UNIVERSITY OF NAIROBI

Effect of chemokine adjuvants on immunogenicity and cross-protective efficacy of serine repeat antigen (SERA) DNA vaccine candidate against *Plasmodium berghei* in mice

RUTH MWENDE MUMO

A research project thesis submitted to the School of Medicine, Department of Biochemistry, University of Nairobi in partial fulfilment of the requirement for the award of the degree of Master of Science in Biochemistry.

2013

DECLARATION

This thesis has not been submitted for award of a degree in any other university

Ruth Mwende Mumo
Reg. No.: H56/76587/09

Signature: ……. Date: ……

With our approval as supervisors:

Prof. Charles Omwandho,
Department of Biochemistry,
University of Nairobi.
NAIROBI.

Signature: ……. Date:……

Dr. Hastings Ozwara,
Department of Tropical and Infectious Diseases,
Institute of Primate Research,
NAIROBI.

Signature: ……. Date: ……

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who made it possible to complete this thesis. I want to firstly thank my project supervisors, Dr. Hastings Ozwara of the Institute of Primate (IPR) Research and Prof. Charles Omwandho of the University of Nairobi, for their guidance in ensuring the work was done well. I thank the malaria team at IPR Margaret Mendi, Faith Onditi, Esther Kagasi, Wycliffe Onkoba and the late Maina Ichagichu for their great assistance during sample collection, processing and analysis. Special thanks to Tom Adino for his invaluable assistance with animal handling and Dr. Thomas Egwang' and Med Biotech Laboratories, Kampala for availing the research funds from World Bank through the Uganda National Council of Science and Technology and providing the DNA vaccine constructs. I also wish to acknowledge my family and friends for their unwavering support and encouragement. Last, but most especially, I am grateful to God for seeing me through this project. May God bless you.

DEDICATION

I dedicate this work to my parents, Mr. and Mrs. Mumo, my brothers Muthoka and Kambale and my best friends Lucy, Isabel and Kevin.

Table of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CCL5	Chemokine (C-C motif) ligand 5
CCL20	Chemokine (C-C motif) ligand 20
CSP	Circumsporozoite protein
DALYs	Disability Adjusted Life Years
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
GDP	Gross Domestic Product
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GNP	Gross National Product
HBsAg	Hepatitis B Surface Antigen
HRP	Horseradish peroxidase
HSV-1	Herpes Simplex Virus 1
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL	Interleukin
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
LLINs	Long-lasting Insecticidal Nets
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
pIRES	Internal Ribosome Entry Site plasmid
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RPMI	Rosswell Park Memorial Institute
RT-PCR	Reverse Transcriptase PCR
RTS,S	Central repeat region of CSP, T-cell epitopes of CSP and HBsAg combined in a single fusion protein (RTS) co-expressed with free HBsAg
SERA	Serine Repeat Antigen
SERP	Serine Rich Protein
TE	Tris EDTA
TMB	Tetramethylbenzidene
TNF-α	Tumor Necrosis Factor alpha
WHO	World Health Organization

ABSTRACT

Malaria remains a major global health challenge with more than 300 million reported clinical cases and between one and three million deaths per year despite concerted efforts to disrupt the mosquito-*Plasmodium*-human triad. This has prompted interest in developing a safe, efficacious, affordable and accessible vaccine to complement other malaria control measures. *Plasmodium*-derived antigens expressed during the asexual blood stage, such as serine repeat antigen (SERA), are viable vaccine candidates. It has been shown that antibodies raised against SERA inhibit growth of malaria parasites *in vitro*. However, there is a need to come up with safe and appropriate adjuvants to improve immunogenicity of such subunit vaccines. In this study the effect of CCL5 and CCL20 as adjuvants, on immunogenicity and cross-protective efficacy of SERA DNA vaccine was evaluated in addition to their safety, in a murine malaria model. BALB/c mice (N=132) were randomly distributed in six (6) groups which were treated as follows; SERA only (n=24), SERA+CCL5 (n=24), SERA+CCL20 (n=24), pIRES plasmid backbone (plasmid control) (n=24), Tris EDTA buffer pH 7.2 (buffer control) (n=24). The remaining 12 mice were used for pre-immunization baseline data (n=6) and non-vaccinated controls (n=6). Mice were injected with 100µg of DNA intramuscularly into each anterior quadriceps muscle in three doses at 3-week intervals (days 0, 21, and 42). Immunization did not elicit any vaccine related adverse reactions at the injection site. Low cytokine and recall responses were observed from ELISA and mononuclear cell proliferation assays respectively. Three weeks after the last immunization, mice were infected with *Plasmodium berghei* blood stage parasites to determine cross protective efficacy. All mice developed patent parasitaemia with the SERA+CCL5 group exhibiting parasitaemia suppression of upto 68.69% and all of the SERA+CCL20 group surviving upto 9 days post-infection. These findings show cross-protection of *Pf*SERA in addition to illustrating potential of immunomodulatory molecules such as CCL20 and CCL5 in improving protection conferred through DNA vaccines while maintaining their safety.

CHAPTER ONE: INTRODUCTION & LITERATURE REVIEW

1.1 Malaria

Malaria is an ancient disease and references to what was almost certainly malaria occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC. Early Greeks, including Homer in about 850 BC, Empedocles of Agrigentum in about 550 BC and Hippocrates in about 400 BC, were well aware of the characteristic poor health, malarial fevers and enlarged spleens seen in people living in marshy places. Scientific studies only became possible after discovery of the parasites by Charles Louis Alphonse Laveran in 1880 and incrimination of mosquitoes as the vectors, first for avian malaria by Ronald Ross in 1897 and later for human malaria by Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900 (Cox, 2010).

Malaria is caused by obligate intracellular apicomplexa parasitic protozoa that are members of the genus *Plasmodium* which contains 172 species that infect birds, reptiles, and mammals. It is transmitted via the bite of an infected female *Anopheles* sp mosquito (Leclerc *et al.*, 2004). Five species of *Plasmodium* commonly infect humans namely: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi.* Although each type of infection causes debilitating febrile illness, only *P. falciparum* carries a substantial risk of death and accounts for majority of morbidity and mortality associated with malaria (Baird, 2005). In Africa, *P. falciparum* is transmitted by *Anopheles* mosquitoes, *Anopheles gambiae* and *Anopheles funestus* (Pierce and Miller, 2009).

Plasmodium malaria is a devastating human parasitic disease claiming numerous lives annually. Malaria has been estimated to represent 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats, after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%) (Engers and Godal, 1998). An estimated 216 million malaria cases were reported globally in 2010, 81% of which were in Africa, resulting in approximately 655,000 deaths, 91% of which were in Africa (WHO, 2011). The global population at risk of malaria is 3.3 billion people, translating to approximately 50% of the

world's population (WHO | Malaria, www.who.int/entity/mediacentre/factsheet/en Retrieved 24th August 2011).

Malaria burden is not evenly distributed with most deaths occurring in sub-Saharan Africa and Asia. Climatic features such as rainfall and humidity affect stability of transmission, with seasonal temperature variation being a predominant factor in explaining the geographical distribution of the disease (Sachs and Malaney, 2002). At the global level, malaria incidence is concentrated in the tropical and subtropical zones in five continents, the same geographical regions with high poverty levels (Worall, *et al*., 2005). The disease is endemic in 109 countries, 45 of which are within the African continent, where it accounts for 20% of all childhood deaths, as illustrated in figure 1 below.

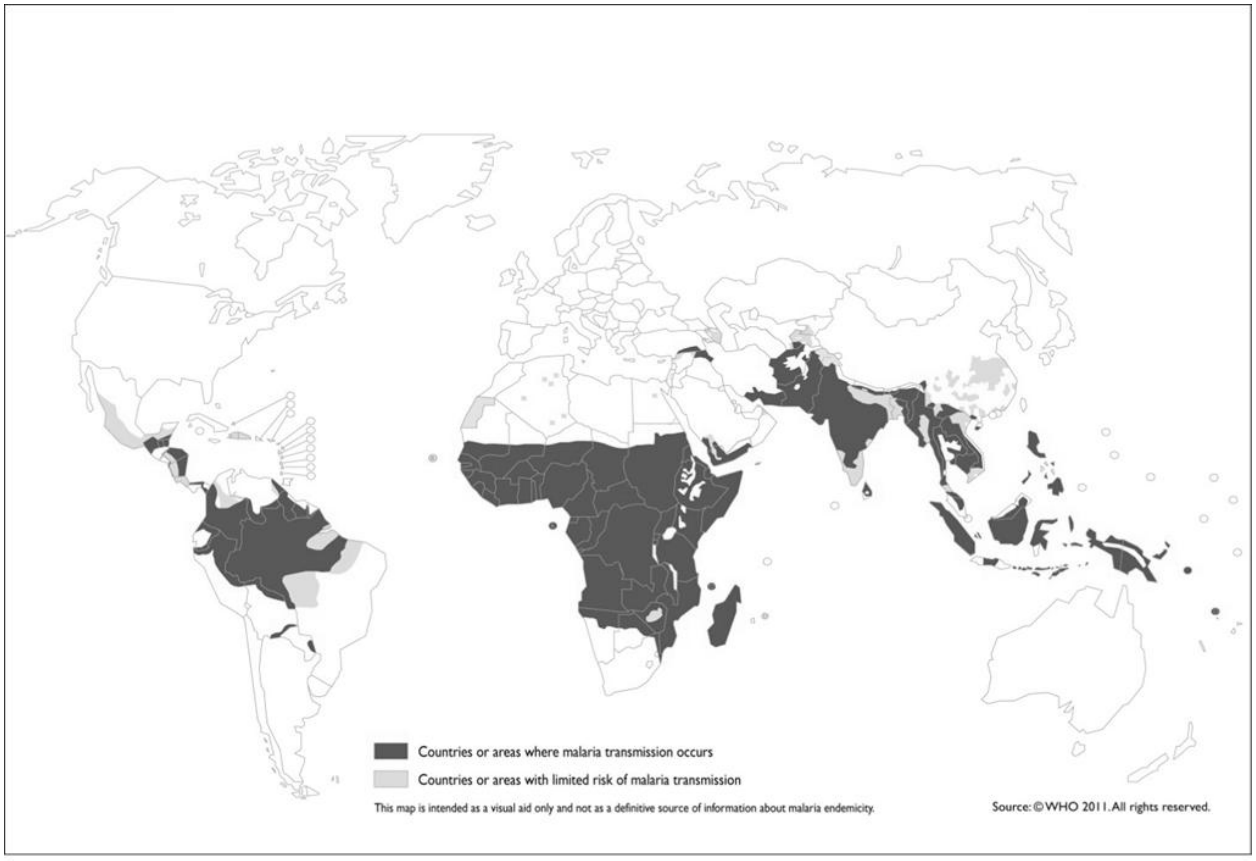

Figure 1: Areas at risk for transmission of malaria in 2010

More than 70% of Kenya's population live in areas where malaria is endemic, making malaria a leading cause of morbidity and mortality responsible for approximately 30% of out-patient visits, requiring more than eight million out-patient treatments each year, and 20% of all hospital admissions (Malaria control programme Kenya, Division of Malaria control, http://www.nmcp.or.ke, Retreived 5th July 2011). At least 14,000 children are hospitalized annually due to malaria, and there are an estimated 34,000 deaths among children under-five each year. An estimated 6,000 pregnant women suffer from malaria-associated anaemia annually, and 4,000 babies are born with low birth weight as a result of malaria associated maternal anaemia (USAID/CDC President's Malaria Initiative, 2010).

The enormous impact of malaria on mortality in humans can readily be appreciated by considering the high prevalence of the haemoglobin S allele (Hb-S) in Africa. Despite the fact that Hb-S homozygosity causes sickle cell anaemia and death in children, Hb-S is maintained at a gene frequency of nearly 18% in West Africa because in the heterozygous state, Hb-SA confers protection against severe malaria. In essence, natural selection demonstrates the burden of the disease, where the chance of death from malaria was so high as to justify welcoming a potentially fatal mutation into the gene pool (Pierce and Miller, 2009). Given this evolutionary backdrop, it would be surprising if the economic and demographic toll of malaria were not comparable. In fact, a comparison of income in malarious and non-malarious countries indicates that average GDP (adjusted to give parity of purchasing power) in malarious countries in 1995 was US$1,526, compared with US$8,268 in countries without intensive malaria; a more than fivefold difference (Sachs and Malaney, 2002).

The economic burden of malaria to a country, the family and individuals is immense. This has been estimated to cause a reduction of 1.3% in annual per capita economic growth in malaria endemic countries and the long term impact of this is a reduction of the GNP by more than a half (WHO, 2005). Prevention and treatment consumes scarce household resources; the burden on the public health sector impacts allocation of government resources; child health, school attendance, performance and cognitive development reduces accumulation of human capital, thereby reducing long-term growth potential; while the burden on households and individuals is

substantial due to time spent caring for the ill or seeking care as well as direct reduction in well-being from sickness (Lutz, 2006).

The global estimate of direct losses due to malaria is USD 12 billion annually and approximately 35.4 million disability adjusted life years (DALYs) are lost in sub-Saharan Africa alone due to the mortality and morbidity of malaria. In the years 2009 and 2010, USD 5.335 billion and 6.180 billion, respectively were spent globally in direct costs for diagnosis, treatment and prevention of malaria (Fernando *et al.*, 2010).

1.2 Current control measures

Conventional approaches of combating malaria involve both prevention and treatment, directed at three targets namely, host, parasite and vector as presented on figure 2 below. The measures targeted at the vector, include:

i) Prevention of bites by mosquitoes using insecticide treated bed nets (ITNs) and more recently, long-lasting insecticidal nets (LLIN)

ii) Prevention of entry of the vector into the house through indoor residual spraying (IRS),

iii) Limitation of mosquito breeding through destruction of mosquito larvae using larvicides and draining swamps (Wellcome trust, http://malaria.wellcome.ac.uk/ 15th July 2011) and wearing protective clothing (Kakkilaya, 2010)

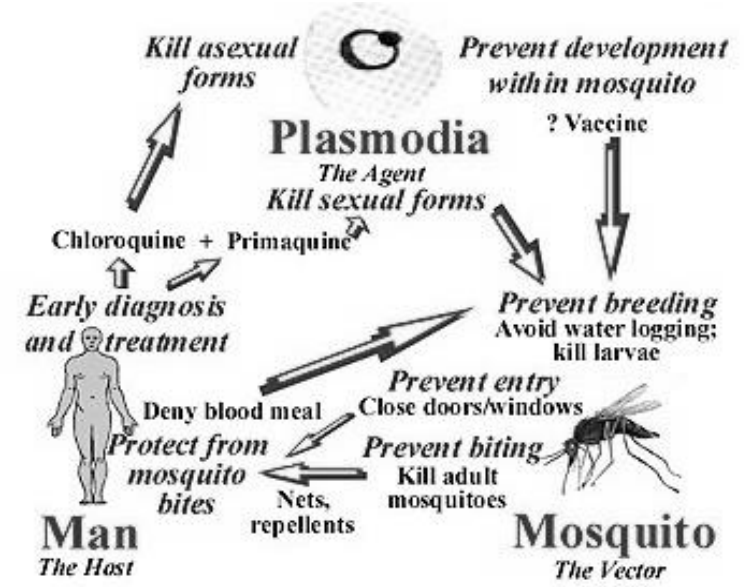

Figure 2: Control strategies for malaria (Kakkilaya, 2010)

IRS involves application of insecticides to the inner surfaces of dwellings, where endophilic *anopheline* mosquitoes often rest after taking a blood meal. An insecticide for IRS is usually selected on the basis of data on insecticide resistance to be effective. Other factors considered are residual efficacy of insecticide, cost, safety and the type of surface to be sprayed. Special attention is usually given to preserving susceptibility to pyrethroids, the only class of insecticide used on ITNs (WHO, 2009).

Treatment using antimalarial chemotherapy has been the primary option in the fight against malaria in many countries (Oyakhirome *et al*., 2007). Antimalarial drugs include chloroquine, amodiaquine (AQ), sulfadoxine-pyrimethamine (SP), quinine, mefloquine and artemisinin derivatives. Reduced susceptibility to chloroquine has been reported in Kenya, as is the case with SP. Resistance to SP has been associated with point mutations in the genes for enzymes involved in obligatory parasite-folate biosynthesis pathway (Mbugi, *et al.*, 2006). AQ is more effective than chloroquine. However, there is cross-resistance to amodiaquine in the chloroquine resistant strains. There have also been isolates with reduced susceptibility to mefloquine in west and central Africa with more pronounced resistance in the border areas between Cambodia, Myanmar and Thailand. The African strains still remain sensitive to quinine with decreasing sensitivity being reported in South-East Asia (RBM/WHO, 2001).

Ever since the discovery of the first case of chloroquine resistance along the Thai-Cambodian border in the late 1950s, Southeast Asia has played an important role as a focus for development of drug resistance to *Plasmodium falciparum* infection management. Although the first case of quinine resistance had been reported much earlier from South America, the onset of chloroquine resistance marked the beginning of a new chapter in the history of malaria in Southeast Asia and by 1973 chloroquine finally had to be replaced by the combination of sulphadoxine and pyrimethamine (SP) as first line drug for treatment of uncomplicated malaria in Thailand and more than 10 African countries also switched their first line drug to SP. In 1985, SP was eventually replaced by mefloquine (Farooq and Mahajan, 2004).

Rapid development of resistance to this new drug led to the introduction of artemisinin as a combination drug (ACT) in mid-1990s. However, resistance to ACT is already developing and although not yet fully characterized, there is evidence of decreased efficacy of the artesunate-mefloquine combination (Mugittu *et al.*, 2006, Laufer, 2009). Intermittent preventive treatment (IPT) is a control strategy in endemic areas that involves providing all pregnant women with at least two preventive treatment doses of an effective antimalarial drug during routine antenatal clinic visits. This approach has been shown to be safe, inexpensive and effective.

Traditional (herbal) medicines have also been used to treat malaria for thousands of years. This class of medicines is accessible and affordable to the rural-poor where health services are beyond reach. However, these herbal remedies have limitations. There is limited clinical data on safety and efficacy, in addition to having unpredictable interactions with biological pathways, with no consensus, even among traditional healers, on which plants, preparations and dosages are the most effective. Moreover, concentration of active ingredients in plant species varies considerably, depending on several factors including environmental factors and soils, among others (Willcox and Bodeker, 2004).

Despite these measures, the burden of malaria is still heavy mainly due to lack of an effective vaccine coupled with rampant drug resistance to effective affordable drugs. This has prompted the need to develop vaccines to complement the drugs in use. Various vaccine approaches have,

and are being explored including whole parasite and subunit vaccines. The different life-stages of the parasite have been studied in order to develop stage-specific vaccine candidates (Bourgon *et al.*, 2004).

1.3 Malaria vaccine development

Hope for vaccine development lies in the ability of *P. falciparum* infection to induce clinical immunity. In areas of intense *P. falciparum* transmission, immunity to severe, life-threatening disease is usually acquired (Crompton *et al.*, 2010). This acquired immunity however, tends to be short-lived, partially strain-specific and dependent on active exposure such that movement away from an endemic area reverses such acquired immunity. This is probably due the complexity of the parasite, which has epitopes which are immuno-dominant, sub-dominant, variant and conserved (Wykes and Good 2007). Further evidence to suggest that protective immunity to malaria is possible is that passive transfer of purified immunoglobulins from such "immune" individuals can protect children as demonstrated by transfer of sera obtained from "immune" African individuals to Thai children experiencing recrudescence of malaria infection (Webster and Hill, 2003).

A perfect malaria vaccine would induce lifelong sterilising immunity, provide cross-species immunity, be protective in the very young and be compatible with the expanded programme on immunisation (EPI) so that it could be administered as part of routine immunisation (Greenwood and Alonso, 2002).

The *Plasmodium* parasite shows great complexity with a genome bearing approximately 5400 protein-coding genes, presenting a myriad of antigens which vary throughout the different stages of its life cycle, and against which sequential consecutive immune responses are required (Holder, 2009). Exactly which of the antigens encoded by the *P. falciparum* parasite produces the key protective immune responses is not known, although some evidence implicates about 20 (Moorthy *et al.*, 2004). Moreover, many parasitic proteins exhibit high polymorphism, and a single parasitic clone may have up to 50 different copies of the gene coding for an essential protein, expressing a different version of such protein in each successive wave of parasitaemia. This antigenic variation appears critical for parasite's survival, and a disadvantage for the

infected individual and scientists aiming to design a vaccine (Aide *et al*, 2007). Thus currently there is no safe and efficacious malaria vaccine licensed for use in humans.

Three main approaches have been adopted for developing these malaria vaccines classified according to the life-cycle stage targeted. These categories are transmission blocking or "altruistic" vaccines (TBV), pre-erythrocytic vaccines (PEV) and blood stage or erythrocytic vaccines (EV).

By inducing antibodies against sexual stage antigens, TBV aim to prevent development of infectious sporozoites in the salivary glands of *Anopheles* mosquitoes. They are intended to protect communities from infection, by preventing transfer of parasites and spread of disease. Antibodies against sexual stage antigens do not however protect individuals from disease if already infected (Aide, *et al.,* 2007). This type of vaccines can be divided into two antigen target categories: (i) pre-fertilization antigens and (ii) post-fertilization antigens. Pfs48/45 and Pfs230 are pre-fertilization antigens found at the surface of the male and female gametes of malaria parasites while Pfs25 and Pfs28 are post-fertilization antigens. The major advantages of post-fertilization target antigens are the strong immunogenicity and limited pre-existing antigenic polymorphism (Kumar, 2007).

Pre-erythrocytic vaccines aim to protect against malaria infection and ideally should provide sterilizing humoral immunity by eliciting antibodies that target invading sporozoites and preventing them from invading the liver. Additionally, or as an alternative, the vaccine should induce a cell-mediated immune response able to inhibit sporozoite maturation (Girard *et al.*, 2007). It has been argued that a pre-erythrocytic vaccine would be valuable even if it did not completely protect against infection, as long as it reduced the number of parasites entering the blood stream and thereby lessened the risk of severe disease (Kwiatkowsky and Marsh, 1997).

One of the earliest proteins that were investigated was the circumsporozoite (CS) protein, found at the surface of the sporozoite, the initial *Plasmodium* stage in humans upon transfer from mosquito. This CS protein received positive attention due to sterile immunity generated from protective antibodies against CS proteins that acted against mosquito-injected sporozoite

challenges following vaccination using intact attenuated sporozoites. In addition to this, CD4+ and CD8+ cells generated could recognize and act against hepatocytes invaded by sporozoites (Vanderberg, 2009). Malaria vaccine candidates against pre-erythrocyte stages include circumsporozoite protein (CSP) positioned intra-vascularly, the liver stage antigens (LSA-1 and LSA-3) which are intra-hepatocytic, Thrombospondin-related anonymous protein (TRAP) found within micronemes of the mosquito sporozoite stage and CSP and TRAP-related protein (CTRP) (Chowdhury *et al.*, 2009).

Currently CSP-based sub-unit vaccine RTS,S/AS02A (recombinant hepatitis B surface antigen-circumsporozoite fusion protein formulated in a proprietary adjuvant, AS02A) is considered as being at the leading edge of malaria vaccine development and a potential vaccine candidate against *P. falciparum* (Chowdhury, *et al.*, 2009). RTS,S is a polypeptide corresponding to amino acids 207 to 395 of the CS protein from *P. falciparum* fused to hepatitis B surface antigen and expressed in the form of virus-like particles in yeast cells. Clinical studies are currently underway with reports of success rates of up to 50% being reported. While the performance of this vaccine candidate is good, it is still far from what an ideal vaccine should be which includes ability to protect up to 90% or more of the population. To this end, further research is on-going to identify better performing vaccine candidates.

