ELICITATION OF ANTI-MOSQUITO IMMUNE RESPONSES IN BALB/c MICE IMMUNIZED WITH PLASMIDS CONTAINING *ANOPHELES GAMBIAE* MUCIN 1 (AgMUC1), GM-CSF AND IL-12 GENES

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OCTOBER, 2013

DECLARATION

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

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DEDICATION

To my wife, Dorothy, whose love, care, encouragement and support saw me through and children, Humphrey, Linda, Collins and Michael whose unspoken understanding I will forever cherish.

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ABBREVIATIONS AND ACRONYMS

ACT Artemisinin Combination Therapy

ADCC Antibody dependent cellular cytotoxicity

AeIMUC 1 *Aedes aegypti* mucin 1

AgMUC 1 *Anopheles gambiae* Mucin 1

APCs Antigen presenting cells

Bti *Bacillus thurigensis isrealensis*

Bs *Bacillus sphaericus*

cDNA Clonal Deoxyribonucleic Acid

CS Circumsporozoite protein

CTL Cytotoxic lymphocyte

DDT Dichloro-diphenyl trichloroethane

ELISA Enzyme linked immunosorbent assay

EM Electron Microscopy

GM-CSF Granulocyte Macrophage colony stimulating factor

HIV Human Immunodeficiency Virus

ICIPE International Center for Insect Physiology and Ecology

IFN-γ Intetrferon gamma

IL-4 Interleukin 4

IL-5 Interleukin 5

IL-12 Interleukin 12

ILRI International Livestock Research Institute

ITN Insecticide treated net

MVTR Malaria Vaccine Technology Roadmap

NK cells Natural Killer cells

OCT Optimal Cutting Temperature

PBS Phosphate Buffered Saline

pUMVC plasmid University of Michigan Vector Core

SIT Sterile insect technique

SEM Scanning Electron Microscopy

SSM Antigen Sexual Stage Mosquito Antigen

TEM Transmisssion Electron Microscopy

TNF-α Tumour necrosis factor alpha

ABSTRACT

Various strategies that block malaria transmission from an infected human host to a female Anopheline mosquito that involve killing of the blood fed mosquito have been studied as a potential strategy to control malaria. These include the immunization of animal hosts with either whole homogenized blood fed mosquito midguts or their extracts. In 1939, William Trager first demonstrated anti-vector immunity by immunizing guinea pigs and rabbits with extracts of the tick *Dermacentor variabilis*. This immunological targeting of tick midgut antigens led to the commercial development of a vaccine against *Boophilus microplus* ticks licenced in 1985.This study investigated the mosquitocidal effects of an immune response elicited by immunization of BALB/c mice with a gene, *Anopheles gambiae* mucin 1 gene (AgMUC1 gene) alone or in combination with immunostimulatory cytokine genes GM-CSF or IL-12. The AgMUC1 genes were cloned from the midgut of blood fed female *Anopheles gambiae* mosquitoes. Four groups of BALB/c mice, six mice per group, were immunized with AgMUC1 gene alone or in combination with plasmids containing AgMUC1, Interleukin 12 (IL-12) or Granulocyte-macrophage colony stimulating factor (GM-CSF) genes. Another group of mice was primed with AgMUC1 cDNA and boosted with recombinant mucin protein. Anti-mucin antibody secreting hybridomas were prepared from the fusion of spleen cells from AgMUC1 cDNA immunized mice and myeloma cells. Groups of female *Anopheles gambiae* mosquitoes were fed on the immunized mice and the anti-mucin antibody containing hybridoma supernatants and their cumulative survival to day seven post blood feeding determined by life table analysis and the Kaplan Meier log rank statistic analysis. Cryostat sections from the midguts of the blood fed mosquitoes were examined by bright light, scanning and transmission electron microscopy. Spleen cells from the immunized mice were stimulated with mucin protein. A significantly higher mortality was observed in the groups of mosquitoes fed on the groups of mice given three or four injections of AgMUC1 cDNA (p=0.00331 and 0.0173 respectively). A highly significant increase in mortality was observed in the groups of mosquitoes fed on the groups of mice immunized with AgMUC1/IL-12 cDNA (p=4.05e-10). Mean anti-mucin antibody levels varied from 1:3,000 in the AgMUC1 cDNA immunized group, 1:6,000 in the AgMUC1/IL-12 cDNA and AgMUC1/GM-CSF cDNA immunized groups to >1:100,000 in the AgMUC1 cDNA immunized / mucin protein boosted group. Cytokine production profiles from *invitro* stimulated spleen cells from AgMUC1 cDNA immunized mice could not be associated with a particular type of immune response. On microscopy, white blood cells were adhering onto the midgut epithelial cell lining and activated hemocytes were observed. Under the transmission electron microscope features characteristic of apoptotic and necrotic processes were observed in the epithelial cells lining the midgut. The results of this study show that the immune response elicited following AgMUC1 cDNA immunization kills blood feeding female *An. gambiae* mosquitoes and that the mechanisms leading to mosquito death could probably be associated with antibody dependent cellular cytotoxicity (ADCC) induced apoptosis or necrosis. In this strategy, the killing of blood feeding adult female mosquitoes has the potential to reduce mosquito density and biting intensity, an attribute required in malaria control. This strategy, combined with other anti-malaria parasite strategies aimed at killing the parasite and the responsible use of insecticides, could reduce malaria transmission in endemic areas.

CHAPTER 1: INTRODUCTION

1.1 Background information

Malaria is an infectious parasitic disease of man transmitted from an infected to a non infected individual by a female Anopheline mosquito. It is caused by five species of parasites of the genus *Plasmodium* that affect humans (*P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*). Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa. Globally, an estimated 3.3 billion people were at risk of malaria in 2011, with populations living in sub-saharan Africa having the highest risk of acquiring malaria (World Malaria Report, 2012). Approximately 80% of cases and 90% of deaths are estimated to occur in WHO African Region, with children under five years of age and pregnant women most severely affected. According to the latest WHO estimates, there were about 219 million clinical cases and an estimated 660,000 deaths globally in the year 2011 (World Malaria Report, 2012). Previuos estimates had 66 million infants exposed to the disease resulting in the death of approximately 500,000 children under the age of 5 years (Snow *et al.,* 1999a, b; 2005).

The strategic approaches to malaria control come within two major domains involving prevention and case management (World Malaria Report, 2012). The WHO recommended malaria control measures include long-lasting insecticidal nets (LLIN), indoor residual spraying (IRS) programmes and access to artemisinin combination therapy (ACT) (World Malaria Report, 2010). There has also been a shift towards use of rapid diagnostic tests and away from presumptive treatment of malaria. Together, these

strategies work against the transmission of the parasite from the mosquito vector to humans and from humans to the mosquito vector, and the development of illness and severe disease. Current efforts in the control of malaria involve diagnosis and treatment of infected persons, larval control, adult mosquito control and the reduction of man-vector contact. These general strategies are mainly targeted towards the elimination of the adult mosquitoes or their larvae from the environment and the elimination of the malaria parasites from the human host.

These two strategies however entail the use of chemical based insecticides, larvicides and anti-malarial drugs. The effectiveness of these strategies is hampered by the rapid development of resistance by the mosquito vector to the insecticides (Quinones *et al.*, 1998; Hargreaves *et al.*, 2000) and the malaria parasites to the anti-malarial drugs (Ridley, 2002). This is complicated by the fact that insecticides and larvicides are harmful to the environment while anti-malarial drugs are toxic to the human body. Further complications are brought about by climatic changes that have enlarged areas of disease transmission (Nchinda, 1998).

Alternative malaria control strategies that are not based on the use of chemicals are required. One of the five pillars of the global plan for insecticide reistance management in malaria vectors launched in 2012 is the development of new, innovative vector control tools (World Malaria Report, 2012). One such tool could be malaria control based on vaccination strategy. This involves blocking the progression of the disease-causing malaria parasite in the human host through the induction of stage specific anti-parasite

transmission blocking immunity (Hoffman, 1996). This immunity prevents the transformation of the malaria parasite stages in the mosquito vector or human host by inducing anti-vector transmission blocking immunity (Alger and Cabrera, 1972) that diminishes the vector's capability to transmit the malaria parasites. This is achieved by either killing the vector or reducing its fecundity.

This dissertation involved the use of the anti-mosquito vector based immunization strategy using a unique protein, an *Anopheles gambiae* mucin 1 protein (AgMuc1) (Shen *et al.,* 1999) that is expressed on the surface of the epithelial cells lining the midgut of the female mosquito after a blood meal. It is hypothesized that this protein helps protect the midgut epithelia from harmful substances and microbes taken in with the blood meal or it may shield the midgut lining from the corrosive effects of the secreted digestive enzymes. The goal of this study was to characterize the mosquitocidal immune response elicited after immunization with plasmid AgMUC1 and determine the pathological processes in the mosquito midgut that may lead to mosquito death.

1.2 Problem statement

The overarching goals of malaria vector control are two-fold: to protect individual people against infective malaria mosquito bites and to reduce the intensity of local malaria transmission at community level by reducing the longevity, human vector contact and density of the local vector mosquito population (World Malaria Report, 2012). Because of the long extrinsic incubation time of *Plasmodium* parasites in their Anopheline vectors, the most effective vector control strategies currently in use rely on insecticide interventions that reduce vector daily survival rates (Enayati and Hemingway, 2010).

The most powerful and most broadly applied interventions are the long-lasting insecticidal nets (LLINs) and the indoor residual spraying (IRS). These interventions work by reducing human-vector contact and by reducing the lifespan of adult female *Anopheles* mosquitoes so that they do not survive long enough to transmit the parasite (World Malaria Report, 2012). For many malaria-endemic regions, these tools can make substantial contributions to malaria control. However, regions where existing interventions will not be sufficiently effective include those where high rates of transmission occur. An example of such a region is sub-saharan Africa where the entomological inoculation rates have been reportedly estimated to be approaching 1000 infective bites per person per year (Kelly-Hope and McKenzie, 2009; Shaukat *et al* 2010).

With the major challenge to the current vector-targeted interventions being insecticide resistance and considering that resistance monitoring efforts are not available to most vector control programs, there is need for development of new tools for vector control as envisaged in the global plan for insecticide resistance management in malaria vectors (World Malaria Report, 2012). These include the development of novel approaches that will reduce the very high vectorial capacities of the dominant malaria vectors, *An. gambiae,* in sub-Saharan Africa.

1.3 Justification of the study

An effective vaccine against malaria has long been envisaged as a potentially valuable addition to the available tools for malaria control. As yet there are no licenced malaria vaccines (World Malaria Report, 2012). A number of candidate vaccines are being evaluated in clinical trials, with one candidate vaccine currently being assessed in phase 3 clinical trials and approximately 20 others in phase 1 or phase 2 clinical trials. The RTS,S/AS01 vaccine targets *P. falciparum* and is currently undergoing phase 3 clinical trials (World Malaria Report, 2012). Many of the vaccines under development or clinical trials are aimed at either neutralizing sporozoites thus halting their development in the human host; destroying developing liver forms thus suppressing blood stage parasitemia in the infected host or blocking further development of gametocytes in the mosquito midgut. Because of the ever-changing form of the parasite during its lifecycle in the human host, the parasite has developed means of evading the host immune mechanisms. This has rendered the anti-parasite approach to malaria control technically complex, in

the sense that an effective anti-parasite vaccine has to incorporate all the malaria parasite developmental stage antigens.

The use of insecticides, larvicides and anti-malarial drugs to control malaria has also proven to be a problem mainly because of the emerging problems of resistance of the adult mosquitoes to the available insecticides, the mosquito larvae resistance to larvicides and the malaria parasites resistance to anti-malarial drugs. These are compounded by the concerns over the safety of insecticides and larvicides by environmentalists and the issue of toxicity of the anti-malarial drugs to the human body. This makes the approach of using chemical control methods difficult as resistance testing, environmental assessment and toxicity monitoring have to be conducted for every new chemical coming into the market. Already there are reports that insecticide resistance in malaria vectors affects all currently used insecticides and most of these reports concern resistance to pyrethroids. If resistance to pyrethroids were to reach a level at which they become ineffective in all areas, in Africa, an estimated 26 million malaria cases and 120,000 malaria deaths averted by the current vector control efforts (LLINs and IRS) would occur (World Malaria Report, 2012).

Based on these limitations, the development of an anti-mosquito vector based vaccine becomes a viable alternative strategy to mosquito and ultimately malaria control. Anti-mosquito based vaccines will aim at killing the adult blood feeding female mosquitoes and/or reduce their fecundity, regardless of whether the vectors are infected or not. Anti-mosquito vaccines have a further potential of effectively reducing the transmission of

several *Plasmodium* species at once as they are transmitted by the same vectors (Beier *et al.,* 1990). This is desirable especially in communities with high malaria transmission resulting from high mosquito densities.

Ticks, like mosquitoes, are ectoparasites whose control is solely dependent on the use of chemicals, acaricides. The one-host tick, *Rhipicephalus (Boophilus) microplus* is an economically important ectoparasite of cattle involved in the transmission of *Babesia bovis,* the aetiological agent of bovine babesiosis (Friedhoff *et al*., 1988). The control of *R. microplus* relies mostly on the use of acaricides and to a less extent by the use of commercial vaccines based on the Bm86 antigen (de la Fuente *et al.,* 2007). Bm86, just like the AgMUC1 antigen of the *An. gambiae* mosquito, is a membrane-bound glycoprotein expressed mainly on the surface of the digestive tract of *R. microplus* (Gough and Kemp 1993). Bm86 based vaccines have shown some effect on controlling *R. microplus* and other tick species, and they can also reduce the use of acaricides (Vargas *et al*., 2010) although their efficacy as stand-alone solution for tick control has been a matter of debate (Willasden 2006). Vaccine controlled field trials in combination with acaricide treatments have demonstrated that an integrated approach resuts in control of tick infestations while reducing the use of acaricides (de la Fuente *et al.,* 2007). These trials have further demonstrated that control of ticks by vaccination has the advantages of being cost-effective, reducing environmental contamination and preventing the selection of drug resistant ticks that result from repeated acaricide application. In addition these vaccines may also prevent or reduce transmission of pathogens by reducing tick populations and/or affecting tick vectorial capacity (Rodriguez Valle *et al.,* 2004).

If both the anti-parasite and the anti-vector vaccine control strategies could similarly become successful in malaria control programs and are then combined and coupled with the responsible use of insecticides and insecticide treated bed nets, larvicides and anti-malarial drugs, morbidity and mortality in communities with high malaria endemicity would be significantly reduced. This reduction would greatly contribute to the WHO Roll Back Malaria program whose aim is to decrease mortality due to malaria (Nabarro and Taylor, 1998; WHO, 2005).

1.4 Research Questions

i. What is the optimum immunization schedule that will elicit significant mosquito killing immune response following AgMUC1 cDNA immunization of Balb/c mice?

ii. What is the effect of co-imunization of AgMUC1 cDNA with immunostimulatory cytokines IL-12 and GM-CSF cDNA on the mosquito killing immune response?

iii. Which type of mosquito killing immune response is elicited following AgMUC1 cDNA immunization?

iv. What are the immune pathological features in the mosquito midgut that could be associated with mosquito death following a blood meal on the AgMUC1 cDNA immunized mice?

v. What would be the effect of anti-mucin antibodies secreted by hybridoma cell lines made from the fusion of spleen cells from AgMUC1 cDNA immunized mice and a mouse myeloma cell line on mosquitoe survival?

vi. What would be the killing mechanisms for mosquitoes fed on anti-mucin antibodies secreted by hybridoma cell lines?

1.5 Null Hypothesis

Targeting critical molecules in the mosquito midgut for immune attack will kill adult blood feeding female *An. gambiae* mosquitoes.

1.6 Objectives

1.6.1 General Objective

To elicit anti-mosquito immune responses in BALB/c mice following immunization with AgMUC1 cDNA and immunostimulatory cytokines IL-12 and GM-CSF and determine the immune mechanisms and immune pathologic features responsible for the mosquitocidal effects on blood feeding female *Anopheles gambiae* mosquitoes.

1.6.2 Specific Objectives

i. To determine the optimal AgMUC1 cDNA immunization schedule, that will elicit an immune response in BALB/c mice that kill blood feeding female *Anopheles gambiae* mosquitoes.

ii. To determine if co-immunization of AgMUC1 cDNA mixed with plasmids containing immunostimulatory cytokine, GM-CSF or IL-12 genes would elicit an enhanced mosquitocidal immune response.

iii. To determine the type of mosquito killing immune response elicited following immunization of female BALB/c mice with plasmid containing AgMUC1 cDNA.

iv. To determine the immune pathological features responsible for the death of *Anopheles gambiae* mosquitoes fed on mice immunized with plasmid containing AgMUC1 cDNA.

v. To prepare mosquitocidal anti-mucin antibody secreting hybridoma cell lines from the fusion of spleen cells from BALB/c mice immunized with plasmid containing AgMUC1 cDNA and myeloma cell lines.

vi. To characterize and determine the mechanisms by which anti-mucin antibodies secreted by hybridoma cell lines kill mosquitoes.

1.7 Expected outputs

This study is expected to stimulate research and understanding of the science behind genetic immunization using genes that target critical molecules that are expressed in the mosquito midgut after a blood meal. AgMUC1 is one such molecule that is expressed in the *An. gambiae* midgut after a blood meal for the purposes of protecting the midgut lining against harmful substances that may come in with the blood meal. If this targeted mosquito killing strategy is to be perfected and made applicable to human populations, it could lead to measurable reduction in mosquito densities and biting intensities and thus lower cases of new malaria infections in communities with high malaria transmission rates in line with the milleneum development goal 6 of reversing the incidence of malaria. It is worth noting that this approach is still in the formative scientific stage of eliciting an optimal mosquito killing immune response that will need to be refined further for it to move to the next stages of vaccine development.

1.8 Limitations of the study

i. The number of injections required to produce an effective mosquito killing immune response could be a hinderance to further developments in this strategy if not reduced.

ii. Most vaccination programs are targeted against children under the age of five. This age group is the one most at risk for malaria attack. Under the EPI this age group is currently being subjected to over five different vaccines hence the

acceptability of adding on more vaccinations might complicate compliance unless this strategy is coulpled to the current vaccines in use as the RTS,S/AS01 currently under phase 3 clinical trials has.

iii. Immune responses in mice are not necessarily similar to those in the primates hence the effects observed in the mouse model might not be similar to those occuring in the primate model.

iv. To-date there is no commercially licenced human gene based vaccine hence it could take long to have such a vaccine actualized.

CHAPTER 2: LITERATURE REVIEW

2.1 Malaria

Malaria is a complex infectious disease whose severity is a function of the integration between the parasites, the *Anopheles* mosquito vector, the human host and the environment. Malaria is caused by the *Plasmodium* parasite which spends its life in both humans and certain species of mosquitoes. Five species of *Plasmodium* cause malaria in humans: *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi.* Of these, *P. falciparum* is the most important in the tropics and is responsible for severe illnesses and death (World Malaria Report, 2012). The risk of malaria infection is determined by the number of vectors, their survival rate, the incubation rate for both the vector and the parasite and the probability of the vector feeding off a human host (Eckhoff, 2011).

Malaria accounted for an estimated 219 million cases (range 154-289 million cases) and 660,000 deaths (range 610,000-971,000 deaths) in 2010 (World Malaria Report, 2012). Globally, an estimated 3.3 billion people were at risk of malaria in 2011, with populations living in sub-saharan Africa having the highest risk of acquiring malaria. Approximately 80% of cases and 90% of deaths are estimated to occur in the WHO African region, with children under five years of age and pregnant women being the most severely affected (World Malaria Report, 2012). Young children under the age of five in sub-saharan Africa have been previously reported as the most affected affected, dying at the rate of nearly 3,000 every day (Anon, 1996). In Africa malaria reportedly causes

approximately 20% of all child deaths, 30-50% of all out-patient visits and up to 50% of hospital admissions (WHO, 2000). Malaria has also been reported to be contributing to increased maternal morbidity and mortality (World Malaria Report, 2012). During pregnancy, malaria is reported to be the major cause of low birth weight in sub-saharan Africa.

2.2 The malaria parasite life cycle

The life cycle of the malaria parasite is divided into three different phases – one in the mosquito (the sporogonic cycle) and two in the human host that is the erythrocytic cycle (in the human blood cells) and the exo-erythrocytic cycle (outside the blood cells). When the mosquito ingests the male and female gametocytes during blood meal the gametocytes form the spermatocytes and oocytes within the mosquito's midgut. These gametes unite to form the zygote which transforms into the motile ookinete. The ookinete penetrates the wall of the mosquito midgut and transforms into a round oocyst. Inside the oocyst the nucleus divides repeatedly resulting into the formation of a large number of sporozoites that causes the enlargement of the oocyst. When the sporozoites are fully formed the oocyst bursts releasing the sporozoites into the mosquitos' body cavity (haemocoel). The sporozoites then migrate to the salivary glands thereby completing this developmental cycle (Aly *et al*., 2009).

The sporozoites (the infective stage of *Plasmodium*) are injected with the saliva when the mosquito next feeds (Beier, 1998; Al-Olayan *et al., 2002*). The time necessary for the

development of the sporozoites varies with the temperature and humidity and generally takes 8-15 days. The sporozoites are injected into the skin as the mosquito takes a blood meal. These cross the endothelium of blood capillaries, enter the host's blood system and migrate to the liver cells where they invade the hepatocytes. In the hepatocytes, each sporozoite undergoes extensive replication within a parasite derived vacuole, essentially walling off the parasite from the liver-cell cytoplasm. Over a period of 7-12 days, the parasite multiplies until the infected liver cell bursts releasing the parasites (merozoites) into the blood stream. The merozoites invade the red blood cells where they multiply again and end up destroying the infected red cells. The released parasites invade fresh red blood cells and the cycle is repeated (Aly *et al*., 2009). Failure in this parasite developmental process would prevent transmission between the mosquito and the vertebrate host. The failure in the malaria parasite developmental stages form one of the goals of vaccine based strategies for blocking malaria transmission (Miller *et al.*, 2002).

2.3 The *Anopheles* mosquito

The female *Anopheles* mosquito is responsible for transmitting malaria while taking a blood meal for its nourishment and the development of her eggs (Nayar and Sauerman, 1975). *Anopheles* mosquitoes usually bite from dusk to dawn and in many localities the principle vectors of malaria are the late night biters and older mosquitoes, which are more likely to be infected (Noor *et al.*, 2009). Different species of *Anopheles* have different peak biting times, different host preferences (animals or humans) and different resting

habits (indoor or outdoor). These factors influence the choice of anti-mosquito based control methods.

Most Anopheline mosquitoes bite at night with some biting shortly after sunset. Mosquitoes that enter houses to bite are called endophagic while those that mostly bite out side are called exophagic. After their blood meal, endophagic mosquitoes rest inside the house and are refered to as endophilic while exophagic mosquitoes will usually rest out side and are refered to as exophilic. Some mosquitoes prefer to take blood from humans and are described as being anthropophagic while those that prefer animal blood are refered to as zoophagic. It is the endophagic, endophilic and anthropophagic mosquitoes that are dangerous as they are more likely to transmit disease from man to man. It is known that to maintain the chain of human-to-human transmission of malaria, a vector must strongly prefer human blood meals to animal blood meals and *An. gambiae* will choose the human host at least 90% of the time for its blood meal (Beier *et al.,* 1988).

