Effect of Chemokine Adjuvants on Safety and Immunogenicity of Serine Repeat Antigen (SERA) DNA Malaria Vaccine Candidate in Olive Baboons (*Papio anubis*)

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2012

DECLARATION

I, Onkoba Wycliffe Nyamongo, do hereby declare that this is my original work and it has not been presented for an award of a degree in any other university.

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DEDICATION

I dedicate this work to all those who think malaria is a global burden, my little jewel, Zendai Moti, my lovely wife Zubeda Shamasdin and my father, friend and hero Bernard Tinega Onkoba.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based combination therapy
ADCI	Antibody-Dependent Cellular Inhibition
ALAT	Alanine aminotransferase
AMA	Apical Membrane Antigen
ANDi	African Network for Drugs and Diagnostics Innovation
ANOVA	Analysis of Variance
AS02A	GlaxoSmithKline Adjuvant System
ASAT	Aspartate aminotransferase
BC	Before Christ
CCL20	C-C Chemokine Ligand 20
CFA	Complete Freud's' Adjuvant
CK-MB	Creatinine Phosphokinase
CO_2	Carbon dioxide
CPM	Cells per Minute
DNA	Deoxyribonucleic Acid

EBA	Erythrocyte-Binding Antigen
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Expanded Programmes of Immunizations
FACS	Fluorescence Activated Cell Analysis
FITC	Fluorescein-isothiocyanate
GC-rich	Guanine-Cytosine rich motifs
GDP	Gross Domestic Product
GLURP	Glutamate-Rich protein
GMP	Good Manufacturing Practice
HIV	Human Immunodeficiency Virus
ID	Identity
IgG	Immunoglobulin G
IM	Intramuscular
IRC	Institutional Review Committee

IRS	Insecticide Residual Spraying
ITN	Insecticide Treated Nets
MSP	Merozoite Surface Protein
NaCl	Sodium Chloride
NHP	Non-human primate
N_2	Nitrogen
O_2	Oxygen
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PE	Phycoerythyrin
PerCP	Peridinin-chlorophyll
PH	Potenz of Hydrogen
RANTES	Regulated upon Activation Normal T cell Expressed and presumably Secreted or also known as CCL5
RAP	Rhoptry Associated Protein

RBC	Red Blood Cell
RESA	Ring Infected Erythrocyte Surface Antigen
RNA	Ribonucleic Acid
RPMI 1640	Roswell Park Memorial Institute 1640 media
RTS, S	Malaria Antigen Vaccine Candidate
SEM	Standard Error of Mean
SERA	Serine Repeat Antigen
SERP	Serine Rich Protein
TA-rich	Thymine-Adenine rich motifs
TE	Tris EDTA buffer
TRAP	Thrombospondin Related Anonymous Protein
USD	United States Dollar
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

Malaria as a disease is a major cause of human death in tropical countries. More than 300 million clinical cases with between one and three million deaths per year are attributed to malaria. Efforts to control this disease are by treatment of infected persons as well as physical or chemical strategies to control the mosquito vector. A safe and effective blood stage malaria vaccine could be a novel weapon in ameliorating the impact of malaria in endemic areas and reinforce hope for malaria eradication. Despite the identification of a number of candidate *Plasmodium falciparum* antigens, serine repeat antigen (SERA) being one such promising target antigen. The realization of an effective vaccine has been complicated due to lack of an appropriate adjuvant for human use. This study evaluated the effect of chemokine adjuvants CCL5 and CCL20 on tolerability, safety, immunogenicity of SERA DNA malaria vaccine constructs and cross protective efficacy against *Plasmodium knowlesi* H strain in the olive baboon *(Papio anubis)* model of malaria. Nine male malaria naïve olive baboons were randomly allocated into three experimental groups of three animals each depending on vaccine regimens (SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone alone). All baboons were immunized intramuscularly with a total medium dose of 1mg/ml of respective vaccines at 0-, 28-, and 56-days schedule. On day 84 all baboons were challenged with *P. knowlesi* H strain blood stage parasites to determine vaccine cross protective efficacy. There were no abnormal changes in animal health status, haematological, kidney and liver function parameters. Immunization site local reactogenicity did not show any vaccine related adverse reactions. Analysis of cellular responses showed that the vaccines were immunogenic with a significant increase in T cell responses. Humoral immune responses showed an increase in anti SERA5 IgG titres throughout the vaccination phase. All experimental baboons developed patent parasitaemia showing that there was no vaccine efficacy as measured by delay in time to parasitaemia and development of clinical malaria disease. SERA DNA vaccine constructs co-expressed with either CCL5 or CCL20 as chemokine adjuvants are safe, well tolerated, and immunogenic with no vaccine related serious adverse events in olive baboons. However, the vaccine did not show cross protection efficacy against *P. knowlesi in vivo.* The current research results provide an initial preclinical validation of CCL5 and CCL20 as immunomodulatory chemokine adjuvants for malaria blood stage vaccines. In addition, provide validated preclinical evidence that the olive baboon can be utilised as an experimental model for evaluating safety and immunogenicity of malaria blood stage vaccines.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Malaria

Malaria is a deadly mosquito-borne infectious disease caused by *Plasmodium spp.* that was first clinically documented by Hippocrates between 470 BC to 370 BC (Hippocrates, 1923). Malaria is still amongst the important parasitic causes of death and suffering today. As far as malaria is concerned, the world can be divided into those regions that are malarious and those that are not, and from the point of view of both public health and economic development, these regions often resemble separate worlds (Malaney *et al.*, 2004). Although malaria burden is not evenly distributed, observations indicate that areas where malaria prospers most, human societies have prospered least (Sachs and Malaney, 2002).

Malaria can impose great economic costs on the communities that it impacts. It affects economic development through changes in household behaviour in response to the disease. This may result in broad social cost influenced by factors such as schooling, demography, migration and saving. Macroeconomic costs such as the impact of malaria on trade, tourism and foreign investment may arise as a result of the pandemic nature of the disease (Sachs and Malaney, 2002).

According to the World Health Organization (WHO) malaria report 2009, approximately 300 million people were infected with malaria in 2008 in 109 countries, 45 of which are in Sub-Saharan Africa with direct economic burden calculated at $12 billion per annum, or 1.3% of GDP growth. Where malaria is highly endemic, adults tend to develop partial immunity to the symptoms of the disease. However, young children bear the burden in terms of morbidity and mortality. The overall impact of malaria on human capital development in children remains largely unexplored and unquantified. In Kenya, an estimated 11% of school days are lost by primary school children every year, while secondary school students miss up to 4.3% school days because of malaria (Sachs and Malaney, 2002). Other studies have reported up to 50% of medically related school absenteeism to malaria (Sachs and Malaney, 2002). In addition, malaria affects the cognitive development and learning ability since children with

malaria have a poorer nutritional status than non-malarial children, an outcome that can impair brain development (Malaney *et al.*, 2004). Long term demographic impacts of malaria affect saving rates. The direct cost of prevention (buying of mosquito nets, insecticides and mosquito coils) and treatment of the disease eat into the disposable income of poor families, as do the cost of lost productivity (Sachs and Malaney, 1993).

Malaria distribution is based on vector distribution and climatic factors such as temperature, humidity and rainfall. Malaria is mainly transmitted in tropical and subtropical areas where *Anopheles* mosquitoes can survive and multiply (Figure 1.1) with the highest transmission found in sub-Saharan Africa. In cooler regions, transmission is less intense and more seasonal. In such climatic areas, *Plasmodium vivax* might be more prevalent because it is more tolerant of lower ambient temperatures (World Health Organization, 2009; Waters *et al.*, 1991).

Plasmodium falciparum is found in tropical and subtropical areas (Figure 1.1). On the other hand *Plasmodium vivax* is found mostly in Asia, Latin America and in some parts of Africa. While *Plasmodium ovale* is found mostly in Africa (especially West Africa) and the Islands of the Western Pacific (World Health Organization, 2009; Waters *et al.*, 1991).

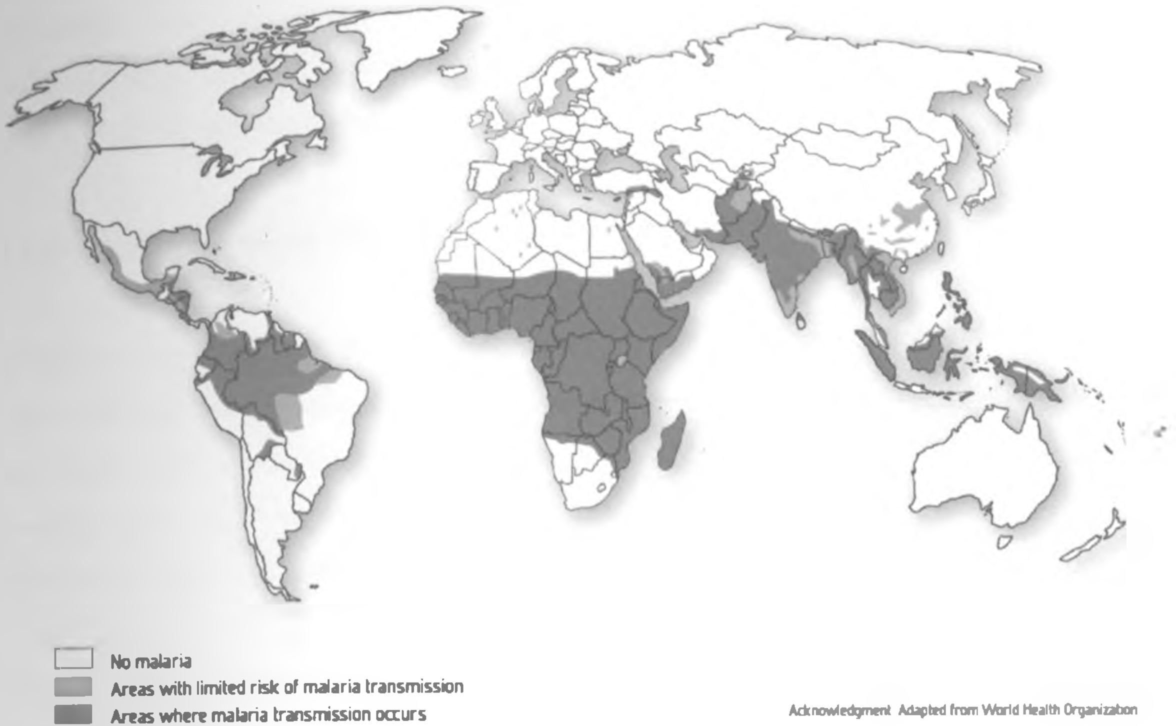

Figure 1.1: Global distribution of malaria transmission risk (World Health Organization, 2009).

1.2 Malaria Parasites

Malaria is transmitted by female *Anopheline* mosquitoes *Anopheles gambiae* and *Anopheles funestus* which are the main vectors of the disease in Africa. Human malaria is caused by *Plasmodium* protozoa. Five species are involved *P. vivax, P. falciparum, P. malariae, P. ovale* (Duffy *et al.*, 1998) and *P. knowlesi* (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008). The disease-causing organisms all belong to the genus *Plasmodium,* a group of parasites that infect vertebrates ranging from ancestral reptiles to man and other primates. Amongst the five malarial species infecting man the most pathogenic is *P. falciparum,* a parasite that differs from most other *Plasmodium spp.* in the regulation of its sexual phase of development. The second most important human pathogen is *P. vivax,* which (together with

P. ovale and *P. cynomolgi*) has a dormant phase in the liver (the hypnozoite) that creates significant challenges in attempts to eradicate the disease(s) because it persists as a metabolically down regulated cell and is therefore difficult to target directly with drugs or possibly vaccines. Other species naturally infecting man include *P. malariae* and the zoonotic infection *P. knowlesi* (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008).

1.3 Life cycle of Human Malaria Parasites, *Plasmodium falciparum*

The life cycle of the malaria parasite is complex (Figure 1.2). Sporozoites (1) are transmitted to the vertebrate host by the bite of an infected female mosquito of the genus *Anopheles*. Once in the blood circulation, they enter human liver cells (2) where they develop into the pre-erythrocytic (exo-erythrocytic) schizonts (3). The period of pre-erythrocytic schizont development varies from one species of parasite to another. Some species like *P. vivax*, *P. ovale* and *P. cynomolgi* have a dominant stage called the hypnozoite stage where the parasite remains dormant in the liver for weeks or even years before they develop into pre-erythrocytic schizonts. The pre-erythrocytic schizonts usually contain millions of merozoites which are released into the blood circulation where they invade the red blood cells. Merozoites develop within the erythrocytes through ring, trophozoite and schizont stages (erythrocytic schizogony). Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite (http://www3.niaid.nih.gov/topics/Malaria/lifecycle.htm). A small portion of the parasites differentiates from newly invaded merozoites into sexual forms (4), which are macrogametocyte (female) and microgametocyte (male). The mosquito ingests the gametocytes during blood feeding. In early mosquito stage, mature macrogametocytes, taken into the midgut of the *Anopheles* mosquito (5), escapes from the erythrocyte to form macrogametes while the microgametocytes exflagellate to form motile microgametes after a few minutes in the mosquito midgut. When the microgamete fertilizes a macrogamete, a zygote is formed which further develops into an ookinete eventually into an oocyst in the late mosquito stages (6). After infection, depending on the *Plasmodium* species and ambient temperature, thousands of sporozoites are released and they invade the salivary gland

epithelium. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again (Silvie *et al.*, 2008).

In primates, malaria infection life cycle and developmental stages of *P. knowlesi* are similar to *P. falciparum* except the course of infection which is a 24 h asexual- blood stage (Garnham, 1966) which is dependent on the host. In its natural host, the long-tailed macaque, infection results in prolonged low-level parasitaemia, whereas in rhesus monkeys and *Papio anubis* parasitaemia rise rapidly and the infection is lethal (Garnham, 1966; Eyles *et al.*, 1962c, Coatney *et al.*, 1971, Ozwara *et al.*, 2003).

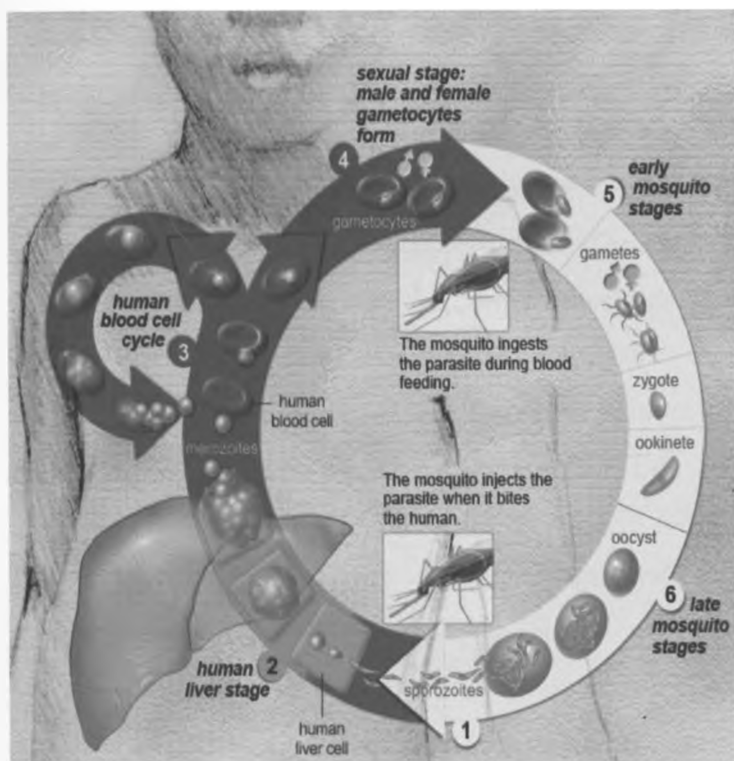

Figure 1.2: Life cycle of *Plasmodium falciparum* (http://www.niaid.nih.gov/topics/malaria/pages/lifecycle.aspx)

1.4 Animal Models of *Plasmodium knowlesi*

The natural vertebrate hosts for *P. knowlesi* are *Macaca fascularis* and *M. nemestrina*. Experimental infection with *P. knowlesi* to study the *in vivo* host parasite interaction has been described in a number of non-human primates such as *M. mulatta* (Coatney *et al.*, 1971), *M. radiate* (Dutta *et al.*, 1982), *M. assamensis* (Dutta *et al.*, 1978), *Presbytis entellus* (Dutta *et al.*, 1981), *Callithrix jacchus* (Langhorne and Cohen, 1979) *Aotus trivigatus* (Garnham, 1966), *Saimiri sciureus* (Collins *et al.*, 1978) and *Papio anubis* (Ozwara *et al.*, 2003; Ozwara *et al.*, 2005). Non-human primates are the only animal models susceptible to the human disease under study because of its similarities in biological mechanisms in humans (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008).

In most experimental models, *P. knowlesi* infection is acute, whereas in their natural *host M. fascicularis*, it generally induces a chronic infection (Coatney *et al.*, 1971; Butcher, 1996). It was shown that the course of *P. knowlesi* infection can differ in *M. fascicularis* from different geographical origins (Schmidt *et al.*, 1977). Since *P. knowlesi* is now considered as the fifth malaria parasite affecting humans (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008). In humans it causes mild infection seldom exceeding 1% parasitaemia. The availability of natural and experimental hosts for *P. knowlesi* offers the possibility to study the biology of malaria parasite and its antigens in a natural host-parasite combination and in hosts whose systems are predictable of the human situations (King, 1988). In addition, the dichotomous disease profile of *P. knowlesi* (a chronic infection in natural hosts and an acute disease in artificial hosts) provide opportunities for understanding the mechanisms of immunity to malaria (Butcher, 1996).

1.5 *Plasmodium knowlesi* as Model Parasite for Malaria Research

Plasmodium knowlesi a parasite that shares many of the vaccine candidate molecules with *P. vivax* has been instrumental in the discovery and characterisation of various blood stage vaccine molecules (Ozwara, 2005). 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. The parasites cannot be maintained in convenient small laboratory animals. As a result, numerous species of rodent (*P. berghei*) and avian (*P. gallinaceum*) malaria parasites have been widely used as laboratory models to study the biology of *Plasmodia* (Langhorne *et al.*, 2011). Although there is proximal phylogenetic relationship between *P. falciparum* and avian *Plasmodia* such as *P. gallinaceum*, differences in their life cycles, insect hosts and in the immune systems of their vertebrate hosts limits their usefulness as models for human malaria (Langhorne *et al.*, 2011).

In biomedical research, animal models are clearly indispensible and are proven tools for discovery. In particular, many of the anti-malarial drugs currently in use emerged from small molecules, whose potencies were assessed in animal models (Fidock *et al.*, 2004). 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.

The olive baboon (*Papio anubis*) is ranked highly in the primate phylogenetic tree compared with other non human primates (NHPs) used in biomedical research. It is phylogenetically close to human with 98% protein homology (http://ca-biomed.org/links/factsheet/). It is fully susceptible to experimental infection with *P. knowlesi* leading to either severe malaria or controlled parasitaemia that result in mild infection (Ozwara *et al.*, 2003). Humans and olive baboons have similar immunopathophysiology and host parasite interactions (Ozwara *et al.*, 2003). The use of olive baboons is a pivotal undertaking in expanding the number of primate hosts for *in vivo* determination of vaccines' safety and immunogenicities.