Blood-stage vaccines have received most attention because symptomatic malaria is caused by blood-stage infection and acquired immunity in humans largely targets blood-stage antigens (Richards and Beeson, 2009). They are aimed at primarily protecting against severe malaria disease, and not against infection, on the assumption that inhibition of parasite invasion cycles will lead to reduced parasite burden and decreased morbidity and mortality (Girard *et al.*, 2007). Sometimes a distinction is made between strategies that are antiparasitic (aiming to inhibit parasite replication) as opposed to antitoxic (aiming to inhibit the harmful by-products of parasite replication). However, an effective blood-stage vaccine should limit parasite replication, whatever other effects it may have (Kwiatkowsky and Marsh, 1997).

Malaria vaccine candidates against erythrocyte stages have focused on antigens responsible for red cell invasion, antigens that are either expressed on or associated with the surface of the

merozoite. These are aimed at generating antibodies that block invasion and curtail parasite replication in blood, reducing the risk or severity of clinical illness. The merozoite surface protein 1, or MSP1, was the first and best characterized of many proteins on the merozoite surface that are being targeted for vaccine development. Others include, MSP2, MSP3, glutamate rich protein (GLURP). Other antigens are located in apical organelles such as apical membrane antigen 1(AMA1) which is thought to play a role in erythrocyte and hepatocyte invasion (Silvie, *et al.*, 2004). Naturally acquired antibodies to AMA1 inhibit erythrocyte invasion *in vitro* and are associated with protection in field studies (Hodder, *et al.,* 2001). Other antigens that are being considered under this category of vaccine candidates include erythrocyte binding antigen (EBA175), Ring-infected erythrocyte surface antigen (RESA), and serine repeat antigen (SERA) (Mahanty *et al.*, 2003, Jones and Hoffman, 1994).

Traditional vaccines were based on whole killed or attenuated microorganisms. Vaccination with radiation-attenuated sporozoites formed the starting point for investigations into vaccine development. Complete protection was achieved in humans against *P. falciparum* and *P. vivax* by exposing them to bites of mosquitoes that had been irradiated to attenuate their sporozoites. Though highly immunogenic and protective, whole cell vaccines are associated with reversal to pathogenesis, risk of contamination from the infectious material, storage problem, and batch to batch variation. Thus a shift to synthetic design and genetic-engineering of sub-unit vaccines (Uadia, 2007, Wykes and Good, 2007, Targett and Greenwood, 2008) is plausible.

Sub-unit vaccines are safer compared to traditional vaccines and pharmaceutical industry can mass-produce consistent formulations of these vaccines using Good Manufacturing Practice (GMP) (Wykes and Good, 2007). Unfortunately, they are known to be poorly immunogenic as they lack known pathogen-associated molecular patterns found in attenuated viral or bacterial vaccines to stimulate dendritic cells and initiate strong adaptive immune responses. It is therefore generally accepted that sub-unit vaccines for malaria will require potent adjuvants to enhance protective immune responses (Kurella, *et al.,* 2000, Coler, *et al.*, 2009, Othoro, *et al.*, 2009).

Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe, and immunogenic vaccines. For the majority of vaccine researchers, this information is not readily available, nor is access to well-

characterized adjuvants (Coler, *et al.,* 2009). The development of the RTS,S/AS02A vaccine demonstrated the critical importance of appropriate adjuvants and formulations for the efficacy of sub-unit vaccines. This vaccine, had failed to provide protection in numerous trials. However, formulation of that same antigen in a new adjuvant platform radically changed the outlook for this product, which has shown safety, immunogenicity, and induction of protection in challenge models with non-immune volunteers, hyper-immune adults, and semi-immune children living in malaria-endemic areas of Africa (Guinovart and Alonso, 2007, Girard, *et al.*, 2007).

DNA vaccines are a 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. They were introduced in 1990 through a study that demonstrated induction of protein expression upon direct intramuscular injection of plasmid DNA in myocytes (Ivory and Chadee, 2004). The historical basis for DNA vaccines rests on the observation that direct *in vitro* and *in vivo* gene transfer of recombinant DNA by a variety of techniques resulted in expression of protein (Gurunathan, *et al*., 2000). Genetic immunization is a novel vaccine strategy that conceptually combines some of the most desirable attributes of standard vaccine approaches (Shedlock and Weiner, 2000).

Vaccines made of DNA are being developed as a form of gene therapy that uses a patient's own cellular machinery to make foreign proteins that stimulate immune response. DNA-based vaccines are being considered due to their ease of production, low cost, long shelf-life, lack of requirement for a cold chain, and ability to induce both humoral and cellular immune responses. These stretches of 'naked DNA' contain genes that are expressed when DNA is taken up by muscle and other cells in the body (Tuteja, 2002).

There is still evidence of limited immunogenicity of plasmid DNA vaccines especially in larger animals such as non-human primates and humans. This results, at least in part, from rapid clearance of vaccine antigen expression by antigen-specific immune responses (Geiben-Lynn, *et al*. 2008). One of the approaches used to improve immunogenicity of such vaccines is the use of genetic adjuvants. Genetic adjuvants are expression vectors encoding biologically active molecules such as cytokines, chemokines and co-stimulatory molecules. These adjuvants can be

encoded on the same vector as the antigen or expressed on a separate vector and co-injected with the vaccine. This method provides adjuvant activity at the site of antigen production, with lasting effect from transfected cells (Ivory and Chadee, 2004).

1.4 *Plasmodium falciparum* SERA5 vaccine candidate

SERA5, also known as SERP and p126, is a 989 amino acid protein with a 35 serine-residues repeat. It is synthesized as a 120kDa precursor in late trophozoites, with a signal peptide that is cleaved upon translocation through the endoplasmic reticulum and then exported into the lumen of the parasitophorous vacuole (Fairlie, *et al.*, 2008). Supporting this is expression analysis that revealed SERA genes, particularly SERA5, are abundantly expressed in *P. falciparum* late trophozoites and schizonts, the parasite stages preceding parasite exit from host erythrocytes (Aoki *et al.*, 2002). Upon merozoite release, it is processed into a 47kDa (an N-terminal fragment), a 50kDa (an interior fragment that contains a strong homology to the active-site domain of cysteine/serine proteinases), an 18kDa C-terminal, and a 6kDa domain. The complex of 47 and 18kDa peptides is associated with merozoite (Belperron, *et al.*, 1999) as illustrated in figure 3 below.

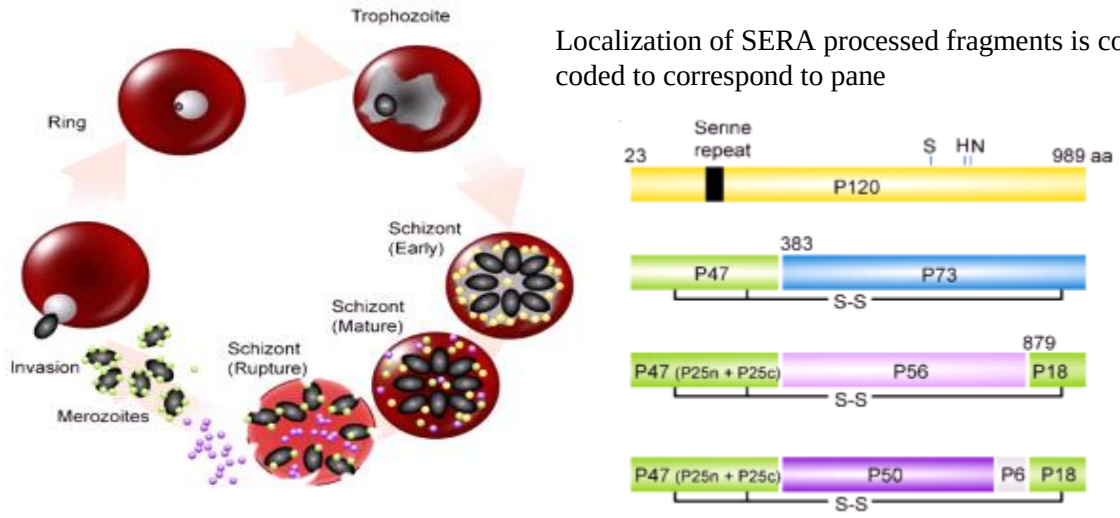

Figure 3: Presentation of SERA5 processing (Palacpac *et al.*, 2011)

A portion of the SERA protein remains unprocessed and associates as a non-integral membrane protein with the surfaces of free infectious merozoites indicated in Figure 4 below, with capacity

to bind to inner-leaflet erythrocyte plasma membrane phospholipids. The 120kDa SERA protein also associates with high-molecular-weight rhoptry proteins. These findings have resulted in the dual hypothesis that SERA is likely to play a role in parasite invasion (Aoki, *et al.*, 2002) and exit from infected host cells rendering it an attractive drug and vaccine target (Putrianti, *et. al*, 2010).

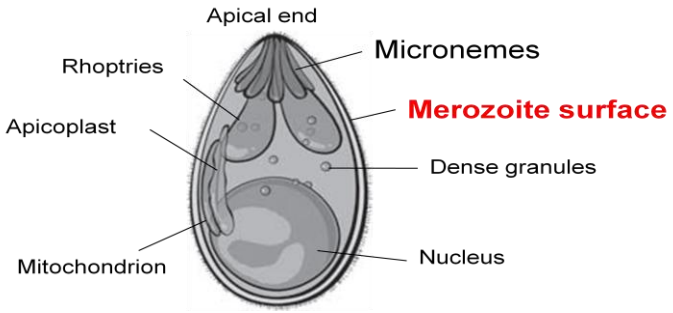

Figure 4: *Plasmodium falciparum* merozoite structure (Richards and Beeson, 2009)

SERA was originally identified by a mouse monoclonal antibody (immunoglobulin M [Ig M]) that inhibits parasite growth *in vitro*. Additional studies have shown that antibodies against *Pf*SERA5 inhibit parasite growth and parasite invasion *in vitro*. Polyclonal antibodies from rodents against amino acids 24 to 285 or 47kDa domain of the SERA protein have also shown to inhibit parasite growth *in vitro* (Pang *et al.*, 1999, Sato *et al.* 2005). The mechanism of this inhibition seems to result from agglutination of ruptured schizonts and merozoites in combination with complement-mediated lysis of late-stage schizonts, or inhibition of parasite invasion of erythrocytes.

Several lines of evidence suggest the importance of SERA in protective immunity to *falciparum* malaria. Most importantly, immunization with purified SERA protein partially protects Saimiri monkeys from both heterologous and homologous challenge with blood-stage parasites. A *Saccharomyces cerevisiae*-expressed SERA peptide derived from the N terminus is also capable of inducing partial protection from homologous challenge in Aotus monkeys (Inselburg *et al.*, 1991). SERA and anti-SERA antibodies are also found in immune complexes that form *in vitro* when schizonts rupture in presence of immune monkey sera. It has been demonstrated that lymphocytes from individuals previously exposed to *P. falciparum* can proliferate *in vitro* in

response to some SERA peptide fragments and that immune sera from humans in malaria-endemic regions contain SERA-specific antibodies. An added advantage is that the amino acid sequence of SERA is conserved sequence in *P. falciparum* isolates from different geographical origins including Asia, Africa, and South America (Bhatia *et al.*, 1987). Collectively, these results have generated interest in SERA as a candidate vaccine immunogen (Tine *et al.,* 1993).

Epidemiological studies in a malaria-endemic area in Uganda indicated that high titres of IgG against *P. falciparum* SERA5 correlated with protection against severe malaria in children where lower parasitaemia is related to increased level of IgG_3 using the recombinant protein SE36. SE36 is identical to SE47' but lacks the run of serine repeats. This proves that the N-terminal domain of SERA is a promising candidate for a malaria vaccine (Okech, *et al.*, 2006). There is a remarkable differential IgG subclass response to the amino terminal and central parts of SERA4, with IgG_3 and IgG_1 being the predominant subclass responses against SE47' and SE50A, respectively, and that higher levels of IgG_3 anti-SE47' were associated with lack of fever and lower parasitaemia in children aged ≤15 years (Okech, *et al.*, 2001).

1.5 Immunomodulatory vaccine adjuvants

Advances in genomics and proteomics have accelerated identification of recombinant and synthetic vaccine molecules, and have also heightened the need for improved adjuvants and formulations beyond those currently available. In conjunction with these advances, recent insights into how immune responses are activated have facilitated the discovery of new and improved adjuvants (Coler *et al*., 2009). Complement-fixing IgG subclasses, IgG_{2a}, IgG_{2b}, and IgG_3, inhibit *Plasmodium* parasite growth more than the non-complement-fixing IgG_1 (Pang and Horii, 1998). Thus, regulation of SERA-specific IgG subclasses is an important factor in SERA vaccine strategy. Co-immunization of SERA and cytokine expressing plasmids encoding IFN-γ, GM-CSF, and IL-12 enhances humoral immune response, inducing SERA-specific IgG_{2a} (Sakai, *et al.*, 1999).

In addition to these cytokines, other immunologically important molecules can be used as molecular adjuvants. (Kim *et al.,* 2000). Co-immunization of mice with SERA plasmid DNA and a plasmid expressing the hepatitis B surface antigen (pCMV-s) by intramuscular route resulted in

higher anti-SERA titres than those generated in mice immunized with the SERA DNA plasmid alone. Furthermore, higher titres were generated in mice immunized intradermally via gene gun than by intramuscular injection. The predominant subclass of the antibodies generated to the SERA DNA vaccines by both gene gun and i.m. immunization was IgG_1. Immunization of mice with the SERA 17-382 DNA vaccine by i.m. injection also produced a moderate level of IgM antibodies (Belperron *et al.*, 1999).

Activation of dendritic cells (DCs) is paramount to effective adjuvants as this results in enhanced antigen up-take, migration to draining lymph nodes, acquisition of co-stimulatory molecules, and presentation of antigenic peptides on MHC class I and II to the T-cell receptor (TCR) (Coler, *et al.,* 2009). DCs, antigen-specific T-helper 1 cells and memory T-lymphocytes are crucial for development of immunity in malaria (Kanoi and Egwang, 2007).

More clinically effective vaccine**s** may need to elicit a more specific immune response against the targeted pathogen. It would be a distinct advantage to design immunization strategies that can be focused according to the correlates of protection known for the particular pathogen**.** Controlling the magnitude and direction of the immune response using molecular adjuvants could be advantageous (Kim *et al.,* 2000). Immature dendritic cells express a number of chemokine receptors such as CXCR3, CCR2, CCR5, and CCR6 that facilitate DC recruitment to sites of inflammation following generation of chemokines such as macrophage inflammatory protein (MIP) 1-α, MIP 1-β, monocyte chemotactic protein (MCP)-1/CCL2, RANTES/CCL5 or interferon γ induced protein 10 (IP-10)/CXCL10, which can modulate both the nature and magnitude of immune response at different levels. This property, in addition to lymphocyte trafficking, indicates that exogenous chemokines, given as recombinant proteins or in their genetic form in expression vectors as genetic adjuvants, might be effective in amplifying and shaping immune response to vaccines (Pinto, *et al.,* 2003).

Regulated on activation normal T-cell expressed and secreted protein (RANTES)/CCL5 is a CC-chemokine and a natural ligand for CC-chemokine receptors (CCRs) 1, 3, 4, and 5. CCR5, the main receptor of CCL5, is expressed mainly on subsets of monocytes, macrophages, NK cells, and T lymphocytes predominantly associated with T helper type-1 (Th1) responses (Shimizu *et*

al., 2007). CCL5 regulates inflammation by promoting leukocyte activation, angiogenesis, antimicrobial effects and haematopoiesis. CCL20/macrophage inflammatory protein 3α (MIP-3α) recruits and activates immature APCs expressing its cognate receptor CCR6 to peripheral sites where they may encounter microbes or infected cells and memory T lymphocytes (Schutyser *et al.*, 2003).

Perturbations in production of CCL5 may affect host immune parameters and the erythropoietic response in blood-borne infections such as malaria (Were *et al.*, 2006). Up-regulated expression of CCL5 and its receptors (CCR3 and CCR5) in the cerebella and cerebral regions of post-mortem human cerebral malaria (CM) brains has been reported. Additionally, increased migration of CCR5+ leukocytes into the brain in experimental murine CM models has been reported (Belnoue *et al.*, 2003). The trafficking of inflammatory Th1 cells into the brain is mediated partly by CCL5 interactions with CCR5 (Sarfo *et al.*, 2005). In contrast, circulating CCL5 was shown to be decreased in children during severe malarial anaemia suggesting that malaria-induced thrombocytopenia may be a source of reduced CCL5. This may contribute, to suppression of erythropoiesis and increased severity of malarial anaemia (Were, *et al.*, 2006). Similarly, levels of CCL5 were observed to be significantly lower in children with cerebral malaria, and these low levels were associated with mortality (Chandy, *et al.*, 2006).

The inherent ability to produce CCL5 appears important in conditioning susceptibility to severe malaria. A study in Gabon revealed that healthy children with prior mild malaria produced significantly higher CCL5 transcripts and protein than children with a history of severe malaria (Ochiel, *et al*., 2005). On the other hand, it has been shown that serum CCL20 levels are not only increased in severe malaria, but correlate with serum levels of IgG antibodies against a recombinant construct of *P. falciparum* SERA5 associated with protection against severe malaria in Ugandan children. Thus vaccine constructs incorporating CCL5 and CCL20 and other biological adjuvants hold promise, and should be investigated (Kanoi and Egwang, 2007).

1.6 Problem statement

Morbidity and mortality as well as economic losses associated with malaria persist in spite of efforts to control the disease. There is also concern about the spread of malaria to regions that

previously did not have malaria resulting from global warming and increased population movements from endemic regions. Current preventive and curative measures targeting vector and parasite control face mounting resistance from both vector and parasite. Misdiagnosis of most fevers as malaria and lack of adherence by patients to completing prescribed doses has contributed to drug resistance. A new approach must be sought to aid the eradication of malaria or reducing the burden. A malaria vaccine is a desirable approach but the complex life cycle of *Plasmodium* with highly polymorphic antigen expression has hampered the vaccine's development. Most efforts are geared towards the blood-stage vaccines. This being the pathogenic stage and the stage against which immunity to malaria would impact most.

To confer long lasting immunity for a disease like malaria, both cell-mediated and humoral immune responses are required. This cannot be achieved via the conventional inactivated or attenuated pathogen vaccines which depend solely on development of antibodies. Thus the DNA approach is being sought as a way of eliciting both forms of immune response. However, alone, DNA vaccines do not elicit sufficiently high immune responses, hence they require use of adjuvants. Unfortunately, there is limited information on adjuvants that can be used in humans. Moreover, lack of information on appropriate adjuvant and parasite antigen combinations that induce protective immunity in humans has resulted in lack of a safe and effective vaccine against malaria to date. This study seeks to determine the effect of including immunomodulatory chemokines CCL5 and CCL20 as adjuvants into a plasmid encoding *Plasmodium falciparum* SERA blood-stage malaria vaccine candidate in a mouse model.

1.7 Justification and significance of the research

Malaria is a deadly disease with most *P. falciparum* malaria cases and a high proportion of malaria deaths originating from Africa. Conventional modes of malaria control are currently facing draw backs with the growing development of resistance to antimalarial drugs and insecticides. A vaccine for malaria has thus become of interest as a new measure to aid in the control of this disease alongside the current control strategies. A malaria vaccine can potentially save millions of lives in endemic countries and significantly reduce the malaria burden.

The importance of a blood-stage malaria vaccine is that clinical illness occurs only during blood-stage infection, with the liver stage of the infection being asymptomatic. One major challenge for vaccine candidates targeting the blood is strain-specificity of the vaccines antigens and the extent to which they would cover parasite polymorphisms encountered in field coupled with the variability displayed in the cell invasion pathways of *P. falciparum* (Chilengi and Gitaka, 2010). SERA, an immunogenic antigen, has been shown to be conserved among field isolates. However, SERA as both a sub-unit and DNA vaccine has been found to require an adjuvant to enhance its immunogenicity. In this study, CCL5 and CCL20 were investigated for their potential to improve SERA DNA vaccine candidate as a result of their chemotactic property of attracting dendritic cells for improved antigen presentation to the immune system.

The rodent malaria model is suitable for this study because *P. berghei* SERA orthologues with *Pf*SERA5 illustrate an overall amino acid sequence similarity of approximately 35% to the human malaria protein (Putrianti, *et al.*, 2010). If a vaccine based on any one of the four *plasmodium* species that infect humans can be shown to be effective against any or all of the others, there would be enormous economic advantages. Manufacturing costs would be lower, since for the price of production of a vaccine for one species, vaccines for the other species could be developed. If a vaccine based on cross-species immunity does not prevent *P. falciparum* infection but proves effective in preventing mortality, morbidity, and disease-related symptoms, it would still represent a significant achievement that should be strongly encouraged.

1.8 Research questions

1. Do immunomodulatory chemokines CCL20 and CCL5 boost immune response against SERA DNA vaccine candidate?
2. Are DNA vaccine formulations for *P. falciparum* SERA cross-protective against *P. berghei* ANKA?
3. Is intramuscular injection with the candidate SERA DNA vaccine formulations safe in mice?

1.8.1 Research hypothesis

Inclusion of chemokines CCL20 and CCL5 in the candidate SERA DNA vaccine improves immunogenicity and cross-protects against *P. berghei* infection in mice

1.9. Objectives

1.9.1 General objective

To evaluate immunogenicity and efficacy of SERA DNA vaccine candidate with and without immunomodulatory chemokines CCL20 and CCL5 against *P. berghei in vivo*

1.9.2 Specific objectives

i. To determine safety of DNA vaccine formulations for SERA through observation of local reactions at the sites of injection, mobility, physical appearance and activity and weight changes in BALB/c mice in the experiment
ii. To determine cellular immune response following immunization with SERA DNA vaccine formulations in experimental mice
iii. To evaluate parasitaemia and survival as determined by number of days survived before death following challenge with blood-stage *P. berghei* ANKA parasites

CHAPTER TWO: MATERIALS AND METHODS

2.1 Study site

This study was conducted at the Institute of Primate Research (IPR), a World Health Organization (WHO) collaborating centre in Human Reproduction and Tropical Disease Research, located near Karen, approximately 20 kilometres from Nairobi (National Museums of Kenya, 2012).

2.2 Animals and parasites

One hundred and thirty-two BALB/c mice of either gender 4 to 8 weeks old were used for the vaccine trials in this study, including an additional 5 that were used for preparation of the crude *P. berghei* lysate antigen. 5 Swiss albino mice weighing 25g were used for propagation of the *P. berghei* ANKA parasites to be used for the challenge infection. The mice were obtained from the animal breeding facility of Chiromo campus, University of Nairobi and the rodent facility of the Institute of Primate Research (IPR) where they were maintained throughout the experimental duration. Mice were exposed to 12 hours of light and darkness daily and allowed free access to mouse pellets and water.

BALB/c and Swiss albino mice were used in this study due to their susceptibility to *Plasmodium berghei* infection. The blood-stage parasites of *Plasmodium berghei* ANKA strain were used in this study to induce malaria infection in experimental mice.