Among the several species of mosquitoes found in Kenya, *Anopheles gambiae* Giles, *Anopheles arabiensis* Patton and *Anopheles funestus* Giles are the principal vectors for the transmission of human malaria (White 1972; Mbogo *et al.*, 1993, 1995). *An. gambiae* breeds in temporary habitats such as pools, puddles, hoof prints, borrow pits, rice fields and irrigation sites. It is highly anthropophilic (prefers to bite humans) and both exophagic (bites outdoors) and endophagic (bites indoors). It has preference for nocturnal feeding. It is also predominantly endophilic (rests indoors after feeding) although it also exhibits partial exophily (resting outdoors after feeding) (Sinka *et al.*, *2010). An. funestus*

breeds in swamps, marshes, edges of streams, rivers, ditches, irrigation sites and other stagnant waters especially along the coast line. It prefers shaded habitats and is predominantly anthropophilic although it also exhibits zoophilism (prefers to bite animals). It is both exophagic and endophagic and has preference for nocturnal feeding. It is predominantly endophilic (Sinka *et al.*, *2010).* *An. arabiensis* breeds in swamps, marshes, and edges of streams, rivers and ditches. It prefers sunlit habitats and may be both anthropophilic and zoophilic although it shows greater tendency towards zoophily. It may be both exophagic and endophagic. It shows greater tendency towards exophily although it may also be endophilic (Sinka *et al.*, *2010).* Apart from these three, there is also *An. melas* which is a salt water breeder and occurs along the coastal areas. It is common in lagoons and mangrove swamps. It breeds heavily in areas colonized by the black mangrove. It is anthropophilic and may show some zoophily in some areas. It is both exophagic and endophagic. It is predomintly endophilic and occasionally exophilic (Sinka *et al.*, *2010).*

2.4 The mosquito lifecycle

The life cycle of a mosquito goes through four separate and distinct stages: the egg, the larva, the pupa and the adult. The whole process can take between 7-16 days and is heavily influenced by nutritional factors, humidity and temperature of their environment; the higher the temperature and humidity the shorter the life cycle period.

Only the adult female mosquitoes require a blood meal, which is used for nourishment and egg development (Nayar and Sauerman, 1975). A female Anopheline mosquito normally mates only once in her life time. It requires a blood meal after mating and before the eggs can start to develop. Digestion of the blood meal and the simultaneous development of the eggs take about two to three days during which time the mosquito does not usually bite. Blood meals are taken every 2-3 days before the next batch of eggs is laid (Killeen *et al.*, 2000). About 100-150 eggs are laid on the surface of water during oviposition. Under the best conditions in the tropics, the average life span of a female Anopheline mosquito is about three to four weeks. A female mosquito continues to lay eggs throughout her life time laying about 1-3 batches of eggs.

A larva hatches from the egg after about 1-2 days. It feeds by taking up food from the water. There are four larval stages or instars. The small larva emerging from the egg is the first instar. It shades its skin after 1-2 days to become the second instar followed by the third and fourth instars at further intervals of about two days each. From the fourth instar it changes into a pupa, the stage during which a major transformation takes place. The pupa stage does not feed and lasts for 2-3 days after which the skin of the pupa splits and the adult mosquito emerges. Mating takes place soon after the adult emerges from the pupa. Normally the female takes her first blood meal only after mating. The first batch of eggs develops after one or two blood meals. Usually successive batches only requires one blood meal (Armstrong and Bransby-Williams, 1961).

2.5 Mosquito control

2.5.1 Mosquito control strategies

Since the discovery by Ronald Ross (1897) that mosquitoes are the vectors of the malaria parasite, vector control became an important part of malaria control programs. Historically, the control of vectors has been a critical, and sometimes the only means to control vector-borne diseases. The goals of malaria vector control are geared towards protecting individual people against infective malaria mosquito bites and reducing the intensity of local malaria transmission at community level by reducing the longevity, human-vector contact and density of the local vector mosquito population (World Malaria Report, 2012). Several strategies have been designed to reduce the mosquito population or render the mosquito vector less competent to transmit malaria. The feeding and resting habits of mosquitoes are of great importance in mosquito control programs (Gillies, 1956). Different intervention strategies are required in order to reduce the propensity of a given female mosquito species to feed on the human host, reduce the average longevity of a mosquito species and finally reduce mosquito vector competence; the innate ability of a mosquito species to permit development of the parasite (Alger, 1972; Srikrishnaraj, 1993). Among these strategies are environmental management, insecticide treatments, and molecular entomology approaches.

2.5.2 Environmental management

Environmental management to control malaria consists of environmental modification and manipulation, and changes in human habitations and activities (Walker *et al.,* 2007). Initial vector control strategies in this area invoved limiting the mosquito population through the draining of wetlands, removal of potential breeding habitats, installation of house screens, and use of larvivorous fish (Shiff, 2002). This approach has had some success in controlling the mosquito population in some countries and is still used and recommended as an alternative approach in a few areas (Killeen *et al.,* 2002). However, the wide array of vectors and their diverse habitat requirements make this strategy impractical in some situations. For example, the draining of wetlands is not suitable in areas where it might adversely affect biodiversity and conservation efforts (Utzinger *et al.,* 2002) and the formation of large numbers of temporary pools during the rainy season in some areas can make this strategy impractical (Shiff, 2002).

2.5.3 Mosquito control

Past efforts in mosquito management relied on the use of Paris green (copper acetoarsenite) and petroleum by-products which were discontinued because of their high toxicity and pollution of water sources (Rozendaal, 1997; Walker and Lynch, 2007). With the discovery of DDT, the focus of malaria control strategies shifted to managing the adult mosquito population whose widespread application lead to reduction of malaria in some parts of the world (Pardo *et al.,* 2006). This gain was eroded with the appearance

of insecticide-resistant mosquitoes (Morgan *et al.,* 2010), an increased public rejection of the application of DDT because of its ecological impact and changes in the feeding behaviors of certain vectors (Potikasikorn *et al.,* 2005).

Vector control through ITNs and IRS is a core component of malaria control programs today and the success of these interventions is dependent upon the continued effectiveness of the insecticides used (World Malaria Report, 2012). Currently, insecticides used for IRS come from only 4 classes: pyrethroids (the most commonly used class), organochlorines (of which DDT is the only compound in use), organophosphates and carbamates. All WHO recommended LLINs use pyrethroids. Increasing resistance of the malaria vectors to pyrethroids is now reported to be widespread and is jeopardizing global malaria control efforts (World Malaria Report, 2012).

2.5.4 Insecticide treated bednets

Insecticide treated nets (ITNs), which include both LLINs and conventional nets that are later treated with an insecticide, work both by protecting the person sleeping under the net (individual level) and by extending the effect to an entire area (community level). Since 2007 WHO has recommended universal coverage with ITNs as high coverage rates are needed to realize the full potential of vector control (WHO, 2007). WHO further recommends one LLIN for every two persons in order to meet the target of universal access, LLINs be provided either free or be highly subsidized and only LLINs

recommended by the WHO Pesticide evaluation scheme be used (World Malaria Report, 2012). Insecticide treated bednets alone have been regarded as an excellent tool to reduce malaria transmission in highly endemic countries, especially by reducing child mortality and morbidity (WHO, 2007). In Asia the use of Pyrethroid treated bednets has been shown to have successfully helped control malaria transmission (Hung *et al., 2002).*

The strategy involving insecticide use plays a significant role in malaria control programs that involve the use of indoor residual spraying as well as Insecticide treated bednets (WHO, 2006). These strategies work by reducing human-vector contact and by reducing the lifespan of adult female *Anopheles* mosquitoes so that they do not survive long enough to transmit the parasite (World Malaria Report, 2012). Both strategies are based on the feeding behavior of the *Anopheles gambiae,* which is anthropophilic and endophagic and in which the reduction in transmission is attained by reducing the lifespan of the mosquito vector (Shiff, 2002). IRS involves the application of residual insecticides to the inner surfaces of dwellings where many vector species of anopheline mosquitoes tend to rest after taking a blood meal (WHO, 2006). IRS is applicable in many epidemiological settings and requires specialized spray equipment and techniques. IRS is effective in rapidly controlling malaria transmission, hence in reducing the local burden of malaria morbidity and mortality provided that most houses and animal shelters (>80%) in targeted communities are treated (WHO, 2006). Currently 12 insecticides are recommended by WHO Pesticide evaluation scheme for IRS on the basis of data on resistance, residual efficacy of the insecticide, costs, safety and the type of surface to be sprayed (WHO, 2011). DDT has a comparatively long residual efficacy as an insecticide

for IRS and can be used as long as necessary provided that the guidelines and recommendations of WHO and the Stockholm convention are all met (WHO, 2011).

In order to achieve a greater efficacy in Africa, insecticide-treated bednets need to be widely distributed among the population and insecticide re-impregnation services have to be provided at a relatively low cost if continuous protection is to be maintained (Curtis and Mnzava, 2000). The same is true for indoor residual spraying which requires appropriate application to be effective (WHO, 2007). The strategy involving the use of insecticides as indoor residual insecticide house spraying and long-lasting insecticidal nets (LLINs) that reduce vector daily survival rates to control the adult blood feeding female mosquitoes has however already met with the problem of rapid development of resistance by the mosquitoes (Hemingway, 1992, Enayati and Hemingway, 2010). Detection of Insecticide resistance affecting all major vector species and all classes of insecticides led to the launch of the global plan for insecticide resistance management in malaria vectors strategy by WHO (World Malaria Report, 2012). This was necessary because large scale implementation of ITN programs is part of an integrated approach for malaria control under the Roll Back Malaria (RBM) program. The spread of insecticide resistance, especially pyrethroid resistance in Africa, is a major threat to vector control programs because with it comes the expensive institution of resistance management measures and intensification of resistance monitoring using both bioassay (susceptibility) tests and genetic methods (World Malaria Report, 2012). Pyrethroids have a repellent effect on most vector species and the lack of an alternative class of insecticides for ITN's has always been a cause of concern because of the potential for insecticide resistance.

2.5.5 Entomopathogenic Fungi

The use of entomopathogenic fungi, widely used against agricultural pests, is currently being tested against mosquito adults. This strategy involves spraying mosquito' resting places with a suspension of fungal spores that readily invades and multiplies inside the mosquito, killing it within 15 days, approximately the amount of time that the malaria parasite takes to develop into its infective form (sporozoite) (Scholte *et al.,* 2003). This is aimed at reducing the parasite's transmission intensity (Scholte *et al.,* 2006). Co infection with *Plasmodium* malaria parasite and entomopathogenic fungi has been shown to inhibit the malaria parasite development in the malaria vector mosquitoes thus reducing their potential for malaria transmission (Blanford *et al.,* 2005).

Two fungal species, *Beauveria bassiana* and *Metarhizium anisopliae,* have been found to be effective against insecticide resistant and insecticide susceptible malaria vector populations (Kikankie *et al.,* 2010), although permethrine resistance in the malaria vector, *An. gambiae,* has been associated with increased susceptibility to fungus infection (Howard *et al.,* 2010). Further reports show that infection with these fungi attenuates the level of insecticide resistance in malaria vector species (Farenhost *et al.,* 2009), enhancing the potential use of these fungi for insecticide resistance management. Exposure to *Beauveria bassiana,* an agricultural pesticide, was reported to not only cause higher mortality rates in malaria-infected mosquitoes but also to have reduced the proportion of surviving mosquitoes carrying sporozoites in their salivary glands (Kanzok

and Jacobs-Lorena, 2006). Further observations show that fungus infected mosquitoes were found to less likely take subsequent bloodmeals than were uninfected mosquitoes.

Unlike the action of most insecticides, entomopathogenic fungi are slow killing, allowing for reproduction prior to death, which is likely to circumvent the development of resistance to fungal infection in target mosquito populations (Read *et al.,* 2009). Although this approach has potential for reducing parasite transmission, there are barriers to be addressed before its widespread application in mosquito control. The barriers include fungal spore viability, fungal specificity and the development of resistance in the mosquitoes (Kanzok and Jacobs-Lorena, 2006).

2.5.6 Biological control

Larval Bio-control strategies involving larvivorous fish (*Gambusia affinis* and *Poecilia reticulate*) and bacterial pathogens such as *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Fillinger *et al.,* 2003; Russell *et al.,* 2003) despite being harmless have yet to be applied on a large scale to prove their efficacy (French-Constant, 2005). The use of larvivorous fish *Gambusia affinis* was initially implemented as a biological control tool throughout the world but its negative impact on the native fauna discouraged its further use (Walker *et al.,* 2007). Currently such efforts have been replaced by the use of native fish with relative success (Fletcher *et al.,* 1992).

The microbial agents *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Bs) are environmentally friendly alternatives, given that the toxins they produce are non-toxic to other species and do not persist for a long period of time in the environment (Walker *et al.,* 2007). Bti and Bs are characterized by their ease of handling, cost-effectiveness and capability of being produced locally. Furthermore, their application does not require expensive equipment. The great advantage of Bti over all other microbial agents is the low probability of developing resistance (Charles and Nielson-LeRoux, 2000).

Larval control is indicated as the sole method of vector control if a high proportion of the breeding sites can be located, accessed and managed (Pampana, 1969). Larval control affects only the vector density and requires high coverage to be effective. It is most feasible where breeding sites are limited in number, easily recognizable and accessible.

2.5.7 Sterile insect technique

A recent strategy considered environmentally friendly but not currently being applied to mosquitoes is the sterile insect technique (SIT). SIT is species-specific and environmentally benign, and increases in effectiveness as the size of the target population declines. This technique involves the mass rearing and release of sterile males which upon mating with the native population are unable to produce viable offspring and thereby drive the native population into decline (Dyke *et al.,* 2005). The major requirement for successful application of SIT is the ability to deliver to the field population, over large areas, large numbers of sexually active genetically sterile males

(Benedict and Robinson, 2003). Because the presence of female mosquitoes in releases reduces the efficiency, effectiveness and itself posses a risk of disease transmission, efforts are made to eliminate the females early in development and as thoroughly as possible.

The technologies used to rear, sterilize and distribute the males might all have a negative impact on final field fitness, indicating that larger numbers of insects must be released than those predicted by simple models. The first successful programme against an anopheline mosquito was the elimination of an isolated *An. albimanus* population using chemosterilized males released in 15 km^2 area in El Salvador (Lofgren *et al.,* 1974). The challenges that impede the widespread use of SIT include loss of male fitness after sterilization, the need to produce sufficient numbers of sterile individuals and the biology of the mosquito population (Phuc *et al.,* 2007).

2.5.8 Genetic control

Genetic manipulation of the mosquito vector strategy (Ito *et al.,* 2002; Marshall, 2009) is yet to materialize although it might be speeded up with the recent completion of the *Anopheles gambiae* genome (Holt *et al.,* 2002). One of the most appealing strategies in the genetic control efforts is the strategy where by the genes for refractoriness to *Plasmodium* are spread into the wild vector population via the release of genetically modified (GM) strains (Carlson, 1996; James *et al.,* 1999). Transgenics released into the wild could enhance naturally occurring defence mechanisms against malaria parasites in

mosquitoes (Ito *et al.,* 2002). A major concern regarding this strategy is the possibility that the GM strains cannot be integrated into the natural malaria vector population because of barriers to gene flow (Lanzaro and Tripet, 2003).

To date, none of the classical approaches to malaria control have proven effective in Africa and the incidence of malaria is still increasing (Marshall, 2001). Among other factors, this is due to the break down of mosquito control programs due to lack of funds (World Malaria Report, 2011), a ban on DDT (Taverne, 1999) and the evolution of mosquito resistance to insecticides (Coetzee *et al.,* 1999). New methods will therefore be continuously needed to control mosquitoes. The use of the host immune response against the mosquito vector therefore becomes one such appealing alternative strategy (Kay, 1994; Noden, 1995). This strategy is aimed at blocking or stopping the transmission of the blood borne pathogens to the mammalian hosts by killing the mosquito vector hence reducing their densities, their capacity to transmit the pathogen effectively, and their ability to replicate (fecundity) (Ramasamy, 1992; Almeida, 1998).

The mosquito life cycle characteristics show that targeting the mosquito would be one of the most effective ways to reduce malaria transmission. There are several general characteristics of the malaria transmission cycle that may allow the disease to be controlled through anti-mosquito vaccines. The first one is that mosquitoes and man are the only two hosts of the four species of the human malaria parasites. Secondly, malaria parasites are only transmitted laterally amongst man and the mosquito. Thirdly, members of the *An. gambiae* complex derive >95% of their blood meals from humans (Beier *et al*.,

1988; Takken *et al.,* 1999). The sporogony of *P. falciparum* in *An. gambiae* requires approximately 10-14 days for completion (Beier, 1998). They often take 2-3 blood meals per gonotrophic cycle (Beier *et al.,* 1996) and have the ability to feed on more than ten different hosts in their lifetime (Colluzi, 1992). These characteristics make mosquitoes more vulnerable to anti-mosquito vaccine than any other strategy. Since *An. gambiae* must feed upon at least three different hosts before they are able to transmit sporozoites and that most transmission is carried out by mosquitoes that have taken between 4 and 12 blood meals (Killeen *et al*., 2000a), vaccine coverage and efficacy in generating an immune response in such a strategy need not be that high.

Mosquito killing control strategies would produce the largest impact on malaria transmission, as they do not allow mosquitoes that feed on an infected host to live to transmit the infective parasite stage to the next host (Billingsley, 1994; Foy *et al*., 2002). This means that a mosquito killing vaccine will not allow a mosquito feeding on an immunized host whether infected or not to live to feed on the next host. Though not attained through vaccination, the most successful malaria control strategy to date has primarily relied on intensive mosquito control efforts (Killeen *et al.,* 2002; Nahlen *et al.,* 2003; World Malaria Report, 2011). Thus with the current global resurgence of malaria, novel mosquito control strategies incorporating the use of vaccination against both the parasite and the vector responsible for the transmission of the parasite will be required.

2.6 Genetic vaccines

Eradication of diseases by immunization generally constitutes the greatest achievement of modern medicine. Vaccines are the most cost effective tools for public health and have been instrumental in previous campaigns against smallpox (Henderson, 1987), polio (John, 2009), and measles (Breman *et al.,* 2011). Vaccines have also been used for sustained control of diseases such as neonatal tetanus (Ropper *et al.,* 2007). Other vaccines such as *Haemophilus influenza* type b conjugate vaccine have the potential to lead to the elimination of *Haemophilus influenza* type b infection in some settings (Adegbola *et al.,* 2005). Whereas many effective viral and bacterial vaccines are available, no effective anti-parasite vaccine has yet been licenced for use in human beings. A gap therefore exists between diseases with successful vaccines, mostly of viral and bacterial origin, and those without a vaccine, mostly parasitic and vector. In order to bridge this gap, alternative approaches to vaccination are required.

So far, small pox is the only disease that has been eradicated through successful vaccination and most of the diseases currently lined up for eradication are of viral origin. Thus, for any successful vaccine initiative, the vaccine may have to be presented to the immune system in the same way viral proteins are. Currently the approach that resembles this virus model of antigen presentation is to develop a vaccine out of the genetic material derived from either the pathogen responsible for the particular disease or the vector responsible for the transmission of the pathogen (Johnston *et al*., 1986; Willadsen *et al.,* 1995; Lal *et al.,* 1998; Shi *et al*., 1999). The current candidate vaccine for malatia, the

RTS,S/AS01, is a hybrid virus like particle containing the C terminus of the circumsporozoite antigen fused to hepatitis B surface antigen (WHO, 2005). Most studied genetic vaccines consist of plasmids which are small rings of double stranded DNA originally derived from bacteria but totally unable to produce an infection (Hooffman, 1996). The plasmids used for immunization incorporate genes that express one or more antigenic proteins normally made by a selected pathogen or transmission vector.

DNA immunization is known to stimulate a potent cellular and humoral immune response (Gurunathan *et al.,* 2000) as opposed to protein immunization which predominantly stimulates humoral immune responses (Lal *et al.,* 1998). An intra-muscular or intra-dermal injection of saline DNA solution leads to an uptake of the DNA by the muscle, skin or antigen presenting cells (APCs) in the vicinity of the injected site (Wolff, 1990; Gurunathan, 2000). The transfected cells synthesize the encoded antigenic protein, akin to the way viral proteins are transcribed in a virus-infected cell.

The ways in which the synthesized proteins are displayed on the surface of the transfected cell determine the type of immune response that will be elicited. A humoral immune response is elicited when the synthesized proteins escape from the cell nucleus into the cytoplasm where they are broken down into peptides that are then picked up by the major histocompatibility complex (MHC) class 2 molecules for display on the cell surface. A cell-mediated immune response is elicited when the synthesized proteins are shuffled from the cell nucleus to the rough endoplasmic reticulum (RER) where they are

broken down into peptides that are picked up by the MHC class 1 molecule for display on the cell surface (Leifert *et al,.* 2004).

The peptides, whether on MHC class 1 or 2 molecules on a professional APC, are displayed alongside critical co-stimulatory molecules that are necessary for the initiation of the immune response. When non-antigen presenting cells transcribe the proteins, the professional APCs in which a similar process takes place for the initiation of the immune response will phagocytose them. In this case, the proteins expressed in the non-APCs are broken down in the APCs' cytoplasm and presented on the surface of the APCs on MHC class 2 molecules for initiation of humoral immune response (Heath *et al.,* 2004)

2.7 Current anti-malaria vaccine strategies

Many lines of evidence indicate that humans can be vaccinated against malaria. Individuals born in endemic areas who survive the first years of exposure continue to develop parasitaemia on natural exposure, but become resistant first to severe, life-threatening malaria and then to clinical disease. Frequent re-exposure is required to maintain this condition of immunity with infection (concomitant immunity). Transfer of gamma-globulin fractions from semi-immune to naïve humans mitigates malaria disease (Bouharoun-Tayoun *et al.,* 1990) demonstrating that clinical protection from malaria is possible and that immunoglobulin targeting malaria antigens can play a critical role. Inoculation of humans with irradiated sporozoites by mosquito bite can prevent the emergence of blood-stage infection after subsequent experimental challenge (Clyde *et al.,*

1973), demonstrating the possibility of inducing high level protection against infection under experimental conditions.

Current efforts to develop malaria vaccines are primarily directed towards reducing morbidity and mortality that are associated with malaria and focus primarily on *Plasmodium falciparum* where over 40 vaccine projects have reached the clinical trials stage with several others in the pre-clinical stage (Schwartz *et al.,* 2012). The Malaria Vaccine Technology Roadmap 2006 has a strategic goal of developing a vaccine with 50% efficacy against severe disease and malaria related mortality protecting for more than 12 months, and secondly a longer-lasting vaccine with 80% protective efficacy against *Plasmodium falciparum* induced clinical malaria by 2020 (MVTR final report, 2006). The primary refinement introduced by the malaria eradication agenda setting process is the confirmation that for elimination and global eradication, impact on transmission rather than morbidity is the paramount efficacy outcome. There is general agreement that if malaria vaccines are to contribute to programs for malaria eradication they will need to have an impact on malaria transmission and the currently available tools are inadequate. Development of a highly efficacious malaria vaccine which dramatically reduces transmission would be a transformative tool that could enable future eradication (Schwartz *et al.,* 2012).

One way of introducing such transmission blocking vaccines would be to develop vaccines that are targeted against the mosquito vector as opposed to the malaria parasite. Despite the potential advantages of anti-vector vaccines, only one anti-vector vaccine has

been licenced against the *Rhipicephalus (Boophilus) microplus* ticks (De la Fuente *et al*., 2007). One of the main performance characteristics of the vaccine is a reduction in acaricide usage. The major benefit to the use of anti-mosquito vaccine control strategy lies not only in reducing the number of vectors but also in reducing their longevity hence the need for insecticides. Infective malaria parasites develop near the end of a mosquito life span in nature so even a small decrease in survival can have a large epidemiologic effect (Vernik *et al.,* 2004).

