Bioassay

In this cell-based assay ninety-six-well microtiter plates (LSBio $^{TM}$) manufactured for cell culture were coated with 100 µl of 10 µg/ml poly-L-Lysine placed into each well of the 96-well plates for 30 minutes at 37°C prior to adding 200 µl of 20,000 extracted splenocytes/well (Figure 3.1). Overnight incubation of the cells was performed at 37°C,

5% $CO_2$. The cell culture medium was then removed and rinsing done with with 200 µl of 1x TBS, twice. Fixing of the cells was then done by incubating with 100 µl of fixing solution (8% formaldehyde) for 20 minutes at room temperature. During the incubation, the plates were sealed with parafilm. Due to the volatility of the fixing solution, appropriate personal wear and protection equipment (mask, gloves and glasses) were worn when using this chemical. The fixing solution was then removed and the plates washed 4 times with 200 µl 1× wash buffer for five minutes each time with gentle shaking on the orbital shaker. For all wash steps, the plates were tapped gently on absorbent papers to remove the solution completely. One hundred microliters of quenching buffer were added and incubation followed for 20 minutes at room temperature. The plates were then washed 3 times with 1× wash buffer for 5 minutes at a time, with gentle shaking on the shaker. One hundred microliters of blocking buffer (3 % BSA in PBS) were then added followed by incubation for 1 hour at room temperature. Washing was then done 4 times with 200 µl of 1× wash buffer for 5 minutes at a time, with gentle shaking on the shaker. Fifty microliters of 1× primary antibodies (IgG Anti-AhR Antibody (LSBio $^{TM}$) were added to the corresponding wells, and covered with Parafilm and incubated for 16 hours (overnight) at 4°C. Wash was then done 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker. Fifty microliters of 1× secondary antibodies (HRP-Conjugated Anti-Mouse IgG Antibody) were added to corresponding wells and incubated for 1.5 hours at room temperature with gentle shaking on the shaker. The plates were then washed 3 times with 200 µl of 1× wash buffer for 5 minutes at a time, with gentle shaking on the shaker. Fifty microliters of ready-to-use TMB substrate were then added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking on the shaker (the ready-to-use substrate was a light-sensitive reagent and had to be kept away from light. Fifty microliters of Sulphuric acid stop solution were then added into each well and read OD at 450 nm immediately using the microplate reader.

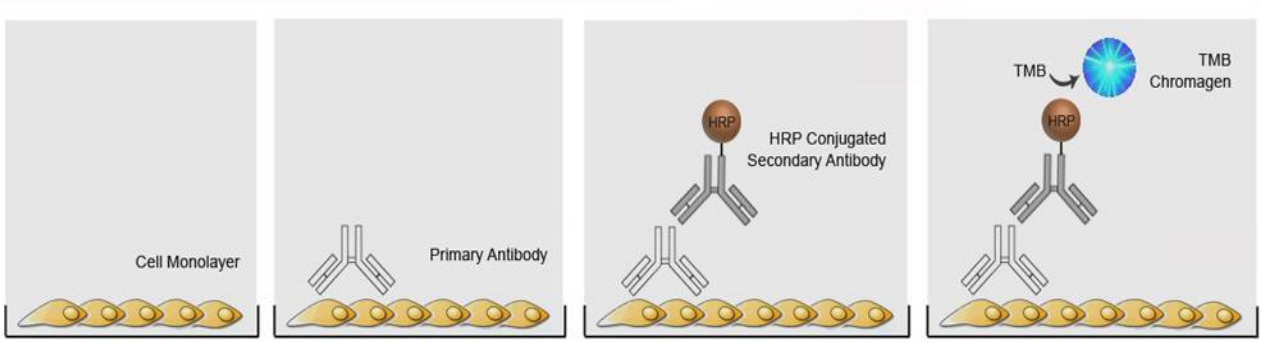



**Figure 3.1: The AHR cell-based bioassay (LSBio TM).**

This bioassay involved the attachement of cells onto the plate using poly-L-Lysine, followed by detection of AHR using a detection system with primary antibody and secondary HRP-conjugated antibodies.

### 3.9 Physiologic Factor Bioassays

#### 3.9.1 Sources of Antibodies and Kits for Physiologic Factor Bioassays

Bioassay kits for adiponectin and angiopoietin-2 detection were obtained from Abcam® (Cambridge, Massachusetts, USA) while angiopoietin-1 kits were purchased from Biocompare® (San Fransisco, California, USA). The kits for angiogenin detection were purchased from Elabscience® (New York, USA), while those for NRP-1 were acquired from Cloud-clone Corp® (Houston, Texas, USA). For quantifying COX-2, MMP-8 and MMP-9 bioassay kits from Cusabio® (San Mateo, California, USA) were used.

#### 3.9.2 Adiponectin, Angiopoietin-1, Angiopoietin-2 and Bioassays

All materials and prepared reagents were equilibrated to room temperature (18 - 25°C) prior to use. Standards, controls and samples were assayed in triplicate and the assays were performed at room temperature (18- 25°C). Excess microplate strips were removed from the plate frame and returned immediately to the foil pouch with desiccant inside.

The pouch was resealed securely to minimize exposure to water vapor and stored in a vacuum desiccators. Fifty microliters of adiponectin or angiopoietin-1 or angiopoietin-2 standards or samples were then pipetted per well and the wells of the 96-well pre-coated (with anti-mouse adiponectin or anti-mouse angiopoietin-1 or anti-mouse angiopoietin-2 antibodies) and pre-blocked microtiter plates (Abcam® and Biocompare®) were covered with sealing tapes and incubated for two hours at 37°C. The timer was started after the last sample addition. The plates were then washed six times with 300 µL of 1× wash buffer automatically and the plates inverted each time and contents decanted; tapping it 4-5 times on absorbent paper towel to completely remove the liquid. Fifty microliters 1× biotinylated anti-adiponectin or anti-mouse angiopoietin-1 or anti-mouse angiopoietin-2 antibodies were added to each well and incubated for one hour. Washing was then done as described above and 50 µL of 1x Streptavidin HRP Conjugate added to each well and incubated for 30 minutes.

The Dynatech MRX ELISA microplate reader programme was then set up in advance. Washing was then done as given above and 50 µL of TMB chromogen substrate added per well and incubated for about 10 minutes or till the optimal blue colour density became developed. Gentle tapping of the plate to ensure thorough mixing and breaking of the bubbles was done in the well with pipette tips and 50 µL of stop solution added to each well. Colour change occurred from blue to yellow and photo-absorbance on the microplate reader at a wavelength of 450 nm read immediately. The mean values of the triplicate readings for each standard and sample were calculated and used to generate a standard curve, using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line was determined by regression analysis using log-log or four-parameter logistic curve-fit. The unknown sample adiponectin, angiopoietin-1, angiopoietin-2 concentrations were determined from standard curves and the value multiplied by the dilution factor.

### 3.9.3 Angiogenin, NRP-1, COX-2, MMP-8 and MMP-9 Bioassays

One hundred microliters of standards, blank, or sample were dispensed per well. The blank wells received the reference standard and sample diluent. Solutions were added to the bottom of the pre-coated (with anti-mouse angiogenin or anti-mouse NRP-1 or anti-mouse COX-2 or anti- mouse MMP-8, or anti- mouse MMP-8 antibodies) and pre-blocked 96-well micro ELISA plate well (Elabscience®, Cloud-clone Corp.®, Cusabio®), touching of the inside walls and foaming was avoided as much as possible and mixing was gentle. The plates were then covered with the sealers provided and incubated for 90 minutes at 37℃. Liquids from each well were removed and, without washing, 100μL of biotinylated anti-mouse angiogenin, anti-mouse NRP-1, anti-mouse COX-2, anti-mouse MMP-8, or anti-mouse MMP-9 detection antibodies working solutions were delivered into each of the wells followed by coverage of the microtiter plates with the provided plate sealer. Gentle tapping of the plates was done to ensure thorough mixing and incubation done for 1 hour at 37°C. The plates were washed six times with 300 μL of 1× wash buffer automatically and then inverted each time and contents decanted; tapping was done 4-5 times on absorbent paper towel to completely remove the liquid. One hundred microliters of streptavidin HRP conjugate working solution were added into each well and the plates covered with plate sealers followed by incubation for 30 minutes at 37°C and a repeat of the washing process described above for five times. Ninety microliters of TMB substrate solution were then added into each well and covered with new plate sealers. Incubation was done for about 15 minutes at 37°C whilst plates were being protected from direct light. The reaction times were shortened or extended according to the actual color change, but not more than 30 minutes; when apparent gradient appeared in standard wells, the reactions were terminated using 50μL of stop solution dispensed into each well causing the color to turn from blue to yellow immediately. The micro-plate reader was opened and activated with set parameters in advance. The optical density (OD value) of each well was determined at once, using a Dynatech MRX ELISA micro-plate reader set to 450 nm. Generated standard curves were then used to determine sample angiogenin, NRP-1, anti-COX-2, MMP-8 and MMP-9 concentrations.

### 3.10 Chemokine and Splenocyte Recall Proliferation Bioassays

#### 3.10.1 Sources of Antibodies and Kits for Chemokine Bioassays

Chemokine bioassay kits for detection of murine CXCL-10 was purchased from Abbexa® (Cambridge, UK), CXCL-5, CXCL-12, CCL-3 kits were from RnD Systems® (Minneapolis, Minnesota, USA), CXCL-16, CX3CL-1, CXCL-1 testing kits were from Abcam® (Cambridge, Massachusetts, USA), CCL-2 and CCL-5 detection reagents obtained were from Abnova® (Walnut, California, USA).

#### 3.10.2 Chemokine Bioassays Procedures

All materials and prepared reagents were equilibrated to room temperature (18 - 25°C) prior to use. Standards, controls and samples were assayed in triplicate and the assays were performed at room temperature (18- 25°C). Fifty microliters of CXCL-10, CCL-2, CCL-5, CXCL-16, CX3CL-1, CXCL-1, CXCL-5, CXCL-12, CCL-3 standards or samples were then pipetted per well and the wells of the 96-well pre-coated (with anti-chemokine antibodies) and pre-blocked microtiter plates (Abcam® and Biocompare®) were covered with sealing tapes and incubated for two hours at 37°C. The plates were then washed six times with 300 µL of 1x wash buffer automatically and the plates inverted each time and contents decanted; tapping it 4-5 times on absorbent paper towel to completely remove the liquid. Fifty microliters 1 (Cambridge, Massachusetts, USA) biotinylated anti-chemokine antibodies were added to each well and incubated for one hour. Washing was then done as described above and 50 µL of 1 (Cambridge, Massachusetts, USA) Streptavidin HRP Conjugate added to each well and incubated for 30 minutes at 37°C.

The Dynatech MRX ELISA microplate reader programme was then set up in advance. Washing was then done as given above and 50 µL of TMB chromogen substrate added per well and incubated for about 10 minutes or till the optimal blue colour density became developed. Gentle tapping of the plate to ensure thorough mixing and breaking of

the bubbles was done in the well with pipette tips and 50 µL of stop solution added to each well. Colour change occurred from blue to yellow and photo-absorbance on the microplate reader at a wavelength of 450 nm read immediately. The mean values of the triplicate readings for each standard and sample were calculated and used to generate a standard curve, using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line was determined by regression analysis using log-log or four-parameter logistic curve-fit. The unknown CXCL-10, CCL-2, CCL-5, CXCL-16, CX3CL-1, CXCL-1, CXCL-5, CXCL-12, CXCL-12 concentrations were determined from standard curves and the value multiplied by the dilution factor.

### 3.10.3 Extraction of Splenocytes from Mice for Proliferation Assays

The fur on left side of sacrificed mouse was wetted using 70% ethanol and the spleen cut out open from the body cavity. Removal of the spleen was then done using the forceps; the spleen is red in color, shaped like a kidney bean; it is longer and flatter than the kidney and is located in the left superior abdominal quadrant. The spleen was placed into a cell strainer. Using the plunger end of a syringe, the spleen was mashed through the cell strainer into the petri dish and the cell strainer rinsed with 5mL DMEM-10 before discarding the strainer. The suspended cells were then transferred into a 15mL conical tube and spun at 800× g for 3 minutes. The supernatant was discarded and the pellet resuspended in 1mL ACK buffer for RBC lysis, followed by incubation at RT for 5-10 minutes. A 9mL volume of DMEM-10 was added and spinning done as before. The supernatant was discarded and pellet resuspended in 3mL DMEM-10, discarding any dead cell mass. Cells were then taken for counting (diluted 10µL cell suspension in trypan blue, and counted with a hemoytometer).

### 3.10.4 Splenocyte White Blood Cell Enumeration

Enumeration of splenocytes was done in order to perform accurate dilution for proliferation assays and have $2 \times 10^5$ cells/well distribution. Trypan blue solution (90 µl; Sigma, St. Louis, USA) was added to a 96 well microtitre plate well. Ten µl of each of the resuspended cells for enumeration was transferred from an eppendorf tube to the respective 96 well plate well. Gentle aspiration was performed in order to mix the cells evenly with trypan blue. The dilution factor of 10 was noted for use in calculating the number of cells per ml. Cells were allowed to stain for 3-5 minutes. Trypan blue stained non-viable cells blue and left viable cells unstained. With the cover slip in place, a P 20 pipetteman was used to transfer a small amount of trypan blue-cell suspension mixture to both chambers of the haemocytometer. Each chamber was allowed to fill by capillary action. The chambers were not overfilled. Starting with chamber 1 of the haemocytometer, all the cells in the four 1 mm corner squares were counted and average number per 1 mm square calculated by dividing by 4. A manual laboratory counter was used in counting. Cells touching the middle line at bottom and right sides were not counted. The procedure was repeated for chamber 2. Accuracy was ensured by taking a second sample and repeating the counting procedure. Calculations were done as follows: Cells per ml = Average count per square × Dilution factor × $10^4$ (count 10 squares). Total cells = Cells per ml × Original volume of fluid from which cells were picked (10ml). Enumerated cells were then resuspended in the right concentration for cryopreservation or for AhR and proliferation bioassays. The coverslips and hemacytometer were decontaminated by rinsing with 70% ethanol and then deionized water before being air dried and stored for future use (Gicheru *et al.,* 2001).

### 3.10.5 Splenocyte Recall Proliferation Assays

Proliferation assays were necessary for the determination of the infected mice' cell mediated specific acquired immune responses. This was done, with modifications, using previously communicated methods (Gicheru *et al.,* 2001. Ozwara *et al.,*2003). Ninety-six

well round bottom microtitre plates (Nunclon, Roskilde, Denmark) were purchased and used for the assays. Briefly, splenic single cell suspensions processed from Alsever (appendix I) solution-diluted splenocytes were resuspended for delivery of 2 x $10^5$ cells/well dispensed in 100 µl of complete RPMI 1640 (RPMI with 10 % FBS, 2 mM L-glutamine, 100µg/ml gentamycin (Life Technologies/BRL), 0.05 mM 2-mercaptoethanol (Sigma). The cells were then stimulated in triplicate wells with $10^9$ parasites/ml *P. berghei* ANKA crude parasite antigen per well or 10µg/ml final concentration of Concanavalin A (Con A; for positive control). Triplicate control background wells received 50µl of complete media. The plates were then covered and taped around to prevent rapid evaporation. Cultures were then incubated at 37ºC in a humidified incubator for 5 days for *P. berghei* ANKA antigen cultures and for 3 days for Con A cultures. Cells were then pulsed with 0.5 µCi of [methyl-3] thymidine (Amersham, Aylesbury, UK; 20 µl delivery/well) over the last 18 hours and then harvested on a fiber glass filter paper (Packard, Wallac, Milton Keynes, UK) using Micromate 196 harvester (Packard). Incorporation of radionuclide into DNA was measured by scintillation spectrometry in a TriCarb 6000 liquid scintillation analyser. Readings were performed as counts per minute and printed using an Epson™ printer.

### 3.10 Cytokine and Immunoglobulin M Measurements

#### 3.10.1 Determination of Cytokine Responses in Sandwich ELISA

Cytokine ELISA procedures were performed in order to measure the levels of cytokine mediated immune responses induced in the mice. Basically, techniques designed previously (Gicheru *et al.,* 1995) were used. Briefly, ninety six-well flat bottomed ELISA microtiter plates were coated with 5 µg/ml of anti-mouse cytokine (IFN-γ, TNF-α,IL-4, IL-23a, IL-10, and IL-17) capture monoclonal antibodies (Becton Dickinson, USA) delivered 50 µl/well. These were incubated overnight at 4 ºC. Excess coating buffers were then flicked off and the wells blocked with 100 µl/well blocking buffer (3% BSA in PBS) followed by 1 hr incubation at 37 ºC. After washing the plates six times, plasma

samples and serially diluted recombinant cytokine standards were dispensed in duplicate, 50 µl/well and plates incubated for 2 hr at 37 ºC. The plates were then washed as before and detector donkey biotinylated anti-mouse cytokine (IFN-γ, TNF-α, IL-4, IL-23a, IL-10, and IL-17) monoclonal antibodies (Becton Dickinson, USA) added 50 µl/well at dilutions of 1:2000. This was followed by a one hr incubation period at 37 ºC then washing as before. Streptavidin Horse Radish Peroxidase (HRP; Sigma USA) was added 50 µl/well and incubated 1 hr at 37 ºC followed by washing six times. Colour development was achieved by adding 50 µl/well of Tetramethylbenzidene (TMB) substrate (Sigma, USA) and optical densities read using a Dynatech MRX machine (Barasa, Ng'ang'a, Kagasi, Gicheru, & Ozwara, 2012).

### 3.10.2 Immunoglobulin M ELISA

Immunoglobulin M (IgM) concentrations were measured using ELISA in order to determine the level of humoral immune responses provoked in the mice. Basically, procedures utilized previously were used (Barasa, Gicheru**,** , Kagasi, & Ozwara, 2010b). Briefly, ninety six well flat bottomed ELISA microtiter plates (Nunclon, Roskilde, Denmark) were coated using 50 µl/well of carbonate bicarbonate buffer containing $10^9$ parasites/ml saponin-fragmented *P. berghei* parasite antigen. These were incubated overnight at 4 ºC for adsorption. Excess coating buffer was then flicked off and 100 µl/well of blocking buffer (3% BSA; Sigma, in PBS) added followed by incubation for 1 hour at 37 ºC. The plates were then washed six times using wash buffer (0.05% tween in PBS; appendix I) and murine sera samples added in triplicate (1:100 dilution in 50 µl/well) on ice before incubation for one hour as before. The plates were then washed as before and 50 µl/well of 1:2000 dilution of anti-mouse Ig M HRP (Sigma, USA) were added. A one hr incubation at 37 ºC followed then the plates were washed ten times. Colour development was achieved by adding 50 µl/well of Tetramethylbenzidene (TMB) substrate (Sigma, USA) and optical densities read using a Dynatech MRX machine.

### 3.11 Data Analysis, Management and Communication

Data entry, management, preliminary summaries such as averages, transformations, percentages and other descriptive statistics were performed using Microsoft Excel® 2013 and the Graphpad Prism-6®, 2015 softwares. Group mean values of parasitaemia, clinical, haematological, immunological, transcription and physiologic biomolecular parameters were compared using one-way Analysis of Variance (ANOVA). Tabulated probability values of $P < 0.05$ were considered significant. Advanced colour-coded matrices (Section 5.6) were developed in order to simplify the complex interrelatioships elucidated amongst TF, physiologic biomolecule, chemokine, cytokine, IgM, and splenocyte recall proliferation parameters and how they affect parasitisation and infection severity. Thus, from the developed colour-coded matrices any reader can instantly decipher an association between a measured factor and parasitaemia and infection severity.

# CHAPTER FOUR

# RESULTS

## 4.1 Introduction

This study was conducted to determine the clinical, haematological, parasitological, physiologic biomolecular and immunological responses elicited in the *P. berghei* - murine (BALB/c) model of malaria following separate co-inoculation with the proinflammatory cytokines IL-12 and IL-18 with immunostimulatory CpG ODN motifs. These two groups of mice intraperitoneally received $1\times 10^4$ virulent wild type *P. berghei* ANKA-parasitized red blood cells (on day zero), before being therapeutically injected with the cytokine – CpG ODN motif solutions the following day (day 1 postinfection). These cytokine-combining gene therapies were performed repeatedly for a total of 5 consecutive days. Six other control groups of mice with different treatments were analysed in the experiments, giving a total of eight mice groups in the investigation; the CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei*; IL-18/ *P. berghei*; IL-12/ *P. berghei*; CpG / *P. berghei*; *P. berghei;* CpG ODN and uninfected mice groups. The mice were monitored for ten days after incoculation before being humanely sacrificed for extraction of samples for bioassays.

A multiplicity of bioassays were utilized in measuring concentrations of various molecules to meet the study's objectives. The results' were analysed using the Graphpad Prism ® via the one-factor ANOVA method and visualizations were done using box-plots, and line graphs where necessary. The outcomes from these experiments demonstrate that cytokine-CpG ODN co-inoculation is a highly potent antimalarial combination and can be further investigated for possible inclusion into therapy and vaccination protocols. The results from these experiments presented herein are clustered as outcomes per target objective: Clinical, haematological and parasitological responses; transcription factor and physiologic biomolecular responses; chemokine and PBMC recall proliferation responses; cytokine and IgM responses.

## 4.2 Parasitological, Clinical and Haematological Responses

### 4.2.1 General Total and Differential Parasitaemia Development Trends

Beginning from day one post-infection, all mice groups on average became parasitaemic with the *P. berghei* group recording the highest parasitaemia levels of all mice groups. The uninfected mice maintained their status quo throughout the study. There were significant 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 parasitemia (5.591 %) experienced through out the experiments by the malaria infected mice that were given cytokine-CpG ODN co-inoculations were three times lower than in the parasitaemia average (11.826 %) in the control experiment groups (Figure 4.0). The highest detected parasitaemia in the CpG/IL-18/*P. berghei* group of mice was 8.0 % while in the CpG/IL-12/*P. berghei*, levels rose to a peak concentration of 9.0 %, much lower compared to the *P. berghei* 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. berghei* infected group and the CpG/*P. berghei* group experienced stronger upturns in parasitaemia trends beginning from day six postinfection than the rest of the groups. The CpG/IL-18/*P. berghei* group and the CpG/IL-12/*P. berghei* groups experienced relatively equivalent levels of average total parasitaemia; 5.727% and 5.455 % respectively. Generally, parasitemia levels of below 10 % were associated with less severe clinical symptoms compared to levels above 10 %. Infected co-inoculation non-recipients experienced dramatic parasitaemia upturns from day 6 post-infection onwards, unlike the co-inoculation groups.

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 postinfection and generally levels started increasing more rapidly beginning from day six postinfection. Just like for total parasitemia, the experimental groups CpG/IL-18/*P.*

*berghei,* and CpG/IL-12/*P. berghei* both experienced lower differential parasitaemia than the rest of the groups (details given in sections starting from 4.2.3). With the exception of the two uninfected groups, all control groups had average total parasitaemia levels above the 9.4 % level. The *P. berghei* group had the highest peak total parasitaemia at 70.2 % with a sharp up-turn taking place beginning from day 6 postinfection. The CpG/*P. berghei* group had the second steepest upward acceleration in parasite count while less steeper intermediate total parasitaemia development trends were witnessed in the CpG/ODN, IL-18/*P. berghei* and IL-12/*P. berghei* groups (Figures 4.1 – 4.3).

Both total and differential parasitaemia trends in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups remained strongly non-dramatic 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. While ring levels remained low in the CpG/IL-18/*P. berghei,* and CpG/IL-12/*P. berghei* groups, they shot to a maximal 26.70 % level in the *P. berghei* group with the other groups experiencing intermediate levels. All mice groups maintained ring parasitaemia levels below the 5 % level up to day 5 when divergent outcomes started as shown in Fig 4.1. While trophozoite levels remained in the CpG/IL-18/*P. berghei* fluctuated between 0 % and 4.8 % and in the CpG/IL-12/*P. berghei* groups, they never exceededthe 3.1 % level. The CpG/IL-12/*P. berghei* and CpG/IL-18/*P. berghei* groups experienced the lowest average schizont levels though out the the ten days of parasitaemia sampling.

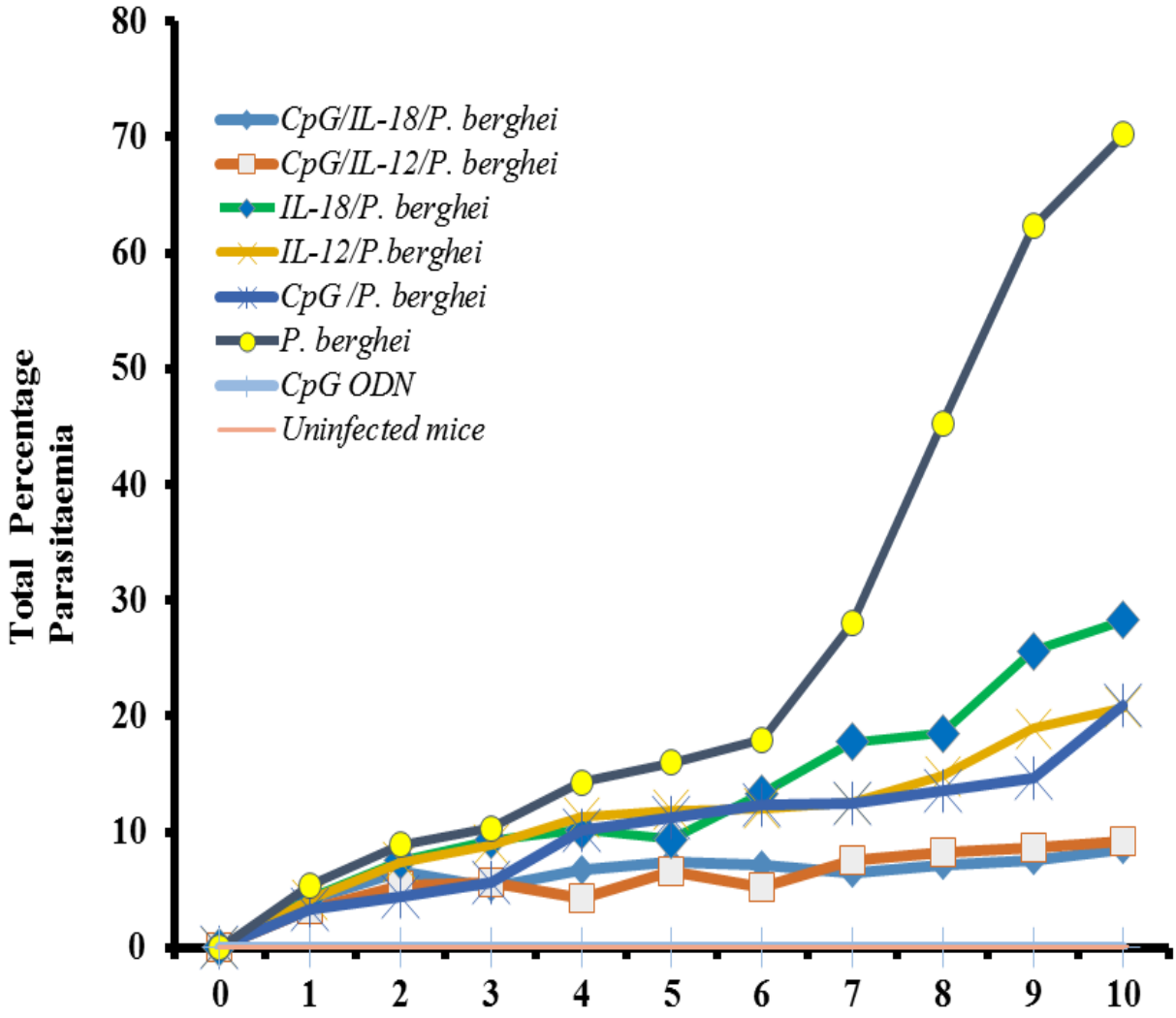



**Figure 4.0: General trend of Overall Parasitaemia Development. The co-inoculations effectively inhibited overall parasitaemia development throughout experimentation.**

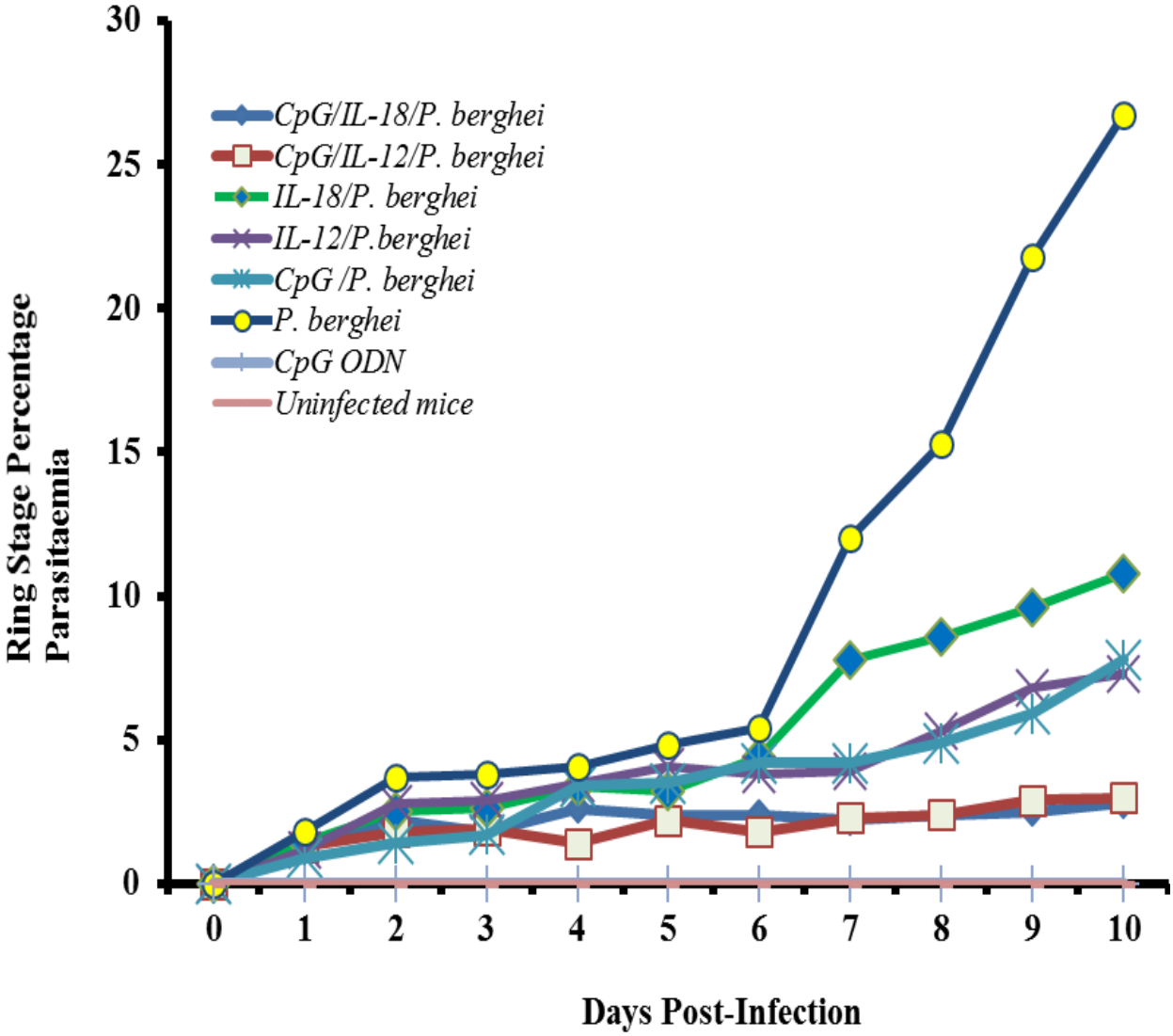



**Figure 4.1: Ring Stage Parasitaemia Development. Ring parasitaemia levels were maintained below the 5 % ceiling in the co-inoculation groups**

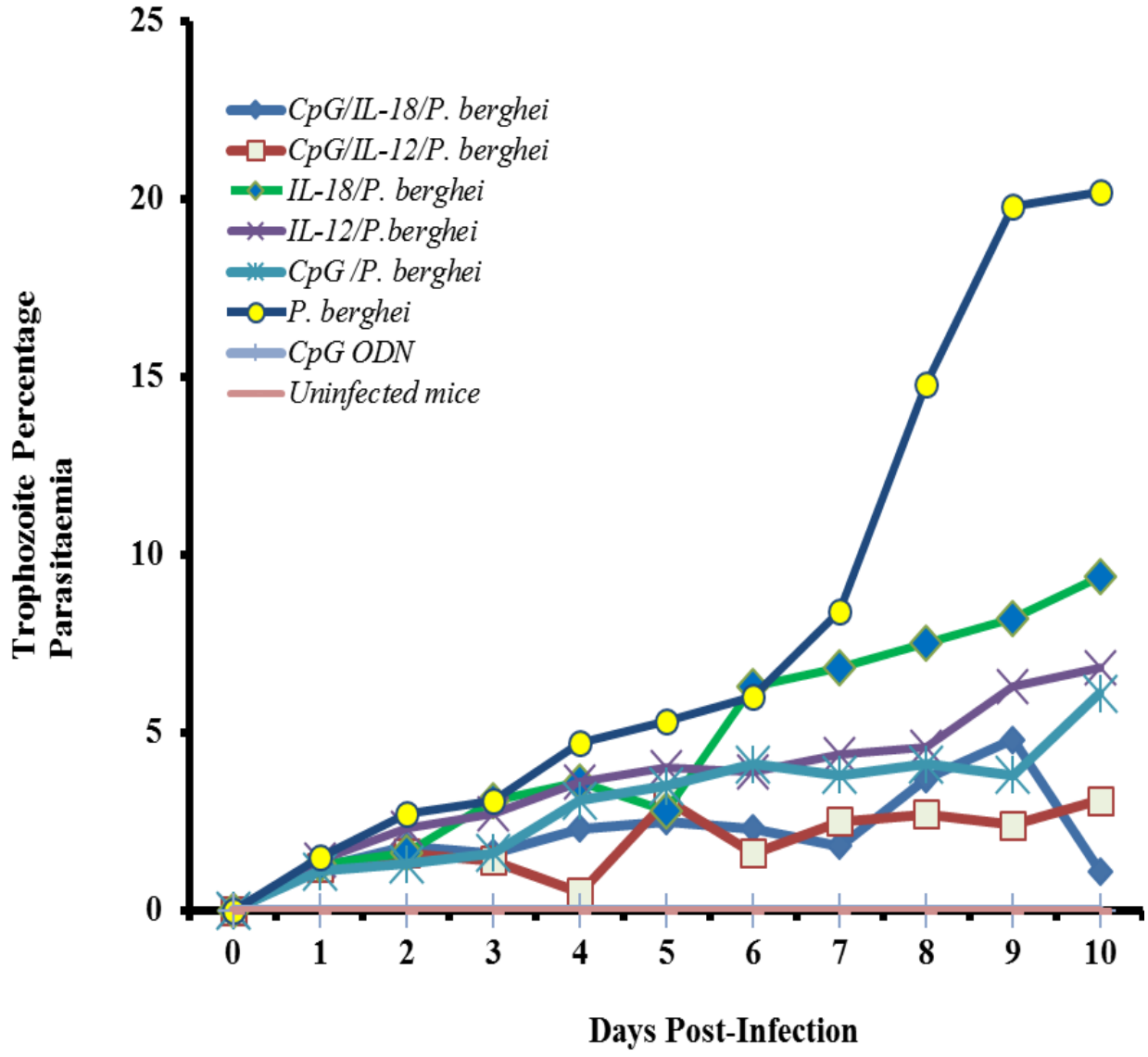



**Figure 4.2: Trophozoite Stage Parasitaemia Development. Trophozoite parasitaemia levels remained below the 5 % level through out experimentation in the co-inoculation groups.**

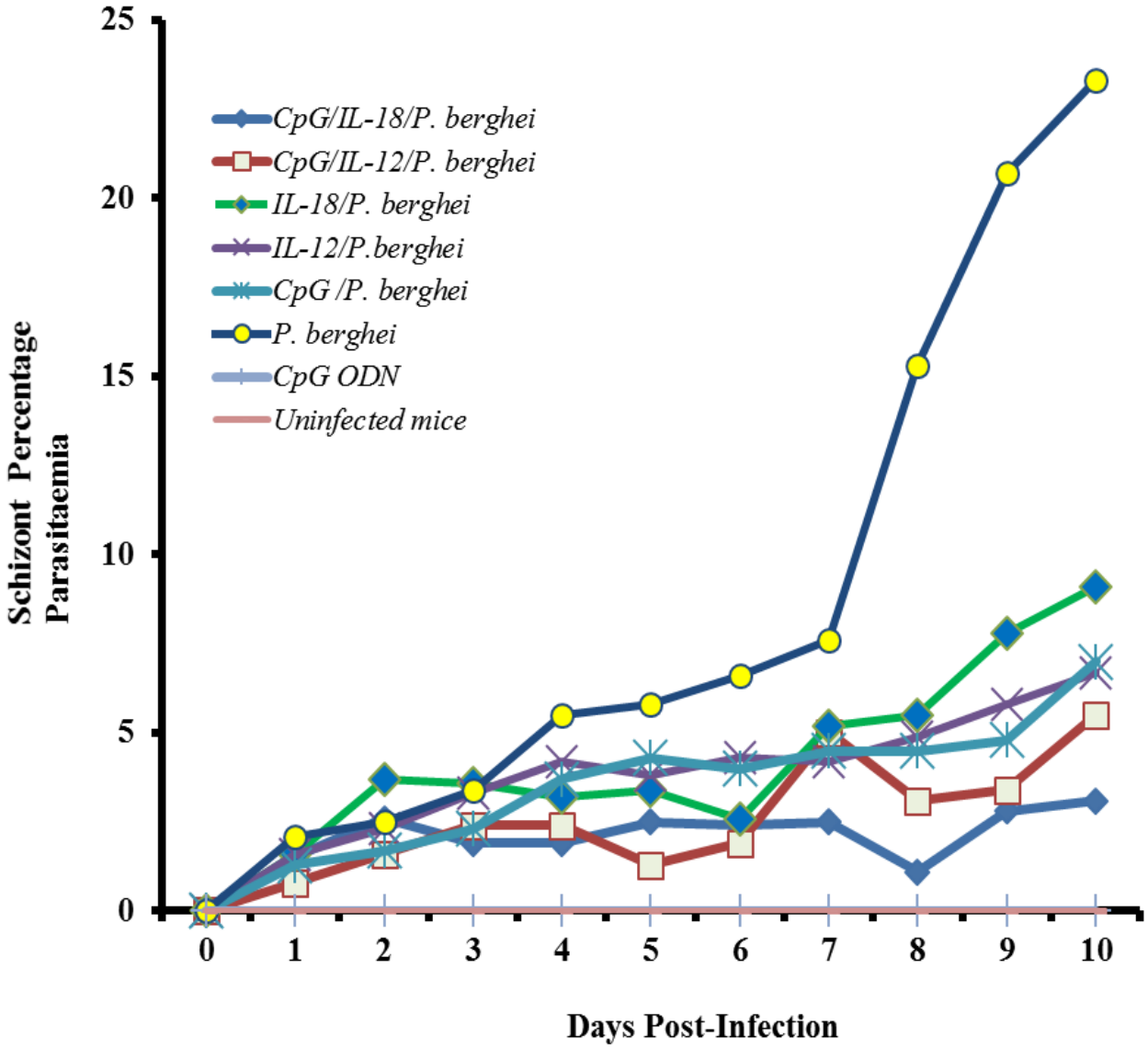



**Figure 4.3: Schizont Stage Parasitaemia Development. The Schizonticidal effects of the co-inoculations sharply limited parasitaemia development beyond the 5 % upper barrier.**

### 4.2.2 Total Parasitaemia Levels

The highest mean total parasitaemia, 25.34 % was recorded in the *P. berghei* group. This was over five times higher than the parasiteamia measured in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups of mice; 5.727 and 54.55, respectively. One-way ANOVA analysis showed that the parasitaemia differences between the groups were highly significant at $P< 0.05$, F (1.064, 10.64) = 10.23. The CpG/ *P. berghei* group (the second highest total parasitaemia group) had a total parasitaemia level of 15, five times higher than the CpG/IL-18/ *P. berghei* group of mice. The IL-12/ *P. berghei* group a mean level of 10.64, two times higher than the CpG/IL-12/ *P. berghei* group. Intermediate concentrations of parasitaemia were recorded in the IL18 / *P. berghei* and IL-12/*P. berghei* groups of mice; 9.88 and 9.455 respectively. No parasitaemia were detected in the CpG ODN and uninfected group of mice. The *P. berghei* group recorded the highest peak total parasitaemia of 70.2 %. The lowest mean totals of 8.0 % and 9.0 % were recorded in the IL-18/CpG/ *P. berghei* and IL-12/CpG/ *P. berghei* groups respectively. The '+' signs in the box-plots represent the mean values recorded.

### 4.2.3 Ring Stage Parasitaemia Levels

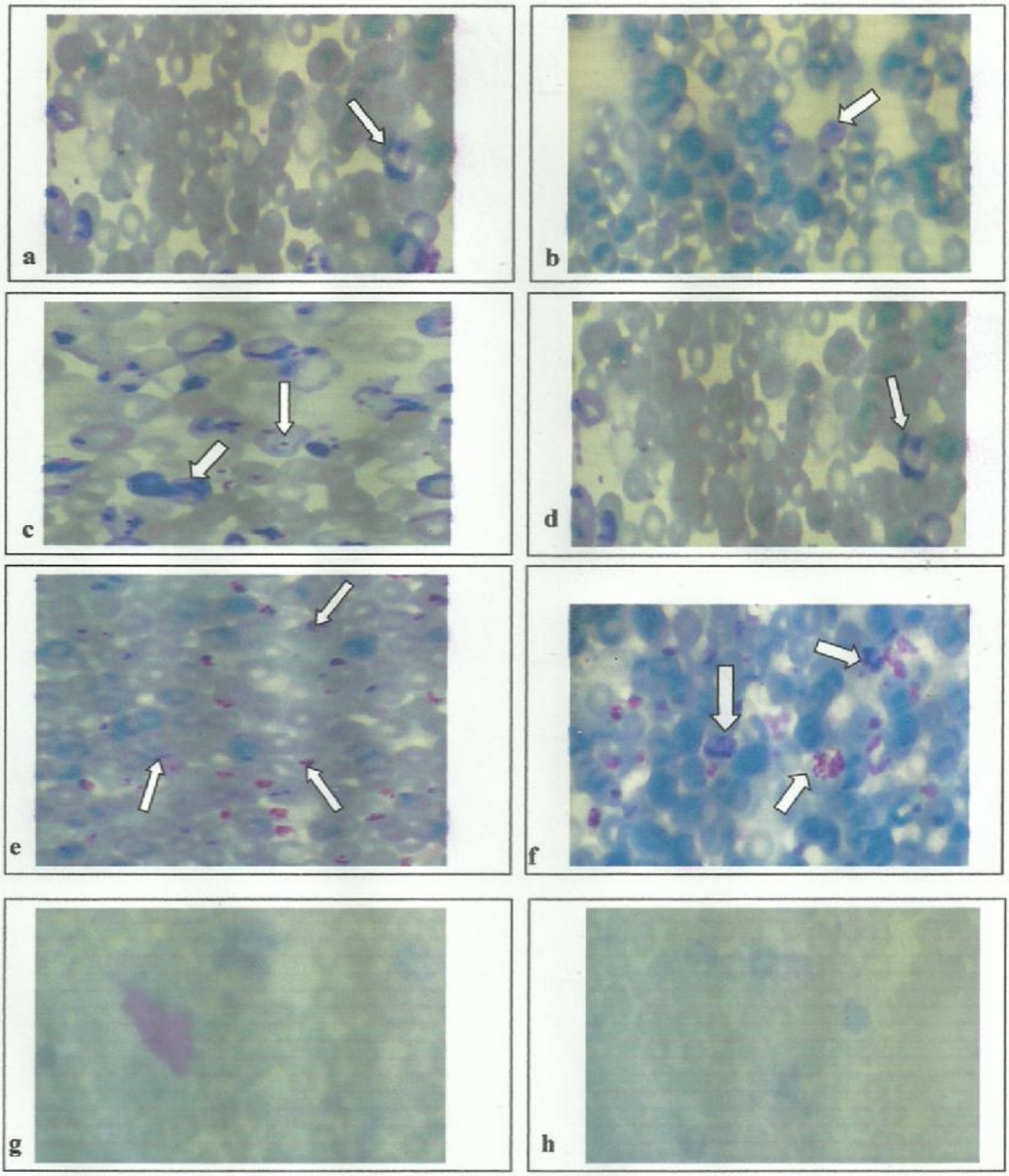
The *P. berghei* group had highest mean levels of ring stage parasitaemia, 9.036 %. This level was over six times higher than the parasiteamia measured in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups of mice; 2.061 and 1.909, respectively. One-way ANOVA analysis showed that the ring parasitaemia differences between the groups were highly significant at $P< 0.0001$, F (7, 70) = 9.3429. The CpG/ *P. berghei* group had ring parasitaemia level of 5.578, almost 3-fold higher than the two cytokine-CpG co-inoculation groups of mice. The IL-12/ *P. berghei* group a total mean ring parsitaemia levels were at 3.78, for the IL-18 / *P. berghei* levels were at 3.4, while the CpG/ODN and uninfected groups remained as expected at 0.0 and 0.0 respectively. The *P. berghei* group recorded the highest peak ring parasitaemia of 26.70 %. The IL-18/CpG/ *P. berghei* and IL-12/CpG/ *P. berghei* groups had the lowest total ring parasitaemia levels.

### 4.2.4 Trophozoite Stage Parasitaemia Levels

The *P. berghei* 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. Data analysis showed that the trophozoite parasitaemia differences were significant at $P<0.0001$, $F(7, 70) = 11.68$. For the CpG / *P. berghei* group of mice parasitaemia levels were 5.145 (second highest trophozite levels) while in the CpG ODN and uninfected groups trophozoite parasitaemia levels were both at 0 %. 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. The *P. berghei* group recorded the highest peak ring parasitaemia of 20.20%. The IL-18/CpG/ *P. berghei* and IL-12/CpG/ *P. berghei* had the lowest mean total differential parasitaemia levels. No parasites were detected in the uninfected group.