2.3 Experimental design

One hundred and thirty-two BALB/c mice were randomly grouped as indicated in Figure 5 below. Spleen samples were collected for proliferation and cytokine assays while blood for determination of parasitaemia was obtained by tail snip. Experimental groups were: SERA+CCL20 in pIRES, SERA+CCL5 in pIRES and SERA in pIRES while the control groups included: Tris EDTA (buffer control), pIRES (plasmid control), and a non-vaccinated control. The baseline group was for determination of pre-immunization parameters. After allocation of groups, mice were labelled using yellow picric acid.

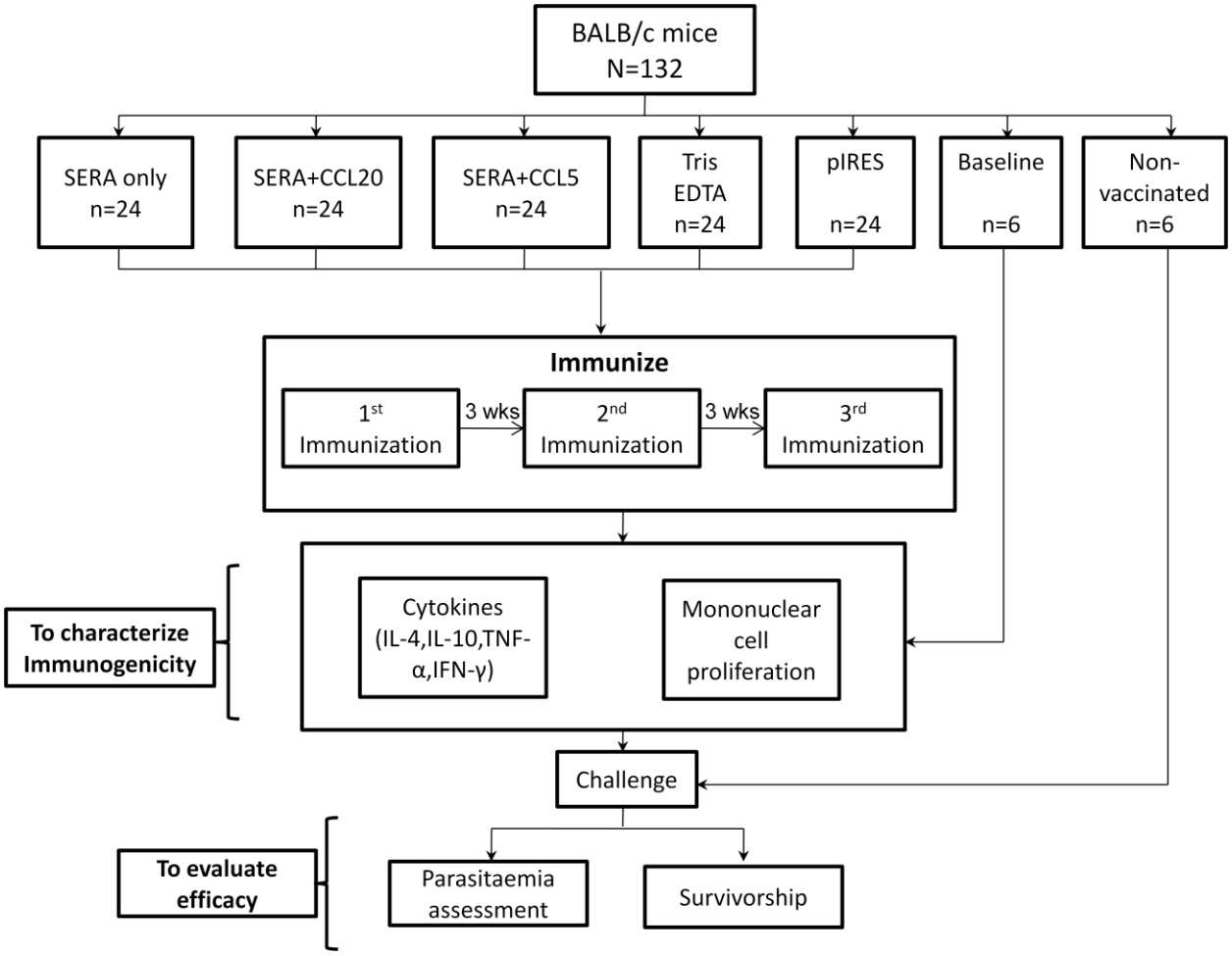

Figure 5: Flow diagram of the experimental design

2.4 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 (10mM Tris 1mM EDTA, 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.

The constructs were received as follows and maintained at -20°C:

- pIRES vector in a colourless plastic reagent bottle labelled: 50mls Gene Art, 1mg/ml none 0936193 in pIRES
- SERA in pIRES construct in a colourless plastic reagent bottle labelled: 50mls Gene Art 508616, 1mg/ml Amp Protein_1 0918836
- SERA and CCL5 in pIRES construct in a colourless plastic reagent bottle labelled: 50mls Gene Art 513602, 1mg/ml Amp Protein_2 0918838 in 09188836
- SERA and CCL20 in pIRES construct in a 50ml centrifuge tube (Fisher Scientific, UK, Sarstedt Cat. No.50809219) labelled: Gene Art 595711E, 1mg/ml Amp Protein_3 0943930 in 0918836

The constructs were, thawed at room temperature and 10ml aliquots transferred into sterile 15ml centrifuge tubes (Corning®, USA, Product No. 430055) to avoid repeated thawing and freezing and stored at -20°C.

2.5 Immunizations

100µl of 1mg/ml DNA in Tris EDTA buffer pH 7.2 was injected using sterile disposable 1ml syringes with 29G x ½" needles (Ken Healthcare, China) into quadriceps muscle of both legs at 3-week intervals for 9 weeks; for a full dose of 600µg of DNA as described by Belperron *et al*., 1999. Immunizations were staggered across 2 weeks to allow for one group to be injected per day with a break in between. In the first week buffer control (TE), plasmid control (pIRES) and SERA in pIRES groups and in the second week SERA and CCL20 in pIRES and SERA and CCL5 in pIRES groups were immunized. On immunization days, the required construct aliquot was thawed at room temperature.

2.6 Safety determination

All mice were observed at the site of immunization for local reactions in terms of erythema, skin swelling and ulceration. Mobility, appearance of fur and general behaviour were also observed

daily for any abnormality after the immunizations. Changes in body weight were taken on immunization days through the experimental duration. The mice were weighed by individually placing them in an open small carton box whose weight had been tared on an electronic weighing balance (Mettler PM3000, USA, Item No. 8773).

2.7 Preparation of crude *P. berghei* lysate antigen

Retrieval, infection and monitoring of parasitaemia of mice was done following IPR standard operating procedures (SOPs) (IPR, 2002). Cryopreserved *P. berghei* ANKA stabilates were retrieved from 9 x 9 cryoboxes (Nalgene®, Thermo Fisher Scientific, UK, Cat. No. CRY-180-020U) from liquid nitrogen storage (Thermolyne Locator 4 Cryobiological Storage System, USA, Product No. CY50935), quickly thawed in a 37°C water bath (Precision Scientific, USA, Cat No. 66798) and diluted with an equal volume of sterile 1x phosphate buffered saline (PBS) pH 7.2. This preparation was then loaded into 1ml syringes with 29G x ½" needles (Ken Healthcare, China) and 100µl administered intraperitoneally into each of 5 BALB/c mice. Parasitaemia was assessed by microscopic examination of Giemsa-stained thin smears of tail blood starting day 3 post-infection.

On day 3 post-infection, tail blood was obtained by snipping 1-2mm off the tip of the mouse's tail using a pair of dissection scissors swabbed with 70% ethanol and on subsequent days, a cotton gauze swab was used to disturb the wound. Thin blood smears were prepared on frosted ended 1" x 3" microscope slides (IMED, China, Cat. No. 7105), fixed with absolute methanol and stained with 10% Giemsa accustain solution (BDH, VWR International Ltd, England, Product No. 352603R) for 10 minutes. Excess stain was washed off under running tap water and the stained smears air dried. Thin blood smears were examined using a binocular laboratory microscope (Zeiss Standard 20, Germany) at x100 objective lens under oil immersion (Cargille, USA, Cat No. 1648). Percent parasitaemia was calculated as follows:

$$\text{Parasitaemia (\%)} \quad \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes counted}} \quad \text{x } 100$$

Where the total number of erythrocytes counted was at least 2000

Mice were sacrificed when parasitaemia reached 5-8% and bled via cardiac puncture. The blood was pooled and taken through a freeze-thaw process 3 times followed by sonication (MSE Soniprep 150, UK), filtered through a 0.45µm pore-size membrane (Sartorius Stedim Biotech, USA, Minisart®, VWR, Cat No. 514-7013), diluted 10 times in RPMI 1640 (Gibco, BRL) and stored at -20°C.

2.8 Immune response determination

Splenocyte cultures were used to determine cellular responses to the vaccine preparations using cytokine and splenocyte proliferations assays.

2.8.1 Isolation of splenocytes

Splenocytes were prepared from mouse spleens as described by Yole *et al*., 2007. Mice were anaesthetized by subcutaneous injection of ketamine-xylazine (0.5ml xylazine/0.5ml ketamine in 5ml saline, 0.1ml/10g) using a 1ml syringe with a 29G x ½" needle (Ken Healthcare, China). Once unconscious, mice were individually placed on a dissecting board, the abdomens swabbed with 70% ethanol, limbs pinned down to the board using 21G x 1½" needles (BD Microlance™ 3, Spain, Cat No. 304432) and an incision made to expose the spleen with a sterile pair of dissecting scissors and dissecting toothed forceps. Spleens were removed and crushed using a 20ml sterile syringe plunger (BD Discardit™ II, USA, Product No. BDAA300296) through an autoclaved fine wire mesh placed over a sterile petri dish in 3mls incomplete RPMI 1640 medium (Gibco, BRL). Erythrocytes in spleen cell suspensions were lysed by drop wise addition with shaking of 2mls laboratory prepared RBC lysing solution (4.15g Ammonium chloride (NH_4Cl), 50ml 0.1M Tris HCl, made up to 500ml with distilled H_2O, pH 7.5 and filtered with 0.22µm pore size filter unit (Millipore Co., USA)). Cells were then transferred to 50ml centrifuge tubes (Corning®, USA, Product No. 430290) and centrifuged at 2,440g for 10 min at 4°C (Hettich Zentrifugen, Rotanta 460 R, Germany). The cell-containing pellets were washed three times with RPMI 1640 supplemented with 5% FBS (Gibco, USA, Product No. 10270) that had been heat inactivated at 56°C for 1 hour in a water bath (Precision Scientific, USA, Cat No. 66798). Splenocytes were resuspended in complete medium (RPMI 1640 supplemented with

10% FBS, gentamicin (Gibco, Auckland, NZ) [50µg/ml], and 5 x 10^{-5}M 2-mercaptoethanol (Sigma, USA, Product No. M3148).

2.8.2 Cytokine ELISAs

Cytokine activity was determined as described in the IPR SOPs (IPR, 2002). Splenocyte cultures resuspended to a concentration of $2x10^5$cells/ml in 48-well flat bottom culture plates (Corning). Cells were counted using Trypan blue (Sigma, Germany, Product No. T8154) and a haemocytometer (Hausser Scientific, Reichert Bright-Line®, USA, Cat. No. 1492). Cultures in duplicate were stimulated with crude *P. berghei* lysate antigen. Unstimulated cells in complete media were used as a control. After 72 hours in a humidified 5% CO_2 incubator at 37°C, cell supernatants were harvested and stored at -80°C.

2.8.2.1 Tumor necrosis factor alpha (TNF-α)

TNF-α levels were determined using a mouse TNF-α ELISA kit (U-CyTech biosciences, The Netherlands, CT303) following manufacturer's instructions. Lyophilized coating antibodies were reconstituted by injecting 250µl of sterile distilled water into the vial, the solution gently mixed for 15 seconds and allowed to stand at room temperature for 2 minutes. Of this stock, 25µl was pipetted out and mixed into 2.5mls of phosphate buffered saline (PBS, prepared by dissolving 96.0g $Na_2HPO_4.2H_20$ and 17.5g KH_2PO_4 in 1L distilled water of which 10ml was further diluted in 1L of distilled water containing 8.8g NaCl and pH adjusted to 7.4) and 25µl added to each well of a 96-well ELISA plate (Greiner Bio-one, Microlon, Cat No. 655061, Austria) and the wells filled up to 50µl with PBS. Plates were then sealed and incubated overnight at 4°C. The coating antibody solution was dispensed off, plates washed 6 times with 0.05% Tween 20 in PBS (washing buffer) using an automatic ELISA plate washer (MRW DYNEX) and blocked with 100µl of blocking buffer (1ml of 10% bovine serum albumin (BSA) stock solution added to 9ml PBS for 1 ELISA plate). Plates were incubated for 1 hour at 37°C. Blocking buffer was dispensed off and undiluted supernatant samples and standards added. Standards were serially diluted by transferring 50µl from well to well with mixing beginning with a concentration of 320pg/ml. Plates were left to incubate for 2 hours at 37°C then washed as above. Lyophilized biotinylated detector antibodies were reconstituted by injecting 0.5ml of sterile distilled water

into the vial with gentle mixing for 15 seconds, and allowed to stand for 2 minutes at room temperature. 50µl of stock detector antibody solution was pipette out of the vial and mixed into 5ml of dilution buffer (1ml of 10% BSA stock solution added to 19ml washing buffer). To each ELISA plate well, 50µl of diluted detector antibody solution was added and the plates incubated at 37°C for 1 hour. After washing the plates six times using washing buffer, streptavidin-horseradish peroxidase polymer (SPP) conjugate was added. This was prepared by injecting 0.5ml of sterile distilled water into the vial containing lyophilized SPP. The SPP solution was gently mixed for 15 seconds and allowed to stand for 1 minute at room temperature. 50µl of SPP solution was pipette out of the vial and mixed into 5mls dilution buffer and added to all wells and the plates incubated for 1 hour at 37°C. Plates were then washed as before. For every 2 plates, one 3,3',5,5' tetramethylbenzidine (TMB) tablet was dissolved in 1ml dimethyl sulfoxide (DMSO) to which 10ml substrate buffer was added. This substrate solution was mixed thoroughly and 50µl immediately dispensed into each well and incubated in darkness at room temperature to allow for the blue colour development. Optical densities were read at 10 minute intervals for 1 hour using an ELISA plate reader (Dynatech MRX) at 630nm.

2.8.2.2 Interferon gamma (IFN-γ)

Interferron gamma levels were determined using an ELISA kit for mouse IFN-γ, following manufacturer's instructions (Mabtech, Sweden, Product Code: 3321-1A-6). High protein binding 96-well ELISA plates (NUNC Maxisorp®, Denmark) were coated with monoclonal antibody AN18 (mAb AN18) diluted to a concentration of 1µg/ml in phosphate buffered saline (PBS, prepared by dissolving 400g NaCl, 10g KCl, 72g Na_2HPO_4, 12g KH_2PO_4 in 500ml distilled water, which was then diluted 1:10 in distilled water for use and pH adjusted to 7.4) by adding 50µl per well. The plates were incubated overnight at 4°C. After washing each plate two times with PBS, the plates were blocked by adding 100µl per well of incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) and incubated for 1 hour at room temperature. The blocking solution was dispensed off and plates washed 5 times with washing buffer. 50µl per well undiluted cell supernatants and standards were then added to the plates. Standards were serially diluted in incubation buffer by transferring 50µl from well to well with mixing beginning with a concentration of 400pg/ml. Plates were then incubated for 2 hours at room temperature

and washed as before. Biotinylated monoclonal antibody R4-6A2 (mAb R4-6A2-biotin) diluted to a concentration of 0.5µg/ml in incubation buffer was added, 50µl to each well, and incubated for 1 hour at room temperature. Plates were washed as before, 50µl streptavidin-alkaline phosphatase conjugate diluted 1:1000 added to each well and incubated at room temperature for 1 hour. One tris buffer tablet (SIGMAFAST, No. T8915) and one p-nitrophenyl-phosphate (pNPP) tablet (SIGMAFAST, No N9389) were dissolved in 5ml double distilled deionised water for each plate. After washing the plates 6 times using the automatic ELISA plate washer, 50µl of substrate solution was added to each well and left to incubate in the dark at room temperature for colour development. Optical densities were read at 10 minute intervals for 1 hour using an ELISA plate reader (Dynatech MRX) at 405nm.

2.8.2.3 Interleukin 10 (IL-10)

Interleukin 10 levels were determined using an ELISA kit for mouse IL-10, following manufacturer's instructions (Mabtech, Sweden, Product Code: 3431-1A-6). High protein binding 96-well ELISA plates (NUNC Maxisorp®, Denmark) were coated with monoclonal antibody 2A5 (mAb 2A5) diluted to a concentration of 2µg/ml in PBS (prepared as for interferon gamma) by adding 50µl per well. Plates were incubated overnight at 4°C. After washing each plate two times with PBS, the plates were blocked by adding 100µl per well of incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) and incubated for 1 hour at room temperature. The blocking solution was dispensed off and plates washed 5 times with washing buffer. 50µl per well of undiluted cell supernatants and standards were then added to the plates. Standards were serially diluted in incubation buffer by transferring 50µl from well to well with mixing beginning with a concentration of 2000pg/ml. Plates were then incubated for 2 hours at room temperature and washed as before. Biotinylated monoclonal antibody 16E3 (mAb 16E3-biotin) diluted to a concentration of 0.1µg/ml in incubation buffer was added, 50µl to each well, and incubated for 1 hour at room temperature. Plates were then washed as before, 50µl streptavidin-alkaline phosphatase diluted 1:1000 added to each well and incubated at room temperature for 1 hour. One tris buffer tablet (SIGMAFAST, No. T8915) and one p-nitrophenyl-phosphate (pNPP) tablet (SIGMAFAST, No N9389) were dissolved in 5ml double distilled deionised water for each plate. After washing the plates 6 times using the automatic ELISA plate washer, 50µl of

substrate solution was added to each well and incubated in the dark at room temperature for colour development. Optical densities were read at 10 minute intervals for 1 hour using an ELISA plate reader (Dynatech MRX) at 405nm.

2.8.2.4 Interleukin 4 (IL-4)

Interleukin 4 levels were determined using an ELISA kit for mouse IL-4, following manufacturer's instructions (Mabtech, Sweden, Product Code: 3311-1A-6) was used to determine IL-4 levels. High protein binding 96-well ELISA plates (NUNC Maxisorp®, Denmark) were coated with monoclonal antibody 11B11 (mAb 11B11) diluted to a concentration of 2µg/ml in PBS (prepared as for interferon gamma) by adding 50µl per well. The plates were incubated overnight at 4°C. After washing each plate two times with PBS, the plates were blocked by adding 100µl per well of incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) and incubated for 1 hour at room temperature. Blocking solution was dispensed off and plates washed 5 times with washing buffer. 50µl per well undiluted cell supernatants and standards were then added to the plates. Standards were serially diluted in incubation buffer by transferring 50µl from well to well with mixing beginning with a concentration of 400pg/ml. Plates were then incubated for 2 hours at room temperature and washed as before. Biotinylated monoclonal antibody BVD6-24G2 (mAb BVD6-24G2-biotin) diluted to a concentration of 0.1µg/ml in incubation buffer was added, 50µl to each well, and incubated for 1 hour at room temperature. Plates were washed as before, 50µl streptavidin-alkaline phosphatase conjugate diluted 1:1000 added to each well and left to incubate at room temperature for 1 hour. One tris buffer tablet (SIGMAFAST, No. T8915) and one p-nitrophenyl-phosphate (pNPP) tablet (SIGMAFAST, No N9389) were dissolved in 5ml double distilled deionised water for each plate. After washing the plates 6 times using the automatic ELISA plate washer, 50µl of substrate solution was added to each well and incubated in the dark at room temperature for colour development. Optical densities were read at 10 minute intervals for 1 hour as before.

2.8.3 Splenocyte proliferation assay

Proliferation was determined by measuring incorporation of [^{3}H] thymidine (Perkin Elmer, NEN®, USA, Product No. NET027Z005MC) into the DNA of actively dividing cells as described by Yole *et al*., 2007. Isolated splenocytes were cultured at 2 x 10^5 cells per well in 96-well round-bottom microwell plates (Nunc, Denmark, Nunclon® Δ Surface). To determine the number of cells that initiated the culture, cells were stained using Trypan blue (Sigma, Germany, Product No. T8154) and counted using a haemocytometer (Hausser Scientific, Reichert Bright-Line®, USA, Cat. No. 1492). Cultures in triplicate were stimulated with crude *P. berghei* lysate antigen. Concanavalin A (ConA) (Sigma, USA, Product No. C7642.) at a concentration of 0.5µg per well and RPMI 1640 were used as positive and unstimulated controls, respectively. After 72 hours in a humidified 5% CO_2 incubator at 37°C, cells were pulsed with 0.5µCi of [^{3}H]thymidine for 18 hours and harvested onto 10.2 x 25.4cm glass filter sheets (Whatman, England, Cat No. 1822-849). Tritiated-thymidine incorporation was counted using a scintillation counter (PerkinElmer Life Sciences, USA, Tri-Carb 2800R). The mean counts per minute and standard error of the mean for each triplicate set of cells were determined.

2.8.4 Storage of splenocytes

The number of remaining cells was calculated by subtracting the number of cells cultured from the total number previously counted. These splenocytes were cryopreserved according to the IPR standard operating procedure for freezing of cells. Since they were still suspended in complete media (RPMI 1640 supplemented with 10% FBS, gentamicin [50µg/ml], and 5 x 10^{-5}M 2-mercaptoethanol), the cells were spun down at 2,440g for 10 minutes at 4°C (Hettich Zentrifugen, Rotanta 460 R, Germany). The supernatant was then poured off and the pellet dislodged. These cells were then stored in 2ml sterile cryovials (Greiner Bio One, CRYO.S™, Germany) at a concentration of 18-22 x 10^6 cells/ml in a freezing solution of 90% FBS in Dimethyl Sulfoxide (Sigma, USA, Product No. D-5879) for 15 minutes in a freezing chamber (Mr Frosty Freezing container, Nalgene®, Thermo Scientific, USA Part No. 5100-0001), an alcohol-filled container designed to slowly freeze vials (-1°C/min), at 4°C, then overnight at -80°C. The vials were subsequently transferred into 9 x 9 cryoboxes (Nalgene®, Thermo Fisher

Scientific, UK, Cat. No. CRY-180-020U) which were placed in a liquid nitrogen tank (Thermolyne Locator® 4 Cryobiological Storage System, USA, Product No. CY50935).

2.9 Cross-protective efficacy determination

Cross-protective efficacy of the vaccine preparations was determined through heterologous challenge infection to determine parasitaemia progression and hence suppression and survivorship.

2.9.1 Parasite propagation

The parasites were propagated using the same procedure as described previously under title 2.7. When mean percent parasitaemia of 5-8% was achieved, mice were sacrificed and bled via cardiac puncture. Blood was then pooled to determine pooled parasitaemia. The approximate number of RBCs in mouse blood is $1x10^7$/µl. The following calculation was used to determine the dilutions necessary to give the required number of parasitized RBCs for the challenge infection of 1 x 10^5/100µl in RPMI 1640 (Gibco, BRL).