There are three areas in which vaccine development against malaria is carried out; vaccines against the pre-erythrocytic or liver stage, the blood stage and the sexual stages of the parasite in the mosquito vector. The use of a pre-erythrocytic malaria vaccine would protect against the infectious form of the parasite (sporozoite) injected by a mosquito and inhibit parasite development in the host liver (Hoffman *et al.,* 2002). Pre-erythrocitic malaria vaccines designed to produce sterile protection can be terminated at the challenge trial stage if no efficacy is demonstrated. The inadequacies with this kind of a vaccine is that, in a previously unexposed individual, if a few parasites escape the immune defenses induced by this stage specific vaccine, they could eventually multiply and result in full blown malaria disease and foster the spread of resistance.

The use of an erythrocytic or blood stage vaccine inhibits parasite multiplication in the red blood cells thus preventing severe disease (Kumar *et al.,* 2002). Blood stage vaccines generally have to progress to field evaluation for proof of concept of clinical effect. Thus the timeliness to reach proof of concept for blood-stage vaccines is much longer than for

pre-erythrocytic vaccines. This may explain why there are more potential blood-stage vaccines than other life-cycle stages in clinical evaluation. However this vaccine will not inhibit development of the erythrocytic stage into the next stage (gametocyte) as this does not leave the erythrocyte. Lastly, sexual stage and mosquito antigen vaccines are receiving renewed attention but are still grossly under-represented in clinical project portfolios. The use of the sexual stage vaccine (gametocyte) will not protect the person being vaccinated but will block transmission to the next host through the next mosquito bite.

Plasmodium falciparum is a highly immune-evasive, multi-stage protozoal parasite with several antigenically distinct mosquito vector and human stages. Molecular understanding of naturally acquired immunity remains in its early stages. Despite the success of the *Pl. falciparum, An. gambiae* and human genome projects, there has been little translation of antigenic targets from post-genomic antigen discovery to clinical evaluation, partly because of the problems of selecting appropriate targets, and the lack of robust and reliable predictive animal models (Schwartz *et al.,* 2012). Thus for a successful malaria parasite based vaccine strategy, all molecules that are critical in each stage of malaria parasite development have to be identified and incorporated into the vaccine (Kumar *et al.,* 2002). An optimal malaria parasite based vaccine must therefore have the ability to elicit protective immunity that block transmission of infection, prevent pathology in the infected host and interrupt transmission of parasites to the mosquito vector (Kumar *et al.,* 2002). This vaccine would most likely be a combination of

molecules from different malaria parasite developmental stages put together as one combined vaccine (Holder, 1999).

The first human anti-parasite vaccine might be considered for licensure by regulators in the next few years as the RTS,S/AS01E progresses through clinical evaluation in a pivotal phase 3 trial (Schwartz *et al.,* 2012). The RTS,S/AS01E has demonstrated 51% efficacy in reducing the rate of all episodes of clinical malaria over 15 months of follow-up in a phase 2 trial in children aged 5-17 months resident in Kilifi, Kenya (Olotu *et al.,* 2011). The full trial results of the ongoing pivotal phase 3 trial will include safety and reactogenicity of a vaccine containing a novel adjuvant, co-administration data with pentavalent DTwP/HepB/Hib and OPV, efficacy in multiple transmission settings, efficacy data over 30 months of follow-up, an 18 month booster dose and efficacy against severe, life-threatening malaria (RTS,S Cinical trials partnership, 2011; White, 2011). The major lessons from the RTS,S trials include the major contribution of the sprozoite challenge trials, the importance of adjuvant, dose and schedule optimization, and the need to use particulate structures to enhance immunogenicity (Schwartz, *et al.,* 2012). Multiple other projects have yielded a degree of efficacy, including the prime-boost pre-erythrocytic projects (Langhorne *et al.,* 2008), the CS protein (Nussenzweig and Nussenzweig, 1989), the Adenovirus (Ad35) vectored CS (http://clinicaltrials.gov/ct2/show/NCT01018459), the Ad35 vectored CS in prime-boost with RTS,S/AS01E (Stewart *et al.,* 2007), the multiple epitope constructs (Reyes-Sandoval *et al.,* 2010), the AdCh63/MVA ME-TRAP (http://clinicaltrials.gov/ct2/show/NCT00890019), Polyepitope DNA EPI300 (http://clinicaltrials.gov/ct2/show/NCT00890760), PfSPZ:

metabolically active, non-replicating malaria sporozoite vaccine (Epstein *et al.,* 2011) and the genetically attenuated sporozoites (VanBuskirk *et al.,* 2009) .

The recent reductions in malaria transmission in Africa, if sustained, will render field efficacy trials more difficult in many current research settings. This could see a shift in emphasis away from blood-stage vaccines towards pre-erythrocytic and sexual stage/mosquito (SSM) antigen vaccine development, emphasizing the role they could play in reduction of transmission. Clinical evaluation of SSM vaccines will however be challenging because these vaccines confer efficacy to humans only at the population level and thus traditional individually randomized trial designs will not apply without major modifications (MALVAC, 2010). An additional problem is the lack of knowledge of the relationship between the effect of a vaccine at an individual level, such as rendering an individual 80% less infectious to mosquitoes, and the effect on transmission (Schwartz *et al.,* 2012).

Two commercial anti-vector vaccines are available for *Boophilus microplus,* both based on the Bm86 antigen: TickGARD in Australia and GAVAC in Cuba. These vaccines are based on the membrane bound glycoprotein, Bm86, located on the tick digestive cells. Ingestion by the feeding tick of antibody to Bm86 leads to destruction of the digestive cells, disruption of the gut and leakage of bovine blood into the tick haemolymph leading to low engorgement weight of the female ticks, low egg laying and death of ticks on the host cattle. One of the main performance characteristics of these vaccines has been the reduction in acaricide usage, which has been significant in both Australia and Cuba,

reduction in tick infestations, reduction in transmission of some tick-borne pathogens and increase in animal production (de la Fuente *et al.,* 2007).

To date, outside the human host, malaria control through transmission blocking vaccine development strategy have primarily focused on the parasite developmental stages in the mosquito midgut (Tang *et al.,* 1992; Sedegah *et al*., 1994; Schneider 1998; Kim *et al,*, 1998). It is therefore important to differentiate between anti-vector immunity that can kill or impair the vector and anti-vector immunity that blocks pathogen development in the vector. Anti-vector immunity that can kill the vector involves the use of crude protein preparations from the mosquito midgut to induce this immune response (Hoffman *et al*., 1997). The anti-vector vaccine so far licensed for tick control is based on this strategy where a midgut protein, Bm86 is targeted. However, in mosquitoes, this strategy has recently been refined through the switch to the use of cDNA encoding critical proteins expressed in the mosquito midgut after a blood meal (Kumar *et al.,* 2002).

The advantages of using cDNA from mosquito midgut derived proteins includes the potential to act against different species of the mosquito vectors, the potential of the immune response to decrease survivorship and fecundity in case the vector is not killed and the potential to disrupt mosquito digestion and absorption. Furthermore, the use of mosquito midgut cDNA library circumvents the traditional problem of obtaining enough crude mosquito midgut protein mixtures that has to be screened for their ability to induce anti-mosquito immunity (Foy *et al*., 2003).

The first immunization of BALB/c mice with *An. gambiae* midgut cDNA library induced host immunity that increased mortality in blood feeding mosquitoes (Foy *et al*., 2003). Further immunization of BALB/c mice with a gene that encodes for mucin protein in the midgut of blood fed *An. gambiae* mosquito, AgMUC1 cDNA (Shen *et al*., 1999), also resulted in an increase in mortality of mosquitoes fed on the immunized mice. When adult female *An. gambiae* mosquitoes were fed on groups of mice immunized with plasmid containing AgMUC1 gene, a consistent and significant increase in mortality was observed (Foy *et al.,* 2003).

2.8 The *Anopheles gambiae* Mucin protein

In the midgut of a blood fed female *An. gambiae* mosquito, a membrane bound mucin protein, the *An. gambiae* mucin 1 (AgMUC1) protein, has been characterized and cloned (Shen *et al.,* 1999). This protein is attached to the lumenal surface of midgut cells. AgMUC1 is an abundant, highly glycosylated protein on the lumenal midgut epithelium hence a potential target antigen for transmission-blocking vaccines.

Mucins are most commonly known in their secreted form where they constitute the main component of the mucous that covers mucosal surfaces. The *An. gambiae* mucin protein is membrane bound and is solely expressed on the lumenal midgut epithelium of the adult female mosquitoes. It is linked to the midgut epithelium via glycosyl-phosphatidylinositol anchor (Shen *et al.,* 1999).

Membrane bound mucins have hydrophobic tails that anchor them to the apical plasma membranes of the epithelial cells (Rayms-Keller *et al.,* 2000). In the *Aedes aegypti* larva and adult mosquito midgut, a protein containing mucin-like domains was identified (Rayms-Keller *et al.,* 2000; Morlais *et al*., 2001). Designated *Aedes aegypti* mucin 1 protein (AeIMUC1), the most recent evidence suggests that in the adult mosquito it functions to bind toxic heme released in the mosquito midgut when hemoglobin is digested (Devenport *et al*., 2003). No such function has yet been ascribed to AgMUC1. If the same function could be accomplished by the AgMUC1 protein then interference with this function through an immune response could further lead to mosquito death even where there are no visible microscopic pathologic features inflicted in the cells lining the midgut epithelium.

2.9 Cytokines Interleukin-12 or Granulocyte Macrophage Colony Stimulating Factor

Cytokines play a key role in the initiation and regulation of the immune and inflammatory responses. The co-injection of immune-modulatory plasmids that encode cytokines, chemokines or co-stimulatory molecules is a promising strategy to improve the efficacy of DNA vaccines. The aim of incorporating plasmid cytokines is either to expand the APC pool or to enhance its potency with these adjuvants without the adverse effects that have been observed with the administration of purified cytokine proteins (Ingolotti *et al.,* 2010). Cytokines can recruit and activate macrophages and dendritic cells and promote antigen presentation to T cells.

Among the many cytokines that can be co-administered with the engineered DNA vaccine, each one of them is useful for a different aspect of the immune response thus providing flexibility in modulating the type of the desired immune response.

2.9.1 Interleukin-12

Interleukin-12 (IL-12) is a pleotropic cytokine secreted by a wide variety of cells including dendritic cells, Macrophages, Monocytes and Neutrophils and is thought to provide a functional bridge between innate resistance and antigen specific adaptive immunity (Trinchieri, 1995). IL-12 is part of a family consisting of IL-23, IL-27 and IL-35 all of which play distinct cellular and functional roles in Th1 development (Beadling *et al.,* 2006). IL-12 plays a critical role in Th1 immune response by inducing the production of IFN-γ by CD4+ T cells and NK cells (Schoenhaut *et al.,* 1992; Bonecchi *et al.,* 1998). It activates and enhances the cytotoxic activities of NK and CD8+ T cells. IL-12 is known to regulate and promote Th1 type immune responses and enhance IFN-γ production (Doolan and Hoffman, 1999). IFN-γ production precedes and initiates the production of IL-12 which induces IFN-γ production in a positive feedback loop that represents an important amplifying mechanism (Trinchieri *et al.,* 2003). NK cells are the major producers of IFN-γ (Trinchieri, 1989). IL-12 has also been shown to be essential for T cells and NK cell production of IFN-γ (Scharton-Kersten and Sher, 1997). IL-12 has been reported to modulate the immune response by promoting cell mediated immune mechanisms that protect against pre-erythrocytic stage malaria (Doolan *et al.,* 1999). The

co-delivery of IL-12 genes with a DNA vaccine has also been reported to have resulted in a reduction in specific antibody response (Kim *et al*., 1997).

2.9.2 Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a cytokine that is a hemopoietic growth factor for stem cells of the macrophage and granulocyte lineage (Metcalf *et al*., 1985). It is known to stimulate neutrophils, monocyte/macrophages differentiation and proliferation, antigen presenting cells and eosinophil formation (Disis *et al.,* 1996). It also increases the antibody dependent cell mediated cytotoxicity of neutrophils, eosinophils and macrophages. GM-CSF has been shown to enhance DNA vaccine elicited immune response in a variety of animal models (Ahlers *et al.,* 2002). GM-CSF protein has been shown to have a short half life ranging from 0.9-2.5 hours in plasma (Stute *et al.,* 1992). In order to achieve local and sustained delivery, many studies have used GM-CSF plasmid DNA instead of recombinant protein because of the instability of the cytokine protein (Sedegah *et al.,* 2004).

Co-administration of cytokines with antigens in varying configurations has been shown to be a promising approach for amplifying vaccine elicited immune responses (Kim *et al.,* 1997). Co-administration of *Plasmodium yoelli* circumsporozoite DNA with a DNA plasmid that express murine GM-CSF was shown to increase the concentration of antibodies produced to *plasmodium yoelii* sporozoite eight fold (Weiss *et al.,* 1998). Murine GM-CSF was also reported to have amplified HIV antigen specific poly-

functional memory CD8+ T cells and thus enhancing not only the magnitude but also the quality of the immune response (Xu *et al.,* 2008). GM-CSF recombinant protein has been used as a vaccine adjuvant with Hepatitis B vaccine in humans where it was reported to have led to increased antibody production after a single immunization (Tarr *et al*., 1996). In rhesus monkeys, the addition of GM-CSF plasmids to the malaria vaccine plasmids was found to be associated with a modest increase in antibody levels (Kumar *et al.,* 2002).

2.9.3 Interleukin-12 / Granulocyte Macrophage Colony Stimulating Factor Plasmids

The data regarding the use of plasmid based molecular adjuvants are promising and prolific and such data suggest that these techniques could help to augment the previously weak responses conferred by DNA vaccines. Already both GM-CSF and IL-12 are being used in humans for medical purposes and their plasmids have been reported to be useful enhancers of DNA based vaccines. To fully realize the potential of DNA vaccines, cytokines are the prime candidates for use in enhancing or redirecting the immune response induced by DNA vaccines.

The plasmid approach is advantageous in being simple and producing the bioactive cytokine. It also has its disadvantages. It is difficult to control and predict the amount, duration and concentration profile of the cytokine at the vaccination site. There is also the uncertain and potential adverse effect of long term expression of cytokine should a rare event of integration into the host genome occur.

In this study, GM-CSF and IL-12 were administered as plasmids in order to exert their mechanisms through expression of the cytokine proteins by the transfected cells. This system might not be as vigorous in stimulating the immune response as administering the cytokine protein itself but it was envisaged that the transfected cells would secrete the cytokine over a longer period of time, hence eliminating the need for boosting in order to continue stimulating the immune response as is the case with the use of recombinant proteins. Furthermore while using plasmids, some cells may be transfected with both the cytokine and the AgMUC1 gene thus enhancing the immune response due to their proximity.

AgMUC1 cDNA was co-delivered with plasmids containing either IL-12 or GM-CSF in order to manipulate the immune response such that the biological effect of antibody and cell mediated immune responses on mosquito survival could be assessed separately. The aim was to manipulate the immune response to AgMUC1 cDNA immunization such that in the case of IL-12 co-immunization, the response is predominantly cell mediated while in the case of co-immunization with GM-CSF the response is predominantly antibody mediated. Co-inoculation of AgMUC1 cDNA with cytokine plasmids has an added advantage in that it can result into the co-transfection of individual cells with both plasmids.

AgMUC1 cDNA was therefore administered alone, with IL-12, GM-CSF, both IL-12 and GM-CSF cDNAs or with a final recombinant mucin protein boost. Primary immunization with DNA plasmid followed by recombinant protein has been shown to result into

superior immunogenicity and protection compared with DNA immunization alone (Jones *et al.,* 2001).

2.10 The mosquito midgut structure

The mosquito midgut is the organ into which the blood meal passes and in which, within a peritrophic membrane secreted by the epithelium, the blood is retained during digestion and absorption. The monolayer of the mosquito midgut epithelium rests on a network of longitudinal and circular muscles that are entirely covered with an extra cellular basal membrane (lamina) (Richards, 1975; Dow, 1986; Chapman, 1999). As the midgut expands in the course of ingesting a blood meal, the distance between the longitudinal muscles increase and the single layered epithelium is distended to give a flat and thin epithelium. In addition to providing integrity to the gut, the muscles also confer the peristaltic actions of the gut allowing the movement of ingested food to the hindgut. There is no zonal segregation of different types of epithelial cells in the midgut of the female mosquito. This single layer of columnar epithelial cells with an apical brush border and a basal laminar is highly specialized to accomplish all the functions of secretion and absorption attributed to the midgut of the adult female mosquito (Bertram and Bird, 1961; Reinhardt and Hecker, 1973).

The mosquito midgut is lined with a single layered epithelium consisting of irregularly shaped cubical to columnar cells whose apical side is folded into numerous actin filled microvilli. It is these microvilli that are normally exposed to the harsh environment of

the gut lumen and are subjected to damage caused by food particle abrasion, digestive hydrolases and attack by pathogens and parasites. These microvilli have however evolved mechanisms that protect them from these effects. These mechanisms include the peritrophic matrix, the glycocalyx and the mucin proteins that line their epithelial surfaces (Willadsen, 1996; Antonyraj, 1998; Shen, 1999).

The midgut morphology is drastically altered after blood feeding. There is need to have a very clear view of the normal midgut structure and compare to the midgut structures that have been altered through immune components interactions from the blood meal. There are no reports about the fine structure of the mosquito midgut epithelium interacting with immune factors such as antibodies or immune cells. Knowledge of such a midgut structure is of special significance for interpreting early events in the interaction between the mosquito midgut lining and the specific immune components present in the blood of BALB/c mice immunized with AgMUC1/IL-12 cDNA. The results of such an examination will have to be interpreted in the context of the fine structure of the midgut epithelial cells derived from the midgut of mosquitoes fed on mice immunized with empty vector cDNA compared to midguts of mosquitoes fed on mice immunized with AgMUC1/IL-12 cDNA.

This study therefore aims to define the immune-pathological features that lead to mosquito death after feeding on AgMUC1/IL-12 cDNA immunized mice with particular attention to the events taking place on the epithelial cells of the blood fed mosquito midgut.

2.11 Anti-mucin antibody producing hybridoma cell lines

A monoclonal antibody is a homogeneous population of antibody molecules of defined specificity secreted by the descendants of a single B lymphocyte. B lymphocyte hybridomas are produced by fusing B-lymphocytes with myeloma cells (Kohler and Milstein, 1975). The B-lymphocytes contribute specific antibody producing genes to the hybridoma cell line while the myeloma cells provide genes that allow the hybridoma cell line to divide indefinitely while continuing to express the immunoglobulin genes (Kennet *et al.,* 1980). The B-lymphocytes are obtained from animals, usually mice, which have been immunized with a specific antigen.

With mosquito killing anti-mucin antibodies secreted from hybridoma cell lines, the antibody mediated mechanisms responsible for mosquito death following a blood meal on an AgMUC1/IL-12 cDNA immunized mice can be determined *invitro*. The purpose of preparing hybridoma cell lines secreting mosquitocidal antibodies is to determine the efficacy of an antibody in mediating pathology in the mosquito midgut.

Studies in which mosquitoes are fed to AgMUC1/IL-12 cDNA immunized mice show that antibodies alone are not sufficient in mediating mosquito death. High anti-mucin antibody titers do not to correlate with the corresponding cumulative survival of the mosquitoes surviving to day 7 after feeding on the immunized mice. This may mean that for effective mosquito killing, either antibodies are not sufficient on their own, or specific

anti-mucin antibodies effectiveness requires augmentation from the other immune components found in the blood of an immunized mouse.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Site

Adult *An. gambiae* G3 strain that were used were reared in the insectary of the Department of Vivarial sciences at Tulane University Medical School USA or the insectary unit at ICIPE. The mosquitos were fed on 10% Karol syrup at 28° C, 75% humidity and a 12-12 hour light - dark cycle. The ICIPE *An. gambiae* strain used in these studies was adapted to feeding on mouse and human blood before being used in the experiments.

3.2 BALB/c Mice

Female BALB/c mice, 6-8 week old, were used. The mice were obtained from either Charles River laboratories, Maine, USA and maintained in the small animal unit at the Tulane University Medical School according to the Universities established protocols or from the UK by ILRI Nairobi and maintained at its pathogen free facility according to the institutions established protocols.

3.3 *Anopheles gambiae* Mucin 1 plasmid

The cDNA coding for AgMUC1 gene was donated by Jacobs Lorena's Laboratory at Case Western University, USA. It was sub-cloned into Ampicillin resistant pcDNA3.1 (+) plasmid vector (Invitrogen) with Xba1 and Hind III restriction enzymes and

electroporated into XL1-Blue MRF'E. coli (Foy et al., 2003). Plasmid DNA for immunization was harvested from E .coli broth cultures using Qiagen endotoxin-free Giga prep kits (Valencia, CA). The cDNA was diluted appropriately in endotoxin-free PBS for use in the immunization protocols.

3.4 Granulocyte Macrophage Colony Stimulating Factor and Interleukin-12 plasmids

Murine GM-CSF cDNA in pUMVC1 and murine IL-12 cDNA in pUMVC3 kanamycin resistant plasmid vectors were procured commercially (Aldevron, North Dakota USA). They were electroporated into XL1-Blue *MRF'E. coli* and cultured on LB agar plates containing Kanamycin. Single colonies were harvested from the LB agar plates. Large-scale cultures from the single colonies were prepared in LB broth containing Kanamycin. Plasmid DNA was harvested from *E.coli* using commercially procured Qiagen endotoxin-free Giga preparation kits (Valencia, CA) and diluted appropriately in endotoxin-free PBS for use in the immunization protocols.

3.5 General immunization protocol

It was a pre-requisite that a pre-immunization blood sample be collected from all the BALB/c mice destined for use in these experiments and tested for anti-mucin antibody reactivity. Immunizations were done via the mouse footpad. For the immunization protocol, the cDNA solution containing 200 µg of AgMUC1 cDNA or empty vector cDNA in endotoxin free PBS was administered. The cytokines were administered a

solution cDNA containing 100 µg of either GM-CSF or IL-12 cDNA. The recombinant mucin or ovalbumin proteins were administered in a solution of 10 mgs of either mucin or ovalbumin in endotoxin free PBS. For multiple immunizations, the cDNA solutions were administered at fourteen (14) days interval. The immunized mice were ready for mosquito bioassays fourteen (14) days after the final immunization schedule. Before mosquito bioassays, blood samples were collected from the immunized mice via the tail bleed for analysis of anti-mucin antibodies in an ELISA based testing protocol (3.8.3)

3.6 Optimization of AgMUC1 immunization schedule

3.6.1 Mice immunization groups

Four groups of six BALB/c mice per group were immunized with AgMUC1 cDNA to form the experimental group and empty vector cDNA to form the control group. The experimental group comprised of three mice while the control group comprised of another three mice. The cDNA solutions were given at a concentration of 200 µg while the multiple injections were given at 14 day intervals as shown (Table 3.1).

Day 14 after the last injection in each group, adult female *An. gambiae* mosquitoes were fed on each of the six mice comprising the experimental and control groups. Immediately after feeding the mosquitoes on the mice, the mice were sacrificed. Cardiac blood and the spleens were collected. Serum was separated from the cardiac blood and frozen at –80 ^{o}C until testing for anti-mucin antibodies ELISA (3.8.3). Spleen cell suspensions were

prepared from the spleens and tested in a lymphocyte proliferation assay using recombinant mucin protein (3.8.1).