### 4.2.5 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 parasiteamia 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 a mean schizont level of 3.736, was about one and a half times higher than in 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 parasitaemia levels were at 3.464, 0 %, and 0 % respectively (Table 4.0; Plates 4.0 [a-h]).




**Plates 4.0 (a-h): Peripheral blood parasitisation differences visualized on microscope slides at × 100 magnification. a. The IL-18/CpG/*P. berghei* b. IL-12/CpG/*P. berghei* c. IL-18/*P. berghei* d. IL-12/ *P. berghei* e. CpG/*P. berghei* f. *P. berghei* g. CpG ODN and h. Uninfected mice group. Thick arrows point to ring and trophozite differential stages while thin arrows point to schizont**

**Table 4.0: Parasitaemia data summary for all the mice groups.**

| | | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|---|
| Total Parasitaemia | Means | 5.727 | 5.455 | 9.455 | 10.64 | 15.73 | 25.34 | 0.0 | 0.0 |
| | SD | 2.195 | 2.583 | 5.837 | 5.784 | 12.76 | 23.63 | 0.0 | 0.0 |
| | SE | 0.6618 | 0.7789 | 1.760 | 1.744 | 3.847 | 7.124 | 0.0 | 0.0 |
| | ANOVA | F (1.064, 10.64) = 10.23, P < 0.05 | | | | | | | |
| Ring Stage | Means | 2.061 | 1.909 | 3.445 | 3.782 | 5.578 | 9.036 | 0.0 | 0.0 |
| | SD | 0.7878 | 0.8336 | 2.320 | 2.161 | 4.559 | 8.766 | 0.0 | 0.0 |
| | SE | 0.2375 | 0.2513 | 0.6995 | 0.6515 | 1.375 | 2.643 | 0.0 | 0.0 |
| | ANOVA | F (7, 70) = 9.342, P < 0.0001 | | | | | | | |
| Trophozoite Stage | Means | 2.100 | 1.827 | 2.955 | 3.245 | 5.145 | 7.864 | 0.0 | 0.0 |
| | SD | 1.294 | 1.028 | 1.761 | 1.739 | 3.919 | 7.175 | 0.0 | 0.0 |
| | SE | 0.3901 | 0.3099 | 0.5309 | 0.5244 | 1.182 | 2.163 | 0.0 | 0.0 |
| | ANOVA | F (7, 70) = 11.68, P < 0.0001 | | | | | | | |
| Schizont Stage | Means | 2.030 | 2.491 | 3.464 | 3.736 | 5.700 | 8.436 | 0.0 | 0.0 |
| | SD | 0.8894 | 1.679 | 1.965 | 1.895 | 4.882 | 7.810 | 0.0 | 0.0 |
| | SE | 0.2682 | 0.5062 | 0.5926 | 0.5715 | 1.472 | 2.355 | 0.0 | 0.0 |
| | ANOVA | F (7, 70) = 11.15, P < 0.0001 | | | | | | | |

### 4.2.6 Clinical Manifestations

Clinical parameters measured were scored on a scale of 1 to ten and each one to ten range score was represented on a clinical parameter score table as a single '+'. The most severe malarial symptoms were detected in the *P. berghei* group and no other group scored any higher clinical symptom index than 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 experiences 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 IL-12/*P. berghei* and 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 rest of the groups never experienced this clinical symptom. Turgidity of the skin sharply dropped in the *P. berghei* and CpG/*P. berghei* groups, and while this characteristic occurred mildly in the CpG/IL-18/*P. berghei* and CpG/IL-12/*P. berghei* groups, the CpG ODN and uninfected mice groups did not exhibit any skin abnormality, including turgidity reduction.

Reduced ocular tension was detected only in the *P. berghei* (extreme reduction) and CpG/IL-18/*P. berghei groups* (mild 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, the CpG/IL-18/*P. berghei* group had mild levels of this feature. The rest of the mice groups never had convulsions. 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 while the rest of the mice groups had no roll over movements in their cages. Severe diarrhea 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 diarrhea. 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 4.1).

**Table 4.1: A comparison table showing the severity clinical manifestations observed in the eight groups of mice.**

| | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|
| Lethargy | + + + | ++ | ++ | ++ | + | + + + | - | - |
| Hair Ruffling | - | - | + | + | ++ | + + + | - | - |
| Appetite Distortion | - | - | + | + | +++ | + + + | + | + |
| Urine Colour | ++ | +++ | + | - | - | + + + | - | - |
| Skin Turgor | + | + | + | + | ++ | + + + | - | - |
| Ocular Tension | + | - | - | - | - | + + + | - | - |
| Limb Paralysis | - | - | + | ++ | + | + + + | + | - |
| Convulsions | - | - | + | - | - | + + + | - | - |
| Roll-over movements | - | - | + | ++ | ++ | ++ | - | - |
| Diarrhoea | - | - | + | ++ | ++ | + + + | - | - |
| Piloerection | + | - | + | + | - | + + + | - | - |

Clinical observations were quantified using arbitrary scale and reported as either absent (–), mild (+), moderate (++) or severe (+++).

### 4.2.7 Weight Changes

The uninfected mice group had a mean body weight level of 22.45. The CpG/IL-12/*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 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 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 4.4 and 4.5). There was a sharper decline in body weight in the *P. berghei* group compared to the rest of the mice groups. Significant ($P < 0.0001$) decline in mean body weight was recorded in the *P. berghei* group compared to the rest of the groups ($P < 0.0001$).

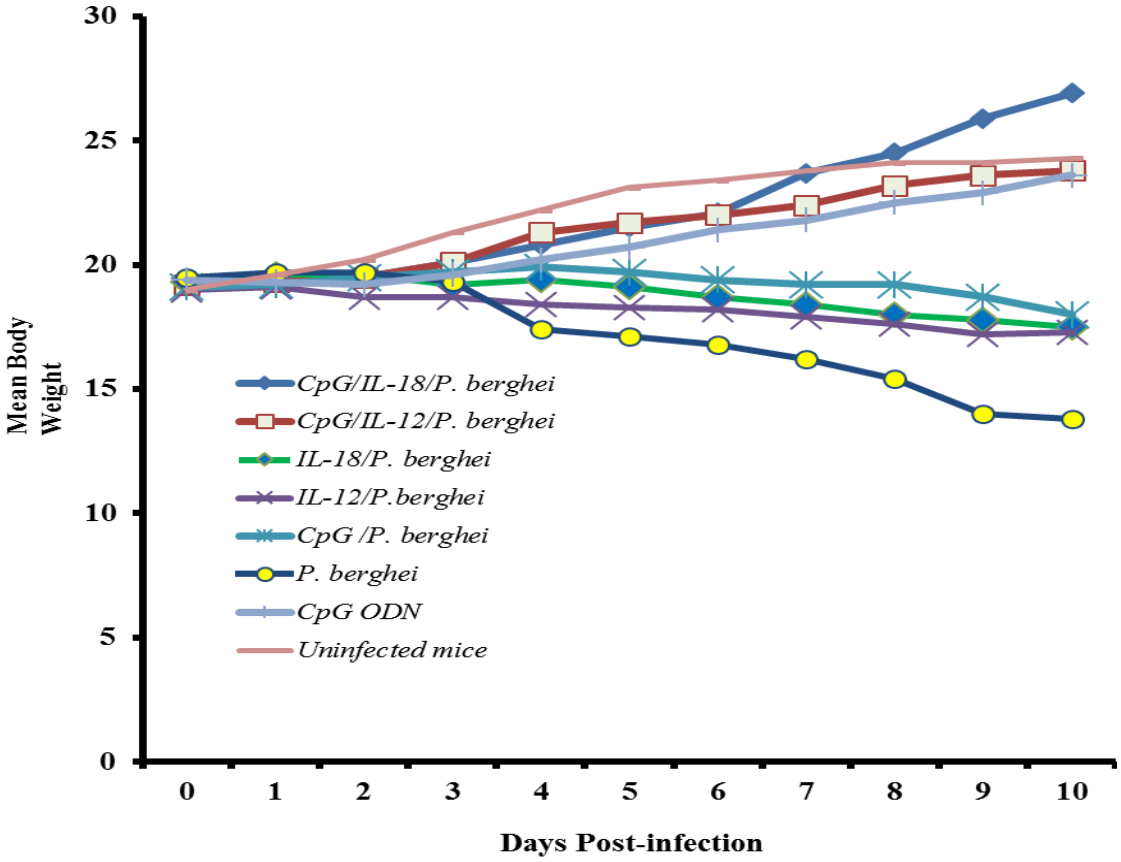



**Figure 4.4: Daily Body Weight Measurements in the Mice Groups.**

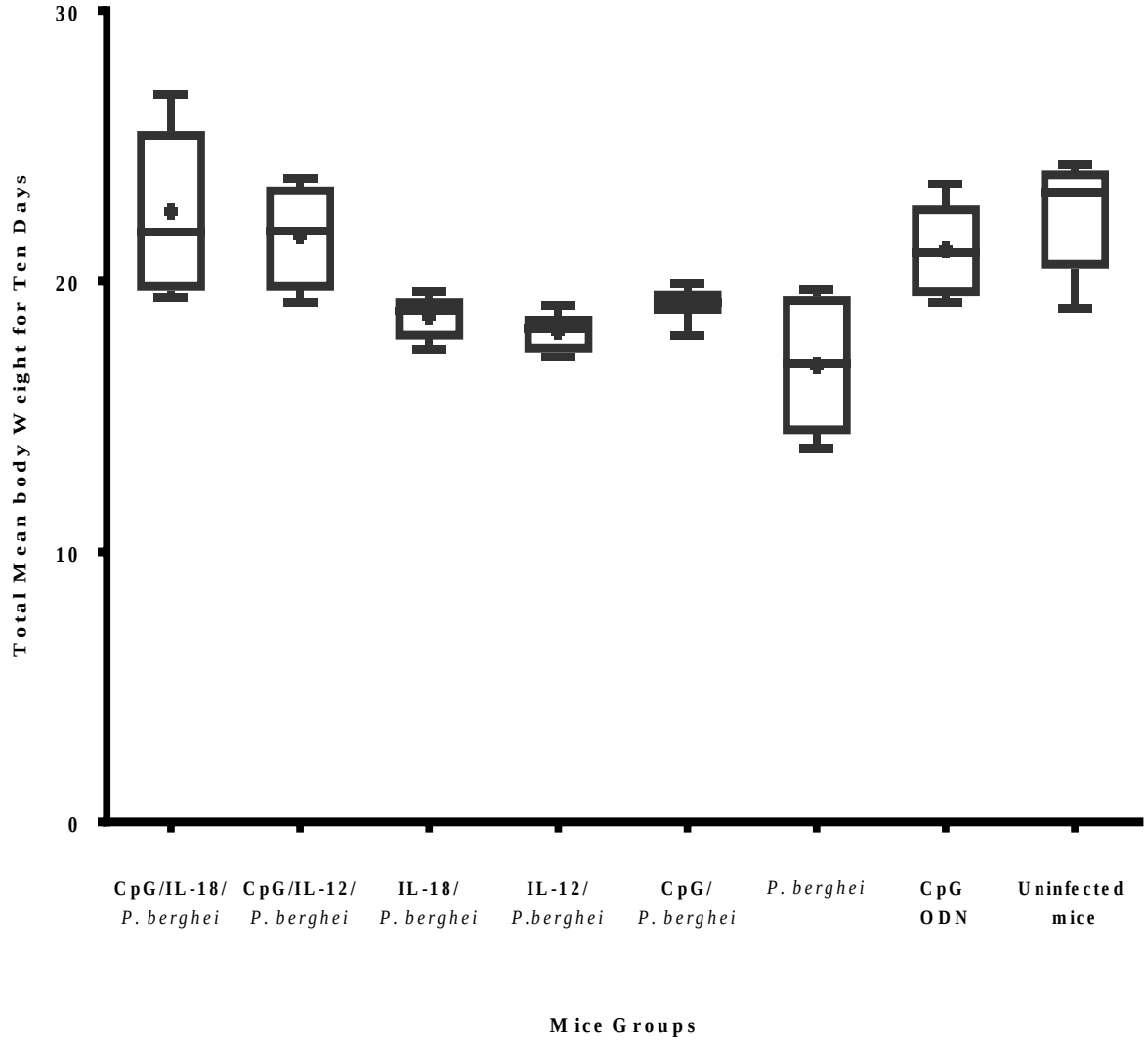



**Figure 4.5: Body Weight Measurements in the Mice Groups**

### 4.2.8 Haematological Responses

With regard to all the RBC indices (PCV, Hgb, MCV, MCH, MCHC, and RBC), the *P. berghei* group recorded the lowest values; 26.9 %, 9.2 g/dL, 44.2fL, 12.6 pg, 34.2 g/dL, 6.08 × 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 recorded significantly ($P< 0.05$) higher values for these indices; 43.2 %, 16.3 g/dL, 16.5 pg, 42.3 g/dL, 10.3 × 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 g/dL, 10.1× 1000/mL, respectively. The MCV value was 43.7 fL. The IL-18/*P. berghei,* IL-12/*P.berghei,* CpG /*P. berghei,* groups had intermediate levels of PCV ranging from 37.7% to 38.9%, while the CpG ODN, and uninfected mice groups had45.2 % and 45.6 % respectively. Similar intermediate levels were recorded for the IL-18/*P. berghei,* IL-12/*P.berghei,* CpG /*P. berghei* groups for the rest of the RBC indices (table 4.2) 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 ODNgroups had significantly higher levels of WBCs, segmented neutrophils, band neutrophils, lymphocytes, mononuclear cells, and eosinophils compared to the rest of the groups, while the percentagevalues 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 uninfectedgroups once again registered mid-level concentrations of the WBC cell types (table 4.3). There were significant variations in the numbers of platelets measured with the *P. berghei* group recording the lowest levels, 126 × 1000, and the highest levels being recorded in the IL-18/*P. berghei* and CpG ODN groups, 302× 1000 and 315 × 1000, respectively.The uninfected mice recorded slightly lower platelet values that averaged to 276 × 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 the highest concentrations of alkaline phosphatase 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 uninfectedgroups, 170.1, 173.1 and 175.2 respectively, and the *P. berghei* and CpG/*P. berghei* 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* 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 glusoce, 100.9 g/dL, 107.6 g/dL, 114.3 g/dL were recorded in the CpG/ *P. berghei,* IL-18/*P. berghei,* CpG/ODN groups.

**Table 4.2: Haematological and clinical chemistry parameter levels analysed in the 8 groups of mice**

| | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|
| PCV (%) | 43.2 | 44.2 | 37.7 | 38.7 | 38.9 | 26.9 | 45.2 | 45.6 |
| Hgb (g/dl) | 16.3 | 15.4 | 10.3 | 11.4 | 12.4 | 9.2 | 18.9 | 18.4 |
| MCV (fl) | 42.1 | 43.7 | 45.2 | 40.2 | 41.7 | 44.2 | 44.3 | 43.3 |
| MCH (pg) | 16.5 | 16.3 | 38.2 | 36.9 | 18.4 | 12.6 | 16.9 | 17.9 |
| MCHC (g/dl) | 42.3 | 43.1 | 37.9 | 38.1 | 38.6 | 34.2 | 47.5 | 46.5 |
| RBC (× 1000/ml) | 10.3 | 10.1 | 8.34 | 9.6 | 9.32 | 6.08 | 10.2 | 10.53 |
| WBC (× 1000) | 13.3 | 13.4 | 6.4 | 6.8 | 6.3 | 5.8 | 09.5 | 10.5 |
| Segmented Neutrophils (%) | 14.2 | 9.7 | 10.5 | 8.5 | 8.8 | 7.6 | 10.3 | 11.3 |
| Band Neutrophils (%) | 4.2 | 1.2 | 2.0 | 2 | 1 | 1 | 2.1 | 2.2 |
| Lymphocytes (%) | 79.4 | 69.1 | 42.2 | 38.2 | 37.4 | 36.3 | 75.4 | 57.3 |
| Mononuclear cells (%) | 4.1 | 2.4 | 2.3 | 2 | 1.2 | 0.9 | 3.3 | 2.1 |
| Eosinophils (%) | 1.9 | 1.7 | 1.9 | 1.2 | 1.2 | 1.1 | 2.1 | 2.0 |
| Basophils (%) | 1 | 1 | 1.1 | 1.1 | 0 | 1.2 | 1.0 | 1.0 |
| Total Bilirubin (mg/dl) | 0.6 | 0.8 | 1.2 | 1.1 | 1.2 | 1.4 | 0.7 | 0.8 |
| Platelets (× 1000) | 302 | 139 | 134.2 | 136.2 | 130.1 | 126 | 315 | 276 |
| Alkaline phosphatase (U/l) | 61.3 | 61.9 | 43.9 | 45.1 | 58.9 | 58.2 | 64.2 | 62.8 |
| ALT (U/I) | 43.7 | 44.6 | 42.2 | 41.1 | 43.3 | 43.4 | 43.3 | 44.4 |
| AST (U/l) | 170.1 | 173.1 | 180.2 | 176.1 | 190.4 | 195.2 | 164.8 | 175.2 |
| Creatinine (mg/dl) | 1.1 | 0.82 | 1.4 | 1.6 | 1.2 | 1.9 | 0.6 | 0.9 |
| Glucose (g/dl) | 119.8 | 120.4 | 107.6 | 117.4 | 100.9 | 92.4 | 114.3 | 117.3 |

### 4.3 Transcription Factors and Physiologic Biomolecules' Levels

Multiple transcription factors and bioactive molecules were measured in these experiments. The transcription factors quantified were NF-kB, NFATc, FOX-P3, IRF-5, STAT-6, AhR and the Kruppel-like factor. Detected physiologic biomolecules included adiponectin, angiogenin, angiopoietin 1, angiopoietin 2, NRP-1, Cox-2, MMP-8 and MMP-9. One significant outcome of these studies was that the concentrations of all transcription factors and bioactive molecules differed significantly among the groups (Tables 4.3, 4.4 and 4.5). Generally, murine recipients of the cytokine-CpG ODN co-inoculation bitherapy manifested with increased levels of NF-kB, NFATc, IRF-5, AhR, KLF and reduced levels of FOXp3 and STAT-6 TFs. The measured STAT-6 levels were reduced in the CpG/IL-12/*P. berghei*, IL-18/*P. berghei* and CpG/*P. berghei* and increased in the *P. berghei* and IL-12/*P. berghei* groups. As for the physiologic biomelecules, adiponectin, angiopoietin 1, NRP-1 and COX-2 were notably increased in the cytokine-CpG ODN combination groups, while angiogenin, angiopoietin 2, MMP-8 and MMP-9 were downregulated. Also, as shown in table below, a variety of general outcomes were noted when the concentrations of the IL-18 / CpG / *P. berghei* and IL-12 / CpG / *P. berghei* groups were compared to the control groups in the experiments:

**Table 4.3: Comparison of transcription factors and bioactive molecules' concentrations:**

| **Quantified Factors** | **Quantitative difference relative to other groups' physiologic biomolecules** | |
|---|---|---|
| Transcription Factors | Quantity in<br><br>IL-18 / CpG / *P. berghei* group | Quantity in<br><br>IL-12 / CpG / *P. berghei* group |
| 1. NFk-B | ↑ Than in all groups | ↑ Than in all groups except group 1 |
| 2. NFATc | ↑ Than in all groups except group 2 | ↑ Than in all groups |
| 3. FOXP-3 | ↓ Than in all other groups | ↓ Than in all except group 1 |
| 4. IRF-5 | ↑ Than in all other groups | ↑ Than in all except group 1 |
| 5. STAT - 6 | ↑ Than in all groups except 4 and 6 | ↓ Than in all except groups 3, 5 and 8 |
| 6. AhR | ↑ Than in all groups except 2 | ↑ Than in all except group |
| 7. KLF | ↑ Than in all groups | ↑ Than in all except group 1 |
| Physiologic Biomolecules | Quantity in<br><br>IL-18 / CpG / *P. berghei* group | Quantity in<br><br>IL-12 / CpG / *P. berghei* group |
| 1. Adiponectin | ↑ Than in all groups | ↑ Than in all except group 1, 4 and 7 |
| 2. Angiogenin | ↓ Than all groups except group 2 | ↑ Than in all groups |
| 3. Angiopoietin 1 | ↑ Than in all groups except group 2 | ↑ Than in all groups |
| 4. Angiopoietin 2 | ↓ Than all except 3,7 and 8 | ↑ Than in all except group 1, 3, 4, 7,8 |
| 5. NRP-1 | ↑ Than all except groups 2 and 4 | ↑ Than in all except groups |
| 6. Cox - 2 | ↑ Than in all other groups | ↑ Than in all except group 1 |
| 7. MMP-8 | ↑ Than in all except groups 5 and 6 | ↓ Than in all except groups 3 and 4 |
| 8. MMP-9 | ↑ Than in all except groups 2 and 4 | ↓ Than in all except group 4 |

The symbol '↑' stands for 'mean concentration greater' while '↓' stands for 'mean concentration lower'. In this table, the groups CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei*; IL-18/ *P. berghei*; IL-12/ *P. berghei*; CpG / *P. berghei*; *P. berghei;* CpG ODN and uninfected mice groups are represented by the numbers 1, 2, 3, 4, 5, 6, 7 and 8 respectively.

**Table 4.4: Transcription Factor Concentrations**

| | | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|---|
| | Means | 221.9 | 141.0 | 67.51 | 58.58 | 85.00 | 41.06 | 63.60 | 30.00 |
| | SD | 19.87 | 12.57 | 1.846 | 3.460 | 7.488 | 2.149 | 2.679 | 2.150 |
| 1. NFk-B | ANOVA | F (7, 336) = 4274, P < 0.0001 | | | | | | | |
| | Means | 4.229 | 4.700 | 0.9573 | 2.858 | 0.6340 | 0.6961 | 2.441 | 0.5214 |
| | SD | 1.246 | 2.098 | 1.239 | 0.3434 | 0.5565 | 0.8212 | 0.2495 | 0.5159 |
| 2. NFATc | ANOVA | F (7, 336) = 219.9, P < 0.0001 | | | | | | | |
| | Means | 0.6757 | 0.7086 | 1.491 | 14.91 | 14.90 | 17.07 | 1.464 | 5.171 |
| | SD | 0.1269 | 0.2646 | 0.8836 | 0.9698 | 2.301 | 1.597 | 0.4143 | 0.5447 |
| 3. FOXP-3 | ANOVA | F (7, 336) = 3964, P < 0.0001 | | | | | | | |
| | Means | 5.743 | 4.357 | 1.601 | 2.774 | 1.041 | 0.7617 | 1.193 | 1.859 |
| | SD | 1.240 | 1.069 | 0.3413 | 0.9748 | 0.3801 | 0.09583 | 0.5747 | 0.2734 |
| 4. IRF-5 | ANOVA | F (7, 336) = 736.3, P < 0.0001 | | | | | | | |
| | Means | 3.676 | 1.321 | 0.7957 | 6.543 | 0.9743 | 7.886 | 3.386 | 0.7000 |
| | SD | 0.7960 | 0.4855 | 0.2080 | 0.9809 | 0.2186 | 0.8546 | 0.4962 | 0.06874 |
| 5. STAT-6 | ANOVA | F (7, 336) = 2920, P < 0.0001 | | | | | | | |
| | Means | 6.381 | 9.114 | 2.301 | 2.551 | 2.079 | 0.7157 | 3.649 | 1.692 |
| | SD | 1.508 | 0.4655 | 0.3648 | 0.3365 | 0.2995 | 0.3899 | 0.8453 | 0.9277 |
| 6. AhR | ANOVA | F (7, 336) = 1838, P < 0.0001 | | | | | | | |
| | Means | 66.30 | 51.16 | 24.94 | 7.771 | 4.657 | 0.6543 | 18.69 | 9.186 |
| | SD | 7.150 | 4.797 | 4.006 | 1.435 | 0.2820 | 0.1050 | 1.489 | 0.9974 |
| 7. KLF | ANOVA | F (7, 336) = 3978, P < 0.0001 | | | | | | | |

**Table 4.5: Physiologic biomolecule concentrations**

| | | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|---|
| | Means | 12.24 | 9.743 | 3.600 | 10.49 | 4.814 | 3.714 | 10.36 | 4.271 |
| | SD | 1.181 | 1.603 | 0.8808 | 0.9076 | 0.4021 | 0.3873 | 0.5260 | 0.5115 |
| 1.Adiponectin | ANOVA | F (7, 336) = 3136, P < 0.0001 | | | | | | | |
| | Means | 64.20 | 51.50 | 94.03 | 84.21 | 276.7 | 715.0 | 190.3 | 166.9 |
| | SD | 7.941 | 3.114 | 4.157 | 5.595 | 26.59 | 38.61 | 29.49 | 8.464 |
| 2. Angiogenin | ANOVA | F (7, 336) = 9980, P < 0.0001 | | | | | | | |
| | Means | 15.74 | 17.57 | 9.400 | 8.343 | 7.486 | 1.769 | 6.143 | 6.457 |
| | SD | 0.6318 | 0.4971 | 1.188 | 0.4514 | 0.6000 | 1.045 | 0.4610 | 0.4383 |
| 3. Angiopoietin 1 | ANOVA | F (7, 336) = 11862, P < 0.0001 | | | | | | | |
| | Means | 1.644 | 3.743 | 1.326 | 3.329 | 14.56 | 16.31 | 1.386 | 1.274 |
| | SD | 0.5630 | 0.6997 | 0.08696 | 0.5936 | 0.9796 | 0.6914 | 0.3521 | 0.2900 |
| 4. Angiopoietin 2 | ANOVA | F (7, 336) = 23989, P < 0.0001 | | | | | | | |
| | Means | 12.66 | 14.30 | 3.086 | 14.17 | 2.214 | 2.003 | 3.086 | 4.143 |
| | SD | 0.4735 | 0.5208 | 0.6096 | 0.5447 | 0.6262 | 0.4644 | 0.3873 | 0.4856 |
| 5. NRP-1 | ANOVA | F (7, 336) = 99045, P < 0.0001 | | | | | | | |
| | Means | 139.6 | 129.9 | 39.04 | 100.4 | 39.56 | 27.41 | 47.67 | 57.60 |
| | SD | 6.534 | 3.954 | 1.983 | 6.030 | 2.293 | 0.8930 | 1.109 | 1.623 |
| 6. Cox - 2 | ANOVA | F (7, 336) = 16655, P < 0.0001 | | | | | | | |
| | Means | 64.07 | 46.29 | 45.31 | 26.31 | 298.0 | 296.9 | 58.97 | 56.89 |
| | SD | 13.88 | 11.22 | 10.98 | 8.463 | 10.97 | 16.58 | 3.719 | 4.178 |
| 7. MMP-8 | ANOVA | F (7, 336) = 25568, P < 0.0001 | | | | | | | |
| | Means | 227.2 | 215.6 | 277.0 | 198.9 | 402.9 | 591.0 | 261.9 | 265.7 |
| | SD | 9.542 | 14.33 | 15.41 | 7.174 | 51.07 | 40.63 | 5.004 | 10.39 |
| 8. MMP-9 | ANOVA | F (7, 336) = 2726, P < 0.0001 | | | | | | | |

### 4.3.1 Transcription Factor Concentrations

#### 4.3.1.1 NF-kB Levels

The NF-kB transcription factor was detected in all investigated mice groups and overall its concentrations spanned from 30.0 ng/ml (in the uninfected mice group) to 221.9 ng/ml (in the CpG/IL-18/ *P. berghei* group). For analytical purposes concentrations were arbitrarily categorized into high, intermediate and low level concentrations. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two high level concentration groups and had a mean NF-kB level of 181.45 ng/ml. The CpG/IL-18/ *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 4274$) concenrations than the rest of the groups in these investigations. This group had a mean NF-kB concentration of 221.9 ng/ml (SD 19.87, SE 2.839). The CpG/IL-12/*P. berghei* group produced significantly higher concentrations of NF-kB than the rest of the groups except group 1 and its mean levels were at 141.0 ng/ml (SD 12.57 SE 1.796). Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded to have generated NF-kB levels of more than 140 ng/ml.

Intermediate levels of NF-kB were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei*, and in the CpG ODN groups; 67.51 (SD 1.846, SE 0.2637), 58.58 (SD 3.460, SE 0.4943), and 63.60 (SD 2.671 and SE 0.3827) respectively and these groups had a mean NF-kB amount of 63.23 ng/ml with values ranging from 58.58 ng/ml to 667.1 ng/ml. In the CpG/ *P. berghei* group NF-kB levels were slightly higher than in the intermediate groups; 85.00 (SD 7.488, SE 1.070). The lowest levels of NF-kB were found in the *P. berghei* and uninfected mice groups; 41.06 ng/ml and 30.00 ng/ml and these two groups had a mean NF-kB amount of 35.53 ng/ml. The NF-kB levels in the CpG/IL-18/ *P. berghei* group were about one 1½ times higher than levels in the IL-18/ *P. berghei* group, 2½ times higher than levels in the CpG/ *P. berghei* group, 3½ times higher than the intermediate concentration groups collectively (described above) and 6 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group

NF-kB levels were 2½ times higher than in the IL-12/ *P. berghei* group, more than 1½ times higher than in the CpG/ *P. berghei* group, 2 times higher than the mean levels of the intermediate concentration groups and 4 times higher than the two lowest concentration groups (Figure 4.6).

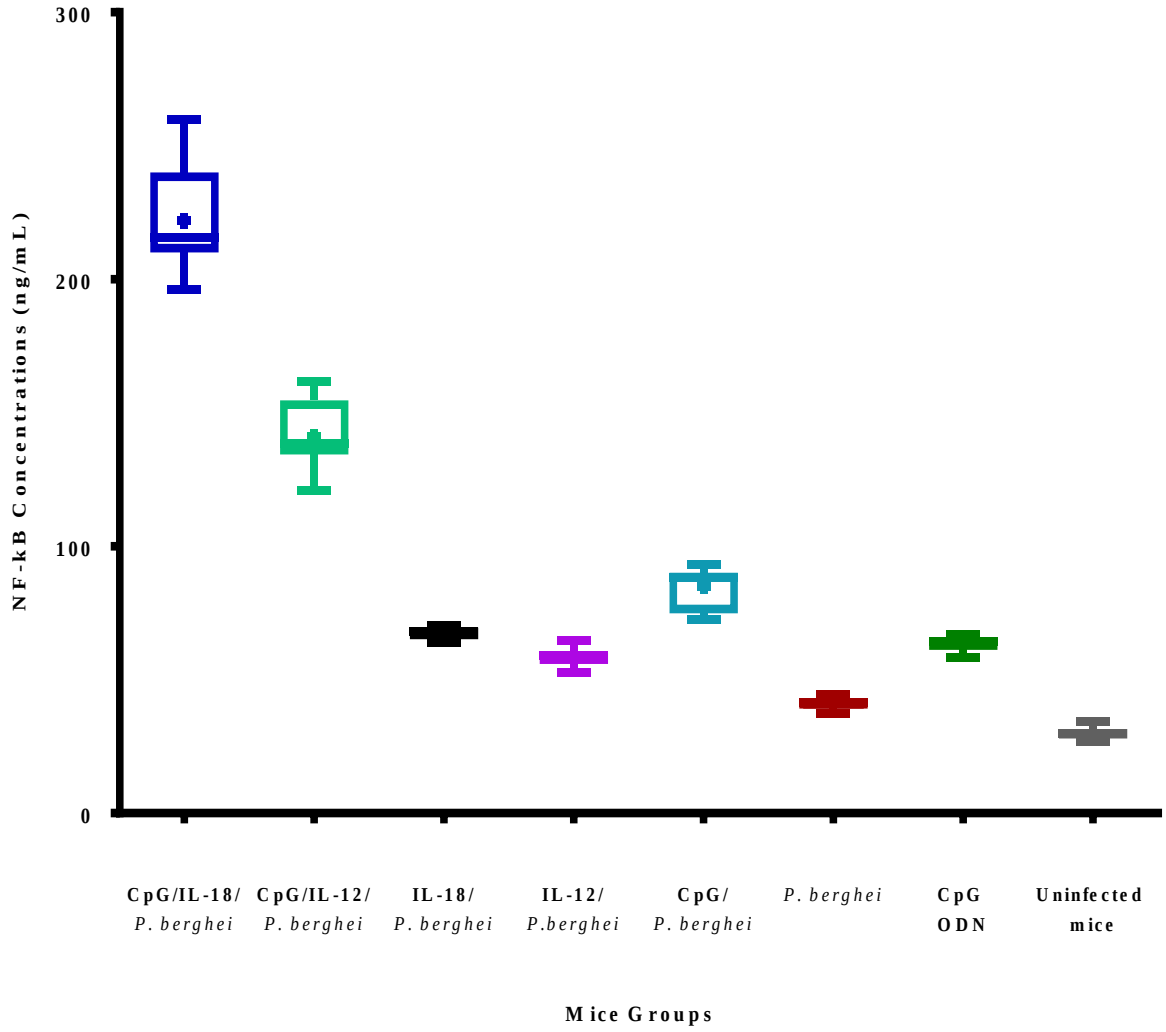



**Figure 4.6: NF-kB levels.**

Both the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups of mice generated the highest ($P < 0.0001$) concentrations of NF-kB, while the *P. berghei* and uninfected groups were noted to have the lowest concentrations.

#### 4.3.1.2 NFATc Levels

The NFATc transcription factor was also measured in all mice groups and its concentrations ranged from 0.5214 ng/ml (in the uninfected mice group) to 4.700 ng/ml (in the CpG/IL-12/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups had the two highest NFATc concentrations and they had a mean NFATc level of 4.4645 ng/ml. The CpG/IL-18/ *P. berghei* group released significantly higher ($P < 0.0001$; $F(7, 336) = 219.9$) concenrations than the rest of the groups in these experiments. This group had a mean NFATc concentration of 4.229 ng/ml (SD 1.246, SE 0.1781). The second main experimental group, CpG/IL-12/*P. berghei* group was detected to have significantly higher concentrations of NFATc than all other groups with the exception of the group CpG/IL-18/ *P. berghei* group and its mean NFATc concentrations were 4.700 ng/ml (SD 2.098 SE 0.2997). It was only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups that were found to have generated mean NFATc levels of more than 4.20 ng/ml.

Intermediate mean levels of NFATc were detected in the IL-12/ *P. berghei,* and in the CpG ODNgroups; 2.858 (SD 0.3434, SE 0.0490), and 2.441 (SD 0.2495 and SE 0.0356) respectively and together, these two groups had a mean NFATc amount of 2.6495 ng/ml. The lowest mean levels of NFATc were recorded in the IL-18/ *P. berghei,* CpG/ *P. berghei, P. berghei* and uninfected mice groups; 0.9573 ng/ml, 0.6340 ng/ml, 0.6961 ng/ml and 0.5214 ng/ml respectivley and these 4 groups had a mean NFATc amount of 0.7022 ng/ml. The NFATc levels in the CpG/IL-18/ *P. berghei* group were about 5 times higher than levels in the IL-18/ *P. berghei* group, 2 times higher than the intermediate concentration groups collectively (as described above) and 6 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group NFATc levels were approximately 2 times higher than in the IL-12/ *P. berghei* group, more than 1½ times higher than the mean levels of the intermediate concentration groups and about 7 times higher than the four lowest concentration groups (Figure 4.7).

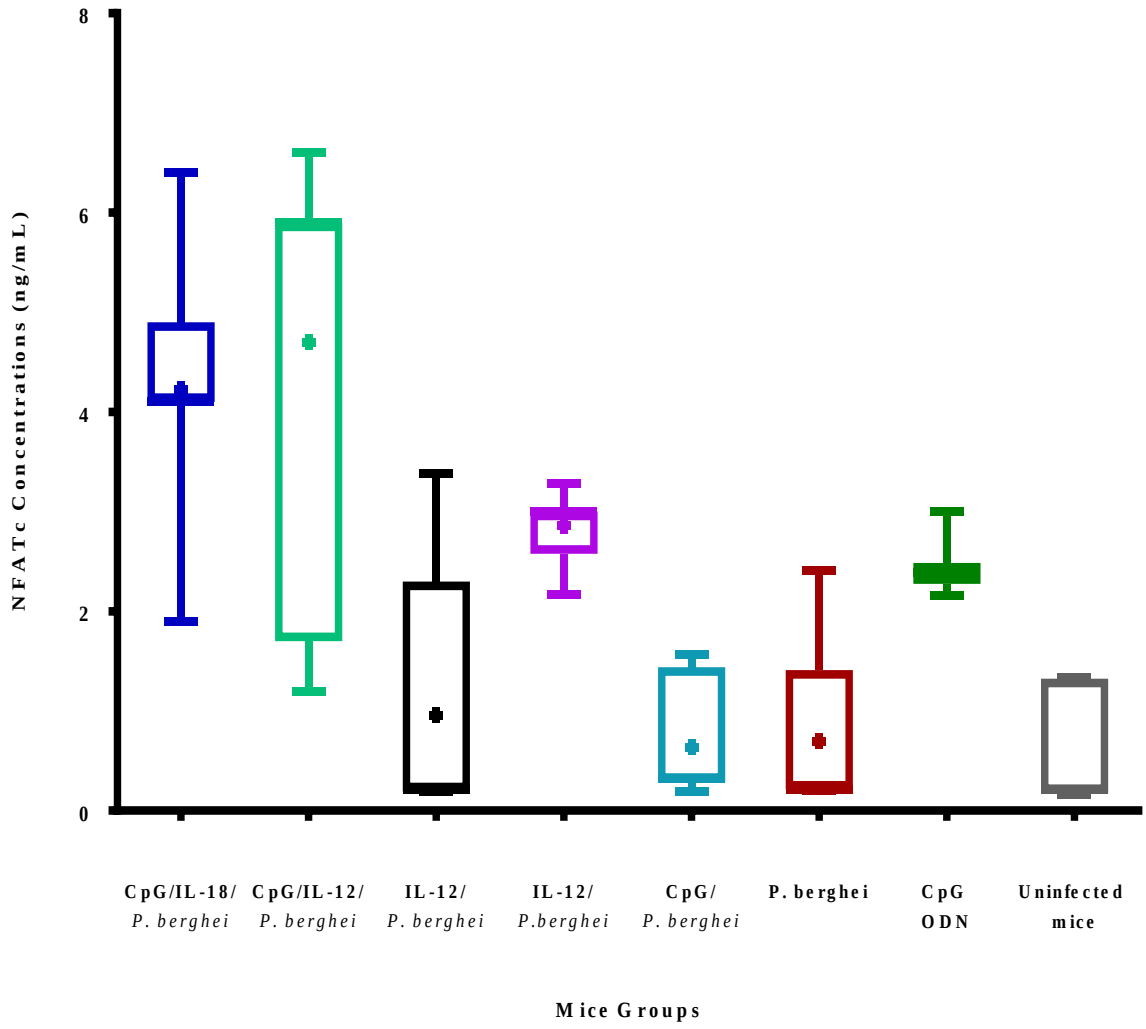



**Figure 4.7: NFATc levels**

The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups of mice were detected with the highest mean 4.4645 ng/ml ($P < 0.0001$) concentrations of NFATc, while intermediate mean levels of NFATc were detected in the IL-12/ *P. berghei*, and in the CpG ODN groups. Both the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were found to have more than six times higher concentrations of NFATc that the four lowest concentrations (mean of 0.7022 ng/ml).

#### 4.3.1.3 FOXP-3 Levels

The mean FOXP-3 transcription factor concentrations ranged from 0.6757 ng/ml (in the CpG/IL-18/ *P. berghei* group) to 14.91 ng/ml (in the IL-12/ *P. berghei* group). The IL-12/ *P. berghei,* CpG/ *P. berghei* and *P. berghei* groups had the three highest FOXP-3 concentrations with a mean FOXP-3 level of 15.627 ng/ml. The *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 3964$) FOXP-3 concentrations than all other groups in the study. This group had a mean FOXP-3 concentration of 17.07 ng/ml (SD 1.597, SE 0.2282). The IL-12/ *P. berghei* and CpG/ *P. berghei* groups, with FOXP-3 concentrations of 14.91 ng/ml and 14.90 pg/ml respectively, were found to have significantly higher concentrations of FOXP-3 than all other groups, with the exception of the *P. berghei* group and their mean FOXP-3 concentration was 14.91 ng/ml (SD 15.36 SE 2.194). Only the IL-12/ *P. berghei,* CpG/ *P. berghei* and *P. berghei* groups were detected with mean FOXP-3 levels of more than 14.00 ng/ml. The rest of the mice groups had concentrations ranging below 5.2 ng/ml. Medium mean levels of FOXP-3 were detected in the uninfected mice group; 5.171 (SD 5.447, SE 0.778).

The lowest mean levels of FOXP-3 were recorded in the CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* and CpG ODN mice groups; 0.6757 ng/ml, 0.7086 ng/ml, 1.491 ng/ml and 1.464 ng/ml respectively and these 4 groups had a mean FOXP-3 amount of 1.084 ng/ml. The FOXP-3 levels in the *P. berghei* group (the highest in the study) were twenty-six times higher than levels in the CpG/IL-18/ *P. berghei* group, twenty-four times higher than in the CpG/IL-12/ *P. berghei* group, three-fold higher than the medium concentration uninfected mice group. The FOXP-3 levels in the *P. berghei* group were eighteen times higher than the mean FOXP-3 level in the 4 lowest concentration groups given above. The mean FOXP-3 levels (15.627 ng/ml) in the CpG/IL-12/ *P. berghei* and CpG/*P. berghei* groups (second highest in the study) were twenty-three times higher than levels in the CpG/IL-18/ *P. berghei* group, twenty-two times higher than in the CpG/IL-12/ *P. berghei* group, three-fold higher than the medium concentration uninfected mice group. The FOXP-3 levels in these two groups were fourteen times higher than the mean FOXP-3 level in the 4 lowest concentration groups (Figure 4.8).

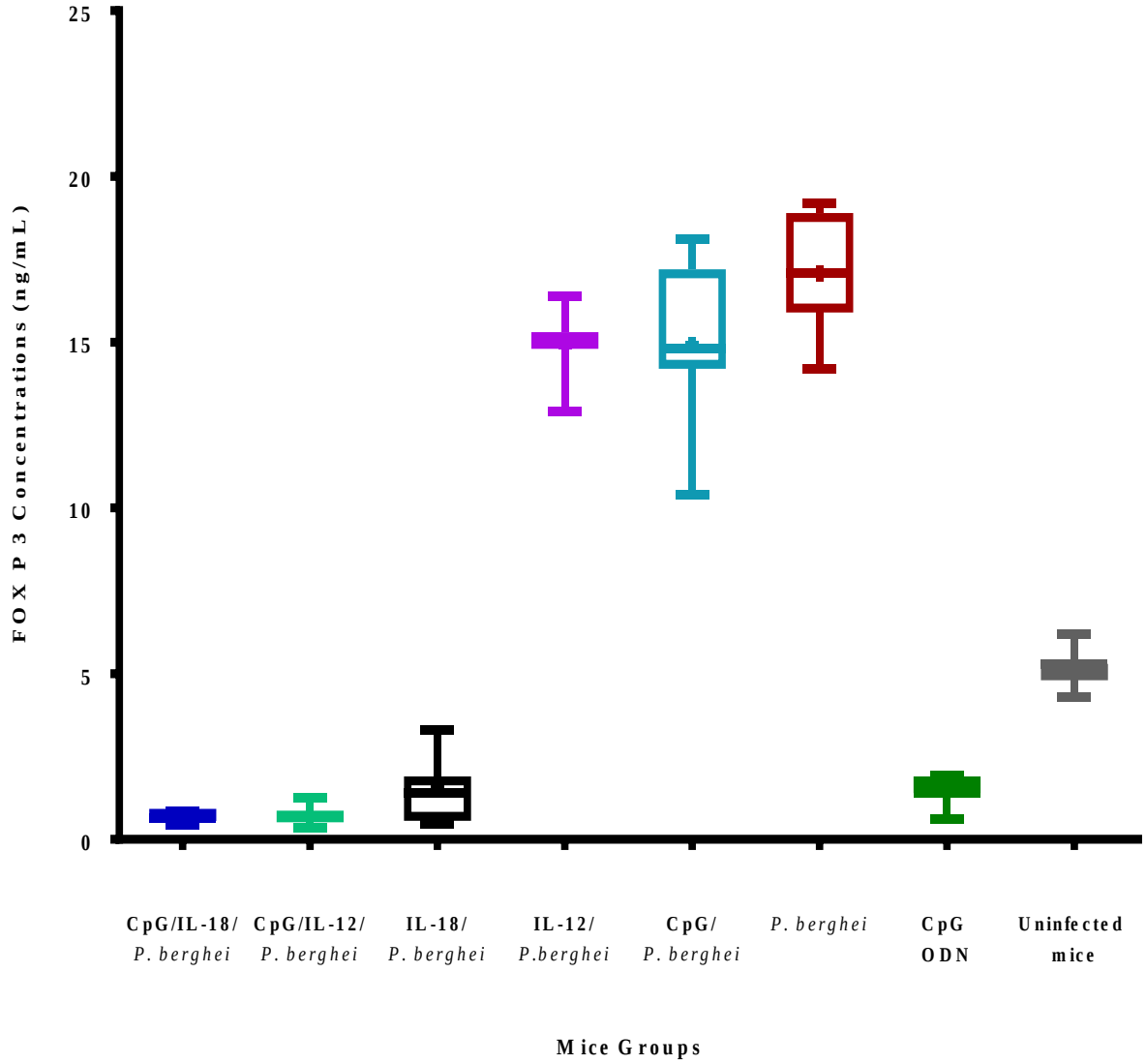



**Figure 4.8: FOXP-3 levels**

The IL-12/ *P. berghei*, CpG/ *P. berghei* and *P. berghei* groups had significantly higher mean FOXP-3 concentrations (15.627 ng/ml; $P < 0.0001$) than the rest of the mice groups.