Total number of RBCs = $1x10^7$/µl x pooled blood volume
Number of parasitized RBCs = Pooled % Parasitaemia x Total number of RBCs
Number of parasitized RBCs required = 1 x 10^5 x Number of mice to be infected
Volume of parasitized RBCs required = (Number of parasitized RBCs required x Total volume of pooled blood) / number of parasitized RBCs
The volume of parasitized RBCs required was then diluted to a final concentration of 1 x 10^5/100µl using RPMI 1640 media (Gibco, BRL).

2.9.2 Preservation of *P. berghei* parasites

The excess volume of *P. berghei* infected blood from the parasite propagation experiment was determined. An equal volume of autoclaved freezing solution (30% Glycerol in 1 x PBS) was added to the infected blood and gently mixed in. This suspension was transferred into cryovials (Greiner Bio One, CRYO.S™, Germany) and stored in a cryobox (Nalgene®, Thermo Fisher

Scientific, UK, Cat. No. CRY-180-020U) in a liquid nitrogen tank (Thermolyne Locator® 4 Cryobiological Storage System, USA, Product No. CY50935).

2.9.3 Challenge infection

Each mouse was injected intraperitoneally with 100µL RBC suspension containing $1x10^5$ RBCs parasitized with *P. berghei* ANKA for malarial infection using disposable 1ml syringes with 29G x ½" needles (Ken Healthcare, China). This was done to determine whether there was suppression of infection progression in vaccinated groups compared to the non-vaccinated and negative control groups, and also to compare the preparation without adjuvant to those with the chemokine adjuvants. Infected mice were followed for parasitaemia by microscopic examination of Giemsa-stained smears of tail blood daily from day 3 post-infection till the day they died. On day 3 post-infection, tail blood was obtained by snipping 1-2mm off the tip of the mouse's tail using a pair of dissection scissors swabbed with 70% ethanol and on subsequent days, a cotton gauze swab was used to disturb the wound to make it bleed. Thin blood smears were prepared on frosted ended 1" x 3" microscope slides (IMED, China, Cat. No. 7105), fixed with absolute methanol and stained with 10% Giemsa accustain solution (BDH, VWR International Ltd, England, Product No. 352603R) for 10 minutes. Excess stain washed off under running tap water and the stained smears air dried. Thin blood smears were examined using a binocular laboratory microscope (Zeiss Standard 20, Germany) at x100 objective lens under oil immersion (Cargille, USA, Cat No. 1648). Percent parasitaemia was calculated as follows:

$$\text{Parasitaemia (\%)} \quad \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes counted}} \quad \text{x } 100$$

Where the total number of erythrocytes counted is at least 2000

2.9.4 Mouse survivorship determination

Survivorship was used to determine how long the mice would survive with infection following administration of the various vaccine preparations. Infection with *Plasmodium berghei* ANKA is

usually lethal. Survivorship was calculated as a percentage of mice still alive per group following infection until all died, and improved survivorship was attributed to the effect of the vaccines.

2.10 Data management and statistical analysis

Data was stored on data sheets as both hard copy and as electronic copy in Microsoft Office 2007 software. Hard copies included laboratory journals, and data print-outs. These were locked in safety cabinets accessible only by authorized persons. Soft copies were stored in computers and memory sticks. Backups made in both hard copy and electronic copy, were stored in a lockable cabinet. Graphs and charts were presented using MS Excel spreadsheet. Statistical significance was calculated using the Mann-Whitney test for non-parametric comparisons intra-group and Kruskal-Wallis one-way analysis of variance (ANOVA) for inter-groups comparisons. p values ≤ 0.05 were considered significant at 95% confidence intervals.

CHAPTER THREE: RESULTS

3.1 Safety

Safety of vaccine delivery mode and formulation following injection into mice was determined through observation of the injection sites and general animal behaviour. No erythema, skin swelling or ulceration were observed at injection sites. In addition, fur appearance and behaviour were normal including mobility and general animal health appeared good with the mice increasing in weight. Figure 6 below presents changes in body weight for control and experimental groups.

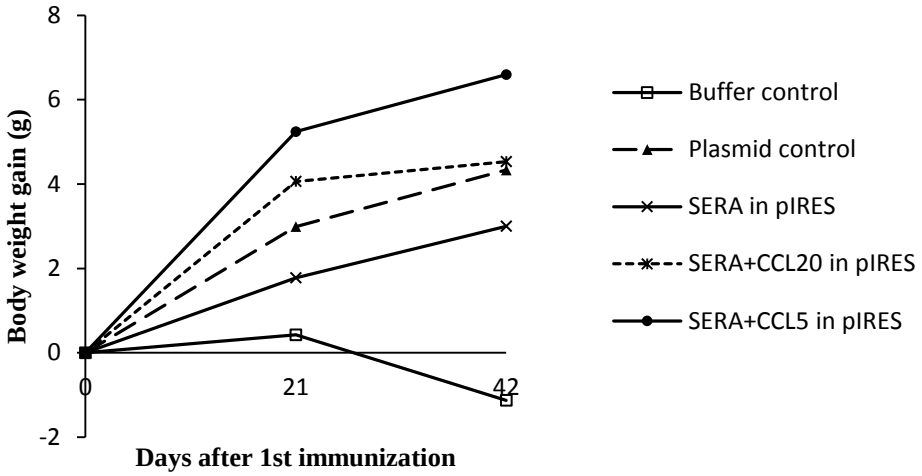

Figure 6: Average change in body weights during immunization period

The average weight increased in all groups except the buffer control group (TE pH 7.2) where there was a decrease. Significant increases in weight were noted in the plasmid control, SERA+CCL20 and SERA+CCL5 groups (p=0.0011, p=0.0023, p<0.0001 respectively). In SERA in pIRES and buffer control groups, there was no significant weight change (p>0.05). These results suggest that general health of the mice remained good, and that the vaccine preparation given i.m. was well tolerated and therefore safe.

3.2 Propagation of *P. berghei* parasites for crude lysate antigen preparation

Crude *P. berghei* lysate antigen was prepared using blood from BALB/c mice infected with *P. berghei* ANKA for determination of cross-reactive recall responses. All mice used in this

experiment were successfully infected with *P. berghei* ANKA and had a significant (p=0.0018) increase in parasitaemia with time as shown in Figure 7.

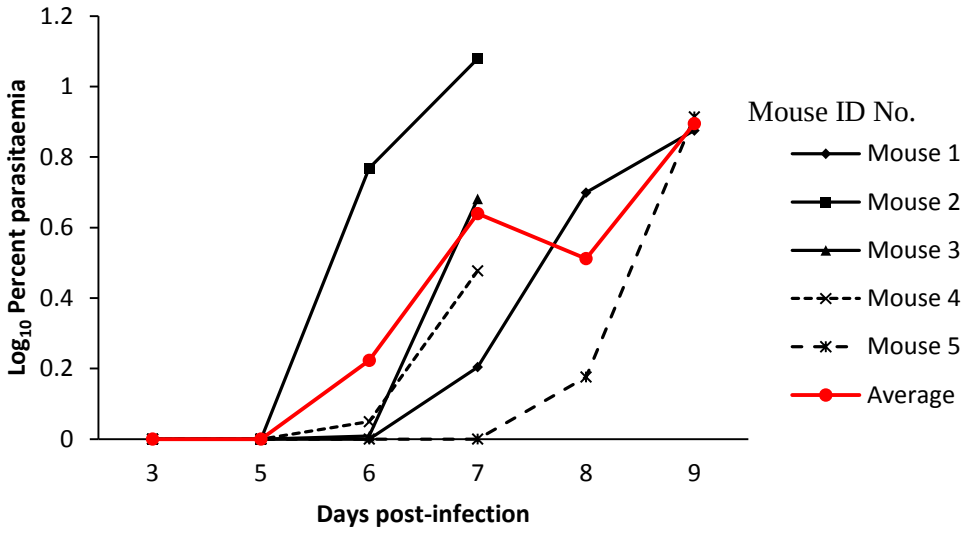

Figure 7: Propagation of *P. berghei* in BALB/c

Mice 2, 3 and 4 were sacrificed on day 7 post-infection, bled and their blood pooled for preparation of the crude lysate antigen. Parasitaemia was not high enough in mice 1 and 5. These were sacrificed on day 9 post-infection and their blood used to prepare stabilates of *P. berghei.* The development of patent parasitaemia shows that the procedure of *P. berghei* stabilate preparation retains parasite viability.

3.3 Propagation of *P. berghei* ANKA parasites

Vaccine induced cross-species protection was determined through challenge infection. This required *in vivo* propagation of *P. berghei* ANKA parasites in mice. Of the 5 mice infected with *P. berghei* ANKA, 2 were excluded from the rest of the experiment. Mouse 5 died before parasitaemia determination began and mouse 2 failed to pick up infection. The remaining 3 picked up infection as presented on Figure 8.

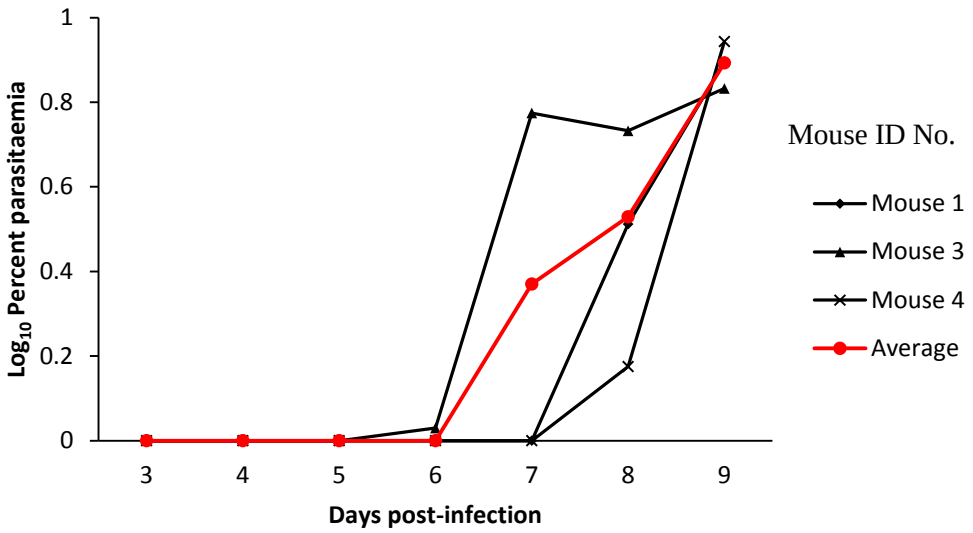

Figure 8: Swiss mice parasitaemia profiles

In mice 1, 3 and 4 parasitaemia increased significantly during the duration of infection (p=0.0083). These mice were sacrificed and bled on day 9 post-infection and their pooled blood had a parasitaemia of 8% which was used for challenge infections. This confirmed that swiss white mice are also susceptible to *P. berghei* infection.

3.4 Immunological responses

Following injection with the DNA vaccine preparations, splenocyte proliferation assays and cytokine ELISAs were used to determine antigen recall and cytokine responses to crude *P. berghei* lysate antigen. These responses were compared across groups to evaluate the effect of including CCL5 and CCL20 as chemokine adjuvants in the SERA DNA vaccine.

3.4.1 Cytokine ELISAs

To evaluate the ability of the *Pf*SERA DNA vaccine preparations to stimulate secretion of the cytokines TNF-α, IFN-γ, IL-10 and IL-4 cytokine ELISAs were used.

To get the overall cytokine response induced by the vaccine preparations, comparisons were made by plotting the titres across all time-points for all four cytokines on a single graph. Baseline and unstimulated titres were also subtracted from each of the time-points for a zeroed

start point and elimination of non-specific reactions.

In the buffer control (TE) group, there was a notable IFN-γ response which waned with time (Figure 9).

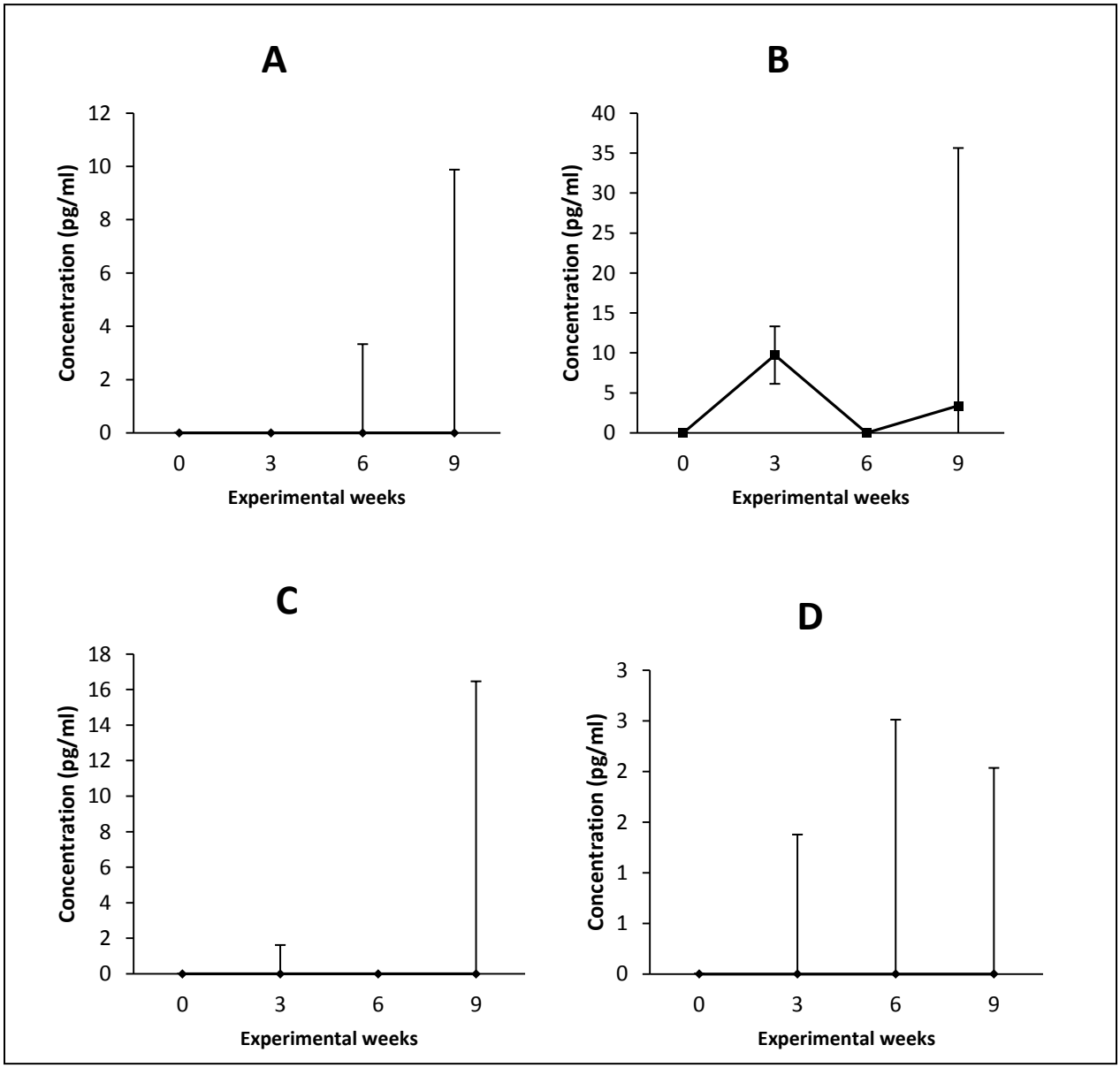

Figure 9: Buffer control group titres for (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4

Despite the variation in the IFN-γ titres over time, there was no significant difference in levels of the 4 cytokines with exception of IFN-γ at 3 weeks (p=0.0067). This indicates poor immunogenicity of Tris EDTA buffer in the mice.

There appeared to be an increase in IL-4 levels in the plasma control group up to week 6 after which they declined. TNF-α and IFN-γ levels increased marginally at week 9. Presented in Figure 10 are the plasmid control (pIRES) group cytokine responses

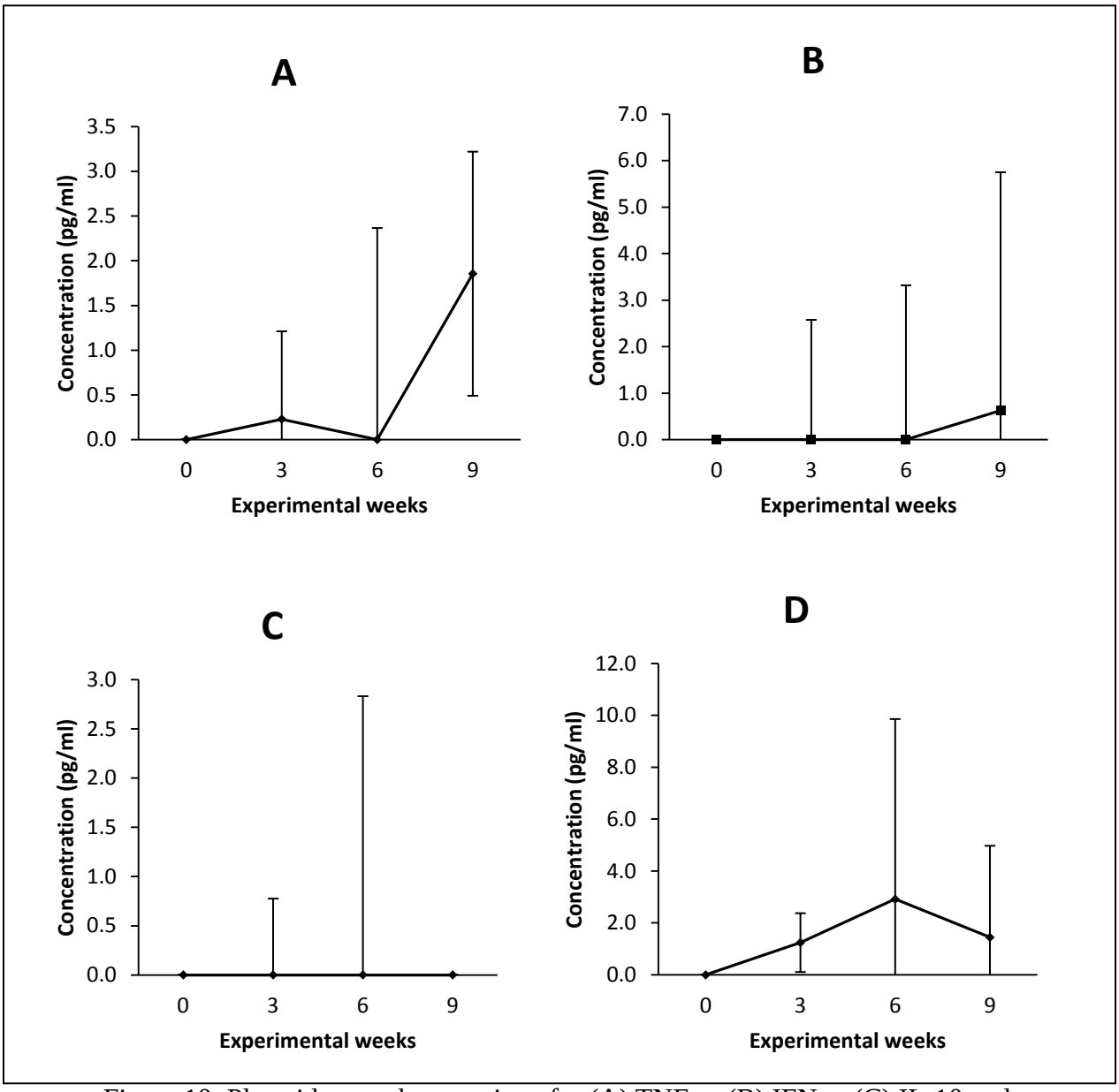

Figure 10: Plasmid control group titres for (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4

However, the four cytokines did not differ significantly at any point (3 weeks p=0.9290; 6 weeks p=0.9500; 9 weeks p=0.9752). This suggests as expected, a low immune response against *P. berghei.*

While the rest of the cytokines in the SERA in pIRES group barely changed throughout the experimental period, IFN-γ was marginally increased at 3 weeks then declined and rose again at 9 weeks as shown on Figure 11.

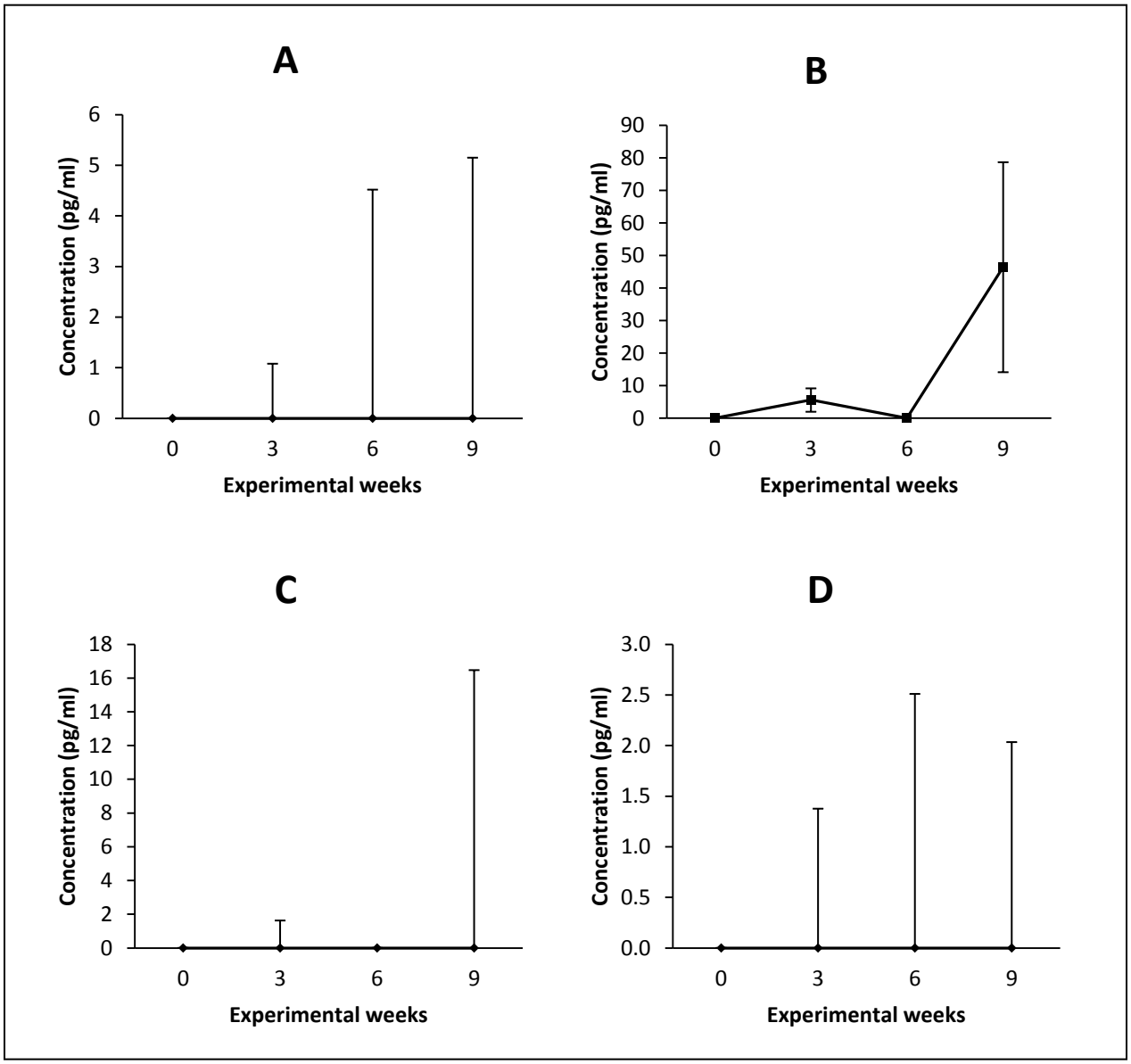

Figure 11: SERA in pIRES group titres for (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4

The increased level of IFN-γ was however not significantly higher than the remaining 3 cytokines namely TNF-α, IL-10 and IL-4 (p=0.2200).