Table 3.1: AgMUC1 cDNA Mice immunization groups

	Group1	**Group 2**	**Group 3**	**Group 4**
Day 0	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA
Day 14	Feed mosquitoes Collect blood and spleen	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA
Day 28	N/A	Feed mosquitoes Collect blood and spleen	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA
Day 42	N/A	N/A	Feed mosquitoes Collect blood and spleen	Exp: 200 ug AgMUC1 cDNA. Cont: 200 ug empty pcDNA
Day 56	N/A	N/A	N/A	Feed mosquitoes Collect blood and spleen

3.6.2 Mosquito Bioassay

Groups of approximately 40-60 adult female *Anopheles gambiae* mosquitoes, 4-6 days post pupation, in clean 500ml size plastic containers were fed on the immunized BALB/c mice for duration of 15-20 minutes. Mosquitoes that did not feed were removed at the end of the feeding session. The mosquito cages were examined daily to day 7 post blood feeding for the number of dead mosquitoes. The number of dead mosquitoes was recorded and removed from each cage daily. The mosquitoes in the cages were maintained on fresh sugar water pads that were changed daily. After recording the dead mosquitoes on day 7, the remaining live mosquitoes were chilled to death, counted and recorded as the number of mosquitoes surviving to day 7 post blood feeding. At day 7 post blood feeding the mosquitoes have completely digested the food bolus and are ready for their next blood meal. Survival analysis was performed on the data as descibed (3.13).

3.7 Enhancencement of the immune response to AgMUC1 cDNA immunization using immunostimulatory cytokines GM-CSF or IL-12 cDNA

Pre-immune serum was collected via tail bleed from all the mice prior to immunization for anti-mucin specific antibody ELISA (3.8.3). The non-reactive anti-mucin antibody ELISA mice were divided into five groups (Table 3.2). Each group comprised of five mice in the experimental group and five in the control group of the study. Each mouse in the group was injected four times with the immunization cDNA at 14 days interval. Each injection comprised of either 200 µg AgMUC1 cDNA alone or mixed with 100 µg of either IL-12 cDNA or GM-CSF cDNA or both cDNAs. The AgMUC1 cDNA and the

cytokine cDNA were mixed such that 20µl - 50 µl of the mixture contained 200 µg of AgMUC1 cDNA and 100µg of the cytokine cDNA. This mixture was given as described (3.5).

Table 3.2: Groups of mice immunized with AgMUC1, IL-12, GM-CSF or Mucin protein

	Group 1	Group 2	Group 3	Group 4	Group 5
Experimental group	200µg AgMUC1 cDNA only	200µg AgMUC1 cDNA + 100 µg GM-CSF cDNA	200µg AgMUC1 cDNA + 100 µg IL-12 cDNA	200µg AgMUC1 cDNA + 100 µg IL-12 cDNA + 100 µg GM-CSF cDNA	200µg AgMUC1 cDNA followed by 10mg recombinant mucin protein boost
Control group	200µg Empty vector cDNA only	200µg Empty vector cDNA+ 100 µg GM-CSF cDNA	200µg Empty vector cDNA + 100 µg IL-12 cDNA	200µg Empty vector cDNA + 100 µg IL-12 cDNA + 100 µg GM-CSF cDNA	200µg Empty vector cDNA followed by 10mg Ovalbumin protein boost

Immediately after mosquito feeding, the mice were sacrificed and serum collected. The sera were isotyped using the BD biosciences mouse isotyping kit following the manufacturer's protocol (3.10.10).

3.8 Characterization of the type of mosquito killing immune response

3.8.1 Cytokine profiles following *invitro* Lymphocyte proliferation assays

Lymphocytes from the spleens of the study mice were collected immediately after mosquito blood feeding session. The mice were sacrificed to obtain the spleens. Spleen cell suspensions were obtained by gently teasing the spleens over a 40µm cell strainer followed by density gradient separation over Histopaque to separate the immune lymphocytes from the other spleen cells. The final spleen cell suspension was made in tissue culture medium after washing in plain RPMI 1640. The cell suspension was seeded in the 96 well flat-bottomed plates at $2x10^5$ cells per well as follows:

i. Plate A contained 15 µg of recombinant mucin protein in 100 µl of growth medium per well to which 100-µl spleen cell suspension was added. This forms the experimental lymphocyte stimulation assay.

ii. Plate B contained 10 µg of ovalbumin in 100 µl of growth medium per well to which 100-µl spleen cell suspension was added. This forms the non-specific lymphocyte stimulation assay.

iii. Plate C contained 10 µg of Con-A in 100 µl of growth medium per well to which 100 µl spleen cell suspension was added. This forms the positive lymphocyte stimulation assay.

iv. Plate D contained 100 µl of growth medium per well to which 100-µl spleen cell suspension was added. This forms the negative control lymphocyte stimulation assay.

All the seeded tissue culture plates were incubated at 37 ^{o}c in 5% CO_2 and >80% humidity. The tissue culture supernatants were collected from each well plate on day 4 for determination of selected cytokine levels from antigen stimulated spleen cells by a solid phase sandwich-ELISA (3.8.2). Cytokine determination was used as a marker of lymphocyte proliferative activities.

3.8.2 Determination of cytokine levels in supernatants collected following *invitro* spleen cells stimulation

The cytokines selected as markers of lymphocyte proliferative activity were IFN-γ and IL-2. These two cytokines are some of the determinants of cell mediated immunity. The cytokine levels were determined using a commercially available quantitative ELISA based test kit using the manufacturers protocol.

Briefly, Immulon micro-ELISA plates (96 well) were coated with an appropriate anti-cytokine antibody (2µg/ml, Pharmigen), diluted in ELISA coating buffer, pH 9.6. After overnight incubation at 4 ^{o}C, plates were washed three times in ELISA wash buffer and

blocked with ELISA blocking buffer for 1 hour at room temperature. The control samples and the tissue culture supernatants were added to their appropriate wells in the plates and incubated for two hours at room temperature. The plates were again washed as above and a mixture of biotinylated anti-mouse cytokine antibody and avidin horseradish peroxidase diluted in the ELISA blocking buffer was added followed by a further incubation for 1 hour at room temperature. After washing the wells, 100μl of freshly prepared TMB substrate solution (50:50 mixtures of A & B) was added to each well and the plate incubated in the dark for 30 minutes. The reaction was stopped using 50***μ***l of 1M Sulphuric acid. Absorbance was read in a micro plate reader at 405 nM. The concentrations of the cytokines were calculated by interpolation from standard curves based on recombinant cytokine dilutions run in parallel on the same plate.

3.8.3 Detection of Anti-mucin antibodies in mice sera using the ELISA

An ELISA to detect anti-mucin antibodies in the mice sera was performed as follows: Immulon micro-ELISA plates (Dynatech, Alexandria, VA) were coated with 100μl per well of 2.5μg/ml recombinant mucin protein in ELISA coating buffer and incubated at 4 ^{o}C overnight. The plates were washed twice with ELISA wash buffer, blocked with100μl per well of ELISA blocking solution for 1 hour at room temperature, then washed three times. Test samples (100μl per well) were added to each well and incubated at room temperature for 2 hours and the plate washed once again five times with wash buffer. After draining the plate off the wash buffer completely, 100μl of horseradish peroxidase (HRP)-conjugated anti-mouse Ig antibody diluted 1:6000 was added then incubated at

room temperature for 1 hour. The plate was finally washed five times after which a final 100µl of freshly prepared TMB substrate solution (50:50 mixture of A & B) was added to each reaction well. The plate was then incubated at room temperature for 30 minutes in the dark. The reaction was stopped with the addition of 50µl of 1M Sulphuric acid to each reaction well. Plates were read with a micro plate reader at 405 nM. The cut-off for this in-house ELISA was calculated using the non-parametric method of two times the mean positive percent of the OD of the pre-immune sera, representing two standard deviations. The ODs above the cut-off value were reported as anti-mucin antibody reactive.

3.8.4 Determination of the duration of AgMUC1/IL-12 cDNA induced immune response

Immunization of mice with AgMUC1/IL-12 has demonstrated the most significant mosquito killing over AgMUC1 alone or with GM-CSF. One group of five BALB/c mice was therefore immunized with AgMUC1/IL-12 cDNA as described (3.6.1). Groups of *An. gambiae* mosquitoes were fed on this group of mice at 5.5, 15 and 36 weeks following the final immunization dose (3.6.2). Mortality was recorded daily from day 1 post blood feeding up to day 7. The data was analyzed as described (3.12).

3.9 Determination of Immune pathological features in the mosquito midgut following a blood meal on AgMUC1 cDNA immunized mice

3.9.1 Preparation of blood fed mosquito midguts

Mosquitoes were fed on the experimental AgMUC1/IL-12 cDNA and the control empty vector/IL-12 cDNA immunized mice groups and their midguts dissected out at 8 hours post blood feeding and processed as below (3.9.2 – 3.9.4).

3.9.2 Preparation of blood fed mosquito midgut sections for bright light microscopy

Dissected midguts for preparation of sections for bright light microscopy were frozen in OCT compound medium at –20 ^{o}C immediately they were dissected out. Cryostat sections (10µm) were made and stained in 10% Giemsa stain in phosphate buffered saline for 15 minutes. The stained sections were examined under the bright light microscope with the aim of identifying differential features appearing in the midgut sections from mosquitoes fed on the experimental AgMUC1/IL-12 cDNA immunized mice compared to those from the midgut sections of mosquitoes fed on the control empty vector/IL-12 cDNA immunized mice.

3.9.3 Preparation of sections for scanning electron microscopy (SEM)

Dissected midguts for preparation of sections for scanning electron microscopy were fixed in the electron microscopy (EM) fixative immediately they were dissected out and

stored at 4 ^{o}C until processing. The midguts were removed from the fixative, cut longitudinally into two pieces and mounted onto tissue blocks. The pieces were impregnated with Gold and examined on the scanning electron microscope with the aim of identifying differential features appearing in the midgut of mosquitoes fed on the experimental AgMUC1/IL-12 cDNA immunized mice compared to those from mosquitoes fed on the control empty vector/IL-12 cDNA immunized mice.

3.9.4 Preparation of sections for Transmission Electron Microscopy (TEM)

Dissected midguts for TEM are prepared in the same way as those for SEM. From the EM fixative the midguts were washed in distilled water then post fixed in 1% Osmium tetroxide in phosphate buffered saline. The midguts were then washed in distilled water, dehydrated through ascending concentrations of ethanol (50% → 70% → 80% → 90% → 96% and lastly absolute ethanol). The midguts were kept in each concentration for 10 minutes. From absolute ethanol, the tissues were transferred to a 50:50 mixture of ethanol and propylene oxide for 10 minutes then transferred to propylene oxide for another 10 minutes. They were then transferred to a 50:50 mixture of propylene oxide and epoxy (araldite) overnight. The tissues were then moved through two changes of araldite each lasting 24 hours. The tissues were then embedded in pure araldite in embedding moulds and incubated at 60 ^{o}C for 72 hours to allow the araldite to harden. Ultra thin sections were cut, stained with 50% uranyl acetate in ethanol for 30 minutes then washed in distilled water. They were then transferred to lead citrate for 10 minutes then washed in distilled water. The sections were then dried and examined on Transmission Electron

Microscope with the aim of identifying differential features appearing in the sections from mosquito midguts fed on the experimental AgMUC1/IL-12 cDNA immunized mice compared to those sections from mosquito midguts fed on the control empty vector/IL-12 cDNA immunized mice.

3.10 Preparation of anti-mucin antibody secreting hybridoma cell lines

3.10.1 Immunization of BALB/c mice for spleen cells isolation

A group of five BALB/c mice that tested negative in the pre-immune serum anti-mucin ELISA test were injected in the hind footpad as described (3.5). Fourteen days after the final third injection, post immune sera was collected via the tail bleed and tested for anti-mucin antibody activity using the ELISA (3.8.3). Two mice with the highest anti-mucin antibody reactivity were selected for the fusion experiment by a modification of the Kohler and Milstein (1975) technique.

3.10.2 Preparation of mouse lymphocytes

Spleen and lymph node cell suspensions were prepared from the two AgMUC1/IL-12 cDNA immunized mice selected on the basis of their high OD readings in the anti-mucin ELISA. The two immunized mice with the highest OD values on the anti-mucin ELISA were sacrificed after being anaesthetized in CO_2, cardiac blood collected and their spleens, draining and mesenteric lymph nodes collected. 70% ethyl alcohol is used to disinfect the mouse skin. The skin on the front of the left thigh is nicked with sterile

scissors. Using sterile forceps, peel and remove the skin, enter the abdominal wall and carefully cut it off including the chest and hold over the head far away from the spleen. Grasp the spleen and pull it off the mesentery and place in a Petri dish with medium. Grasp the draining lymph nodes and the mesenteric lymph nodes and pull off into the petri dish. The spleens and the lymph nodes were processed separately by gentle teasing. The teased organs were then sieved through a 0.45u filter.

3.10.3 Preparation of myeloma cells

Myeloma cells suspension was prepared from the myeloma cell cultures. Myeloma cells are normally maintained in 75 cm^2 tissue culture flasks. They are seeded at $5x10^4$ cells per milliliter in 25 ml of growth medium and incubated at 37 ^oC in humidified 5% carbon dioxide. They are sub cultured every 2-3 days. Sub culturing is accomplished by vigorously pippetting to detach the cells, discarding one-half to three-fourth of the culture and adding fresh medium. For use in fusion, one day after sub culturing, the cells are washed off the growth medium and held in plain RPMI 1640 until use.

3.10.4 Cell Fusion procedure

The fusion of the immunized mouse spleen cells and the lymph node cells suspensions with myeloma cells was carried out as follows:.

The myeloma cells were washed off growth medium and held in plain RPMI 1640 medium with Pen/Strept for 1-2 hours prior to fusion. A count of the myeloma cells, spleen cells and lymph node cells was performed. The spleen / lymph node cell suspension were mixed with the myeloma cells at a ratio of 2 spleen cells to 1 myeloma cell and centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded, the cell pellet warmed to 37 ^{0}C, and 0.5 ml of warm PEG I added to the cell pellet. This was mixed gently by stirring for 1 minute to allow the cells to fuse. After exactly 1 minute, 0.5 ml of warm PEG II was added, stirred again gently for 3 minutes to stop the fusion reaction. 5 ml of HAT medium was added and the cell suspension continued to be mixed gently before a further 20 ml of HAT medium was added. The whole cell suspension was transferred to a T75 flask and incubated at 37 ^{0}C, 5% CO_2 in a humidified incubator. The spleen and lymph node cells were fused separately following the same procedure.

3.10.5 Preparation of feeder cells

Seventy two hours after fusion prepare thymocyte cell suspension from 10 – 20 day old BALB/c mice and seed 96 well plates at $5x10^3$ cells per well. The neonate mice are killed by cervical dislocation, placed in 70% ethyl alcohol and the thymus removed into a Petri dish by the same surgical procedure as for the spleens except that the thymus is located just on top of the heart. The thymus is teased the same way as the spleen.

The debris was removed by letting the cell suspension stand for 5 minutes. The cells were decanted into a fresh tube and washed once by centrifugation. The cell pellet was resuspended in 10 ml of growth medium. A cell count was performed and the cell

concentration adjusted to 10^7 cells per ml so as to be seeded at 10^6 cells per well in 100ul into the 96 well tissue culture plates.

3.10.6 Seeding fused cells

Hybridoma cell count was performed before seeding into the 96 well tissue culture plates. The following cell concentrations were prepared in HT medium and added to their respective 96 well tissue culture plates containing the thymocytes:

i. 40 ml of 10^4 cells per ml and 4 plates containing thymocytes seeded with 100 µl per well ie 10^3 cells per well. The final hybridoma concentration per well is 10^3 cells per well.

ii. 40 ml of 10^3cells per ml and 4 plates containing thymocytes seeded with 100 µl per well ie 10^2 cells per well.

iii. 40 ml of 10^2cells per ml and 4 plates containing thymocytes seeded with 100 µl per well ie 10^1 cells per well.

iv. 40 ml of 10 cells per ml and 4 plates containing thymocytes seeded with 100 µl per well ie 1 cell per well.

The plates were incubated at 37 ^{0}C, 5% CO_2 in a humidified incubator. The plates were inspected macroscopically by viewing them against a light source from below in order to identify single white hybridoma colonies after 10 days. Supernatants were collected from wells with a single growing mass of hybridoma cells for anti-mucin antibody ELISA (3.8.3).

3.10.7 Cloning

The anti mucin secreting hybridoma MF1/LYMPH/122 was cloned in order to isolate the individual clones producing only one specific antibody isotypes. This hybridoma secretes anti-mucin antibody isotypes IgM, IgG1, IgG2a and IgG3. Cell suspensions of this hybridoma were prepared at concentrations of 10^3 cells per ml, 10^2 cells per ml and 10 cells per ml. Each cell suspension was plated at 100µl per well to give a set of 96-well tissue culture plates containing 10^2 cells per well, 10 cells per well and 1 cell per well. Thymocytes were added to all the wells at a concentration of 10^6 cells per well.

The plates were incubated at 37 ^{0}C, 5%CO_2 in a humidified incubator. The plates were inspected macroscopically by viewing them against a light source from below in order to identify single white mass of hybridoma colonies after 10 days. Supernatants were collected from the identified wells for anti-mucin antibody ELISA (3.8.3), anti-mucin antibody isotype determination (3.10.10) and mosquito bioassay (3.6.2).

3.10.8 Cryopreservation

The mouse myeloma cells and the hybridoma cells were frozen by gently pelleting a 24-hour culture, resuspending in freezing medium at a cell concentration of $3x10^6$ cells per ml and distributing into 1 ml cryovials. Freezing is done by gradually lowering the vials in the vapor phase of the liquid nitrogen storage tank every hour until the vials are immersed in the liquid nitrogen. This process takes approximately three hours.

3.10.9 Harvesting Anti-mucin antibody secreting hybridomas

Anti-mucin ELISA reactive wells were sub cultured into twenty-four well plates followed by expansion into 25 cm^2 then 75 cm^2 tissue culture flasks from where the final supernatant was collected. The supernatants were then concentrated to 10 times their original volume using Amicon concentration apparatus at 10,000 molecular weight cut-off.

3.10.10 Determination of Anti-mucin antibody isotypes

The reactive hybridomas were isotyped using the mouse immunoglobulin isotyping ELISA kit (BD Biosciences) following the manufacturers protocol as per the insert. Briefly, an appropriate amount of each isotype-specific rat anti-mouse monoclonal antibody was diluted in coating buffer and plated on the ELISA plates at 50 µl per well. The plate was incubated at $4^{o}c$ overnight. After washing three times with PBS-Tween, 200 µl blocking buffer was added to each well and the plates incubated at RT for 30 minutes. Again the plates were washed as before then 100 µl of hybridoma supernatants, positive and negative controls added to their respective wells. The plates were incubated at RT for 1 hour, and then washed five times. 100 µl of horseradish peroxidase labelled anti-mouse immunoglobulin monoclonal antibody solution was added, incubated for 1 hour, and then washed five times. Finally 100 µl of TMB substrate A and B (equal volumes mixed prior to use) was added and the plates incubated for 30 minutes at RT in the dark. Positive reaction wells will turn greenish-blue color while negative wells will

remain colorless. 50 µl of stop solution (1 m sulphuric acid) was added to each well. Positive wells will turn yellow. The plates were read in an Elisa reader at 405 nm.

3.11 Determination of mechanisms by which anti-mucin antibodies kill blood feeding mosquitoes

3.11.1 Determination of complement effect in antibody mediated mosquito killing using immune mice sera

Mice sera containing different anti-mucin IgG antibody subtype (3.10.10) were pooled as either the sera containing only IgG1, IgG1 and 2a or IgG1, 2a and 3 and fed to groups of *An. gambiae* mosquitoes in an *in vitro* membrane feeding system (Rutledge *et al.,* 1964). Sera from AgMUC1/GM-CSF, AgMUC1/IL-12 and AgMUC1/Ovalbumin immunized mice contained IgG1 anti-mucin antibodies only and were pooled together. Sera from AgMUC1 and AgMUC1/GM-CSF/IL-12 contained a mixture of IgG1 and IgG2a anti-mucin were pooled together. Sera from AgMUC1/Mucin protein boosted mice contained a mixture of IgG1, IgG2a and IgG3 (Table 3.3)

One group of mosquitoes was fed on mice sera containing complement while the other group was fed on mice sera depleted of complement. The sera were fed to the mosquitoes mixed with fresh naïve mouse red blood cells depleted of complement by washing off the plasma-containing complement using phosphate buffered saline. Complement was depleted from the mice sera by inactivating one aliquot of the pooled sera at 56 ^{o}C for 60 minutes. This aliquot was mixed with fresh naïve mouse blood. The feeding process was carried out as shown (Table 3.3).

Table 3.3: Groups of mosquitoes fed on pooled immune mice sera containing various anti-mucin IgG antibody Subtypes

	Group 1	Group 2	Group 3
Experimental group	Pooled anti-mucin IgG1 containing mice sera mixed with fresh naïve mouse blood	Pooled anti-mucin IgG1 and IgG2a containing mice sera mixed with fresh naïve mouse blood	Pooled anti-mucin IgG1, IgG2a and IgG3 containing mice sera mixed with fresh naïve mouse blood
Control group	Inactivated pooled anti-mucin IgG1 containing mice sera mixed with washed fresh naïve mouse red blood cells	Inactivated pooled anti-mucin IgG1 and IgG2a containing mice sera mixed with washed fresh naïve mouse red blood cells	Inactivated pooled anti-mucin IgG1, IgG2a and IgG3 containing mice sera mixed with washed fresh naïve mouse red blood cells

Mortality was recorded daily from day 1 post blood feeding up to day 7. The data was analyzed as described (3.12).

3.11.2 Determination of anti-mucin antibody effect in antibody mediated mosquito killing using anti-mucin antibody secreting hybridoma supernatants

The concentrated anti-mucin ELISA reactive supernatants were fed to adult *An. gambiae* mosquitoes in an *in vitro* membrane feeding system mixed with fresh naive mice blood as follows:

Approximately 40-60 mosquitoes were fed on an aliquot of each of the isotyped hybridoma supernatant mixed with an equal volume of fresh mice blood through *in vitro* membrane feeders lined with mouse skin through which the mosquitoes fed. The feeders were interconnected to a 37 ^{o}C circulating water bath to keep the blood meal warm. Feeding took 15-30 minutes. Mosquitoes that did not feed were removed from the cages. Mortality was recorded daily upto day 7 when the surviving mosquitoes were killed and counted. Survival analysis was analysed (3.12)

3.11.3 Determination of complement effect in anti-mosquito antibody mediated mosquito killing using anti-mucin antibody secreted hybridoma supernatant MF1/LYMPH/122

The concentrated IgM, IgG1, IgG2a and IgG3 anti-mucin antibody containing supernatant from MF1/LYMPH/122 hybridoma cell line was concentrated to ten times its original volume and fed to adult *An. gambiae* mosquitoes in an *in vitro* membrane feeding system mixed with fresh naive mice blood containing complement and that depleted of complement activity by washing the blood with normal saline before mixing with the suparnantant (Table 3.4).