Plasmodium knowlesi is an attractive experimental system for malaria research. This is because it is a parasite of monkeys that have immune and metabolic systems very similar to those of humans (King, 1988) and *P. knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante *et al.*, 1998). *Plasmodium knowlesi*, whose genome has been sequence entirely (Pain *et al.*, 2008) is an ideal experimental system for

malaria research. It is a pathogen known to infect humans (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008) making it an excellent model parasite for drug, vaccine and malaria in pregnancy studies (White, 2008).

1.6 Malaria Control

As malaria control intensifies, it is vital to monitor malaria burden and trends, to track the coverage and impact of interventions. The government of every country affected by malaria has a national control policy covering prevention and care management. Methods of control are mainly the use of anti-malaria treatment and vector control (World Health Organization, 2008).

Malaria control strategies are aimed at interfering with various aspects of the vector-parasite-host transmission dynamics. Broadly, they include environmental breeding site management, vector control with area and residual insecticide-treated nets (ITNs), insect repellents and wearing appropriate clothing (Walther and Walther, 2007) and parasite control that is through treatment of infected persons. The objective of anti-malarial treatment is to ensure cure of the infection, reduce morbidity and mortality of the disease, prevent progression of uncomplicated malaria into severe and potentially fatal disease, reduce the impact of malaria infection and prevent drug resistance. Currently, this is achieved by use of artemisinin-based combination therapy (ACT), (World Health Organization, 2008).

However, there is gradual emergence of parasite strains resistant to antimalarial drugs, as well as of insecticide-resistant *Anopheles* mosquito populations. A lasting solution to the control of malaria is the development of an effective vaccine that will confer long-lasting protection against malaria infection. The vaccine should enable wide coverage and affordable to the susceptible populations that need it most. An ideal human malaria vaccine must be safe, easy to manufacture in large scale, easy to administer especially through expanded programmes of immunization (EPI). It should confer long-lasting immunity against all forms of the disease (Richie, 2006).

1.7 Vaccine Adjuvants

Purified soluble proteins or polysaccharides are not well recognized by the immune system during vaccination and sometimes they even induce tolerance rather than immunity, so-called adjuvants are often added to vaccines to enhance immunogenicity (Calabro *et al.*, 2011).

Adjuvants comprise all substances that have the ability to enhance the immune responses against a co-injected antigen. However, many adjuvants were found empirically, and progress to understand their mechanism of action has been slow, which partly explains why the number of adjuvants approved for human use is still low. For instance, aluminium salts - generically named alum - have been used for more than 70 years, but only now are we beginning to understand how alum works (Calabro *et al.*, 2011).

A number of candidate parasite antigens have been identified which have shown to stimulate parasite-specific immune response (Kanoi and Egwang, 2007). While this has led to tremendous progress in defining protective immunity, the existence of multiple, potentially variable parasite antigens and parasite developmental stages has hampered the achievement of a feasible vaccine. Additional complications include lack of available information on appropriate adjuvant for human use and parasite antigen combinations that induce protective immunity in humans (Belperron *et al.*, 1999).

Therefore, identification of an appropriate adjuvant to be used with a potent parasite antigen is crucial in developing an effective malaria vaccine. Immunomodulatory chemokine adjuvants have advantage over the conventional adjuvants because they have the capability of enhancing pro-inflammatory responses, recruiting and activating dendritic cells and sustaining immunological memory. Many malaria vaccine candidate antigens, adjuvants, and virus-vectored systems have been under development over the past twenty years. Presently, RTS,S antigen formulated with either the AS02A or AS01E adjuvant system has consistently been shown to confer partial protective immunity (30-35%) against infection by *P. falciparum* in humans (Bejon *et al.*, 2004; Alonso *et al.*, 2005).

Identifying potent adjuvant formulations for current *Plasmodium* antigens under investigation as possible vaccine candidates will contribute towards the advancement of malaria vaccine research.

The ability to induce an immune response to a protein antigen by administration of plasmid DNA encoding the antigen has been successfully demonstrated in many different parasitic infections and animal models (Ivory and Chadee 2004). For some antigens, a single immunization suffices in eliciting long-lasting immunity. However, for other antigens, repeated administration of DNA is required to attain either long-lasting or any detectable immune response (Belperron *et al.,* 1999). Effective human adjuvants should be capable of inducing antibody responses, to boost pro-inflammatory responses and sustain immunological memory that is sufficient to confer protection against the blood stages of *Plasmodium*. Therefore, there is need to evaluate the effect of immunomodulatory chemokines CCL5 and CCL20 as possible chemokine adjuvants.

1.8 Malaria Vaccine Development

To complement existing means of controlling malaria, effort is being put into development of a vaccine. Vaccine development against human malaria currently focuses on three types based upon aspects of the parasite life cycle. This vaccine approaches can be broadly grouped into pre-erythrocytic, blood stage and transmission blocking.

1.8.1 Pre-erythrocytic Malaria Vaccines

These are vaccines directed at sporozoites and/or liver stages aimed at protecting against onset of blood stage infection. Whole organism approaches have a range of potential advantages. In short, they have the benefit of not requiring the precise determination of epitopes or antigens. Rather the whole organism enables a vast array of antigens to be delivered, essentially providing a multi-epitope vaccine, with a high probability of antigens being in their native conformation. There are several important challenges to this approach.

Safety is a major concern, as current technology requires that parasites are cultured in human erythrocytes, which is accompanied by a risk of serious blood-borne infections (Richards and Beeson, 2009).

Large-scale production of whole parasites ensuring consistent quality and dose is more challenging than for recombinant or synthetic vaccines. With live-attenuated parasites there is also the concern that attenuated parasites might revert to a non-attenuated state, although genetic attenuation by targeting multiple genes would make this unlikely. There are likely to be significant challenges in producing and delivering live blood-stage parasites for widespread use (Richards and Beeson, 2009).

In vitro culture of *P. vivax* is difficult to sustain and large-scale production is not currently feasible. It seems highly probable that any current generation of pre-erythrocytic vaccine will also require a blood stage component to control breakthrough infections (Richards and Beeson, 2009). Similarly to pre-erythrocytic vaccines, a case has been made for the development of whole-parasite-based blood stage vaccines, although logistical and safety issues remain pertinent (Pinzon-Charry and Good, 2008).

1.8.2 Blood Stage Malaria Vaccines

Blood stage vaccines against *P. falciparum* are aimed at preventing complications of disease, such as cerebral malaria or anaemia. Both *P. falciparum* and *P. vivax* can cause severe anaemia, but only *P. falciparum* causes the many complications of cerebral malaria, hypoglycaemia, metabolic acidosis and respiratory distress (Mackintosh *et al.*, 2004). *Plasmodium falciparum* is responsible for the great majority of deaths and, for this reason; most effort has been devoted to *P. falciparum* vaccines. Most blood stage vaccines have focused on antigens responsible for red cell invasion, antigens that are either expressed on or associated with the surface of the merozoite (MSP1, MSP2, MSP3 and GLURP) or in apical organelles (RAP1, RAP2, AMA1 and EBA175) (Genton, 2008; Pinzon-Charry and Good, 2008; Epstein *et al.*, 2007).

The design of these vaccines employs a combination of mechanisms to ameliorate clinical disease, including direct attack on erythrocyte-stage parasites, blockade of erythrocyte invasion, inhibition of cytoadherence, induction of antibody-dependent cellular inhibition (ADCI) and possibly, neutralization of malarial toxins (Schofield, 2002). The recent recognition of the importance of T-cell help in the generation of long-term antibody responses has spurred the development of vaccine strategies that enhance cellular immunity (Good, 2001). Although immunity induced by antigens expressed in asexual blood stages of the parasite does not achieve complete clearance of the parasite, or sterile immunity (Hoffman *et al.*, 1987), repeated infections and the associated re-exposure to these antigens are likely to provide a natural boost to vaccine-specific immunity. Unlike vaccines for pre-erythrocytic stages, the goal of erythrocytic (asexual blood stage) vaccines would, therefore, be to either reduce the parasite load by preventing invasion of red cells or parasite replication/growth after invasion or to prevent clinically apparent complications, such as cerebral malaria. Therefore, asexual blood-stage vaccines would act as a disease-ameliorating vaccine (Moorthy and Hill, 2002). It has also been argued that blood stage vaccines could work alone as disease-controlling vaccines (Epstein *et al.*, 2007), although this would be only be feasible if such vaccines were much more effective than any so far assessed in humans. Leading candidates MSP 119, MSP142 and AMA1 have shown modest, if any, anti-parasitic effects (Genton, 2008; Pinzon-Charry and Good, 2008; Epstein *et al.*, 2007). The Combination B vaccine candidate containing MSP1, MSP2 and RESA showed limited strain specific anti-parasitic effects evidenced by an excess of infections by strains containing the allelic form of MSP2 absent from the vaccine (Genton, 2002).

1.8.3 DNA Vaccines

DNA vaccines are new type of sub-unit vaccines allowing protein expression in mammalian cells after introduction of plasmid or recombinant viral vectors encoding the selected protective antigen. Protective immunity conferred by DNA vaccines has been shown in many animal models of various diseases including HIV, tuberculosis and cancer (Lee *et al.*, 2004; Taracha *et al.*, 2003; Pavlenko *et al.*, 2004). DNA vaccines induce strong humoral and

cellular immunity and have the potential to increase immunogenicity through modifications of the vector or incorporation of adjuvant-like cytokine genes (Kanoi and Egwang, 2007). Application of this new vaccination technology with regard to parasitic infection provides new hope for significant advances in anti-parasitic vaccine research. Parasites have complex life cycles and host immunity to stage-specific antigens may not overlap with other later stages or vector-borne stages. Antigenic variation and other immune evasion mechanisms also complicate the development of vaccines against parasites (Ivory and Chadee, 2004).

However, with recombinant DNA technology and the versatility of DNA vaccination, it is now possible to take rational parasite specific strategies to vaccine design and overcome the obstacles presented by parasitic diseases (Reyes and Ertl, 2001). Improving DNA vaccine efficacy against parasitic disease can be achieved by: single or several immunizations, use of immunomodulatory chemokine adjuvants and multivalent vaccines or codon optimization (Ivory and Chadee, 2004). An advantage of this approach is the ability to direct immune responses to a specified region or epitope. This has the potential to maximize protective responses whilst minimizing undesired responses. A further advantage is the suitability for large-scale antigen production under good manufacturing practice. The inherent difficulty lies in identifying protective target antigens for development, or specific domains of proteins, and the likelihood that multiple antigens would be required to induce an effective response and overcome antigenic diversity. Expression of many *Plasmodium* proteins is problematic because they are often conformationally complex and/or large. Most recombinant antigens require some form of adjuvant to elicit sufficient immune responses. There is much uncertainty about which protein–adjuvant combinations will be well- tolerated, yet elicit an effective immune response with sustained duration (Richards and Beeson, 2009).

1.9 Serine Repeat Antigen (SERA)

Plasmodium falciparum SERA also known as SERP (Knapp *et al.*, 1989) or p126 (Delplace *et al.*, 1985) is a 120 kDa protein (Figure 1.3) that is highly expressed in early trophozoite and schizont blood stages of the *P. falciparum* life cycle (Fox and Bzik, 1994). In schizont

stages, SERA is abundantly synthesized and secreted into the parasitophorous vacuole (Delplace *et al.*, 1987; Lyon and Haynes 1986). At the time of parasite egress from infected erythrocytes, a fraction of the accumulated 120-kDa pool of SERA protein is proteolytically processed into 47 kDa (an N-terminal fragment), 50-kDa (an interior fragment that contains a strong homology to the active-site domain of cysteine/serine proteinases), and 18-kDa fragments which accumulate in culture medium (Debrabant and Delplace 1989; Debrabant *et al.*, 1992; Delplace *et al.*, 1985; Eakin *et al.*, 1989). A pool of full-length SERA protein (the 120-kDa form) remains unprocessed, associates as a non integral membrane protein with the surfaces of free infectious merozoites (Pang *et al.*, 1999; Perkins, 1986; Perkins and Zeifer 1994), and retains the capacity to bind to inner-leaflet erythrocyte plasma membrane phospholipids (Perkins and Zeifer 1994). The 120 kDa SERA protein also associates with high-molecular-weight rhoptry proteins (Perkins and Zeifer 1994).

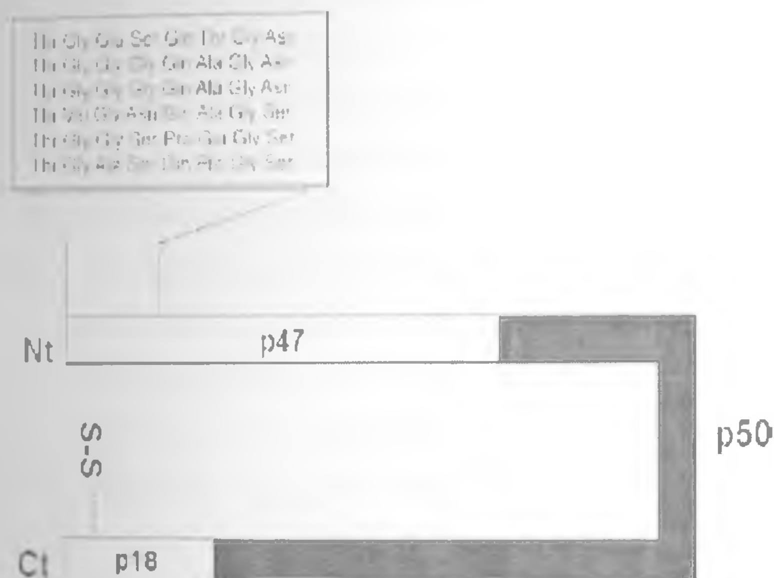

Figure 1.3: Sequence of the amino-terminal region of *P. falciparum* serine repeat antigen (SERA). At the end of asexual blood stage SERA is proteolytically processed into a 47 kDa N-terminal domain (p47/repeat motifs), a 50 kDa central domain (p50) and a 18kDa C-terminal domain (p18) (Adapted from Riccio *et al.*, 2005).

From previous studies, the serine repeat antigen ranks as a candidate antigen for inclusion as a subunit in a polyantigen malaria vaccine because:

(i) Specific monoclonal and polyclonal antibodies against SERA can inhibit the *in vitro* growth of the parasite (Chulay *et al.*, 1987; Bzik *et al.*, 1988; Knapp 1989);

(ii) Immunization with SERA can induce partial protection against parasite challenge in *Saimiri* and *Aotus* monkeys (Perrin *et al.*, 1984; Delplace *et al.*, 1985; Inselburg *et al.*, 1991; Enders *et al.*, 1992; Knapp *et al.*, 1992);

(iii) A positive association between infection induced antibody response and the degree of protective immunity has been reported. Increased level of IgG against 47 kDa peptide correlates with lower parasitaemia in the peripheral blood and absence of fever in a group of children (Banic *et al.*, 1998; Okech *et al.*, 2001);

(iv) Mouse and rat antibodies against the N-terminal 47 kDa domain have been shown to inhibit the intra-erythrocytic proliferation of parasites *in vitro* (Pang *et al.*, 1999;1996; Sugiyama *et al.*, 1996; Fox *et al.*, 1997);

(v) Recombinant proteins corresponding to the 47 kDa domain of SERA conferred protective immunity in *Aotus* and squirrel monkeys against the parasite challenges (Inselburg *et al.*, 1991; 1993; Suzue *et al.*, 1996);

(vi) It is highly conserved among field and clinical isolates of *P. falciparum* (Bhatia *et al.*, 1987; Coppel *et al.*, 1988; Fox and Bzik 1994; Morimatsu *et al.*, 1997) and

(vii) Animal protection after vaccinations with the SERA protein in Complete Freund's adjuvant (CFA) in primate model was protective though it was non sterile (Inselburg *et al.*, 1991; 1993a; 1993b; Suzue *et al.*, 1997).

1.10 Role of Immunomodulatory Chemokine Adjuvants in Malaria Vaccine Development

Novel concepts in malaria vaccine development must include intervention strategies that entail the generation and maintenance of specific memory T cells in addition to vaccine antigen presentation (Kanoi and Egwang, 2007). It has been reported that alternating immunizations with recombinant attenuated fowl pox 9 virus or modified vaccinia virus

Ankara encoding pre-erythrocytic malaria antigens enhance memory responses (Bejon *et al.*, 2006). For the development of immunity in malaria dendritic cells, antigen-specific T helper 1 cell and memory T lymphocytes are crucial (Liu *et al.*, 2006; Pouniotis *et al.*, 2004; Seixas *et al.*, 2001; Perlmann *et al.*, 2000; Bergmann *et al.*, 1997; Jangpatarapongsa *et al.*, 2006).

Chemokines are pro-inflammatory molecules involved in immunoregulatory and inflammatory processes. They predominantly chemoattract and activate leukocytes. Leukocyte trafficking is a highly controlled processes that also involves adhesion molecules such as selectins and integrins (Von and Mackay, 2000). The migration of leukocytes is not only an important event during inflammatory reactions but is also essential in normal immune surveillance (Moser *et al.*, 2004). Chemokines such as CCL5 enhance pro-inflammatory responses and CCL20 recruit and activate dendritic cells and memory T lymphocytes (Schutyser *et al.*, 2003), have been incorporated into vaccine constructs (Shimizu *et al.*, 2006; Chavan *et al.*, 2006).

New malaria vaccine constructs could incorporate CCL5 and CCL20 in order to boost pro-inflammatory responses and sustain immunological memory. The design of such vaccines must be based on a solid understanding of the molecular and cellular components of immunity and memory in *P. falciparum* malaria. The role of CCL5 in clinical immunity has emerged over the past 5 years (Ochiel *et al.*, 2005; Were *et al.*, 2006; John *et al.*, 2006), but that of CCL20 is virtually unknown. Kanoi and Egwang (unpublished data) have shown that serum CCL20 levels are not only increased in severe malaria, but are correlated with serum levels of IgG antibodies against a recombinant construct of *P. falciparum* SERA5; serum titres of these antibodies are associated with protection against severe malaria in Ugandan children (Okech *et al.*, 2006). Vaccine constructs which incorporate CCL5 and CCL20 and other biological adjuvants hold promise, and should be urgently investigated and tested in malaria clinical trials in endemic countries (Kanoi and Egwang, 2007).

1.11 Problem Statement

Malaria, which is transmitted by anopheline mosquitoes, is an enormous public health problem worldwide that kills one to three million people annually, majority being in Sub-Saharan Africa (World Health Organization, 2009). Global warming and deterioration of public hygiene caused by natural disasters (cyclones, earthquakes, and tsunami) are major concerns for increased risk of malaria outbreaks in malaria-free areas where residents have no natural immunity against the parasite. Resistance against chemotherapeutics and insecticides in addition to poor economic situation has also contributed to malaria burden (Richie, 2006).

A safe and an effective blood stage malaria vaccine are ne eded as a novel weapon in ameliorating the impact of malaria in endemic areas and reinforce hope for malaria eradication. An ideal malaria vaccine should be safe, easy to manufacture, easy to administer and when administered should confer life-long immunity against all blood stage forms of the disease and generating and maintaining specific memory T cells in addition to antigen presentation (Richie, 2006).