The mice vaccinated with SERA+CCL20 in pIRES showed an increase in levels of the inflammatory cytokine IFN-γ, which wane at 6 weeks as shown on Figure 12.

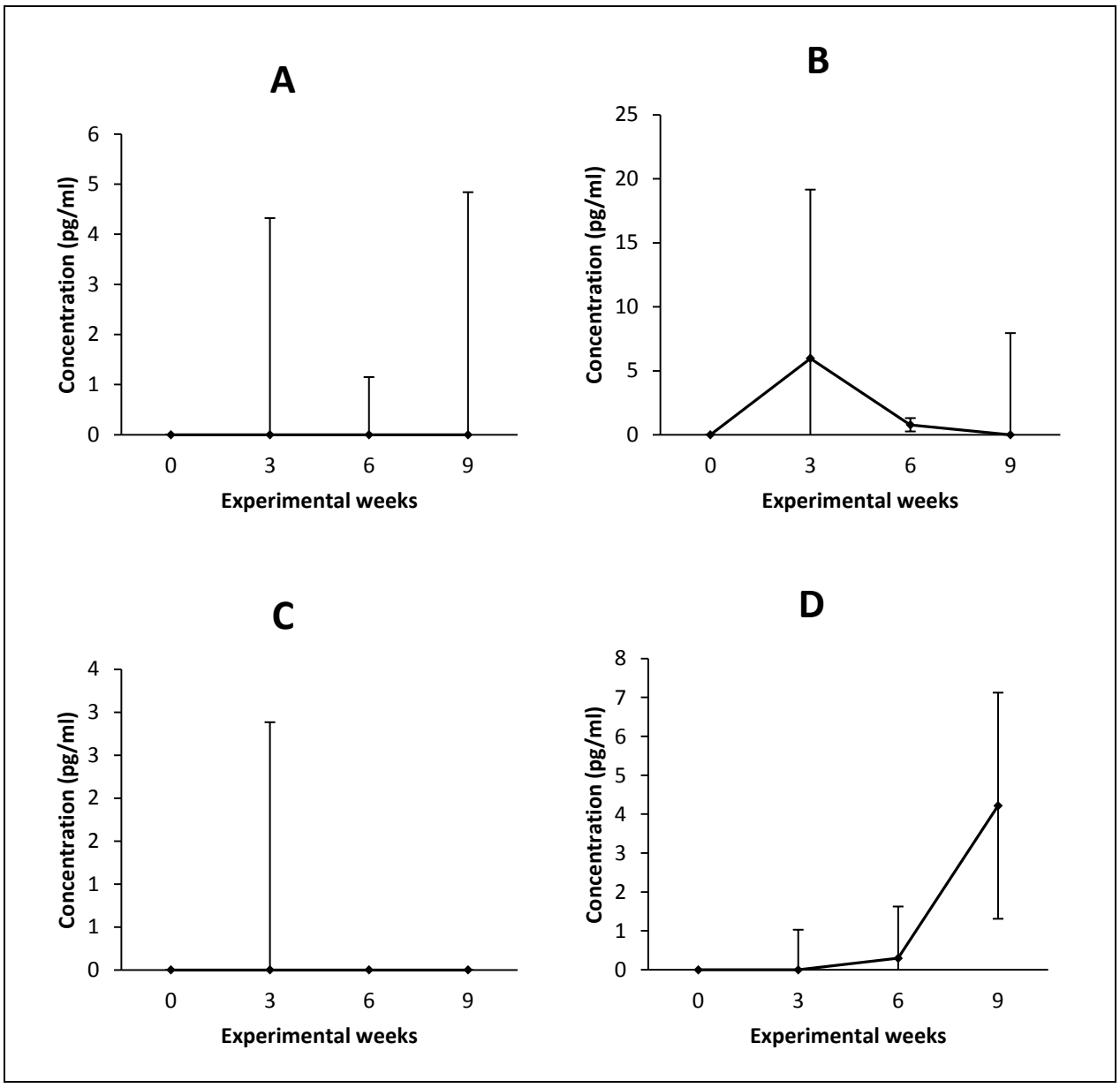

Figure 12: SERA+CCL20 in pIRES group titres for (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4

In addition, IL-4 levels were also seen to rise through the 9 weeks to more than observed with SERA in pIRES and almost twice the amount induced by SERA+CCL5 in pIRES. These differences in levels of the different cytokines observed were not significantly different from

each other at any point (3 weeks p=0.9108; 6 weeks p=0.9159; 9 weeks p=0.9045). However this still suggests an improvement in immunogenicity.

The IFN-γ responses in the SERA+CCL5 in pIRES group increased from week 3 all through to week 9 where it reached its peak. This is presented in Figure 13.

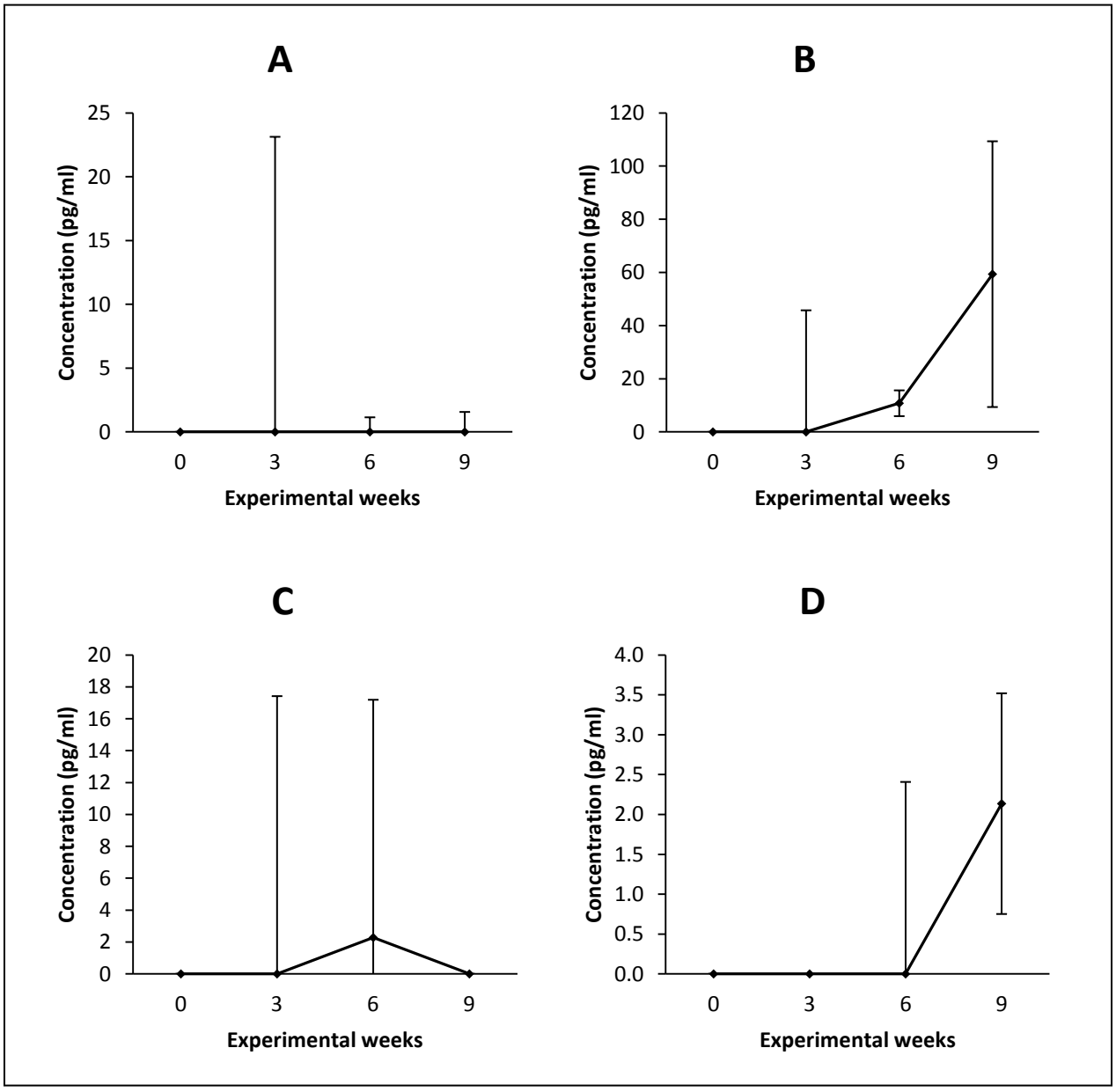

Figure 13: SERA+CCL5 in pIRES group titres for (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4

TNF-α remained relatively unchanged throughout the course of sampling while IL-10 response induced was not sustained. On the other hand, there was a marginal increase in IL-4 at 9 weeks. Given that there were no significant differences between the cytokines (3 weeks $p>0.9999$; 6 weeks $p=0.7426$; 9 weeks $p=0.2790$), it was still noted that the final level of IFN-γ was higher than that of SERA in pIRES group. This suggests a marginal increase in immunogenicity.

3.4.2 Splenocyte proliferation assay

Pre-immunization stimulation indices (SI) were significantly higher for splenocytes under concanavalin A (Con A) stimulation as compared to the splenocytes under crude *P. berghei* lysate antigen (P. b. Lysate Ag) stimulation ($p=0.0022$). This indicates the system was working. Figure 14 below presents pre-immunization SI counts using Con A and crude *P. berghei* lysate antigen stimulation.

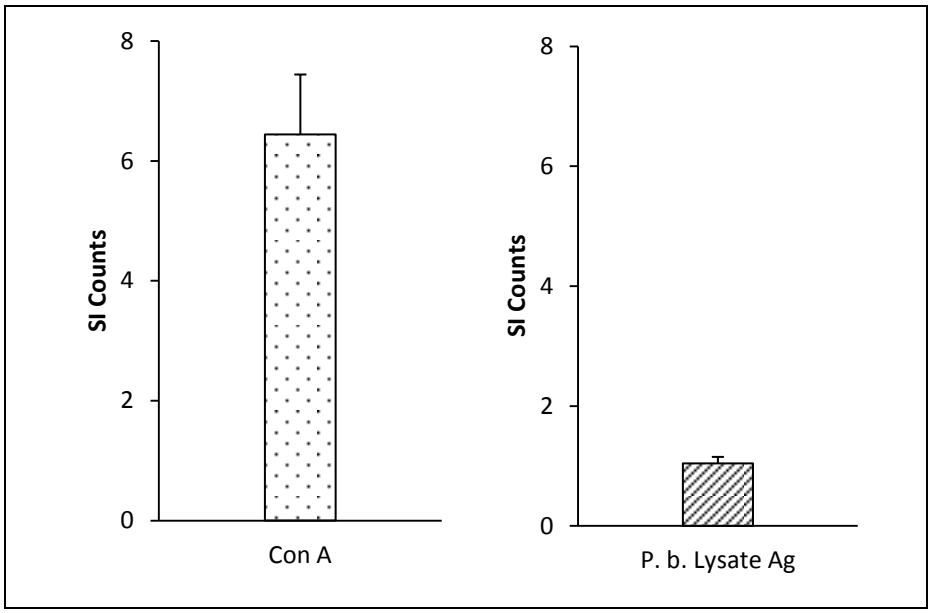

Figure 14: Average pre-immunization stimulation

The pre-immunization group showed a significantly higher reading in the Con A stimulated cells as compared to the cells stimulated by crude *P. berghei* lysate antigen ($p<0.0001$). This shows that the cells were viable and the system was working and the low SI counts observed under *P. berghei* antigen stimulation indicate a low recall response.

Stimulation indices from the buffer control groups were significantly higher for cells under Con A stimulation compared to *P. berghei* lysate antigen (p=0.0079). The SI counts for splenocytes under crude *P. berghei* lysate antigen stimulation demonstrated a low recall response. These observations are presented in Figure 15.

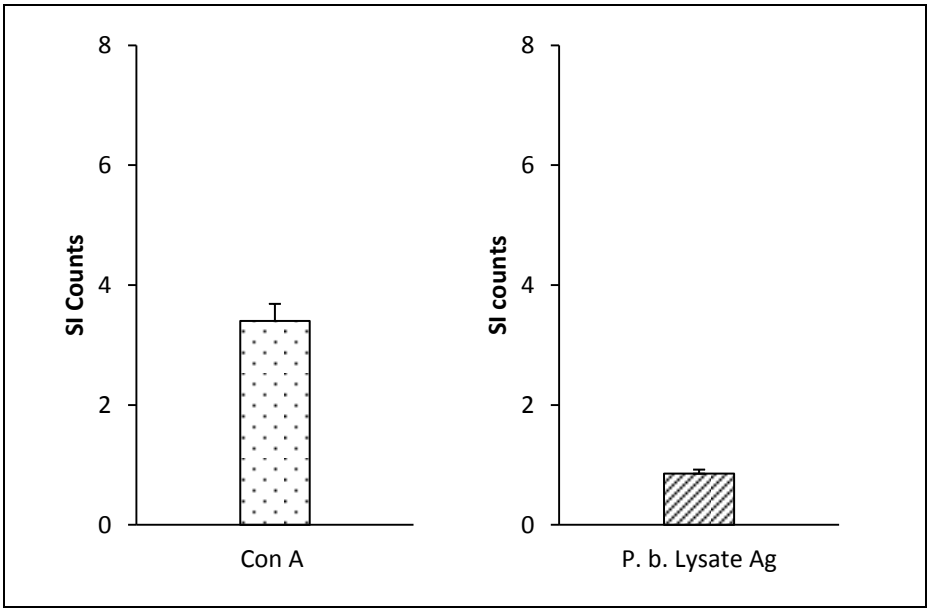

Figure 15: Stimulation indices for the buffer control group after second boost

Stimulation indices obtained in this buffer control (TE pH 7.2) group under Con A stimulation ranged from 2.6 to 4.4 although the differences were not marked and had no significant difference (p>0.9999). Under crude *P. berghei* lysate antigen stimulation, the SI counts ranged from 0.7 to 1.1 but the differences were not significant (p>0.9999).

In the plasmid control group, Con A stimulation led to significantly higher counts than crude *P. berghei* lysate antigen (p=0.0317) as presented in Figure 16. This indicates a low recall response to the *P. berghei* antigen.

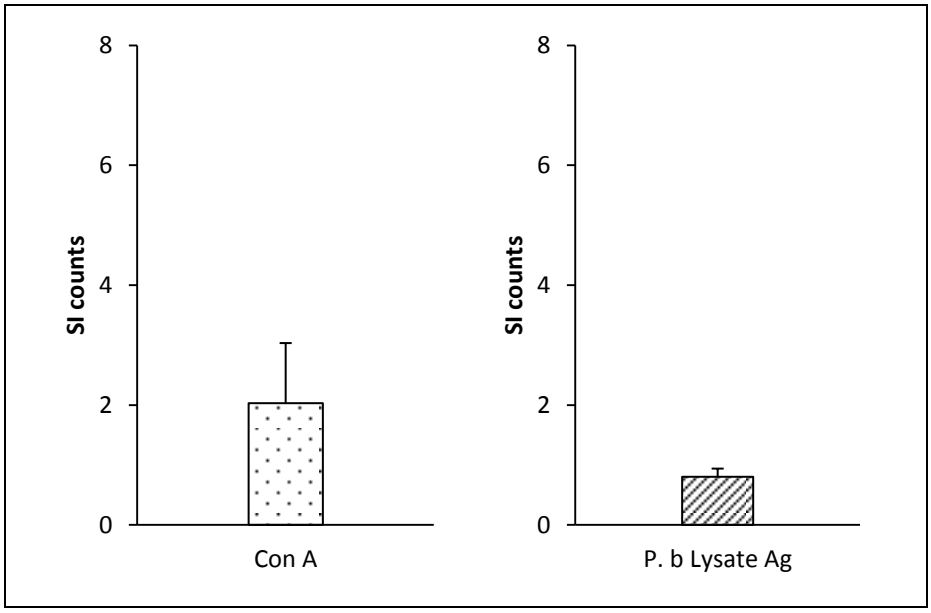

Figure 16: Stimulation indices for the plasmid control group after second boost

For Con A stimulation, SI counts ranged from 0.9 to 3.0. These differences were however not significant (p>0.9999). The lowest stimulation count under crude *P. berghei* lysate antigen stimulation was 0.5 while the highest was 1.1. The variation in the counts however are also not significant (p>0.999).

In the group that received the plasmid encoding SERA alone, Con A stimulation counts of this group were significantly higher than those of crude *P. berghei* lysate antigen (p=0.0087) as shown in Figure 17. This result also indicates a low recall response to the *P. berghei* antigen.

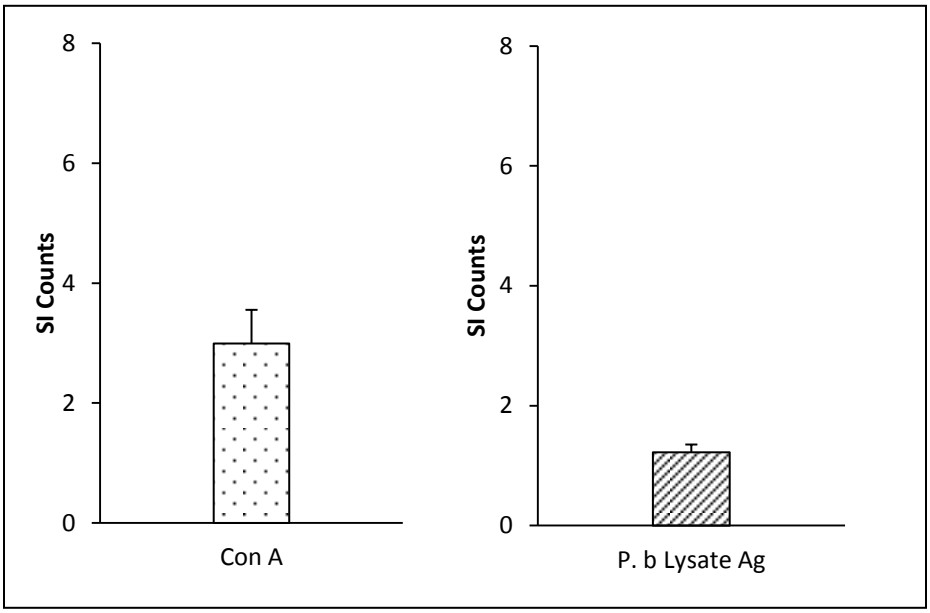

Figure 17: Stimulation indices for SERA in pIRES group after second boost

The SERA in pIRES group's highest stimulation counts under Con A stimulation was 5.2 while the lowest reading was 1.5. These variations are however not significant (p>0.9999). Under crude *P. berghei* lysate antigen stimulation, the highest count was 1.7 and the lowest was 0.9. Similarly, the differences were not significant (p>0.9999).

Significantly higher counts were observed in the Con A stimulated cells in the group that was injected with the construct that contained SERA with CCL20 as an adjuvant, compared to the cells stimulated with the crude *P. berghei* lysate antigen (p=0.079). Figure 18 presents the stimulation indices obtained after the second boost of the SERA+CCL20 DNA vaccine.

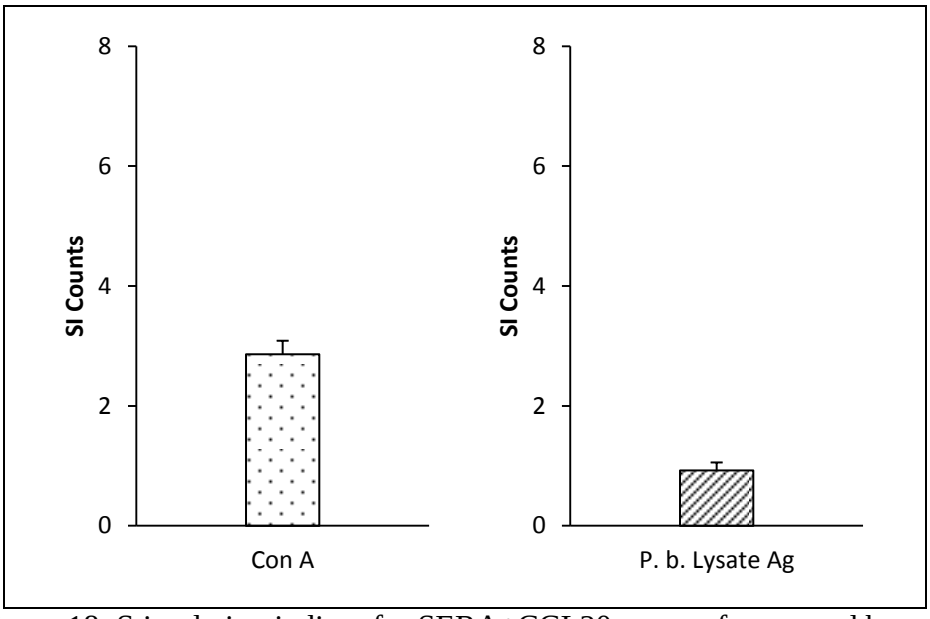

Figure 18: Stimulation indices for SERA+CCL20 group after second boost

The highest stimulation indices under Con A stimulation was 3.6 while 2.4 was the lowest but the differences among the individual mice were not significant (p>0.9999). Under crude *P. berghei* lysate antigen, mouse 9 had the highest stimulation counts while the rest were lower but comparable to each other and just like with the Con A counts, the variations observed were not significant (p>0.9999). The low SI counts indicate a low recall response to crude *P. berghei* lysate antigen.

In the group that received plasmid encoding SERA and CCL5 proliferation assays showed a significant difference between the indices obtained from the Con A and crude *P. berghei* lysate antigen (p=0.0260), with the Con A indices higher as presented on Figure 19.