Mosquito feeding comprised of approximately 40-60 mosquitoes per group. The feeders were interconnected to a 37 ^{o}C circulating water bath to keep the blood meal warm. Feeding took 15-30 minutes. Mosquitoes that did not feed were removed from the cages. Mortality was recorded daily upto day 7 when the surviving mosquitoes were killed and counted. Survival analysis was analysed (3.12)

Table 3.4 Groups of mosquitoes fed on concentrated supernatant containing anti-mucin antibodies secreted by hybridoma cell line MF1/LYMPH/122

	Group 1	Group 2	Group 3
Experimental group	Concentrated MF/LYMPH/122 supernatant mixed with fresh naïve mouse blood	Concentrated MF/LYMPH/122 supernatant mixed with washed fresh naïve mouse red blood cells	Concentrated MF/LYMPH/122 supernatant mixed with fresh naïve mouse blood
Control group	Concentrated myeloma cell line supernatant mixed with fresh naïve mouse blood	Concentrated myeloma cell line supernatant mixed with washed fresh naïve mouse red blood cells	Concentrated MF/LYMPH/122 supernatant mixed with washed fresh naïve mouse red blood cells

3.11.4 Determination of Cross species mosquito killing effects using anti-mucin antibodies secreted by hybridomas

Groups of approximately 40-60 *An. arabiensis* mosquitoes were fed on MF1/LYMPH/122 hybridoma and X63.Ag myeloma supernatants. The supernatants were mixed with an equal volume of fresh mice blood. Feeding was done through invitro membrane feeders lined with freshly isolated mouse skin. The mosquitoes fed through the mouse skin. The feeders were interconnected to a 37 ^{o}C circulating water bath to keep the blood meal warm. Feeding took 15-30 minutes. Mosquitoes that did not feed were

removed from the cages. Mortality was recorded daily upto day 7 when the surviving mosquitoes were killed and counted. Survival analysis was analysed (3.12).

3.12 Data analysis

Survival analysis was performed using life tables to determine the daily cumulative survival of mosquitoes surviving to day 7 after feeding on the BALB/c mice. The Kaplan-Meier statistical analysis and the Log-Rank test were used to determine the differences and the significance of the differences in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding within the immunization groups and between the experimental and control groups. Bivarriate correlations using the Pearson's correlation coefficient and the two-tailed test of significance were performed to establish the relationship and significance of the relationship between the daily cumulative survival of the blood fed mosquitoes surviving to day 7, the specific anti-mucin IgG antibody titers and the cytokine profiles. Analysis was performed on R-statistical soft ware version 2.14.2 (The R-foundation for statistical computing)

CHAPTER 4: RESULTS

4.1 Optimization of AgMUC1 cDNA immunization schedule

4.1.1 The daily cumulative survival of the mosquitoes surviving to day 7 after feeding on the immunized AgMUC1 cDNA immunized mice

The daily cumulative survival of mosquitoes surviving to day 7 post blood feeding was analyzed by constructing life table survival curves that were compared by the Kaplan Meier log rank analysis in order to obtain the difference and significance of the difference in the survival of mosquitoes fed on different mice groups. No significant differences were observed in the daily cumulative suvival of the blood fed mosquitoes surviving to day 7 between the groups of mosquitoes fed on each of the replicate mice within each immunization groups. The data from all the six mice comprising of each group were pooled and analyzed using life tables. In the life table analyses, no significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between the groups of mosquitoes fed on the mice immunized once or twice with AgMUC1 cDNA and their controls similarly immunized with empty vector cDNA (p=0.122; Figure 4.1 and p=0.127;.Figure 4.2). Significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between the groups of mosquitoes fed on the experimental mice immunized three or four times with AgMUC1 cDNA and those fed on the control mice similarly immunized with empty vector cDNA (, p=0.00331; Figure 4.3 and p=0.0173; Figure 4.4).

The general trend observed with this schedule of immunization was that more deaths occur with increasing number of injections starting at three injections (Figure 4.5).

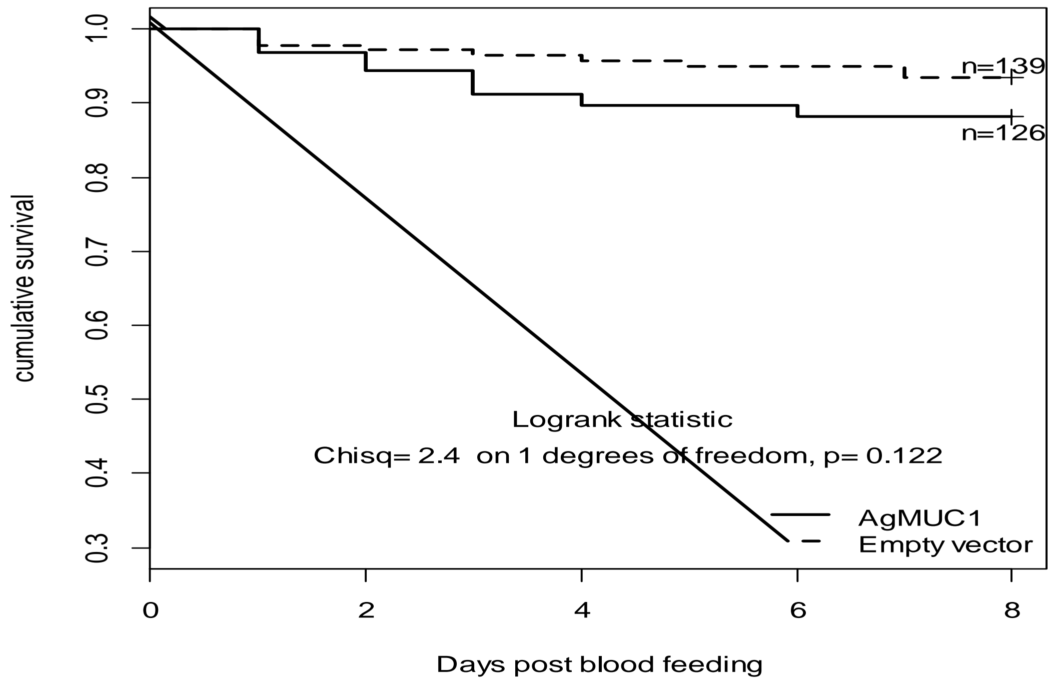

Figure 4.1: Comparison of the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on BALB/c mice immunized once with 200 µg AgMUC1 cDNA or Empty vector cDNA, plotted from life tables.

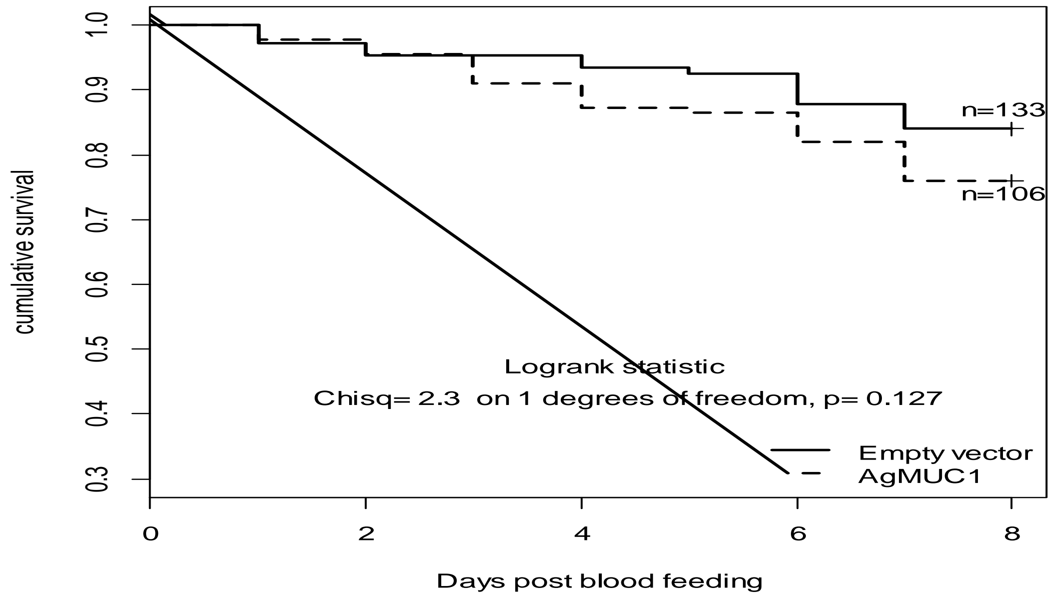

Figure 4.2: Comparison of the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on BALB/c mice immunized twice with 200 µg AgMUC1 cDNA or Empty vector cDNA per immunization, plotted from life tables.

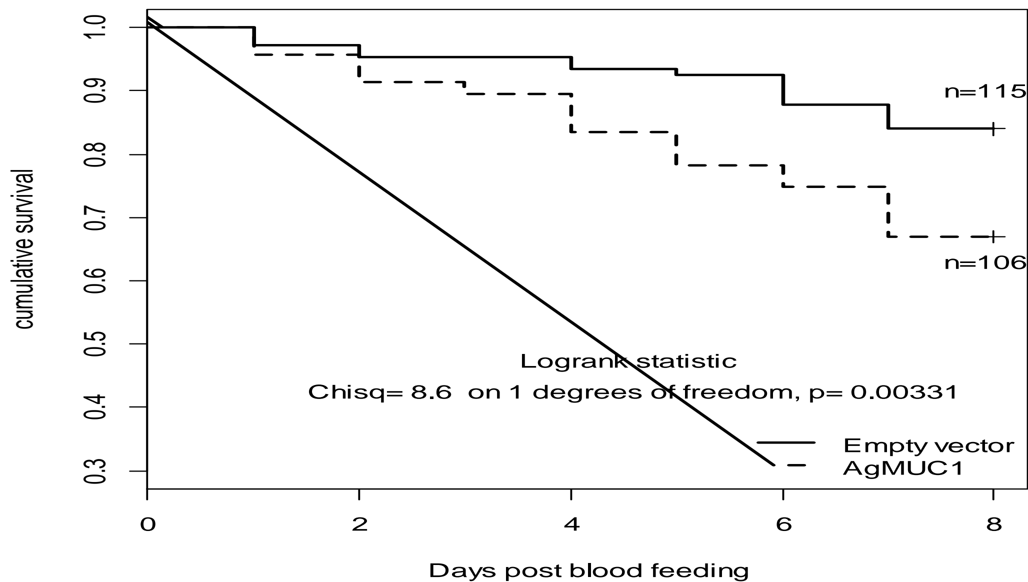

Figure 4.3: Comparison of the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on BALB/c mice immunized three times with 200 µg AgMUC1 cDNA or Empty vector cDNA per immunization, plotted from life tables.

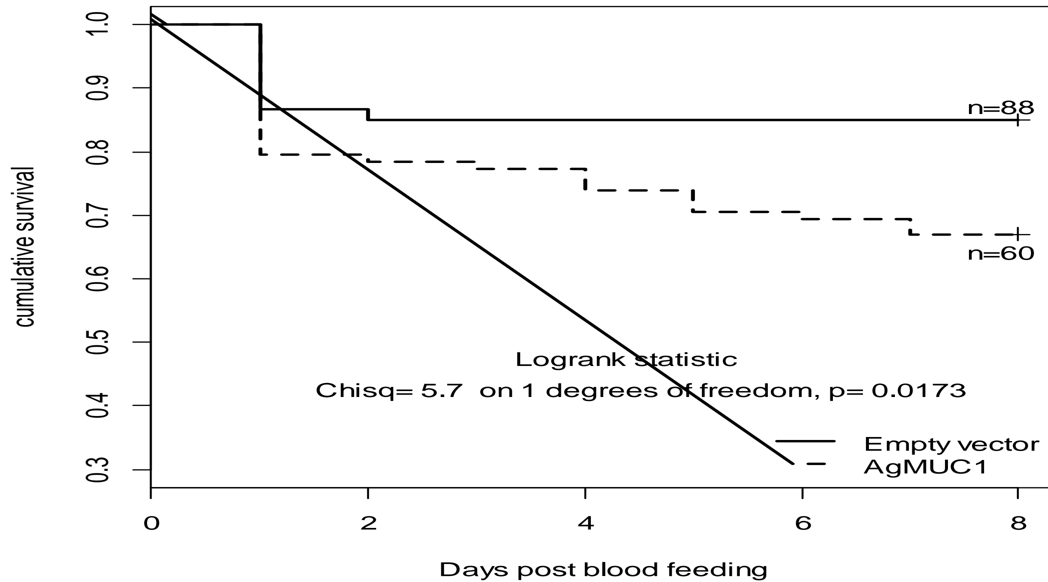

Figure 4.4: Comparison of the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on BALB/c mice immunized four times with 200 µg AgMUC1 cDNA or Empty vector cDNA per immunization, plotted from life tables.

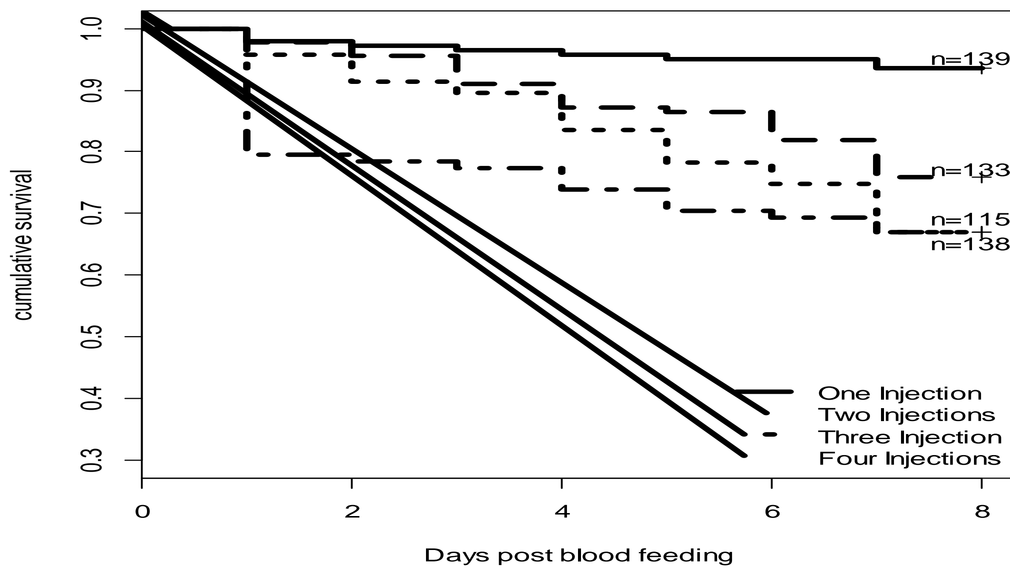

Figure 4.5: Comparison of the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on BALB/c mice immunized once, twice, thrice and four times with 200 µg AgMUC1 cDNA.

4.2 Enhancement of immune responses to AgMUC1 cDNA immunization of BALB/c mice using immunostimulatory cytokines: GM-CSF and IL-12 cDNA

4.2.1 Survival analyses

The daily cumulative survival of mosquitoes surviving to day 7 post blood feeding was analyzed by constructing life table survival curves that were compared by the Kaplan Meier log rank analysis in order to obtain the difference and significance of the difference in the survival of mosquitoes fed on different mice groups. No significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between groups of mosquitoes fed on individual mice within their immunization groups (Table 4.1). The individual data from the mice in each group was pooled for subsequent group analyses.

Table 4.1 Chi-sq statistical analysis of the significance of the differences in the cumulative survival of groups of mosquitoes fed on individual mice within the different AgMUC1 cDNA immunized mice groups

Immunization group	Chi-sq statistic	p-value	Significance
AgMUC1 cDNA immunized group	1.5 on 4 degrees of freedom	0.834	Non-significant
Empty Vector immunized group	3.3 on 4 degrees of freedom	0.511	Non-significant
AgMUC1/GM-CSF cDNA immunized group	3.2 on 4 degrees of freedom	0.531	Non-significant
Empty Vector/GM-CSF cDNA immunized group	7.1 on 4 degrees of freedom	0.13	Non-significant
AgMUC1/IL-12cDNA immunized group	6.1 on 3 degrees of freedom	0.108	Non-significant
Empty vector/IL-12 cDNA immunizedgroup	4.7 on 4 degrees of freedom	0.322	Non-significant
AgMUC1cDNA immunized/Mucin protein boosted group	2.7 on 4 degrees of freedom	0.102	Non-significant
AgMUC1cDNA immunized/Ovalbuminprotein boosted group	0.2 on 1 degrees of freedom	0.62	Non-significant
AgMUC1/GM-CSF/IL-12 cDNA immunized group	4 on 3 degrees of freedom	0.262	Non-significant
Empty vector/GM-CSF/IL-12 cDNA immunized group	3.7 on 4 degrees of freedom	0.454	Non-significant

In the survival analysis of the groups of mosquitoes fed on the different groups of AgMUC1 cDNA immunized mice, significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between groups of mosquitoes fed on the experimental AgMUC1 cDNA immunized and control empty vector cDNA immunized mice groups (p=6.72e-08*;* Figure 4.6), the experimental AgMUC1 + IL-12 cDNA immunized and control empty vector +IL-12 cDNA immunized mice groups (p=4.06e-10*;* Figure 4.8) and the experimental AgMUC1 + IL-12 + GM-CSF cDNA immunized and control empty vector +IL-12 + GM-CSF cDNA immunized mice groups (p=6.48e-09*;* Figure 4.10).

No significant differences were observed in the cumulative survival of the groups of mosquitoes fed on the experimental AgMUC1 + GM-CSF cDNA immunized and the control empty vector + GM-CSF cDNA immunized mice groups (p=0.068*;* Figure 4.7). In the groups of mosquitoes fed on the experimental AgMUC1 cDNA immunized + recombinant mucin protein boost and control AgMUC1 cDNA immunized + ovalbumin protein boost mice group, no significant differences were observed in their cumulative survival. (p=0.314*;* Figure 4.9).

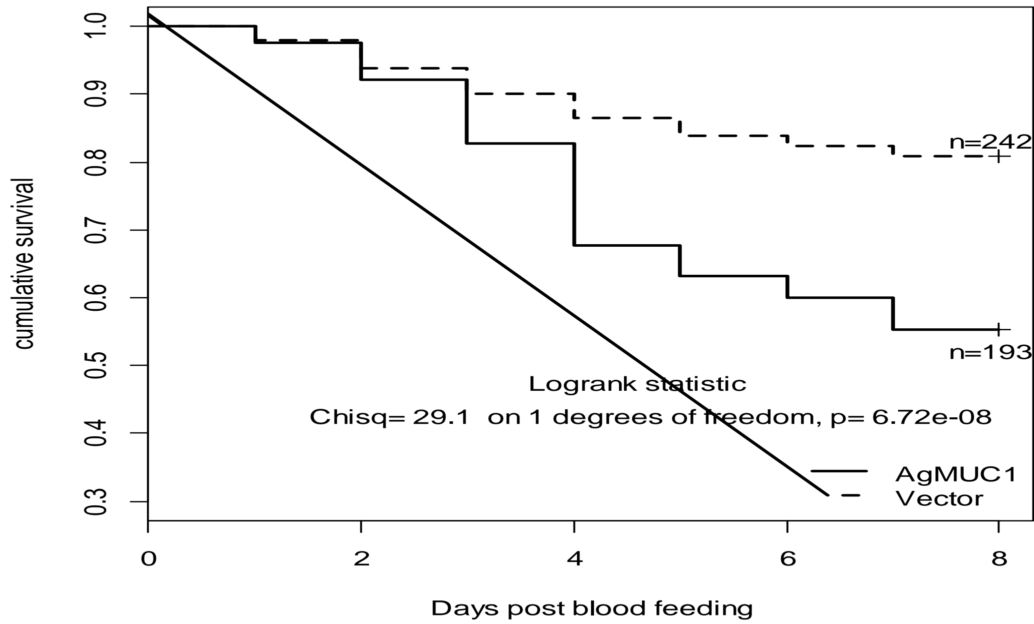

Figure 4.6: Comparison of the survival of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 cDNA with those fed on mice immunized with Empty vector cDNA.

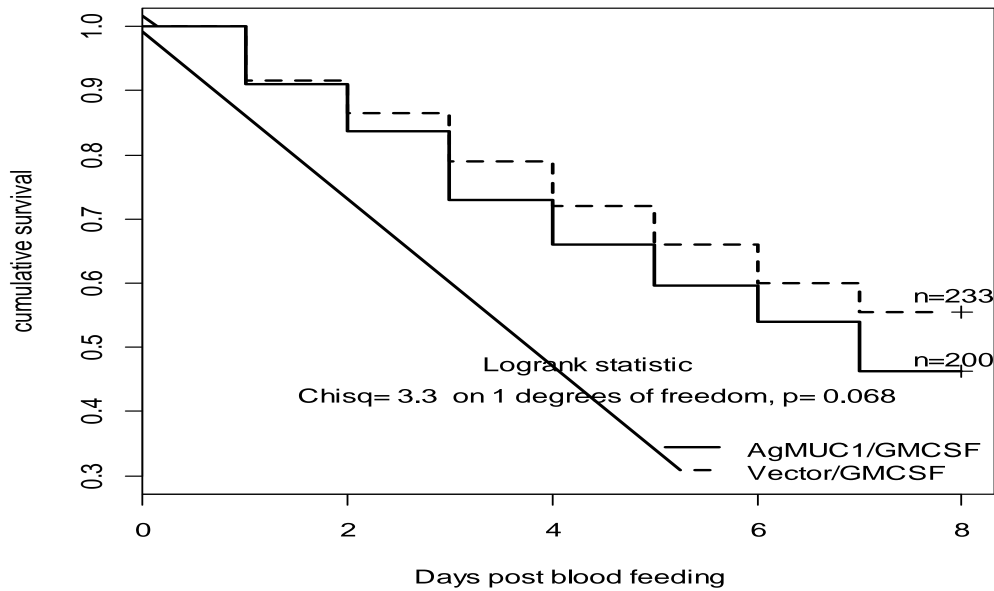

Figure 4.7: Comparison of the survival of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 + GM-CSF cDNA with those fed on mice immunized with Empty vector + GM-CSF cDNA.

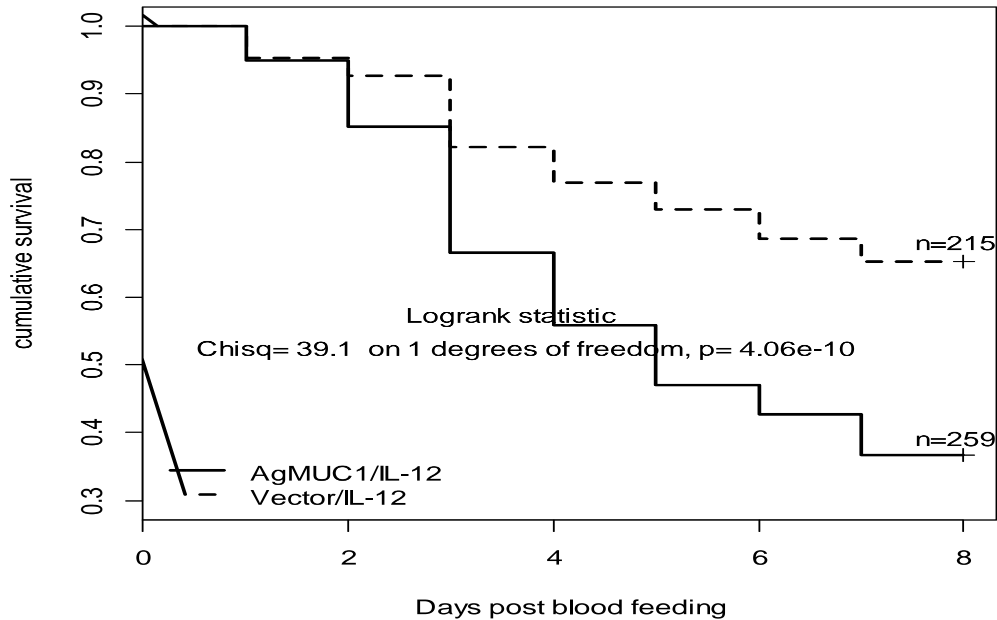

Figure 4.8: Comparison of the survival of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 + IL-12 cDNA with those fed on mice immunized with Empty vector + IL-12 cDNA.