#### 4.3.1.4 IRF-5 Levels

The IRF-5 transcription factor was measured in all mice groups and its concentrations spanned from 0.7617 ng/ml (in the *P. berghei*mice group) to 5.743 ng/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were found to have generated the highest concentrations of IRF-5 and these two groups and had a mean IRF-5level of 5.505 ng/ml. The CpG/IL-18/ *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 736.3$) concenrations than the rest of the groups in these investigations. This group had a mean IRF-5 concentration of 5.743 ng/ml (SD 1.240, SE 1.1771). The CpG/IL-12/*P. berghei* group had significantly higher concentrations of IRF-5 than the rest of the groups (with the exception of group 1) and its mean levels were at 4.357 ng/ml (SD 1.069 SE 0.1527). Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded with IRF-5levels of more than 4.300 ng/ml.

Intermediate levels of IRF-5, 2.774 ng/ml, were detected in the IL-12/ *P. berghei* group while the lowest levels of IRF-5 were found in the IL-18/ *P. berghei,* CpG/ *P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 1.601 ng/ml, 1.041 ng/ml, 0.7617 ng/ml, 1.193 ng/ml and 1.859 ng/ml respectively and these five groups had a mean IRF-5 amount of 1.2911 ng/ml. The IRF-5 levels in the CpG/IL-18/ *P. berghei* group were about one 4 times higher than levels in the IL-18/ *P. berghei* group, 2 times higher than levels in the IL-12/ *P. berghei* group (intermediate concentration group) and 4 times higher than the average level, 1.2911 ng/ml, of the five lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group IRF-5 levels were 1½ times higher than in the IL-12/ *P. berghei* group (this was also the midlevel concentration group), more than 3 times higher than in the five lowest concentration groups (Figure 4.9).

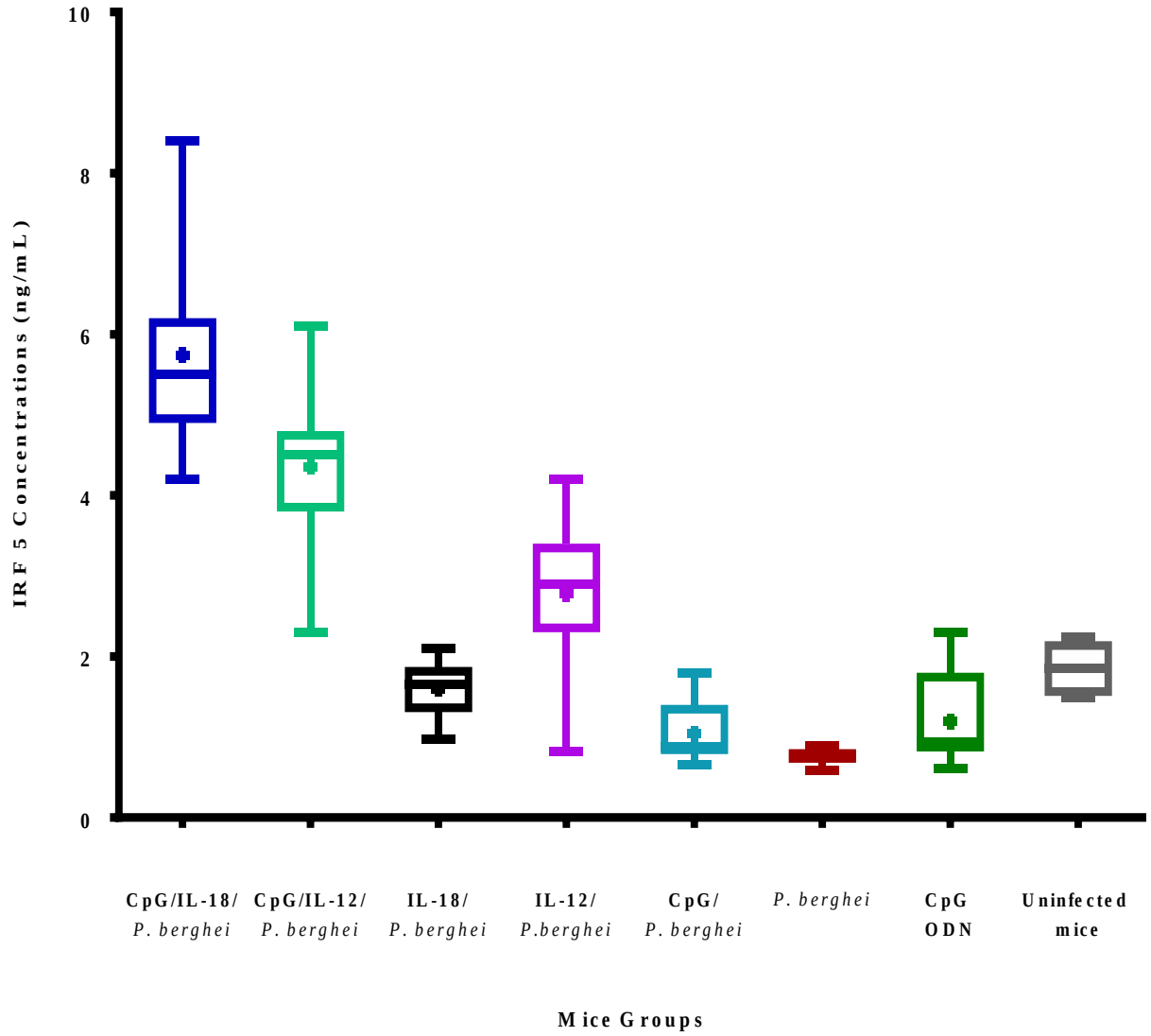



**Figure 4.9: IRF-5 levels**

The mean IRF-5 concentrations in both the CpG/IL-18/ *P. berghei* and the CpG/IL-12/ *P. berghei* groups, were significantly higher than in the rest of the mice groups.

#### 4.3.1.5 STAT-6 Levels

The mean STAT-6 transcription factor concentrations ranged from 0.700 ng/ml (in the uninfected mice group) to 9.40 ng/ml (in the *P. berghei* group). The IL-12/ *P. berghei* and *P. berghei* groups had the highest STAT-6 concentrations with a mean STAT-6 level of 8.65 ng/ml. The *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 2920$) STAT-6 concentrations than all other groups in the study. This group had a mean STAT-6 concentration of 9.400 ng/ml (SD 0.0687, SE 0.0098). TheIL-12/*P. berghei* group, with STAT-6 concentrations of 7.900 ng/ml (SD 0.485, SE 0.0693) was found to have a significantly higher concentration of STAT-6 than all other groups (except the *P. berghei* group). Only two these two groups, IL-12/ *P. berghei* and *P. berghei* had STAT-6 measurements of more than 7.900 ng/ml. The rest of the mice groups had concentrations ranging below 4.90 ng/ml. Intermediate levels of STAT-6 were detected in the CpG/IL-12/ *P. berghei* mice and in the CpG ODN groups of mice; 4.890 ng/ml (SD 3.676, SE 0.7960) and 4.200 ng/ml (SD 3.386, SE 0.496) respectively, giving a mean value of 4.5 ng/ml. The lowest levels of STAT-6 were measured in the CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* CpG/ *P. berghei* and in the uninfected mice groups; 1.321ng/ml, 0.7957 ng/ml, 0.9743 ng/ml and 0.700 ng/ml respectively and these 4 groups had a mean STAT-6 amount of 0.948 ng/ml. The STAT-6 levels in the *P. berghei* group (the highest in the study) were about 2 times higher than levels in the CpG/IL-18/ *P. berghei* group, 3 times higher than in the CpG/IL-12/ *P. berghei* group and 2-fold higher than in the intermediate concentration groups. The STAT-6 levels in the *P. berghei* group were also10 times higher than the mean STAT-6 level in the 4 lowest concentration groups above. The mean STAT-6 levels (15.627 ng/ml) in the IL-12/ *P. berghei* group, second highest in the study, were about 2 times higher than levels in the CpG/IL-18/ *P. berghei* group, 3 times higher than in the CpG/IL-12/ *P. berghei* group, about 2-fold higher than the intermediate concentration groups. The STAT-6 levels in these two groups were 8 times higher than the mean STAT-6 level in the 4 lowest concentration groups. The mean STAT-6 level in the CpG/IL-18/ *P. berghei* group 5 times exceeded the mean level in the IL-18/ *P. berghei* group, while levels in the CpG/IL-12/ *P. berghei* group were 4-fold lower than those in the IL-12/ *P. berghei* group (Figure 4.10).

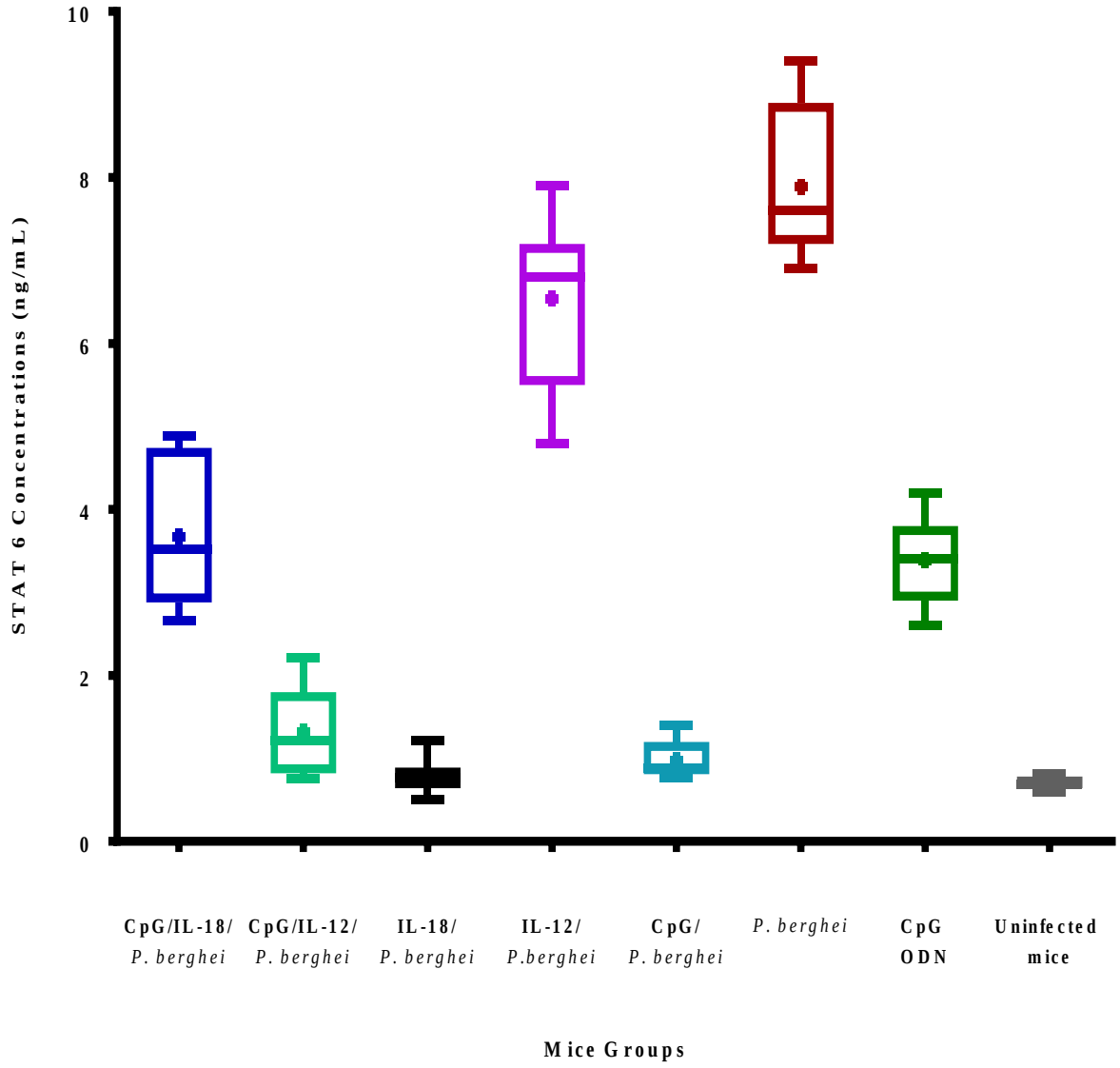



**Figure 4.10: STAT-6 levels**

The IL-12/ *P. berghei* and *P. berghei* produced significantly higher ($P < 0.0001$; $F(7, 336) = 2920$) STAT-6 concentrations than the rest of the mice groups in the study and had a mean STAT-6 level of 8.650 ng/ml.

#### 4.3.1.6 AhR Levels

The measured concentrations of the AhR transcription factor ranged from 0.7157 ng/ml (in the *P. berghei* mice group) to 9.114 ng/ml (in the CpG/IL-12/ *P. berghei* group). High level concentrations were found in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups and these had a mean AhR level of 9.05 ng/ml. The CpG/IL-12/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 1838$) AhR concentrations than the rest of the groups in these investigations. This group had a mean AhR concentration of 9.114 ng/ml (SD 0.465, SE 0.665). The CpG/IL-18/*P. berghei* group generated significantly higher concentrations of AhR than the rest of the groups (except the CpG/IL-18/ *P. berghei* group) and its mean levels were at 6.381 ng/ml (SD 1.508 SE 0.215). Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded withAhR levels of more than 6.300 ng/ml.

Intermediate levels of AhR were detected in the IL-18/ *P. berghei,* IL-12/ *P. berghei* and in the CpG/ *P. berghei* ODN groups; 2.301 ng/ml (SD 1.846, SE 0.2637), 2.551 ng/ml (SD 3.460, SE 0.4943), and 2.079 ng/ml (SD 2.671 and SE 0.3827) respectively and these groups had a mean AhR amount of 2.310 ng/ml with values ranging from 2.301 ng/ml to 2.551 ng/ml. In the CpG ODN group AhR levels were slightly higher than in the intermediate groups; 3.649 ng/ml (SD 0.845, SE 0.120). The lowest levels of AhR were detected in the *P. berghei* and uninfected mice groups; 0.7157 ng/ml and 1.692 ng/ml and these two groups had a mean AhR amount of 1.203 ng/ml. The AhR levels in the CpG/IL-18/ *P. berghei* group were more than 2½ times higher than levels in the IL-18/ *P. berghei* group, 3 times higher than the intermediate concentration groups collectively (described above) and 5 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group AhR levels were 3½ times higher than in the IL-12/ *P. berghei* group, 4 times higher than the mean levels of the intermediate concentration groups and 8 times higher than the meanconcentration of lowest level groups (Figure 4.11).

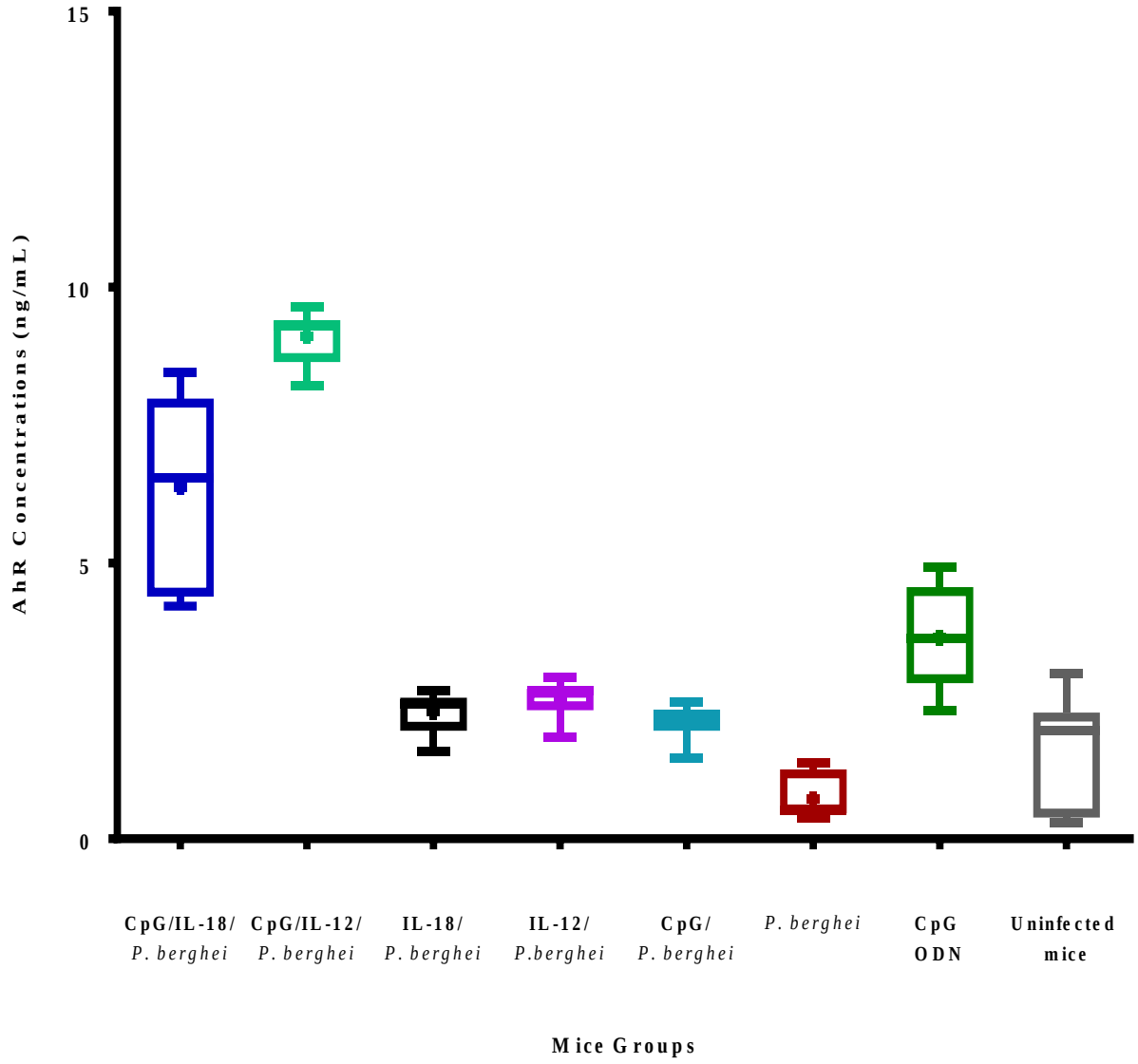



**Figure 4.11: AhR levels**

The concentrations AhR in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups (a mean AhR level of 9.05 ng/ml) were significantly higher ($P < 0.0001$; $F(7, 336) = 1838$) than in the rest of the mice groups.

#### 4.3.1.7 KLF Levels

The measured concentrations of the KLF transcription factor ranged from 0.654 ng/ml (in the *P. berghei* mice group) to 66.30 ng/ml (in the CpG/IL-18/ *P. berghei* group). Significantly ($P < 0.0001$; $F(7, 287) = 3978$) higher levels of KLF concentrations were found in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups than the rest of the groups and these two groups had mean KLF levels of 66.30 ng/ml and 51.16 ng/ml respectively. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded with KLF levels of more than 50.00 ng/ml.

Medium levels of KLF were detected in the IL-18/ *P. berghei,* and CpG ODN groups; 24.94 ng/ml and 18.69 ng/ml respectively and these two groups together had a mean KLF amount of 21.815 ng/ml. In the CpG ODN group KLF levels were slightly higher than in the intermediate groups; 3.649 ng/ml. The lowest levels of KLF were detected in the IL-12/ *P. berghei,* CpG/ *P. berghei, P. berghei* and uninfected mice groups; 7.771 ng/ml, 4.657 ng/ml, 0.6543 ng/ml and 9.186 ng/ml and these four groups had a mean KLF amount of 5.5670 ng/ml. It was noticeable that of all the lowest KLF levels recorded, only the *P. berghei* group had concentrations (0.6543 ng/ml) below the 1.00 ng/ml level, 100 times lower that the highest recorded concentration (66.30 ng/ml in the CpG/IL-18/ *P. berghei* group). The KLF levels in the CpG/IL-18/ *P. berghei* group were more than 2½ times higher than levels in the IL-18/ *P. berghei* group, 3 times higher than the medium concentration groups (collectively described above) and 11 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group KLF levels were 6½ times higher than in the IL-12/ *P. berghei* group, 2 times higher than the mean levels of the intermediate concentration groups and 9 times higher than the mean concentration of the groups (Figure 4.12).

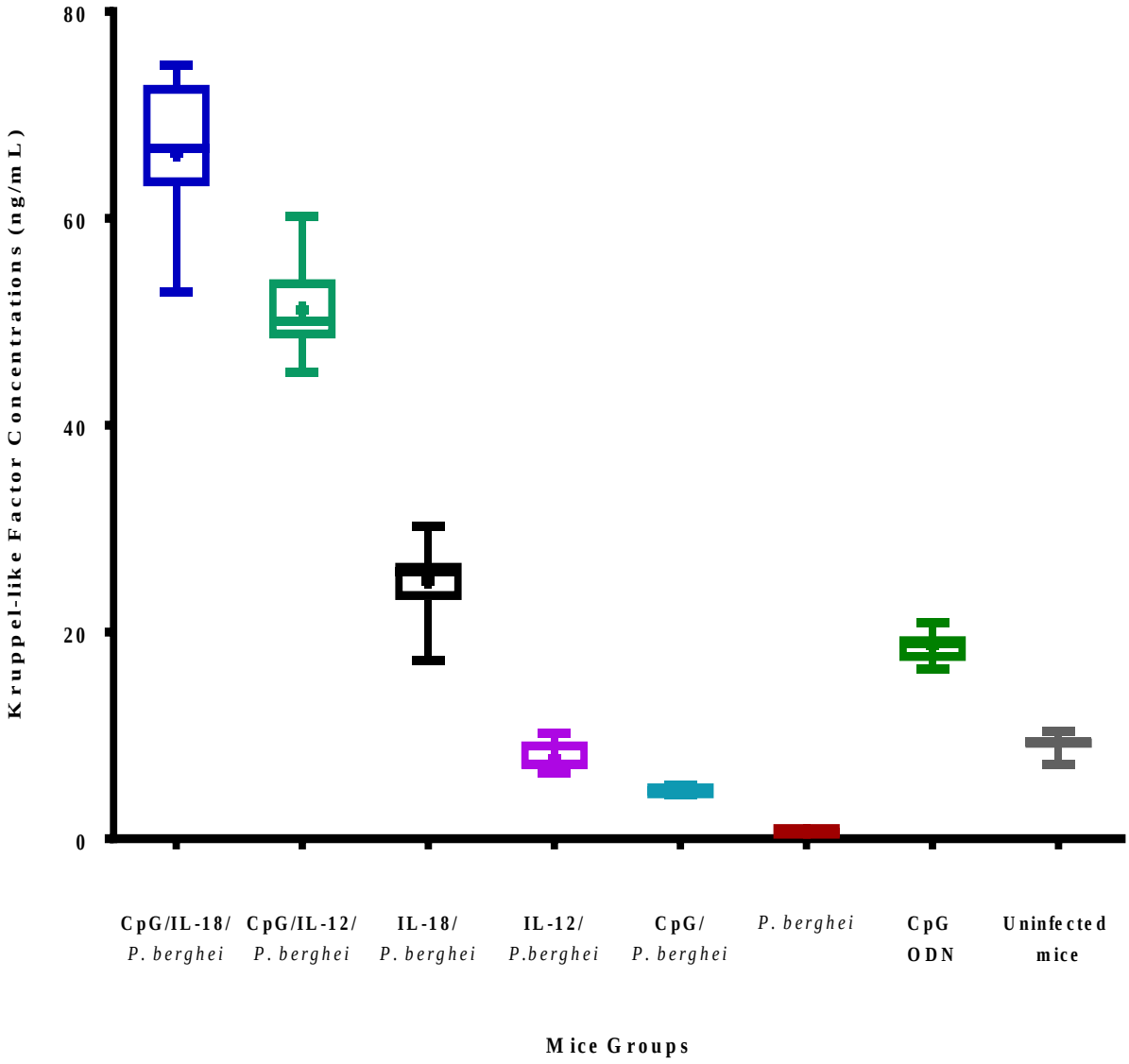



**Figure 4.12: KLF levels**

Significantly ($P < 0.0001$; $F(7, 287) = 3978$) higher levels of KLF concentrations were found in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups than the rest of the groups.

### 4.3.2 Physiologic Biomolecules' Concentrations

#### 4.3.2.1 Adiponectin Levels

The concentrations of the adiponectin molecule ranged from 3.600 ng/ml (in the IL-18/ *P. berghei* mice group) to 12.24 ng/ml (in the CpG/IL-18/ *P. berghei* group). Significantly ($P < 0.0001$; $F (7, 336) = 3136$) high level concentrations were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-12/ *P. berghei,* CpG ODN groups; 12.24 ng/ml, 9.743 ng/ml, 10.49 ng/ml, and 10.36 ng/ml. These four groups together had a mean adiponectin level of 10.708 ng/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 3136$) adiponectin concentrations than the rest of the groups in these investigations. This group had a mean adiponectin concentration of 12.24 ng/ml. The IL-12/*P. berghei* group generated significantly higher concentrations of adiponectin than the rest of the groups (except the CpG/IL-18/ *P. berghei* group) and its mean levels were at 10.49 ng/ml. It was followed closely by the CpG ODN group which had 10.36 ng/ml mean adiponectin concentration. Only the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-12/ *P. berghei* and CpG ODN groups were found to have adiponectin levels of more than 9.500 ng/ml.

Lower levels of adiponectin were detected in the IL-18/ *P. berghei,* CpG/ *P. berghei, P. berghei* and uninfected mice groups; 3.600 ng/ml, 4.814 ng/ml, 3.714 ng/ml and 4.271 ng/ml and these four groups all together had a mean adiponectin amount of 4.099 ng/ml. The 3.600 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration. The adiponectin levels in the CpG/IL-18/ *P. berghei* group were about3½ times higher than levels in the IL-18/ *P. berghei* group (lowest detected) and 3 times higher than the lowest concentration groups as provided above. In the CpG/IL-12/ *P. berghei* group adiponectin levels were one-fold lower than in the IL-12/ *P. berghei* group and 2 times higher than the mean levels of the lower concentration groups (Figure 4.13).

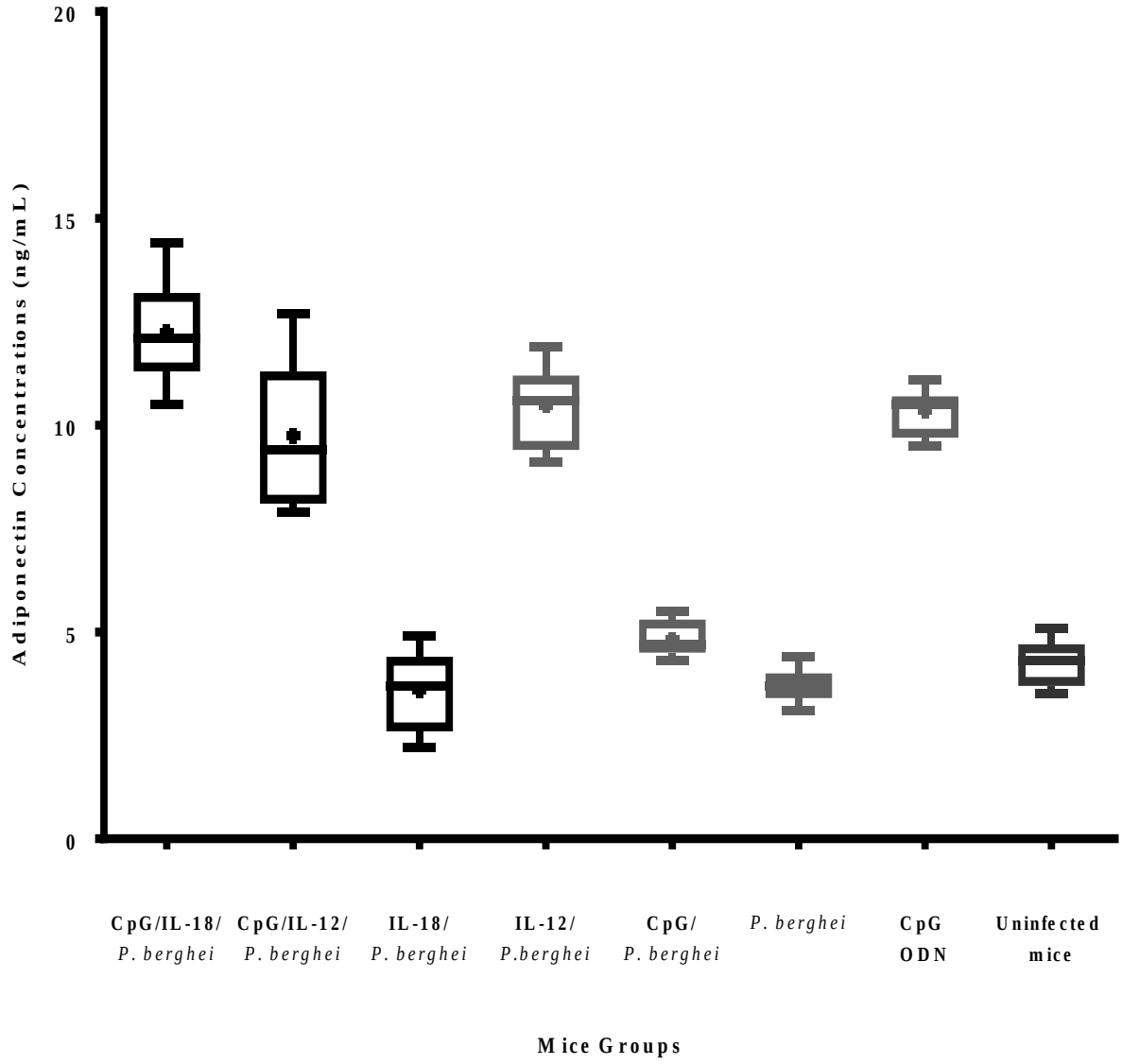



**Figure 4.13: Adiponectin levels**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 3136$) adiponectin concentrations than the rest of the groups in these investigations. The adiponectin levels in the CpG/IL-18/ *P. berghei* group were about 3½ times higher than levels in the IL-18/ *P. berghei* group (lowest detected amount).

#### 4.3.2.2 Angiogenin Levels

Quantified angiogenin concentrations ranged from 51.50 ng/ml (in the CpG/IL-12/ *P. berghei* mice group) to 715.00 ng/ml (in the *P. berghei* group). The *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 9980$) angiogenin concentrations than all other groups in the study. This group had a mean angiogenin concentration of 715.00 ng/ml. This group had 2½ times higher angiogenin levels than the second highest group, the CpG/ *P. berghei* group, which had a concentration of 276.7 ng/ml.

Intermediate levels of angiogenin were detected in the CpG/ *P. berghei,* CpG ODN, and in the uninfected groups of mice; 276.7 ng/ml, 190.3 ng/ml and 166.9 ng/ml respectively, giving a mean value of 211.3 ng/ml. The lowest levels of angiogenin were measured in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei* and IL-12/ *P. berghei* mice groups; 64.20 ng/ml, 51.50 ng/ml, 94.03 ng/ml and 84.21 ng/ml respectively and these 4 groups had a mean angiogenin amount of 73.485 ng/ml. The angiogenin levels in the *P. berghei* group (the highest in the study) were about 11 times higher than levels in the CpG/IL-18/ *P. berghei* group, 13 times higher than in the CpG/IL-12/ *P. berghei* group,3-fold higher than in the intermediate concentration groups andapproximately 10 times higher than the mean angiogenin level in the 4 lowest concentration groups above.

The mean angiogenin levels (276.7 ng/ml) in the CpG/ *P. berghei* group, second highest in the study, were about 4 times higher than levels in the CpG/IL-18/ *P. berghei* group, 5 times higher than in the CpG/IL-12/ *P. berghei* group, about 1-fold higher than the intermediate concentration groups and about 4 times higher than the mean angiogenin level in the 4 lowest concentration groups. The mean angiogenin level in the CpG/IL-18/ *P. berghei* was exceeded 1.5-fold by the mean level in the IL-18/ *P. berghei* group, where as the mean levels in the CpG/IL-12/ *P. berghei* group were 1.6-fold lower than in the IL-12/ *P. berghei* group (Figure 4.14).

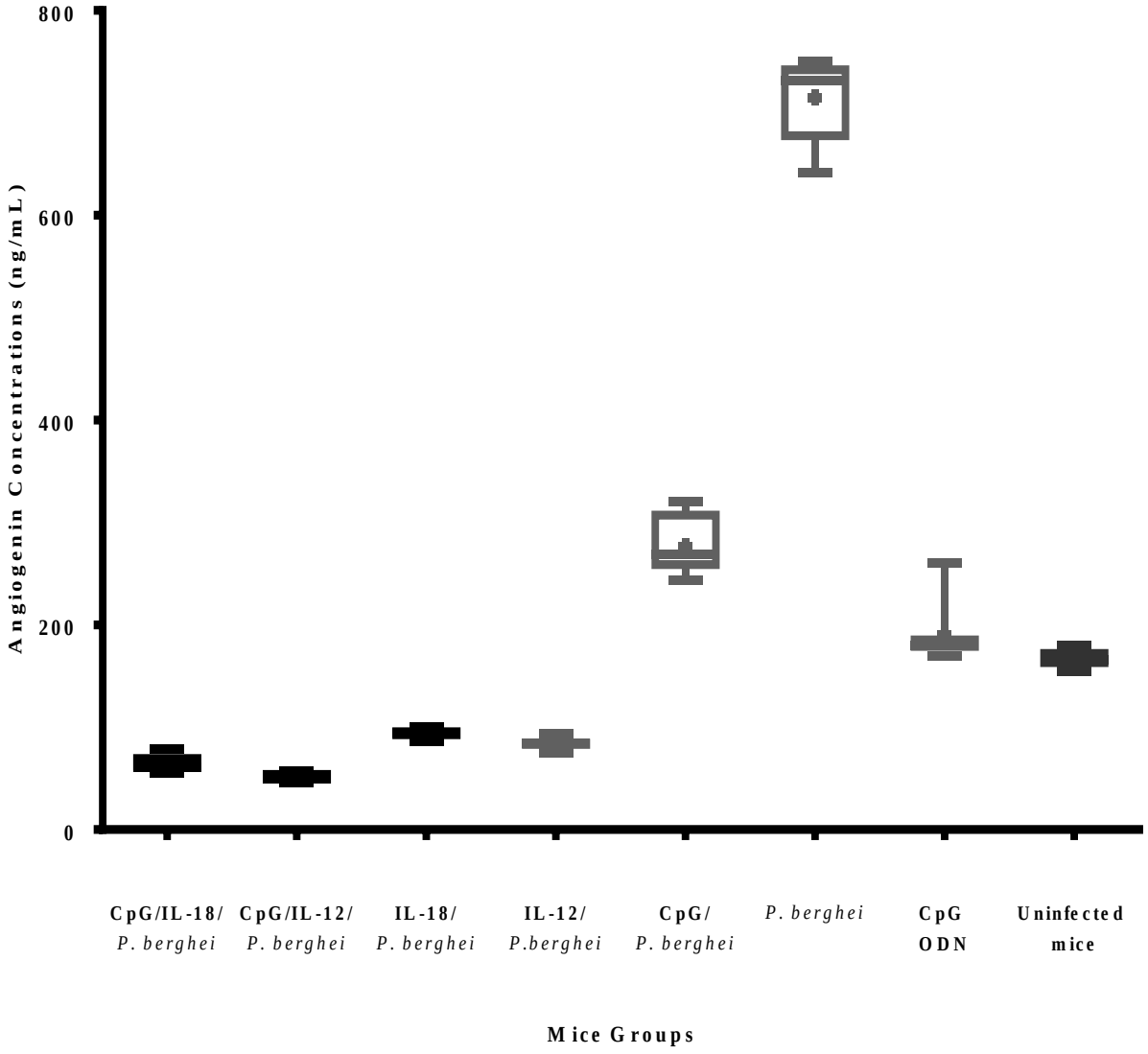



**Figure 4.14: Angiogenin levels**

The *P. berghei* group generated significantly higher ($P < 0.0001$; $F(7, 336) = 9980$) angiogenin concentrations than all other groups.

#### 4.3.2.3 Angiopoietin 1 Levels

Angiopoietin 1was detected in all investigated mice groups and overall its concentrations spanned from 1.769 ng/ml (in the *P. berghei* mice group) to 17.57 ng/ml (in the CpG/IL-12/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F (7, 336) = 11862$) high level concentration groups andthey had a mean angiopoietin 1level of 16.655 ng/ml. The CpG/IL-12/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 11862$) angiopoietin 1 concentrations than the rest of the groups in these investigations. This group had a mean angiopoietin 1 concentration of 17.57 ng/ml. The CpG/IL-18/*P. berghei* group produced significantly higher concentrations of angiopoietin 1 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 15.74 ng/ml. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded with angiopoietin 1levels of above15.5 ng/ml.

Intermediate levels of angiopoietin 1 were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei*, CpG/ *P. berghei*, CpG ODN and uninfected groups; 9.400 ng/ml, 8.343 ng/ml, 7.486 ng/ml, 6.143 ng/ml and 6.457 ng/ml respectively and these 5 groups had a mean angiopoietin 1 level of 7.566 ng/ml with values ranging from 6.143 ng/ml to 9.400 ng/ml. The lowest mean level of angiopoietin 1was found in the *P. berghei* group; 1.769 ng/ml. The angiopoietin 1 levels in the CpG/IL-18/ *P. berghei* group were about one 1½ times higher than levels in the IL-18/ *P. berghei* group, 2 times higher than the intermediate concentration groups collectively (described above) and 9 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group angiopoietin 1levels were 2 times higher than in the IL-12/ *P. berghei* group, more than 2 times higher than the mean levels of the intermediate concentration groups and 10 times higher than the two lowest concentration groups (Figure 4.15).

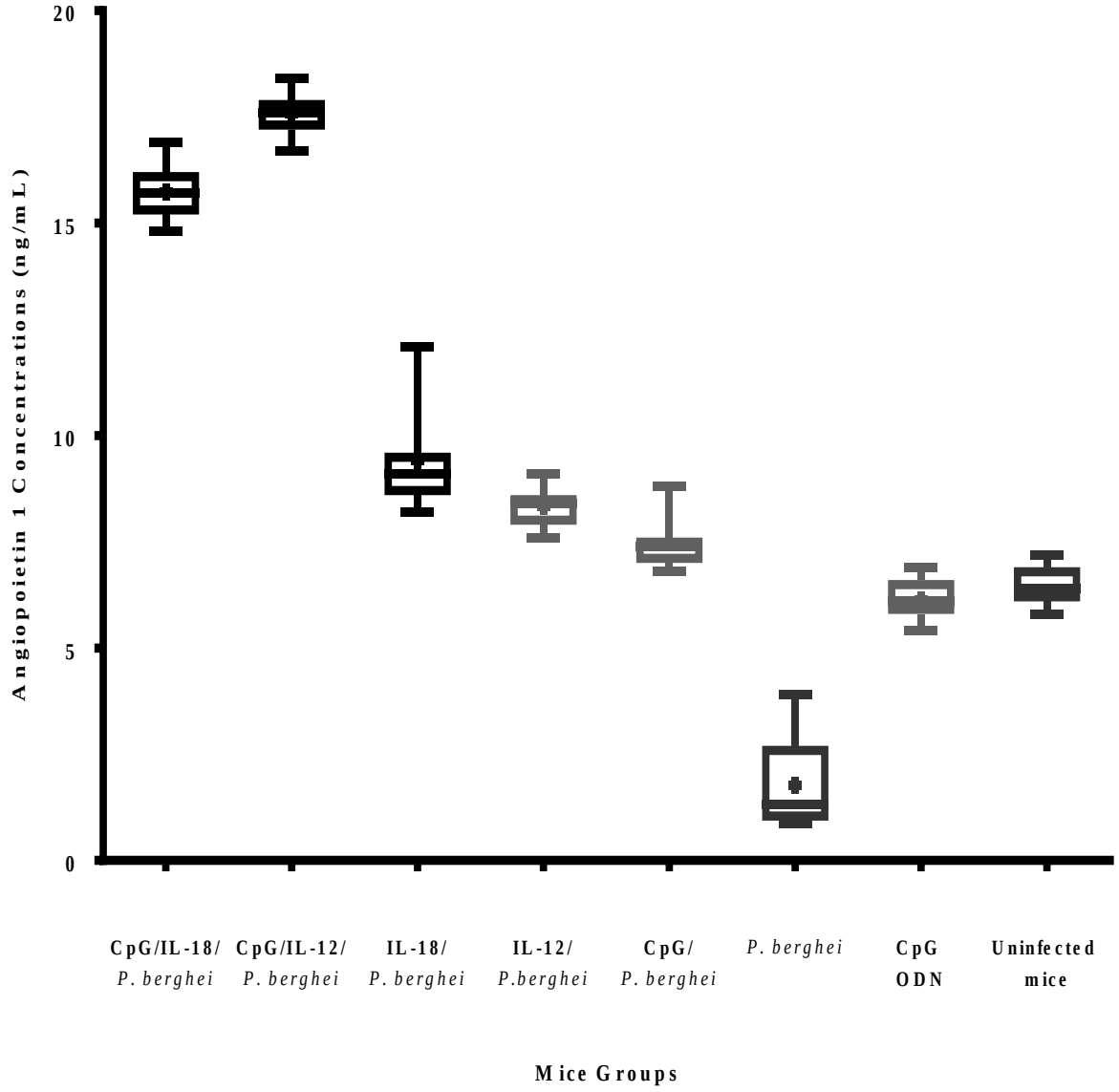



**Figure 4.15: Angiopoietin-1 levels**

The CpG/IL-12/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 11862$) angiopoietin 1 concentrations than the rest of the groups in these investigations.

#### 4.3.2.4 Angiopoietin 2 Levels

Angiopoietin 2 concentrations ranged from1.274 ng/ml (in the *P. berghei* mice group) to 16.31 ng/ml (in the IL-18/ *P. berghei* group). The outcomes in angiopoietin 2 levels were polar in nature with one pole having two highest concentration groups while the other pole had 6 low concentration groups. Significantly ($P < 0.0001$; $F(7, 336) = 23989$) high level angiopoietin 2 concentrations were found in the CpG/ *P. berghei* and *P. berghei* groups; 14.56 ng/ml and 16.31 ng/ml. These two groups together had a mean angiopoietin 2 level of 15.435 ng/ml. With a 16.31 ng/ml angiopoietin 2 mean level, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 3136$) angiopoietin 2 concentrations than the rest of the groups in these investigations. The CpG/*P. berghei* group generated significantly higher concentrations of angiopoietin 2 than the rest of the groups (except the *P. berghei* group) and its mean levels were at 14.56 ng/ml. Only the CpG/ *P. berghei* and *P. berghei* groups were were found to have angiopoietin 2 levels of more than 14.500 ng/ml.

Lowest levels of angiopoietin 2 were detected in the CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei*, IL-18/ *P. berghei*, IL-12/ *P. berghei*, CpG ODN and uninfected mice groups; 1.644 ng/ml, 3.743 ng/ml, 1.326 ng/ml,3.329 ng/ml, 1.386 ng/ml and 1.274 ng/ml respectively and all these six groups all together had a mean angiopoietin 2 amount of 1.905 ng/ml. The 1.274 ng/ml level in the uninfected group was the lowest detected concentration. The angiopoietin 2 levels in the *P. berghei* group were about 10 times higher than levels in the CpG/IL-18/ *P. berghei* group, 4 times higher than in the CpG/IL-12/ *P. berghei* and 9 times higher than the lowest concentration groups as mentioned above. The angiopoietin 2 levels in the CpG/*P. berghei* group were about 9 times higher than levels in the IL-18/ *P. berghei* group, about 4 times higher than in the CpG/IL-12/ *P. berghei* and 8 times higher than the lowest concentration groups as given above. In the CpG/IL-18/ *P. berghei* group angiopoietin 2 levels were one-fold lower than in the IL-18/ *P. berghei* group and 0.5-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group angiopoietin 2 levels were one-fold lower than in the IL-12/ *P. berghei* group and approximately 2 times higher than the mean levels of the lowest concentration groups (Figure 4.16).

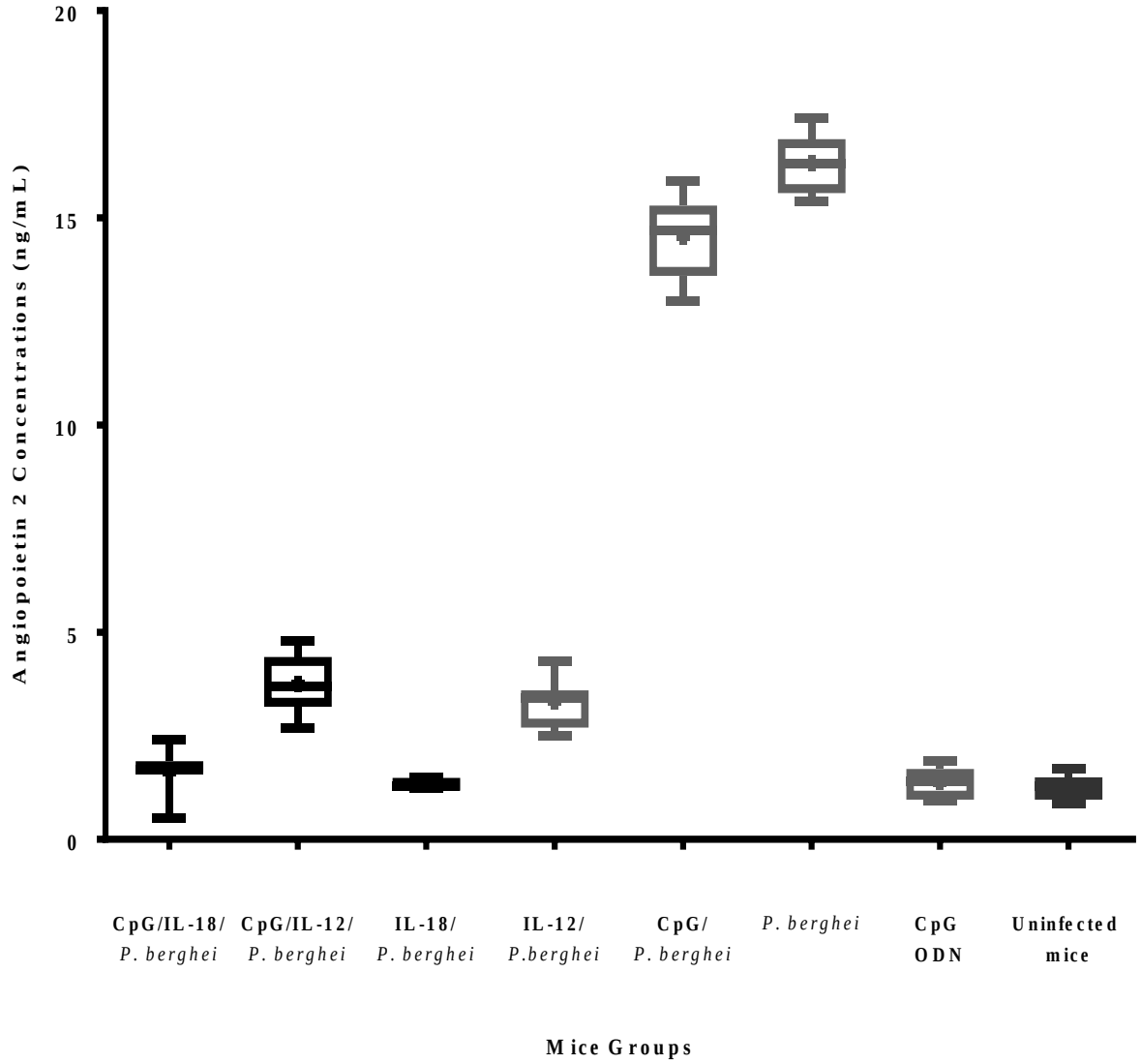



**Figure 4.16: Angiopoietin 2 levels**

The *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 3136$) mean angiopoietin 2 concentrations, 16.31 ng/ml, than the rest of the groups in these investigations.