Malaria vaccine candidate antigens, adjuvants, and virus-vectored systems have been under development over the past twenty years. Presently, RTS,S antigen formulated with either the AS02A or AS01E adjuvant system has consistently been shown to confer partial protective immunity (30-35%) against infection by *P. falciparum* in humans (Bejon *et al.*, 2004; Alonso *et al.*, 2005). Other potential antigens like *P. falciparum* serine repeat antigen (SERA) has also shown partial protection but using a non-appropriate adjuvant, Complete Freund's Adjuvant (CFA) (Inselburg *et al.*, 1991; 1993a;1993b). At present, there is lack of knowledge on appropriate adjuvants and parasite antigen combinations that can induce protective immunity in humans and it clearly represent an area for further studies. The search for more effective human adjuvants, that are capable of inducing antibody responses that are of sufficient magnitude to protect humans against the blood stages of *Plasmodium*, must continue. Further improvement of vaccine efficacy using SERA and other candidate antigens

may be achieved by developing novel adjuvants and/or vector systems. Consequently, lack of promising adjuvants will have adverse effects on malaria vaccine development that require very strong immunogenicity for protection.

1.12 Justification of the study

The incorporation of immunomodulatory chemokine adjuvants (CCL5 and CCL20) to SERA DNA vaccine holds the promise of a feasible human malaria blood stage vaccine. CCL5 has been used as adjuvants for cancer immunotherapy for boosting anti-tumour immunity (Lapteva and Huang, 2010).

A highly effective malaria vaccine will need to be multivalent incorporating antigens of several life cycle stages. There is a strong rationale for developing blood-stage vaccines as part of this strategy. Pathogenesis of malarial disease results from blood-stage infection and studies in humans and animal models have clearly established that immune responses targeting blood-stage antigens can protect against disease or facilitate control of parasitaemia (Richards and Beeson, 2009). Immunization with blood-stage antigens, mainly merozoite antigens, has been shown to be protective in a number of animal models using different antigens and there was some protective effect with one blood-stage vaccine tested in humans (Richards and Beeson, 2009). At present, the leading blood-stage vaccine candidates are all merozoite proteins, either located on the merozoite surface or contained within the apical organelles.

DNA vaccines induce strong humoral and cellular immunity and have the potential to increase immunogenicity through modifications of the vector or incorporation of adjuvant-like cytokine genes. An advantage of DNA vaccines is the ability to direct immune responses to a specified region or epitope. This has the potential to maximize protective responses whilst minimizing undesired responses. A further advantage is the suitability for large-scale antigen production under good manufacturing practice. The inherent difficulty is identifying protective target antigens for development, or specific domains of proteins, and the

likelihood that multiple antigens would be required to induce an effective response and overcome antigenic diversity. Expression of many *Plasmodium* proteins is problematic because they are often conformationally complex and/or large. Most recombinant antigens require some form of adjuvant to elicit sufficient immune responses. There is much uncertainty about which protein-adjuvant combinations will be well-tolerated, yet elicit an effective immune response with sustained duration (Richards and Beeson, 2009). Improving DNA vaccine efficacy against parasitic disease can be achieved by: single or several immunizations, use of immunomodulatory chemokine adjuvants and multivalent vaccines or codon optimization (Ivory and Chadee, 2004).

To improve safety, efficacy and immunogenicity of SERA DNA malaria vaccine candidate immunomodulatory chemokine adjuvants CCL5 and CCL20 have been co-expressed as possible adjuvant systems. These C-C chemokines such as CCL5 enhance T helper 1 responses, and CCL20 recruit and activate dendritic cells and memory T lymphocytes (Schutyser *et al.*, 2003).

The olive baboon model of malaria was used because it is ranked highly in the primate phylogenetic tree compared to other non human primates (NHPs) used in biomedical research. It is fully susceptible to experimental infection with *P. knowlesi* leading to either severe malaria or controlled parasitaemia that result in mild infection (Ozwara *et al.*, 2003), a human malaria parasite (Chin *et al.*, 1965; White, 2008) and can mimic the human disease state (Onderdonk *et al.*, 1998) and immunological response to infection (Kennedy *et al.*, 1997). The olive baboon is readily available in Kenya where the study was conducted. Therefore, as an ideal experimental system, it will be the best platform to generate preclinical data on the effect of chemokine adjuvants on the safety and immunogenicity of SERA DNA malaria vaccine candidate.

1.13 Research Questions

1. Is SERA DNA vaccine co-expressed with chemokine adjuvant safe in the baboon?
2. Does the use of immunoregulatory chemokines as adjuvants enhance host immune responses to SERA DNA vaccine?
3. Does SERA DNA vaccine confer cross protection against *P. knowlesi* challenge infection in the baboon model of malaria?

1.14 Research Hypothesis

SERA DNA vaccine co-expressed with immunomodulatory chemokine adjuvants is safe, tolerable, immunogenic and confers cross protection against *P. knowlesi* infection in the baboon

1.15 OBJECTIVES

1.15.1 General Objective

To evaluate the effect of chemokine adjuvants on safety and immunogenicity of SERA DNA malaria vaccine candidate in the olive baboon.

1.15.2 Specific Objectives

1. To determine the safety of SERA DNA vaccine co-expressed with CCL5 as chemokine adjuvant in the baboon
2. To determine the safety of SERA DNA vaccine co-expressed with CCL20 as chemokine adjuvant in the baboon
3. To determine host cellular and humoral immune responses elicited by SERA DNA vaccine co-expressed with immunomodulatory chemokine adjuvant CCL20 and CCL5 in the baboon

4. To establish whether SERA DNA vaccine co-expressed with either CCL20 or CCL5 as immunomodulatory chemokine adjuvant confers cross protection against *P. knowlesi* malaria infection in the baboon

CHAPTER TWO: MATERIALS AND METHODS

2.1 Study site

This study was carried out at the Institute of Primate Research (IPR), Karen-Nairobi. Kenya. Its mandate is to undertake biomedical research using non-human primates (NHPs) as experimental models for human diseases and infections. The IPR is located in the pristine environment of Oloolua forest with diverse flora and fauna. It is a WHO collaborating centre for research in reproductive health and infectious diseases and centre of excellence for African network for drugs and diagnostics innovation (ANDi).

2.2 Experimental Animals

Nine male malaria naïve *P. anubis* (olive baboons) weighing eight kilos and above were used in the study. Only male baboons were allocated to the study because sex is not a risk factor of vaccination and infection outcome (Ozwara *et al.*, 2003). Prior to inclusion in the study, the baboons were trapped and maintained in the quarantine facility of IPR for a period of 90 days. In quarantine they were fed on commercial non-human primate diet (Unga Millers Limited, Nakuru), supplemented with fruits, vegetables and additional ascorbic acid. Minerals and water were provided *ad libitum* (Olobo *et al.,* 1990). During quarantine, the baboons were screened for haemoprotozoan parasites, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV). Microbiological examination of effusions, pus, ulcer material and skin scrapping specimens were done to detect pathogenic agents that cause infection in wounds and the skin (Eley and Bambra, 1993). Animals were housed singly in cages to facilitate frequent handling, observation and to avoid injury from fighting with cage mates. Their general health status was monitored throughout the experimental period. Animal restraint was accomplished by administering mixture of 10 mg/kg of ketamine hydrochloride (Agrar, Holland) and 0.5 mg/kg xylazine (Agrar, Holland) intramuscularly (IM) for physical health examinations, phlebotomies and vaccinations. No paralytics were used. The

anaesthesia was used as per to the European Union approved and IPR standard operating procedures all experimental animal work in this study was carried out under protocols approved by the Institutional Research Committee (IRC) of Institute of Primate Research.

2.3 Study Limitations

1.2 Under ideal conditions, all data to be used for this study would have been obtained conclusively. However, this was not the case due to lack of the required number of experimental animals. The shortcoming was due to from logistical problems in issuance of NHPs capture permits by Kenya Wild life Service for biomedical research.

2.4 Parasites

Plasmodium knowlesi H strain, Pk1 (A+) clone (Barnwell *et al.*, 1983) blood stage parasites for inducing baboon malaria infection were retrieved from liquid nitrogen and cultured *in vitro* overnight. The parasites were originally isolated by Chin *et al.*, (1965), Barnwell and his team successfully cloned the parasite by micro manipulation and subsequent passage in rhesus monkey (Barnwell *et al.*, 1983; Pain *et al.*, 2008). *Plasmodium knowlesi*, whose genome has been entirely sequenced (Pain *et al.*, 2008) and is an ideal experimental system for malaria research, it is a pathogen known to infect humans (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; White, 2008) and an excellent model parasite for drug, vaccine and malaria in pregnancy studies (White, 2008).

Plasmodium knowlesi H strain cryopreserved stocks were used to initiate blood stage infection in olive baboons. Baboon red blood cells were used to culture *P. knowlesi*. Baboon peripheral blood was collected in alsevers' solution and spun at 1500 rpm, 20°C for 10 minutes (Hettich Zentriffugen, Rotanta 460 R, Germany). The plasma was discarded and the red blood cells (Packed cell volume) washed twice with RPMI 1640 (GIBCO, BRL). Then red blood cells were reconstituted to 50% PCV and stored at 4°C for up to 2 weeks.

Baboon whole blood was collected for separation of serum. The blood was left on the bench for 1 hour and kept at 4°C for the clot to form. The clot was dislodged with an applicator stick and the blood spun at 1500 rpm, 4°C for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany). Serum was collected and heat inactivated at 56°C for 1 hour and stored at -20°C until use.

Cryopreserved parasites stocks were retrieved from liquid nitrogen and used to initiate *in vitro* cultures. They were quickly thawed at 37°C in a water bath. Inside a biosafety hood, the parasites were transferred into a sterile 50 ml centrifuge tubes and equivalent volume (1X) of 3.5% NaCl was added drop wise. The parasite-NaCl suspension was spun at 1500 rpm, 20°C for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany). The supernatant was discarded and the parasite pellet dislodged. The parasites were washed again with ½X volume of 3.5% NaCl and respun as before. The parasite pellet was washed again with 1X and 5X volumes of 10% baboon serum RPMI 1640 and respun as before. The parasite pellets were transferred into T25 cm^3 tissue culture flasks with complete culture medium (20% baboon serum, 15µg/ml gentamycin, 2.5% PCV and rest RPMI 1640). The parasite cultures were gassed with 90% N_2, 5% CO_2 and 5% O_2 for 30 seconds and incubated at 37°C for about 18 hours. After incubation, about 200 µl of the culture suspension were transferred into a clean eppendorf tube and spun at 2000 rpm for 2 minutes (Eppendorf centrifuge 5414). The supernatant was discarded and the parasite pellet was used to make duplicate thin blood smears.

The thin blood smears were methanol fixed and stained with 10% Giemsa accustain solution (BDH, VWR international Ltd, England) for 10 minutes. The thin blood smears were then washed under running tap water and air dried. The thin blood smears were examined by microscopy under oil immersion x100 objective lens. The slides were checked for bacterial contaminations, parasite viability and percentage parasitaemia determination by counting the number of parasitized red blood cells in at least 10, 000 uninfected red blood cells. The *in vitro* parasite culture suspension was spun down at 1500 rpm, 20°C for 10 minutes. The supernatant was discarded and the pellet volume determined. The parasite concentration was adjusted to 2×10^5 *P. knowlesi* parasites.

2.5 DNA Vaccine Constructs

The DNA vaccine constructs were manufactured according to Good Manufacturing Practice (GMP) regulations by Gene Art (Regensburg, Germany). Formulated by combining SERA in pIRES (plasmid Internal Ribosomal Entry Site), SERA with CCL5 as an adjuvant in pIRES plasmid, SERA with CCL20 as an adjuvant in pIRES plasmid and pIRES plasmid alone and dissolved in Tris EDTA buffer (TE pH 7.2) at a concentration of 1 mg/ml. The codon usage was adapted to the codon bias of *Homo sapiens* gene. Regions of very high (> 80%) or very low (< 30%) GC content was avoided where possible. During the optimization process, the following cis-acting sequence motifs were avoided where applicable: internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or GC-rich sequence stretches, RNA instability motifs, repeat sequences and RNA secondary structures and (cryptic) splice donor and acceptor sites in higher eukaryotes.

2.6 Study Design.

This was an open-label study on evaluating the effect of chemokine adjuvants on safety, tolerability, immunogenicity of SERA malaria DNA vaccine candidate and cross protective efficacy against *P. knowlesi* in olive baboons. The vaccine regimens were administered IM on a 0-, 28-, and 56-days schedule (Table 2.1).

Table 2.1: Immunization schedule and doses

Treatment Group	n (baboons)	Full dose strength
SERA +CCL5 in pIRES plasmid	3	1mg
SERA +CCL20 in pIRES plasmid	3	1mg
pIRES plasmid backbone alone	3	1mg

In accordance with ethical guidelines regarding use of non-human primates in biomedical

research, only the minimal number of baboons required to obtain valid and reproducible data were included in this study. This is in compliance with the concept of '3Rs' (Reduce, Replace and Refine) used to minimize the use of animals in research and any associated suffering without affecting the quality of scientific work (Russel and Burch, 1959).

2.7 Experimental Design

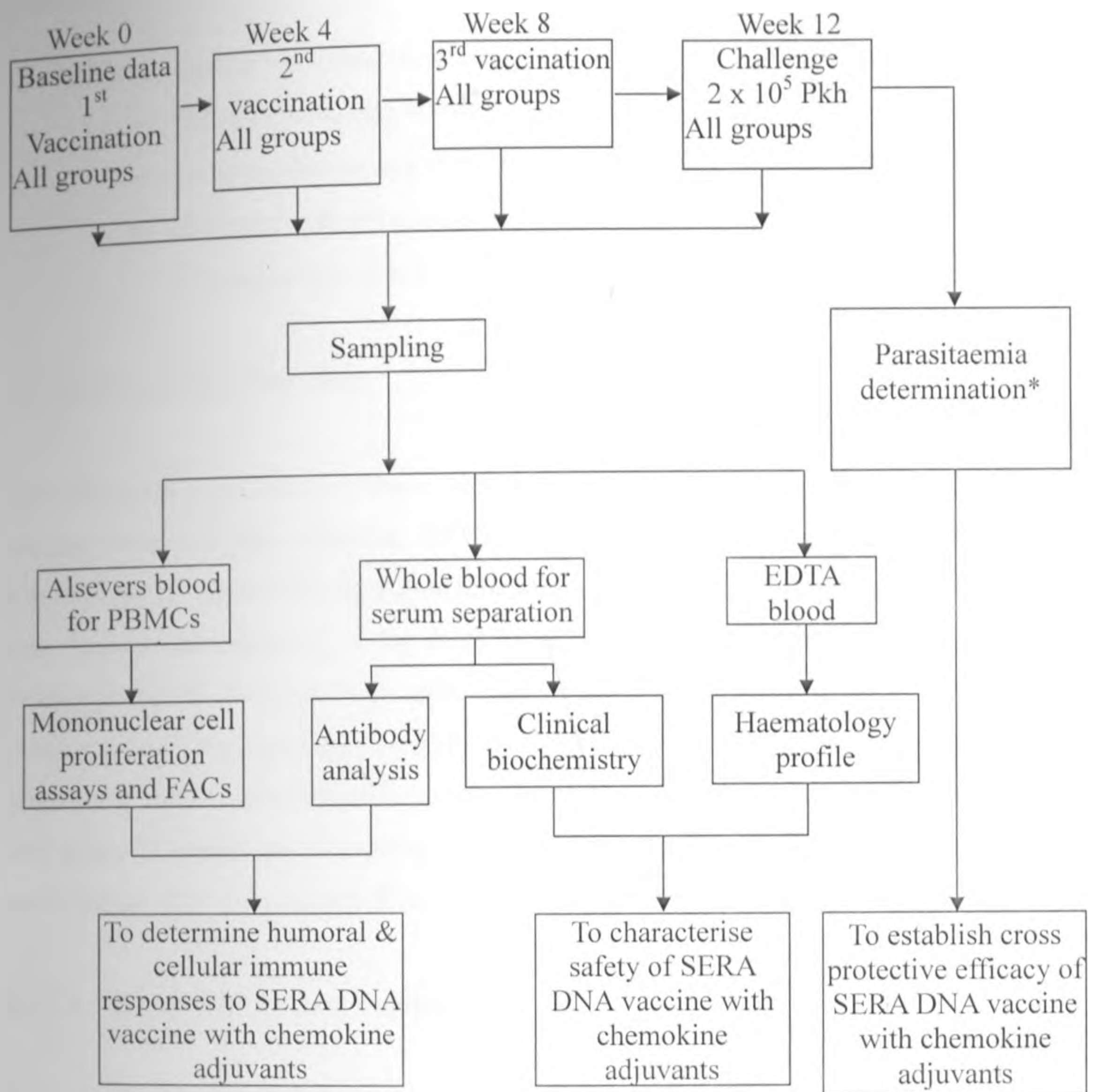

Figure 2.1: Experimental Design

Key: Treatment Groups; (n=3; SERA+ CCL5 in pIRES); (n=3; SERA+CCL20 in pIRES) and (n=3; pIRES backbone alone) *Treat at 5% Parasitaemia; Pkh: *Plasmodium knowlesi* H strain; pIRES; plasmid of internal ribosomal entry site.

2.8 Inclusion of the Study

The following criteria were used to select experimental animals for inclusion in the study;

- Baboons weighing above 8 kgs
- Baboons that are not infected with haemoprotozoan parasites
- Baboons with *P. knowlesi* whole parasite antigen recall proliferation indices of < 2 counts per minute (cpm)

2.9 Vaccine Administration

Immunizations were done in three time points which were four weeks apart. The DNA vaccine constructs were stored at -20°C and thawed on ice and reconstituted to 333.3μg/ml with Tris EDTA buffer (TE pH 7.2) before administration. At each time point, animals' thighs were shaved and midpoint of the *rectus femoris* muscle determined using a 30 cm ruler. Peripheral blood obtained from each animal prior to vaccinations and used for clinical chemistry and haematology analysis. Body temperature and weight of animals were determined. All immunizations were done on both legs by perpendicular injection with a ½-inch gauge 21 needle into the *rectus femoris* at the mid-anterior thigh. Each animal received a total medium dose of 1 mg/ml of respective candidate DNA vaccine construct.

2.10 Assessing Safety and Tolerability

The animals were assessed for safety and tolerability of the vaccine post immunization via animal physical health, immunization local site reactogenicity, haematological profile and clinical kidney and liver functions.

2.10.1 Post Vaccination Animal Health Monitoring

After vaccinations, the animals were monitored daily for general health status, appetite by closely monitoring feeding habits of the animals and general behaviour. Vaccination sites

were examined for indurations, erythema, skin swelling, warmth, ulceration and regional lymphadenopathy by direct examination 1, 2, 3, and 14 days after each vaccination by investigators unaware of group assignments (Pichyangkul *et al.*, 2008).