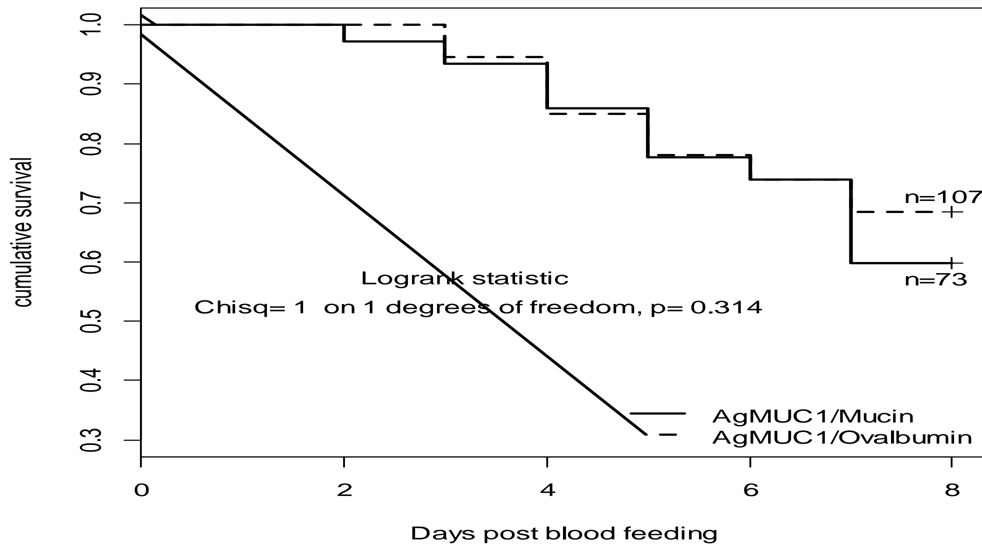

Figure 4.9: Comparison of the survival of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 cDNA and a final boost of recombinant mucin protein with those fed on mice immunized with AgMUC1 cDNA and a final boost of OvalAlbumin.

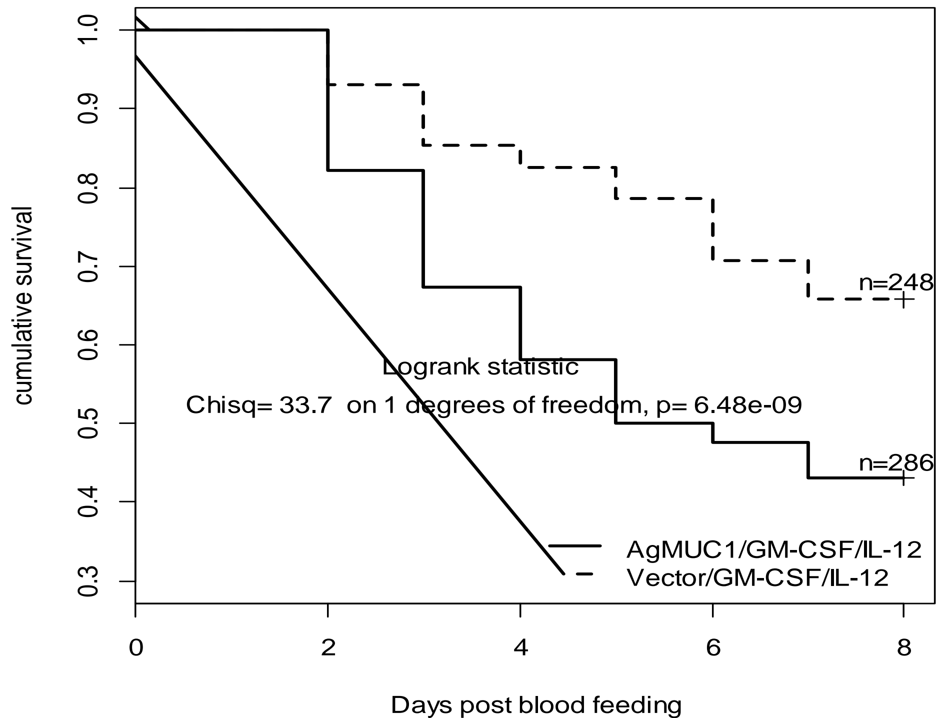

Figure 4.10: Comparison of the survival of *An. gambiae* mosquitoes fed on mice immunized with a mixture of AgMUC1 + GM-CSF + IL-12 cDNAs with those fed on mice immunized with Empty vector + GM-CSF + IL-12 cDNAs.

Analysis of survival by ANOVA on the daily percentage of mosquito survival showed that as early as day 2 post blood feeding there were significant differences in the survival of mosquitoes fed on the experimental and control mice in the AgMUC1/IL-12 cDNA immunized group (F=12.9; *p=0.009*) and the AgMUC1/IL-12/GM-CSF cDNA immunized group (F=*13.1; p=0.024;* Table 4.2).

Table 4.2 One-Way ANOVA of the daily cumulative proportions of the mosquitoes surviving to day 7 after blood feeding on the experimental and control mice groups (F; *p values with one degree of freedom)*

Group	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
AgMUC1	0(0.915)	0.1(0.738)	0.7(0.418)	9.5(0.015)*	9.7(0.014)	10.9(0.011)	10.8(0.011)
AgMUC1/GM-CSF	0.1(0.801)	0.2(0.642)	2.7(0.140)	1.3(0.286)	5.6(0.046)*	6.3(0.036)	3.7(0.104)
AgMUC1/IL-12	0(0.962)	12.9(0.009)*	12.3(0.01)	13.3(0.008)	26.2(0.001)	23.4(0.002)	27.2(0.001)
AgMUC1/Mucin	0.8(0.411)	4.1(0.077)	9.3(0.016)*	19.9(0.002)	15(0.005)	13.1(0.007)	16.2(0.004)
AgMUC1/GM-CSF/IL-12	ND	7.7(0.024)*	13.1(0.007)	10.7(0.011)	9.3(0.016)	6(0.04)	4.6(0.064)

* First day on which the differences in the daily cumulative proportion of mosquitoes surviving were significantly different between the mosquitoes fed on the experimental and control mice groups. ND – Not determinable

4.3 Characterization of the type of mosquito killing immune response elicited by AgMUC1 cDNA immunization

4.3.1 Cytokine production profiles in mice immunized once, twice, thrice and four times with AgMUC1 cDNA

In vitro IFN- γ and IL-2 production from the immunized mice spleen cells suspension stimulated by recombinant mucin protein were used as markers of lymphocyte proliferation. No specific pattern of cytokine production was observed from the *in vitro* stimulation assay that could be associated with either a particular pattern of antibody production or the daily cumulative survival of the mosquitoes surviving to day 7 after feeding on either the experimental or control groups of immunized mice. IFN- γ levels were very low on the second and third AgMUC1 cDNA injections compared to the control group. IL-12 levels were also low but only on the second AgMUC1 cDNA injection. It is important to note that there was a switch to IgG1 antibody production at the time IFN- γ and IL-12 levels appeared low.

Table 4.3 Comparison of mean cytokine levels (pg/ml) in spleen cells stimulated in vitro with recombinant mucin protein from the experimental AgMUC1 cDNA immunized mice groups and the control empty vector cDNA immunized mice groups

Group	**MEAN IFN- γ (pg/ml)**		**MEAN IL-2 (pg/ml)**	
	Mucin	***Vector***	***Mucin***	***Vector***
1 injection	1004	1233	13	12
2 injections	51	859	9	29
3 injections	517	1636	17	11
4 injections	1296	1284	19	20

4.3.2 Cytokine production profiles in mice co-immunized with immunostimulatory cytokines GM-CSF and IL-12

Spleen cells from AgMUC1 cDNA immunized mice on *in vitro* stimulation with the recombinant mucin protein produced higher levels of IL-2, IFN- γ, IL-5 and TNF-α. Levels of IL-4 were undetectable (Table 4.4). In the AgMUC1/GMCSF cDNA immunized mice there were no differences in the levels of IL-2 and IL-4 between the

experimental and control groups. However slightly lower levels of IFN- γ, IL-5 and TNF-α were detected in the experimental group (Table 4.4).

Spleen cells from AgMUC1/IL-12 cDNA immunized mice on *in vitro* stimulation with the recombinant mucin protein produced slightly higher levels of IFN- γ, IL-4 and TNF-α and lower levels of IL-2 and IL-5 in the experimental group (Table 4.4). In the AgMUC1 cDNA immunized / Mucin protein boosted mice, generally lower levels of IL-2, IFN- γ, IL-5, IL-4 and TNF-a respectively were detected compared to AgMUC1 cDNA immunized / Ovalbumin protein boosted mice (Table 4.4).

Table 4.4 Cytokine profiles and their Mean concentrations from recombinant mucin protein stimulated spleen cells from the immunized mice

IMMUNISATION GROUP	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-5 (pg/ml)	IL-4 (pg/ml)	TNF-α (pg/ml)
AgMuc1	33±15	4208±1533	4.0±3.8	0	689±199
Empty vector	20±13	1428±2030	1.4±2.2	2.0±2.0	443±143
AgMuc1+GM-CSF	35±13	2020±947	4.0±2.0	0.9±1.4	469±113
Empty vector + GM-CSF	35±10	3680±1280	6.0±2.0	0.9±1.2	568±107
AgMuc1+IL-12	31±17	4633±604	1.5±3.0	1.1±1.3	470±184
Empty vector + IL-12	41±14	4153±604	2.7±2.8	0.5±1.2	320±75
AgMuc1 + Mucin protein	24±16	2652±1133	0	0.5±1.1	356±50
AgMuc1 + Ovalbumin	33±20	3474±1734	5.7±6.0	0.9±0.8	366±134

4.3.3 Anti-mucin antibody isotype production profiles in mice immunized with one, two, three and four injections of 200 µg AgMUC1 cDNA

The group of mice that received only one injection of AgMUC1 cDNA and the group that received two injections had no difference in their mean total IgG anti-mucin antibody titers (Table 4.5). Similarly, in the group of mice that received three and four injections respectively the mean total IgG anti-mucin antibody titers were same; a three-fold increase in the total IgG anti-mucin antibody levels (Table 4.5). No anti-mucin antibodies were detected in the control groups of mice immunized with empty vector cDNA.

The IgG anti-mucin antibody isotype detected after one and two AgMUC1 cDNA injections was predominantly IgG2a anti-mucin antibody (Table 4.5). After three and four AgMUC1 cDNA injections both IgG1 and IgG2a anti-mucin antibodies predominated (Table 4.5).

Table 4.5 Anti-mucin IgG antibody subtypes titres as determined from groups of mice receiving 200µg AgMUC1 cDNA once, twice, thrice or four times at intervals of 14 days

Immunization Group	Total IgG Antibody Titre	IgG1 Antibody Titre	IgG2a Antibody Titre
One Injection	1000	0	1000
Two Injections	1000	200	1000
Three Injections	3000	6000	3000
Four Injections	3000	6000	3000

4.3.4 Anti-mucin antibody isotype production profiles in mice co-immunized with AgMUC1 cDNA, GM-CSF and IL-12

The co-immunization of AgMUC1 cDNA with either IL-12 or GM-CSF increased the mean total IgG anti-mucin antibody titers two fold from 1:3,000 in the AgMUC1 cDNA immunized group to 1:6,000 in both the AgMUC1/GM-CSF and AgMUC1/IL-12 cDNA immunized groups. A final recombinant mucin protein boost of AgMUC1 cDNA immunized mice increased the mean total IgG anti-mucin antibody titers to over

1:100,000. With a final ovalbumin protein boost of the AgMUC1 cDNA immunized mice, the mean anti-mucin antibody titers remained at the 1:3000 levels (Table 4.6).

The daily cumulative survival of the blood fed mosquitoes surviving to day 7 post-blood feeding correlated negatively with increased total IgG anti-mucin antibody titers in the AgMUC1/GM-CSF cDNA immunized group (*r =-0.759*), the AgMUC1/IL-12 cDNA immunized group (*r =-0.782*) and the AgMUC1/IL-12/GM-CSF cDNA immunized group (*r =-0.832*) . No correlation was observed in the AgMUC1 cDNA immunized group (*r=0.350)* and the AgMUC1/Mucin protein boosted group (*r=0.387*). Significant mosquito killing was observed where modest anti-mucin antibody titres were detected as in the AgMUC1 cDNA immunized mice group compared to where very high total IgG anti-mucin antibodies were detected as in the AgMUC1 cDNA immunized/recombinant mucin protein boosted group. In the AgMUC1 cDNA immunized / Ovalbumin protein boosted group there was a positive correlation between total IgG anti-mucin antibody titers and the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding (*r=0.617)* (Table 4.6).

Of the IgG anti-mucin antibody subtypes IgG1, IgG2a and IgG3, only IgG1 anti-mucin antibody subtype showed a negative correlation with the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding in all the immunization groups (Table 4.6). This could imply that it is the IgG1 anti-mucin antibody subtype that is responsible for mosquito killing in the AgMUC1 cDNA immunized mice (Table 4.6). No correlation was observed between IgG2a and IgG3 anti-mucin antibody subtypes with

the daily cumulative proportions of the blood fed mosquitoes surviving to day 7 in the immunized groups implying that these antibody subtypes may not be associated with mosquito killing (Table 4.6).

Table 4.6 Correlations between the Mean titers of IgG anti-mucin antibody subtypes and the daily cumulative proportions (%) of the blood fed *Anopheles gambiae* mosquitoes surviving to day 7 post-blood feeding

GROUP	**Total IgG Titre (*r*)**	**IgG1 Titre (*r*)**	**IgG2a Titre (*r*)**	**IgG3 Titre (*r*)**
AgMUC1	3,000 (0.350)	3,000 (-0.582)	640 (0.444)	0 (***)
AgMUC1/GM-CSF	6,000 (-0.759)	4000 (-0.914)	0 (***)	0 (***)
AgMUC1/IL-12	6,000 (-0.782)	3000 (-0.606)	0 (***)	0 (***)
AgMUC1/Mucin Protein	102,400 (0.387)	66600 (-0.671)	82000(0.384)	1840 (0.144)
AgMUC1/Ovalbumin	3,000 (0.617)	3000 (-0.758)	0 (***)	0 (***)
AgMUC1/IL-12/GM-CSF	6,000 (-0.832)	4000 (-0.706)	500 (0.498)	0 (***)

*** Not determinable because the antibody subtype was undetectable

NB: Correlations > 0.5 are considered significant while correlations <0.5 are considered non-significant.

4.3.5 Correlations between Antibody titres and Cytokine production profiles in the mice immunized with AgMUC1 mixed with GM-CSF or IL-12

In the group of mice that were immunized with only AgMUC1 cDNA, there was no correlation between total IgG antibody production and the levels of all the cytokines analysed (Table 4.7).

In the group of mice that were immunized with AgMUC1/GM-CSF, there was a strong positive correlation (*r=0.902*) between IL-4 levels and the total IgG anti-mucin antibody titer. No significant correlations were observed between antibody production and all the other cytokines determined in this group of immunized mice (Table 4.7).

In the group of mice that were immunized with AgMUC1/IL-12 cDNA, strong positive correlations were observed between the total anti-mucin IgG antibody production and the levels of IL-5 (*r=0.995*), IL-4 (*r=0.697*) and IL-2 (*r=0.965*) (Table 4.7).

In the group of mice that were immunized with AgMUC1 cDNA and boosted with recombinant mucin protein, the total anti-mucin IgG antibody titers correlated positively with TNF-α (*r=0.731*), IFN-γ (*r=0.712*) and IL-5 (*r=0.965*) (Table 4.7).

In the group of mice that were immunized with AgMUC1 cDNA and boosted with ovalbumin protein, there were strong positive correlations between anti-mucin IgG antibody titers and IFN-γ (*r=0.713*), IL-4 (*r=0.874*) and IL-2 (*r=0.969*) (Table 4.7).

Table 4.7 Correlations between anti-mucin IgG antibody titers and cytokine profiles

IMMUNISATION GROUP	Total IgG anti-mucin antibody titer	TNF-α (*r*)	IFN-γ (*r*)	IL-5 (*r*)	IL-4 (*r*)	IL-2 (*r*)
AgMUC1	1:3000	0.557	0.231	-0.530	***	0.150
AgMUC1/GM-CSF	1:6000	-0.106	-0.439	-0.329	0.902	-0.383
AgMUC1/IL-12	1:6000	-0.076	0.405	0.995	0.697	0.965
AgMUC1/Mucin Protein	>1:100,000	0.731	0.712	0.965	-0.688	0.442
AgMUC1/Ovalbumin	1:3000	0.036	0.713	***	0.874	0.969

*** - Not determinable as the cytokines were undetectable

4.3.6 Correlations between Anti-mucin IgG antibody subtype profiles and Mosquito survival

Of the IgG anti-mucin antibody sybtypes, only the IgG1 anti-mucin antibody subtype demonstrated negative correlation with mosquito survival in the AgMUC1 (*r*=-0.582), AgMUC1/GMCSF (*r*=-0.914), AgMUC1/IL-12 (*r*=-0.0.606) and AgMUC1/Mucin protein (*r*=-0.671) immunized groups (Table 4.8)

Table 4.8 Correlations between anti-mucin IgG antibody subtypes and Mosquito survival

Immunization Group	Total IgG		IgG1		IG2a		IgG3	
	Mean	***r***	***Mean***	***r***	***Mean***	***r***	***Mean***	***r***
AgMuc1	3000	0.350	2400	-0.582	640	0.444	0	0
AgMuc1/GM-CSF	6000	-0.759	4200	-0.606	0	***	0	0
AgMuc1/IL-12	6000	-0.782	3500	-0.776	0	***	0	0
AgMuc1/Mucin protein	>100000	0.387	60000	-0.671	82000	0.384	1840	0.144
AgMuc1/Ovalbumin	3000	0.317	2400	0.358	0	***	0	0

4.3.7 Correlations between IgG anti-mucin antibody subtypes and the cytokine profiles in the recombinant mucin protein stimulated spleen cells from the group of mice that were immunized with AgMUC1 cDNA and boosted with recombinant mucin protein

In the group of mice that were immunized with AgMUC1 cDNA and boosted with recombinant mucin protein high levels of IgG anti-mucin antibody subtypes were detected. Only the IgG2a anti-mucin antibody subtype demonstrated a positive correlation with TNF-α (*r=0.684*), IFN-γ (*r=0.653*) and IL-5 (*r=0.954*) (Table 4.9). Negative correlations were observed between IgG1 anti-mucin antibodies and TNF-α (*r=-0.565*) and IFN-γ (*r=-0.501*). A negative correlation was also observed between IgG3 anti-mucin antibodies and IL-4 (*r=-0.*575).

Table 4.9 Correlations between IgG anti-mucin antibody subtypes and the cytokine profiles in the recombinant mucin protein stimulated spleen cells from the group of mice that were immunized with AgMuc1 cDNA and boosted with recombinant mucin protein

Antibody Subtype	TNF-α (r)	IFN-γ(r)	IL-5(r)	IL-4(r)	IL-2(r)
IgG1 (1:66600)	-0.565	-0.501	-0.378	-0.292	-0.360
IgG2a (1:82000)	0.684	0.653	0.954	-0.347	0.345
IgG3 (1:1840)	0.159	0.279	-0.095	-0.575	0.480

4.3.8 Determination of anti-mucin antibody subtypes killing effect in the presence and / or absence of complement

Generally no significant differences were observed between the daily cumulative survival of the blood fed mosquitoes surviving to day 7 after feeding on fresh mouse blood containing IgG anti-mucin antibody subtypes IgG1, IgG1 + IgG2a and IgG1 + IgG2a + IgG3 in the presence or absence of complement; $p>0.05$ in all groups (Figures 4.11, 4.12 and 4.13). This could be due to the fact that immunized mice sera fed to *An. gambiae* in an *in vitro* membrane feeding system is not as effective in mosquito killing as the mosquitoes directly feeding on the immunized mice. This shows that the presence/absence of complement in the blood meal has no effect on mosquito survival.

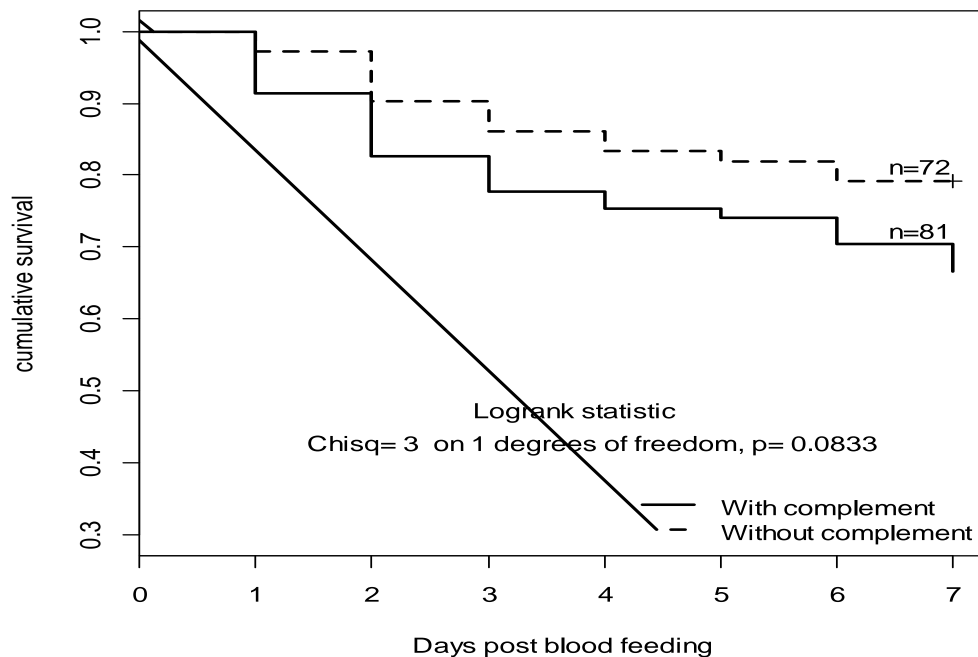

Figure 4.11: Comparison of the daily cumulative survival of the blood fed *An. gambiae* mosquitoes surviving to day 7 after feeding on pooled mice sera containing anti-mucin IgG1 antibody in the presence and absence of complement.