#### 4.3.2.5 NRP-1 Levels

The neuropilin-1 (NRP-1) molecule concentrations ranged from 2.003 ng/ml (in the *P. berghei* mice group) to 14.30 ng/ml (in the IL-18/ *P. berghei* group). Polar outcomes in NRP-1 levels were noted with one pole having three highest concentration groups while the other pole had 5 low concentration groups. Significantly ($P < 0.0001$; $F (7, 336) = 23989$) high level NRP-1 concentrations were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* and IL-12/ *P. berghei* groups; 12.66 ng/ml, 14.30 ng/ml, and 14.17 ng/ml respectively. These three groups together had a mean NRP-1 level of 13.71 ng/ml. With a 14.17 ng/ml NRP-1 mean level, the IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 99045$) NRP-1 concentrations than the rest of the groups in these investigations. The CpG/IL-12/*P. berghei* group generated significantly higher concentrations of NRP-1 than the rest of the groups (except the IL-12/*P. berghei* group) and its mean levels were at 14.30 ng/ml. Only the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* and IL-12/*P. berghei* groups were were found to have NRP-1 levels of more than 12.500 ng/ml.

Lowest levels of NRP-1 were detected in the IL-18/ *P. berghei,* CpG/ *P. berghei, P. berghei,* IL-12/ *P. berghei,* CpG ODN and uninfected mice groups; 3.086 ng/ml, 2.214 ng/ml, 2.003 ng/ml, 3.086 ng/ml, 4.143 ng/ml respectively and all these five groups all together had a mean NRP-1 amount of 2.906 ng/ml. The 2.003 ng/ml level in the *P. berghei* group was the lowest detected concentration. The NRP-1 levels in the IL-12/*P. berghei* group were about 0.5 times higher than levels in the CpG/IL-18/ *P. berghei* group, 1-fold higher than in the CpG/IL-12/ *P. berghei* and about 5 times higher than the lowest concentration groups above. In the CpG/IL-18/ *P. berghei* group NRP-1 levels were 4-fold higher than in the IL-18/ *P. berghei* group and also 4-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group NRP-1 levels were one-fold higher than in the IL-12/ *P. berghei* group and approximately 5 times higher than the mean levels of the lowest concentration groups (Figure 4.17).

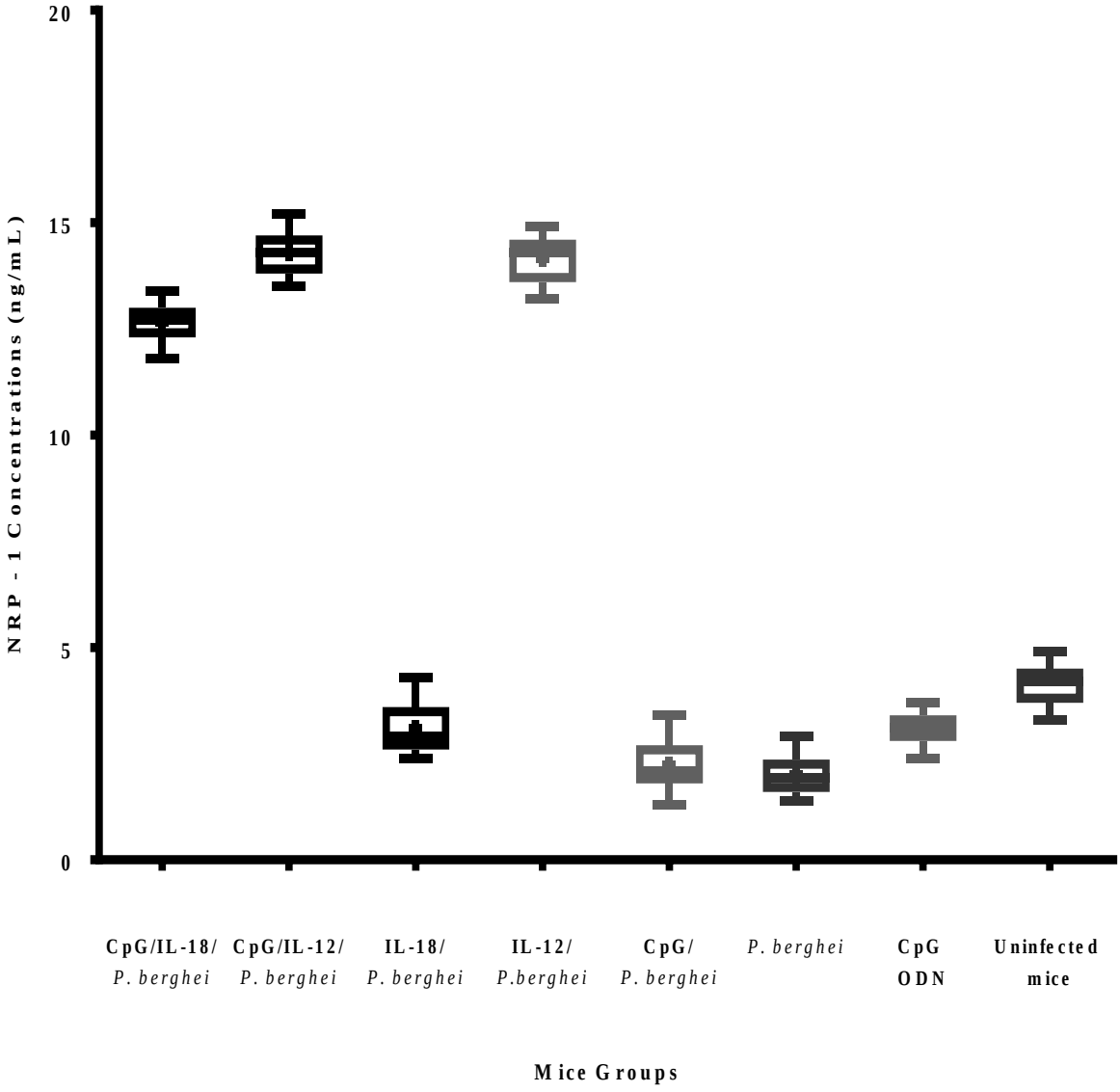



**Figure 4.17: NRP-1 levels**

With a 14.17 ng/ml NRP-1 mean level, the IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 99045$) NRP-1 concentrations than the rest of the groups in these investigations.

#### 4.3.2.6 COX -2 Levels

Levels of COX -2 detected in these experiments ranged from 27.41 ng/ml (in the *P. berghei* mice group) to 139.6 ng/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were found to have significantly ($P < 0.0001$; $F(7, 336) = 16655$) higher concentrations of COX -2 and taken together, both groups had a mean COX -2 level of 134.75 ng/ml. With a level of 139.6 ng/ml, the CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 16655$) COX -2 concentrations than the rest of the groups in these investigations. The CpG/IL-12/*P. berghei* group produced significantly higher concentrations of COX -2 than the rest of the groups except group 1 and its mean level was at 129.9 ng/ml. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were the only groups recorded with COX -2 levels higher than 129.00 ng/ml.

Mid-levels of COX -2 were detected in the IL-12/ *P. berghei* group which averaged at 100.4 ng/ml. Low-levels of COX -2 was found in the IL-18/ *P. berghei,* CpG/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 39.04 ng/ml, 39.56 ng/ml, 27.41 ng/ml, 47.67 ng/ml and 57.60 ng/ml, with the uninfected group having a slightly higher amount than the other low-level groups. Low-level groups together generated an average COX -2 concentration of 42.256 ng/ml. The COX -2 levels in the CpG/IL-18/ *P. berghei* group were about 4 times higher than levels in the IL-18/ *P. berghei* group, one-fold higher than the mid-level concentration group and 3 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group COX -2 levels were 1.2-fold higher than in the IL-12/ *P. berghei* group (this was also the COX -2 mid-level group) and 3-fold higher than the two lowest concentration groups (Figure 4.18).

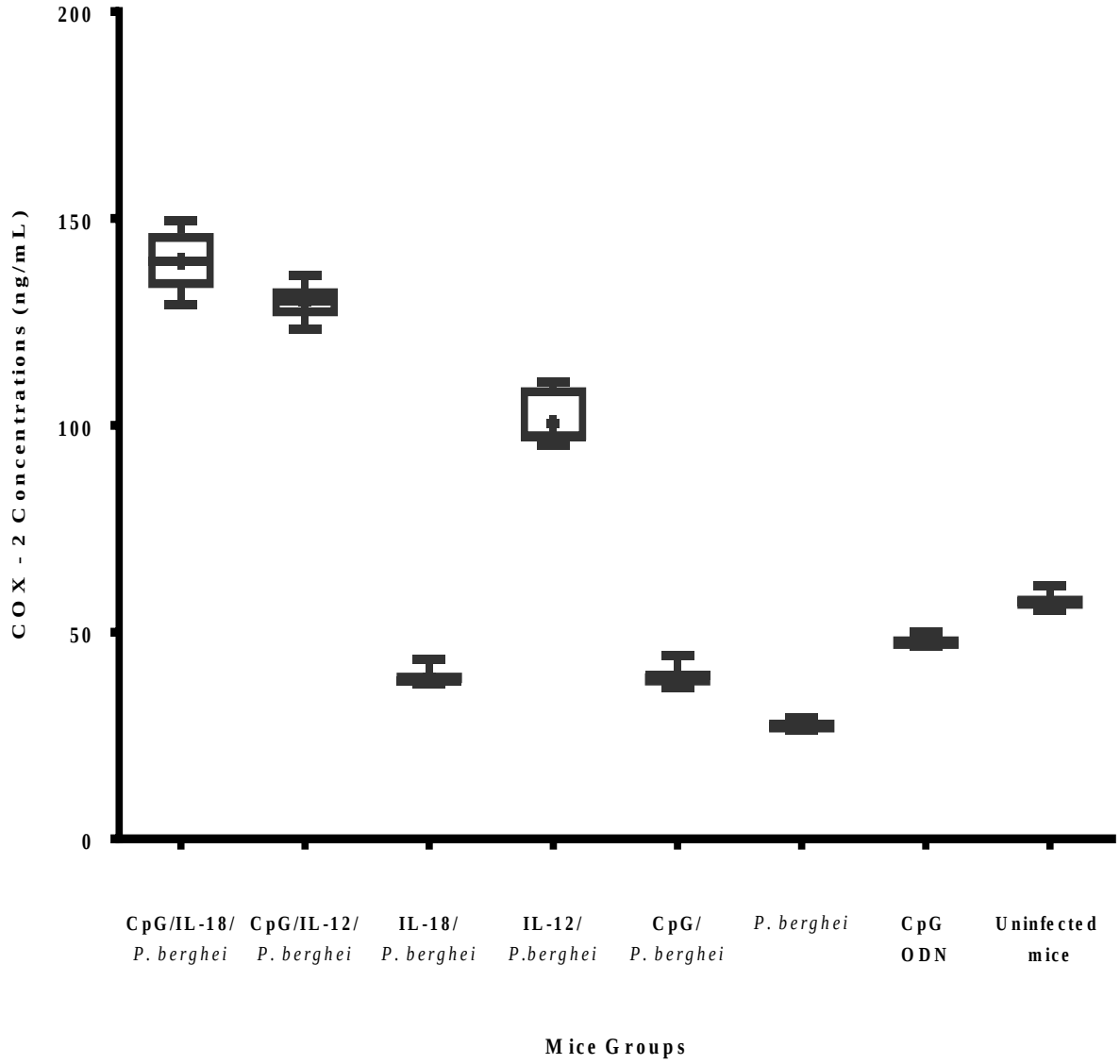



**Figure 4.18: COX -2 levels**

At a mean level of 139.6 ng/ml, the CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 16655$) COX -2 concentration than the rest of the groups while the *P. berghei* group had the lowest mean concentration, 27.41 ng/ml.

#### 4.3.2.7 MMP - 8 Levels

Concentrations of MMP – 8 ranged from 26.31 ng/ml (in the IL-12/*P. berghei* mice group) to 298.0 ng/ml (in the CpG/*P. berghei* group). Findings indicated MMP – 8 levels also had polar distribution with one pole having two highest concentration groups while the other pole had 6 low concentration groups. Significantly ($P < 0.0001$; $F(7, 336) = 25568$) high level MMP – 8 concentrations were found in the CpG/*P. berghei* and *P. berghei* groups; 298.0 ng/ml and 296.9 ng/ml. These two groups together had a mean MMP – 8 level of 297.45 ng/ml. With a 298.0 ng/ml MMP – 8 mean level, the CpG/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 25568$) MMP – 8 concentrations than the rest of the investigated groups. The *P. berghei* group generated significantly higher concentrations of MMP – 8 than the rest of the groups (except the *P. berghei* group) and its mean levels were at 296.90 ng/ml. Only the CpG/*P. berghei* and *P. berghei* groups yielded MMP – 8 levels of greater than 296.0 ng/ml.

Lowest levels of MMP – 8 were detected in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* IL-12/ *P. berghei,* CpG ODN and uninfected mice groups; 64.07 ng/ml, 46.29 ng/ml, 45.31 ng/ml, 26.31 ng/ml, 58.97 ng/ml and 56.89 ng/ml respectively and all these six groups all together had a mean MMP – 8 amount of 49.64 ng/ml. The 26.31 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration. The MMP – 8 levels in the CpG/*P. berghei* group were about 4.7 times higher than levels in the IL-18/ *P. berghei* group, 6.4 times higher than in the CpG/IL-12/ *P. berghei* and 6-fold higher than the lowest concentration groups as given above. The MMP – 8 levels in the *P. berghei* group were about 4.5 times higher than levels in the CpG/IL-18/ *P. berghei* group, 6.4 times higher than in the CpG/IL-12/ *P. berghei* and 9 times higher than the lowest concentration groups as mentioned above. In the CpG/IL-18/ *P. berghei* group MMP – 8 levels were 1.4-fold higher than in the IL-18/ *P. berghei* group and 1.2-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group MMP – 8 levels were 1.7-fold higher than in the IL-12/ *P. berghei* group and approximately 0.9-fold higher than the mean levels of the lowest concentration groups (Figure 4.19).

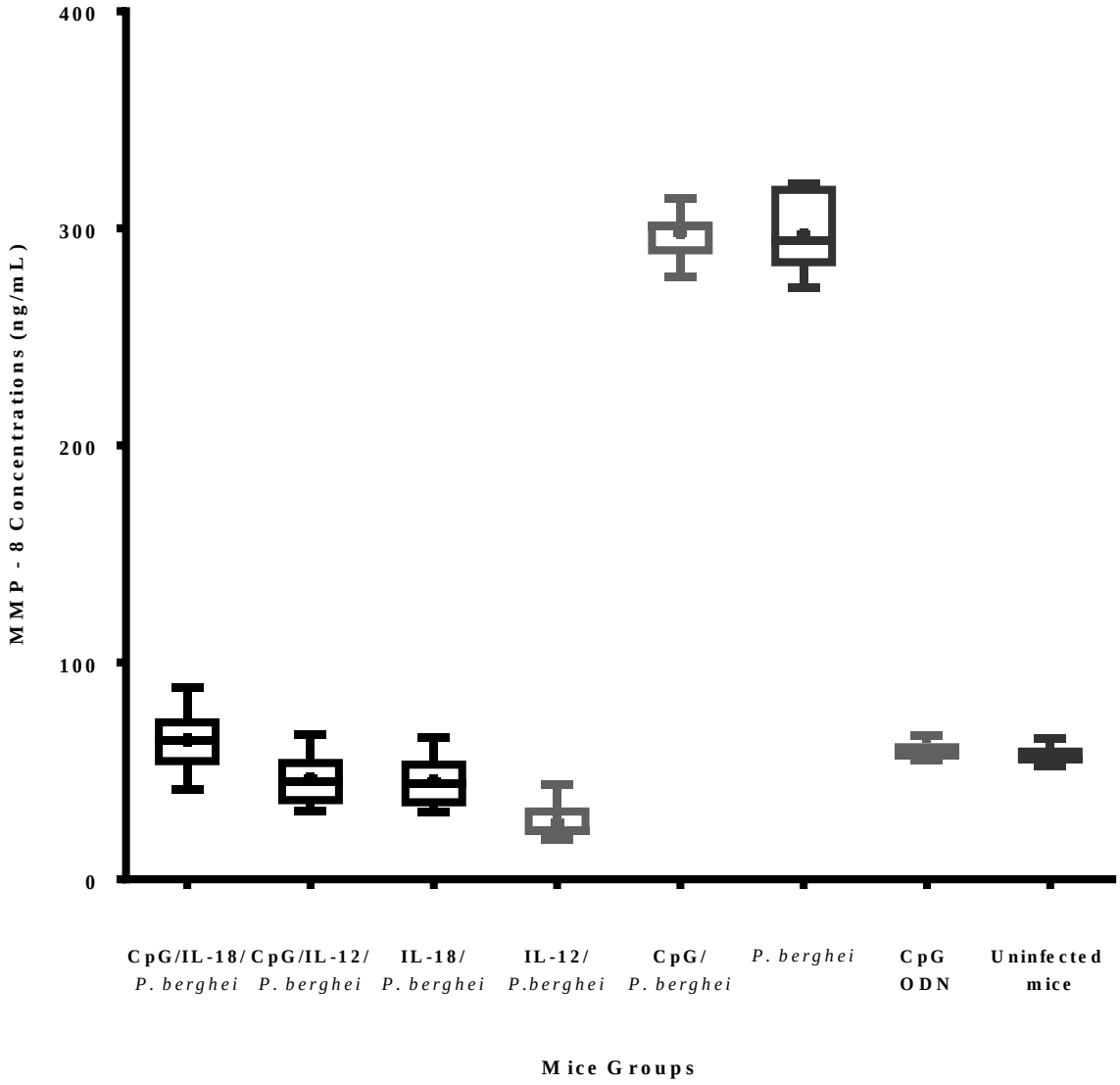



**Figure 4.19: MMP - 8 levels**

With a 298.0 ng/ml MMP – 8 mean level, the CpG/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 25568$) MMP – 8 concentrations than the rest of the investigated groups. The 26.31 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration.

#### 4.3.2.8 MMP - 9 Levels

Levels of MMP – 9 detected in these experiments ranged from 198.9 ng/ml (in the IL-12/*P. berghei* mice group) to 591.0 ng/ml (in the *P. berghei* group). The *P. berghei* and CpG/ *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 2726$) higher concentrations of MMP – 9 than the other mice groups and taken together, both groups had a mean MMP – 9 level of 496.95 ng/ml. With a level of 591.00 ng/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 2726$) MMP – 9 concentrations than the rest of the groups in these investigations. The CpG/*P. berghei* group produced significantly higher concentrations of MMP – 9 than the rest of the groups except the *P. berghei* group and its mean level was at 402.9 ng/ml. The *P. berghei* and CpG/ *P. berghei* groups were the only groups recorded with MMP – 9 levels higher than 400.00 ng/ml (Figure 4.20).

Medium levels of MMP – 9 were detected in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* CpG ODN and uninfected group; 227.2 ng/ml, 215.6 ng/ml, 277.0 ng/ml, 261.9 ng/ml and 265.7 ng/ml respectively and these averaged at 207.9 ng/ml. Low-levels of MMP – 9 were found in the IL-12/ *P. berghei* group; 198.9 ng/ml. The MMP – 9 levels in the *P. berghei* group were about 2.6 times higher than levels in the CpG/IL-18/ *P. berghei* group, 2.7 times higher than in the CpG/IL-12/ *P. berghei,* 3-fold higher than the medium level average concentration and also 3-fold higher than the lowest concentration group (the IL-12/ *P. berghei* group). The MMP – 9 levels in the CpG/*P. berghei* group were about 1.7 times higher than levels in the CpG/IL-18/ *P. berghei* group, 1.8 times higher than in the CpG/IL-12/ *P. berghei,* 2-fold higher than the medium concentration groups and also 2-fold higher than the average concentration of the lowest concentration group (the IL-12/ *P. berghei* group). In the CpG/IL-18/ *P. berghei* group MMP – 9 levels were 1.2-fold lower than in the IL-18/ *P. berghei* group, 1.09-fold higher than the mean of the medium level groups and 1.1-fold higher than the mean levels of the lowest concentration group. In the CpG/IL-12/ *P. berghei* group MMP – 9 levels were 1.08-fold higher than in the IL-12/ *P. berghei* group (this was also the lowest recorded concentration), 1.03-fold higher than the average level of the medium level groups (Figure 4.20).

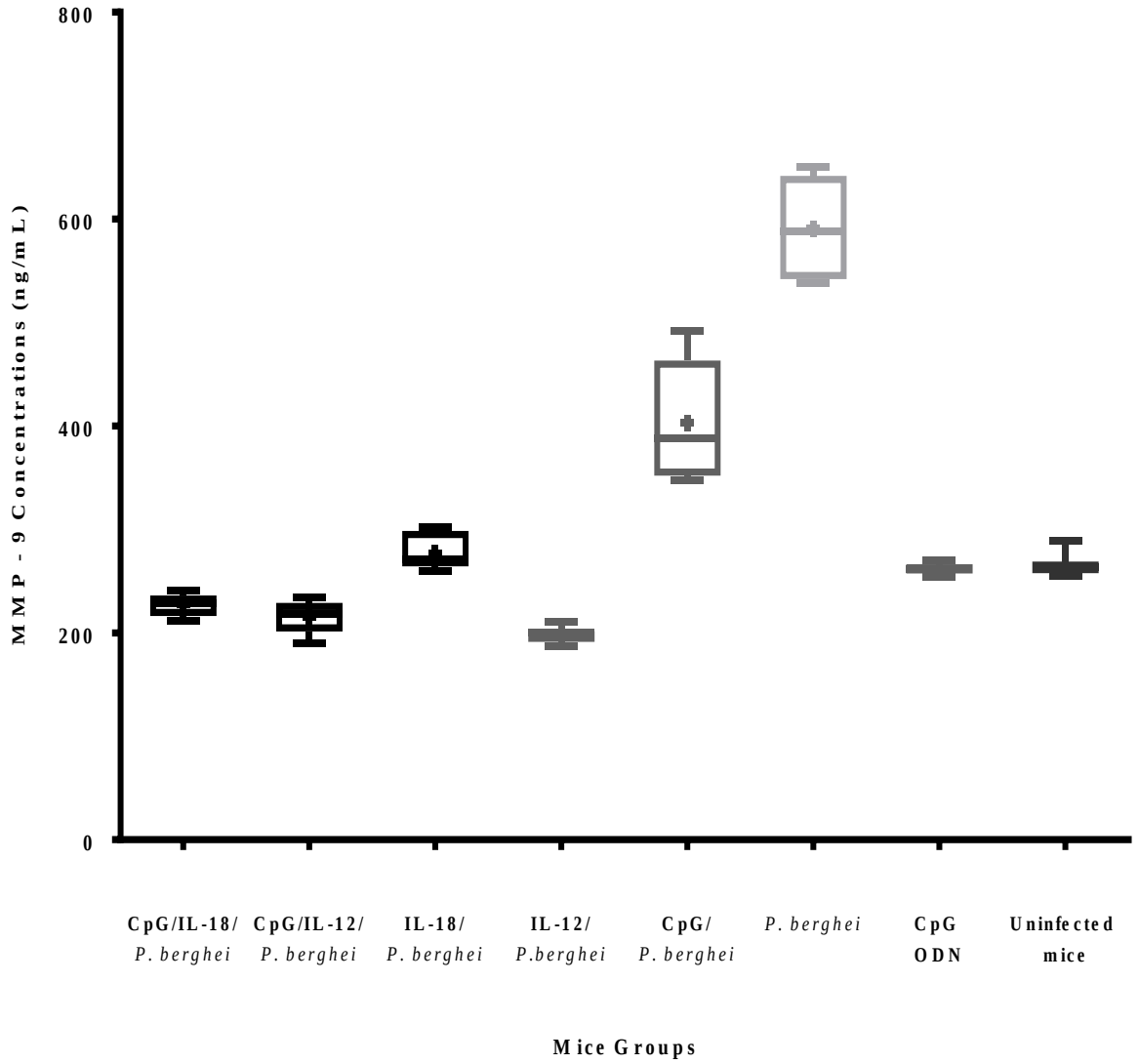



**Figure 4.20: MMP - 9 levels**

The *P. berghei* and CpG/ *P. berghei* were found to have significantly ($P < 0.0001$; $F(7, 336) = 2726$) higher concentrations of MMP – 9 than the other mice groups.

### 4.4 Chemokine and Splenocyte Recall Proliferation Responses

Various chemokines were quantified in this study and in addition, splenocyte recall proliferation responses in response to crude *P. berghei* parasite antigen were measured. The chemokines detected were CXCL-10, CCL-2, CCL-5, CXCL-16, CX3CL-1, CXCL-1, CXCL-5, CXCL-12 and CCL-3. PBMC recall proliferation responses were calculated in terms of stimulation indices (SI). As revealed by one-factor ANOVA analysis, the concentrations of all measured chemokines differed significantly among the groups (Tables 4.6 and 4.7). Generally, the IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* main experimental groups had significantly increased levels of CCL-2, CCL-5, CXCL-1, CXCL-12, CX3CL-1, and recall proliferation SIs and significantly reduced levels of CCL-3, CXCL-5, CXCL-10 and CXCL-16 than the all other groups.

**Table 4.6: Comparison of chemokine concentrations and proliferation SI:**

| **Chemokines** | **Quantitative difference relative to other groups' concentrations** | |
|---|---|---|
| Chemokine/ SI | Quantity in IL-18 / CpG / *P. berghei* group | Quantity in IL-12 / CpG / *P. berghei* group |
| 1. CXCL-10 | ⬆Than in all groups except 5 and 6 | ⬇Than in all groups 4 |
| 2. CCL-2 | ⬆Than in all groups | ⬆Than in all groups except 1 |
| 3. CCL-5 | ⬆Than in all groups except 2 | ⬆Than in all groups except |
| 4. CXCL-16 | ⬇Than in all except groups2,7,8 | ⬇Than in all except group 7 |
| 5. CX3CL-1 | ⬆ Than in all other groups | ⬆Than in all except group 1 |
| 6. CXCL-1 | ⬆Than in all groups | ⬆Than in all except group 1 |
| 7. CXCL-5 | ⬇Than in all except group 2 | ⬇Than in all groups |
| 8. CXCL-12 | ⬆Than in all groups | ⬆Than in all except group 1 |
| 9. CCL-3 | ⬇Than all groups except group 2 | ⬇Than in all groups |
| 10. Proliferation | ⬆Than in all groups except group 2 | ⬆Than in all groups |

The symbol '⬆' stands for 'mean concentration greater' while '⬇' stands for 'mean concentration lower'.

**Table 4.7: Chemokine Concentrations and Proliferation SI**

| | | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL-18/ *P. berghei* | IL-12/ *P. berghei* | CpG /*P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|---|
| | Means | 216.1 | 66.71 | 101.9 | 57.70 | 491.3 | 602.5 | 96.06 | 179.1 |
| | ANOVA | F (7, 336) = 39643, P < 0.0001 | | | | | | | |
| 1. CXCL-10 | | | | | | | | | |
| | Means | 790.0 | 776.2 | 374.6 | 262.6 | 149.9 | 66.49 | 218.4 | 166.6 |
| | ANOVA | F (7, 336) = 4779, P < 0.0001 | | | | | | | |
| 2. CCL-2 | | | | | | | | | |
| | Means | 139.9 | 143.1 | 38.89 | 88.83 | 48.39 | 40.76 | 75.87 | 64.84 |
| | ANOVA | F (7, 336) = 4927, P < 0.0001 | | | | | | | |
| 3. CCL-5 | | | | | | | | | |
| | Means | 163.3 | 126.5 | 208.0 | 293.3 | 624.9 | 604.4 | 120.8 | 160.2 |
| | ANOVA | F (7, 336) = 2520, P < 0.0001 | | | | | | | |
| 4. CXCL-16 | | | | | | | | | |
| | Means | 659.4 | 517.7 | 201.1 | 151.9 | 222.5 | 96.14 | 151.8 | 111.9 |
| | ANOVA | F (7, 336) = 5656, P < 0.0001 | | | | | | | |
| 5. CX3CL-1 | | | | | | | | | |
| | Means | 551.5 | 462.8 | 223.7 | 262.6 | 141.1 | 76.03 | 137.7 | 105.9 |
| | ANOVA | F (7, 336) = 9955, P < 0.0001 | | | | | | | |
| 6.CXCL-1 | | | | | | | | | |
| | Means | 92.10 | 86.34 | 200.3 | 176.1 | 465.1 | 628.8 | 183.1 | 132.7 |
| | ANOVA | F (7, 336) = 13489, P < 0.0001 | | | | | | | |
| 7. CXCL-5 | | | | | | | | | |
| | Means | 509.7 | 729.45 | 270.4 | 407.1 | 160.9 | 142.3 | 228.7 | 242.2 |
| | ANOVA | F (7, 336) = 7504, P < 0.0001 | | | | | | | |
| 8. CXCL-12 | | | | | | | | | |
| 9. CCL-3 | Means | 41.76 | 37.10 | 66.59 | 66.99 | 117.5 | 122.10 | 55.34 | 58.61 |
| 10. Proliferation | Means | 7.657 | 8.376 | 3.857 | 3.214 | 4.514 | 3.414 | 2.829 | 2.571 |
| | ANOVA | F (7, 336) = 6238, P < 0.0001 | | | | | | | |
| SI | | | | | | | | | |

### 4.4.1 Chemokine Concentrations

#### 4.4.1.1 CXCL - 10 Levels

Levels of CXCL – 10 detected in these experiments ranged from 57.70 pg/ml (in the IL-12/*P. berghei* mice group) to 602.5 pg/ml (in the *P. berghei* group). The *P. berghei* and CpG/ *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 39643$) higher concentrations of CXCL – 10 than the other mice groups and taken together, both groups had a mean CXCL – 10 level of 546.9 pg/ml. With a level of 628.40 pg/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 39643$) CXCL – 10 concentrations than the rest of the groups in these experiments. The CpG/*P. berghei* group generated significantly higher concentrations of CXCL – 10 than the rest of the groups except the *P. berghei* group and its mean level was at 491.3 pg/ml. The *P. berghei* and CpG/ *P. berghei* groups were the only groups recorded with CXCL – 10 levels higher than 490.00 pg/ml. Midlevels of CXCL – 10 were detected in the CpG/IL-18/ *P. berghei* and uninfected group; 216.1 pg/ml, and 179.1 pg/ml respectively and these averaged at 197.6 pg/ml.

Low levels of CXCL-10 were found in the CpG/IL-12/ *P. berghei*, IL-18/ *P. berghei*, IL-12/ *P. berghei*, CpG ODN and uninfected mice group; 66.71 pg/ml, 101.9 pg/ml, 57.70 pg/ml and 96.06 pg/ml respectively. Taken together, the low-level groups had a mean CXCL -10 concentration of 80.60 pg/ml. The CXCL – 10 levels in the *P. berghei* group were about 2.8 times higher than levels in the CpG/IL-18/ *P. berghei* group, 9 times higher than in the CpG/IL-12/ *P. berghei*, 3-fold higher than the medium level average concentration and also 7.4-fold higher than four the lowest concentration groups. The CXCL – 10 levels in the CpG/*P. berghei* group were about 2.2 times higher than levels in the CpG/IL-18/ *P. berghei* group, 7.3 times higher than in the CpG/IL-12/ *P. berghei*, 2.4-fold higher than the medium concentration groups and also 6-fold higher than the average concentration of the four lowest concentration groups. In the CpG/IL-18/ *P. berghei* group CXCL – 10 levels were 2.1-fold higher than in the IL-18/ *P. berghei* group, 1.09 -fold higher than the mean of the medium level groups and 2.7-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei*

group CXCL – 10 levels of 66.71 pg/ml, the lowest recorded, were 1.15-fold higher than in the IL-12/ *P. berghei*, 3-fold higher than the average level of the medium level groups and 1.43-fold lower than the low-level group average (Figure 4.21).

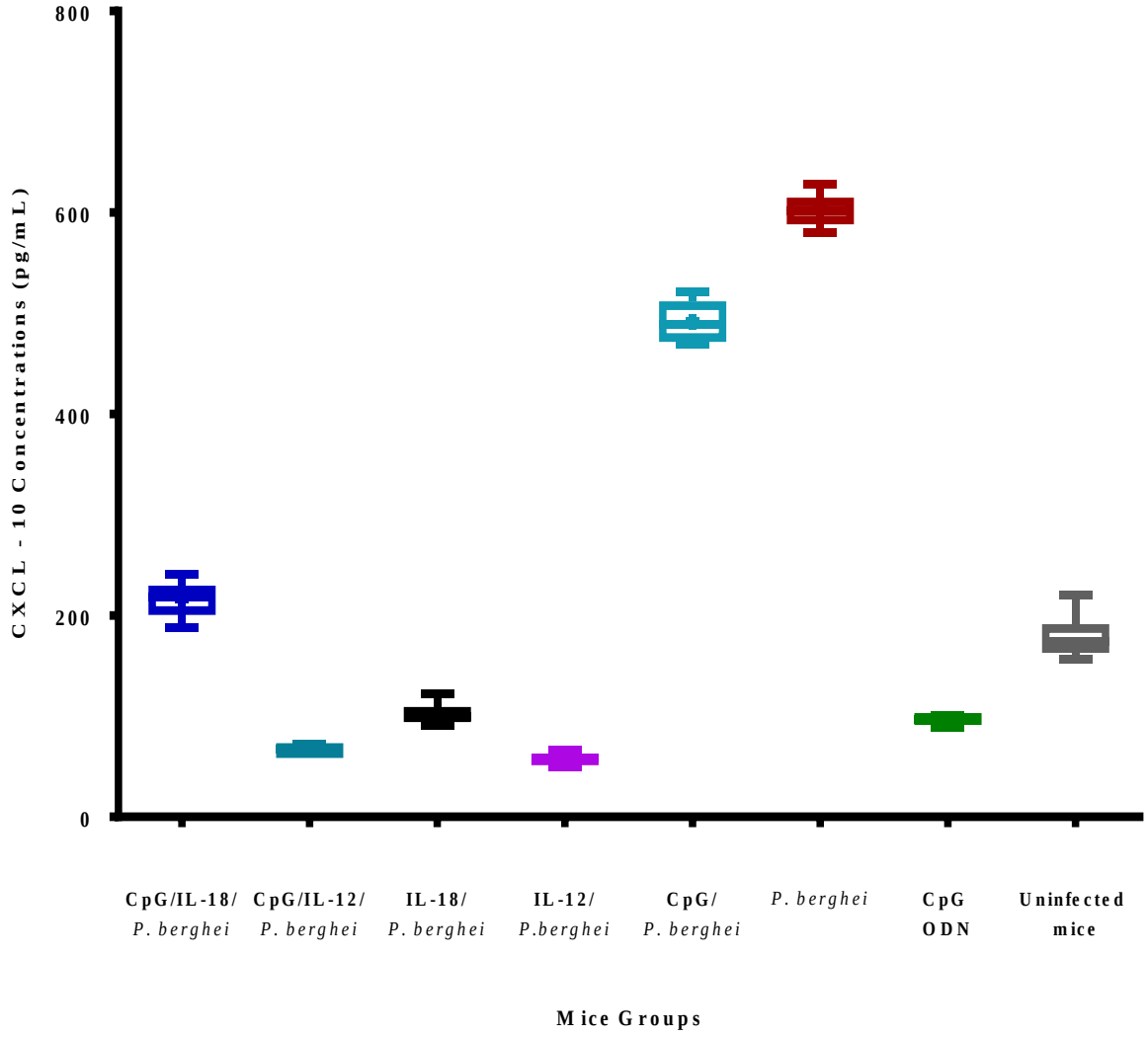



**Figure 4.21: CXCL - 10 levels**

The mean CXCL - 10 concentration of 628.40 pg/ml in the *P. berghei* group was found to be significantly higher ($P < 0.0001$; $F(7, 336) = 39643$) than CXCL–10 concentrations in the rest of the groups in these experiments.

#### 4.4.1.2 CCL - 2 Levels

The CCL – 2 chemokine was detected in all investigated mice groups and overall its concentrations varied from 66.49 pg/ml (in the *P. berghei* mice group) to 790.0 pg/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F (7, 336) = 4779$) high level concentration groups and together they had a mean CCL – 2 level of 783.1 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 4779$) CCL – 2 concentrations than all the other groups, having a mean CCL – 2 concentration of 790.0 pg/ml. The CpG/IL-12/*P. berghei* group produced significantly higher concentrations of CCL – 2 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 776.2 pg/ml. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded with CCL – 2 levels of above 775.0 pg/ml.

Intermediate levels of CCL – 2 were detected in the IL-18/ *P. berghei,* IL-12/ *P. berghei,* and CpG ODN groups; 374.6 pg/ml, 262.6 pg/ml and 218.4 pg/ml respectively and these 3 groups had a mean CCL – 2 level of 285.2 pg/ml with values ranging from 218.4 pg/ml to 374.6 pg/ml. The lowest mean level of CCL – 2 were found in the CpG/*P. berghei, P. berghei* and uninfected mice groups; 149.9 pg/ml, 66.49 pg/ml and 166.6 pg/ml. These 3 groups together averaged at 127.66 pg/ml. The CCL – 2 levels in the CpG/IL-18/ *P. berghei* group were about one 2.1 times higher than levels in the IL-18/ *P. berghei* group, 3 times higher than the intermediate concentration groups collectively and 6 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group CCL – 2 levels were 3 times higher than in the IL-12/ *P. berghei* group, 3 times higher than the mean levels of the intermediate concentration groups and 6 times higher than the two lowest concentration groups (Figure 4.22).

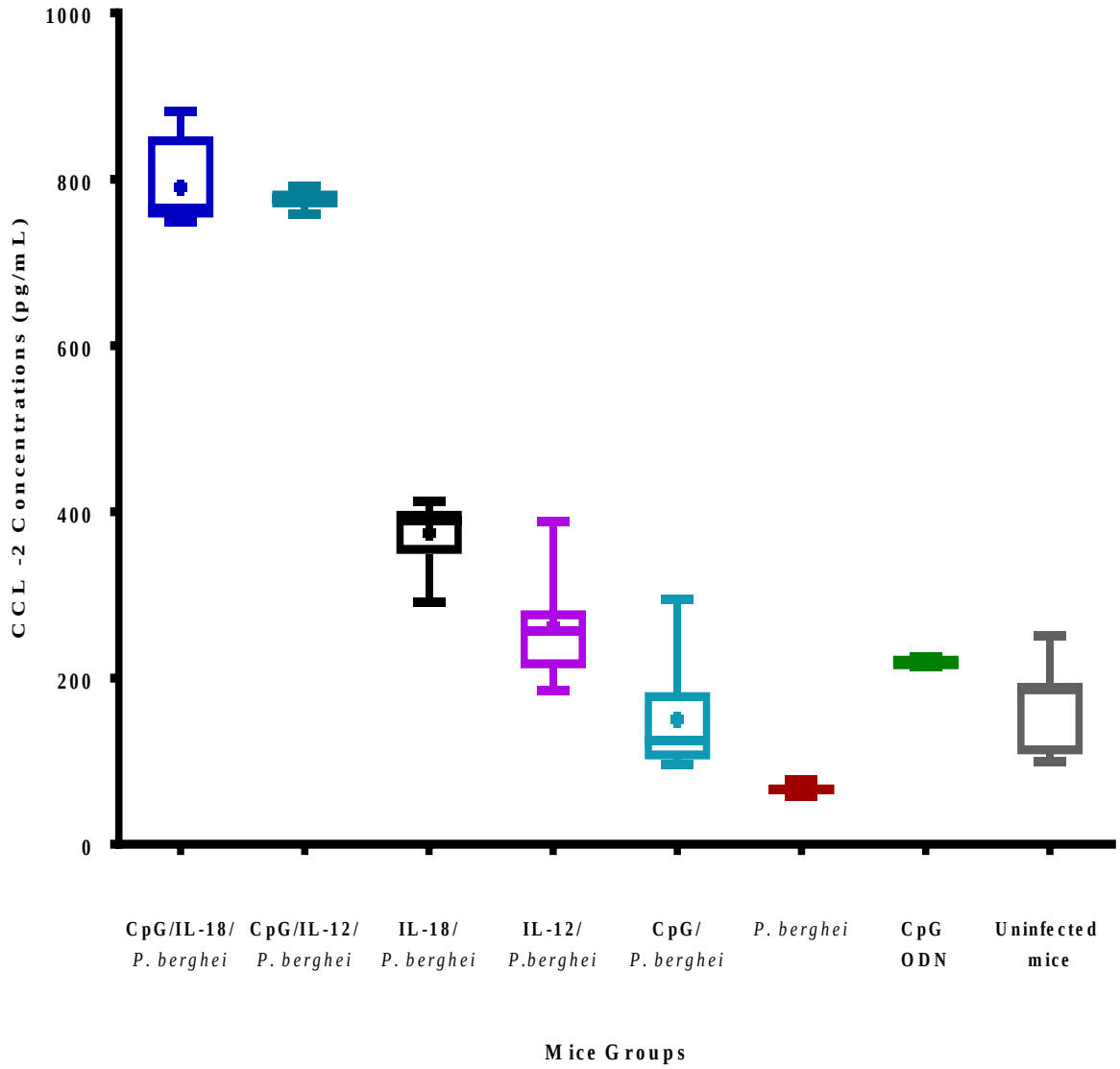



**Figure 4.22: CCL - 2 levels**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4779$) CCL – 2 concentrations than all the other groups, having a mean CCL – 2 concentration of 790.0 pg/ml.

#### 4.4.1.3 CCL – 5 / RANTES Levels

Levels of CCL–5 detected in these experiments ranged from 39.89 pg/ml (in the IL-18/*P. berghei* mice group) to 143.1 pg/ml (in the CpG/IL-12/*P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were found to have significantly ($P < 0.0001$; $F(7, 336) = 4927$) higher concentrations of CCL–5 and taken together, both these groups had a mean CCL–5 level of 141.5 pg/ml. With a level of 143.1 pg/ml, the CpG/IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4927$) CCL–5 concentrations than the rest of the groups. The CpG/IL-18/*P. berghei* group produced significantly higher concentrations of CCL–5 than the rest of the groups except the CpG/IL-12/ *P. berghei* group and its mean level was at 148.4 pg/ml. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were the only groups recorded with CCL–5levels higher than 143.00 pg/ml.

Mid-levels of CCL–5 were quantified in the IL-12/ *P. berghei,* CpG ODN and uninfected groups; 88.83 pg/ml, 75.87 pg/ml, 64.84 pg/ml and these averaged at 76.51 pg/ml. Low-levels of CCL–5 was found in the IL-18/ *P. berghei,* CpG/*P. berghei,* and *P. berghei* mice groups; 39.89 pg/ml, 48.39 pg/ml and 40.76 pg/ml respectively, and low-level groups together generated an average CCL–5 concentration of 43.01 pg/ml. The CCL–5 levels in the CpG/IL-18/ *P. berghei* group were about 4 times higher than levels in the IL-18/ *P. berghei* group (this group also had the lowest detected CCL-5 amount), 1.82-fold higher than the mid-level concentration group and 3.25 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group CCL–5 levels were 3-fold higher than in the IL-12/ *P. berghei* group, 1.8-fold higher than the mid-level groups together and 3.3-fold higher than the two lowest concentration groups (Figure 4.23).