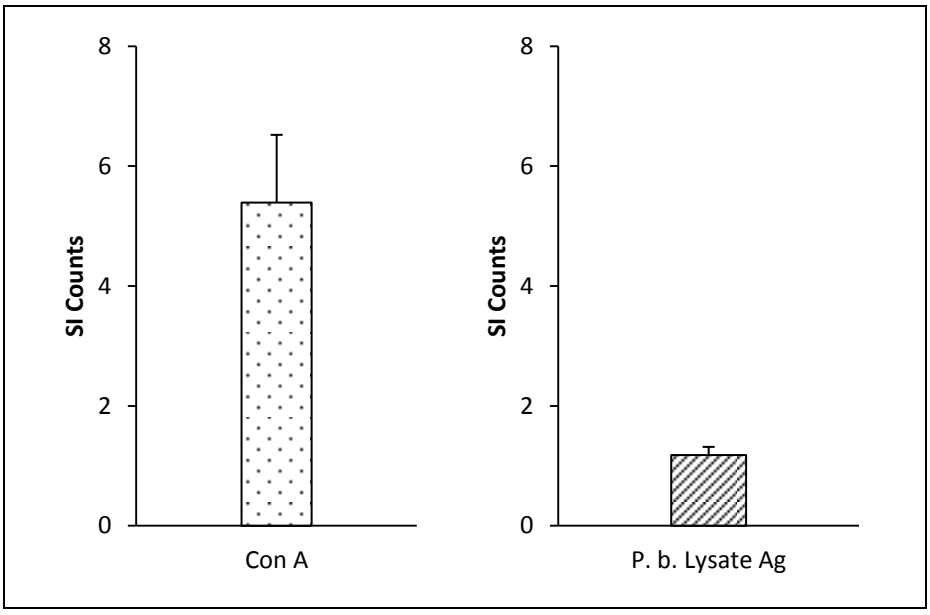

Figure 19: Stimulation indices for SERA+CCL5 group after second boost

Although stimulation indices under Con A stimulation was highest at 9.0 and 1.0 was the lowest the variations within the group were significant (p>0.9999). In the cells stimulated with crude *P. berghei* lysate antigen, stimulation indices varied from 0.7 to 1.4 and the differences were not significant (p>0.9999). However, the SI counts under crude *P. berghei* lysate antigen were low, suggesting a low recall response to the same.

Group comparisons of Con A against crude *P. berghei* antigen are presented in Figure 20.

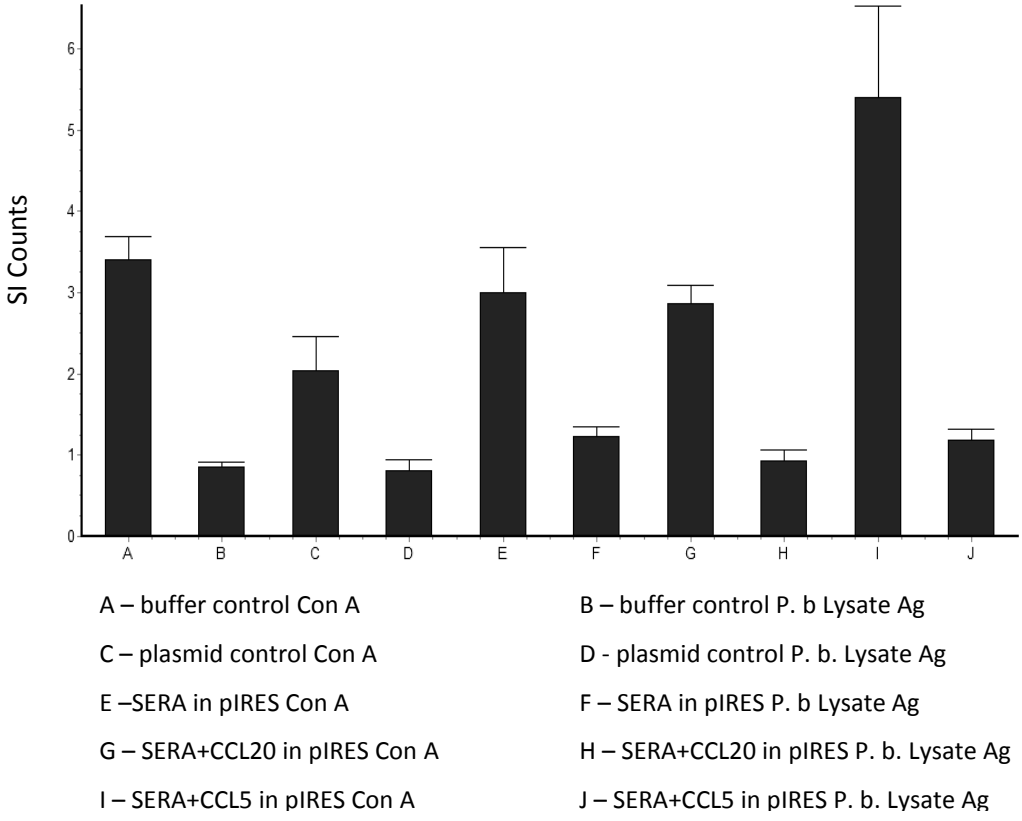

Figure 20: Stimulation indices after second boost

Stimulation indices for cells stimulated with Con A showed that SERA+CCL5 in pIRES had the highest stimulation followed by the buffer control group, SERA in pIRES, SERA+CCL20 in pIRES and finally the plasmid control group in descending order. However analysis by ANOVA showed that the differences observed were not significant with the exception of the comparison between plasmid control group and SERA+CCL5 in pIRES ($p<0.05$).

Inter-group comparison of crude *P. berghei* lysate antigen stimulation was done to determine the effect of inclusion of the immunomodulatory chemokines CCL5 and CCL20 in the SERA DNA candidate vaccine. *Plasmodium berghei* ANKA crude antigen showed greatest stimulation in the group that received SERA in pIRES followed by SERA+CCL5 in pIRES, SERA+CCL20 in pIRES, buffer control and plasmid control. These differences are however not significant

(p=0.3491). Similarly, stimulation with pre-immunization controls, also showed no significant differences with those obtained after the second boost (p=0.1172) despite giving higher readings than the buffer control, plasmid control and SERA+CCL20 in pIRES groups and lower stimulation than SERA in pIRES and SERA+CCL5 in pIRES groups.

These results show low induction of cross-reactive recall responses to *P. berghei* antigen by SERA DNA vaccine candidate even in the presence CCL20 and CCL5.

3.5 Cross-protective efficacy

A vaccine that can induce protection against more than one strain of the *Plasmodium* parasites would save time and resources developing a vaccine for each of them, in addition to protecting individuals in areas where the more than one strain is prevalent. In this case the vaccine preparations were *P. falciparum* based. Cross-protective efficacy of the vaccine preparations was determined through heterologous infection of the vaccinated mice with *P. berghei* ANKA, 3 weeks after receiving the second boost. Both percent parasitaemia and improved survival during infection were determined.

3.5.1 Parasitaemia profiles

Parasitaemia suppression was determined by comparing the parasitaemia profiles of the experimental groups (SERA in pIRES, SERA+CCL20 in pIRES, SERA+CCL5 in pIRES) and control groups (non-vaccinated control, buffer control, plasmid control).

Within the buffer control group (TE pH 7.2), all mice picked infection with the exception of mouse 18 which was excluded. This is presented on Figure 21 below.

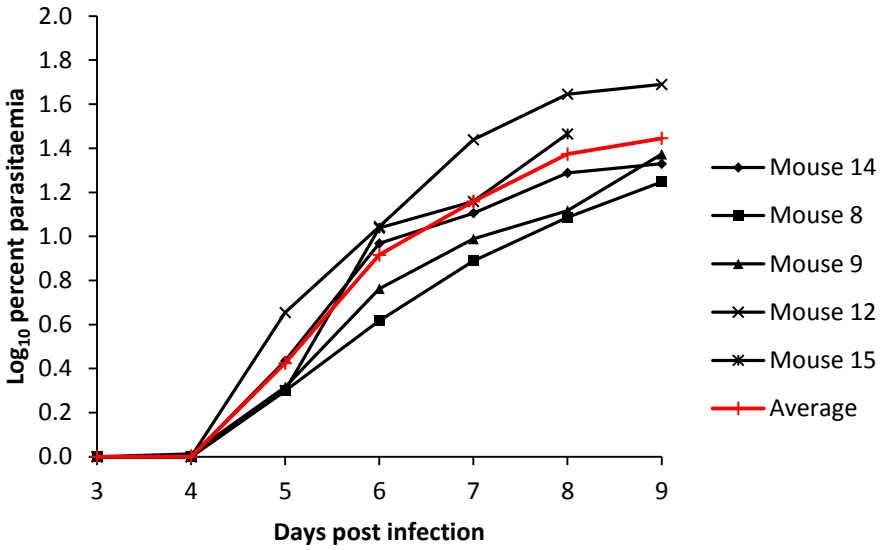

Figure 21: Buffer control group parasitaemia profile

Mouse 12 had the highest progression of parasitaemia peaking at 4,893,300 infected RBCs (iRBCs)/µl of blood while the rest appeared to have a slower progression. However, even with that higher peak, no significant difference was observed among the mice throughout infection (p>0.9999). This shows low suppression of parasitaemia.

The plasmid control group (pIRES vector backbone) were all positive for infection and no significant difference was observed among the mice (p>0.9999). Figure 22 below presents the percent parasitaemia progression from day 3 post-infection.

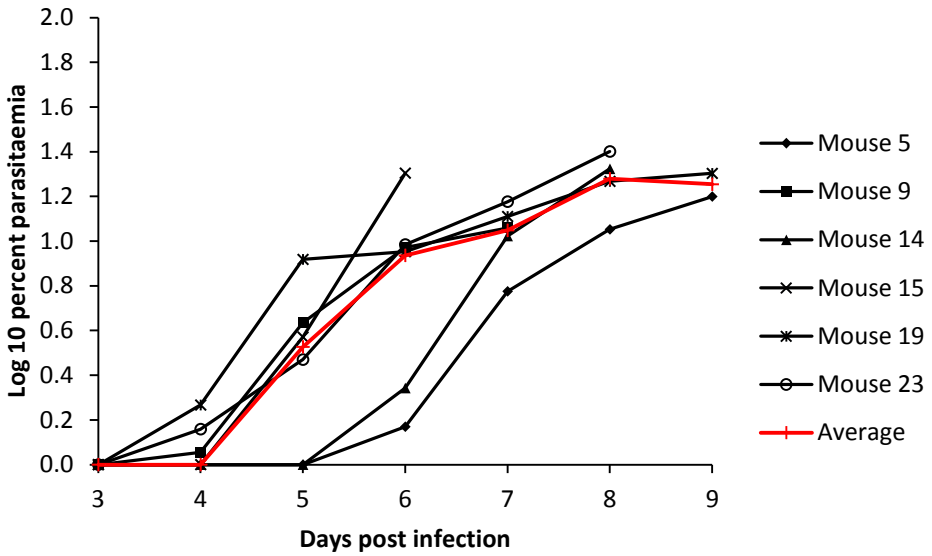

Figure 22: Plasmid control group parasitaemia profile

Mouse 15 had a rapid increase in parasitaemia from day 5 to day 6 post-infection and died on day 6 post-infection at a parasitaemia of 2,014,300 iRBCs/µl of blood. Highest parasitaemia was achieved on day 8 post infection by mouse 23 at 2,517,900 iRBCs/µl of blood.

Infection picked in all the mice in the SERA only group as presented in the parasitaemia profile on Figure 23.

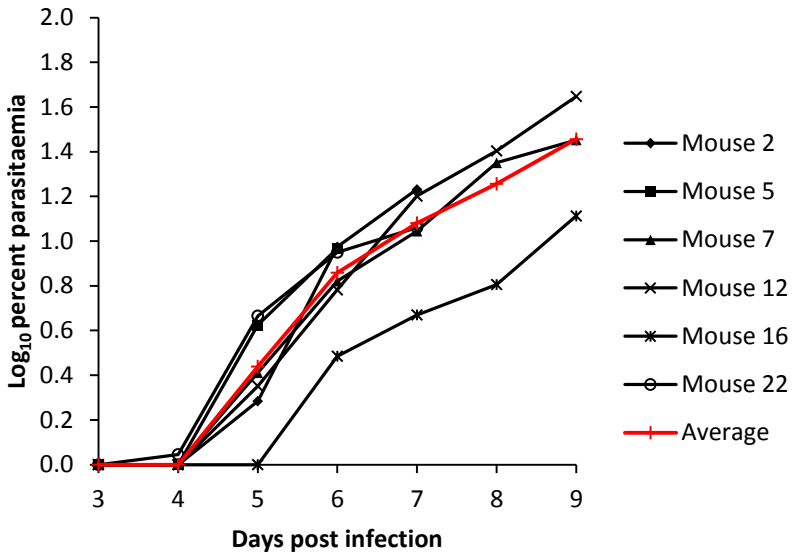

Figure 23: SERA group parasitaemia profile

The mice showed similar progression until day 7 post-infection where mouse 22 appeared to be slower than the rest and chronic towards the end. On the same day, mice 2 and 22 succumbed to infection at 1,703,200 iRBCs/µl of blood and 1,149,000 iRBCs/µl of blood respectively. The highest parasitaemia seen in this group was 4,440,900 iRBCs/µl of blood in mouse 12 on day 9 post-infection. No significant difference was seen among the mice (p=0.6217).

In the group that received SERA with CCL20 as the adjuvant, all mice got infected and had comparable rates of progression of infection with no significant difference observed between individuals (p=0.9037). This data is presented on Figure 24.

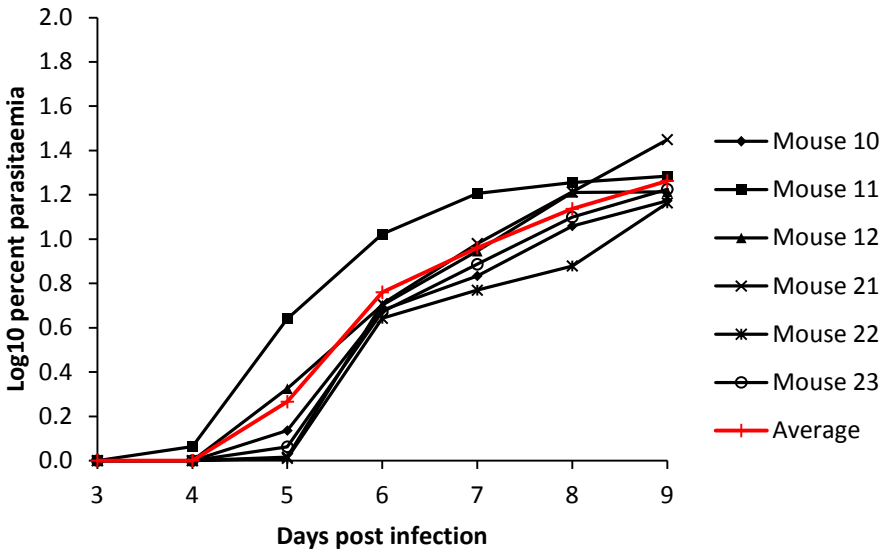

Figure 24: SERA+CCL20 group parasitaemia profile

After day 9 post-infection, all the mice died except mouse 21 and 10 which both appeared chronic and died on day 12 post-infection.

Where CCL5 was used as a potential adjuvant for SERA, mice used for the challenge infection had similar rates of progression of percentage parasitaemia as presented in Figure 25.

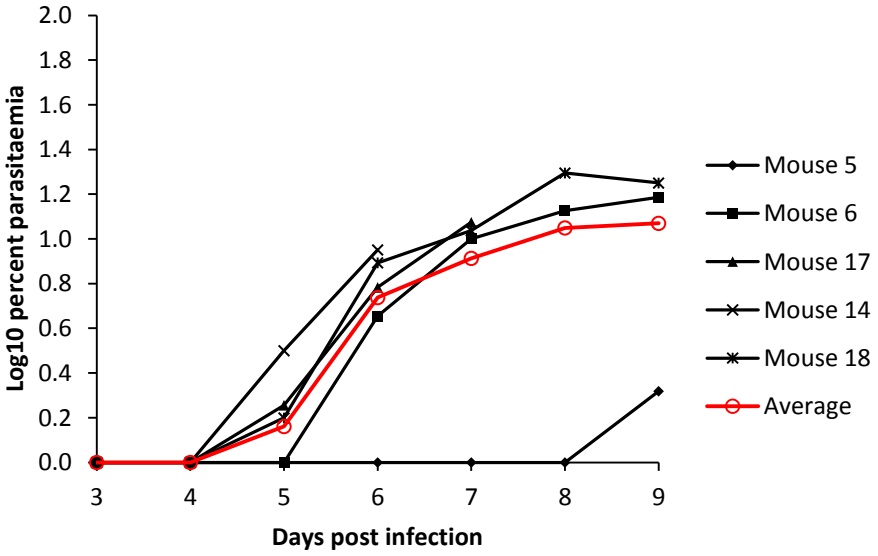

Figure 25: SERA+CCL5 group parasitaemia profile

These mice were all positive for infection by day 3 post-infection, showing no significant difference among them (p=0.4538).

For the non-vaccinated group, mouse 5 had the slowest rate of progression of infection, and outlasted the rest during infection. The other mice in the group had rates of progression more similar to each other and no significant difference was observed among them (p=0.2144). Figure 26 below presents percent parasitaemia progression for the non-vaccinated controls.

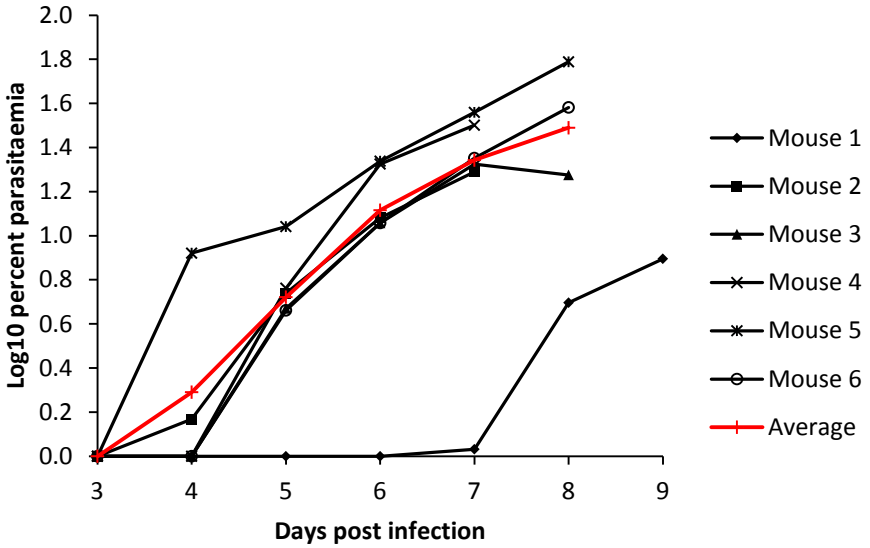

Figure 26: Non-vaccinated control group parasitaemia profile

Mouse 1 reached its highest parasitaemia of 1,692,100 iRBCs/µl of blood on day 11 post-infection and died a day later after parasitaemia dropped to 1,377,200 iRBCs/µl of blood being the longest surviving of the group. Highest parasitaemia in the group was observed in mouse 5 on day 8 post-infection at 6,144,200 iRBCs/µl of blood which was also the highest of all the mice in the challenge infections.

Inter-group comparison of percent parasitaemia progression was done by calculating the averages for each group and presented on Figure 27.

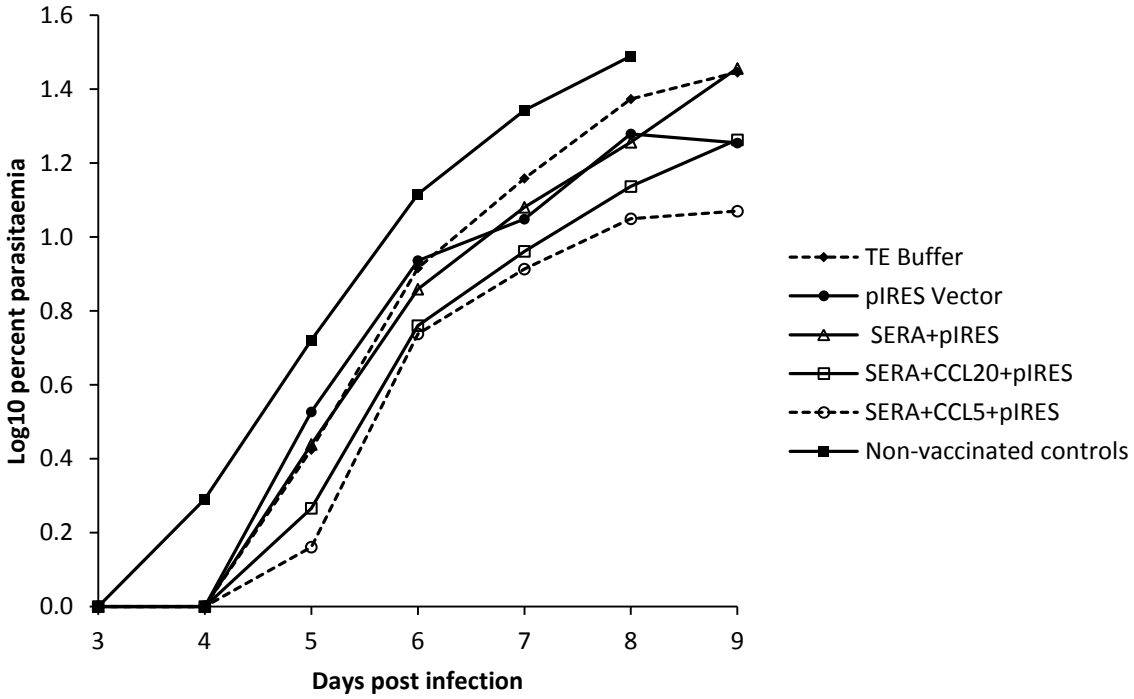

Figure 27: Average *P. berghei* parasitaemia profiles for all groups

There was a significant increase in parasitaemia from infection through the course of infection up to day 8 post-infection (PI). From day 5 post-infection, the profiles diverged to show a rapid increase in parasitaemia in the non-vaccinated control group, followed by buffer control (Tris EDTA ph7.2) group. SERA in pIRES and pIRES groups showed similar rate of progression being slower than those of the non-vaccinated control and Tris EDTA buffer pH7.2 groups but still faster than the SERA+CCL20 in pIRES and SERA+CCL5 in pIRES groups.

In terms of total parasite burden throughout patent infection, it was observed that mean cumulative parasitaemia was significantly lower in the SERA+CCL5 in pIRES group compared to the non-vaccinated group as shown on Figure 28 (p=0.0437).

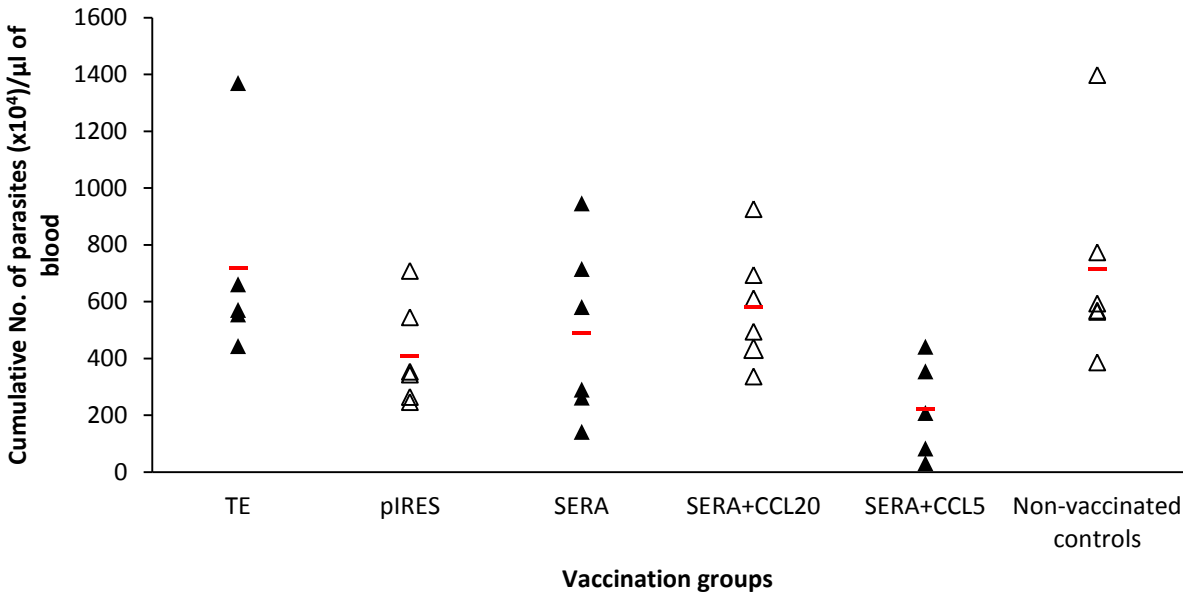

Figure 28: Cumulative parasitaemia

The mean cumulative parasitaemia showed no suppression in the buffer control (TE) group, while pIRES, SERA in pIRES and SERA+CCL20 in pIRES showed suppressions of 42.58%, 31.45% and 18.31% respectively. SERA+CCL5 in pIRES group showed the highest suppression at 68.69%.