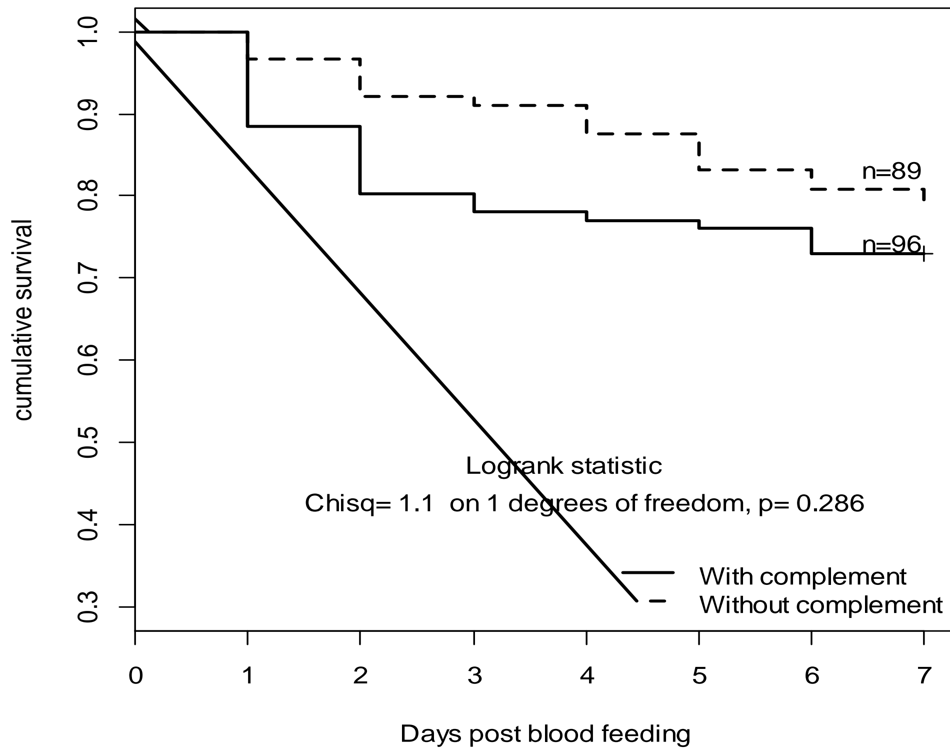

Figure 4.12: Comparison of the daily cumulative survival of the blood fed *An. gambiae* mosquitoes surviving to day 7 after feeding on pooled mice sera containing anti-mucin IgG1 and IgG2a antibody in the presence and absence of complement.

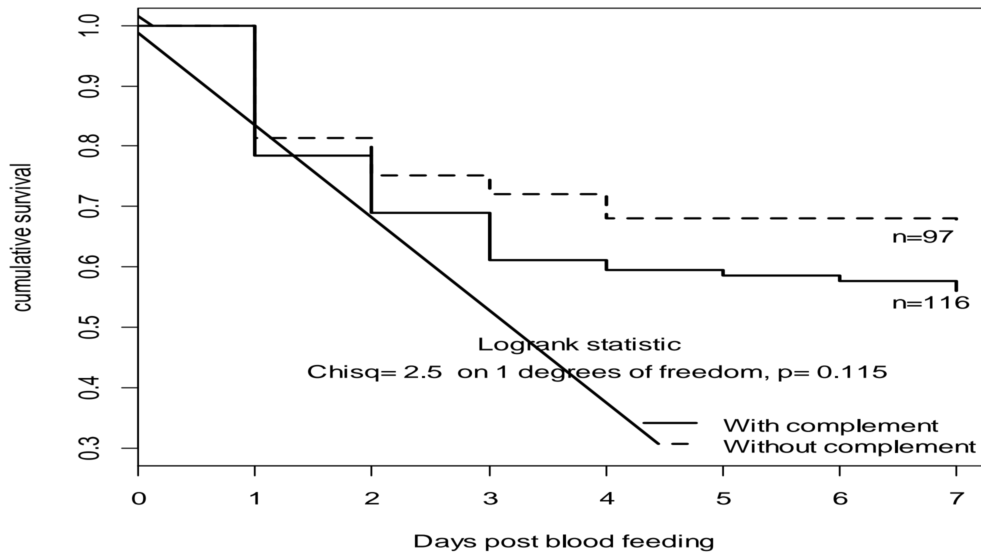

Figure 4.13: Comparison of the daily cumulative survival of the blood fed *An. gambiae* mosquitoes surviving to day 7 after feeding on pooled mice sera containing anti-mucin IgG1, IgG2a and IgG3 antibody in the presence and absence of complement.

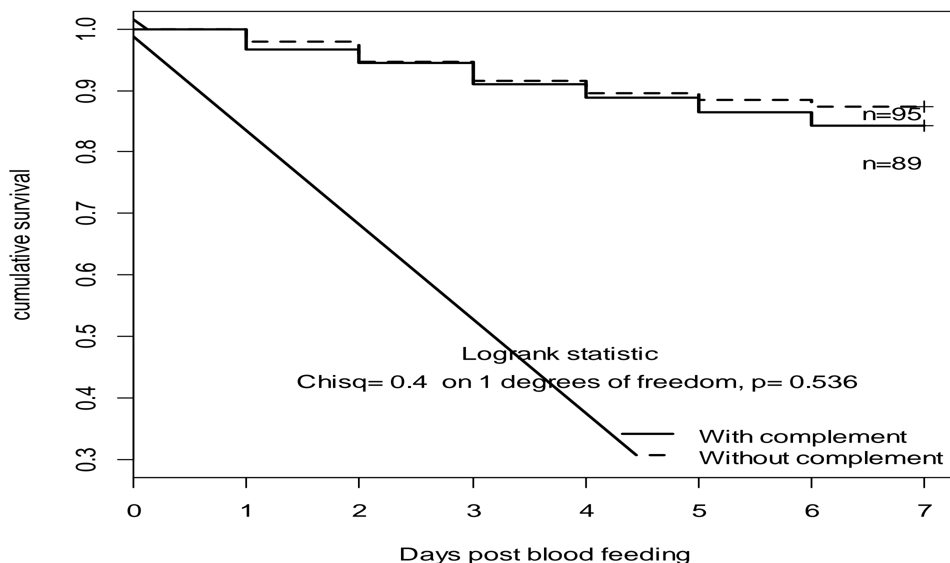

Figure 4.14: Comparison of the daily cumulative survival of the blood fed *An. gambiae* mosquitoes surviving to day 7 after feeding on pooled empty vector immunized mice sera in the presence and absence of complement.

4.3.9 Duration of the mosquitocidal immune response in group of mice that were immunized with AgMUC1/IL-12 cDNA

The mosquitocidal immune response in the AgMUC1/IL-12 cDNA immunized mice was determined at 5.5 weeks, 15 weeks and 36 weeks after the last immunization. The results show the mosquitocidal immune response declines with time. At 5.5 weeks after the last injection in the immunization schedule, highly significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding between the mosquitoes fed on mice immunized with AgMUC1/IL-12 cDNA and those fed on mice immunized with empty vector/IL-12 cDNA (p= 0.00491*;* Figure 4.15). Similarly significant differences were also observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding between the mosquitoes fed on mice immunized with AgMUC1/IL-12 cDNA and those fed on mice immunized with empty vector/IL-12 cDNA at 15 and 36 weeks after the last injection (p= 0.000659*;* Figure 4.16 and p= 0.00101*;* Figure 4.17 respectively). Thus although the mosquitocidal effects seems to decline over time as shown on the life table survival curves (Figure 4.18), the effects are significant when the survival of mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice are compared to those fed on the control empty vector/IL-12 cDNA immunized mice during this period.

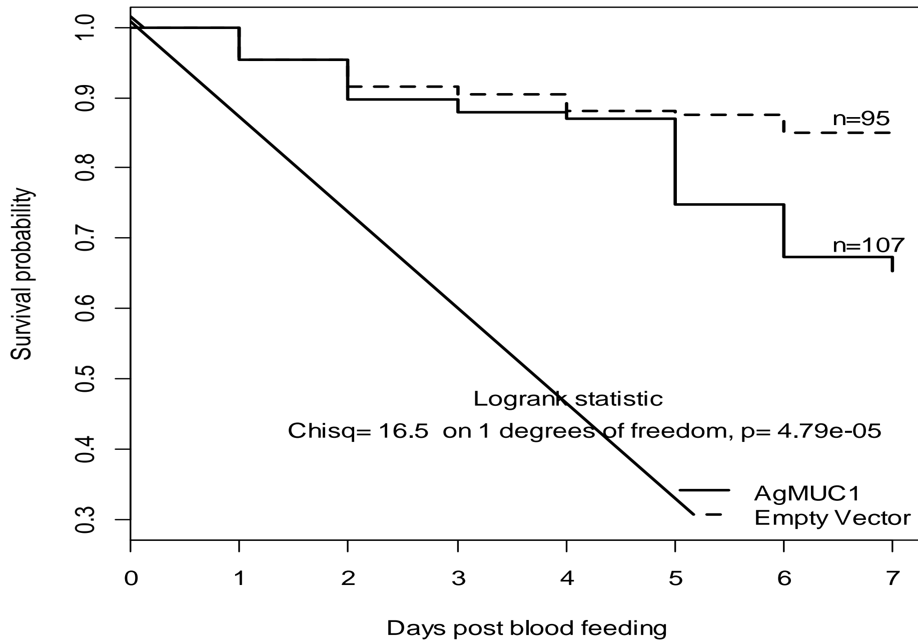

Figure 4.15: Comparison of the daily cumulative survival of the mosquitos' surviving to day 7 after feeding on the group of mice that were immunized with AgMUC1/IL-12 cDNA at 5.5 weeks after the last cDNA injection

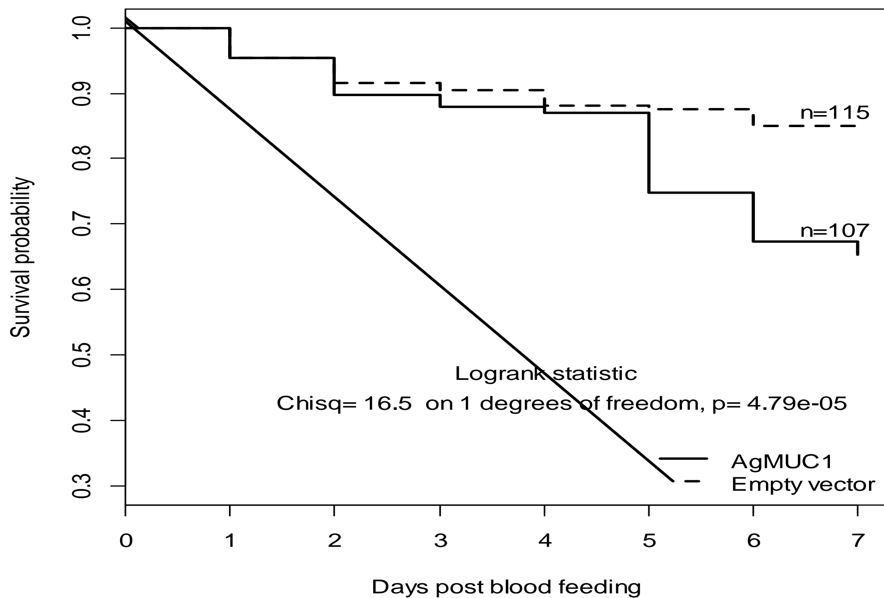

Figure 4.16: Comparison of the daily cumulative survival of the mosquitos' surviving to day 7 after feeding on the group of mice that were immunized with AgMUC1/IL-12 cDNA at 15 weeks after the last cDNA injection.

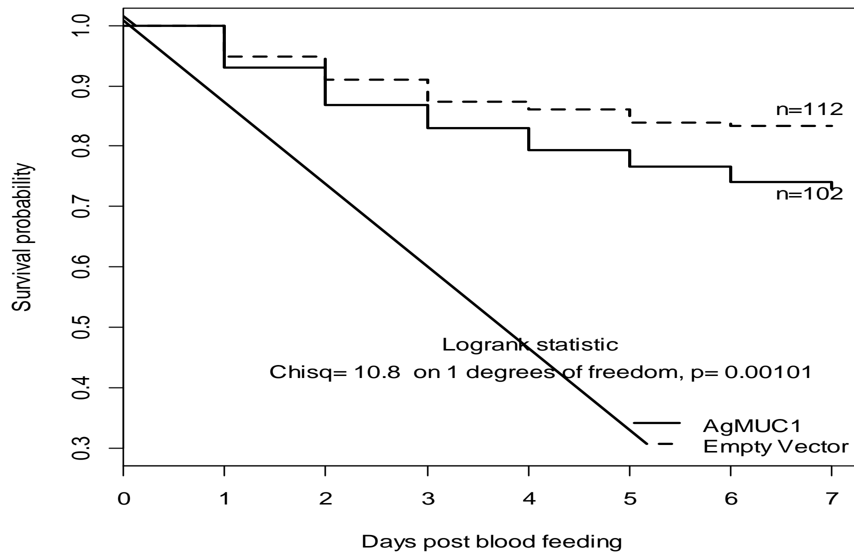

Figure 4.17: Comparison of the daily cumulative survival of the mosquitos' surviving to day 7 after feeding on the group of mice that were immunized with AgMUC1/IL-12 cDNA at 36 weeks after the last cDNA injection.

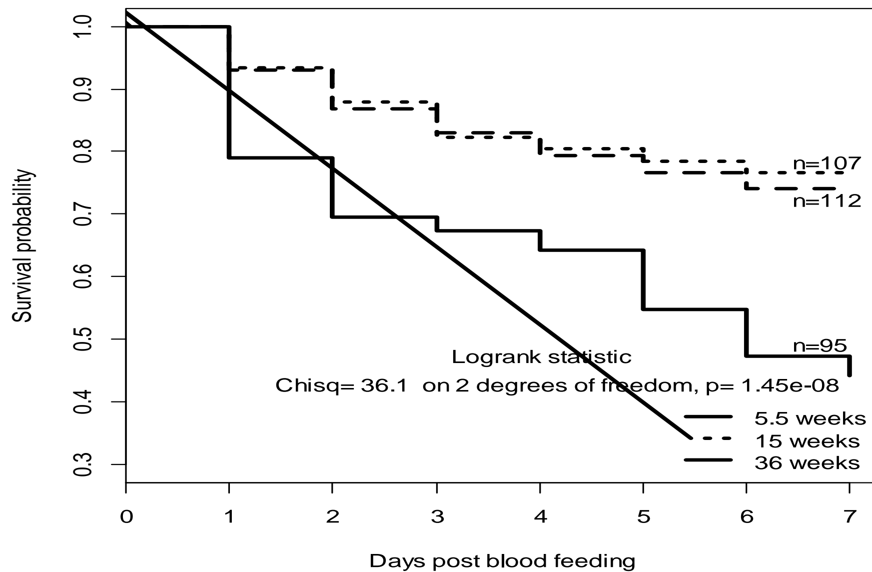

Figure 4.18: Comparison of the daily comulative survival of the mosquitos' surviving to day 7 after feeding on the group of mice that were immunized with AgMUC1/IL-12 cDNA at 5.5, 15 and 36 weeks after the last cDNA injection.

4.4 Determination of immune pathologic features responsible for mosquito mortality after feeding on the group of mice that were immunized with AgMUC1/IL-12 cDNA

4.4.1 Bright light microscopic features

Plates 4.1, 4.2 and 4.3 are giemsa stained mosquito midgut sections of the *An. gambiae* mosquito fed on BALB/c mice immunized with AgMUC1/IL-12 cDNA. A common feature observed in these sections is the adherence of white blood cells on the epithelial surface of the mosquito midgut lining. This feature was not observed in the mosquito midgut sections made from mosquitoes fed on BALB/c mice immunized with empty vector/IL-12 cDNA (Plates 4.4 and 4.5). The other fearures observed included the formation of nodules along the midgut epithelium (Plate 4.6), the appearance of some epithelial cells that appears to be undergoing cell death (Plate 4.7), the appearance of some swollen epithelial cells probably full of midgut contents and which could burst resulting into necrotic features in the midgut lining (Plate 4.8), the appearance of some gaps through the epithelial cell lining (Plates 4.9, 4.10 and 4.11) and the appearance of hemocytes along the basement membrane of the midgut lining (Plates 4.12, 4.13 and 4.14).

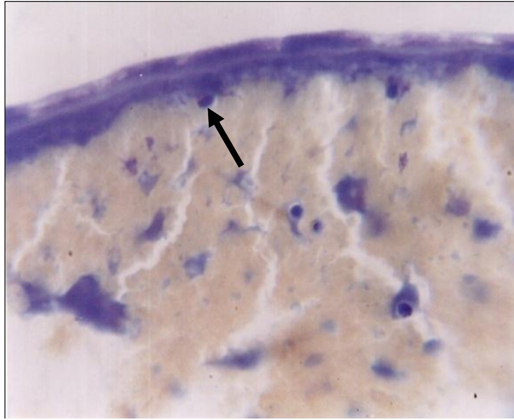

Plate 4.1 Magnification x10

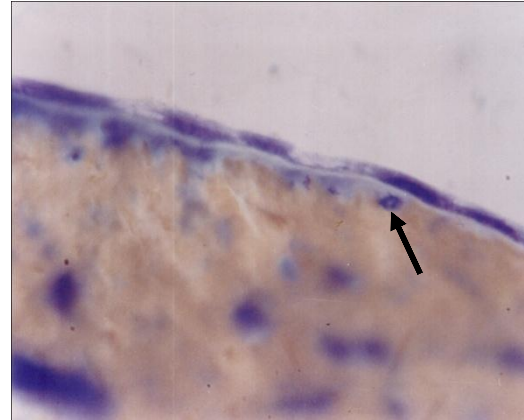

Plate 4.2 Magnification x10

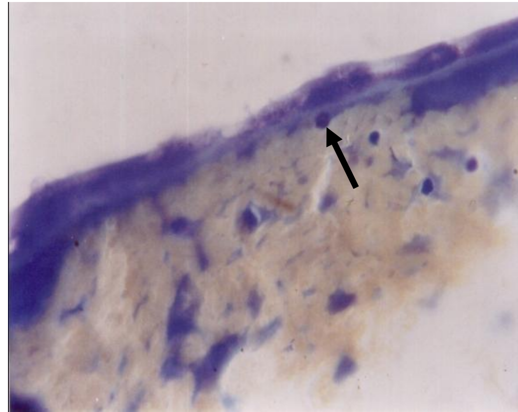

Plate 4.3 Magnification x10

Plates 4.1, 4.2 and 4.3: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on the experimental BALB/c mice immunized with AgMUC1/IL-12 cDNA. The arrows show the adherence of white blood cells along the epithelial lining in mosquitoes fed on the immune mice.

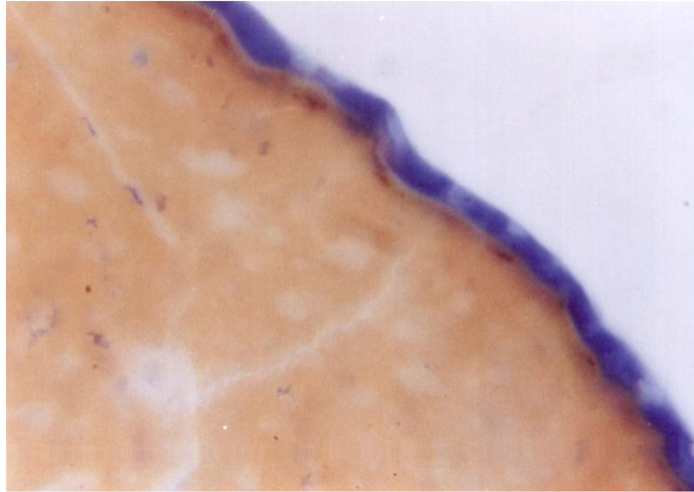

Plate 4.4 Magnification x10

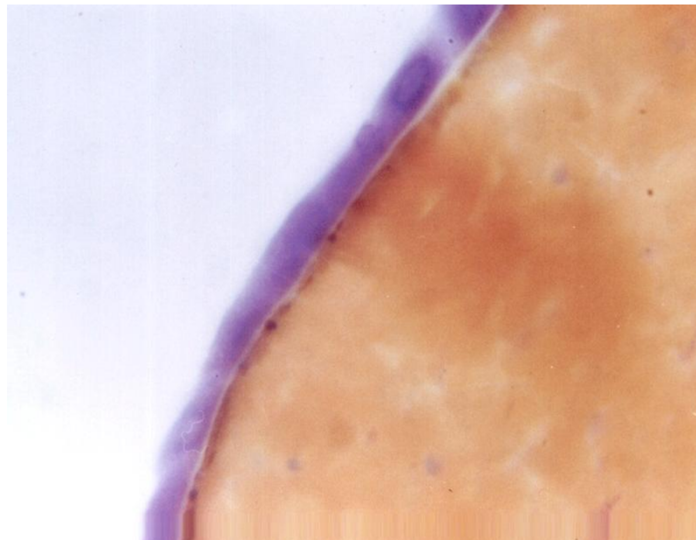

Plate 4.5 Magnification x10

Plates 4.4 and 4.5: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on the control empty vector/IL-12 cDNA immunized mice. No white blood cells were seen adhering onto the epithilial lining as was observed in the sections from mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice.

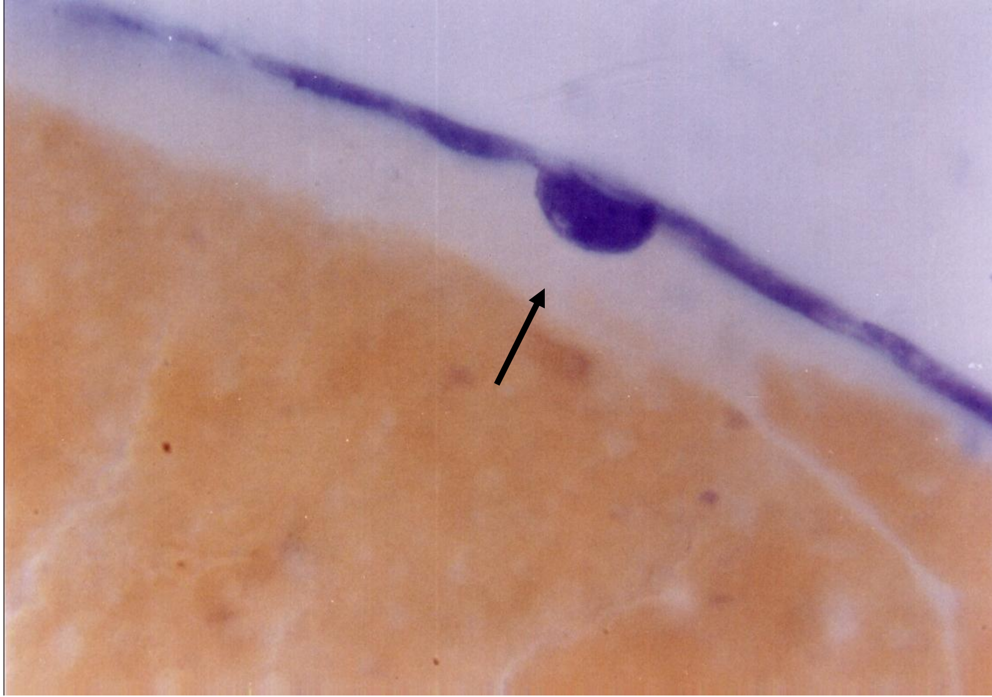

Plate 4.6 Magnification x10: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. Arrow shows a nodule (probably an encapsulated white blood cell).