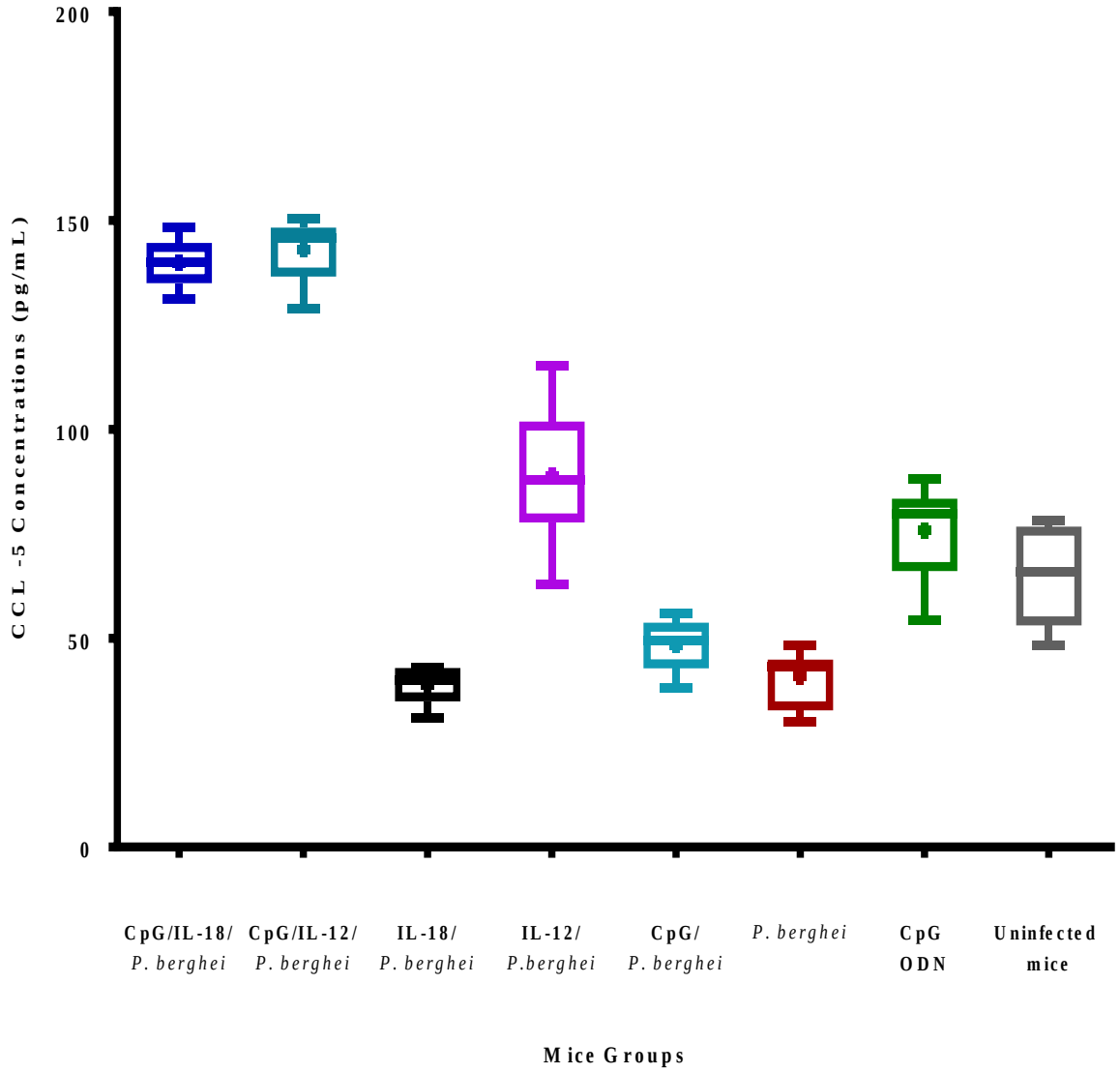



**Figure 4.23: CCL – 5 levels**

The CpG/IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4927$) CCL–5concentrations than the rest of the groups. Lowest concentrations were recorded in the IL-18/*P. berghei* group.

#### 4.4.1.4 CXCL-16 Levels

Concentrations of CXCL-16 detected in these experiments ranged from 120.8 pg/ml (in the CpG ODN mice group) to 624.9 pg/ml (in the CpG/*P. berghei* group). The CpG/ *P. berghei* and *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 2520$) higher concentrations of CXCL-16 than the other mice groupsand taken together, both these groups had a mean CXCL-16 level of 614.65 pg/ml. With a level of 624.9 pg/ml, the CpG/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 2520$) CXCL-16concentrations than all the other analysed groups. The *P. berghei* group produced significantly higher concentrations of CXCL-16 than the rest of the groups except the CpG/*P. berghei* group and its mean level was at 604.4 pg/ml. The CpG/ *P. berghei* and *P. berghei* groups were the only groups recorded with CXCL-16levels higher than 604.0 pg/ml.

Medium levels of CXCL-16 were detected in the IL-18/ *P. berghei* and IL-12/ *P. berghei* groups; 208.0 pg/ml and 293.3 pg/ml respectively and these averaged at 250.65 pg/ml. Low-levels of CXCL-16 were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* CpG ODN and uninfected groups; 163.3 pg/ml, 126.5 pg/ml, 120.8 pg/ml and 160.2 pg/ml respectively, averaging at 142.7 pg/ml. The CXCL-16 levels in the CpG/*P. berghei* group were about 3.8 times higher than levels in the CpG/IL-18/ *P. berghei* group, 4.9 times higher than in the CpG/IL-12/ *P. berghei,* 2.5-fold higher than the medium level average concentration and 4-fold higher than the lowest concentration groups. The CXCL-16 levels in the *P. berghei* group were 3.7 times higher than levels in the CpG/IL-18/ *P. berghei* group, 5 times higher than in the CpG/IL-12/ *P. berghei*, 2.4-fold higher than the medium concentration groups and also 4.2-fold higher than the average concentration of the lowest concentration groups. In the CpG/IL-18/ *P. berghei* group CXCL-16 levels were 1.2-fold lower than in the IL-18/ *P. berghei* group, 1.5 -fold lower than the mean of the medium level groups and 1.1-fold higher than the mean levels of the lowest concentration group. In the CpG/IL-12/ *P. berghei* group CXCL-16 levels were 2.3-fold lower than in the IL-12/ *P. berghei* group, 2-fold higher than the average level of the medium level groups and 1.1-fold lower than the lowest CXCL-16 level groups (Figure 4.24).

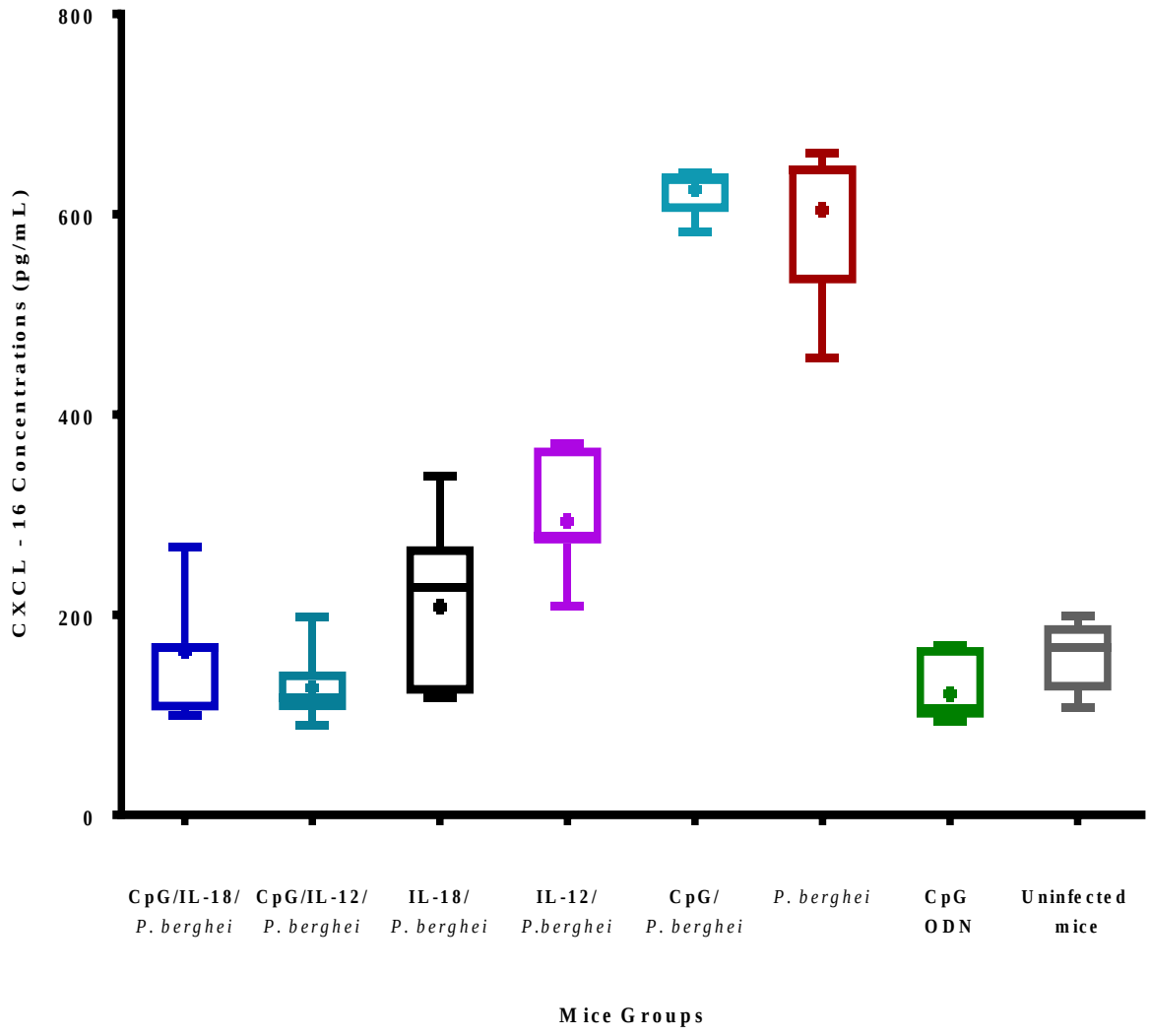



**Figure 4.24: CXCL-16 levels**

With an average concentration of 614.65 pg/ml, the CpG/ *P. berghei* and *P. berghei* groups had significantly ($P < 0.0001$; $F(7, 336) = 2520$) higher concentrations of CXCL-16 than the other mice groups.

#### 4.4.1.5 CX3CL-1/Fractalkine Levels

The CX3CL-1 chemokine wasdetected in all investigated mice groups and overall its concentrations ranged from 96.14 pg/ml (in the *P. berghei* mice group) to 659.4 pg/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F (7, 336) = 5656$) high level concentration groups and together they had a mean CX3CL-1 level of 588.55 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 5656$) CX3CL-1 concentrations than all the other groups, having a mean CX3CL-1 concentration of 659.4 pg/ml. The CpG/IL-12/*P. berghei* group produced significantly higher concentrations of CX3CL-1 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 517.7 pg/ml. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were found with CX3CL-1 levels of above 517.0 pg/ml.

Intermediate levels of CX3CL-1 were detected in the IL-18/*P. berghei* and CpG/*P. berghei* groups; 201.1 pg/ml and 222.5 pg/ml 2 groups had a mean CX3CL-1 level of 211.8 pg/ml. The lowest mean level of CX3CL-1 were found in the IL-12/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 151.9 pg/ml, 96.14 pg/ml, 151.8 pg/ml and 111.9 pg/ml. These 3 groups together averaged at 127.93 pg/ml. The CX3CL-1 levels in the CpG/IL-18/ *P. berghei* group were about one 3.2 times higher than levels in the IL-18/ *P. berghei* group, 3.1 times higher than the intermediate concentration groups collectively and 5.1 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group CX3CL-1 levels were 3.4 times higher than in the IL-12/ *P. berghei* group, 2.4 times higher than the mean levels of the intermediate concentration groups and 4 times higher than the two lowest concentration groups (Figure 4.25).

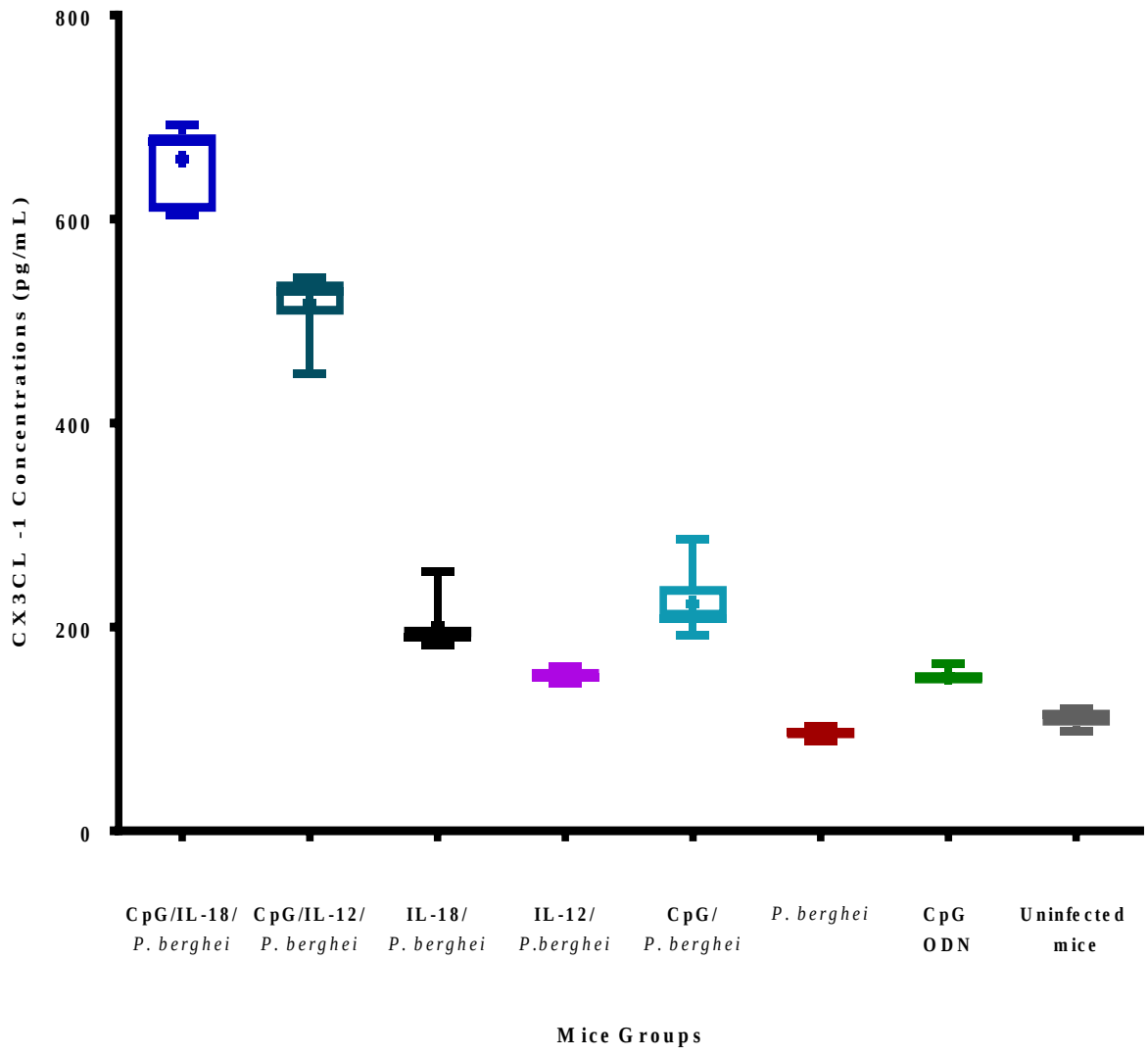



**Figure 4.25: CX3CL-1/Fractalkine levels**

The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F(7, 336) = 5656$) high level concentration groups and together they had a mean CX3CL-1 level of 588.55 pg/ml.

#### 4.4.1.6 CXCL - 1 Levels

The CXCL-1 chemokine wasdetected in all investigated mice groups and overall its concentrations varied from 76.03 pg/ml (in the *P. berghei* mice group) to 551.5 pg/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F(7, 336) = 9955$) high level concentration groups and together they had a mean CXCL-1 level of 507.15 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 9955$) CXCL-1 concentrations than all the other groups, having a mean CXCL-1 concentration of 551.5 pg/ml. The CpG/IL-12/*P. berghei* group had significantly higher concentrations of CXCL-1 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 462.8 pg/ml. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were had CXCL-1 levels exceeding the 460.0 pg/ml value.

Intermediate levels of CXCL-1 were detected in the IL-18/ *P. berghei* and IL-12/ *P. berghei* groups; 223.7 pg/ml, 262.6 pg/ml and 260.5 pg/ml respectively and these 2 groups had a mean CXCL-1 level of 242.1 pg/ml. The lowest mean level of CXCL-1 were found in the CpG/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 141.1 pg/ml, 76.03 pg/ml, 137.7 pg/ml and 105.9 pg/ml. These 4 groups together averaged at 115.18 pg/ml. The CXCL-1 levels in the CpG/IL-18/ *P. berghei* group were about 2.5 times higher than levels in the IL-18/ *P. berghei* group, 2.2 times higher than the intermediate concentration groups collectively, and 4.8 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group CXCL-1 levels were 1.7 times higher than in the IL-12/ *P. berghei* group, about 2 times higher than the mean levels of the intermediate concentration groups and 4 times higher than the two lowest concentration groups (Figure 4.26).

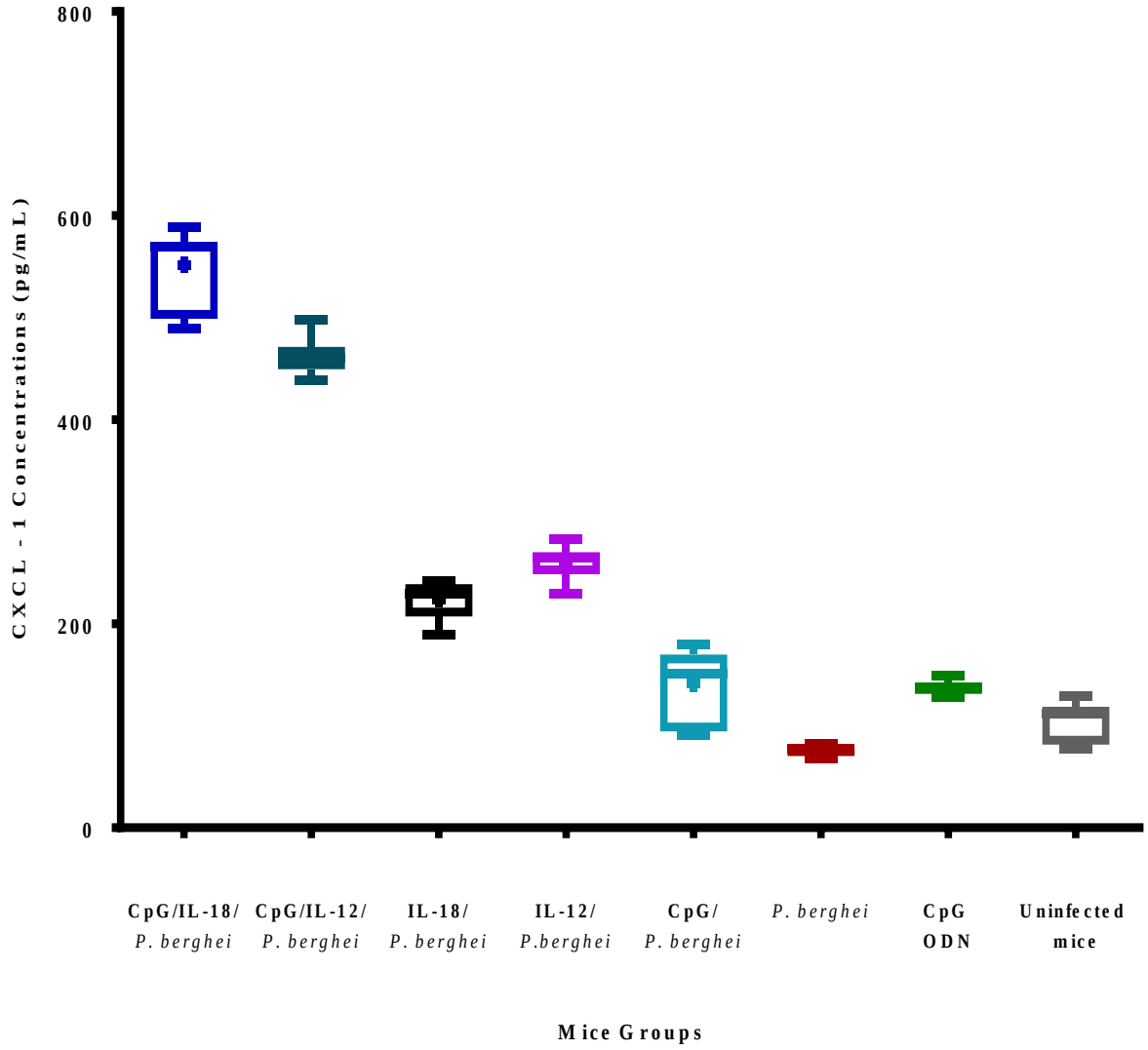



**Figure 4.26: CXCL-1 levels**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 9955$) CXCL-1 concentrations than all the other groups, having a mean CXCL-1 concentration of 551.5 pg/ml.

#### 4.4.1.7 CXCL-5 / ENA-78 Levels

Concentrations of CXCL - 5 / ENA - 78 detected in these experiments ranged from 92.10 pg/ml (in the CpG/IL-18/ *P. berghei* mice group) to 628.8 pg/ml (in the *P. berghei* group). The CpG/ *P. berghei* and *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 13489$) higher concentrations of CXCL-5 than the other mice groups and together both these groups had a mean CXCL-5 level of 545.0 pg/ml. With a level of 628.8 pg/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 13489$) CXCL-5 concentrations than all the other groups. The CpG/*P. berghei* group expressed significantly higher concentrations of CXCL-5 than the rest of the groups except the *P. berghei* group and its mean level was at 465.1 pg/ml. The CpG/ *P. berghei* and *P. berghei* groups were the only groups recorded with CXCL-5 levels higher than 460.0 pg/ml.

Medium levels of CXCL-5 were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei* and CpG ODN mice groups; 200.3 pg/ml, 176.1 pg/ml and 183.1 pg/ml respectively and these averaged at 186.5 pg/ml. Low-levels of CXCL-5 were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* and uninfected groups; 92.10 pg/ml, 86.34 pg/ml, and 132.1 pg/ml respectively, averaging at 103.51 pg/ml. The CXCL-5 levels in the *P. berghei* group were about 6.8 times higher than levels in the CpG/IL-18/ *P. berghei* group, 7.23 times higher than in the CpG/IL-12/ *P. berghei,* 3.3-fold higher than the medium level average concentration and 6-fold higher than the lowest concentration groups. The CXCL-5 levels in the CpG/*P. berghei* group were 5 times higher than levels in the CpG/IL-18/ *P. berghei* group, 5.4 times higher than in the CpG/IL-12/ *P. berghei,* 2.5-fold higher than the medium concentration groups and also 4.5-fold higher than the average concentration of the lowest concentration groups. In the CpG/IL-18/ *P. berghei* group CXCL-5 levels were exceeded by those in the in the IL-18/ *P. berghei* group by an over 2.2-fold difference, 2 -fold lower than the mean of the medium level groups and 1.1-fold higher than the mean levels of the lowest concentration group. In the CpG/IL-12/ *P. berghei* group CXCL-5 levels were 2-fold lower than in the IL-12/ *P. berghei* group, also 2-fold lower than the average level of the medium level groups and 1.1-fold lower than the lowest CXCL - 5 level groups (Figure 4.27).

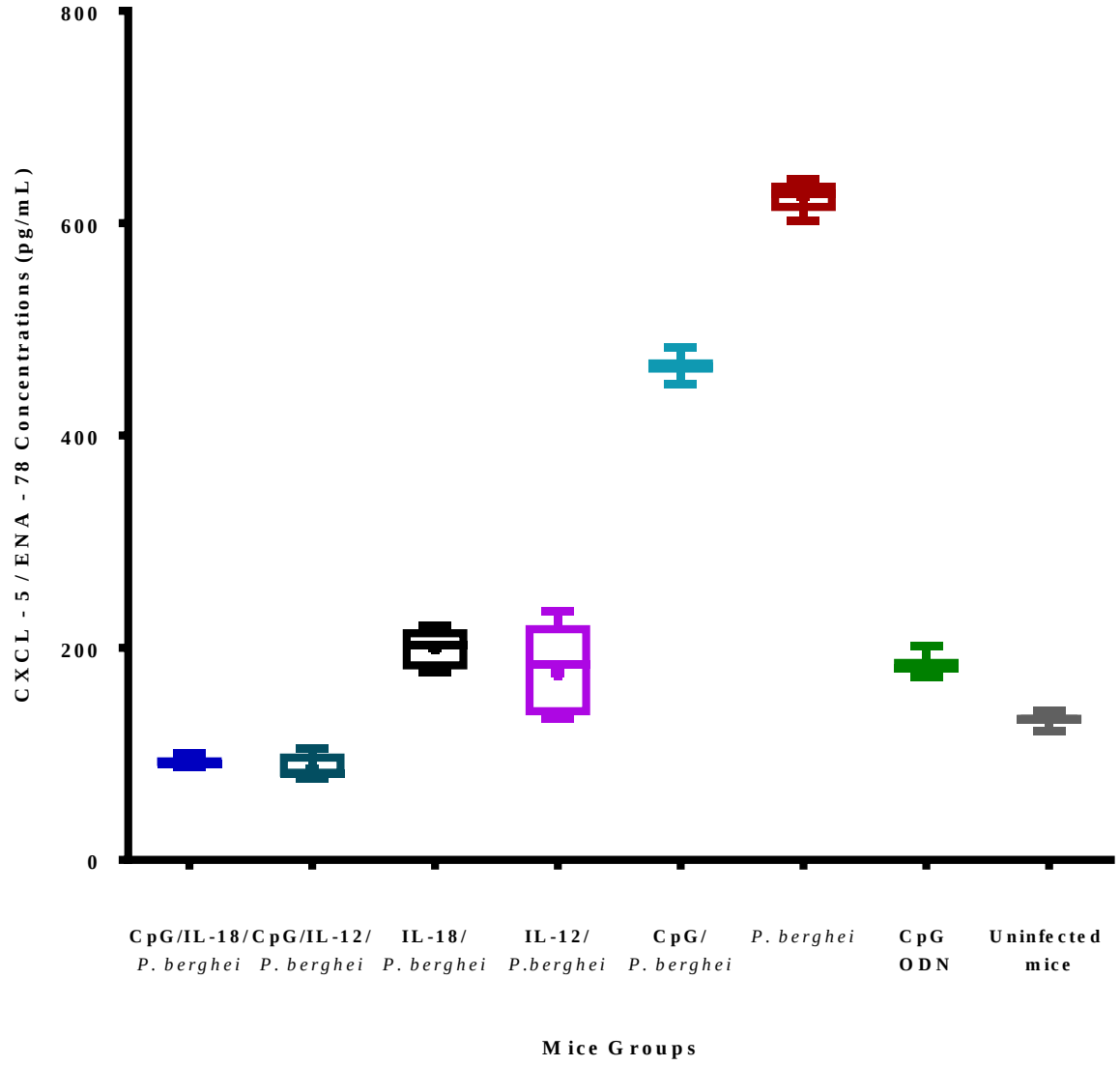



**Figure 4.27: CXCL-5 / ENA-78 levels**

The CpG/ *P. berghei* and *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 13489$) higher concentrations of CXCL-5 than the other mice groupsand together both these groups had a mean CXCL-5 level of 545.0 pg/ml.

#### 4.4.1.8 CXCL-12 Levels

The CXCL-12 concentrations were quantified in all investigated mice groups and overall mean concentrations varied from 142.3 pg/ml (in the *P. berghei* mice group) to 729.4 pg/ml (in the CpG/IL-18/ *P. berghei* group). Significantly high CXCL-12 concentrations were detected in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groupsand together these two groups had a mean CXCL-12 level of 619.55 pg/ml ($P < 0.0001$; $F(7, 336) = 7504$). The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 7504$) CXCL-12 concentrations than all the other groups, having a mean concentration of 729.4 pg/ml. The CpG/IL-12/*P. berghei* group had significantly higher concentrations of CXCL-12 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 509.7 pg/ml. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups had CXCL-12 levels exceeding the 505.0 pg/ml value.

Intermediate levels of CXCL-12 were detected in the IL-12/ *P. berghei* group which had an average amount of 407.1 pg/ml. The lowest mean level of CXCL-12 were found in the IL-18/ *P. berghei,* CpG/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 270.4 pg/ml, 160.9 pg/ml, 142.3 pg/ml, 228.7 pg/ml and 242.2 pg/ml. These 5 groups together averaged at 208.9 pg/ml. In the CpG/IL-18/ *P. berghei* group CXCL-12 levels were about one 2.7 times higher than levels in the IL-18/ *P. berghei* group, 1.8 times higher than the intermediate concentration groups collectively, and 3.4 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group CXCL-12 levels were 1.3 times higher than in the IL-12/ *P. berghei* group, about 1.2 times higher than the mean levels of the intermediate concentration groups and 2.4 times higher than the two lowest concentration groups (Figure 4.28).

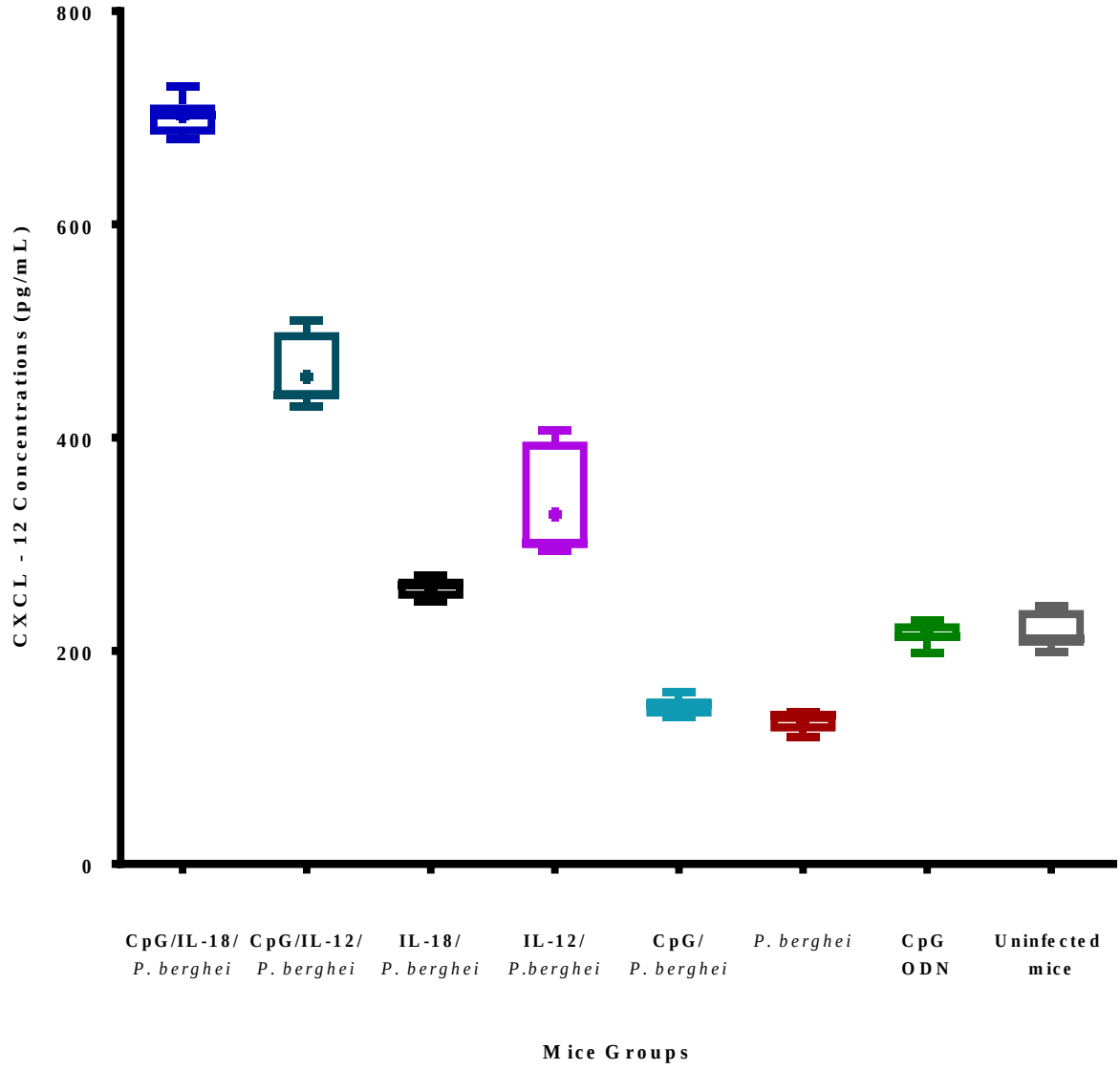



**Figure 4.28: CXCL-12 levels**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 7504$) CXCL-12 concentrations than all the other groups, having a mean concentration of 729.4 pg/ml.

#### 4.4.1.9 CCL-3 Levels

Concentrations of CCL-3 detected in these experiments ranged from 37.10 pg/ml (in the CpG/IL-12/ *P. berghei* mice group) to 122.1pg/ml (in the *P. berghei* group). Significantly higher CCL-3 levels were quantified in the CpG/ *P. berghei* and *P. berghei* groups than in the other mice groups and together both these groups had a mean CCL-3 level of 119.8 pg/ml ($P < 0.0001$; $F(7, 336) = 6697$). With a level of 122.1 pg/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 6697$) CXCL-5 concentrations than all the other groups. The CpG/*P. berghei* group also expressed significantly higher concentrations of CCL-3 than the rest of the groups except the *P. berghei* group and its mean level was at 117.5 pg/ml. The CpG/ *P. berghei* and *P. berghei* groups were the only groups whose CCL-3 levels measured higher than 120.0 pg/ml.

Medium levels of CCL-3 were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei*, CpG ODN and uninfected mice groups; 66.59 pg/ml, 69.99 pg/ml, 55.34 pg/ml, 58.61pg/ml respectively and these averaged at 62.63 pg/ml. Relatively lower levels of CCL-3 were found in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups; 41.76 pg/ml, 37.10 pg/ml, averaging at 39.43 pg/ml. The concentrations of CCL-3 in the *P. berghei* group were about 3 times higher than levels in the CpG/IL-18/ *P. berghei* group, 3 times higher than in the CpG/IL-12/ *P. berghei,* 2 fold higher than the medium level average concentration and 3-fold higher than the lowest concentration groups. The CCL-3 levels in the CpG/*P. berghei* group were 2.8 times higher than levels in the CpG/IL-18/ *P. berghei* group, 3.1 times higher than in the CpG/IL-12/ *P. berghei,* 1.8-fold higher than the medium concentration groups and also 3-fold higher than the average concentration of the lowest concentration groups. The CCL-3 levels in the CpG/IL-18/ *P. berghei* group were exceeded by those in the in the IL-18/ *P. berghei* group by a 1.6-fold difference, they were 1.4 -fold lower than the mean of the medium level groups and 1.05-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group CCL-3 levels were 2-fold lower than in the IL-12/ *P. berghei* group, also 1.6-fold lower than the average level of the medium level groups and 1.01-fold lower than the lowest CXCL - 5 level groups (Figure 4.29).

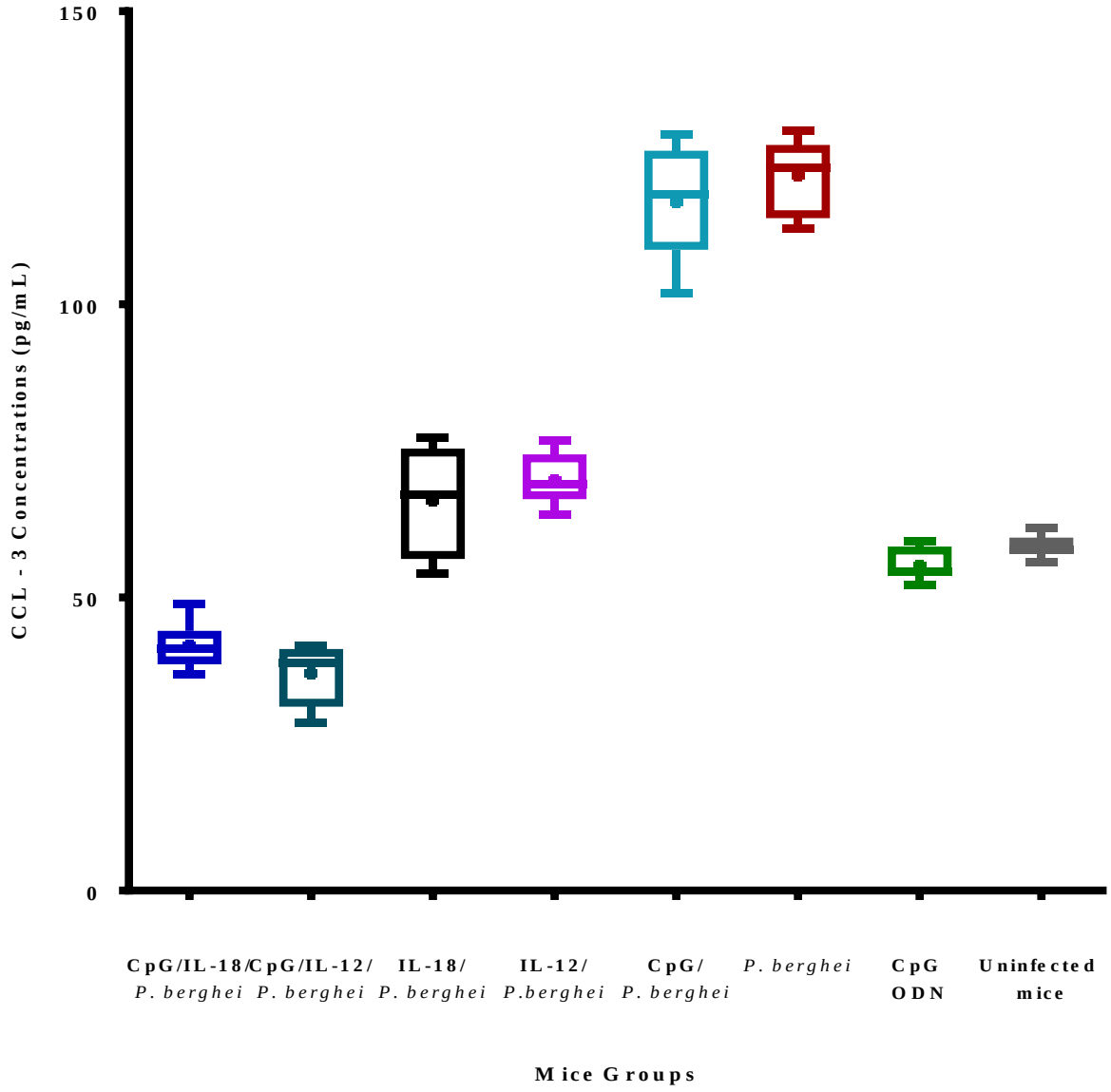



**Figure 4.29: CCL-3 levels**

Significantly higher CCL-3 levels were quantified in the CpG/ *P. berghei* and *P. berghei* groups than in the other mice groupsand together both these groups had a mean CCL-3 level of 119.8 pg/ml ($P < 0.0001$; $F(7, 336) = 6697$).

#### 4.4.1.10 Recall proliferation Response/Stimulation Indices (SI)

Recall proliferation responses were measured by calculating stimulation indices (SI) of splenocyte expansion in response to *P. berghei* crude parasite antigen. Quantities of SI detected in these experiments ranged from 2.571 (in the uninfected mice group) to 8.371 (in the CpG/IL-12/ *P. berghei* group). Significantly higher SI levels were noted in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups than in the other mice groups and together both these groups had a mean SI level of 8.014 ($P < 0.0001$; $F (7, 336) = 6238$). With the SI level of 8.371, theCpG/IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 6238$) SI than all the other groups. The CpG/IL-18/*P. berghei* group also expressed significantly higher SI values than the rest of the groups except the CpG/IL-12/*P. berghei* group and its mean level was at SI 7.657. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were the only groups whose SI levels measured higher than the 8.0 level.

Medium levels of SI were detected in the IL-18/*P. berghei* and CpG/*P. berghei* mice groups; 3.857 and 4.514 respectively and these averaged at 4.18. Relatively lower levels of SI were recorded in the IL-12/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 3.214, 3.414, 2.829 and 2.571, averaging at 3.007. The SI levels in the CpG/IL-18/ *P. berghei* group exceeded those in the in the IL-18/*P. berghei* group by a 2-fold difference, were 1.8-fold higher than the mean SI levels of the medium level groups and 2.5-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group SI levels were 2.6-fold higher than in the IL-12/ *P. berghei* group, also 2-fold higher than the average level of the medium level groups and 2.8-fold higher than the lowest SI level groups (Figure 4.30).

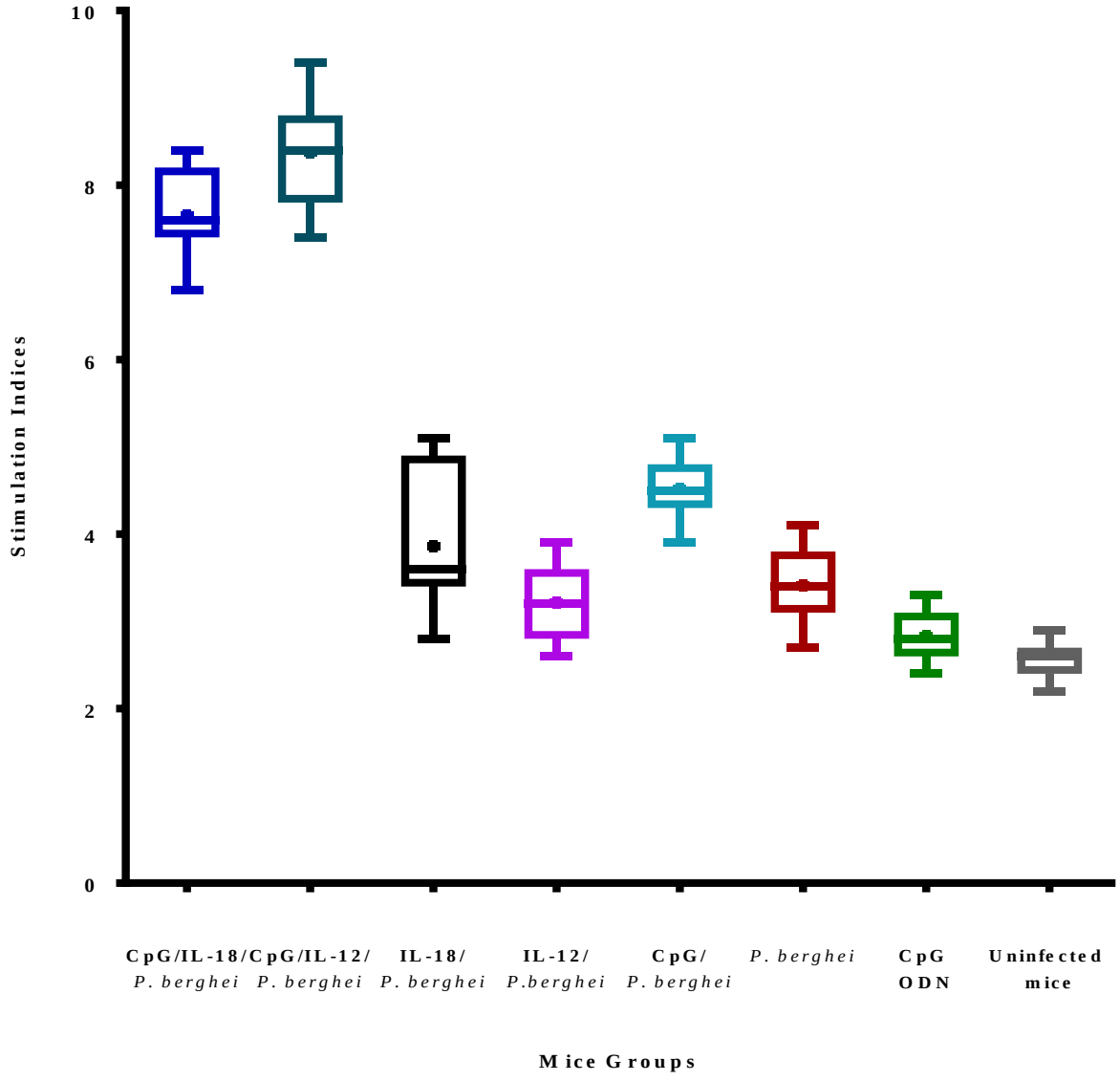



**Figure 4.30: Recall proliferation Response/Stimulation Indices.**

Significantly higher SI levels were noted in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups than in the other mice groupsand together both these groups had a mean SI level of 8.014 ($P < 0.0001$; $F(7, 336) = 6238$).

### 4.5 Cytokine and Immunoglobulin M Responses

A spectrum of cytokines, both proinflammatory and anti-inflammatory, were quantified in these experiments and in addition, anti-malarial antigen immunoglobulin M (IgM) responses were measured. The cytokines analysed were IFN-γ, TNF-α, IL-4, IL-23a, IL-10, and IL-17. One-factor ANOVA analysis indicated that concentrations of all detected cytokines differed significantly amongst the groups (Tables 4.8 and 4.9). The IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* main experimental groups had significantly increased levels of IFN-γ, TNF-α, IL-17, IL-23a and IgM and significantly reduced levels of IL-4, and IL-10 than most of the other groups. A variety of general outcomes were noted:

**Table 4.8: Comparison of cytokine and IgM concentrations:**

| **Quantified Biomolecules** | **Quantitative difference relative to other groups' biomolecules** | |
|---|---|---|
| Cytokine/<br><br>IgM | Quantity in<br><br>IL-18 / CpG / *P. berghei* group | Quantity in<br><br>IL-12 / CpG / *P. berghei* group |
| 1. IFN-γ | ⬆ Than in all groups | ⬆ Than in all groups except group 1 |
| 2. TNF-α | ⬆ Than in all groups | ⬆ Than in all except group 1 |
| 3. IL-4 | ⬇ Than in all except groups 2 and 8 | ⬇ Than in all except group 8 |
| 4. IgM | ⬆⬇ Than in all except group 2 and 4 | ⬆⬇ Than in all groups |
| 5. IL-23a | ⬇ Than in all groups | ⬇ Than in all except group1 |
| 6. IL-10 | ⬇ Than in all except groups 2,3,4 | ⬇ Than in all except group 3 |
| 7. IL-17 | ⬆ Than all groups | ⬆ Than in all except group 2 |

The symbol '⬆' stands for 'mean concentration greater' while '⬆' stands for 'mean concentration lower'.