2.10.2 Haematological Analysis

Peripheral EDTA blood was obtained from all animals via the femoral vein and analysed using Beckman Coulter ACT 5 Diff. CP (USA). Measurements were made for complete blood cell count, haematocrit, haemoglobin, means corpuscular volume, platelet counts, erythrocyte counts and leukocyte counts.

2.10.3 Clinical Biochemistry Assays

To determine kidney and liver functions, serum was processed from part of peripheral blood and analysed using Humalyzer 2000 (Human, Germany) machine to measure creatinine phosphokinase (CK-MB), Total bilirubin, direct bilirubin, alanine aminotransferase (ALAT/GPT), aspartate aminotransferase (ASAT/GOT), urea, and total protein. The tests were conducted according to the manufacturer's instructions (Human, Germany).

2.11 Immunogenicity of SERA

Vaccine immunogenicity was determined by determining humoral and cellular immune responses in baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone alone.

2.11.1 Antibody Analysis

For humoral immune responses, the sera obtained for end point titres, immunoglobulin specificity and avidity was measured using recombinant protein of SERA5 (SE36) in enzyme-linked immunosorbent assay. The synthetic gene construct encoding the recombinant antigen SE36 is identical to the amino terminus of SERA5. The optimal antigen concentrations of 4µg/ml for BK-SE36 were determined by checker board titration and used in direct ELISA.

Ninety six well flat bottomed Maxisorb ELISA plates (Nunc Rockilde, Denmark) were coated overnight at 4°C with 50µl of 4µg/ml of recombinant BK-SE36 protein diluted in bicarbonate coating buffer (15.0mM Na_2CO_3; 34.9mM $NaHCO_3$ PH 9.6). Excess coating buffer was flicked off and the plates washed 2 times with 0.05% tween 20 in PBS (PBST). Non specific binding sites were blocked with 100µl/well of blocking buffer (1% BSA in PBST) and incubation for 1 hour at 37°C. Pre-immunization sera were used as negative controls. Unbound BSA was flicked off. The test sera were added (50 µL per well) in duplicates at dilutions of 1:200 in 0.05% PBST on ice before incubation for 2 hour at 37°C. The plate washed three times with washing buffer (0.05% tween 20 in PBS) delivered by an automatic washing machine (Dynex MRW, Germany). After washing the plates, 50µl/well of 1:4000 dilution of peroxidase-conjugated rabbit anti-human IgG (Sigma, St. Louis, MO) was added and incubated for 1 hour at 37°C. Unbound conjugate was washed three times using washing buffer. Colour development was achieved by adding 50µl/well of 3,3,5,5'-tetramethyl-benzidene (TMB) substrate (KPL, Gaithersburg, MD).

Optical densities were read using a Dynatech MRX micro plate reader (Dynatech Laboratories, Sussex, UK) at 630 nm filter setting after 30 minute incubation at 37°C. Reactions stopped using 50µl/well of 2M sulphuric acid and resulting yellow colour read at 450 nm. Anti BK-SE36 titres were defined as the proportion of serum samples with absorbance greater that the mean absorbance of negative controls plus two standard

deviations of negative controls.

2.11.2 Isolation of Plasma and PBMC from the Alsevers'-Diluted Whole Blood

For cell mediated immune (CMI) responses, 60 ml of whole blood-alsevers' mixture (20 ml alsevers' mixed with 40 ml whole blood) was layered on two 50 ml centrifuge tube containing 10ml lymphocyte separation medium (LSM) each. Then the mixture was then centrifuged at a speed of 3000 rpm for 30 minutes at 20°C (Hettich Zentriffugen Rotanta 460R, Germany). The mononuclear cell layer (buffy coat layer) was transferred into sterile 50 ml centrifuge tube. The isolated PBMCs were then washed twice using sterile 1X PBS and spun at 1500 rpm for 10 minutes at 4°C (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant was discarded and the pellet dislodged. After the second wash the cells were resuspended in 10 ml complete RPMI 1640 [RPMI 1640 (GIBCO, BRL) with 10% FBS (GIBCO, BRL), 1% L-Glutamine (GIBCO, BRL), 80μg/ml Gentamycin (Sigma, St. Louis, MO) 25mM HEPES (GIBCO, BRL)]. Approximately 10μl was then transferred onto a 96 well culture plate mixed with 90μl of trypan blue (Sigma St Louis, MO) for counting and enumeration. The cells were used immediately to set up blast assays and FACs analysis or cryopreserved for later use.

To cryopreserve cells, 18-22 x 10^6 cells/ml were resuspended in freeze mix (10% dimethyl sulfoxide (DMSO, Sigma, St Louis, MO) and 90% heat-inactivated FBS [GIBCO, BRL]), The cells were then placed in cryocell freezing containers filled with cold ethanol, kept at 4°C for 15 minutes, transferred to -80°C overnight. Then cells stored liquid nitrogen until further use.

2.11.3 Retrieving of cryopreserved PBMCs for FACS

The cryopreserved cells were retrieved from liquid nitrogen on ice and quickly thawed 37°C water bath while continuously swirling. Then transferred into the biosafety hood where they

were wiped with 70% ethanol. In the biosafety hood the cells were resuspended in 10ml of incomplete RPMI 1640 (GIBCO-BRL). The cells were washed twice by spun at 1500 rpm for 10 minutes at 4°C (Hettich Zentriffugen Rotanta 460R, Germany). The supernatant was discarded and the pellet dislodged. After the second wash the cells were resuspended in 1ml complete RPMI 1640 [RPMI 1640 (GIBCO, BRL) with 10% FBS (GIBCO, BRL), 1% L-Glutamine (GIBCO, BRL), 80μg/ml Gentamycin (Sigma, St. Louis, MO) 25mM HEPES (GIBCO, BRL)]. Approximately 10μl was then transferred onto a 96 well culture plate mixed with 90μl of trypan blue (Sigma St Louis, MO) for counting and enumeration. The cells were used immediately for FACS.

2.11.4 Fluorescence Activated Cell Analysis (FACS)

Monoclonal antibodies (BD biosciences, UK) against T-cell surface markers were used to determine to $CD3^+$ (All T cells), $CD8^+$ (Cytotoxic T cells), $CD4^+$ (T helper cells), $CD4^+/CD8^+$ and $CD3^+/CD4^+/CD8^+$ populations. Peripheral blood mononuclear cells were retrieved and reconstituted into 1×10^6 cells/ml in 2% BSA in PBS. The cells were stained using a round bottomed 96 well tissue culture microtitre plate. First of all, 2μl per single well of fluorescein-isothiocyanate (FITC) conjugated mouse anti-human $CD3\varepsilon^+$ (BD Biosciences, San Jose, CA), Peridinin-chlorophyll (PerCP) conjugated mouse anti-human $CD4^+$ (BD Biosciences, San Jose, CA) and phycoerythyrin (PE) conjugated mouse anti-human $CD8^+$ (BD Biosciences, San Jose, CA) were added to each well of the microtitre plate. For cases of double and triple-staining another 2μl of the second or third antibody for this concomitantly with the first antibody. Then to the unstained well and stained wells 100μl of the reconstituted wells were dispensed. The microtitre plate was covered with aluminium foil and incubated on ice in the dark while rocking for 30 minutes. After incubation, cells were washed thrice with 100μl of cold 2% BSA in PBS at 1500 rpm for 5 minutes at 4°C (Hettich Zentriffugen Rotanta 460R, Germany) to remove the unbound antibody. After the last wash the cells were re-suspended in 100μl of cold 2% BSA in PBS and storing them on ice.

For measurements, the cells were mixed and transferred into 12 x 75 mm round bottomed polystyrene (FACS) tubes (BD Falcon). Each tube was vortexed before acquisition to make sure that they are thoroughly resuspended and mixed. Fluorescence was measured on a FACS (Becton Dickinson, Mountain View CA). Cells were analysed by forward and right-angle scatter and the lymphocyte population to assess reactivity of the mAb with PBMCs. Data analysis was performed using Cellquest^{TM} software (Becton Dickinson) to determine frequencies and mean fluorescence intensities.

2.12 Cross Protective Efficacy

Cross protective efficacy of the vaccine was determined by challenging the baboons with *P. knowlesi* and the percentage parasitaemia determined.

2.12.1 Challenge Infection with *Plasmodium knowlesi*

At four weeks post the last vaccination, all animals were challenged with 2 x 10^5 *P. knowlesi* H strain blood stage parasites via the saphenous vein using a butterfly needle and flushing with 2ml of warm physiological saline (PH 7.2).

2.12.2 Parasitaemia Determination

Finger prick blood was obtained from challenged animals on a daily basis starting from three days post challenge and continued until end point. The blood was used to make duplicate thin blood smears. The thin blood smears were methanol fixed and stained with 10% Giemsa accustain solution (BDH, VWR international Ltd, England) for 10 minutes. Rinsed under running tap water, air dried and examined by light microscopy under oil immersion X100 objective lens. Percentage parasitaemia was determination by counting the number of parasitized red blood cells in at least 10,000 uninfected red blood cells. At 5% parasitaemia the animals were treated with 1 mg/kg body weight of pyrimethamine in a banana

presentation for three days.

2.13 Data Management and Statistical Analysis

Data generated was both parametric and non-parametric in nature. Results were displayed using Microsoft Excel® and Openoffice.org® databases. Descriptive statistics and summary tables were employed initially to describe the data. Graph Pad Instat® statistical software was used to analyse data. The student t-test and analysis of variance (ANOVA) were used to analyse parametric data comparing two and multiple means, respectively. Statistical significance was calculated by the Mann-Whitney test for non-parametric comparisons of two means and Kruskal-Wallis for comparison of more than two means. P values ≤ 0.05 were considered significant at 95% confidence intervals.

CHAPTER THREE: RESULTS

3.1 Safety Assessment and Tolerability

To assess safety and tolerability of the DNA vaccine constructs, immunization site local reactogenicity and general health status were monitored on a daily basis throughout the experimental period. Data on immunization site reactogenicity, animal physical health status, haematology and clinical biochemistry was collected.

3.1.1 Immunization Site Local Reactogenicity

The injection sites were examined for reactions, including indurations, erythema, skin swelling, skin warmth, abscess, ulceration or other abnormalities at 0, 1, 2, 3 and 14 days after each vaccine injection. Quantitative measurements were not performed, but each site was subjectively graded throughout the study by the same experienced veterinarian unaware of study group assignment using the following numeric grading scale: 0, absent; 1, mild; 2, moderate; and 3, severe (Pichyangkul *et al.,* 2008). No significant or sustained local or systemic abnormalities were observed in any group after any immunization. These suggested that the vaccine was well tolerated at all vaccination time points and doses. The only exception was for PAN 3362 (SERA+CCL20 in pIRES group) and PAN 3551 (pIRES backbone group). These animals exhibited grade 1 or grade 2 local reactions, muscle indurations and cutaneous erythema that resolved without intervention. Additionally, reaction intensity was unrelated to dose or vaccine because it was above the injection site.

3.1.2 Animal Physical Health Status

All experimental animals maintained good body condition with pink and moist mucus membranes, capillary refill time (CRT) of < 2 seconds. Their respiratory rate was within normal clinical range of 22-35 cycles per minute with normal lung sounds. Their heart rate

was regular and within normal clinical range of 80-200 beats per minute. Appetite was normal throughout the experimental period.

3.1.2.1 Mean Body weight of baboons

All the animals across all groups experienced weight differences between before vaccination and end point vaccination (Table 3.1). PAN 3529 (SERA+CCL20 in pIRES group) and PAN 3611 (SERA+CCL5 in pIRES group) died unexpectedly. Post-mortem examination did not reveal any vaccine related pathology.

Table 3.1: Mean body weights (kg) with standard error of means (SEM) of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

Treatment Group	Before vaccination	End point vaccination	Difference
SERA + CCL5 in pIRES	25.066 ± 3.227	20.733 ± 0.8762	-4.33
SERA + CCL20 in pIRES	19.566 ± 0.5457	28.85 ± 3.250	9.284
pIRES vector backbone	20.23 ± 0.7424	21.866 ± 0.1856	1.636

3.1.2.2 Mean Body Temperature of baboons

The mean body temperature of baboons was maintained within the normal clinical range of 37°C to 39°C (Table 3.2). This showed that the vaccines did not fever in the baboons.

Table 3.2: Mean body temperature (°C) with standard error of means (SEM) of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

Treatment Group	Before vaccination	End point vaccination	Difference
SERA + CCL5 in pIRES	38.2± 0.2517	37.63±0.3283	-0.57
SERA + CCL 20 in pIRES	38.47± 0.4842	37.8±0.0881	-0.67
pIRES vector backbone	38.533±0.3180	38.1±0.4	-0.433

3.1.2.3 Lymph Nodes of baboons

Before first immunization (T0), inguinal lymph nodes were observed to be normal. After the first (T1) and second immunizations (T2), inguinal lymphadenopathy ipsilateral with the vaccination site was observed in all study animals. The lymph nodes remained enlarged up to T3 (Table 3.3).

Table 3.3: Lymph nodes of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

Animal identity	Time point			
	T0	**T1**	**T2**	**T3**
Gp A **PAN 3612**	Normal	Auxiliary enlarged	Inguinal enlarged	Inguinal slightly enlarged
PAN 3614	Normal	Inguinal enlarged	Both inguinal and auxiliary enlarged	Inguinal slightly enlarged
PAN 3611	Normal	Inguinal slightly enlarged	Inguinal slightly enlarged	Inguinal enlarged
Gp B **PAN 3351**	Normal	Normal	Inguinal enlarged	Inguinal slightly enlarged
PAN 3362	Normal	Inguinal enlarged	Inguinal slightly enlarged	Inguinal enlarged
PAN 3529	Normal	Inguinal slightly enlarged	Inguinal slightly enlarged	ND*
Gp C **PAN 3200**	Normal	Both inguinal and auxiliary slightly enlarged	Inguinal enlarged	Inguinal enlarged
PAN 3208	Normal	Both inguinal and auxiliary enlarged	Normal	Inguinal enlarged
PAN 3551	Normal	Inguinal slightly enlarged	Inguinal slightly enlarged, auxiliary enlarged	Both enlarged

*ND: Not determined; T0: Baseline; T1: After the first Immunization; T2: After the second immunization; T3: After the third immunization and challenge point.

3.1.3 Haematological Profiles of Experimental Animals

Haematological profiles were conducted to assess changes in haematocrit, haemoglobin, red blood cell counts, RBC morphology, WBC counts, lymphocytes, segmented neutrophils and monocytes. Two way ANOVA was used to compare intragroup and intergroup means.

Giemsa stained blood smears showed essentially normal erythrocytes with hypochromicity, anisocytosis (variation in sizes) and poikilocytosis (variation in shape). Mitotic lymphocytosis and a shift of the polymorphonuclear neutrophils to the left were observed. This indicated an increase in the production of neutrophils. There were no important clinical signs showing toxicity of the DNA vaccine and the immunomodulatory chemokines used.

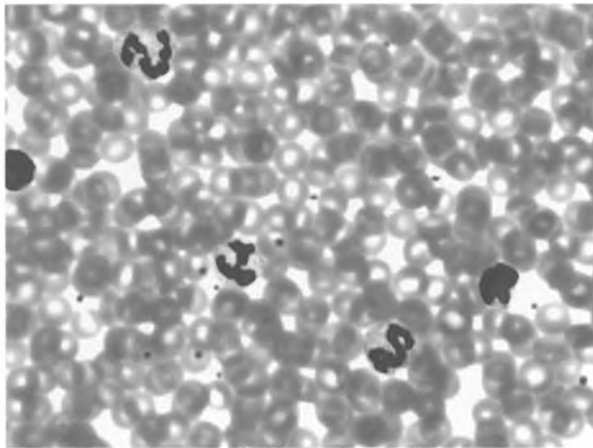

Plate 3.1: Giemsa stained blood smear showing essentially normal erythrocytes with hypochromicity, anisocytosis, poikilocytosis and a shift of the polymorphonuclear neutrophils.

Before vaccination, mean erythrocyte counts for all groups were within the baboon clinical laboratory reference values (4.9 to 6.4 x $10^6/\mu L$). An increase was observed across all experimental groups after the first immunization (T0). At the second immunization (T1), mean erythrocyte counts remained elevated in SERA+CCL5 in pIRES and pIRES vector backbone groups that decreased after the third immunization (T2) before challenge infection (T3). In SERA+CCL20 in pIRES group, there was a decrease in erythrocyte count at T2 and a increase before T3 (Figure 3.1). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

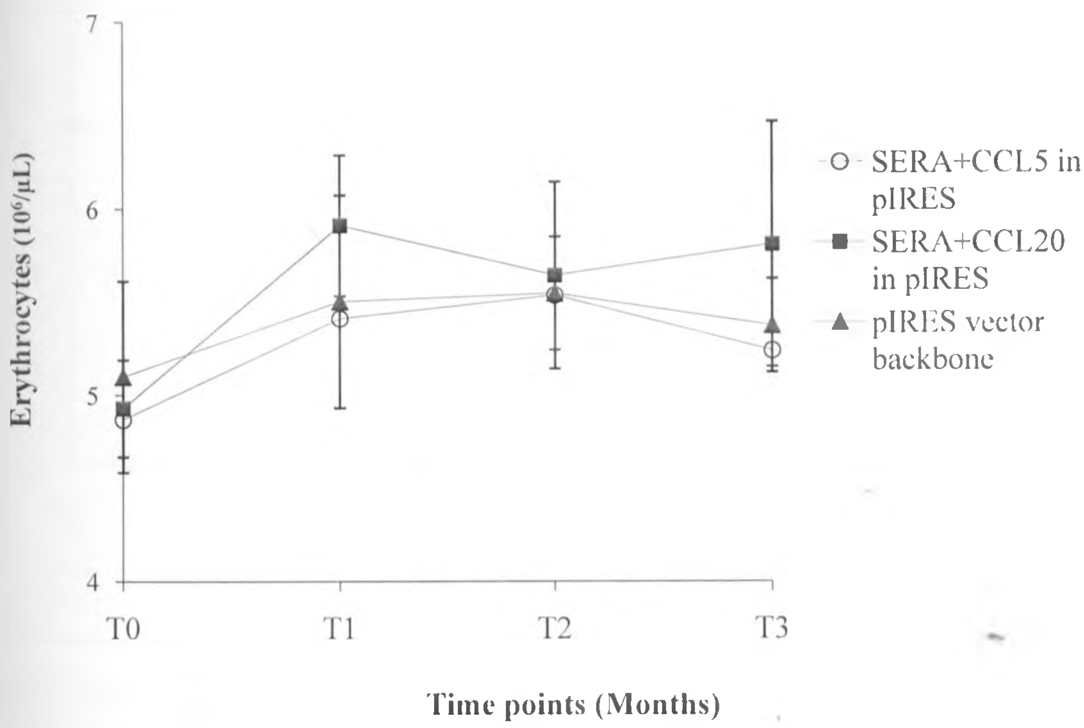

Figure 3.1: Mean erythrocyte counts of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

After T0, a decrease in mean haemoglobin levels was observed in all groups. In SERA+CCL5 in pIRES and pIRES vector backbone groups, an increase was observed after T1 that remained elevated through T2 and decreased before T3. In animals vaccinated with SERA+CCL20 in pIRES, a decrease was observed after T1 and T2 before challenge infection (Figure 3.2). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