In addition to the suppression in cumulative parasitaemia, mean peak parasitaemia was also lower in the SERA+CCL5 in pIRES group by 55.6% compared to non-vaccinated groups as shown in Figure 29.

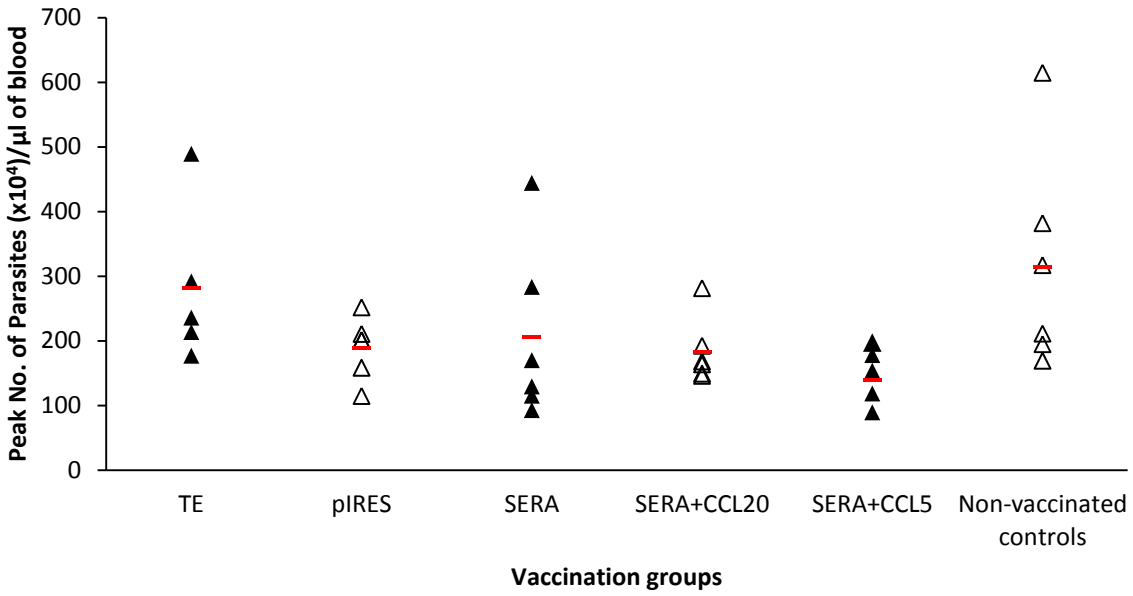

Figure 29: Peak parasitaemia

The other groups also showed some suppression where buffer control (TE) group, plasmid control (pIRES), SERA in pIRES and SERA+CCL20 in pIRES having lower average peak parasitaemia by 10.57%, 39.75%, 34.58% and 41.75% respectively. The differences were however not significant (p=0.0601).

These results indicate parasitaemia suppression was induced most in the SERA+CCL5 in pIRES group.

3.5.2 Mouse survival

Improved survival of mice under *P. berghei* infection was determined using the number of days the mice stayed alive following infection. Mice started dying from day 7 PI with one mouse dying in each of the pIRES, SERA in pIRES and SERA+CCL5 in pIRES groups. SERA+CCL20 in pIRES had the best survival rate with more mice surviving longer while the non-vaccinated control, Tris EDTA buffer pH7.2 and pIRES vector groups had the lowest. These observations are presented on Figure 30.

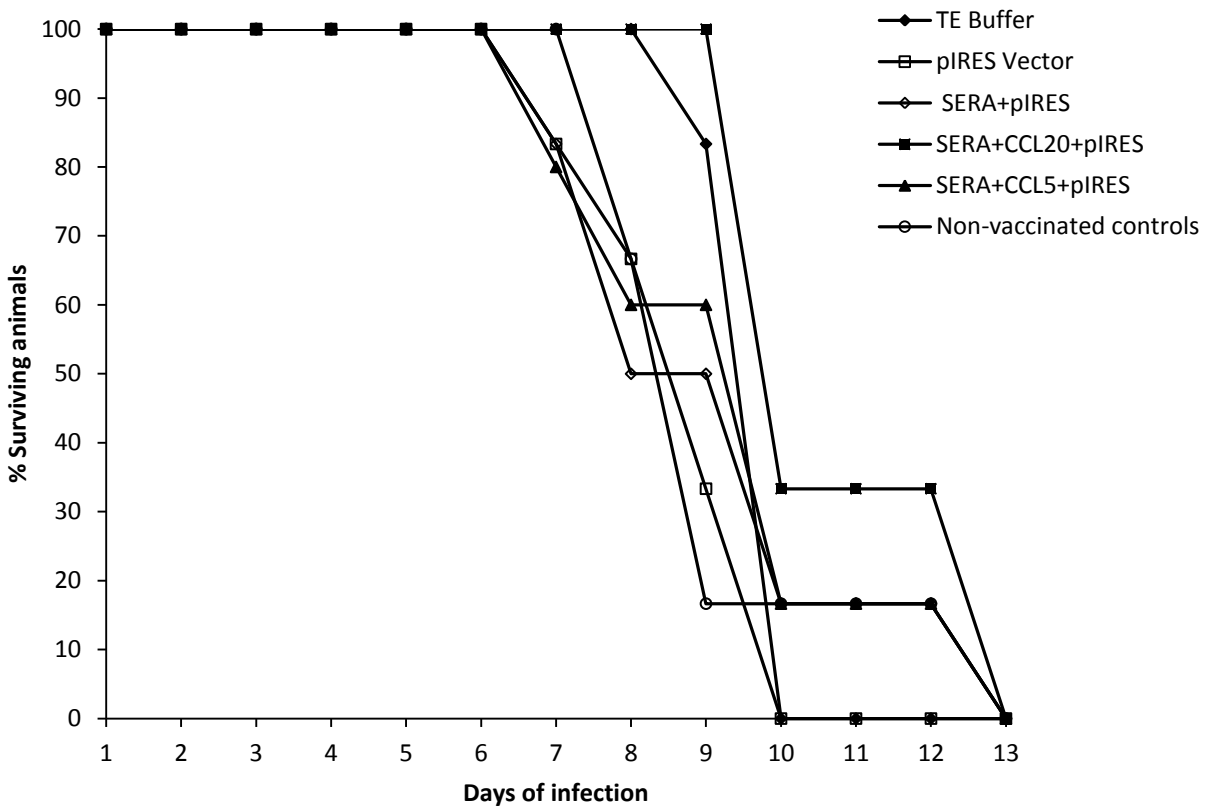

Figure 30: Survivorship curve following *P. berghei* challenge

On day 7 post infection, one mouse died from the plasmid control, SERA in pIRES and SERA+CCL5 in pIRES groups. By day 10 post-infection, all mice in the buffer and plasmid control groups had died while only one remained in the SERA in pIRES, SERA+CCL5 in pIRES and non-vaccinated groups and two in the SERA+CCL20 in pIRES group. These remaining mice died on day 13 post-infection. This suggests plasmid encoding SERA and CCL20 improved survival of infected mice.

CHAPTER FOUR: DISCUSSION

In this study, CCL5 and CCL20 were investigated as chemokine adjuvants for safety and effect on immunogenicity and cross-protective efficacy of SERA in BALB/c mice against *P. berghei*. Both CCL5 and CCL20 are chemotactic for CD45RO memory T lymphocytes but whereas CCL5 is a potent chemoattractant for leukocytes including monocytes, CCL20 is chemotactic for immature dendritic cells (Volin, *et al.*, 1998; Izadpanah, *et al.*, 2001). Results show that the SERA DNA vaccine candidate for malaria administered intramuscularly is safe even in the case of co-expression of CCL20 and CCL5 in mice. Splenocytes from all mice (buffer control, plasmid control, SERA in pIRES, SERA+CCL20 in pIRES and SERA+CCL5 in pIRES groups) registered low cytokine and recall responses to crude *P. berghei* antigen. However plasmid co-expressing SERA and CCL5 showed significant suppression of parasitaemia of up to 68.69% compared to non-vaccinated controls. Moreover, CCL20 co-expression with SERA showed better survival under *P. berghei* ANKA infection where 100% survived up to day 9 of infection as opposed to 16.67% in non-vaccinated controls.

As with biological products introduced into the human organism, all vaccines have potential to cause adverse reactions including at the site of injection, fever or other systemic reactions and sometimes anaphylaxis. In addition to specific infectious disease-related antigens, other components of vaccines may theoretically cause adverse effects, including adjuvants added to enhance vaccine potency (Global Advisory Committee on Vaccine Safety and WHO secretariat, 2009).

Vaccination effectively enables control of many infectious diseases. However, the problem of adverse reactions accompanied by vaccination cannot always be avoided. While most adverse reactions are mild and local, some vaccines have been associated with rare but severe systemic reactions. The body weight of vaccine treated animals can be analyzed as a general safety test for toxicity of vaccines (Momose *et al.*, 2010). There was an increase in body weight made after administration of the various vaccine constructs. There were no local reactions, swellings, ruffled fur or altered gait. These results show that the DNA vaccines administered intramuscularly are safe and that the chemokines CCL20 and CCL5 are safe for use as adjuvants in vaccines.

Developing a vaccine that could induce simultaneous protection against *P. falciparum* and *P. vivax* or even more human *plasmodium* species would have enormous economic, safety, and manufacturing advantages (Douradinha, *et al*., 2008). Cross-reactive cellular recall responses to crude *P. berghei* antigen were low as comparison between the stimulation indices of the vaccinated and non-vaccinated groups did not show a significant difference. Results also showed a low effect of chemokines CCL5 and CCL20 on immunogenicity of SERA against *P. berghei* where no significant difference was noted when compared against pre-immunization stimulation indices. This implies low levels of priming which may be due to differences in the homologies of *P. falciparum* and *P. berghei* SERA antigens.

Results from cytokine assays showed low improvement of immunogenicity. The IFN-γ response induced by SERA in pIRES group was not sustained in contrast to SERA+CCL5 in pIRES which increased till the end attaining a level about 1.3 times higher than SERA in pIRES. Similarly, IL-4 levels in the SERA+CCL20 in pIRES group rose to almost double the titres induced by SERA+CCL5 in pIRES whose response only showed up at the end whereas SERA in pIRES IL-4 remained almost unchanged. Though the differences were not significant, these responses may have been in part responsible for the reduced parasitaemia observed in the two groups. Th1 type pro-inflammatory immune responses are essential for controlling parasite load during the early phase of infection. Protective $CD4^+$ T cells release IFN-γ to activate effector cells such as macrophages, which may exert anti-malarial effects by releasing nitric oxide (NO). NO can reduce parasitaemia during the initial phase of blood-stage malaria infection (Feng *et al*., 2012). In addition, IL-4 production by T cells stimulated by malaria antigens *in vitro* has been associated with increased concentrations of serum antibodies to the same activating malaria antigens *in vivo* (Troye-Blomberg *et al.*, 1990).

Studies with humans have found a correlation between IgG1 and IgG3 levels and protection against severe disease. These two isotypes have been shown to mediate opsonisation. While Nunes and his colleagues (2009) indicated that secreted antibody is essential to adaptive immunity to *P. berghei* in mice, they also noted that passively transferring serum from immune mice did not result in complete clearance suggesting a role for T cells in developing immunity (Nunes, *et al.*, 2009). Studies in both humans and mice have shown that inflammatory cytokines,

including IFN-γ are essential mediators of protective immunity to erythrocytic malaria (Artavanis-Tsakonas and Riley, 2002).

IL-10 levels remained low in all groups and can partially be the reason why there was no parasite clearance in the SERA+CCL5 in pIRES group where significant parasitaemia suppression was observed. Resistance to rodent malaria is absolutely dependent on signals mediated by INF-γ followed by an IL-10 response to completely clear parasitaemia. Similarly in humans IFN-γ secreted by CD4+ T cells is correlated with resistance to reinfection with *P. falciparum* and clinical attacks of malaria (Bakir *et al.*, 2011). IL-4 which is essential in parasite clearance in late infections had low levels. Our results are in line with these findings where parasitaemia developed was not cleared due to low cytokine responses.

In the cross-protective efficacy studies, parasitaemia profiles revealed a cross-species protective effect in the groups vaccinated with plasmid encoding SERA, SERA and CCL20 and SERA and CCL5. The greatest effect was observed in the SERA+CCL5 in pIRES group having a lower mean cumulative parasitaemia and mean peak parasitaemia than other groups, and a significant difference seen between it and the non-vaccinated control group. On the other hand the SERA+CCL20 in pIRES group had better survival. A prominent feature of rodent malaria infections is that survival is linked to ability to control replication of blood-stage parasites (Stevenson and Riley, 2004). However, the exact mechanism for this observation remains unclear.

Cross-species interactions have been observed with plasmodial asexual blood stages, but these interactions are poorly understood and have been less well studied than the cross-species protection associated with exoerythrocytic stages (Douradinha, *et al*., 2008). Immunization with *P. falciparum* sporozoites delivered by the bite of infected mosquitoes protected an average of 60% mice from *P. berghei* sporozoite infection. Similarly, passively transferred immunoglobulin from *P. falciparum* sporozoite-immunized mouse serum protected naive mice against challenge with *P. berghei* sporozoites, indicating that cross-protection is mediated, at least in part, by anti-sporozoite antibody (Sina *et al.*, 1993). Cross-protection against one plasmodial species induced by asexual blood stages of another has also been observed where sera

from mice highly infected with *P. chabaudi* (parasitemia≥50%), inhibited growth of asexual blood stages of *P. falciparum in vitro* (Butcher and Clark, 1990). In this study, we observed suppression in mean cumulative parasitaemia of 68.69% and mean peak parasitaemia of 55.6% in the SERA+CCL5 group compared to non-vaccinated mice.

The overall result of this study is that the vaccine preparations were safe but levels of immune parameters investigated were lower than those required for protection against severe malaria. Immunology results showed low cross-reactivity between *P. falciparum* and *P. berghei* which could be due to differences in homologies of SERA molecules in the two parasites. However, plasmid encoding both SERA and CCL5 showed improved parasite suppression while co-expression of SERA and CCL20 resulted in improved survival. These observations show that the chemokines marginally improved immunogenicity of the SERA DNA vaccine candidate and cross-protective efficacy was improved.

CHAPTER FIVE: CONCLUSIONS

According to the study, we found that the DNA formulations of the *Pf*SERA malaria vaccine candidate:

1. were safe because there were no reactions at injection sites, weight, physical activity, mobility and appearance were normal
2. inclusion of immunomodulatory chemokines CCL20 and CCL5 marginally improved immunogenicity against *P. berghei* infection in mice
3. improved cross-protective efficacy where the chemokines were used as genetic adjuvants

5.1 Study Limitations

- Lack of a *P. falciparum* model to evaluate the vaccine formulations *in vivo.*
- Poorly understood mechanisms of the exact correlates of cross-species immunity against plasmodial infection as well as limited information on appropriate antigen and adjuvant combinations to target the relevant immune responses.
- Time constraints, hence humoral responses to the DNA vaccine formulations of *Pf*SERA were not investigated.

5.2 Recommendations

Further investigations, including humoral responses, should be undertaken to determine the mechanisms behind parasitaemia suppression observed in animals vaccinated with plasmid encoding SERA and CCL5 in addition to the role played by CCL20 in improving survival. Moreover, more efficient modes of administration of the DNA vaccines such as a gene gun or electroporation should be adopted to enhance cellular uptake of the plasmids. These methods reduce the dose required to induce an immune response and improve uptake of plasmid by cells and in turn enhance antigen expression.

REFERENCES

Aide, P., Bassat, Q., & Alonso, L. P. (2007). Towards an effective malaria vaccine. *Archives of Disease in Childhood* **92**, pp. 476-479.

Aoki, S., Li, J., Itagaki, S., Okech, B. A., Egwang, T. G., Matsuoka, H., Palacpac, N. M. Q., Mitamura, T., & Horii, T. (2002). Serine Repeat Antigen (SERA5) Is Predominantly Expressed among the SERA Multigene Family of *Plasmodium falciparum*, and the Acquired Antibody Titres Correlate with Serum Inhibition of the Parasite Growth. *The Journal of Biological Chemistry* **277** (49), pp. 47533-47540.

Artavanis-Tsakonas, K., & Riley, E. M. (2002). Innate Immune Response to Malaria: Rapid Induction of IFN-γ from Human NK Cells by Live *Plasmodium falciparum*-Infected Erythrocytes. *The Journal of Immunology* **169**, pp. 2956–2963.

Baird, J. K. (2005). Review: Drug Therapy Effectiveness Of Antimalarial Drugs. *The New England Journal of Medicine* **352**, pp. 1565-1577.

Bakir, H. Y., Tomiyama, C., & Abo, T. (2011). Cytokine profile of murine malaria: stage-related production of inflammatory and anti-inflammatory cytokines. *Biomedical Research* **32** (3), pp. 203-208.

Belnoue, E, Kayibanda, M., Deschemin, J., Viguier, M., Mack, M., Kuziel, W. A., & Rénia, L. (2003). CCR5 deficiency decreases susceptibility to experimental cerebral malaria. *Blood* **101**, pp. 4253-4259.

Belperron, A. A., Feltquate, D., Fox, B. A., Horii, T., & Bzik, D. J. (1999). Immune Responses Induced by Gene Gun or Intramuscular Injection of DNA Vaccines That Express Immunogenic Regions of the Serine Repeat Antigen from *Plasmodium falciparum. Infection and Immunity* **67** (10), pp. 5163-5169.

Bhatia, A., Delplace, P., Fortier, B., Dubremetz, J. F., & Vernes, A. (1987). Immunochemical Analysis of a Major Antigen of *Plasmodium falciparum* (P126) Among Ten Geographic Isolates. *American Journal of Tropical Medicine and Hygiene* **36**, pp. 15-19.

Bourgon, R., Delorenzi, M., Sargeant, T., Hodder, A. N., Crabb, B. S., & Speed T. P. (2004). The Serine Repeat Antigen (SERA) Gene Family Phylogeny in *Plasmodium*: The Impact of GC Content and Reconciliation of Gene and Species Trees. *Molecular Biology and Evolution* **21** (11), pp. 2161-2171.

Butcher, G. A., & Clark. I. A. (1990). The inhibition of *Plasmodium falciparum* growth *in vitro* by sera from mice infected with malaria or treated with TNF. *Parasitology* **101,** pp. 321–326.

Chandy, C. J., Opika-Opoka, R., Byarugaba, J., Idro, R., & Boivin, M. J. (2006). Low Levels of RANTES Are Associated with Mortality in Children with Cerebral Malaria. *The Journal of Infectious Diseases* **194**, pp. 837–45.

Chilengi, R., & Gitaka, J. (2010). Is vaccine the magic bullet for malaria elimination? A reality check. *Malaria Journal* **9** (Suppl 3):S1, doi:10.1186/1475-2875-9-S3-S1.

Chowdhury, K., Kantor, M., & Sestras, R. (2009). Malaria Vaccine Candidate Diversity Offers Challenges and Opportunities for Effective Vaccine Development. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **37** (1), pp. 9-16.

Coler, R. N., Carter, D., Friede, M., & Reed, S. G. (2009). Review Article: Adjuvants for Malaria Vaccines. *Parasite Immunology* **31,** pp. 520-528.

Cox, F. E. G. (2010). Review: History of the Discovery of the Malaria Parasites and their Vectors. *Parasites and Vectors* **3** (1), pp. 5.

Crompton, **P. D., Pierce, S. K., & Miller, L. H.** (2010). Advances and challenges in malaria vaccine development. *The Journal of Clinical Investigation* **120** (12), pp. 4168-4178.

Douradinha, B., Mota, M. M., Luty, A. J. F., & Sauerwein, R. W. (2008). Cross-Species Immunity in Malaria Vaccine Development: Two, Three, or Even Four for the Price of One?. *Infection and Immunity* **76** (3), pp.873-878.

Engers, H. D., & Godal, T. (1998). Malaria Vaccine Development: Current Status. *Parasitology Today* **14** (2), pp. 56-64.

Fairlie, W. D., Spurck, T. P., McCoubrie, J. E., Gilson, P. R., Miller, S. K., McFadden, G. I., Malby, R., Crabb, B. S., & Hodder, A. N. (2008). Inhibition of Malaria Parasite Development by a Cyclic Peptide That Targets the Vital Parasite Protein SERA5. *Infection and Immunity* **76** (9), pp. 4332–4344.

Farooq, U., & Mahajan, R. C. (2004). Drug resistance in malaria. *Journal of Vector Borne Diseases* **41**, pp. 45–53.

Feng, Y., Zhu, X., Wang, Q., Jiang, Y., Shang, H., Cui, L., & Cao, Y. (2012). Allicin enhances host pro-inflammatory immune responses and protects against acute murine malaria infection. *Malaria Journal* **11**:268, doi:10.1186/1475-2875-11-268.

Fernando, S. D., Rodgrigo, C., & Rajapakse, S. (2010). The 'hidden' burden of malaria: cognitive impairment following infection. *Malaria Journal* **9**:366, doi:10.1186/1475-2875-9-366.

Geiben-Lynn, R., **Greenland, J. R., Frimpong-Boateng, K., Van Rooijen, N., Hovav, A., & Letvin, N. L.** (2008). CD4-T lymphocytes mediate in vivo clearance of plasmid DNA vaccine antigen expression and potentiate CD8+ T-cell immune responses. *Blood* **112** (12), pp. 4585-4590.

Girard, M. P., Reed, Z. H., Friede, M., & Kieny, M. P. (2007) A review of human vaccine research and development: Malaria. *Vaccine* **25**, pp. 1567–1580.

Global Advisory Committee on Vaccine Safety(GACVS) & WHO secretariat. (2009). Global safety of vaccines: strengthening systems for monitoring, management and the role of GACVS. *Expert Reviews* **8** (6), pp. 705-716.

Greenwood, B., & Alonso, P. (2002) Malaria Vaccine Trials. *Chemical Immunology* **80,** pp. 366-395.

Guinovart C., & Alonso, L. P. (2007) Methods for Determining Vaccine Efficacy and Effectiveness and the Main Barriers to Developing a Fully Deployable Malaria Vaccine. *American Journal of Tropical Medicine and Hygiene.* **77**(Suppl 6), pp. 276–281.