**Table 4.9: Mean Cytokine and IgM Concentrations**

| | | CpG/IL-18/ *P. berghei* | CpG/IL-12/ *P. berghei* | IL18/ *P. berghei* | IL-12/ *P. berghei* | CpG / *P. berghei* | *P. berghei* | CpG ODN | Uninfected mice |
|---|---|---|---|---|---|---|---|---|---|
| 1. IFN-γ | Means | 1625 | 1427 | 990.8 | 1279 | 499.7 | 230.0 | 567.3 | 260.3 |
| | SD | 15.80 | 10.48 | 171.8 | 16.89 | 24.39 | 13.55 | 17.83 | 25.40 |
| | ANOVA | F (7, 336) = 4502, P < 0.0001 | | | | | | | |
| 2. TNF-α | Means | 448.0 | 436.5 | 184.3 | 303.5 | 160.3 | 176.4 | 47.27 | 88.76 |
| | SD | 7.572 | 9.568 | 11.45 | 7.975 | 21.43 | 12.57 | 15.77 | 10.49 |
| | ANOVA | F (7, 336) = 30299, P < 0.0001 | | | | | | | |
| 3. IL-4 | Means | 62.99 | 54.76 | 131.5 | 141.2 | 79.71 | 164.4 | 87.77 | 48.39 |
| | SD | 6.192 | 7.971 | 6.004 | 5.096 | 4.435 | 11.12 | 5.332 | 4.403 |
| | ANOVA | F (7, 336) = 5612, P < 0.0001 | | | | | | | |
| 4. IgM | Means | 4.186 | 5.200 | 2.743 | 4.600 | 2.029 | 1.804 | 1.966 | 1.514 |
| | SD | 0.5385 | 0.2700 | 0.2951 | 0.3285 | 0.1768 | 0.1485 | 0.06721 | 0.1472 |
| | ANOVA | F (7, 336) = 3834, P < 0.0001 | | | | | | | |
| 5. IL-23a | Means | 165.6 | 176.7 | 90.70 | 92.09 | 48.30 | 19.87 | 76.40 | 47.27 |
| | SD | 7.820 | 7.124 | 4.533 | 7.171 | 2.513 | 3.171 | 5.116 | 1.803 |
| | ANOVA | F (7, 336) = 16034, P < 0.0001 | | | | | | | |
| 6. IL-10 | Means | 133.0 | 103.6 | 183.5 | 180.3 | 240.6 | 324.5 | 111.3 | 95.63 |
| | SD | 6.986 | 4.375 | 7.792 | 6.679 | 7.689 | 27.65 | 3.361 | 10.66 |
| | ANOVA | F (7, 336) = 4671, P < 0.0001 | | | | | | | |
| 7. IL-17 | Means | 420.5 | 367.5 | 103.6 | 189.2 | 184.4 | 42.94 | 122.7 | 114.9 |
| | SD | 4.013 | 13.65 | 7.056 | 7.792 | 6.679 | 4.853 | 5.447 | 4.243 |
| | ANOVA | F (7, 336) = 67376, P < 0.0001 | | | | | | | |

### 4.5.1 IFN – Gamma Levels

The IFN-γ cytokine was detected in all investigated mice groups and overall its concentrations varied from 230.0 pg/ml (in the *P. berghei* mice group) to 1625 pg/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei* and the IL-12/ *P. berghei* groups had significantly ($P < 0.0001$; $F(7, 336) = 4502$) higher IFN-γ level concentrations of than the other groups and together the three groups had a mean IFN-γ level of 1443.67 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4502$) IFN-γ concentrations than all the other groups, having a mean IFN-γ concentration of 1625 pg/ml. The CpG/IL-12/*P. berghei* group had significantly higher concentrations of IFN-γ than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 1427 pg/ml, while the IL-12/ *P. berghei* group had 1279 pg/ml mean IFN-γ levels. Only the CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei* and IL-12/ *P. berghei* groups were had IFN-γ levels exceeding the 1250 pg/ml value. TheIL-18/ *P. berghei* group followed closely with a mean IFN-γ level of 990.8 pg/ml.

Intermediate levels of IFN-γ were detected in the CpG/*P. berghei* and CpG ODN groups; 499.7 pg/ml, and 567.3 pg/ml respectively and these 2 groups had a mean IFN-γ level of 533.5 pg/ml. The lowest mean level of IFN-γ were found in the *P. berghei* and uninfected mice groups; 230.00 pg/ml and 260.3 pg/ml. These 2 groups together averaged at 245.15 pg/ml. The IFN-γ levels in the CpG/IL-18/ *P. berghei* group were about one 1.6 times higher than levels in the IL-18/ *P. berghei* group, 3 times higher than the intermediate concentration groups collectively, and 6.6 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group IFN-γ levels were 1.1 times higher than in the IL-12/ *P. berghei* group, about 2.6 times higher than the mean levels of the intermediate concentration groups and 5.8 times higher than the two lowest concentration groups (Figure 4.31).

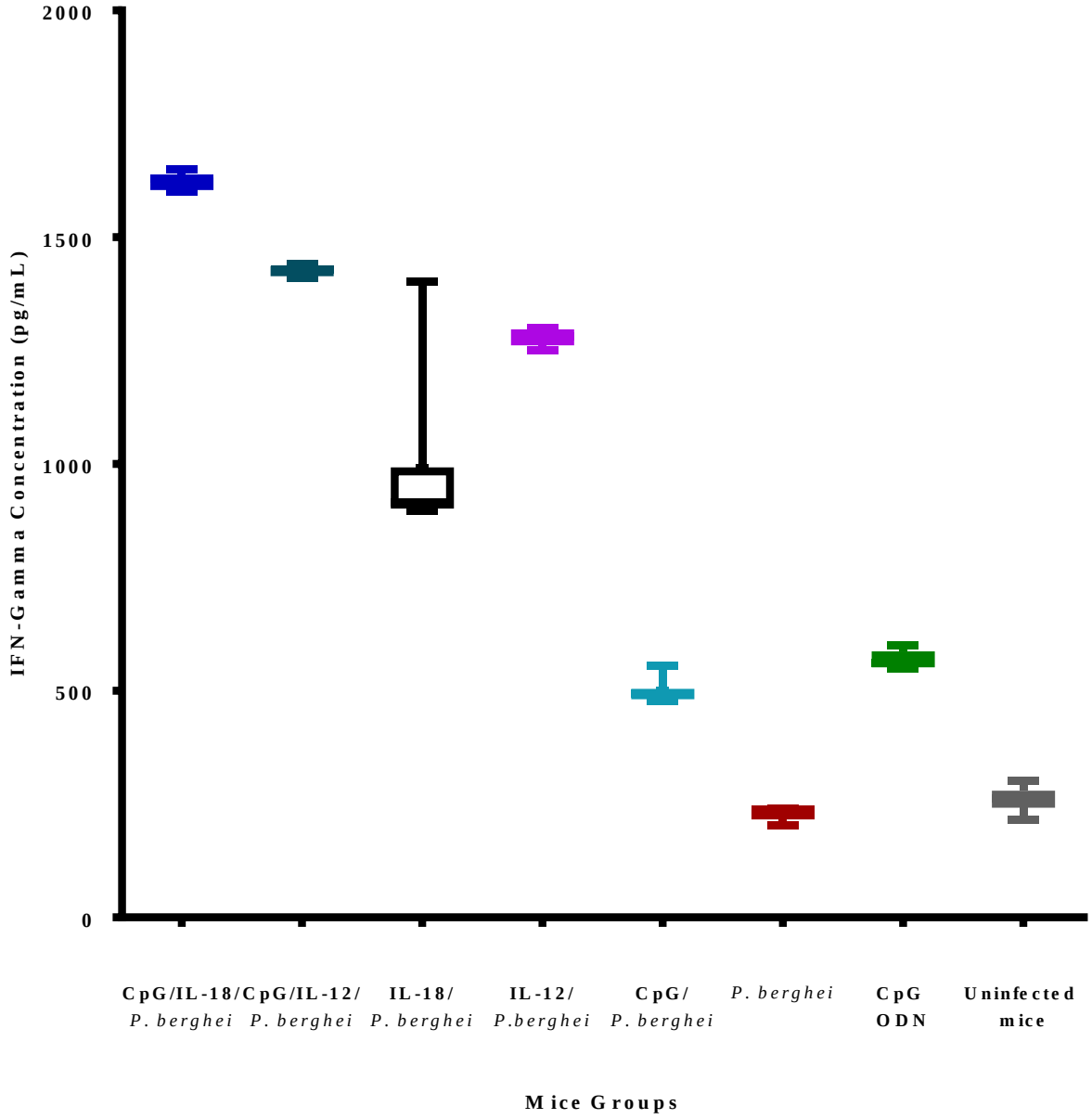



**Figure 4.31: IFN-γ levels**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4502$) IFN-γ concentrations than all the other groups.

### 4.5.2 TNF – Alpha Levels

Levels of TNF-α detected in these experiments spanned from 88.76 pg/ml (in the uninfected mice group) to 448.0 pg/ml (in the CpG/IL-18/*P. berghei* group). The CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei* and the CPG ODN groups had significantly ($P < 0.0001$; $F(7, 336) = 30229$) higher TNF-α level concentrations of than the other groups and together the three groups had a mean TNF-α level of 428.43 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 30229$) TNF-α concentrations than all the other groups, having a mean TNF-α concentration of 448.0 pg/ml. The CpG/IL-12/*P. berghei* group had significantly higher concentrations of TNF-α than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 436.5 pg/ml, while the IL-12/ *P. berghei* group had 400.8 pg/ml mean TNF-α levels. Only the CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei* and CpG ODN groups had TNF-α levels exceeding the 400.00 pg/ml value.

Mid-levels of TNF-α were quantified in IL-12/ *P. berghei* group followed the higher level groups closely with a mean TNF-α level of 303.5 pg/ml. Low-levels of of TNF-α were quantified in the IL-18/ *P. berghei*, CpG/*P. berghei*, *P. berghei* and uninfected groups; 184.3pg/ml, 160.3pg/ml, 176.4pg/ml and 88.76 pg/ml respectively and these averaged at 152.44 pg/ml. The uninfected mice group had significantly ($P < 0.0001$; $F(7, 336) = 30229$) the lowest TNF-α levels than the rest of the groups in the experiment; 88.76 pg/ml. The TNF-α levels in the CpG/IL-18/ *P. berghei* group were about 2.4 times higher than levels in the IL-18/ *P. berghei* group (a group that also had one of the lowest detected TNF-α amount), 1.5-fold higher than the mid-level concentration group and 3 times higher than the 4 lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group TNF-α levels were 1.4-fold higher than in the IL-12/ *P. berghei* group, 1.4-fold higher than the mid-level groups together and 3-fold higher than the two lowest concentration groups (Figure 4.32).

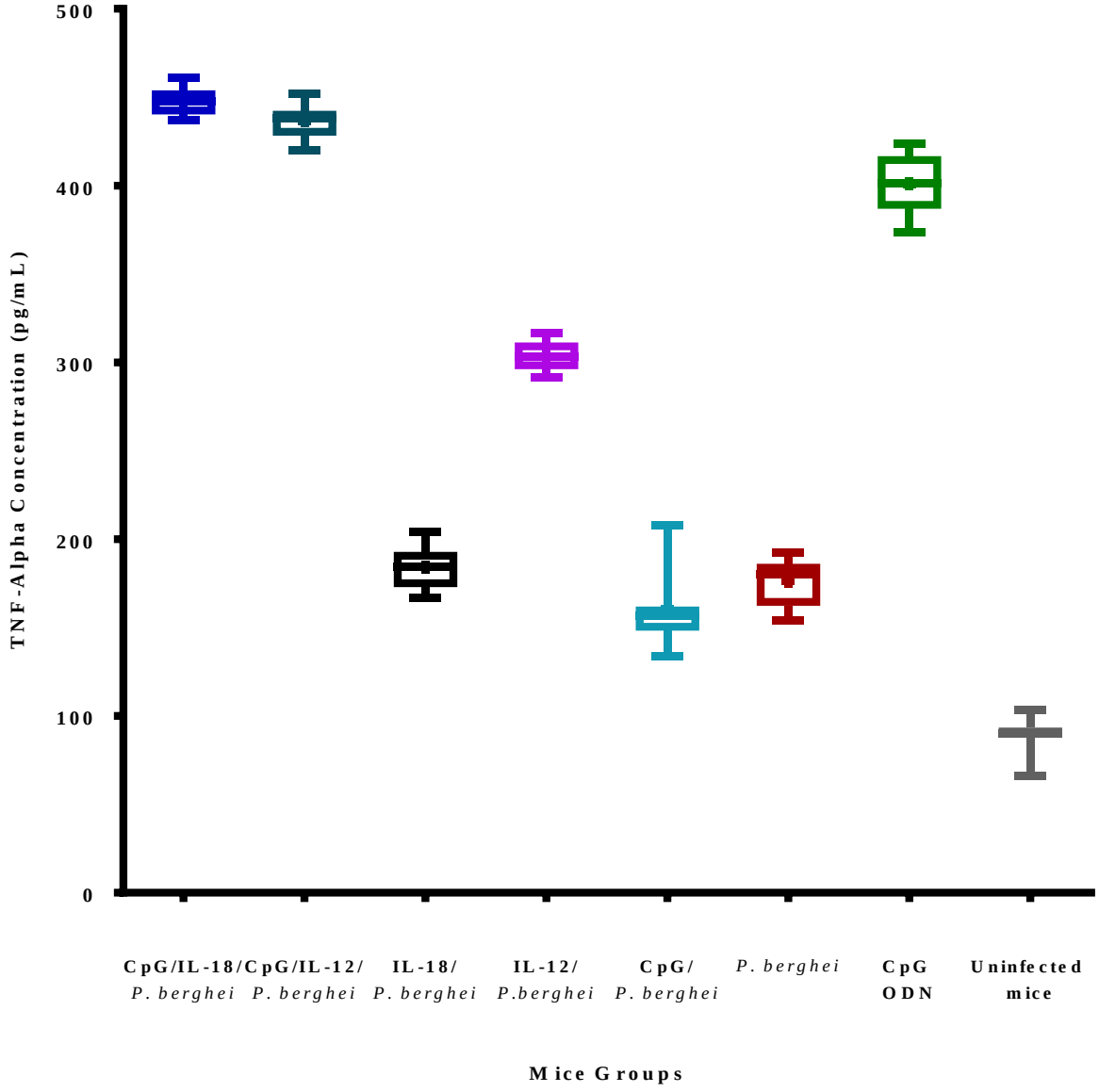



**Figure 4.32: TNF-α levels**

The uninfected mice group had significantly ($P < 0.0001$; $F(7, 336) = 30229$) the lowest TNF-α levels than the rest of the groups in the experiment; 88.76 pg/ml.

### 4.5.3 IL-4 Levels

Levels of IL-4 detected in these experiments varied from 48.39 pg/ml (in the uninfected mice group) to 164.4 pg/ml (in the CpG/IL-18/*P. berghei* group). The IL-18/ *P. berghei*, IL-12/ *P. berghei* and the *P. berghei* groups had significantly ($P < 0.0001$; $F(7, 336) = 5612$) higher IL-4 concentrations of than the other groups and together the three groups had a mean IL-4 level of 145.7 pg/ml. The *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 5612$) IL-4 concentrations than all the other groups, having a mean IL-4 concentration of 164.4 pg/ml. The IL-12/*P. berghei* group had significantly higher concentrations of IL-4 than the rest of the groups except the *P. berghei* group and its mean level was at 141.2 pg/ml, while the IL-12/ *P. berghei* group had 131.5 pg/ml mean IL-4 levels. Only the IL-18/ *P. berghei*, IL-12/ *P. berghei* and the *P. berghei* groups had IL-4 levels exceeding the 130.00 pg/ml.

Mid-levels of IL-4 were recorded in the CpG/*P. berghei* and CpG ODN groups; 79.71 pg/ml and 87.77 pg/ml respectively, with a mean IL-4 level of 83.74 pg/ml. Low-levels of of IL-4 were quantified in the CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei* and uninfected mice groups; 62.99 pg/ml, 54.76 pg/ml and 48.39 pg/ml respectively and these averaged at 55.38 pg/ml. The uninfected mice group had significantly ($P < 0.0001$; $F(7, 336) = 5612$) the lowest IL-4 levels than the rest of the groups in the experiment; 55.38 pg/ml. The IL-4 levels in the *P. berghei* group were 2.6 times higher than in the CpG/IL-18/ *P. berghei* group, 3 times higher than in the CpG/IL-12/ *P. berghei* group,2 times higher than the mid-level expression groups put together and 3 times higher than the mean of IL-4 levels in the 3 lowest groups together. The IL-4 levels in the CpG/IL-18/ *P. berghei* group were about 2 times lower than levels in the IL-18/ *P. berghei* group (a group that also had one of the 3 highest detected IL-4 levels), 1.3-fold lower than the mid-level concentration group and 1.1 times higher than the mean of the 4 lowest concentration groups in which this group also belongs. In the CpG/IL-12/ *P. berghei* group IL-4 levels were 2.6-fold lower than in the IL-12/ *P. berghei* group, 1.5-fold lower than the mid-level groups together and similar (1.0-fold) to the lowest concentration groups where it belonged (Figure 4.33).

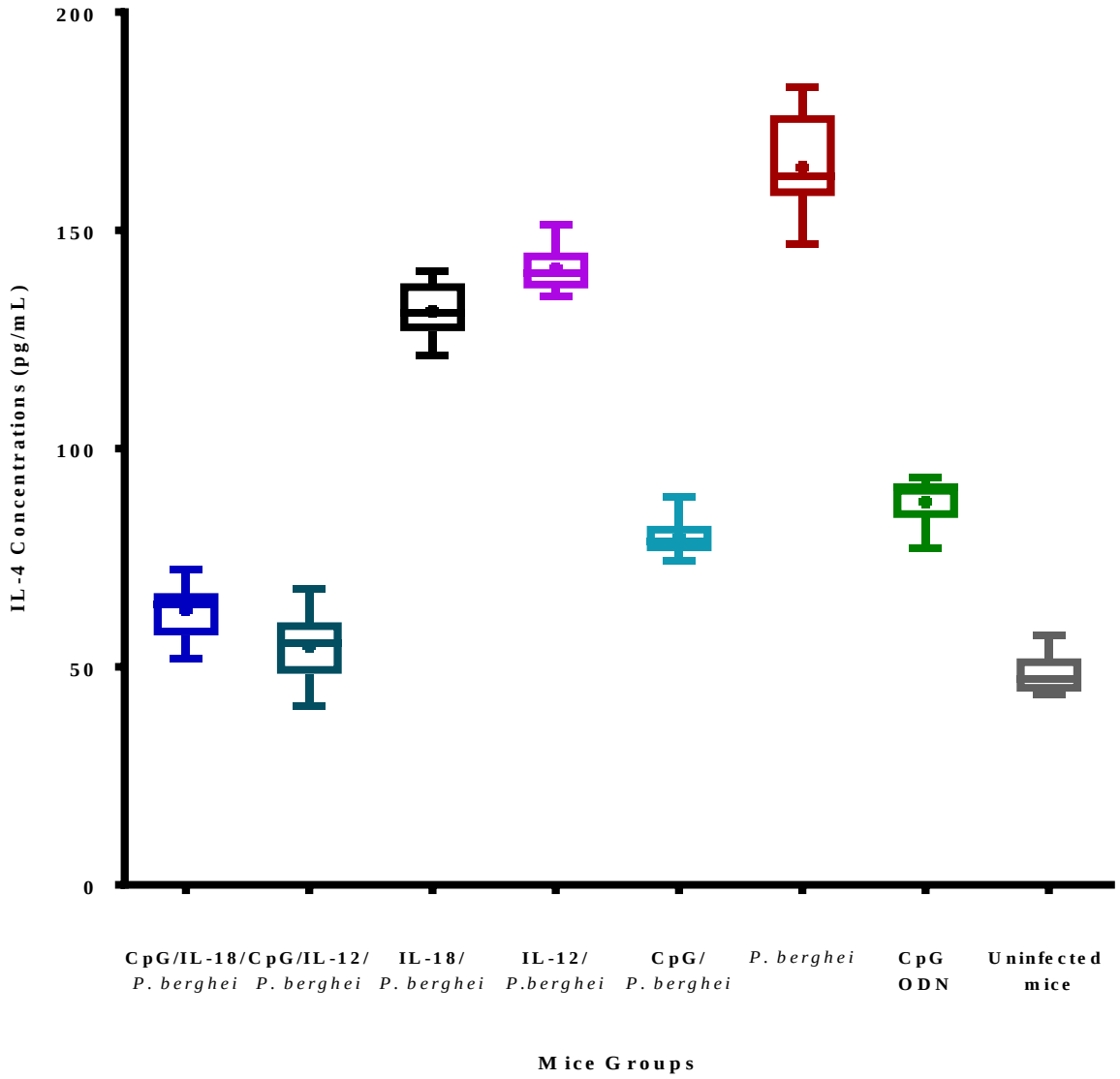



**Figure 4.33: IL-4 levels**

The uninfected mice group had significantly ($P < 0.0001$; $F(7, 336) = 5612$) the lowest IL-4 levels than the rest of the groups in the experiment; 55.38 pg/ml.

### 4.5.4 IL-23a Levels

The IL-23a cytokine was detected in all investigated mice groups and overall its concentrations varied from 77.40 pg/ml (in the *P. berghei* mice group) to 165.6 pg/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly ($P < 0.0001$; $F(7, 336) = 16034$) highest level concentration groups and together they had a mean IL-23a level of 171.15 pg/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 16034$) IL-23a concentrations than all the other groups, having a mean IL-23a concentration of 165.6 pg/ml. The CpG/IL-12/*P. berghei* group had significantly higher concentrations of IL-23a than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 176.7 pg/ml. Only in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups did the IL-23a levels exceed the 175.00 pg/ml level.

Intermediate levels of IL-23a were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei* and CpG ODN groups; 90.70 pg/ml, 92.09 pg/ml and 76.40 pg/ml respectively and these 3 groups had a mean IL-23a level of 86.40 pg/ml. The lowest mean levelc of IL-23a were found in the CpG/*P. berghei, P. berghei* and uninfected mice groups; 48.30 pg/ml, 76.03 pg/ml, 19.87 pg/ml and 47.27 pg/ml. These 4 groups together averaged at 38.48 pg/ml. The IL-23a levels in the CpG/IL-18/ *P. berghei* group were one 1.8 times higher than levels in the IL-18/ *P. berghei* group, 1.9 times higher than the intermediate concentration groups collectively, and 4.3 times higher than the lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group IL-23a levels were 1.9 times higher than in the IL-12/ *P. berghei* group, about 2 times higher than the mean levels of the intermediate concentration groups and 4.6 times higher than the two lowest concentration groups (Figure 4.34).

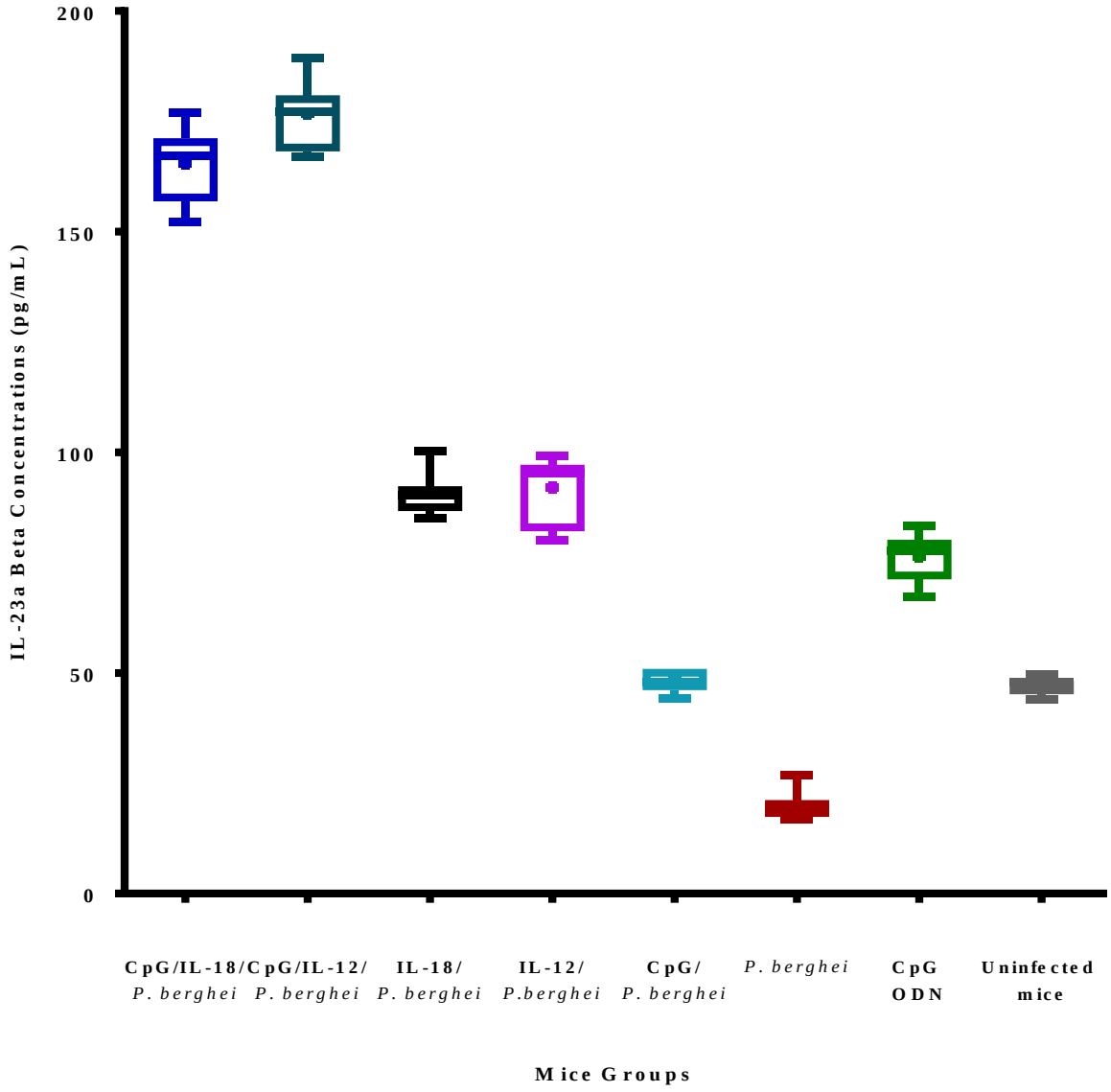



**Figure 4.34: IL-23a levels**

Intermediate levels of IL-23a were detected in the IL-18/ *P. berghei*, IL-12/ *P. berghei* and CpG ODN groups; 90.70 pg/ml, 92.09 pg/ml and 76.40 pg/ml respectively.

### 4.5.5 IgM Levels

Immunoglobulin M was detected in all investigated mice groups and overall its concentrations ranged from anoptical density (OD) of 1.51 (in the uninfected mice group) to OD 5.20 (in the CpG/IL-12/ *P. berghei* group; Fig 4.35). The CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei* and IL-12/ *P. berghei* groups were the three significantly ($P < 0.0001$; $F (7, 336) = 3834$) highest level concentration groups and together they had a mean IgM level of OD 4.66. The CpG/IL-12/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) =3834$) IgM concentrations than all the other groups, having a mean IgM concentration of OD 5.20 and it was followed by the IL-12/ *P. bergheig*roup which had mean IgM levels of OD 4.60. The CpG/IL-18/*P. berghei* group was the third highest and its mean IgM level was at OD 4.18. Only the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei* and IL-12/ *P. berghei* groups had IgM levels exceeding the OD 4.0 level.

Intermediate levels of IgM were detected in the IL-18/ *P. berghei* group; OD 2.743. The lowest mean level of IgM were found in the CpG/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; OD 2.02, OD 1.80, OD 1.96 and OD 1.51. These 4 groups together averaged at OD 1.82. The IgM levels in the IL-12/ *P. berghei* group were about one the same (1.0-fold) as levels in the IL-18/ *P. berghei* group, 1.7 times higher than the intermediate concentration group, and 2.5 times higher than the lowest concentration groups detailed above. The IgM levels in the CpG/IL-18/ *P. berghei* group were about one 1.5 times higher than levels in the IL-18/ *P. berghei* group, which was also the intermediate concentration group, and 2.3 times higher than the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group IgM levels were 1.1 times higher than in the IL-12/ *P. berghei* group, about 2 times higher than the mean levels of the intermediate concentration groups and 2.8 times higher than the 4 lowest concentration groups (Figure 4.35).

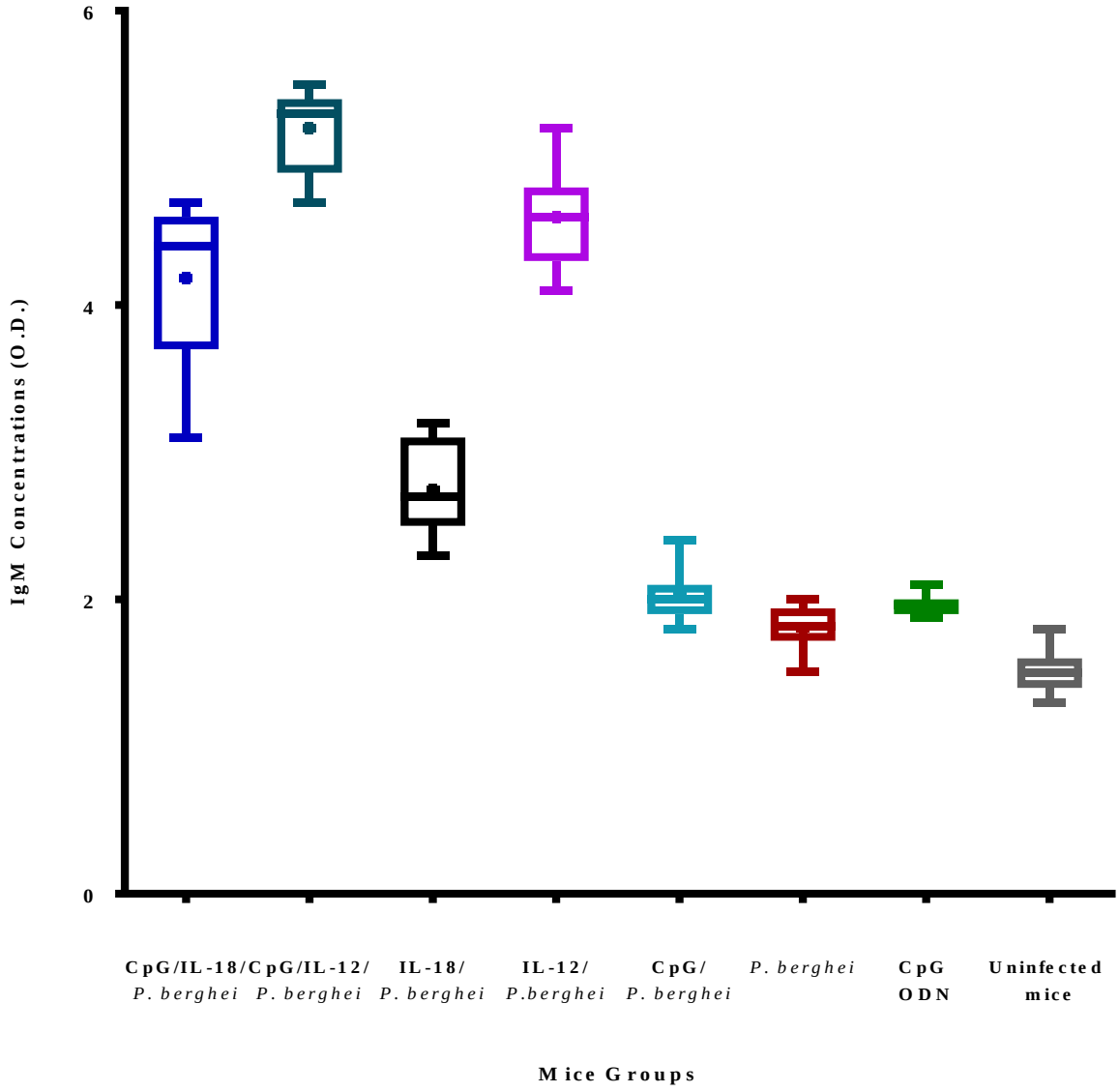



**Figure 4.35: IgM levels.**

The CpG/IL-12/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 3834$) IgM concentrations than all the other groups, having a mean IgM concentration of OD 5.20.

### 4.5.6 IL-10 Levels

Concentrations of IL-10 detected in these experiments ranked from 95.63pg/ml (in the uninfected mice group) to 324.5pg/ml (in the *P. berghei* group). The CpG/ *P. berghei* and *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 4671$) higher concentrations of IL-10 than the other mice groups and taken together, both these groups had a mean IL-10 level of 282.55pg/ml. With a level of 324.5pg/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 4671$) IL-10 concentrations than all the other analysed groups. The CpG/*P. berghei* group produced significantly higher concentrations of IL-10 than the rest of the groups except the CpG/*P. berghei* group and its mean level was at 240.6 pg/ml. The CpG/ *P. berghei* and *P. berghei* groups were the only groups recorded with IL-10 concentrations that exceeded 240.0 pg/ml.

The IL-18/ *P. berghei* and IL-12/ *P. berghei* groups formed the *mid-level cluster* in terms of IL-10 concentraions; they generated 183.5 pg/ml and 180.3 pg/ml respectively and these averaged at 181.9 pg/ml. The CpG/IL-18/ *P. berghei*, CpG/IL-12/ *P. berghei*, CpG ODN and uninfected groups formed the *low-level cluster* producing 133.0 pg/ml, 103.6pg/ml, 111.3 pg/ml and 95.63pg/ml respectively, averaging at 110.88 pg/ml. The IL-10 levels in the *P. berghei* group were about 2.4 times higher than levels in the CpG/IL-18/ *P. berghei* group, 3.1 times higher than in the CpG/IL-12/ *P. berghei*, 1.8-fold higher than the medium level average concentration and 2.9-fold higher than the lowest concentration groups. The IL-10 levels in the CpG/*P. berghei* group were 1.8 times higher than levels in the CpG/IL-18/ *P. berghei* group, 2.3 times higher than in the CpG/IL-12/ *P. berghei*, 1.3-fold higher than the medium concentration groups and also 2.1-fold higher than the average concentration of the lowest concentration groups. In the CpG/IL-18/ *P. berghei* group IL-10 levels were 1.4-fold lower than in the IL-18/ *P. berghei* group, equivalent (1-fold) than the mean of the medium level groups, and about 1.1-fold higher than the mean levels of the lowest concentration groups (where the CpG/IL-18/ *P. berghei* group belonged). In the CpG/IL-12/ *P. berghei* group IL-10 levels were 1.7-fold lower than in the IL-12/ *P. berghei* group,1.8-fold lower than the average level of the medium level groups and equivalent (1.0-fold) to than the lowest IL-10 level groups (Figure 4.36).

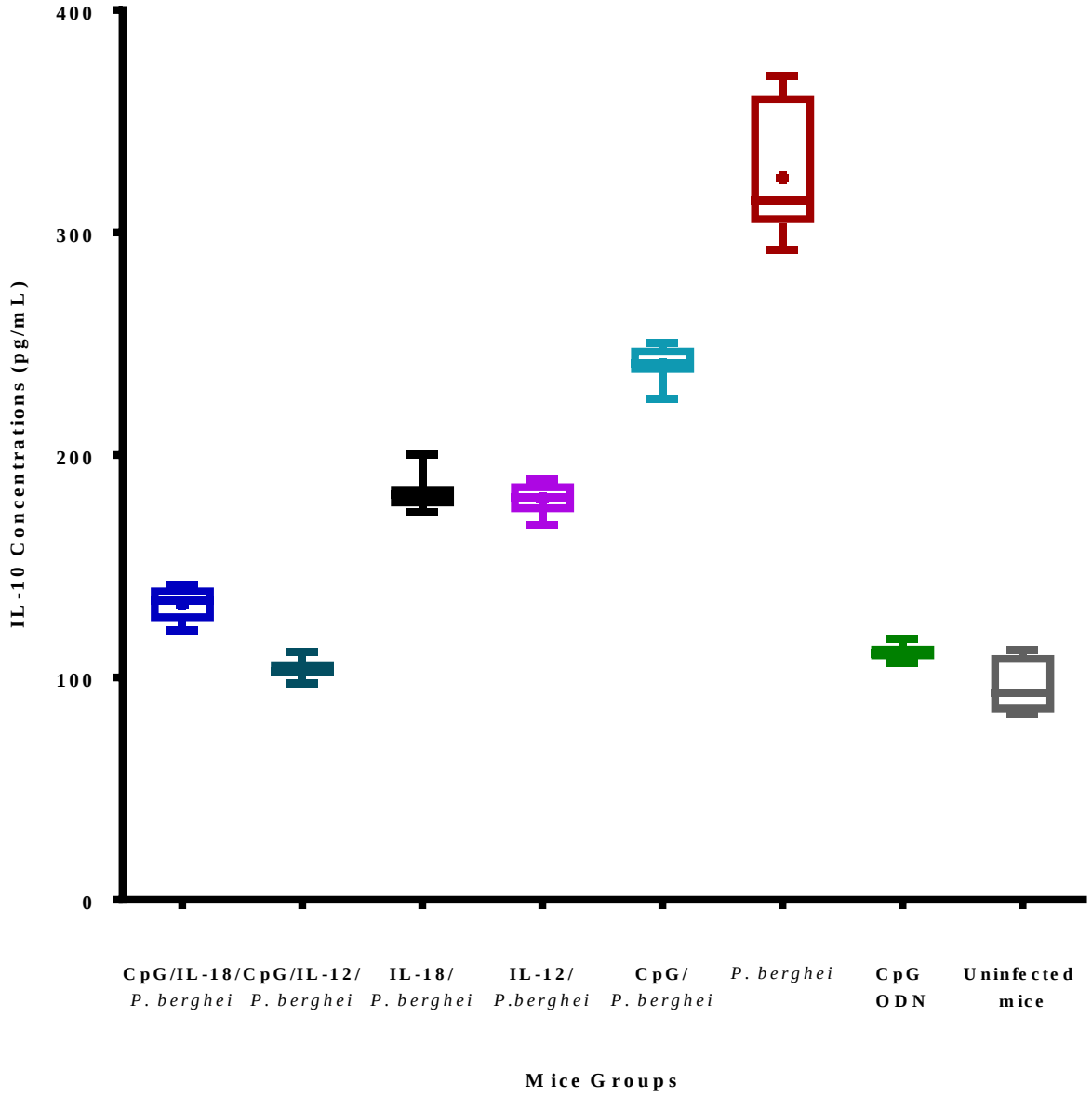



**Figure 4.36: IL-10 levels.**

The *P. berghei* group had significantly higher ($P < 0.0001$) IL-10 concentrations than all the other groups, having a mean IL-10 concentration of 528.4 pg/ml.

### 4.5.7 IL-17 Levels

The IL-17 concentrations were analysed in all mice groups and overall mean concentrations spanned from 42.94 pg/ml (in the *P. berghei* mice group) to 420.5 pg/ml (in the CpG/IL-18/ *P. berghei* group). Significantly high IL-17 concentrations were detected in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups and together these two groups together had a mean IL-17 level of 394.00 pg/ml ($P < 0.0001$; $F(7, 336) = 67376$). The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 67376$) IL-17 concentrations than all the other groups, having a mean concentration of 420.5 pg/ml. The CpG/IL-12/*P. berghei* group was the second highest group and its mean IL-17 quantity was at 367.5 pg/ml. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups had IL-17 levels exceeding the 365.00 pg/ml value.

Intermediate levels of IL-17 were detected in the IL-12/ *P. berghei* and CpG /*P. berghei* groups which had an average amount of 186.8pg/ml. The lowest mean levels of IL-17 were found in the IL-18/ *P. berghei*, *P. berghei*, CpG ODN and uninfected mice groups; 1.3.6 pg/ml, 42.94 pg/ml, 122.7 pg/ml and 114.9 pg/ml. These 4 groups together averaged at 96.03 pg/ml. With an IL-17 concentration of 42.94 pg/ml, the *P. berghei* group had significantly ($P < 0.0001$; $F(7, 336) = 67376$) lower IL-17 levels than the rest of the mice groups. In the CpG/IL-18/ *P. berghei* group IL-17 levels were about one 4 times higher than levels in the IL-18/ *P. berghei* group, 2.3 times higher than the intermediate concentration groups collectively, and 4.4 times higher than the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group IL-17 levels were 2 times higher than in the IL-12/ *P. berghei* group, about 2 times higher than the mean levels of the intermediate concentration groups and 3.8 times higher than the two lowest concentration groups (Figure 4.37).

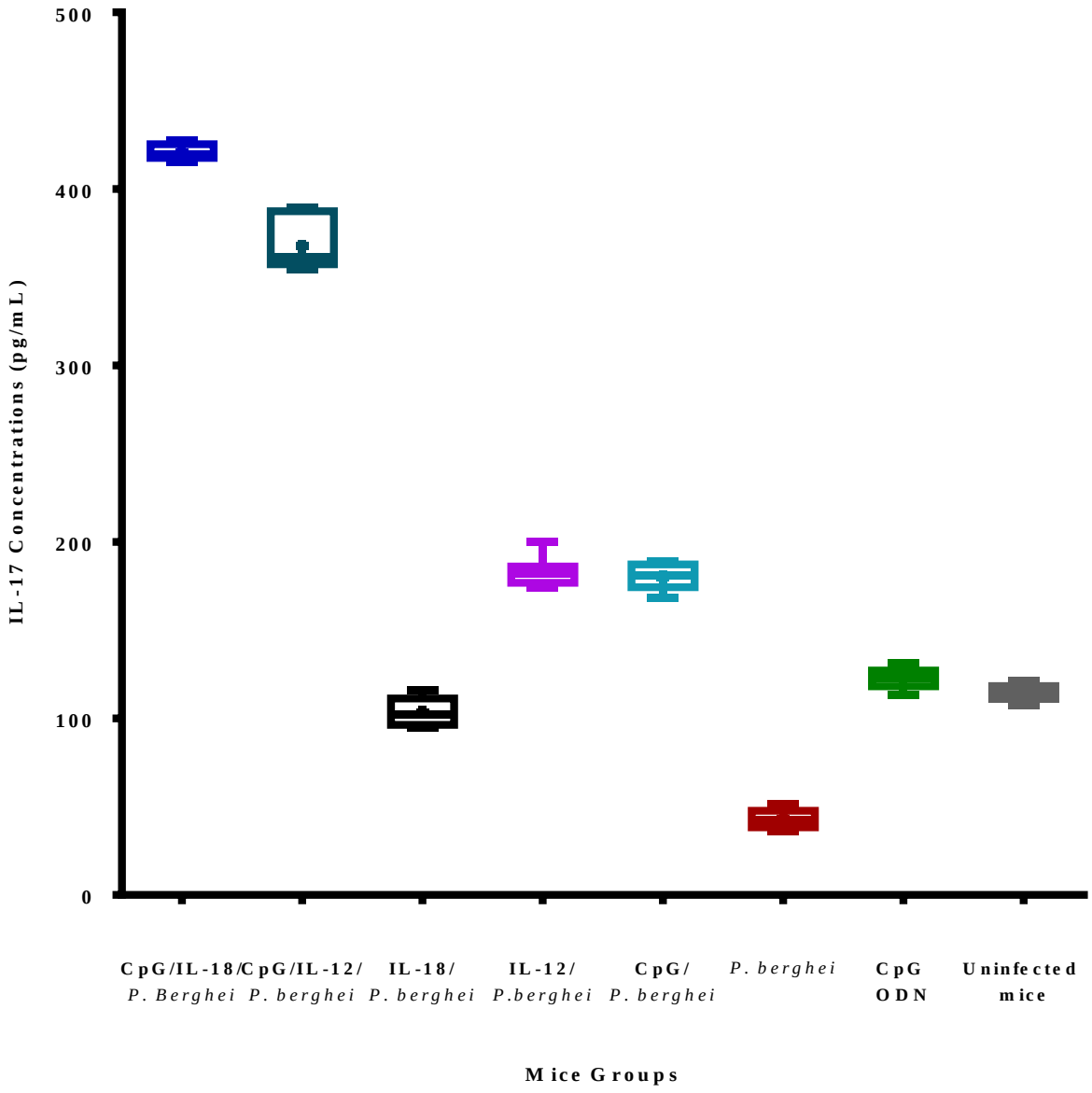



**Figure 4.37: IL-17 levels.**

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 67376$) IL-17 concentrations than all the other groups, having a mean concentration of 420.5 pg/ml.

# CHAPTER FIVE

# DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Clinical, Haematological and Parasitological Responses to Cytokine-CpG Motif ODN Co-inoculation in the BALB/c-*P. berghei* ANKA Model

Generally, cytokine-CpG ODN coinoculation in the 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-l2/ *P. berghei* groups, experienced reduced parasitaemia. They had significantly reduced total and differential parasitaemia levels, lower peak parasitaemia, and less dramatic total and differential parasitaemia trends. This gave an 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, Abdul, Outhayphone, & Mohd, 1999). Treatment of *P. berghei* infected mice with anti-IL-12 was shown to cause increased parasitaemia levels, fatal results, and lower IFN-γ mRNA expression and secretion activities (Yoshimoto, Yoneto, Waki, & Nariuchi, 1998). IL-12 triggers IFN-γ 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 causing early IFN-γ release and parasitaemia reduction (Romero *et al.,* 2007).

Down regulation of IL-12 functions was found to be positively correlated with severity of *P. falciparum* malaria in African children and mild *P. falciparum* malaria was reportedly associated with high IL-12 and IL-18 levels in plasma

(Malaguarnera, Pignatelli, Musumeci, Simporè, & Musumeci 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). 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 interferon gamma inducing factor, also enhances T and NK cell maturation cytokine production, and cytotoxicity (Gracie *et al.,* 2003). The protective roles of IL-18 against the blood stages of both lethal *P. berghei* ANKA and the non-lethal *P. yoelii* strain have been described previously. 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 rIL-18 delayed parasitaemia and increased survival rate. In contrast, injection of anti-IL-18 antibodies exacerbated infection and shortened survival of malaria infected mice (Tominaga *et al.,* 2000). Early production of IFN-γ, a cytokine induced by IL-18, has been linked to protection from murine celebral malaria. Similarly, this would also be expected to occur in mice given 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 increase in both IL-18 and IL-12 has been implicated in defense against *P. falciparum* by modulating the synthesis of inflammatory cytokines (Malaguarnera *et al.,* 2002).