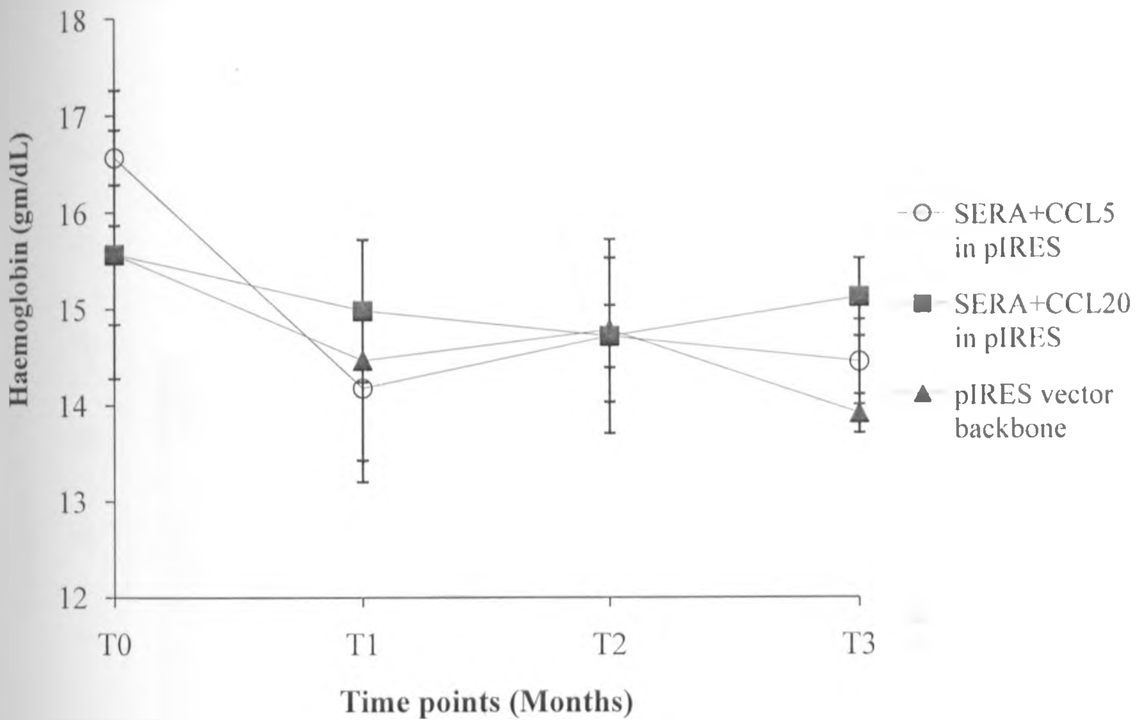

Figure 3.2: Mean haemoglobin levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

After T0, A decrease in mean haematocrit in animals vaccinated with SERA+CCL5 in pIRES was observed that increased after the second vaccination (T1) and decreased before challenge infection (T3). In animals vaccinated with SERA+CCL20 in pIRES an increase was noted after T0. A decreased was later observed after T1 and an increase before the challenge infection. In the pIRES vector backbone group, the fluctuations did not show any increase or decrease in mean haematocrit values from T0 to T3 (Figure 3.3). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

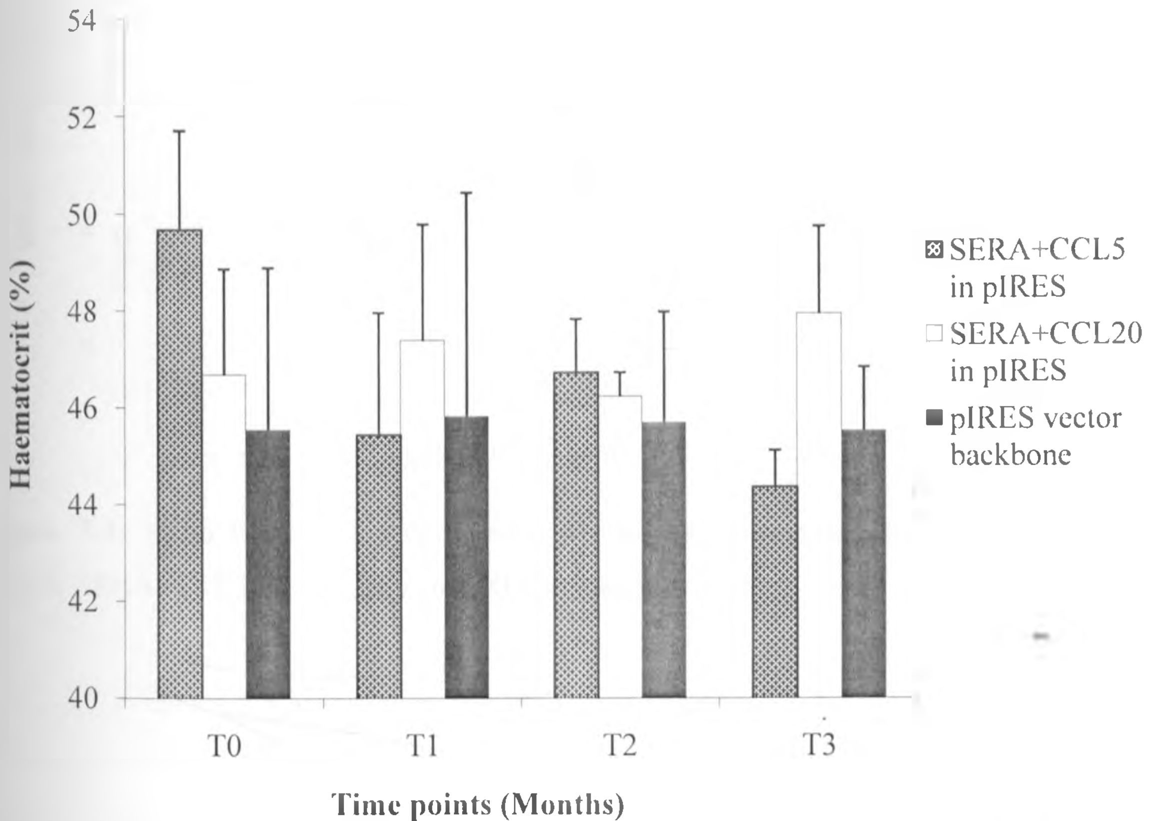

Figure 3.3: Mean haematocrit of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

After T0, all the groups there was an increase in mean white blood cell counts followed by a decrease after T1 and an increased before challenge infection (Figure 3.4). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

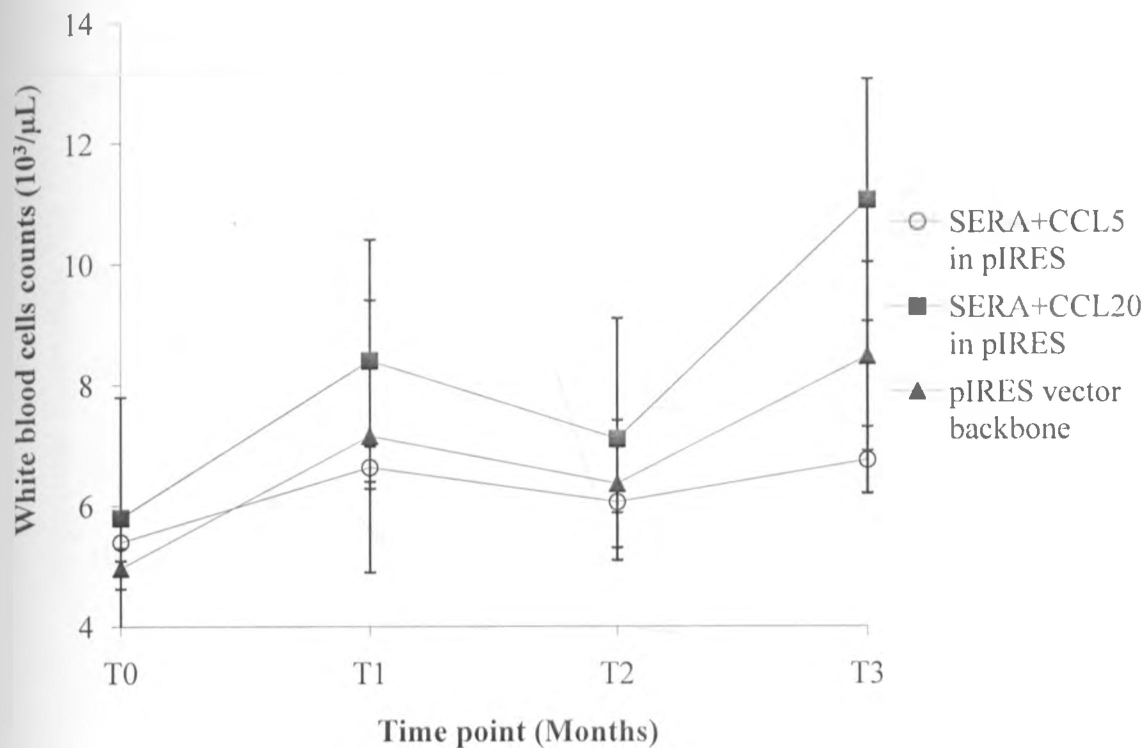

Figure 3.4: Mean white blood cell counts of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In all the groups, a decrease in mean segmented neutrophils was observed across all the immunization time points (Figure 3.5). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

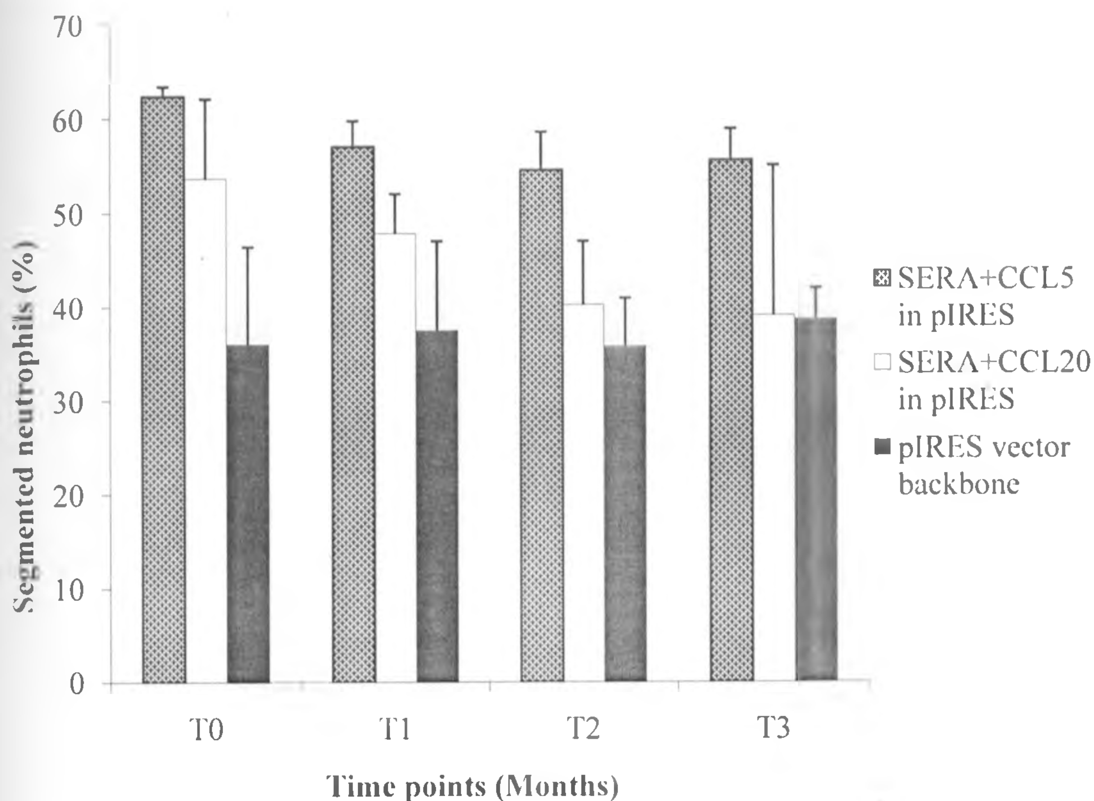

Figure 3.5: Mean segmented neutrophil counts of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In SERA+CCL5 in pIRES and SERA+CCL20 in pIRES groups, there was a increase in mean lymphocyte counts from T0 until T2 when a decrease was observed before challenge infection. In the pIRES vector backbone group, a decrease was observed at all immunization time points (Figure 3.6). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

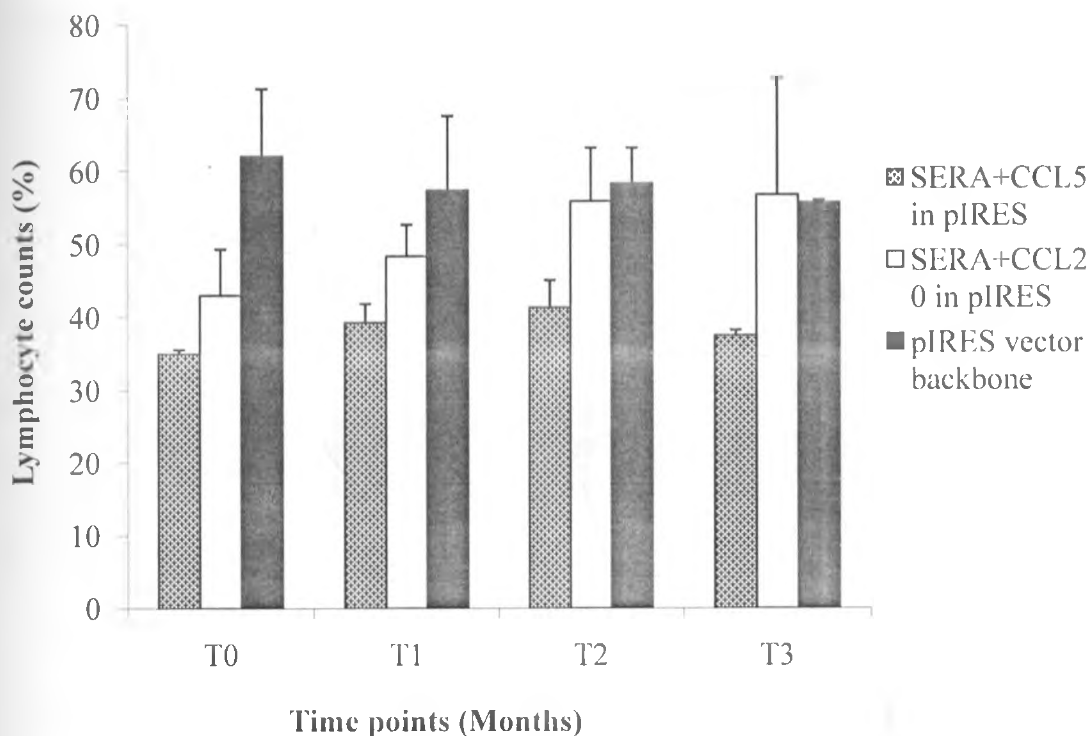

Figure 3.6: Mean lymphocyte counts of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In SERA+RANTES in pIRES and SERA+CCL20 in pIRES groups, there was an increase in the mean monocyte counts after T0 which remained unchanged between T1 and T2. An increase was observed before the challenge infection. In the pIRES vector backbone group, after T0 there was a decrease in the monocyte counts. There was no change between the T1 and T2 (Figure 3.7). An increase was observed before T3. Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

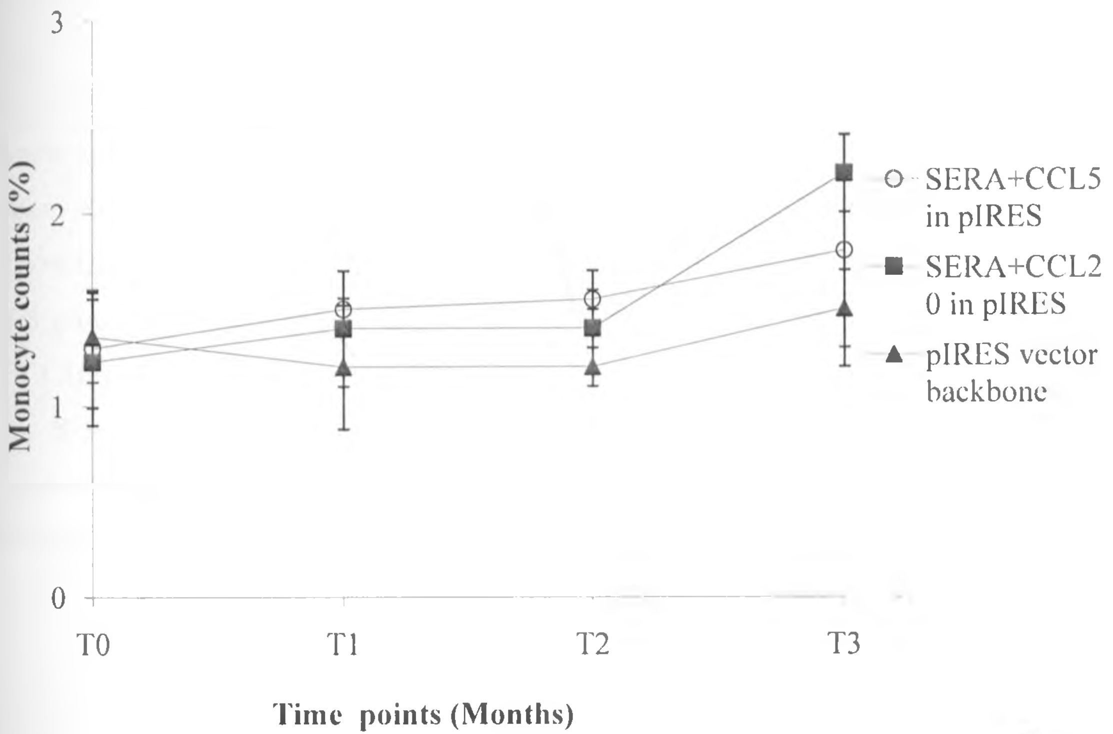

Figure 3.7: Mean monocyte counts of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.1.4 Clinical Biochemistry Assays

Clinical biochemistry assays were conducted to evaluate liver and kidney functions in all vaccinated baboons.

3.1.4.1 Liver Function Tests of Experimental Animals

To evaluate liver functions, assays for aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), creatinine phosphokinase (CK-MB) and total protein were conducted.