Gurunathan, S., Klinman, D. M., & Seder, R. A. (2000). DNA VACCINES: Immunology, Application, and Optimization. *Annual Review of Immunology* **18**, pp. 927–974.

Hodder, A., Crewther, P. E., & Anders, R. F. (2001). Specificity of the Protective Antibody Response to Apical Membrane Antigen 1. *Infection and Immunity* **69** (5), pp. 3286-3294.

Holder, A. A. (2009). Malaria Vaccines: Where Next?. *PLoS Pathogens* **5** (10): e1000638.

Inselburg, J., Bzik, D. J., Li, W., Green, K. M., Kansopon, J., Hahm, B. K., Bathurst, I. C., Barr, P. J., & Rossan, R. N. (1991). Protective Immunity Induced in Aotus Monkeys by Recombinant Sera Proteins of *Plasmodium falciparum*. *Infection and Immunity* **59** (4), pp. 1247-1250.

Institute of Primate Research. (2002). Standard Operating Prodecures. p125-128, 188, 210, 218-219.

Ivory, C., & Chadee, K. (2004). DNA Vaccines: Designing strategies against parasitic infections. *Genetic Vaccines and Therapy* **2**:17, doi:10.1186/1479-0556-2-17.

Izadpanah, A., Dwinell, M. B., Eckmann, L., Varki, N. M., & Kagnoff, M. F. (2001). Regulated MIP-3α/CCL20 Production By Human Intestinal Epithelium: Mechanism For Modulating Mucosal Immunity. *American Journal of Physiology Gastrointestinal Liver Physiology* **280**, pp. G710–G719.

Jones, T. R., & Hoffman, S. L. (1994). Malaria Vaccine Development. *Clinical microbiology reviews* **7**(3), pp. 303-310.

Kakkilaya, B. S. (2010). http://www.malariasite.com/malaria/ControlOfMalaria.htm. Retrieved 18th January 2010.

Kanoi, B. N. & Egwang, T. G. (2007). New concepts in vaccine development in malaria. *Current Opinions on Infectious Diseases* **20**, pp. 311-316.

Kim, J. J., Yang, J-S., Dentchev, T., Dang, K., & Weiner, D. B. (2000) Chemokine Gene Adjuvants Can Modulate Immune Responses Induced by DNA Vaccines. *Journal of Interferon and Cytokine Research* **20**, pp. 487–498.

Kumar, N. (2007). A vaccine to prevent transmission of human malaria: A long way to travel on a dusty and often bumpy road. *Current Science* **92** (11), pp. 1535-1544.

Kurella, S., Manocha, M., Sabhnani, L., Thomas, B., & Rao, D. N. (2000). New Age Adjuvants and Delivery Systems for Subunit Vaccines. *Indian Journal of Clinical Biochemistry* **15** (Suppl. 1), pp. 83-100.

Kwiatkowski, D., & Marsh, K. (1997). Development of a malaria vaccine. *The Lancet* **350** (9092), pp. 1696 – 1701.

Laufer, M. K. (2009). Monitoring Antimalarial Drug Efficacy: Current Challenges. *Current Infectious Disease Reports* **11** (1), pp. 59–65.

Leclerc, M. C., Hugot, J. P., Durand, P., & Renaud, F. (2004). Evolutionary relationships between 15 *Plasmodium* species from New and Old World primates (including humans): an 18S rDNA cladistic analysis. *Parasitology* **129**, pp. 677–684.

Lutz, H. G. (2006). Farmers' Organization's Guide to the Marketing Environment for Small Scale Farmers in Southern Africa: Collecting the money. Harare: Swedish Cooperative Centre Regional Office for Southern Africa. p. 25.

Mahanty, S., Saul, A., & Miller, L. H. (2003). Review: Progress in the development of recombinant and synthetic blood-stage malaria vaccines. *The Journal of Experimental Biology* **206**, pp. 3781-3788.

Mbugi, E. V., Mutayoba, B. M., Balthazary, S. T., Nyambo, T. B., & Mshinda, H. (2006). Drug resistance to sulphadoxine-pyrimethamine in *Plasmodium falciparum* malaria in Mlimba, Tanzania. *Malaria Journal* **5**:94, doi:10.1186/1475-2875-5-94.

Momose, H., Mizukami, T., Ochiai, M., Hamaguchi, I., & Yamaguchi, K. (2010). A New Method for the Evaluation of Vaccine Safety Based on Comprehensive Gene Expression Analysis. *Journal of Biomedicine and Biotechnology*, doi:10.1155/2010/361841.

Moorthy, V. S., Good, M. F., & Hill, A. V. S. (2004). Review: Malaria vaccine developments. *The Lancet* **363**, pp. 150–56.

Mugittu, K., Genton, B., Mshinda, H., & Beck, H. P. (2006). Research Molecular monitoring of *Plasmodium falciparum* resistance to artemisinin in Tanzania. *Malaria Journal* **5**, pp. 126-128.

National Museums of Kenya. http://www.museums.or.ke/content/blogsection/6/57. Retrieved on 6th November 2012

Nunes, J. K., Starnbach, M. N., Dyann F. & Wirth, D. F. (2009). Secreted Antibody Is Required for Immunity to *Plasmodium berghei*. *Infection and Immunity* **77** (1), pp. 414–418.

Ochiel, D. O., Awandare, G.A., Keller, C.C., Hittner, J. B., Kremsner, P. G., Weinberg J. B., & Perkins, D. J. (2005). Differential regulation of β-chemokines in children with *Plasmodium falciparum* malaria. *Infection and Immunology* **73**, pp. 4190-4197.

Okech, **B. A., Nalunkuma, A., Okello, D., Pang, X-L., Suzue, K., Li, J., Horii, T., & Egwang, T. G.** (2001). Natural Human Immunoglobulin G Subclass Responses to *Plasmodium falciparum S*erine Repeat Antigen in Uganda. *American Journal of Tropical Medicine and Hygiene* **65** (6), pp. 912-917.

Okech, B., Mujuzi, G., Ogwal, A., Shirai, H., Horii, T., & Egwang, T. G. (2006). High Titres of IgG Antibodies against *Plasmodium falciparum* Serine Repeat Antigen 5 (SERA5) are

associated with Protection against Severe Malaria in Ugandan Children. *American Journal of Tropical Medicine and Hygiene* **74** (2), pp. 191-197.

Othoro, C., Johnston, D., Lee, R., Soverow, J., Bystryn J., & Nardin, E. (2009). Enhanced Immunogenicity of *Plasmodium falciparum* Peptide Vaccines Using a Topical Adjuvant Containing a Potent Synthetic Toll-Like Receptor 7 Agonist, Imiquimod. *Infection and Immunity* **77** (2), pp. 739–748.

Oyakhirome, S., Pötschke, M., Schwarz, N. G., Dörnemann, J., Laengin M., Salazar, C. O., Lell, B., Kun, J. F. J., Kremsner, P. G., & Grobusch, M. P. (2007). Artesunate – amodiaquine combination therapy for falciparum malaria in young Gabonese children. *Malaria Journal* **6**:29, doi:10.1186/1475-2875-6-29.

Palacpac, N. M. Q., Arisue N., Tougan, T., Ishii, K. J., & Horii, T. (2011). *Plasmodium falciparum* serine repeat antigen 5 (SE36) as a malaria vaccine candidate. *Vaccine* **29**, pp. 5837–5845.

Pang, X-L., & Horii, T. (1998). Complement-mediated killing of *Plasmodium falciparum* erythrocytic schizont with antibodies to the recombinant serine repeat antigen (SERA). *Vaccine* **16** (13), pp. 1299-1305.

Pang, X., Mitamura, T., & Horii, T. (1999). Antibodies Reactive with the N-Terminal Domain of *Plasmodium falciparum* Serine Repeat Antigen Inhibit Cell Proliferation by Agglutinating Merozoites and Schizonts. *Infection and Immunity* **67** (4), pp. 1821-1827.

Pierce, S. K., & Miller, L. H. (2009). World Malaria Day 2009: What Malaria Knows about the Immune System That Immunologists Still Do Not. *The Journal of Immunology* **182**, pp. 5171-5177.

Pinto, A. R., Reyes-Sandoval, A., & Ertl, H. C. J. (2003). Chemokines and TRANCE as genetic adjuvants for a DNA vaccine to rabies virus. *Cellular Immunology* **224**, pp. 106–113.

Putrianti, E. D., Schmidt-Christensen, A., Arnold, I., Heussler, V. T., Matuschewski, K., & Silvie, O. (2010). The *Plasmodium* serine-type *SERA* proteases display distinct expression patterns and non-essential *in vivo* roles during life cycle progression of the malaria parasite. *Cellular Biology* **12** (6), pp. 725-739.

RBM (Roll Back Malaria)/WHO. (2001). The Use of Antimalarial Drugs: Report of an Informal Consultation. Retrieved 8th September, 2010 from http://www.rollbackmalaria.org/cmc_upload/

Richards, J. S., & Beeson, J. G. (2009). The future for blood-stage vaccines against malaria. *Immunology and Cell Biology* **87**, pp. 377-390.

Sachs, J., & Malaney, P. (2002). The economic and social burden of malaria. *Nature* **415**, pp. 680-685.

Sakai, **T., Horii, T., Hisaedaa, H., Zhanga, M., Ishiia, K., Nakanoa, Y., Maekawaa, Y., Izumic, K., Nittad, Y., Miyazakid, J., & Himeno, K.** (1999). DNA immunization with *Plasmodium falciparum* serine repeat antigen: regulation of humoral immune response by coinoculation of cytokine expression plasmid. *Parasitology International* **48**, pp. 27-33.

Sarfo, B. Y., Armah, H. B., Irune, I., Adjei, A. A., Olver, C. S., Singh, S., Lillard J. W., & Stiles, J. K. (2005). *Plasmodium yoelii* 17XL infection up-regulates RANTES, CCR1,CCR3 and CCR5 expression, and induces ultrastructural changes in the cerebellum. *Malaria Journal* **4**:63, doi:10.1186/1475-2875-4-63.

Sato, D., Li, J., Mitamura, T., & Horii, T. (2005) *Plasmodium falciparum* Serine-repeat antigen (SERA) forms a homodimer through disulphide bond. *Parasitology International* **54**, pp. 261-265.

Schutyser, E., Struyf, S., & Van Damme, J. (2003). The C-C chemokine CCL20 and its receptor CCR6. *Cytokine And Growth Factor Reviews* **14**, pp. 409-426.

Shedlock, D. J., & Weiner, D. B. (2000). DNA vaccination: antigen presentation and the induction of immunity. *Journal of Leukocyte Biology* **68**, pp. 793-806.

Shimizu, Y., Inaba, K., Kaneyasu, K., Ibuki, K., Himeno, A., Okaba, M., Goto, Y., Hayami, M., Miura, T., & Haga, T. (2007). A genetically engineered live-attenuated simian-human immunodeficiency virus that coexpresses the RANTES gene improves the magnitude of cellular immunity in rhesus macaques. *Virology* **361** (1), pp. 68-79.

Silvie, O., Franetich, J-F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A.W., Gemert, G-J., Robert W. Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., & Mazier, D. (2004). A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by *Plasmodium falciparum* Sporozoites. *The Journal of Biological Chemistry* **279** (10), pp. 9490–9496.

Sina, B. J., Dorosario, V. E., Woollett, G., Sakhuja, K., & Hollingdale M. R. (1993). *Plasmodium falciparum* Sporozoite Immunization Protects against *Plasmodium berghei* Sporozoite Infection. *Experimental Parasitology* **77** (2), pp. 129-135.

Sinden, R. E., Butcher, G. A., & Beetsma, L. A. (2002). Maintenance of the *Plasmodium berghei* life cycle. *Malaria Methods and Protocols: Methods in Molecular Medicine* **72** (II), pp. 25-40.

Stevenson, M. M., & Riley, E. M. (2004). Innate immunity to Malaria. *Nature Reviews Immunology* **4**, pp. 169-180, doi: 10.1038/nri1311.

Targett, G. A., & Greenwood, B. M. (2008). Malaria vaccines and their potential role in the elimination of malaria. *Malaria Journal* **7** (Suppl 1) S10, doi: 10.1186/1475-2875-7-S1-S10

Tine, J. A., Conseil, V., Delplace, P., De Taisne, C., Camus, D., & Paoletti, E. (1993). Immunogenicity of the *Plasmodium falciparum* Serine Repeat Antigen (p126) Expressed by Vaccinia Virus. *Infection and Immunity* **61** (9), pp. 3933-3941.

Troye-Blomberg, M., Riley, E. M., Kabilan, I., Holmberg, M., Perlmann, H., Ansersson, U., Heusser, C. H. & Perlmann, P. (1990). Production of activated T cells of interleukin 4 but not interferon-gamma is associated with elevated levels of serum antibodies to activating malaria antigens. *Proceedings of the National Academy of Science USA* **87**, pp. 5484–88.

Tuteja, R. (2002). DNA Vaccine Against Malaria: A Long Way To Go. *Critical Reviews in Biochemistry and Molecular Biology* **37** (1), pp. 29–54.

Uadia, **P. O.** (2007). Editorial: Malaria and the Challenges of Vaccine Development. *Tropical Journal of Pharmaceutical Research* **6** (4), pp. 801-802.

Vanderberg, **J. P.** (2009). Reflections on early malaria vaccine studies, the first successful human malaria vaccination, and beyond. *Vaccine* **27**, pp. 2-9.

Volin, M. V., Shah, M. R., Tokuhira, M., Haines, G. K, Woods, J. M., & Koch, A. E. (1998). RANTES expression and contribution to monocyte chemotaxis in arthritis. Clinical **89** (1), pp. 44-53.

Webster, D., & Hill A. V. S. (2003). Progress with new malaria vaccines. *Bulletin of the World Health Organization* **81**, pp. 902-909.

Wellcome Trust. http://malaria.welcome.ac.uk. Retrieved 15th July 2011.

Were, T., Ouma C., Otieno, R. O., & Orago, A. S. (2006). Suppression of RANTES in children with *Plasmodium falciparum* malaria. *Haematologica* **91**, pp. 1396-1399.

WHO, (2005). Malaria control today, current WHO recommendations.

WHO, (2009). World Malaria Report 2009.

WHO, (2011). World Malaria Report 2011.

WHO, (2011). http://who.int/entity/mediacentre/factsheet/en. Retrieved 24th August 2011.

Willcox, M. L., & Bodeker, G. (2004). Clinical review: Traditional herbal medicines for malaria. *British Medical Journal* **329** (7475), pp. 1156-1159.

Worall, E., Basu, S., & Hanson, K. (2005). Is malaria a disease of poverty? A review of the literature. *Tropical Medicine and International Health* **10 (**10), pp. 1047–1059.

Wykes, M., & Good, M. F. (2007). A case for whole-parasite malaria vaccines. *International Journal for Parasitology* **37**, pp. 705–712.

Wykes, M. N., & Good, M. F. (2009). Infectious disease: Malaria, What have we learnt from mouse models for the study of malaria?. *European Journal of Immunology* **39**, pp. 2004-2006**.**

Yole, D. S., Shamala, K. T., Kithome, K., & Gicheru, M. M. (2007). Studies on the nteraction of *Schistosoma mansoni* and *Leishmania major* in experimentally infected BALB/c mice. *African Journal of Health Sciences* **14**, pp. 80-85.

APPENDICES

APPENDIX I: Weights (in grams) of control and experimental mice

Buffer Control			
Mouse No.	Day 0	Day 21	Day 42
1	25.4	24.8	
2	28.6	30.0	
3	29.9	28.8	26.6
4	23.9	24.3	
5	26.5	26.2	27.3
6	30.2	29.0	
7	26.5	26.0	28.6
8	25.8	24.3	25.4
9	27.0	26.8	26.4
10	25.5	25.8	25.9
11	28.9	26.9	27.2
12	32.4	33.0	30.9
13	31.3	32.4	29.6
14	29.6	32.5	29.8
15	34.8	35.8	32.7
16	32.4	33.0	23.3
17	28.8	31.0	28.7
18	32.7	34.0	35.0
19	31.8	32.7	29.1
20	32.5	33.8	29.2
21	30.9	33.0	
22	30.9	33.0	
23	34.7	35.0	
24	33.7	32.7	30.0
Average	29.8	30.2	28.6
Difference	0	0.4	-1.2

Plasmid control			
Mouse No.	Day 0	Day 21	Day 42
1	30.6	31.1	31.5
2	27.2	28.8	27.1
3	25.2	24.9	28.4
4	23.9	23.8	
5	25.0	26.8	24.3
6	26.6	23.0	25.0
7	19.8		
8	25.9	26.0	
9	23.6	22.7	23.4
10	24.8	25.8	
11	23.4	24.8	25.1
12	26.3	31.6	30.2
13	26.6	29.3	28.2
14	28.5	33.0	34.2
15	27.1	32.0	33.3
16	29.6	36.9	38.1
17	28.3	33.9	33.2
18	24.4	30.4	
19	23.3	24.4	37.4
20	28.4	31.3	31.7
21	25.4	34.1	34.91
22	29.7	34.2	
23	27.0	31.4	31.4
24	25.8	28.9	
Average	26.1	29.1	30.4
Difference	0	3.0	4.3

SERA in pIRES			
Mouse No.	Day 0	Day 21	Day 42
1	26.2	30.0	31.5
2	22.3	22.8	26.0
3	29.2	34.0	33.9
4	26.4	29.4	30.6
5	25.0	29.1	30.7
6	28.5	35.2	
7	31.2		36.2
8	23.8	26.7	
9	25.0	26.7	29.5
10	26.6	30.3	31.6
11	29.0	34.6	35.6
12	22.3	24.4	28.1
13	26.8	29.9	
14	20.5	18.0	17.7
15	23.5	23.6	14.8
16	18.4	19.6	21.0
17	25.9	28.6	28.4
18	18.1	19.1	
19	21.4	21.2	22.9
20	18.6	21.1	
21	22.1	21.3	
22	20.6	22.2	23.5
23	21.6	20.4	20.3
24	20.7	22.5	22
Average	23.9	25.7	26.9
Difference	0	1.8	3.0

SERA+CCL20 in pIRES			
Mouse No.	Day 0	Day 21	Day 42
1	25.7	26.4	
2	22.5	27.7	
3	21.4	27.4	28.8
4	22.2	26.1	27.1
5	22.7	26.3	26.2
6	19.7	24.9	25
7	19.6	13.0	
8	24.8	23.8	
9	15.6	20.4	21.5
10	13.4	17.6	19.5
11	15.9	21.0	22.2
12	21.2	26.2	28.4
13	27.8	31.8	32.1
14	31.4	32.2	
15	25.4	31.8	32
16	30.6	33.9	
17	27.1	31.1	
18	17.9	26.8	27.5
19	19.2	24.4	26.7
20	15.9	21.9	21.9
21	16.2	21.2	23.1
22	20.2	27.4	30
23	18.5	24.5	24.7
24	21.6	26.2	26.3
Average	21.5	25.6	26.1
Difference	0	4.1	4.5

SERA+CCL5 in pIRES			
Mouse No.	Day 0	Day 21	Day 42
1	18.9	22.5	25.6
2	16.6	21.3	
3	16.8	21.3	23.2
4	15.8	21.4	23.4
5	14.2	19.3	21
6	12.7	21.7	22.5
7	18.8	21.8	22.5
8	12.3	22.8	25.1
9	12.9	22.8	24.6
10	16.8	21.8	23.6
11	18.6	22.3	29.1
12	18.6	21.8	27.6
13	21.4	26.2	24.8
14	18.8	25.5	28.1
15	16.7	24.2	28.6
16	19.6	25.5	25
17	19.7	25.8	27.5
18	15.5	22.4	28.3
19	12.9	24.9	30.3
20	19.8	25.4	
21	21.5	28.1	
22	30.2	27.1	
23	33.8	30.9	
24	33.1	35.1	
Average	19.0	24.2	25.6
Difference	0	5.2	6.6

APPENDIX II: Percent parasitaemia charts of control and experimental mice

	Buffer Control					
Day PI	Mouse 14	Mouse 8	Mouse 9	Mouse 12	Mouse 15	Average
3	0.13	0.14	0.24	0.15	0.15	0.162
4	0.49	0.42	1.03	0.61	0.48	0.606
5	2.72	1.99	2.07	4.51	2.00	2.658
6	9.29	4.14	5.79	11.08	10.91	8.242
7	12.75	7.74	9.74	27.43	14.39	14.410
8	19.42	12.21	13.10	44.21	29.17	23.622
9	21.35	17.68	23.60	48.93		27.890

	Plasmid Control						
Day PI	Mouse 5	Mouse 9	Mouse 14	Mouse 15	Mouse 19	Mouse 23	Average
3	0.00	0.13	0.00	0.14	0.22	0.25	0.123
4	0.21	1.13	0.19	0.65	1.85	1.44	0.912
5	0.57	4.32	0.34	3.72	8.28	2.95	3.363
6	1.48	9.36	2.20	20.14	8.97	9.65	8.633
7	5.96	11.47	10.54		12.89	15.02	11.176
8	11.29		21.07		18.50	25.18	19.01
9	15.83				20.09		17.96

	SERA in pIRES						
Day PI	Mouse 2	Mouse 5	Mouse 7	Mouse 12	Mouse 16	Mouse 22	Average
3	0.09	0.03	0.04	0.05	0.09	0.04	0.057
4	0.60	0.68	0.38	0.65	0.46	1.11	0.647
5	1.92	4.21	2.58	2.25	0.89	4.63	2.747
6	9.43	9.28	6.62	6.05	3.06	8.91	7.225
7	17.03		11.08	15.90	4.68	11.49	12.04
8			22.41	25.33	6.40		18.047
9			28.37	44.41	12.96		28.58

*Day PI= Day post-infection

	SERA+CCL20 in PIRES						
Day PI	Mouse 10	Mouse 11	Mouse 12	Mouse 21	Mouse 22	Mouse 23	Average
3	0.15	0.00	0.04	0.05	0.05	0.00	0.048
4	0.60	1.16	0.77	0.25	0.17	0.52	0.578
5	1.37	4.37	2.11	1.04	1.02	1.16	1.845
6	4.77	10.53	5.04	5.11	4.39	4.70	5.757
7	6.82	16.10	8.83	9.55	5.88	7.70	9.147
8	11.47	17.99	16.26	16.33	7.57	12.57	13.698
9	14.92	19.23	16.36	28.13	14.57	16.79	18.317

	SERA+CCL5 in pIRES					
Day PI	Mouse 5	Mouse 6	Mouse 17	Mouse 14	Mouse 18	Average
3	0.00	0.09	0.13	0.10	0.05	0.073
4	0.00	0.09	0.90	0.41	0.28	0.337
5	0.00	0.69	1.80	3.16	1.58	1.448
6	0.00	4.52	6.07	8.93	7.82	5.468
7	0.00	10.00	11.87		10.88	8.189
8	0.52	13.37			19.73	11.204
9	2.08	15.37			17.79	11.746

	Non-vaccinated controls						
Day PI	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Average
3	0.00	0.17	0.22	0.05	0.98	0.05	0.245
4	0.00	1.47	0.54	0.70	8.33	0.67	1.952
5	0.05	5.49	4.68	5.78	11.00	4.59	5.265
6	0.46	12.04	11.43	21.15	21.78	11.42	13.047
7	1.08	19.46	21.14	31.70	36.25	22.49	22.02
8	4.97		18.87		61.44	38.18	30.865
9	7.86						7.86