Elevated proinflammmatory IL-18 cytokine levels were also associated with uncomplicated *P. falciparum* malaria limiting progression to life-threatening complications 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 outruled 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 that involved such combinations; the CpG/IL-18/ *P. berghei* and CpG/IL-l2/ *P. berghei* groups. Cytokine-CpG motif ODN co-inoculations 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 & Diggs 1977; Landau & Boulard 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 schizonticidal 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, Fonager, Braks, Khan, & Janse 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. 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 (Sarkar, Ahluwalia, Vijayan & Talwar, 2009). 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 chillness may occur. The fever is usually irregular shivering and mild chills although in advanced infections the pattern of fever becomes less regular (Bartoloni, & Zammarchi, 2012). Malarial complications may involve supervening symptoms such as acute renal failure, dysfunctions of hematopoietic systems (e.g. severe anaemia) pulmonary oedema, generalized convulsions, circulatory collapse, followed by coma and death. Metabolic acidosis and hypoglycemia are also common systemic complications (Bartoloni *et al.*, 2012). 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 and malaria has also been shown to contribute to weight reduction and suboptimal growth especially in children 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-l2/ *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 studies (Kotepui *et al.,* 2015; Sowunmi *et al.,* 2007) and haematological anomalies including anaemia, thrombocytopaenia, and leukocytosis or leukopaenia and are amongst the major characteristics that accompany malaria infections and they are usually worse in cases of *P. falciparum* infection (Manas *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 (Sowunmi *et al.,* 2007). 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, Kager, Vreeken, & White 2000); the CpG/IL-18/ *P. berghei* and CpG/IL-l2/ *P. berghei* groups of mice experienced less severe malarial 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-ellicited eosinophilia has previously been connected to depression of *P. berghei* infection in mice (Zainal-Abidin, Robiah, & Ismail, 1984) and antiplasmodial 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 malaria-infected controls), indicating a possible eosinophilic role in mediation of reduced *P. berghei* severity. Prolonged neutrophil dysfunction after *Plasmodium falciparum* malaria is related to hemolysis and heme oxygenase-1 induction (Cunnington *et al.,* 2012). Similar to the currents cytokine-CpG motif co-inoculation

study's findings, anti-malarial activities of neutrophils were described (Kumaratilake & Ferrante, 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 and a potent eosinophilic response following antimalarial therapy was shown to predicts 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 (Manas *et al.,* 2015) and it is known that animals deprived of T lymphocytes suffer severe *Plasmodial* infections and cannot be immunized against malaria parasites (Allison & Eugui, 1983). CD4+T Cells were shown to expand in *P. berghei* (NK-65) infected and immunized BALB/C Mice leading to protection (Kumar *et al.,* 2002). Antimalarial immunity can be transferred in mice by 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, 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, Eichmann, Kury, Linke & Langhorne, 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 **(**Manas *et al.,* 2015**).** Mononuclear cells are required in elimination of *Plasmodi*a as illustrated by reports of increased nitric oxide production and mononuclear cell nitric oxide synthase activities in malaria-tolerant Papuan adults (Boutlis *et al.,* 2003). The BALB/c mice groups 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) 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 red blood cells 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 byproduct 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. Low albumin (hypoalbuminemia) may be caused by liver disease / dysfunction and this was detected in the hyperparasitised *P. berghei* control group. Contrastingly, the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups had comparable albumin levels to the normal uninfected mice control group, suggesting relatively normal liver functionality.

While ALP and ALT levels did not vary significantly among the mice groups, and 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, AST activities increase, showing positive correlation with liver damage (Abro *et al.*, 2009).

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 transaminase (ALT) is a transaminase enzyme (EC 2.6.1.2). It is also called alanine aminotransferase (ALAT) and was formerly called serum glutamate-pyruvate transaminase (SGPT) or serum glutamic-pyruvic transaminase (SGPT).

The ALT enzyme 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. 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. ALP levels in plasma rise with large bile duct obstruction, intrahepatic cholestasis, or infiltrative diseases of the liver. AST, also called serum glutamic oxaloacetic transaminase or aspartate aminotransferase, 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 that was detected with extremely low glucose levels. Inhibition of glycogenolysis has been implicated in mediation of hypoglycaemia, which constitutes a major complication of severe malaria (van Thien *et al.,* 1997; Blümer *et al.,*

2005). 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, thus providing evidence that cytokine-CpG therapy prevented hypoglycaemic tendencies in the context of murine *P. berghei* malaria.

## 5.2 Transcription factor and Bioactive Molecule Responses

### 5.2.1 Transcription Factor concentrations following anti-malarial Cytokine-CpG Motif Oligodeoxynucleotide Co-inoculation

Overall, this study's murine recipients of the cytokine-CpG ODN co-inoculation biotherapy experienced upregulated levels of NF-kB, NFATc, IRF-5, AhR, KLF and reduced levels of FOXp3 and STAT-6 TFs. The measured STAT-6 levels were reduced in the CpG/IL-12/*P. berghei*, IL-18/*P. berghei* and CpG/*P. berghei* and increased in the *P. berghei* and IL-12/*P. berghei* groups. Mice that were administered with therapeutic cytokine – CpG motif did not only manage to suppress malaria, but were also noted to have generated significantly higher levels of the NF- kB transcription factor which is usually found in a majority of animal cell-types and participates in cellular responses to stimuli like stress, cytokines, oxidized LDL and microbial invasion (Gilmore, 2006). The prototypical pro-inflammatory transcription factor NF- kB plays key roles in the regulation of immune responses to infection and is dysregulation leads to abnormalities such as cancers and improper immune development (Gilmore, 2006) and NF-KB was found to play a central to the regulation of the *P. falciparum*-modulated endothelium transcriptome (Tripathi, Sha, Shulaev, Stins, & Sullivan, 2009). All control mice groups; Uninfected mice, *P. berghei* - infected mice CpG-inoculated mice, IL-12/CpG and IL-18/CpG mice groups all had significantly lower NF-kB levels below the 85 ng/ml level, revealing that stronger NF-kB mechanisms were induced by cytokine-CpG motif ODN

co-inoculation in the murine host. Previous reports indicated that downstream signaling of NF-kB pathways occurs during malaria infections leading to the production of pro-inflammatory cytokines, nitric oxide and chemokines causing and NF-kB has a presence in the enhancer promoter regions of pro-inflammatory cytokine genes (Punsawad *et al.*, 2013).

The boosting of NF-kB-dependent basal immunity of *Anopheles gambiae* was found to cause aborted development of *P. berghei* and PBMCs of patients with complicated *P. falciparum* malaria were shown to display decreased NF-kB activities (Punsawad *et al.*, 2012). Conversely, patients with uncomplicated *P. falciparum* and *P. vivax* malaria were found to have significantly higher NF-kB concentration agreeing with the current findings showing that increased levels of NF-kB triggered in mild malaria scenarios. There was reduced *P. berghei* parasitisation in mice that were co-administered with cytokines and CpG motifs in the current findings and activation of parasite-controlling dendritic cells (DC) during both *P. berghei* and *P. chabaudi chabaudi* infections was shown to involve direct cell-to-cell contact, infected RBC internalization by DC and it involves TLR4, TLR9, MyD88 and signaling via NF-kB (Seixas, Moura Nunes, Matos, & Coutinho, 2009). The cytokines Il-12 and IL-18 were associated with higher levels of detected NF-kB when they were introduced into *P. berghei* infected mice in coincidence with CpG ODN compared to when they were administered independently and the capacities of these cytokines to induce T cells to express neurokinin 1 receptor (NK-1R; an enhancer of IFN-γ production following engagement with substance) was reported to involved NF-kB activation (Weinstock, Blum A, Metwali, Elliott, & Arsenescu, 2003). Ability of the cytokine-CpG ODN combination to promote increased NF-kB concentrations could provide other various NK-kB related benefits to the immune system.

Nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent 1 (NFATc), a protein that is encoded by the *NFATc1* gene, was significantly higher in the two mice groups that received cytokine-CpG ODN combinations, divulging that cytokine-CpG co-inoculation during murine malaria plays a role in inducible gene transcription during immune responses. The outcomes of increased NFATc in the

context of IL-18 and IL-12 (synergistically acting cytokines) co-inoculated in to mice with CpG motifs could be expected since cytokines such as IL-18 and IL-12 have been previously uncovered as being capable of triggering increases in not only NFATc, but also NF-kB leading to increased IFN-γ production in murine T helper 1 cells (Tominaga *et al.,* 1999). The IL-18 and IL-12 synergistic ramifications of NFATc increase would be expected to occur, given the widely reported concerted operations of these two cytokines in causing IFN-γproduction from T-cells (Tsuji-Takayama *et al.,* 1999), induction of cytokine apoptosis and control of Th1/Th2 cytokine expression. The provoking of increases in NFATc levels in mice that received cytokine-CpG motif co-inoculations could possibly lead to promotion of both innate and adaptive anti-parasitic immune mechanisms that limit the progression of malaria. The ability of this transcription factor (TF) to evoke innate and adaptive (Tsuji-Takayama *et al.,* 1998; Kavitha, 2006) immune mechanisms have been reported. Pathogenic binding to pattern-recognition receptors (PRR), such as TLR4, CD14, and dectin 1, induces the activation of calcineurin/NFAT signaling in myeloid cells such as dendritic cells, leading to molecular pathways involved in host protection.

*Plasmodium berghei*-infected mice that received cytokine-CpG combinations had lower amounts of the FOXP3 (forkhead box P3) protein also referred to as scurfin, which involved in various immune system responses including acting as a master regulator transcription factor in the development and function of regulatory T cells (Zhang, & Zhao 2007). Regulatory T cells generally turn the immune response down, and detection of lower FOX-P3 levels could be indicative of less T-cell suppression in the cytokine-CpG co-inoculation groups leading to more robust early-phase anti-malarial immunity than in control groups of mice. The interferon regulatory factor (IRF), is a member of the IRF family, a group of transcription factors with diverse roles, including virus-mediated activation of interferons, and modulation of cell growth, differentiation, apoptosis, and immune system activities, was detected at higher concentrations in the cytokine-CpG motif co-inoculation groups of mice that in the control groups. In malaria infection, IRF-5 is involved in evoking the production of IFN-β by splenocytes in response to stimulation by AT-rich oligonucleotides that resemble those in the malarial

genome (Sharma *et al.,* 2011; Sin, Carla, Kevin & Laurent, 2014). The occurrence of higher concentrations of IRF-5 in cytokine-CpG co-inoculation groups in coincidence with lower malarial severity indicated likely anti-*Plasmodial* roles in during malaria.

Highest concentrations of the STAT-6 TF were detected in the *P. berghei*-infected control mouse group which also experienced the highest parasitaemia and most severe symptoms. This STAT family TF is phosphorylated by receptor-associated kinases, and then forms homo- or heterodimers that translocate to the cell nucleus where they act as transcriptional activators. This STAT-6 protein plays a central role in exerting IL-4 mediated biological responses. From the seven STAT proteins, STAT-6 is activated by IL-4 and IL-13 and plays a predominant role in the immune system (Sin*et al.,* 2014). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups on this occasion had lower levels of STAT 6 compared to the *P. berghei* control group, thereby suggesting that lower STAT-6 concentrations following cytokine-CpG ODN co-inoculation in malaria could mediate parasitaemia-reduction. This outcome was accompanied by lower disease severity and better outcomes in terms of haematological indices compared to the *P. berghei*-infected control group.

The aryl hydrocarbon receptor (AhR or AHR or ahr or ahR) protein which is encoded by the the AHR gene and is a ligand-activated transcription factor involved in the regulation of biological responses to planar aromatic hydrocarbons (Burbach, Poland & Bradfield, 1992) was detected at significantly higher proportions in malaria-infected mice with cytokine-CpG co-inoculations compared to the controls. It regulates xenobiotic-metabolizing enzymes such as cytochrome P450. The aryl hydrocarbon receptor is a member of the family of basic helix-loop-helix transcription factors and it binds several exogenous ligands such as natural plant flavonoids, polyphenolics and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds. AhR is a cytosolic transcription factor that is normally inactive, bound to several co-chaperones. Upon ligand binding to chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the chaperones dissociate resulting in AhR translocating into the nucleus and dimerizing with ARNT (AhR nuclear translocator), leading to changes in gene transcription.

Lower parasitisation and malarial severity experienced by mice groups that received cytokine-CpG co-inoculations is in tandem with previous reports showing that AhR-knockout (KO) mice infected with *P. berghei* Anka manifested with increased parasitemia, earlier mortality, enhanced leukocyte-endothelial cell interactions in the brain microvasculature, and higher brain inflammation (interleukin-17 [IL-17] and IL-6) and liver (gamma interferon [IFN-γ] and tumor necrosis factor alpha [TNF-α]) compared to infected wild-type (WT) mice (Brant *et al.*, 2014). Conversely, and as expected, higher parasitisation and severity of disease as it happened in the *P. berghei* control group coincided with lower AhR concentrations. The aryl hydrocarbon receptor is required for optimal resistance to *Listeria monocytogenes* infection in mice (Shi *et al.,* 2007).

As with NF-kB, NFATc, FOXP-3 and AhR, the concentrations of the Krüppel-like factor (KLF) transcription factor which are a set of zinc finger DNA-binding proteins that regulate gene expression, were significantly higher in the two main experimental mice groups involving cytokine-CpG combination therapy compared to all control groups. Each KLF family member has a characteristic set of three zinc fingers at its C-terminus. These fingers are related to those of the archetypal *Drosophila melanogaster* regulatory protein Krüppel. Hence the family is named after Kruppel and family members numbered roughly in the order in which they were discovered (Turner & Merlin 1999). In mammals there are 17 genes in the KLF family. In addition, there are also 9 related proteins that form the SP1 subfamily. The transcription factors SP1 to SP9 are similar to the KLFs but their zinc fingers are more in the middle of the protein rather than at the C-terminus (Perkins, Sharpe & Orkin, 1995). The higher levels of KLF-1 detected in the IL-18 and CpG ODN control groups compared to the rest of the control mice groups point to capability of independent IL-18 and CpG ODN in activation higher KLF quantities *in vivo*. Krueppel-like factor 1 is a transcription factor that is necessary for the proper maturation of erythroid (red blood) cells (Crispino, & Weiss 2014). The Krüppel-like factor (KLF) family of transcription factors participates in various processes that include proliferation, differentiation, growth, development, survival, and responses to external stress. Seriously symptomatic and high parasitaemia controls had lower concentrations of KLF-1 compared to the cytokine-CpG recipients in the current study.

### 5.2.2 Expression Physiologic Biomolecules following anti-*P. berghei* Cytokine-CpG Motif Oligodeoxynucleotide Co-inoculation

Generally, the physiologic biomolecules, adiponectin, angiopoietin 1, NRP-1 and COX-2 were demonstrably increased in the cytokine-CpG ODN combination groups, while angiogenin, angiopoietin 2, MMP-8 and MMP-9 were downregulated. Adiponectin, alternatively called APN, GBP-28, apM1, AdipoQ and Acrp30 which is a protein that is involved in regulating glucose levels as well as fatty acid breakdown was noted to be more highly expressed in the cytokine-CpG co-inoculation groups and also in two control groups; the IL-12/ *P. berghei* and CpG ODN groups compared to the rest of the control groups. In studies carried out on *P. falciparum* malaria, it was found that although patients with cerebral malaria had greater amounts of adiponectin those with uncomplicated malaria, patients with *P. falciparum* infection who had higher glucose production also had higher adiponectin levels and given adiponectin's ability to downregulate glucose generation, activation of adiponectin release during infection could be a mechanism of restraining the glucose production stimulating properties of hormones and cytokines acting during infection (Blumer *et al.,* 2005). The secretion of glucose counter-regulatory hormones and cytokines can complicate infections by triggering increases in glucose production. The insulin-sensitising, fat-derived adiponectin hormone such as the one detected in the cytokine-CpG coinoculation groups could play a regulatory role that counteracts the extent of parasite-activated glucose production (Blumer *et al.,* 2005).

Higher levels of adiponectin in the IL-12/ *P. berghei* and CpG ODN groups than in the rest of the controls implicate IL-12 and CpG ODN in a triggering role that leads to a higher presence of adiponectin in respective recipient murine hosts. The lower levels of adiponectin recorded in *P. berghei*-infected mice treated with IL-18 could be expected, according to a previous study (Chendrasekar *et al.,* 2007) in which it was discovered that IL-18 suppresses adiponectin expression in $_3T_3$-L1 Adipocytes via a novel signal transduction pathway involving ERK1/2-dependent NFATc4 phosphorylation.

Quantities of the angiogenin (Ang) protein, also known as ribonuclease 5, a potent stimulator of new blood vessels through the process of angiogenesis, were significantly lower in the cytokine-CpG co-inoculated groups compared to the rest of the groups. Although at the time of this writing there was no other report on the role of angiogenin in malaria infection, Ang a hydrolyzer of cellular RNA (causing protein synthesis modulation), and an actor on DNA causing a promoter-like increase in the expression of rRNA (Gao, & Xu 2008), has been associated with cancer and neurological disease through angiogenesis and through activation of gene expression that suppresses apoptosis (Tello-Montoliu A, Patel & Lip 2006). Thus a higher presence of angiogenin in the excessively parasitized *P. berghei* control mice group may not come as a surprise, also because of the implicated roles of Ang in other disease scenariaos like multiple myeloma (Alexandrakis *et al.,* 2004) and neurodegenerative diseases (Gao *et al.,* 2008). Given the outcome with the *P. berghei*-infected control group of mice, the lower Ang levels in the cytokine-CpG co-inoculated groups could be related to the control of parasitaemia in these two mice groups.

Angiogenin is a key protein implicated in angiogenesis in normal and tumor growth. Angiogenin interacts with endothelial and smooth muscle cells resulting in cell migration, invasion, proliferation and formation of tubular structures (Gao *et al.,* 2008). Lower parasitaemia and both independent cytokine and cytokine-CpG combinations significantly influenced lower Ang concentrations while the absence of therapeutic cytokines (IL-12 and IL-18 in this case) or CpG provided conditions that permitted increased parasitaemia, as noted earlier and higher levels of the Ang protein. Cytokine influences on Ang presence have been reported elsewhere (Verselis, Olson & Fett, 1999).

Angiopoietin 1 (ANGPT 1), a member of the angiopoietin family of vascular growth factors that play a role in embryonic and postnatal angiogenesis were higher in the cytokine-CpG co-inoculation groups that in the rest of the groups, thereby supporting previous evidence that increased levels of ANGPT 1 are associated with uncomplicated malaria (UM [Conroy *et al.,* 2009]). Conversely patients exhibiting complicated malaria, including those with severe (non-cerebral) malaria and cerebral malaria had lower levels of ANGPT 1; the current report has shown that ANGPT 1 levels were lowest in the *P.*

*berghei*-infected control mice group. There was a reduced in ANGPT-1 levels in patients with SM (non-cerebral) versus CM and in participants with severe disease, ANGPT-2, but not ANGPT-1, levels correlated with cumulative organ injury scores; however, ANG-1 correlated with the presence of renal dysfunction and coma (Conroy *et al.*, 2009).

Levels of angiopoietin-2 (ANGPT 2) were significantly higher in the CpG/*P. berghei* and the *P. berghei* control groups compared to the rest of the mice groups, including the main experimental cytokine-CpG co-inoculation groups indicating that the cytokine-CpG therapeutic combination not only limits disease severity, but is also accompanied by increased levels of ANGPT 2 which has been widely associated with severe complicated malaria (Conroy *et al.*, 2009). Several studies have indicated that higher levels of ANGPT 2 coinciding with lower levels of ANGPT 1 that give a higher ANGPT 1/ANGPT 2 ratio are associated with complicated and severe malaria including increased CM and PM severity (Conroy *et al.*, 2009). In the context of PM, ANGPT-2 levels in the placenta, and activates a strong increase in the ANGPT-2/ANGPT-1 ratio in the placenta and the serum. Severe *Plasmodium falciparum* infection PM and LBW have all been connected to higher in ANGPT-2:ANGPT-1 ratio (Gomez *et al.*, 2014). The current report gives a similar link in terms of ANGPT 1/ANGPT 2 ratio which was induced in the *P. berghei*-infected mice that were given cytokine-CpG ODN therapeutic co-inoculations. These mice had a significantly higher ANGPT 1/ANGPT 2 ratio compared to the heavily parasitised and severely symptomatic *P. berghei* control mice group in the, a scenario which has been shown to significantly support disease control and limitation of *Plasmodial* severity. Conversely, the CpG/*P. berghei* and the *P. berghei* control groups had a lower ANGPT 1/ANGPT 2 ratio, as a result of the relatively higher concentrations of ANGPT 2 and higher ANGPT 1 concentrations than ANGPT 2 that were detected in these two groups, and indeed this status coincided with highly severely outcomes, both in terms of parasitaemia and symptomatic manifestations.

Neuropilin-1 (NRP1), a membrane-bound co-receptor to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF; MIM 192240) and semaphorin (see SEMA3A; MIM 603961) family members and was found to be significantly increased in murine recipients of cytokine-CpG co-inoculations than control groups in the study.

Although up to the time of this publication there has been no direct indication of the role of NRP-1 in *Plasmodium* infection, NRP-1 has been implicated in versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion (Soker, Takashima, Miao, Neufeld, & Klagsbrun 1998). In the current experiments, lower NRP-1 concentrations were accompanied by higher *P. berghei* parasitaemia and increased severity of malaria while lower higher NRP-1 values accompanied lower parasitaemia and milder malaria, suggesting a disease limiting role of NRP-1 in the context of malaria. In immunity, NRP-1 is important in the formation of successful synapse between dendritic cells (DCs) and T cells, and it was identified as biomarker that provides distinction between naturally occuring Treg cells originating in the thymus and peripheral adaptive Treg cells (Campos-Mora, Morales, Gajardo, Catalán, & Pino-Lagos, 2013).

Cyclooxygenase (COX), also referred to as prostaglandin-endoperoxide synthase (PTGS), which is an enzyme (EC1.14.99.1) that is responsible for formation of prostanoids, including prostaglandins such as prostacyclin and thromboxane occurred at higher concentrations in the mice groups with cytokine-CpG ODN co-administration than in the controls agreeing with other data suggesting that the induction of COX-2 expression and prostaglandin synthesis has a protective effect against malarial conditions including CM (Ball, MacDougall, McGregor, & Hunt, 2006). The neutrophil collagenase, also known as matrix metalloproteinase-8 (MMP-8) or PMNL collagenase (MNL-CL), a collagen-cleaving enzyme which is present in the connective tissue of most mammals, was detected at higher concentrations in the CpG/*P. berghei* and untreated *P. berghei* control groups than in the rest of the mice groups, signifying that elevated MMP-8 levels were correlated with increased parasitaemia and severe malaria (Dietmann *et al.*, 2008), as both these mice groups experienced significantly higher parasitaemia and symptomatic malaria than their cytokine-CpG-receiving counterparts. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases; other family members are adamalysins, serralysins, and astacins. The MMPs belong to a larger family of proteases known as the metzincin superfamily (Van Lint, & Libert 2007). Collectively, these matrixin enzymes are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules. They are known to

be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine inactivation (Van Lint *et al.,* 2007). MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense and MMP 8 has been linked to reduced malarial severity (Dietmann *et al.,* 2008). Increased levels of both MMP-8 mRNAs and MMP-9+ cells were detected in severe *P. berghei* ANKA malaria in the murine model (Van Den Steen *et al.,* 2006), further agreeing with our findings of elevated MMP-8 in the CpG/*P. berghei* and untreated *P. berghei* control groups and downregulated MMP-8 concentrations in the cytokine-CpG combination groups which also had lower parasitaemia and less symptomatic outcomes.

The CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei*; IL-18/ *P. berghei*; IL-12/ *P. berghei* mice groups which all had less parasitaemia and milder malaria outcomes in comparison to the CpG/*P. berghei* and untreated *P. berghei* control groups reflected collectively had significantly downregulated MMP-8 and MMP-9 levels than the later groups further indicating that cytokine and CpG-elicited reduction in MMP-8 was associated with lower infection and parasitaemia characteristics. Previous reports have shown that increases in tissue inhibitors of metalloproteinase-1 (TIMP-1) and MMP-8 levels were associated with severe *P. falciparum* malaria outcomes TIMP-1 levels were increased in patients with severe malaria, compared with those in patients with uncomplicated malaria Raised TIMP-1 levels were strongly correlated with malarial severity, as determined by the simplified multiorgan dysfunction score (Dietmann *et al.,* 2008). Current findings of reduced MMP-9 levels in mice groups that were given the IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* co-inoculations support the previous findings that that MMP-9 levels were strongly upregulated in C57BL/6 mice brain infected with *P. berghei* ANKA, which is a murine model of CM swith similar characteristics to human CM. Upregulated MMP-9 amounts were selective for the central nervous system, where they were linked to the vasculature and parenchyma while immunohistochemistry procedures revealed that higher amounts of MMP-9 were released by monocytic lineage cells (CD11b+ [Van Den Steen *et al.,* 2006]).

## 5.3 Chemokine Expression following anti-malarial Cytokine-CpG Motif Oligodeoxynucleotide Co-administration

Overall, the IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* main experimental groups had upregulated amounts of CCL-2, CCL-5, CXCL-1, CXCL-12, CX3CL-1 and proliferation SI and depressed levels of CCL-3, CXCL-5, CXCL-10 and CXCL-16 than the other mice groups. The C-X-C motif chemokine 10 (CXCL10) also known as Interferon gamma-induced protein 10 (IP-10) or small-inducible cytokine B10, an 8.7 kDa protein, and a small cytokine belonging to the CXC chemokine family was detected at significantly higher concentrations in the CpG/*P. berghei* and untreated *P. berghei* control groups than the rest of the mice groups, including the cytokine-CpG ODN combination recipients, further confirming that CXCL10 promotes adverse malaria outcomes in increased parasitisation and severe complications like CM (Wilson *et al.,* 2011). This report reveals that cytokine-CpG co-inoculation in *P. berghei* infected mice, is accompanied reduced plasma CXCL-10 levels leading to reduced parasitisation and milder malarial manifestations. In support of these findings, it was previouisly shown that pharmacologic inhibition of CXCL10 in combination with anti-malarial therapy reduces severity and eliminates mortality associated with murine model of cerebral malaria (Wilson *et al.,* 2013).

The chemokine (C-C motif) ligand 2 (CCL2) which is also referred to as monocyte chemotactic protein 1 (MCP1) and is a small inducible cytokine A2 that belongs to the CC chemokine family, was notably increased in the CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei* groups compared to the rest of the mice groups in the study, indicating a significant protective role of elevated CCL-2 in this context, given these two groups had the lowest parasitaemia and less symptomatic outcomes. Infection of knockout mice with *Plasmodia* showed critical roles of MCP-1 and IL-10 in parasitemia control and host mortality (Pattaradilokrat *et al.,* 2014). The CCL2 chemokine recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection. CCL2 is a monomeric polypeptide, with a molecular weight of approximately 13 kDa. CCL2 is anchored in the plasma membrane of endothelial cells by glycosaminoglycan side chains

of proteoglycans. CCL2 is primarily secreted by monocytes, macrophages and dendritic cells. Platelet derived growth factor is a major inducer of CCL2 gene. To become activated CCL2 protein has to be cleaved by metalloproteinase MMP-12. CCR2 and CCR4 are two cell surface receptors that bind CCL2 (Craig, & Loberg 2006). Control mice groups exhibiting increased parasitaemia including the CpG/*P. berghei* and untreated *P. berghei* control groups and more adverse symptoms also had significantly reduced CCL-2 quantities suggesting a CCL-2 role in increased susceptibility.

Levels of the CCL-5 chemokine, also known as RANTES (regulated on activation, normal T cell expressed and secreted), were upregulated in the *P. berghei*-infected murine recipients of the parasite-limiting cytokine-CpG co-inoculation. The higher CCL-5 levels in these mice groups were thus accompanied by lower parasitaemia and uncomplicated malaria with milder symptoms. The suppression of RANTES in children with *Plasmodium falciparum* malaria was previously described and it was suggested that thrombocytopenia cold reduce RANTES leading to suppression of erythropoiesis in children with malarial anemia and conversely, increased RANTES levels were linked to reduced malarial complications (Were *et al.*, 2006).

The Chemokine (C-X-C motif) ligand 16 (CXCL16), which is also a small cytokine belonging to the CXC chemokine family and is larger than other chemokines (with 254 amino acids), is composed of a CXC chemokine domain, a mucin-like stalk, a transmembrane domain and a cytoplasmic tail containing a potential tyrosine phosphorylation site that may bind SH2 (Matloubian, David, Engel, Ryan, & Cyster 2000). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* main experimental groups expressed the lowest amounts of this chemokine compared to the rest of the mice groups in the study and these reduced levels were therefore associated with lower parasitaemia and milder malarial outcomes. Parasite-diminishing reductions in CXCL-16 were induced in *P. berghei*-infected BALB/c mice that received therapeutic cytokine-CpG ODN co-inoculations. On the contrary, mice groups that had higher parasitaemia and severe malarial outcomes were found to have upregulated CXCL-16 concentrations.

The Fractalkine cytokine protein, also known as chemokine (C-X3-C motif) ligand 1 (CX3CL1), a large 373 amino acid protein, that contains multiple domains and is the only known member of the $CX_3C$ chemokine family was upregulated in the/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei* groups indicating that the low parasitaemia-associated cytokine-CpG combination was accompanied by increased $CX_3C$ chemokine quantities. In other studies, mature types of *P. falciparum*-infected RBCs were demonstrated to adhere to membrane-bound $CX_3CL1$/fract-alkine, which is located on vascular endothelium (Hatabu, Kawazu, Aikawa, & Kano 2003). This was suggested as a new mechanism through which the *Plasmodium*is able to sequester in blood vessels, especially during CM. The current study on the other hand reveals that free increased CX3CL1 in plasma are associated with reduced parasitaemia and lower clinical severity in murine recipients of cytokine-CpG ODN therapeutic co-administration.

The two cytokine-CpG-co-administered groups manifested with higher CXCL-1 chemokine levels than the rest of the mice groups, showing inductivity if this chemokine by the cytokine-CpG ODN combination and an association between increased levels in the CXCL-1 and antimalarial protection in this scenario. The molecules CXCL2, IL-6, CCL20, CXCL1, and IL-8, were upregulated up to more than 100-fold, thus indicating an important role of blood-brain barrier (BBB) endothelium in the innate protection during *P. falciparum*–infected erythrocyte (Pf-IRBC) sequestration (Tripathi *et al.*, 2009). Current data also demonstrates that separate introduction of the cytokines IL-18 and IL-12 into *P. berghei*-infected BALB/c mice stimulates increases in CXCL-1 and that are accompanied by relatively increased protection in comparison to highly parasitized control mice groups that included the untreated *P. berghei*-infected group that also had lowest CXCL-1 concentrations.

The control mice groups that displayed increased parasitaemia and which also had more adverse malarial symptoms including the CpG/*P. berghei* and untreated *P. berghei* control groups manifested with the highest CXCL-5/ENA-78 chemokine quantities suggesting a CXCL-5 parasitaemia and malaria disease-promoting role. Similarly, studies based on *P. falciparum* infections in Cameroonian women have revealed that depressed serum CXCL5 and raised CCL28 levels occured in malarial positives when compared

with healthy controls (Che, Nmorsi, Nkot, Isaac, & Okonkwo, 2015), and the mean concentration of CXCL11 was higher in symptomatic than asymptomatic group, while CCL28 was lower in symptomatic individuals. The CXCL-5 levels in murine recipients of the cytokine-CpG therapeutic co-administration had the lowest detected levels amongst all mice groups, further supporting the opinion that CXCL-5 reduction is associated with reduced parasitaemia and mild/uncomplicated malaria.

Levels of the chemokine protein called the stromal cell-derived factor 1 (SDF-1) also known as C-X-C motif chemokine ligand 12 (CXCL12), were recorded highest in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups showing that the cytokine-CpG co-inoculation, which is associated with low parasitaemia, also caused upregulation of SDF-1 in the murine hosts. The Malaria-diminishing properties of CXCL-12 were demonstrated in experiments in which supplementation of CXCL12 (SDF-1) was proven to induce homing of CD11c+ dendritic cells to the spleen thereby promoting control of *Plasmodium berghei* malaria in BALB/c mice (GarniGarnica, de Moraes, Rizzo, & de Andrade, 2005). Downregulated expression of CXCL-12 in control mice groups, including the untreated *P. berghei*-infected group were associated with increased parasitaemia more symptomatic/problematic malarial outcomes. This could have arisen as a result of lack of the various potential benefits that CXCL-12 could offer including increased chemoattraction of lymphocytes (Ara *et al.,* 2003).

In the current experiments, the highest CCL-3 concentrations were recorded in the high parasitaemia-experiencing control groups of mice including the untreated *P. berghei*-infected group, reflecting that absence of the cytokine-CpG combination being investigated allowed increases in CCL-3, which promotes *P. berghei* infectivity, hence the resultant high parasitaemia and accompanying adverse outcomes reported herein. In experiments by Belnoue *et al.*, (2003), the brains of wild-type mice with CM exhibited increases in CCR5 leukocytes, particularly CCR5+ CD8+ T cells, and also increases in T-helper 1 (Th1) cytokine production. Although CM effects of high CCL-3 were not analysed in the current experiments, the ability of high peripheral CCL-3 to encourage increased parasitaemia was observed from the higher parasitaemia levels detected in the

control mice groups (these had high CCL-3 levels) as opposed to the mice groups that were given cytokine-CpG co-inoculations.

Higher SI levels, indicating stronger proliferation responses were recorded from the two cytokine-CpG co-inoculation groups compared to all controls, indicating that the IL-12/CpG and IL-18/CpG combinations promoted the ability of splenocytes in the *P. berghei*-infected BALB/c hosts to recall and proliferate against challenging malarial antigens. In contrast, highly parasitized control mice groups, especially the untreated *P. berghei*-infected mice group had the lowest SI levels reflecting downregulated anti-malarial recall proliferation, and low murine anti-*P. berghei* recall proliferation responses have been linked with adverse malarial outcomes like CM and increased CM pathogenesis (Nie, Bernar, Schofield, Hansen 2007).

## 5.4 Induction of anti-*Plasmodial* Immunity via Cytokine-CpG Motif Oligodeoxynucleotide Co-inoculation

In the IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* main experimental groups increased levels of IFN-γ, TNF-α, IL-17, IL-23a and IgM and reduced levels of IL-4, and IL-10 prevailed. Variations in human cytokine responses and their connection to malaria outcomes remain an intensively debated area of immunology. Levels of IFN-γ were significantly higher in the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* mice groups than the rest of the mice groups, reflecting both the widely reported protective nature of this cytokine against *P. berghei* and other malarial parasites and its inductivity by IL-12 and IL-18. These two cytokine-CpG co-inoculated groups were also the ones that had lowest total and differential parasitaemia, lowest peak parasitaemia and least symptomatic malaria, and therefore cytokine-CpG-mediated protection against *P. berghei* in the murine host is indeed accompanied by upregulation of IFN-γ. Interferon gamma (IFNγ) is a dimerized soluble cytokine that is the only member of the type II class of interferons (Gray & Goeddel 1982). Gamma interferon production is critical for protective immunity to infection with blood-stage *Plasmodium berghei* XAT but neither NO production nor NK cell activation is critical (Yoneto *et al.,* 1999). In their experiments, Yoneto *et al.,* (1999) showed that neither NO production nor NK cell activation is critical in protection against *P. berghei* XAT infection and that IFN-gamma plays an crucial role in the elimination of malarial parasites, possibly by the enhancement of phagocytic activity of macrophages. In the current experiments, control mice groups that did not receive the cytokine-CpG combination, suffered increased parasitaemia and more symptomatic outcomes, illustrating, as expected, a link between low levels of IFN-γ and reduced anti-*P. berghei* protection. Furthermore, introduction of IL-18 and IL-12 without any accompanying CpG ODN also resulted in increased IFN-γ production and protection in comparison to control mice that did were not treated with any of these.

TNF-α concentrations of were highest in the two cytokine-CpG ODN co-inoculation groups than in the controls, indicating a protective role in this context. The IL-12/CpG and IL-18/CpG combinations not only induced increased anti-*Plasmodial* protection in BALB/c mice, but also triggered significant upregulation of TNF-α in these two mice

groups. Increased TNF-α levels have previously been linked to protection against *P. berghei* (Mugweru *et al.,* 2013) and also against *P. chabaudi* in experiments in which IL-12-induced protection against blood-stage *Plasmodiumchabaudi* AS was shown to require IFN-gamma and TNF-alpha and it occurs via a nitric oxide-dependent mechanisms (Stevenson MM, Tam, Wolf & Sher 1995). Significantly higher levels of IFN-gamma and TNF-alpha were found in the sera of IL-12-treated mice, which correlated with high levels of the nitric oxide (NO) metabolite, NO3-. Furthermore, CD4+T cell depletion was found to abrogate IL-12-induced resistance. Administration of neutralizing mAb against IFN-gamma or TNF-alpha to IL-12-treated mice showed that simultaneous depletion of both cytokines resulted in 100% mortality (Stevenson *et al.,* 1995). The control mice groups CpG/*P. berghei* and untreated *P. berghei* which had higher parasitaemia than the cytokine-CpG co-inoculation groups, coincidentally had significantly lower TNF-α levels than the cytokine-CpG co-inoculation groups, enhancing the opinion, from these studies that, TNF-α aids in anti-*P. berghei* protection in BALB/c mice. It has also been communicated that TNF-α has an inhibitory effect on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations (Depinay *et al.,* 2011).

Current study outcomes showed that cytokine-CpG ODN co-inoculation, which significantly reduced malarial severity, was accompanied by downregulated IL-4 concentrations, further supporting the opinion that low levels of IL-4 mediate anti-*P. berghei* protection (Saeftel *et al.,* 2004). Mice deficient in interleukin-4 (IL-4) or IL-4 receptor alpha have higher resistance to sporozoite infection with *Plasmodium berghei* (ANKA) than do naive wild-type mice (Saeftel *et al.,* 2004). IFN-gamma-producing NK cells are critical in host resistance against the sporozoite liver stage by inducing NO production, an effective killing effector molecule against *Plasmodium.* The absence of IL-4-mediated functions increases the protective innate immune mechanism which results in immunity against *P. berghei* infection in these mice, with no major role for IL-13 (Saeftel *et al.,* 2004). Untreated *P. berghei* control mice and other control mice groups that had increased parasitaemia, simultaneously expressed higher IL-4 levels than the cytokine-CpG ODN-treated mice, showing the malaria disease-promoting role of high

IL-4 concentrations (White, Jarboe & Krzych, 1994); no IL-4 was detected in splenic cultures from mice during protective immunity.

The cytokine-CpG ODN co-inoculation groups, which significantly reduced malarial severity, was also experienced increased IgM levels compared to the controls. Immunoglobulin M antibodies have been demonstrated to be protective against *P. berghei* malaria (Racine, McLaughlin, Jones, Witmer, McNamara, Woodland, & Winslow, 2011). Immunoglobulin M, or IgM for short, is a basic antibody that is produced by B cells. IgM is by far the physically largest antibody in the human circulatory system. It is the first antibody to appear in response to initial exposure to an antigen. The spleen, where plasmablasts responsible for antibody production reside, is the major site of specific IgM production (Racine *et al.*, 2011). IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure. IgM antibodies do not pass across the human placenta (only isotype IgG). These two biological properties of IgM make it useful in the diagnosis of infectious diseases. Demonstrating IgM antibodies in a patient's serum indicates recent infection, or in a neonate's serum indicates intrauterine infection (e.g. congenital rubella). The development of anti-donor IgM after organ transplantation is not associated with graft rejection but it may have a protective effect (McAlister *et al.*, 2004). The control mice groups in our current experiments, including the highly parasitized untreated *P. berghei*-infected group expressed lower concentrations of IgM compared to their cytokine-CpG-receiving counterparts, indicating that their reduced IgM levels rendered them more susceptible to *P. berghei* malaria.

Amounts of IL-23a, were significantly increased in the cytokine-CpG co-inoculated mice groups compared to the controls, and these were accompanied by reduced parasitaemia and milder malarial clinical symptoms. This cytokine has recently been implicated in the induction of increased protection against *P. berghei* in the murin model (Ishida *et al.*, 2013). Mice deficient in IL-23 (p19KO) had higher parasitemia and died earlier than wild-type (WT) controls. The p19KO mice had lower numbers of IL-17-producing splenic cells than their WT counterparts (Ishida *et al.*, 2013). IL-23 is produced by dendritic cells and macrophages. Interleukin-23 is a heterodimeric cytokine composed

of an IL-12p40 subunit that is shared with IL-12 and the IL-23p19 subunit. A functional receptor for IL-23 (the IL-23 receptor) has been identified and is composed of IL-12R β1 and IL-23R (Oppmann *et al.,* 2001). In the current report, the IL-23a potential anti-*P. berghei* effects are clearly evidenced by the occurrence of increased parasitaemia in the untreated *P. berghei* infected mice group (and in other control groups) under conditions of reduced IL-23a, which can otherwise be reversed via simultaneous introduction of cytokine/CpG inoculum.

Murine recipients that received cytokine-CpG co-inoculations expressed the lowest IL-10 levels, and these were accompanied by reduced parasitaemia and disease severity and high IL-10 concentrations have been associated with increased virulence of murine and human malaria and vice versa. Increased levels of IL-10 and TNF in plasma have been detected in young African children with malarial anemia and high levels of parasitemia (Gopinathan & Subramanian, 1986; Ouma *et al.;* 2008). In common IL-10 promoter variants, the -1082A/-819T/-592A (ATA) haplotype has been associated with increased susceptibility to severe anemia. Current findings indicate that cytokine/CpG-initiated IL-10 downregulation causes parasitaemia reduction and reduced malarial severity in the BALB/c-*P. berghei* murine malaria model.

The cytokine-CpG co-inoculation groups expressed higher concentrations of IL-17 than the controls, thereby accompanying milder malarial outcomes and low parasitaemia. Previous research has shown that IL-23 protection against *Plasmodium berghei* infection in mice is partially dependent on IL-17 from macrophages (Ishida *et al.*, 2013). IL-17 was discovered to be crucial for maintenance of splenic macrophages. Adoptive transfer of macrophages into macrophage-depleted mice revealed that macrophage-derived IL-17 is required for macrophage accumulation and parasite eradication in the recipient mice. Furthermore, IL-17 induced CCL2/7, which recruit macrophages; IL-23, IL-17, and macrophages reduce the severity of infection with blood-stage malaria parasites (Ishida *et al.*, 2013). To elicit its functions, IL-17 binds to a type I cell surface receptor called IL-17R of which there are at least three variants IL17RA, IL17RB, and IL17RC (Starnes, Broxmeyer, Robertson, & Hromas, 2002). Our current findings show that in the absence of cytokine-CpG co-inoculation, relatively lower IL-17 concentrations prevail, and these

are accompanied by increased parasitisation and severe clinical manifestations. The colour matrices (Section 5.5 below) simplify the interrelationships between various analysed factors and parasitaemia and infection severity.

### 5.5 Colour Matrices for interrelationships between Various Analysed Factors and Parasitaemia and Infection Severity

#### 5.5.1 Relationship between TFs and Parasitaemia Profiles and Infection Severity

| | IL18/CpG/P | IL12/CpG/P | IL18 /PB | IL12/PB | CpG/PB | *P. Berghei* | CpG | Uninfecte |
|---|---|---|---|---|---|---|---|---|
| **Parasitaemia** | 5.727 | 5.455 | 9.455 | 10.64 | 15.73 | 25.34 | 0 | 0.0 |
| **Clinical Severity** | ++ | ++ | +++ | +++ | +++++ | +++++++ | + | - |
| NF-kB | 221.9 | 141.0 | 67.51 | 58.58 | 85.00 | 41.06 | 63.60 | 30.00 |
| NFATc | 4.229 | 4.700 | 0.9573 | 2.858 | 0.6340 | 0.6961 | 2.441 | 0.5214 |
| FOXP3 | 0.6757 | 0.7086 | 1.491 | 14.91 | 14.90 | 17.07 | 1.464 | 5.171 |
| IRF-5 | 5.743 | 4.357 | 1.601 | 2.774 | 1.041 | 0.7617 | 1.193 | 1.859 |
| STAT-6 | 3.676 | 1.321 | 0.7957 | 6.543 | 0.9743 | 7.886 | 3.386 | 0.7000 |
| AhR | 6.381 | 9.114 | 2.301 | 2.551 | 2.079 | 0.7157 | 3.649 | 1.692 |
| KLF | 66.30 | 51.16 | 24.94 | 7.771 | 4.657 | 0.6543 | 18.69 | 9.186 |

- Higher Extreme Value
- Medium Lower Value
- Medium Lower Value
- Lower Extreme Value

**Colour Coding**

**How to read these colour matrices:** If for example, parasitaemia in a given column is in lower extreme ( ), while a molecule you are relatiing with is in higher extreme level ( ), then -- denotes an inverse relationship (negative correlation); the higher the molecules' concentration, the lower the parasitaemia. Having -- would mean a positive correlation in the downward direction. Similar deductions can be made between or among the molecules themselves at all concentration levels in different groups and rows.