In SERA+CCL5 in pIRES and pIRES vector backbone groups, there was a decrease in mean aspartate aminotransferase levels after T0. An increase was observed in all groups after T1. A decrease was observed in the pIRES vector backbone group and there was no change in the SERA+CCL5 in pIRES group between T2 and challenge infection. In the SERA+CCL20 in pIRES group, there was an increase between T0 and T2. There was no change between T2 and T3 (Figure 3.8). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

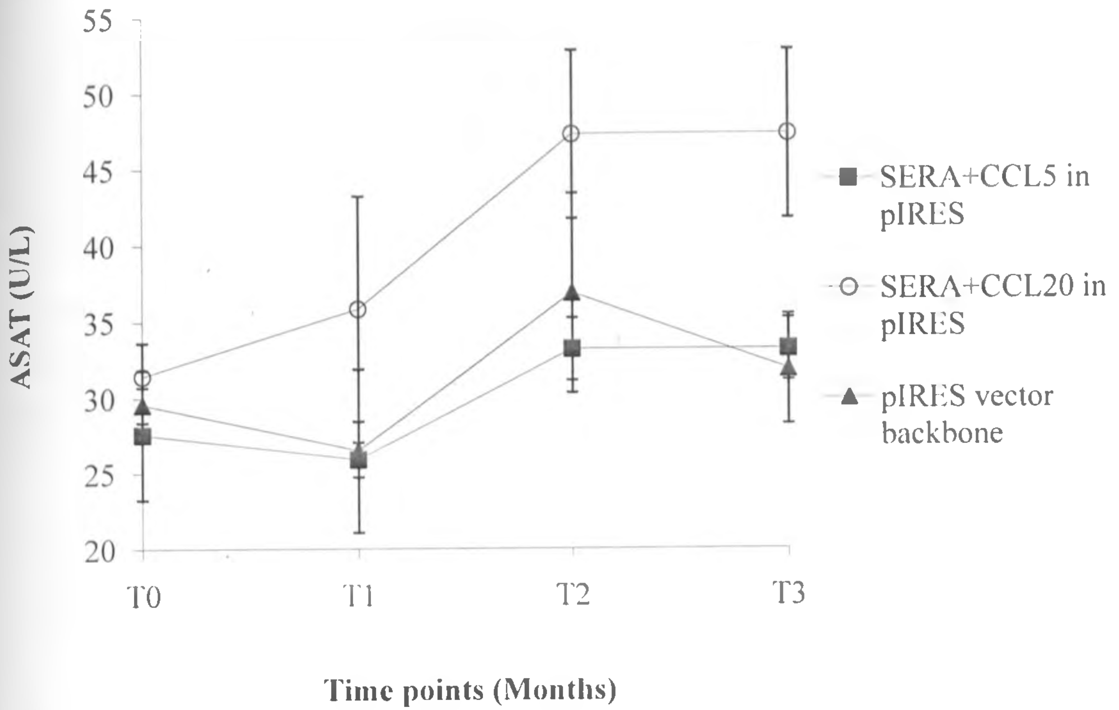

Figure 3.8: Mean aspartate aminotransferase (ASAT) levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In SERA+CCL20 in pIRES group, an increase in the mean levels of alanine aminotransferase was observed between T0 and challenge infection. In SERA+CCL5 in pIRES group, an increase was observed between T0 and T2 followed by a decrease before challenge infection. In pIRES vector backbone group, a decrease was observed between T0 and T1. After T1, there was an increase in mean ALAT levels between T1 to T3 (Figure 3.9). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

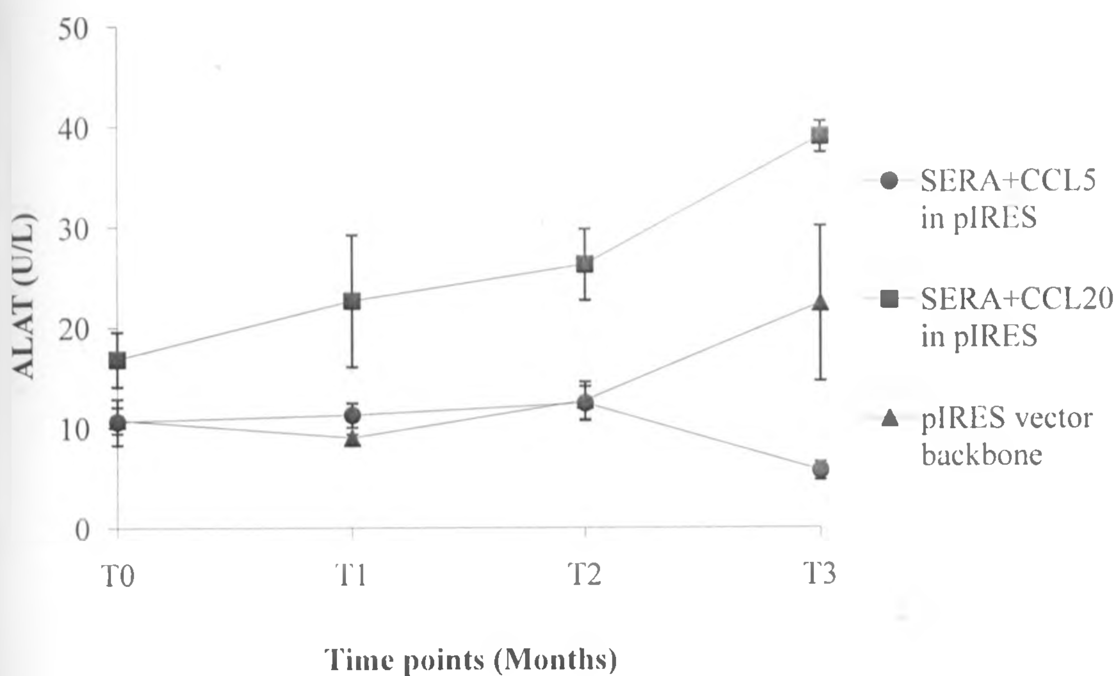

Figure 3.9: Mean Alanine aminotransferase (ALAT) levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In SERA+CCL20 in pIRES and pIRES vector backbone groups, a decrease was observed in mean level of creatinine phosphokinase after T0. Both groups showed an increase between T1 and T2 that later decreased before challenge infection. In the SERA+CCL5 in pIRES group, an increase was observed from T0 to T2 that later decreased before challenge (Figure 3.10). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

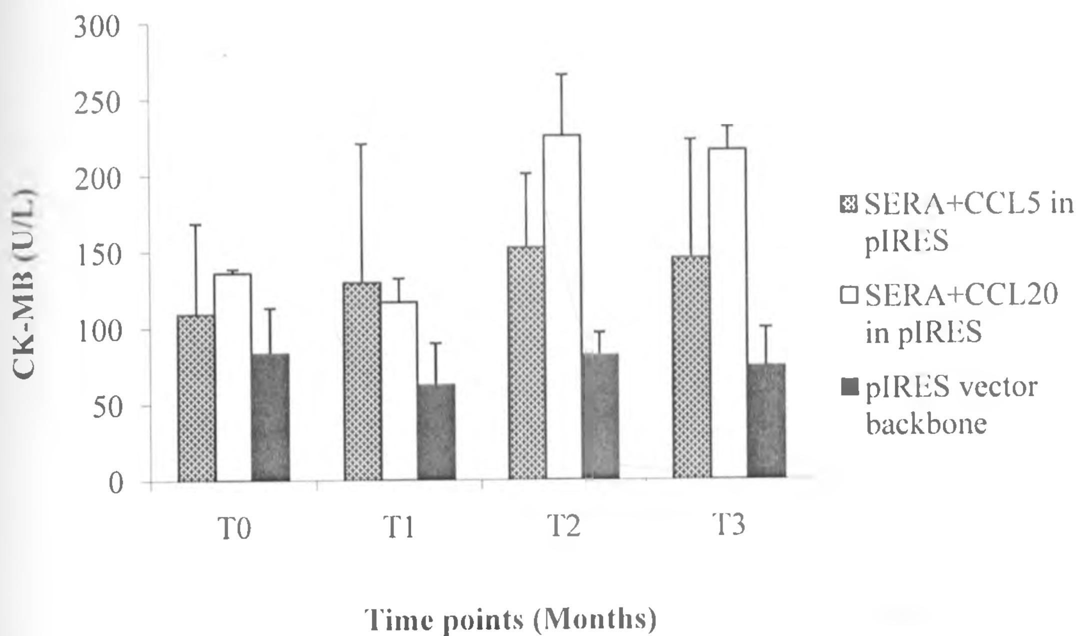

Figure 3.10: Mean Creatinine phosphokinase (CK-MB) levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

Animals vaccinated with SERA+CCL20 in pIRES and pIRES vector backbone showed a decrease in the mean protein levels from T0 to T1 and later an increase from T1 to challenge infection. In the SERA+CCL5 in pIRES group there was an increase between T0 and T1 that later decreased at T2 and eventually increased at T3 (Figure 3.11). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

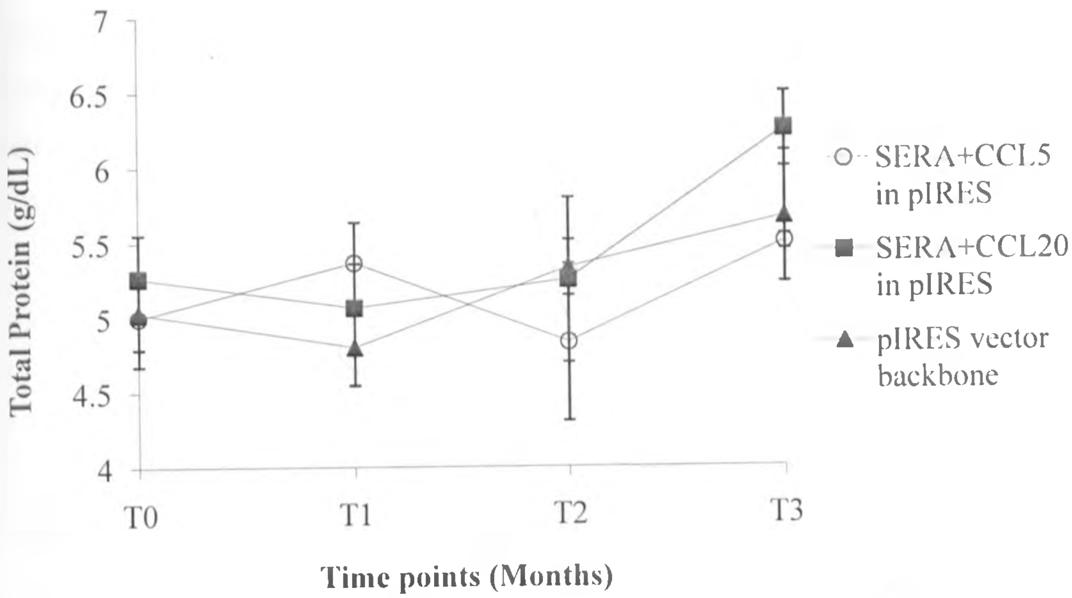

Figure 3.11: Mean Total protein levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.1.4.2 Renal Function Tests of Experimental Animals

To assess renal function, urea, creatinine, total bilirubin and direct bilirubin levels were assayed.

After T0, animals vaccinated with SERA+CCL5 in pIRES and pIRES vector backbone showed an increase in mean urea levels that decreased after T1. In SERA+CCL5 in pIRES group there was no change after T2 to before challenge infection but in the pIRES vector backbone group an increase was observed after T2 to T3. In the SERA+CCL20 in pIRES group, an increase was observed after T0 to T2 that decreased before challenge (Figure 3.12). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The differences at T0 were considered not quite statistically significant (p = 0.080) but in T1 it were statistically significant (p = 0.025) at 95% confidence limits.

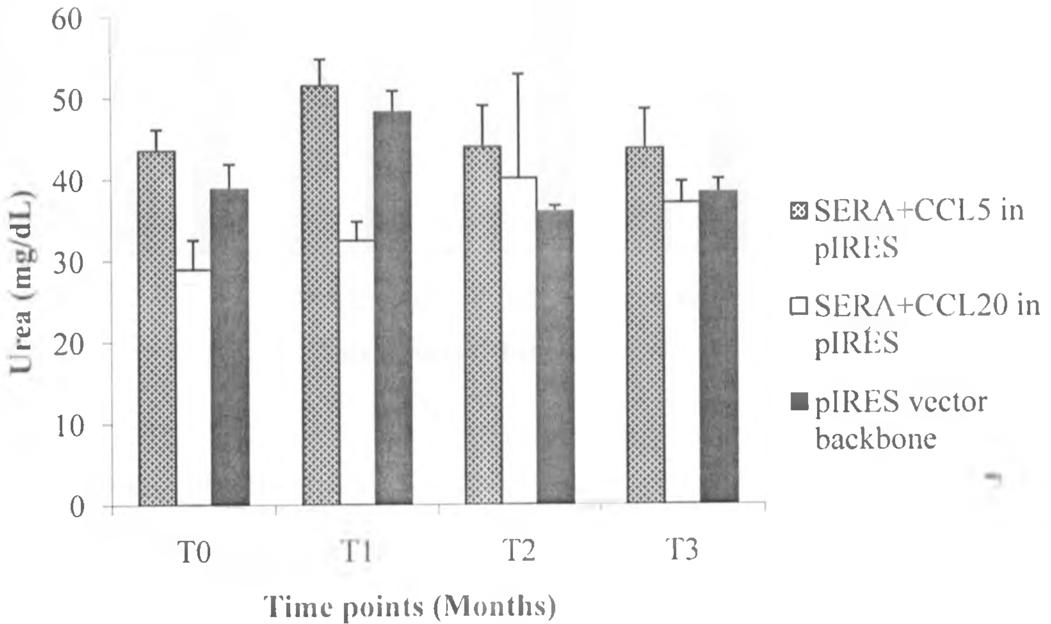

Figure 3.12: Mean Urea levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In SERA+CCL20 in pIRES and pIRES vector backbone groups, an increase in the mean creatinine levels was observed after T0 that decreased after T1 and later increased before challenge infection. In the SERA+CCL5 in pIRES group, an elevation in mean creatinine levels was observed from after T0 to T2 that later decreased before challenge (Figure 3.13). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

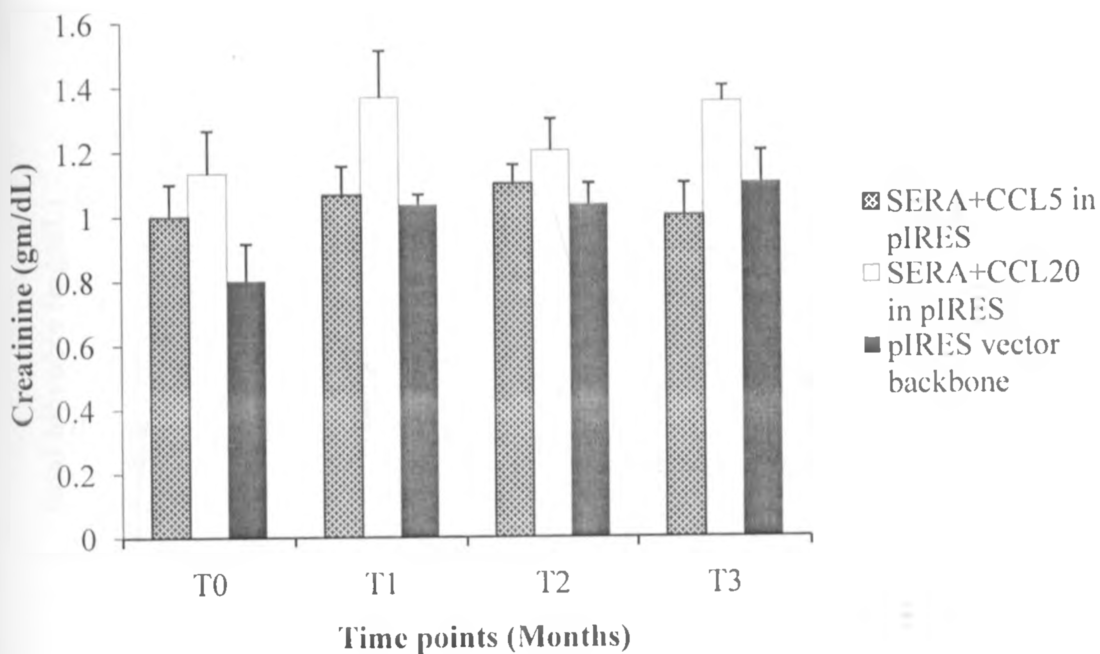

Figure 3.13: Mean Creatinine levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

After T0, in all the groups an elevation in mean total bilirubin levels was observed. In animals vaccinated with SERA+CCL20 in pIRES and pIRES vector backbone showed a decrease after T1 that later was elevated before the before challenge infection. In SERA+CCL5 in pIRES group, an elevation was observed after T0 that remained elevated to T2 and decreased after T2. (Figure 3.14). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

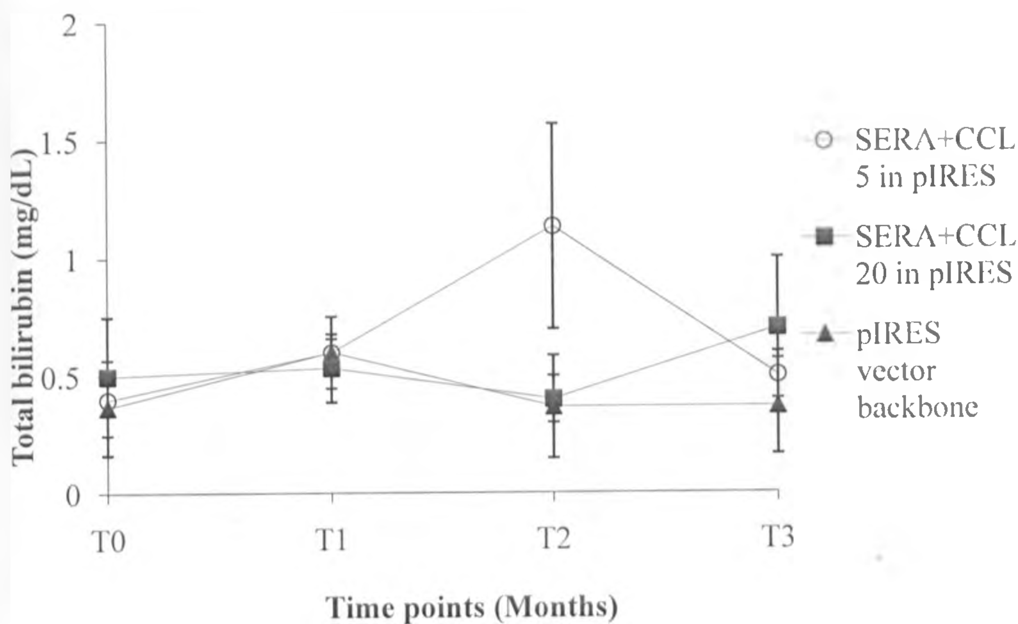

Figure 3.14: Mean Total bilirubin levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

After T0, an increase in mean direct bilirubin levels was observed in animals vaccinated with SERA+CCL5 in pIRES and pIRES vector backbone. That later decreased after T1 in both groups and eventual elevated after T2 to before challenge infection. In the SERA+CCL20 in pIRES group, a decrease was observed after T0 and an elevation after T1 that later decreased before T3 (Figure 3.15). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

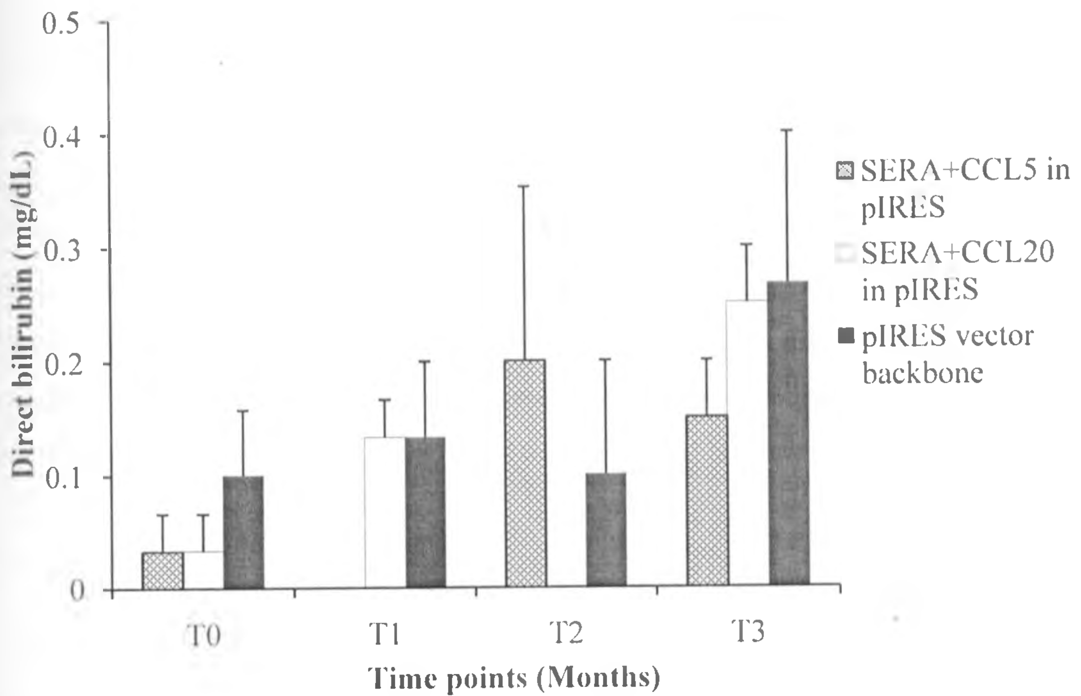

Figure 3.15: Mean Direct bilirubin levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.2 Immunogenicity

Immunogenicity of SERA in immunized baboons was determined through conducting of ELISA using the collected peripheral blood. For humoral responses serum was assayed for anti recombinant BK-SE36 IgG antibody titres. The cellular immune responses were determined by the cellular phenotypes and populations of lymphocytes in the collected peripheral mononuclear cells.