### 5.5.2 Relationship between Physiologic Biomolecules and Parasitaemia Profiles and Infection Severity

| | IL18/CpG/P | IL12/CpG/P | IL18 /PB | IL12/PB | CpG/PB | *P. Berghei* | CpG | Uninfecte |
|---|---|---|---|---|---|---|---|---|
| **Parasitaemia** | 5.727 | 5.455 | 9.455 | 10.64 | 15.73 | 25.34 | 0 | 0.0 |
| **Clinical Severity** | ++ | ++ | +++ | +++ | +++++ | +++++++ | + | - |
| AdipoQ | 12.24 | 9.743 | 3.600 | 10.49 | 4.814 | 3.714 | 10.36 | 4.271 |
| ANG | 64.20 | 51.50 | 94.03 | 84.21 | 276.7 | 715.0 | 190.3 | 166.9 |
| ANGPT 1 | 15.74 | 17.57 | 9.400 | 8.343 | 7.486 | 1.769 | 6.143 | 6.457 |
| ANGPT 2 | 1.644 | 3.743 | 1.326 | 3.329 | 14.56 | 16.31 | 1.386 | 1.274 |
| NRP-1 | 12.66 | 14.30 | 3.086 | 14.17 | 2.214 | 2.003 | 3.086 | 4.143 |
| COX-2 | 139.6 | 129.9 | 39.04 | 100.4 | 39.56 | 27.41 | 47.67 | 57.60 |
| MMP-8 | 64.07 | 46.29 | 45.31 | 26.31 | 298.0 | 296.9 | 58.97 | 56.89 |
| MMP-9 | 227.2 | 215.6 | 277.0 | 198.9 | 402.9 | 591.0 | 261.9 | 265.7 |

### 5.5.3 Relations in Chemokine Expression, Splenocyte Recall Proliferation, Parasitaemia Profiles and Infection Severity

| | IL18/CpG/P | IL12/CpG/P | IL18 /PB | IL12/PB | CpG/PB | *P. Berghei* | CpG | Uninfecte |
|---|---|---|---|---|---|---|---|---|
| **Parasitaemia** | 5.727 | 5.455 | 9.455 | 10.64 | 15.73 | 25.34 | 0 | 0.0 |
| **Clinical Severity** | ++ | ++ | +++ | +++ | +++++ | +++++++ | + | - |
| CXCL-10 | 216.1 | 66.71 | 101.9 | 57.70 | 491.3 | 602.5 | 96.06 | 179.1 |
| CCL-2 | 790.0 | 776.2 | 374.6 | 262.6 | 149.9 | 66.49 | 218.4 | 166.6 |
| CCL-5 | 139.9 | 143.1 | 38.89 | 88.83 | 48.39 | 40.76 | 75.87 | 64.84 |
| CXCL-16 | 163.3 | 126.5 | 208.0 | 293.3 | 624.9 | 604.4 | 120.8 | 160.2 |
| CX3CL-1 | 659.4 | 517.7 | 201.1 | 151.9 | 222.5 | 96.14 | 151.8 | 111.9 |
| CXCL-1 | 551.5 | 462.8 | 223.7 | 262.6 | 141.1 | 76.03 | 137.7 | 105.9 |
| CXCL-5 | 92.10 | 86.34 | 200.3 | 176.1 | 465.1 | 628.8 | 183.1 | 13.7 |
| CXCL-12 | 509.7 | 729.45 | 270.4 | 407.1 | 160.9 | 142.3 | 228.7 | 242.2 |
| CCL-3 | 41.76 | 37.10 | 66.59 | 66.99 | 117.5 | 122.10 | 55.34 | 58.61 |
| Proliferation SI | 7.657 | 8.376 | 3.857 | 3.214 | 4.514 | 3.414 | 2.829 | 2.571 |

### 5.5.4 Relationship between Cytokine Concentrations, IgM Titers, Parasitaemia Profiles and Infection Severity

| | IL18/CpG/P | IL12/CpG/P | IL18 /PB | IL12/PB | CpG/PB | *P. Berghei* | CpG | Uninfecte |
|---|---|---|---|---|---|---|---|---|
| **Parasitaemia** | 5.727 | 5.455 | 9.455 | 10.64 | 15.73 | 25.34 | 0 | 0.0 |
| **Clinical Severity** | ++ | ++ | +++ | +++ | +++++ | +++++++ | + | - |
| IFN-γ | 1625 | 1427 | 990.8 | 1279 | 499.7 | 230.0 | 567.3 | 260.3 |
| TNF-α | 448.0 | 436.5 | 184.3 | 303.5 | 160.3 | 176.4 | 400.8 | 88.76 |
| IL-4 | 62.99 | 54.76 | 131.5 | 141.2 | 79.71 | 164.4 | 87.77 | 48.39 |
| IgM | 4.186 | 5.200 | 2.743 | 4.600 | 2.029 | 1.804 | 1.966 | 1.514 |
| IL-23a | 165.6 | 176.7 | 90.70 | 92.09 | 48.30 | 19.87 | 76.40 | 47.27 |
| IL-10 | 133.0 | 103.6 | 183.5 | 180.3 | 240.6 | 324.5 | 111.3 | 95.63 |
| IL-17 | 420.5 | 367.5 | 103.6 | 189.2 | 184.4 | 42.94 | 122.7 | 114.9 |

## 5.6 Conclusions

One of the major revelations from this project was that cytokine-CpG ODN co-inoculation in the BALB/c-*P. berghei* ANKA drastically reduce parasitaemia progression such immunotherapeutic interventions mediate milder malarial clinical and haematological outcomes. Not only does the cytokine-CpG ODN combinational therapy suppress total *Plasmodial* parasitaemia but it also significantly causes declined differential parasitaemia, causes lower peak parasitaemia, and less dramatic total and differential parasitaemia trends. Co-injection of both the inflammasome complex products IL-18 and IL-12 with CpG ODNs also strongly reduces severe symptoms of malaria and leads to less haematological damage. Cytokine-CpG ODN co-injection impeded hair ruffling, appetite loss, skin turgor reduction, limb paralysis, convulsions and declines in RBCs, RBC indices, and WBCs.

It was also resolved from these investigations that the co-introduction of cytokines with CpG ODNs during malaria triggers upregulation of the transcription factors (TF) NF-kB, NFATc-1, IRF-5, AhR and KLF and downregulation of the FOXP3 and STAT-6 TF. In tandem with other reported findings, these outcomes were associated with less symptomatic malarial severity, reduced parasitisation, les severe haematological implications. Conversely, downregulated expression of the NF-kB, NFATc-1, IRF-5, AhR and KLF TFs were linked to increased predisposition to problematic malaria accompanied by adverse clinical-hematological and parasitological manifestations and this also applied to increases in FOXP3 and STAT-6 expression levels. Intermediate expression levels of these TFs were associated with moderate outcomes, respectively. Physiologic biomolecular analysis indicated that adiponectin, ANGPT1, NRP-1 and Cox-2 concentrations upregulated in malaria-infected cytokine-CpG ODN recipients while angiogenin, ANGPT2, MMP-8 and MMP-9 levels were downregulated thereby being associated with reduced parasitaemia, mild malaria and less mild haematological outcomes. Likewise reductions in adiponectin, ANGPT1, NRP-1 and Cox-2 and increases in angiogenin, ANGPT2, MMP-8 and MMP-9 that prevailed in the absence of the cytokine-CpG ODN simultaneous bitherapeutic intervention were associated with elevated parasitisation, severely symptomatic malaria and haematological damage.

This project uncovered that cytokine-CpG ODNs combinational immunotherapy in the context of malaria activates elevated quantities of the chemokines CCL-2, CCL-5, CXCL-1, CXCL-12 and CX3CL-1 while at the same time it causes decreases in levels of CCL-3, CXCL-5, CXCL-10, CXCL-16 and splenocyte recall proliferation SI. Contrastingly, conditions of depressed CCL-2, CCL-5, CXCL-1, CXCL-12 and CX3CL-1 and simultaneously increased CCL-3, CXCL-5, CXCL-10, CXCL-16 and splenocyte recall proliferation SI, which occurred in non-cytokine-CpG-treated malaria-infected hosts were linked to extreme parasitisation, symptomatic complications and adverse malaria-related haematological states. Cytokine-CpG ODN bitherapeutic co-injection during malaria has also been unraveled as an initiator of increased expression of the cytokines IFN-γ, TNF-α, IL-17, IL-23a and IgM antibodies, and it leads to simultaneous declines in the amounts of IL-4, and IL-10; these were associated with low *Plasmodia* parasitisation, mild malaria and less haematological damage. Moreover, in malaria infected murine hosts that do not receive cytokine-CpG ODN co-immunotherapy expression of IFN-γ, TNF-α, IL-17, IL-23a and IgM becomes reduced while IL-4, and IL-10 levels are elevated, conditions that were linked to intensive *Plasmodial* parasitisation and severely symptomatic malaria.

Inferential decision on the null hypothesis: A decision was reached to reject the null hypothesis that stated that there are no differences in clinical, haematological, parasitological, transcription factor, physiologic biomolecular, and immunological manifestations between BALB/c mice with cytokine-CpG motif ODN co-inoculation and BALB/c mice without cytokine-CpG motif ODN co-inoculation in the context of *P. berghei* ANKA strain malaria infection. Elaborate findings communicated herein demonstrated that indeed there are significant differences in clinical, haematological, parasitological, transcription factor, physiologic biomolecular, and immunological manifestations between BALB/c mice with cytokine-CpG motif ODN co-inoculation and BALB/c mice without cytokine-CpG motif ODN co-inoculation in the context of *P. berghei* ANKA strain malaria infection.

## 5.7 Recommendations

The investigators involved in the current project recommend that further research can be carried out in order to determine whether cytokine-CpG ODN bi-therapeutic modalities can mediate protection similar to the kind reported herein, in alternative malaria-infected hosts including other murine and rodent malaria models and non-human primate (NHP) models of malaria such as the highly popular baboon-*P. knowlesi* model of malaria infection. Similarly, the co-administration of this highly immunopotentiating combination can be investigated for its reproducibility against other murine malaria strains including *P. yoelii*, *P. chabaudi chabaudi* and *P. vinckei*. Current findings largely focused on blood-borne responses to cytokine-CpG ODN co-inoculations. However, in future malaria-infected hosts can be investigated further following cytokine-CpG ODN biotherapies on whether tissue specific malarial complications including hepato-splenic damage, cerebral malaria (CM) and placental malaria (PM) can be countered by such interventions.

Researchers can also investigate the combination of CpG ODNs with physiologic biomolecules, cytokines and nanoscale factors for their efficacies not only against *Plasmodia*, but also against othermicrobial agents including infectious diseases like *Schistosoma*, various *Leishmania* species, numerous bacterial, fungal, viral and parasitological agents. Investigations can also be done to determine whether the immunomodulatory cytokine/biomolecular-CpG ODN combination can impact upon non-communicable conditions such as diabetes, neoplasms, cardiac ailments among many others. Molecular DNA and RNA level responses to cytokine-CpG ODN co-injection can be analysed e.g. via PCR, FACS scan or hybridization techniques to further elucidate the potential of such interventions in alleviating numerous various conditions.

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# APPENDICES

## Appendix I: Buffers and Reagents

**Carbonate-Bicarbonate buffer (pH 9.6)**

| | |
|---|---|
| $Na_2CO_3$ | 1.59g |
| NaH $CO_3$ | 2.93g |
| $NaN_3$ | 0.2g |

Make up to 1 litre with distilled water, store at 4°C for not more than 2 weeks.

**Phosphate Buffered Saline (pH 7.2-7.4)**

| | |
|---|---|
| NaCl | 8.0g |
| $KH_2PO_4$ | 0.2g |
| $Na_2HPO_4.12H_2O$ | 2.9g |
| KCl | 0.2g |
| $NaN_3$ | 0.2g |

Make up to 1 litre with distilled water, store at 4°C

**0.05% Tween 20 in PBS (pH 7.2-7.4)**

| | |
|---|---|
| NaCl | 8.0g |
| $KH_2PO_4$ | 0.2g |
| $Na_2HPO_4.12H_2O$ | 2.9g |
| KCl | 0.2g |
| $NaN_3$ | 0.2g |
| Tween 20 | 0.5 ml |

Make up to 1 litre with distilled water, store at room temperature.

**Giemsa Stain**

| | |
|---|---|
| Giemsa stain | 4 ml |
| Methanol | 4 ml |
| Double distilled water | 92 ml |

Store at room temperature

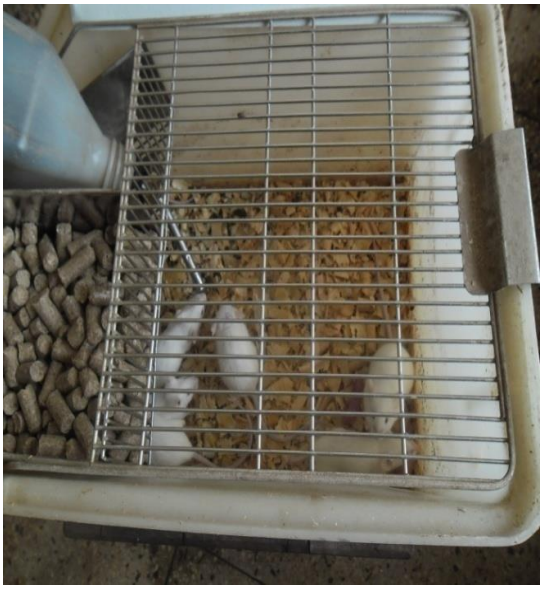
**Alsevers Solution**

| | |
|---|---|
| Dextrose | 10.25 g |
| NaCl | 2.1g |
| Trisodium citrate | 4.0 g |
| Double distilled water | 500 ml |

Sterilise by filtering through 0.2 µm pore size filter, store at 4°C

**Appendix II: Project Pictures Showing Experimental BALB/c in Cages**

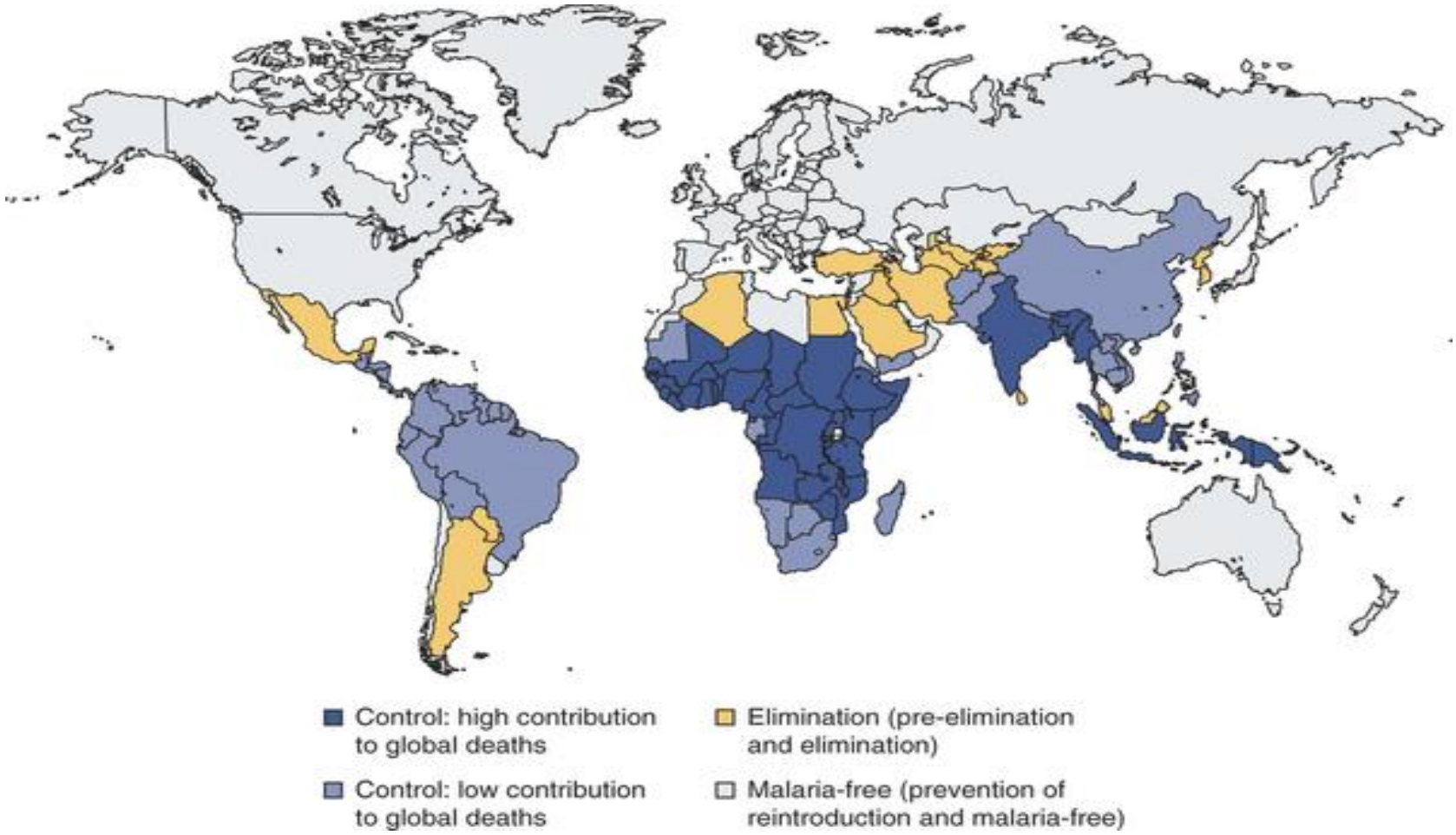

**A** **B**



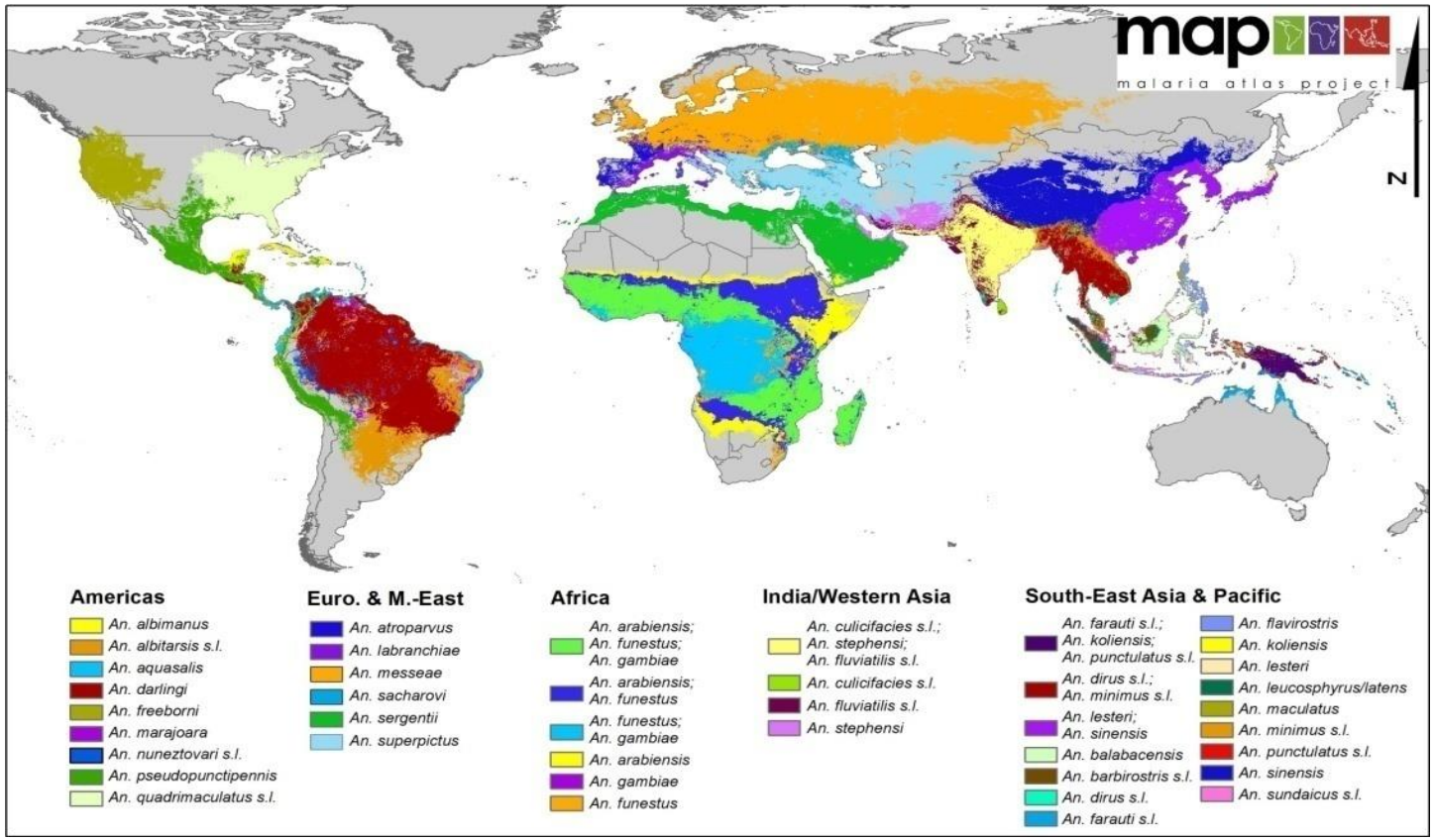
**C** **D**

**A.** The IL-18/CpG/*P. berghei* group being transferred into the infection room. **B.** The IL-12/CpG/*P. berghei* group being transferred back into the animal house after infection. **C.** and **D.** IL-12/*P. berghei* and CpG ODN groups being monitored for clinical manifestations.

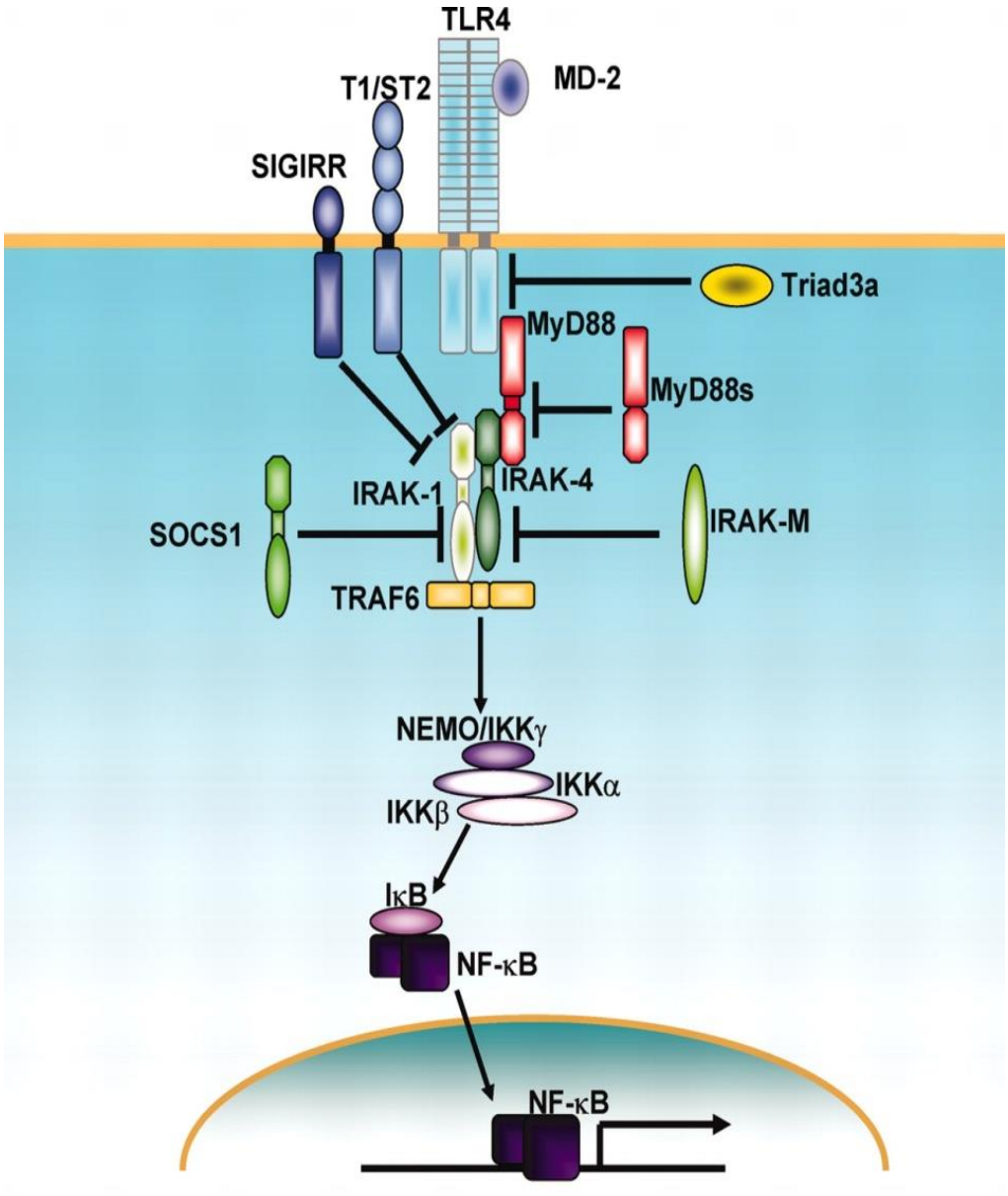
**Appendix III: Global Distribution of Malaria**



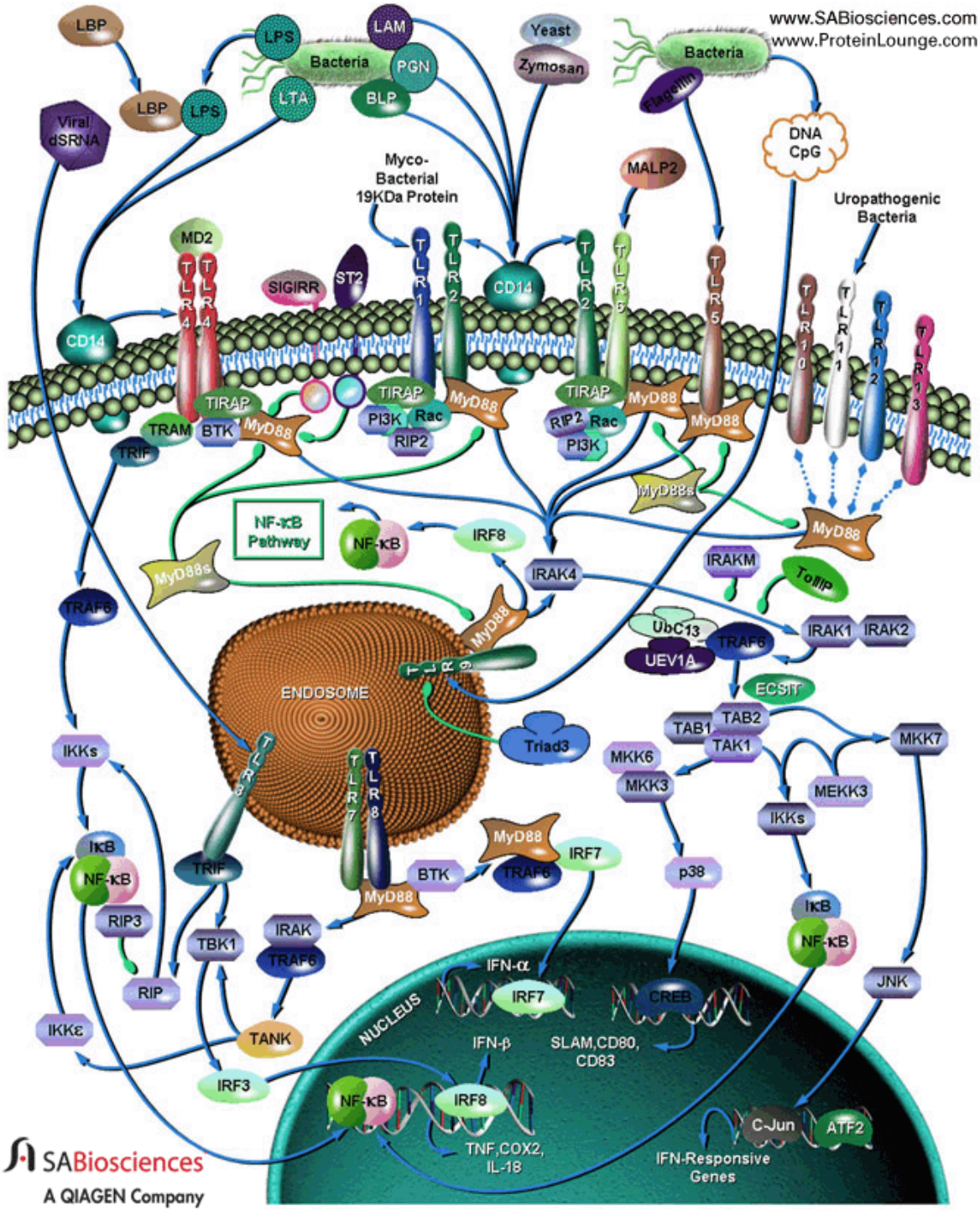
## Appendix IV: Global Distribution of the Dominant Vector Species of Malaria



**Appendix V: Toll Like Receptor Signaling Pathways**

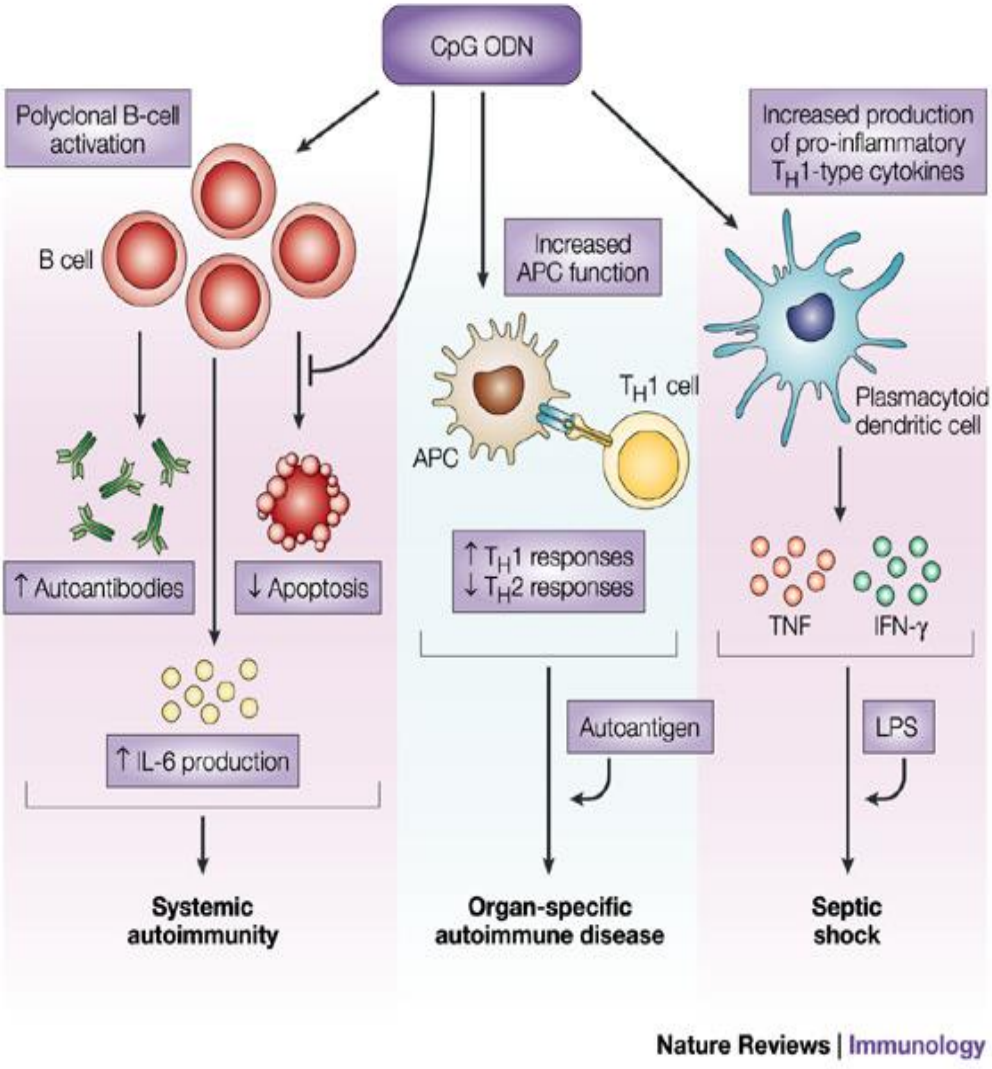


## Appendix VI: Cascades in Direct and Indirect TLR activation by CpG ODN

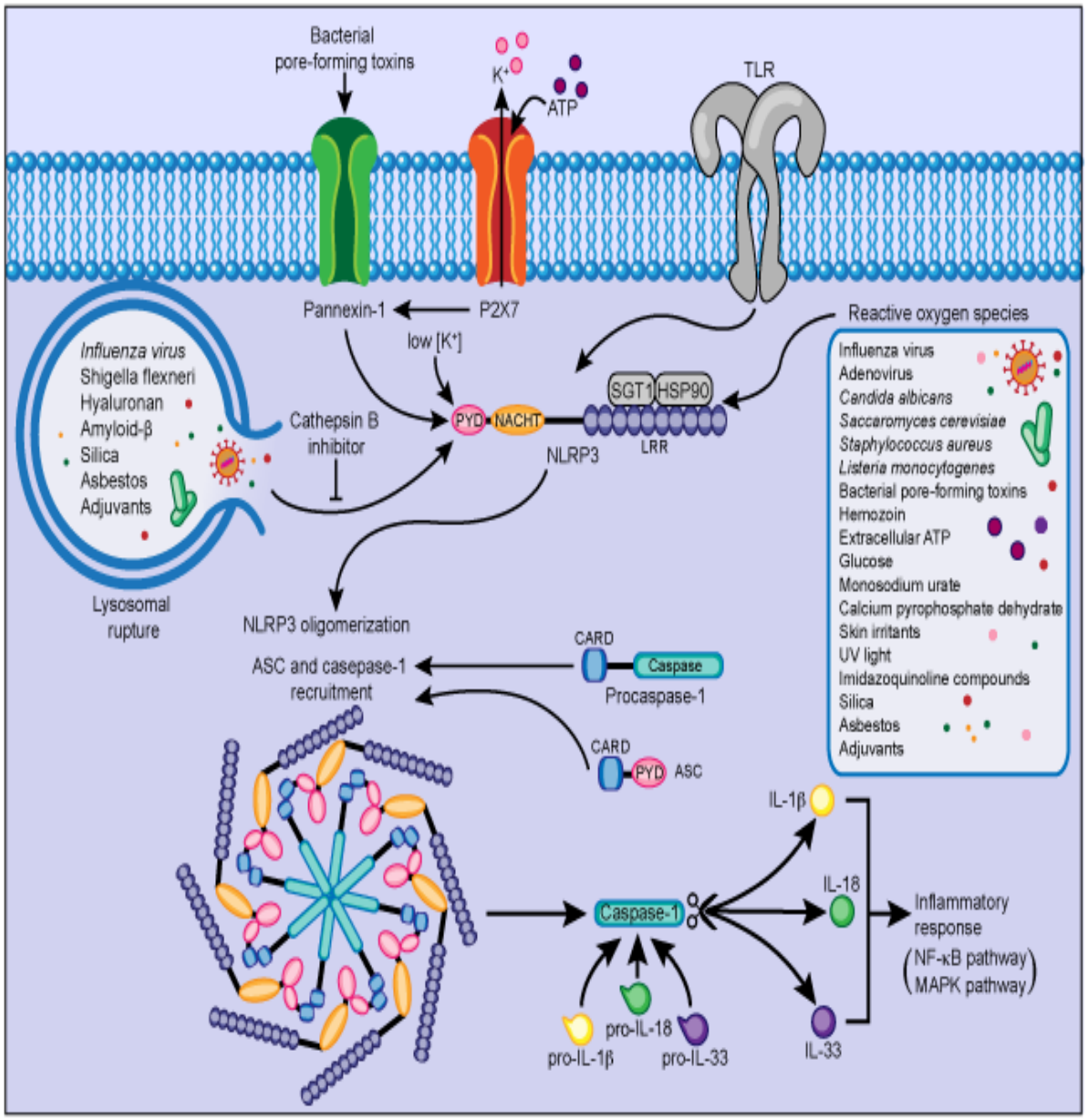


## Appendix VII: Cascades in NF-kB-mediated Signaling

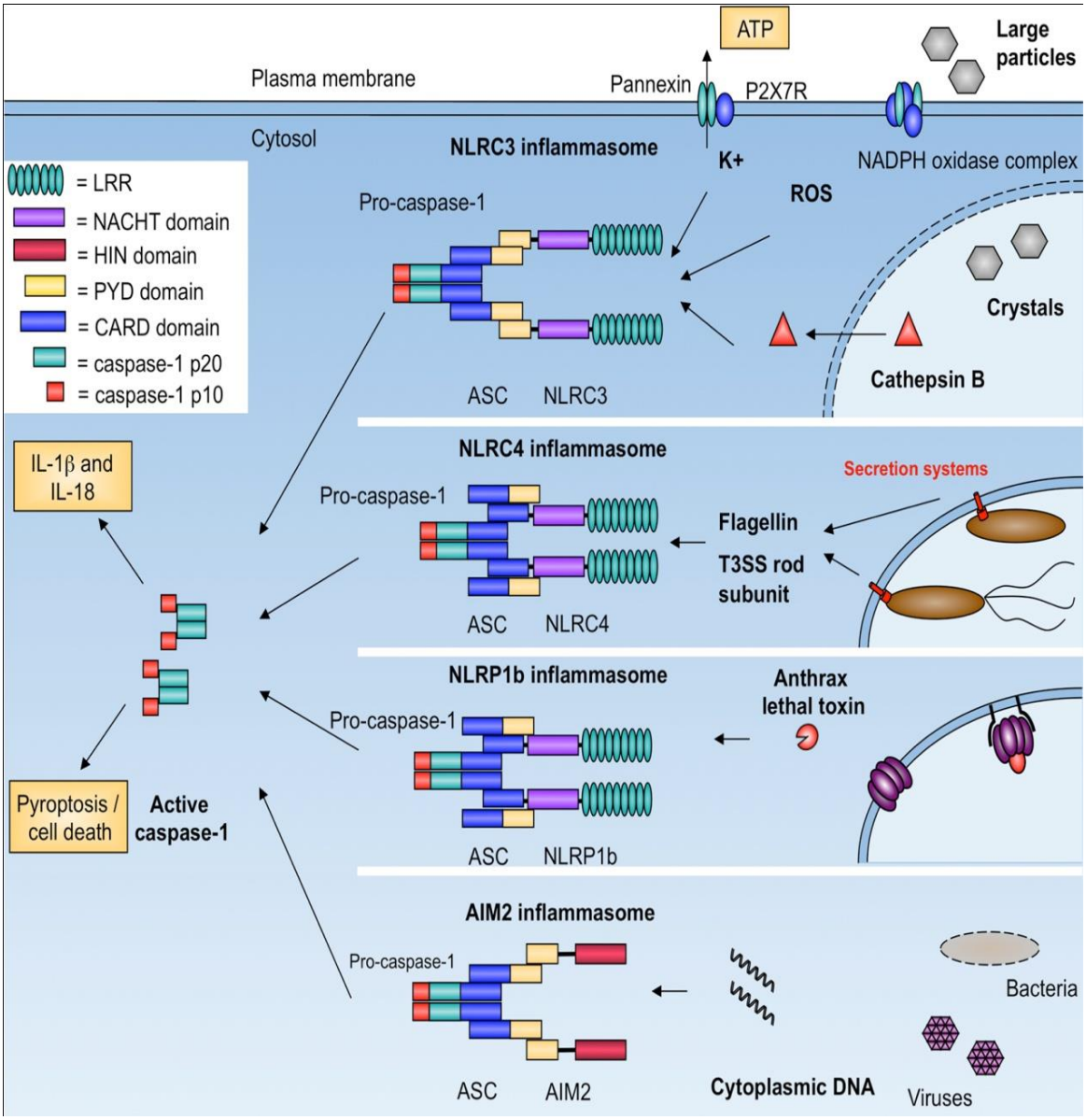
**Appendix VIII: Summary of Further Possibilities that Accompany the Use of CpG motif Oligodeoxynucleotides**



## Appendix IX: Inflammasome Signaling via Caspase and NLRP-Mediated Activities



## Appendix X: Inflammasome Activation Pathways via NLRs



**Appendix XI: Project Approval Documentation from KEMRI**

# KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/SSC/102653 9th April, 2014

Mustafa Barasa

Thro'
Director, CBRD
NAIROBI

REF: SSC No. 2793 (New) – Cytokine-CpG Motif ligodeoxynucleotide Co-inoculation in Balb/c Mice Infected with *Plasmodium berghei* ANKA strain

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 213rd meeting held on 8th April, 2014 has since been approved for implementation by the SSC.

**Kindly submit 4 copies of the new protocol to SSC within 2 weeks from the date of this letter i.e., 23rd April, 2014.**

We advise that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

# KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

**KEMRI/ACUC/ 01.09.14** **24th September, 2014**

Mustafa Barasa,
P. O. Box 2003 - 50100.
KAKAMEGA. KENYA

Mustafa,

**RE: Animal use approval for SSC 2793 - "Cytokine-Cpg Motif Oligodeoxynucleotide Co-Inoculation in Balb/C Mice Infected With *Plasmodium Berghei* Anka Strain" protocol**

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of two years starting from when the final ethical approval will be obtained. The committee expects you to adhere to all the animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 24 SEP 2014 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

# KENYA MEDICAL RESEARCH INSTITUTE

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**KEMRI/RES/7/3/1** **October 27, 2014**

**TO:** **MUSTAFA BARASA,**
**PRINCIPAL INVESTIGATOR**

**THROUGH:** **DR. KIMANI GACHUHI,**
**THE DIRECTOR, CBRD,**
**NAIROBI**

16/1/15

Dear Sir,

**RE: SSC PROTOCOL NO. 2793 (*RESUBMISSION*): CYTOKINE-CPG MOTIF OLIGODEOXYRIBONUCLEOTIDE CO-INOCULATION IN BALB/C MICE INFECTED WITH PLASMODIUM BERGHEI ANKA STRAIN.**

Reference is made to your letter dated October 21, 2014. The ERC Secretariat acknowledges receipt of the revised proposal on October 22, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised by the expedited review team have been adequately addressed.
The study is granted approval for implementation effective this **October 27, 2014**. Please note that authorization to conduct this study will automatically expire on **October 26, 2015.**

If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **September 15, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

**PROF. ELIZABETH BUKUSI,**
**ACTING SECRETARY,**
**KEMRI/ETHICS REVIEW COMMITTEE**

## Appendix XII: Publications

### A. Articles Currently Published Online (Year 2015):

1. Cytokine-CpG Motif Oligodeoxynucleotide Co-Inoculation in BALB/c Mice Infected with *Plasmodium berghei* ANKA Strain - *Journal of Medical and Biological Science Research,* Vol. 1 (10), pp. 145-161, 2015.
2. Expression of physiologic biomolecules following anti-malarial cytokine-CpG motif oligodeoxynucleotide gene therapy - *Merit Research Journal of Medicine and Medical Sciences,* Vol. 3(9) pp. 432-452, 2015.

MERIT RESEARCH JOURNALS
www.meritresearchjournals.org

Merit Research Journal of Medicine and Medical Sciences (ISSN: 2354-323X) Vol. 3(9) pp. 432-452, September, 2015
Available online http://www.meritresearchjournals.org/mms/index.htm


*Original Research Article*

# Expression of Physiologic Biomolecules following Anti-Malarial Cytokine-CpG Motif Oligodeoxynucleotide Gene Therapy

**Barasa Mustafa[1,2,3*], Shaviya Nathan[3], Wamalwa Ronald[3], Mambo Fidelis[3], Okoth Patrick[4], Omedo Asaba Robin[3], Reuben Rutto[2], Kagasi Ambogo Esther[5], Mutai Charles[3], Sowayi Alubokho George[3], Ozwara Suba Hastings[5], Ng'ang'a Waithera Zipporah[1] and Anjili Omukhango Christopher[2]**

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**Abstract**

**According to latest estimates, there were 198 million cases of malaria worldwide in 2013, with 82% of these cases occurring in Africa. There were approximately 584,000 malaria deaths worldwide. The outcome of infection with *Plasmodia* parasites is determined by the activities of various biomolecules, cytokines and other host-specific factors. *Plasmodia* parasites evade immunity and modulate immune systems to their advantage thereby exacerbating infection and disease. When combined in therapy, immunostimulatory unmethylated CpG motif oligodeoxynucleotides (ODNs) synergise with cytokines in the promotion of anti-parasitic mechanisms providing effective protection in various cases. The CpG ODNs enhance immune activities through ligation to plasmacytoid dendritic cell (pDC) Toll-like receptors (TLRs) such as TLR-9 and they activate both pDCs and B-cells, while cytokines modulate cellular behaviour. By altering cellular functionality, the cytokine-CpG ODN immunotherapy combination can alter the expression of physiologic factors and this can influence disease outcomes including severity of infection with malaria parasites. It was previously unclear how physiologic biomolecules like matrix metalloproteinases (MMPs) and angiopoietins (ANGPTs) could be influenced by the coincidental introduction of recombinant cytokines and CpG ODNs during malaria. This project studied cytokine-CpG motif ODN co-inoculation in BALB/c mice infected with *P. berghei* ANKA strain. Two BALB/c mice groups infected with virulent blood stage *P. berghei* ANKA strain parasites were given immunotherapeutic cytokine and CpG ODN combinations for five consecutive days while six other control groups with different treatments were included for comparison. The mice were monitored daily for clinical symptoms and parasitaemia development from day 1 postinfection. At ten days postinfection, all mice were humanely sacrificed for the extraction of EDTA-treated blood and plasma for measuring various physiologic factors. Results unraveled cytokine-CpG-based gene therapy as an enhancer of anti-*Plasmodial* activities accompanied by elevations in adiponectin, ANGPT1, neuropilin-1(NRP-1) and cyclooxygenase-2 (Cox-2) and delevations in ANGPT2, MMP-8 and MMP-9. These physiologic outcomes, which are largely agreeable with data from other studies, favour further investigations on combinatorial cytokine-CpG ODN gene therapy for potential inclusion into preventative and therapeutic anti-malarial interventions.**

**Key Words:** BALB/c Mice, Cytokines, CpG Motif ODN, Malaria, *P. berghei* ANKA, Physiologic Biomolecules.

## INTRODUCTION

There were about 219 million cases of malaria in 2010 accompanied by 660 000 malaria-related deaths. Africa is the most affected continent: about 90% of all malaria deaths occur there and malaria remains inextricably linked with poverty (Keating, 2012). According to the latest approximations, released in December 2014, there

Journal of Medical and Biological Science Research
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ISSN: 2449-1810
Research Paper
http://pearlresearchjournals.org/journals/jmbsr/index.html

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

**Barasa Mustafa[1,2,3*], Ndeti Mula Cosmas[1,2], Wanjala Christine Ludwin[3,4], Ingonga Mwihwa Johnstone[2], Osero Bernard[2], Sarah Shinoko Muyonga[3], Wanyonyi Khakasa Mable[5], Frankline Tireito[6], Drinold Mbete[6], Kokonya Apollo Donald[7], Ozwara Suba Hastings[8], Zipporah Waithera Ng'ang'a[1], and Anjili Omukhango Christopher[2]**

Accepted 29 October, 2015

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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