3.2.1 Antibody Responses in Immunized Baboons

For humoral immune responses, antibody titres were determined by ELISA. M ean IgG responses to BK-SE36 showed an increase in all the groups from the T0 in all groups throughout the experimental period. In SERA+CCL20 in pIRES group, there was a decrease after the T1 that later increased after T3 (Figure 3.16). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were statistically significant ($p < 0.05$) at 95% confidence limits.

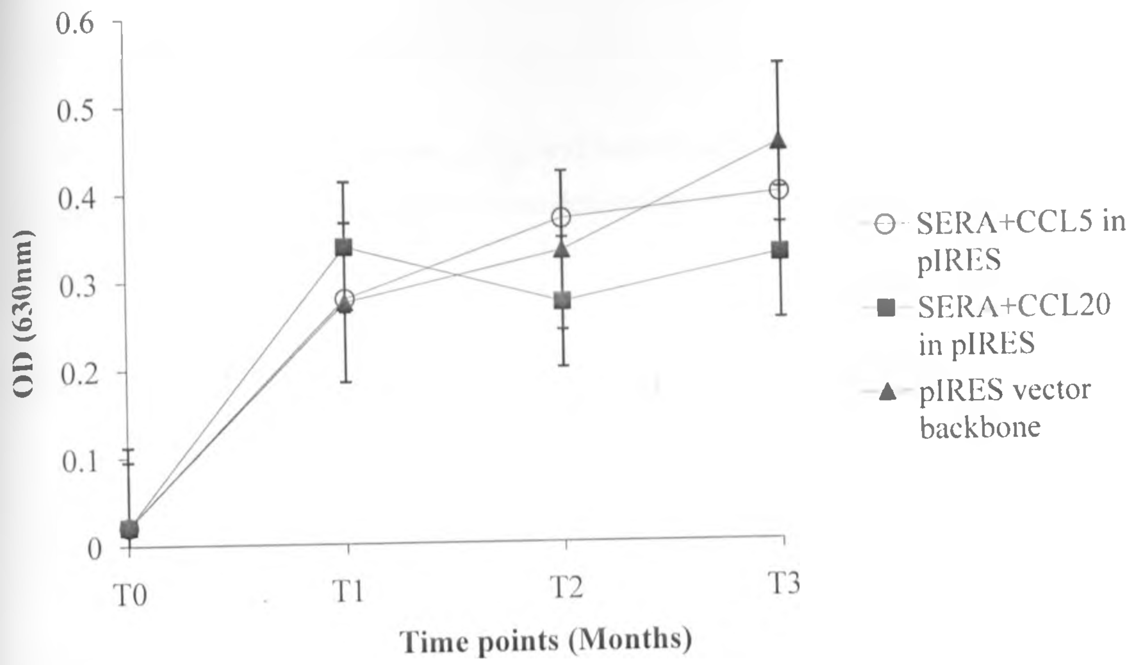

Figure 3.16: Mean anti BK-SE36 IgG levels of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.2.2 Cellular immune responses

For cell mediated immune responses, using anti human CD3, CD4 and CD8 monoclonal antibodies, baboon cellular phenotypes were determined by fluorescence activated cell analysis.

3.2.2.1 $CD4^+$ T cells

In both SERA+CCL5 in pIRES and pIRES vector backbone groups, the $CD4^+$ T cell population increase was noted after T0. A decreased was observed in the SERA+CCL5 in pIRES group and an increase was noted in the pIRES vector backbone group after T1. In the SERA+CCL20 in pIRES group a decrease was noted after T0 that later increased after T1. In all the groups a decrease was noted after T2 (Figure 3.17). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The increases at T2 of vaccination were statistically significant ($p < 0.05$) at 95% confidence limits.

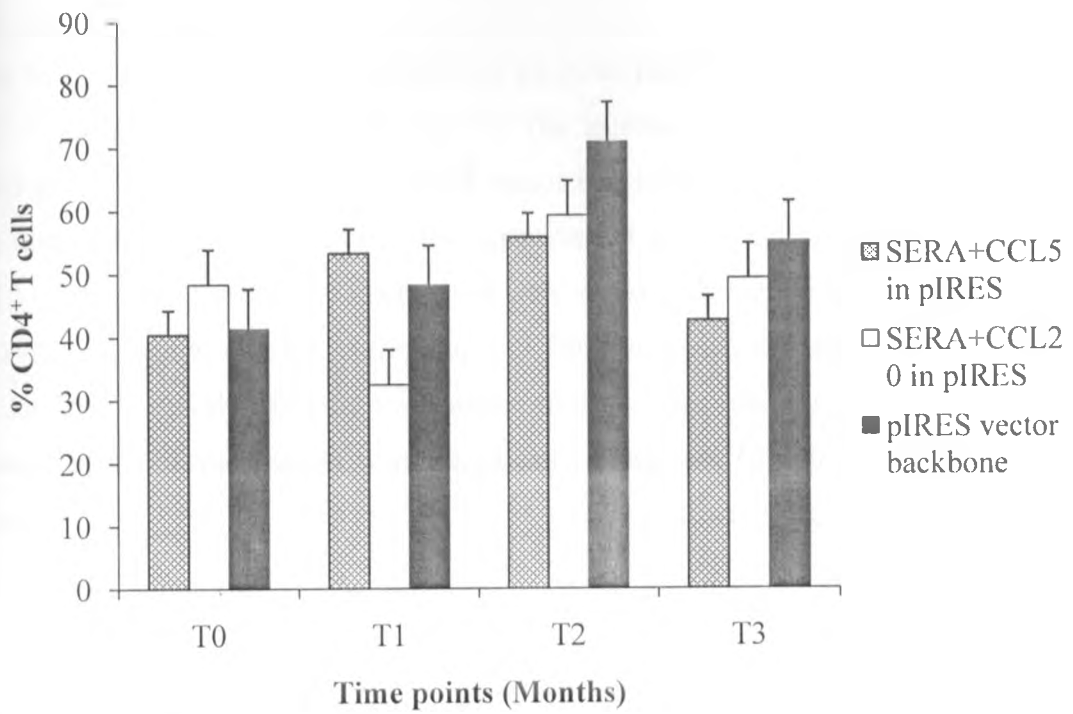

Figure 3.17: Mean percentage $CD4^{+}$ T cells in baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.2.2.2 $CD8^+$ T Cells

In the SERA+CCL5 in pIRES and SERA+CCL20 in pIRES groups an increase in the mean $CD8^+$ T cell population was noted after T0. The increase was observed in SERA+CCL5 in pIRES group until after T1. In the pIRES vector backbone group, a decreased was observed from after the T1 through T2. In all the experimental groups a decrease was observed after the T2 (Figure 3.18). At T2 the fluctuations were statistically significant ($p < 0.05$). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The increases at T2 of vaccination were statistically significant ($p < 0.05$) at 95% confidence limits.

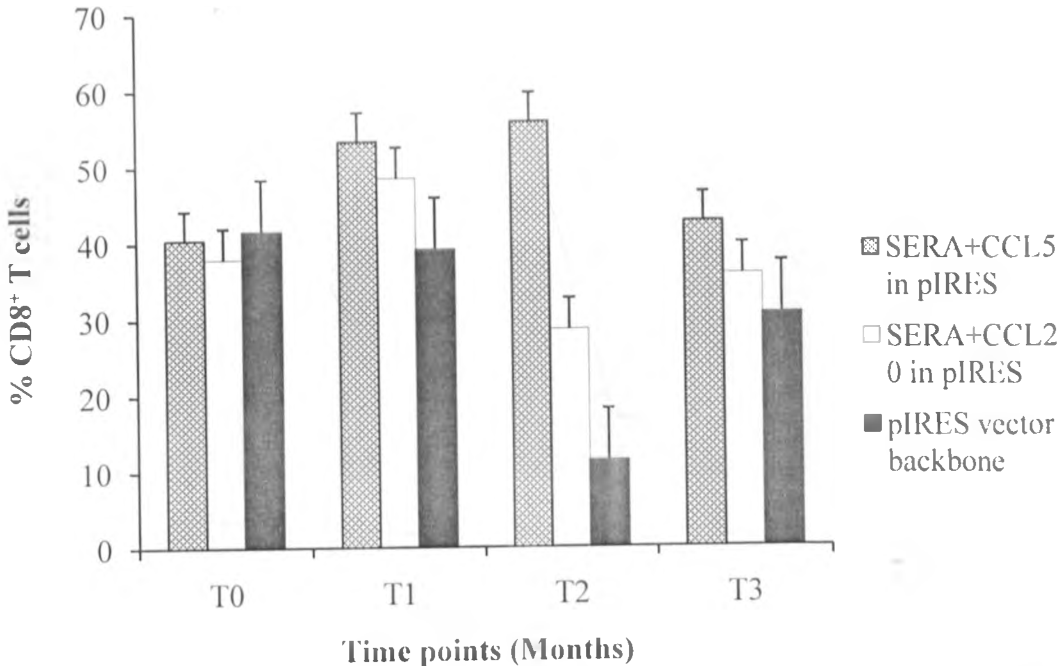

Figure 3.18: Mean percentage $CD8^+$ T cells in baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.2.2.3 $CD4^+CD8^+$ T Cells

In all the groups, an increase in the $CD4^+CD8^+$ T cell populations was observed after T0. A decrease was noted in the SERA+CCL5 in pIRES group at T1 that later increased after T2. In the SERA+CCL20 in pIRES and pIRES vector backbone groups an increase was noted after T1 and a decrease after T2 (Figure 3.19). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The increases at T2 of vaccination were statistically significant ($p < 0.05$) at 95% confidence limits.

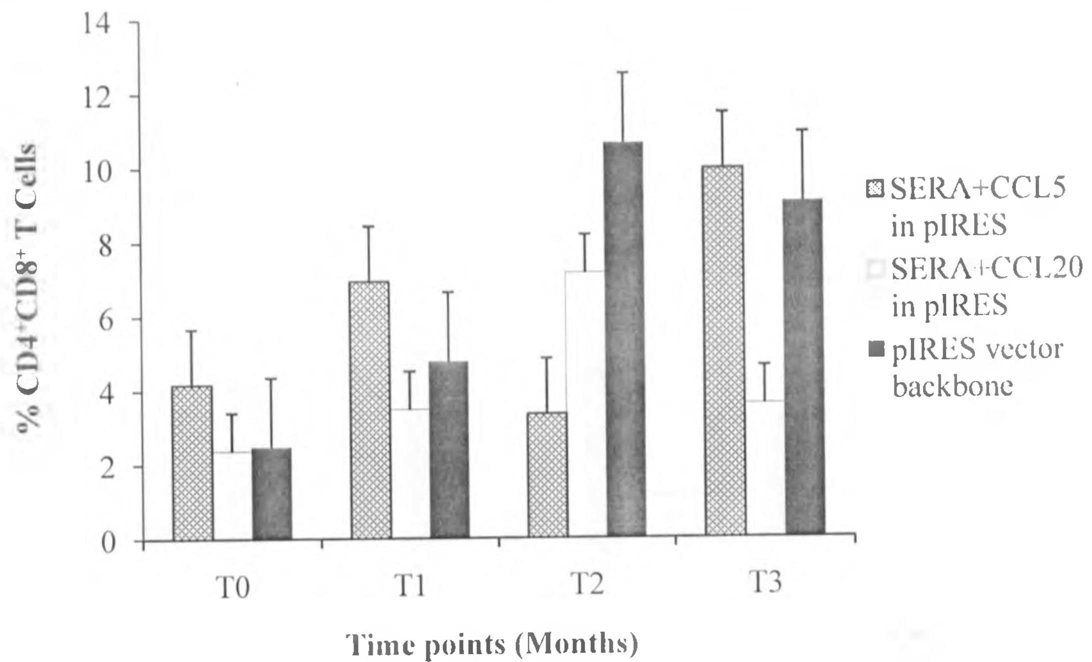

Figure 3.19: Mean percentage $CD4^+CD8^+$ T cells in baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.2.2.4 $CD4^-CD8^-$ T Cells

In all the groups except SERA+CCL20 in pIRES showed a decrease in the $CD4^-CD8^-$ cell population after T0. After T1 and T2 immunizations in all the groups showed a decrease of the $CD4^-CD8^-$ population (Figure 3.20). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases at T2 of vaccination were statistically significant ($p < 0.05$) at 95% confidence limits.

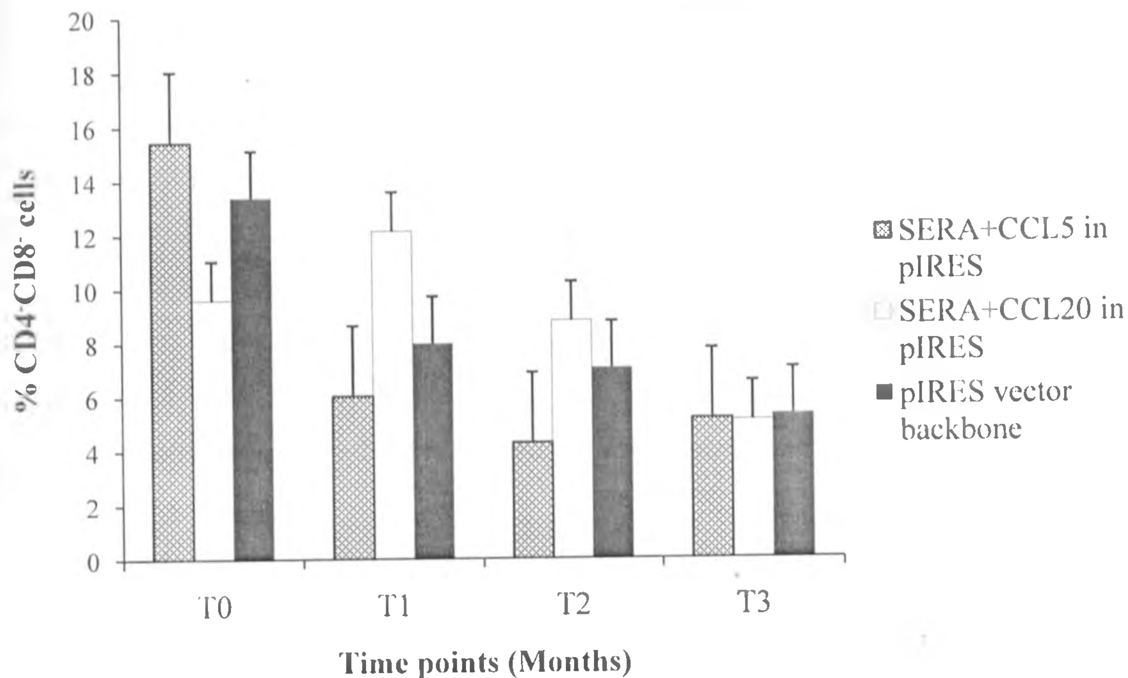

Figure 3.20: Mean percentage $CD4^-CD8^-$ T cells in baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

3.3 Cross Protective Efficacy

All baboons inoculated with 2×10^5 blood stage *P. knowlesi* H strain parasites developed patent parasitaemia by day 5 post-inoculation (Table 3.4).

Two way ANOVA and the student t-test were used to compare intra-group and intergroup mean parasitaemia respectively. Five animals had acute parasitaemia that systematically increased to greater than 500 parasites per 1×10^4 erythrocytes, reaching as high as 1898 parasites per 1×10^4 erythrocytes at the time of treatment. The remaining three animals developed chronic parasitaemia (Table 3.4) with peak levels less than 300 parasites per 1×10^4 erythrocytes at day 9, which therefore decreased to less than 155 parasites per 1×10^1 erythrocytes (Figure 3.21). All baboons with parasitaemia of greater than 500 parasites per 1×10^4 erythrocytes were treated with three doses of 1mg/kg body weight of pyrimethamine. Parasitaemia profiles of the infected baboons were not statistically significant ($p > 0.05$). Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination were not statistically significant ($p > 0.05$) at 95% confidence limits.

Table 3.4: Selected parameters of parasitaemia in baboons infected with *P. knowlesi* H strain

Animal ID	Vaccine construct	Peak parasitaemia [a]		Pre-patent period challenge [b]
		%P (Profile)[c]	Day	
PAN 3614	SERA+CCL5 in pIRES	15.27 (Acute)	6	4
PAN 3612	SERA+CCL5 in pIRES	8.30 (Acute)	6	4
PAN 3351	SERA+CCL20 in pIRES	6.30 (Acute)	8	3
PAN 3362	SERA+CCL20 in pIRES	1.39 (Chronic)	8	5
PAN 3200	pIRES vector backbone	18.98 (Acute)	10	6
PAN 3208	pIRES vector backbone	3.00 (Chronic)	9	4
PAN 3551	pIRES vector backbone	7.01 (Acute)	6	3

[a] The highest level of parasitaemia detected in 10 days the baboons were monitored before treatment.

[b] Period from day of challenge to when the day parasites were first detected in peripheral circulation by thin smear method.

[c] Post-infection parasitaemia when the animal profile was scored acute or chronic.

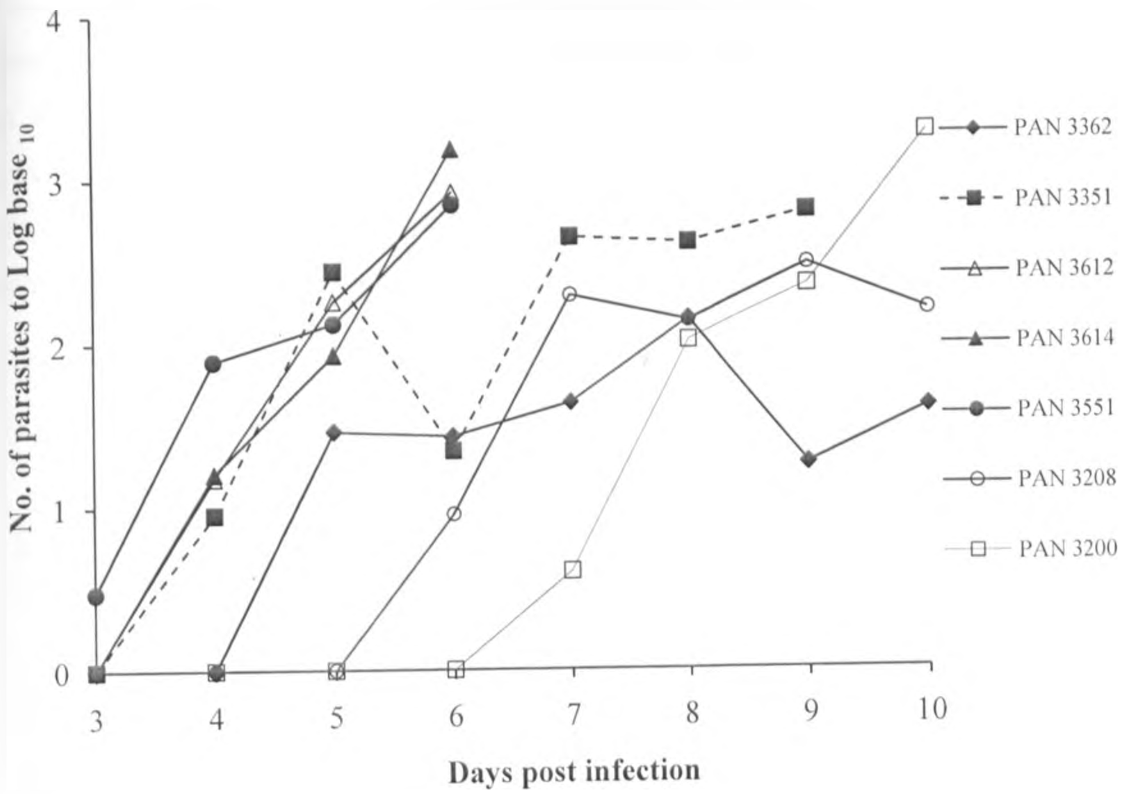

Figure 3.21: Parasitaemia profile of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

In comparison, animals vaccinated with SERA+CCL5 in pIRES developed highest mean parasitaemia of 1178.5 parasites per 1 x 10^4 erythrocytes than pIRES vector backbone group that showed parasitaemia of 1026.5 parasites per 1 x 10^4 erythrocytes (Figure 3.22). However, the comparative mean parasitaemia of the two groups were not statistically different ($p > 0.05$). Animals vaccinated with SERA+CCL20 in pIRES developed a chronic mean parasitaemia of 272.5 parasites per 1 x 10^4 erythrocytes.

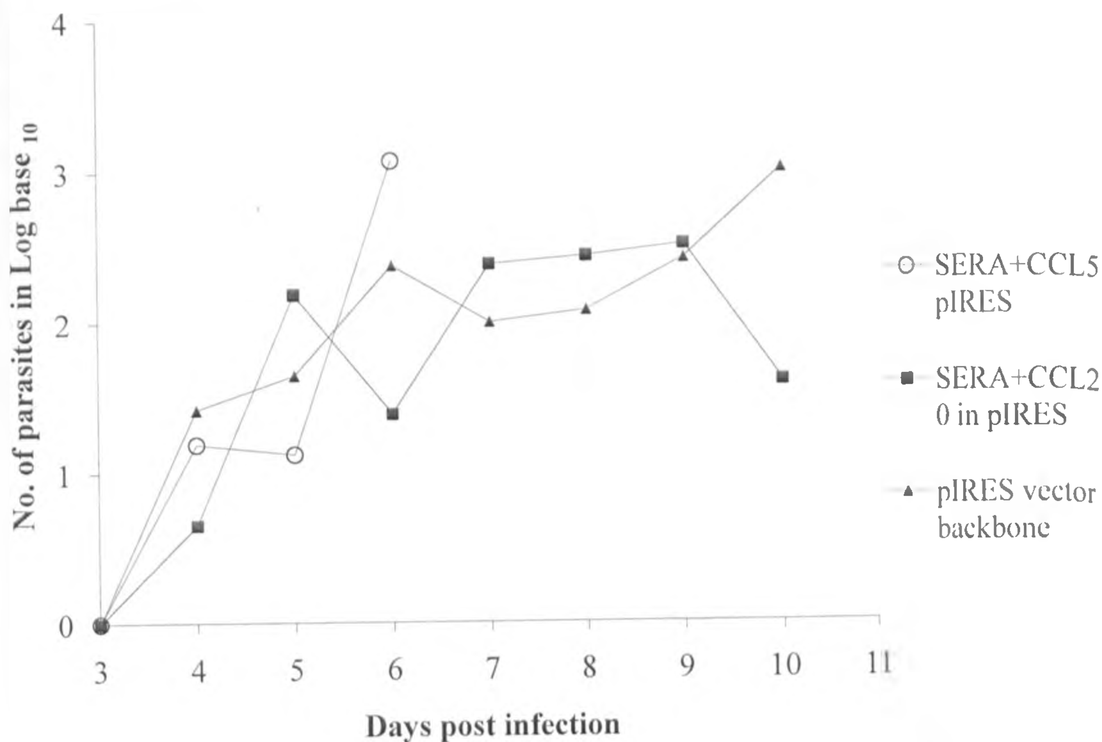

Figure 3.22: Comparative mean parasitaemia profiles of baboons vaccinated with SERA+CCL5 in pIRES, SERA+CCL20 in pIRES and pIRES vector backbone.

CHAPTER FOUR: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

In this thesis, the olive baboon *(P. anubis)* was used to assess the tolerability, safety, immunogenicity and cross protective efficacy induced by SERA DNA vaccine constructs formulated by combining SERA with CCL5 as an adjuvant in pIRES (plasmid Internal Ribosomal Entry Site), SERA with CCL20 as an adjuvant in pIRES plasmid and pIRES plasmid alone. The olive baboon is ranked highly in the primate phylogenetic tree compared with other non-human primates (NHPs) used in biomedical research. It is phylogenetically close to human with 98% protein homology (http://ca-biomed.org/links/factsheet/). It is also fully susceptible to experimental infection with *P. knowlesi* leading to either severe malaria or controlled parasitaemia that result in mild infection (Ozwara *et al.*, 2003). In the end, baboons were infected with *P. knowlesi* to understand cross-protection efficacy of the test vaccine combinations. *Plasmodium knowlesi* is an attractive experimental system for malaria research. This is because 1) it is a natural parasite of monkeys that have immune and metabolic systems very similar to those of humans (King, 1988) and *P. knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante *et al.*, 1998) and 2) it also infects humans leading to a severe life threatening infection (Cox-Singh *et al.*, 2008, White, 2008).

The overall results obtained showed that SERA DNA vaccine constructs were safe, well tolerated, and no serious adverse events related to the vaccines occurred. However, they were immunogenic but unable to offer cross protection against *P. knowlesi in vivo.*

Results reveal that the haematological profiles, the clinical kidney and liver function parameters investigated in all baboons remained within the clinical normal ranges throughout the entire vaccination phase. Indurations, erythema, skin swelling, warmth, ulceration and

regional lymphadenopathy were not observed on the vaccination sites. Toxicities from amyloidosis, anaemia, neutropenia, hepatic failure, pulmonary failure, myocardial infarction, lethargy, fatigue and renal insufficiency were not detected. These implied that the vaccines were well tolerated and safe in the baboons. There were fluctuations in haematological, liver and kidney functions parameters but the values remained within normal clinical ranges. No detrimental changes were recorded on blood, liver and kidney functions. Together, these studies confirm that SERA DNA vaccine constructs were safe and tolerable in olive baboons. These results are in line with the work done by Horii and colleagues involving animal experiments using non-human primates, and a human phase 1a clinical trial assessing SE36 vaccine safety and immunogenicity of SE36 protein and aluminum hydroxyl gel (SE36/AHG). Subcutaneous vaccinations were well tolerated; with no significant safety issues identified, no serious adverse events observed and all adverse events (AEs) resolved. The predominant adverse event was erythema and indurations at the administration site. To a lesser degree itchiness, fever, and fatigue were observed as systemic symptoms. Events were all mild and remitted, suggesting that these were not clinically problematic (Horii *et al.*, 2010).

Naturally acquired protective immunity against blood stage malaria involves both antibodies and $CD4^+$ T cells (reviewed in Langhorne *et al.*, 2009). For the *P. falciparum* vaccine RTS,S both anti-CSP antibody levels and T cell responses have been partially correlated with protection (Kester *et al.*, 2009, Sun *et al.*, 2003). T cell responses have been associated with the control of blood-stage malaria in mice and humans (Goodman *et al.*, 2010). From the $CD4^+$ T cell response in the pIRES vector backbone alone had a general trend towards higher immune responses than groups immunized with the SERA+CCL5 in pIRES and SERA+CCL20 in pIRES. Two way ANOVA was used to compare intragroup and intergroup means. Statistical significance was calculated by Kruskal Wallis for comparison of more than two non-parametric means. The decreases and increases at various time points of vaccination did not show statistical significance ($p > 0.05$) at 95% confidence limits. These created the assumption that either the pIRES vector backbone contained CpG motifs or other immunogenic epitopes. The $CD4^+$ T cell response peaked with the second immunization in the majority of monkeys. Surprisingly, the $CD4^+$ T cell response decreased significantly after

the third immunization. It is unclear why there was a reduction in the number of $CD4^+$ T cells in circulating blood after the final dose of vaccination, but the observed reduction is consistent with the preclinical evaluation of the safety and immunogenicity of a vaccine consisting *P. falciparum* liver-stage antigen 1 with adjuvant AS01B vaccine in rhesus monkeys (Pichyangkul *et al.*, 2008). A direct role of activated $CD4^+$ T cells against infected liver cells is possible, as several cell types present in the liver, such as kupffer cells, liver dendritic cells, endothelial cells and hepatocytes themselves can express MHC Class II molecules necessary for antigen presentation to $CD4^+$ T cells. $CD4^+$ T cells, through the expression of TNF-α and IFN-γ, could contribute to the elimination of intracellular *Plasmodia*, or through other yet uncharacterized effector mechanism(s). CD4-derived IL-2 could also help NK or $CD8^+$ T cells to clear parasites as has been shown in blood-stage infection. Whether or not they display intrinsic protective effector functions, $CD4^+$ T cells are likely to contribute to antibody production (Ansong *et al.*, 2011).

From the $CD8^+$ T cell response results obtained there was a trend towards higher immune responses in groups that were immunized with the SERA+CCL5 in pIRES after the third immunization. In SERA+CCL20 in pIRES group it was higher after the first immunization as compared to those that received pIRES vector backbone alone across the experimental period. However, the differences were not statistically significant in majority of the monkeys, the $CD8^+$ T cell response peaked with the first immunization. Surprisingly, the $CD8^+$ T cell response decreased significantly after the second immunization in baboons vaccinated with SERA+CCL20 in pIRES and pIRES vector backbone. It is unclear why the number of $CD8^+$ T cells in circulating blood decreased after this second dose of vaccination. Though we presume that the effect may be linked to the anaesthesia used for ketamine dampens cellular immunity in postoperative procedures (Beilin *et al.*, 2007). After the last dose of immunization there was an increase in $CD8^+$ T cell responses in baboons immunized with SERA+CCL20 in pIRES and pIRES vector backbone. The discovery that $CD8^+$ T cells mediate significant antiparasitic activity against the liver stage of *P. yoelii* provides an argument that similar mechanisms may occur in human *P. falciparum* malaria (Goodman *et al.*, 2010) . Further suggestion of the role of cellular immunity in protection against *P. falciparum* comes from those studies in humans in which protective immunity has been

associated with significant cellular immune responses to blood-stage parasites, in the absence of strong blood-stage antibody responses (Goodman *et al.*, 2010). The $CD4^{+}CD8^{+}$ T cell responses peaked after the second immunization in baboons vaccinated with SERA+CCL20 in pIRES. In the SERA+CCL5 in pIRES the response peaked after the third immunization. In the control groups where animals were vaccinated with pIRES vector backbone alone had low responses throughout the experimental period as compared to the experimental animals.

The specific total IgG antibodies against recombinant constructs of SERA5 as shown (figure 4.16), were higher after the third immunization comparable to the second immunization. Recombinant proteins corresponding to SERA 47 kDa domain conferred protective immunity in *Aotus* and squirrel monkeys against parasite challenge (Inselburg *et al.*, 1991; 1993; Suzue *et al.*, 1996). Epidemiological study in a holo-endemic area of Uganda revealed that increased level of IgG to 47 kDa peptide correlates with lower parasitaemia in the peripheral blood and absence of fever in a group of children, but IgG levels against 50 kDa peptide does not (Banic *et al.*, 1998; Okech *et al.*, 2001). Therefore from previous studies it shows that the SERA DNA vaccine constructs expressing CCL5 and CCL20 as chemokine adjuvants are immunogenic and evoke acquired immune responses.

In malaria studies, parasitaemia monitoring is the gold standard for measuring the efficacy of a malaria vaccine candidate. In the reported study, animals inoculated with *P. knowlesi* showed patent parasitaemia by day 6 post-inoculation suggesting that SERA DNA vaccine constructs in pIRES did not delay to time in parasitaemia against *P. knowlesi* blood stage parasites. Comparative mean group parasitaemia analysis of animals vaccinated with SERA+CCL20 in pIRES implied that this vaccine had some anti-plasmodial activity. This was confirmed by regulated parasitaemia and low group parasitaemia. However, these differences were not strong as shown by inadequate statistical significance. Further increase in individual animal parasitaemia profiles rules any anti-plasmodial effect of the vaccines. This final individual parasitaemia profile and subsequent disease progression is consistent with untreated with malarial infection in olive baboons (Ozwara *et al.*, 2003).

Ozwara and colleagues (2003) inoculated 10 olive baboons with *P. knowlesi*. The animals

developed patent parasitaemia by day 5 post-inoculation. Seven animals had acute parasitaemia but systematically increased to greater than 500 parasites per 1 x 10^4 erythrocytes, to reach as high as 4,950 parasites per 1 x 10^4 erythrocytes at the time of killing. All baboons with acute infection had become lethargic by day 12 post-infection. The remaining three animals developed chronic parasitaemia with peak levels of less than 300 parasites per 1 x 10^4 erythrocytes by day 16, which thereafter decreased to less than 50 parasites per 1 x 10^4 erythrocytes. In the present study, 5 animals developed acute parasitaemia while 2 had chronic suggesting normal knowlesi malaria progression profile (Ozwara *et al.*, 2003).

The malarial infection was either severe (acute parasitaemia) or mild (chronic parasitaemia). Chronically infected animals appeared to control parasitaemia. This is also seen in other non-human primates (Ibiwoye *et al.*, 1993). The mechanisms that predispose *P. knowlesi*-infected non-human primates to develop either severe or mild infection are unknown. In general, *P. knowlesi* produces chronic self regulating infection in the natural host, *Macaca fascicularis*. However, the course of *P. knowlesi* infection can differ in *M. fascicularis* from different geographic regions (Schmidt *et al.*, 1977). In this study, animals originated from the same area, thus excluding baboon origin as a factor involved in the different infection outcomes. Like humans infected with *P. falciparum*, baboons infected with *P. knowlesi* developed clinical symptoms at the onset of parasitaemia.

In the face of the obtained results, SERA DNA vaccine constructs formulated by combining SERA in pIRES (plasmid Internal Ribosomal Entry Site), SERA with CCL5 as an adjuvant in pIRES plasmid, SERA with CCL20 as an adjuvant in pIRES plasmid and pIRES plasmid backbone have met crucial development requirements of being safe, tolerable and immunogenic in non human primates as shown during this *in vivo* study. However, cross-protection efficacy against *P. knowlesi in vivo* requires further investigation. This study has provided new information on the safety, immunogenicity and cross protective efficacy against *P. knowlesi in vivo* at a medium dose of 1 mg/ml used in the olive baboons as a blood stage vaccine.

In conclusion, it is important to test new adjuvant formulations capable of inducing strong humoral and cellular immune responses that are suitable for human use. This study demonstrates for the first time that the malaria vaccine candidate SERA DNA co-expressed with CCL5 and CCL20 as chemokine adjuvants is safe with no significant local or systemic adverse safety as assessed by general biochemical analysis, as well as haematological assays, and is capable of eliciting antibody, T cell responses and anti SERA5 antibodies in the baboon model hence it can be used to immunomodulate responses to malaria vaccines.

The current research results provide an initial preclinical validation for the future use of olive baboons as experimental models for evaluating safety and immunogenicity of malaria blood stage vaccines.

4.2 CONCLUSIONS

2.2 At a DNA medium dose of 1mg/ml used in this study, SERA DNA vaccine constructs formulated by combining SERA with CCL5 as an adjuvant in pIRES (plasmid Internal Ribosomal Entry Site), SERA with CCL20 as an adjuvant in pIRES plasmid and pIRES plasmid backbone alone showed that:

- *Plasmodium falciparum* SERA DNA vaccine co-expressed with CCL5 as an immunomodulatory chemokine adjuvant was safe and tolerable in the baboon
- *Plasmodium falciparum* SERA DNA vaccine co-expressed with CCL20 as an immunomodulatory chemokine adjuvant was safe and tolerable in the baboon
- Host and cellular immune responses were elicited by SERA DNA vaccine co-expressed with immunomodulatory chemokine adjuvant CCL5 and CCL20 in the baboon
- *Plasmodium falciparum* SERA DNA vaccine co-expressed with either CCL5 or CCL20 as immunomodulatory chemokine adjuvant does not confer cross protection against *P. knowlesi* malaria infection in the baboon model of malaria

4.3 RECOMMENDATIONS

Based on this study, it is recommended that;

- Further studies should be conducted with a large number of baboons to conclusively evaluate the effect of chemokine adjuvants on enhancing immunogenicity of the SERA DNA vaccine.
- The collected samples should be assayed for SERA proteins or precursor molecules, CCL5 and CCL20 chemokine adjuvants to determine whether the DNA construct was transcribed *in vivo*.
- The pIRES vector backbone should be analysed for CpG motifs or other immunogenic epitopes.

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