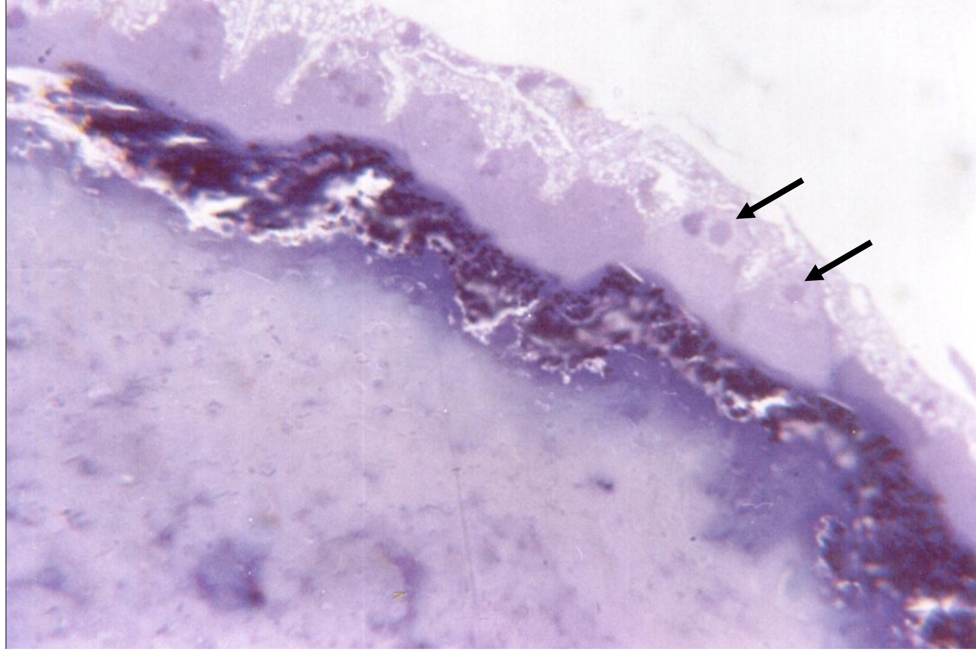

Plate 4.7 Magnification x100

Plate 4.7: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. The arrows show epithelial cells whose shrinking nuclei may be undergoing fragmentation by apoptosis.

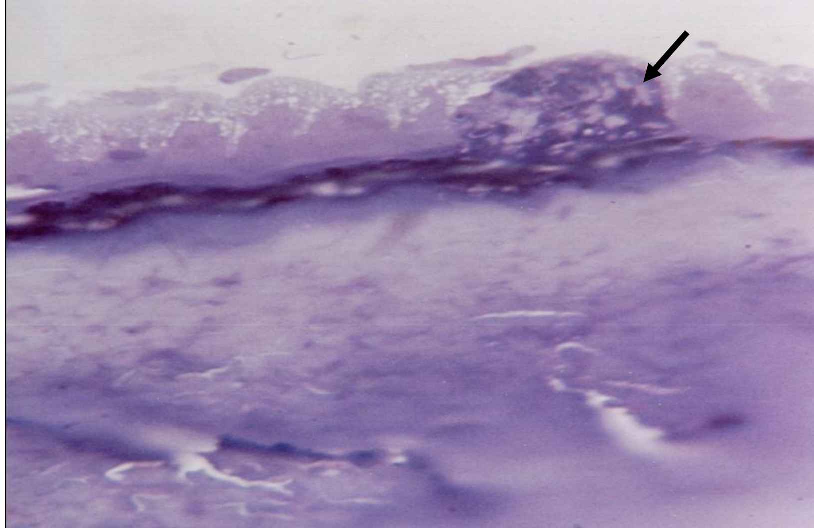

Plate 4.8 Magnification x100

Plate 4.8: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. The arrow shows a swollen epithelial cell where midgut contents could have sipped into the epithelial cell

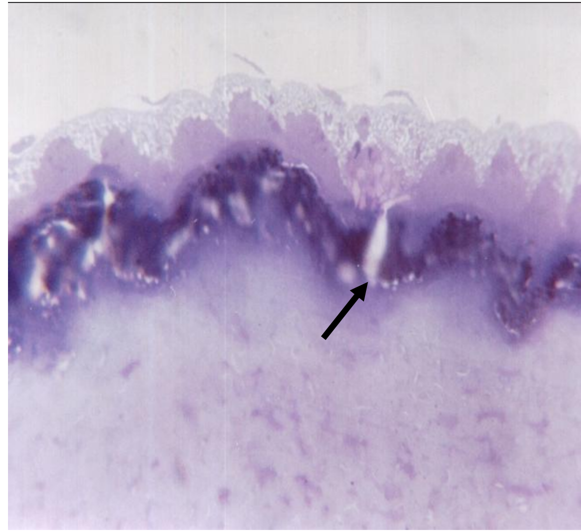

Plate 4.9 Magnification x100

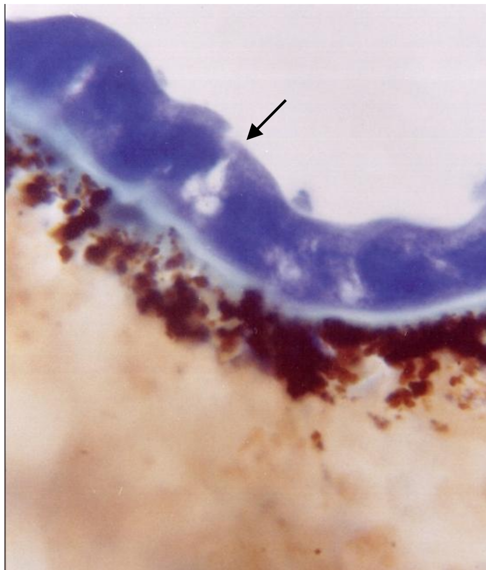

Plate 4.10 Magnification x100

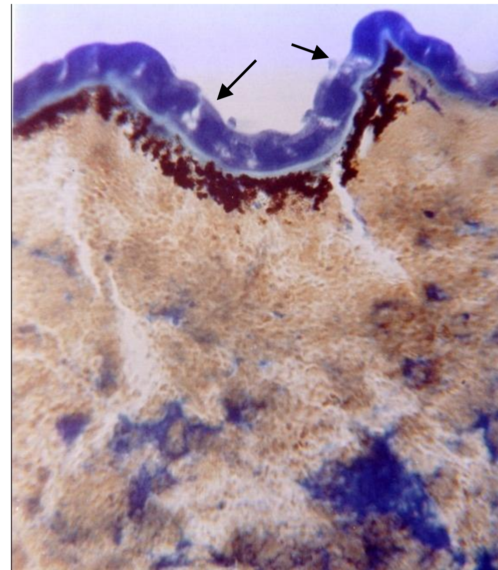

Plate 4.11 Magnification x100

Plates 4.9, 4.10 and 4.11: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. The arrows show gaps in the epithelial cell lining through which the midgut contents could mix with the hemocell and lead to mosquito death.

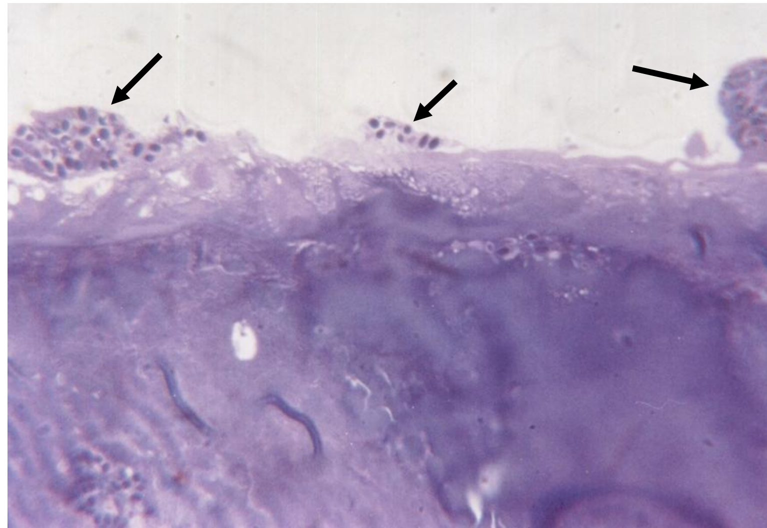

Plate 4.12 Magnification x100

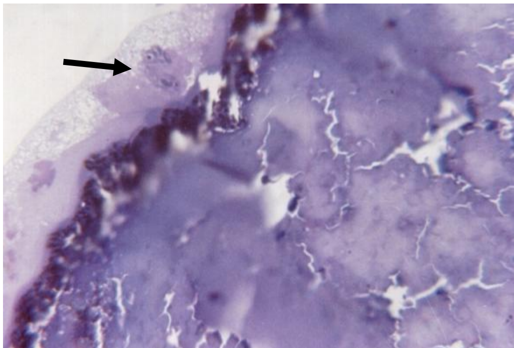

Plate 4.13 Magnification x100

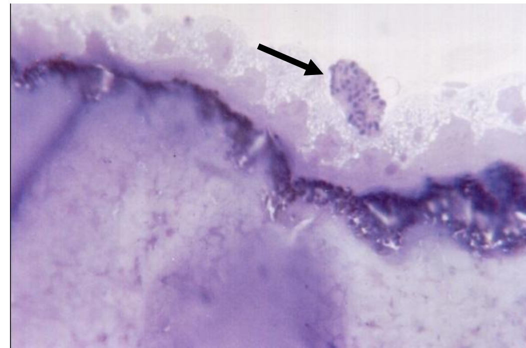

Plate 4.14 Magnification x100

Plates 4.12, 4.13 and 4.14: Giemsa stained cryostat sections prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. Arrows show hightened hemocyte activities along the basement membrane in the midgut sections from mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice.

4.4.2 Scanning Electron Microscopic (SEM) features

SEM features seem to confirm the adherence of white blood cells onto the epithelial lining (Plates 4.15 and 4.16). The adherent cells appear amoeboid in shape indicating that this sort of adherence might be transient.

Encapsulation of the white blood cells as observed under bright light microscopic features (Plate 4.6) was also observed as one of the features under SEM features (Plate 4.17).

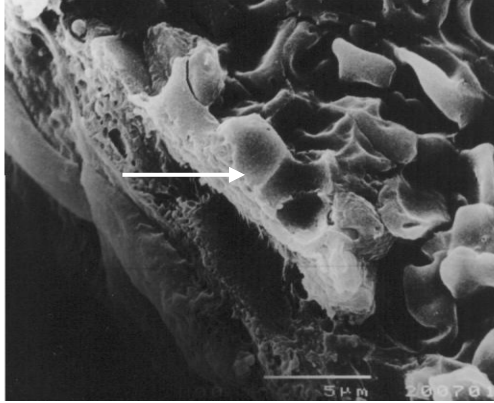

Plate 4.15 Magnification. x 5,000

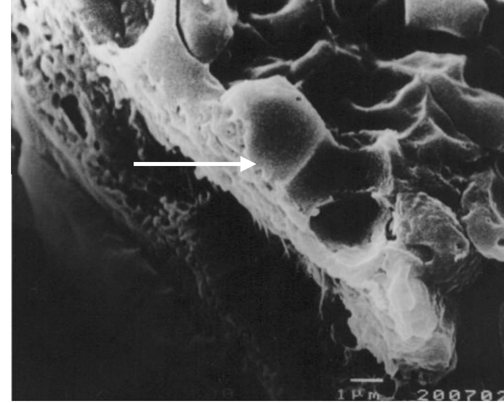

Plate 4.16 Magnifiction. x 7, 500

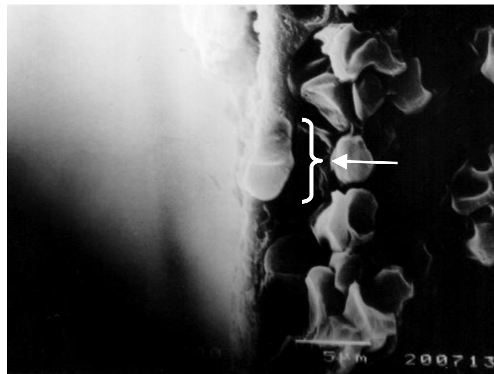

Plate 4.17 Magnification. x3,500

Plates 4.15, 4.16 and 4.17: Scannining Electron Microscopy micrographs prepared from blood fed *An. gambiae* mosquito midgut 8 hours post blood feeding on AgMUCl/IL-12 cDNA immunized mice. The arrows show the attachement of white blood cells onto the epithelial lining of the mosquito midgut. The arrow in Figure 35 show a group of white blood cells buried in the mosquito midgut epithelium.

4.4.3 Transmission Electron Microscopic (TEM) features

In the transverse sections from the midgut of the mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice, epithelial cell nuclei shrinking as a result of probably apoptotic processes were observed (Plate 4.19). The nuclei of the affected cells could be seen to have condensed considerably compared to that seen in the sections from the mosquitoes fed on the group of mice that were immunized with control empty vector/IL-12 cDNA (Plate 4.18). This compares very well with the features observed under bright light microscopy (Plate 4.7). The basement membrane appears thickened and since it is an immunologically active organ, this thickening could be the one causing response from the hemocytes that were observed under bright light microscopy to be activated (Plates 4.12, 4.13 and 4.14). The apoptotic cell was seen to have more prominent mitochondria compared to the normal epithelial cell.

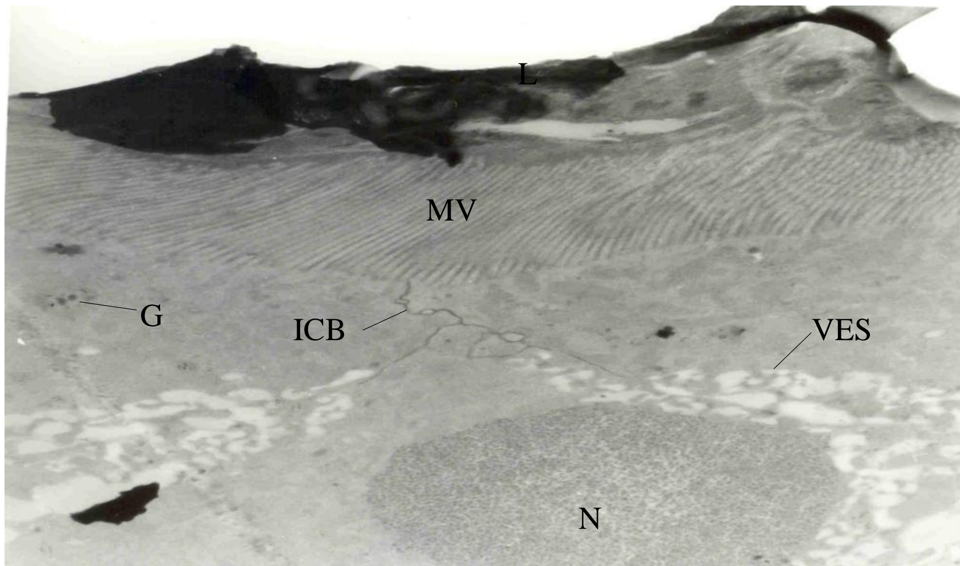

Plate 4.18: Show the general features of fine structure in the midgut epithelial cell. Midgut lumen (L), Microvilli (MV), Intercellular boundary (ICB), Golgi complex (G), Nucleus (N), Vesicles (VES). Mosquito fed on empty vector/IL-12 cDNA immunized mouse

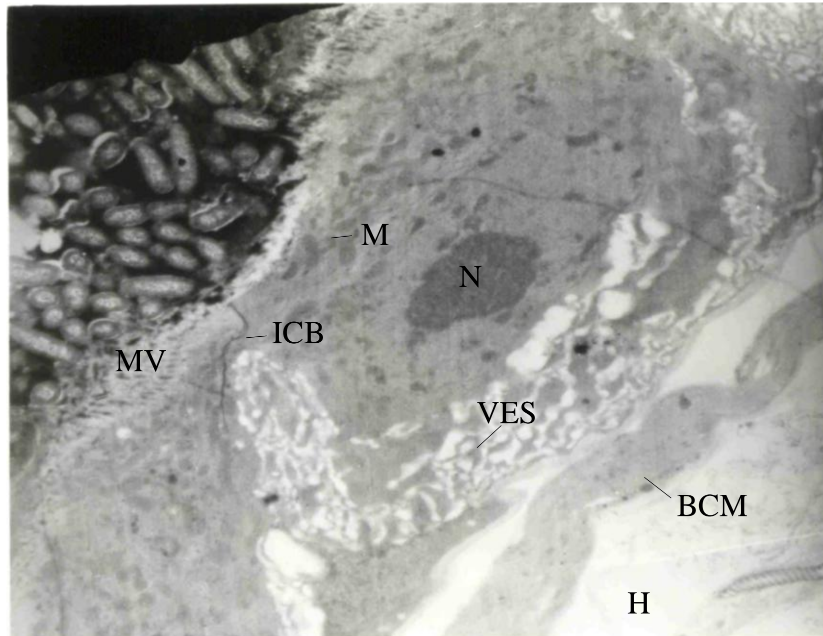

Plate 4.19: Show fine structure in the epithelial cell from the midgut of *An. gambiae* mosquito fed on BALB/c mice immunized with AgMucl/IL-12 cDNA, 8 hrs post blood feeding. Note the shrinking of the nucleus and the thickening of the basement cell membrane (BCM). Hemocel (H).

4.5 Preparation and characterisation of anti-mosquito antibodies secreted by hybridoma cells

4.5.1 Screening for anti-mucin secreting hybridomas

From the original fusion plates, a total of thirty-six wells were reactive in the anti-mucin ELISA, of which one was from the fusion between mouse lymph node cell suspension and the myeloma cells. All the rest were from the fusion of the mouse spleen cells and the myeloma cells. All the thirty-six reactive wells were sub-cultured into the twenty-four well plates and retested in the anti-mucin ELISA. Out of these, 11 were found to maintain their reactivity in the anti-mucin ELISA. These were MF1/LYMPH/122, MF2/SPL/C4, MF2/SPL/F6, MF1.1.B3, MF1.3.B1, MF1.3.A12, MF1.2.A7, MF1.2.H12, MF1.3.A8, MF1.3.B2 and MF2.1.D3.

4.5.2 Determination of hybridoma secreted anti-mucin antibody isotypes

The eleven anti-mucin ELISA reactive hybridoma supernatants were isotyped using the mouse immunoglobulin isotyping kit (BD Biosciences) following the manufacturers protocol (3.10.10). Four of the eleven anti-mucin secreting hybridomas had their antibodies successfully isotyped (Table 4.10).

Table 4.10 Anti-mucin antibody isotypes secreted by four hybridoma cell lines

Hybridoma	MF1/lymph/122	MF1.1.B3	MF1.3.B1	MF1.2.H12
Antibody Isotype	IgM, IgG1, IgG2a, IgG3	IgG1	IgG1, IgG3	I gG1, IgG2a, IgG3

4.5.3 Hybridoma cloning

MF1/LYMPH/122 had a mixture of clones secreting both IgM and all the three IgG subtypes and was further cloned (3.10.7). Sixteen anti-mucin antibody secreting hybridoma isolates were identified. On passaging from their original culture wells nine isolates continued secreting anti-mucin ELISA reactive supernatants with two isolates secreting strongly reactive anti-mucin antibodies. The supernatants from the two isolates, 5H8 and 10D6, were re-isotyped and 10D6 was found to be secreting monoclonal IgM anti-mucin antibodies while 5H8 was found to be secreting a mixture of IgG2a, IgG3 and IgM anti-mucin antibodies. All the other isolates i.e 2B2, 3G11, 13H9, 15D12, 15H5, 4C2 and 17C10 secreted anti-mucin antibody isotype IgM and reacted weakly in the anti mucin ELISA.

4.5.4 Mosquito bioassay

Three anti-mucin antibody secreting hybridomas were selected for mosquito bioassays (3.12.2) based on the types of anti-mucin antibody isotypes they were secreting. These

were MF1/LYMPH/122 monoclonal IgM anti-mucin antibody secreting clone (10D6), MF1.1.B3 monoclonal IgG1 anti-mucin secreting hybridoma and MF1.2.H12 polyclonal anti-mucin IgG1, IgG2a and IgG3 secreting hybridoma. Significant differences were observed between the cumulative survival of the mosquitoes fed on the anti-mucin hybridoma supernatants and the control myeloma cell line supernatants (Figures. 4.19, 4.20 and 4.21 and Table 4.11).

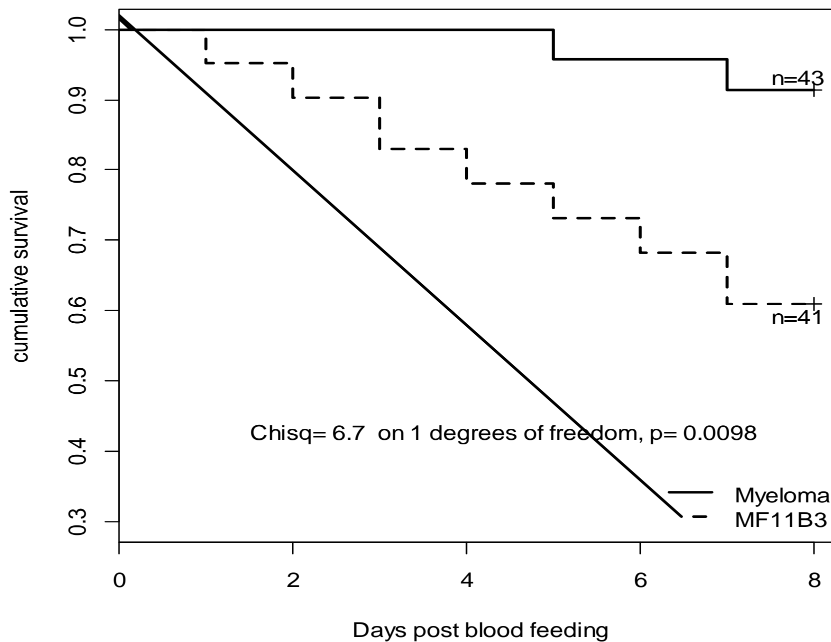

Figure 4.19: Comparison of the daily cumulative survival of the *An. gambiae* mosquitoes surviving to day 7 after feeding on MF1.1.B3 monoclonal IgG1 anti-mucin secreting hybridoma supernatant and the myeloma cell line supernatant. Constructed from life tables and compared by Kaplan Meier log rank analysis.

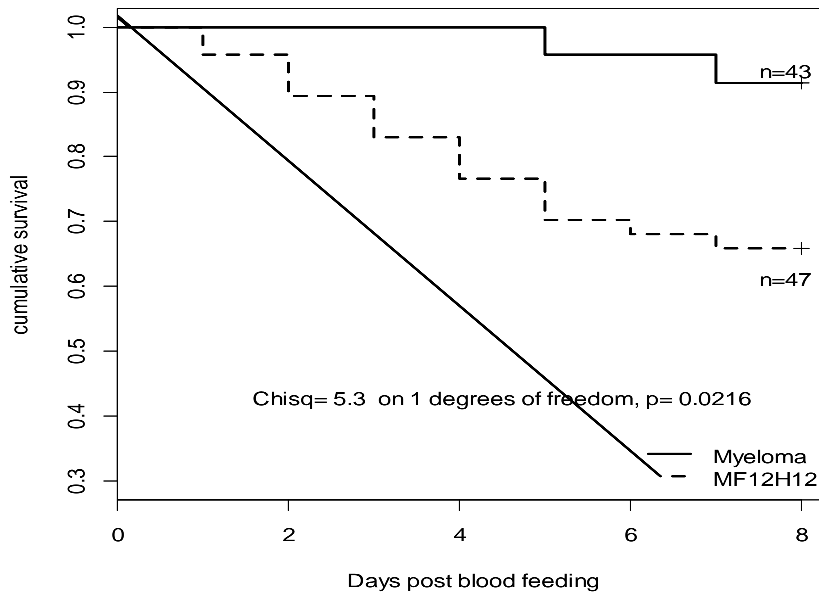

Figure 4.20: Comparison of the daily cumulative survival of the *An. gambiae* mosquitoes surviving to day 7 after feeding on MF1.2.H12 IgG1, IgG2a and IgG3 anti-mucin secreting hybridoma supernatant and the myeloma cell line supernatant. Constructed from life tables and compared by Kaplan Meier log rank analysis.

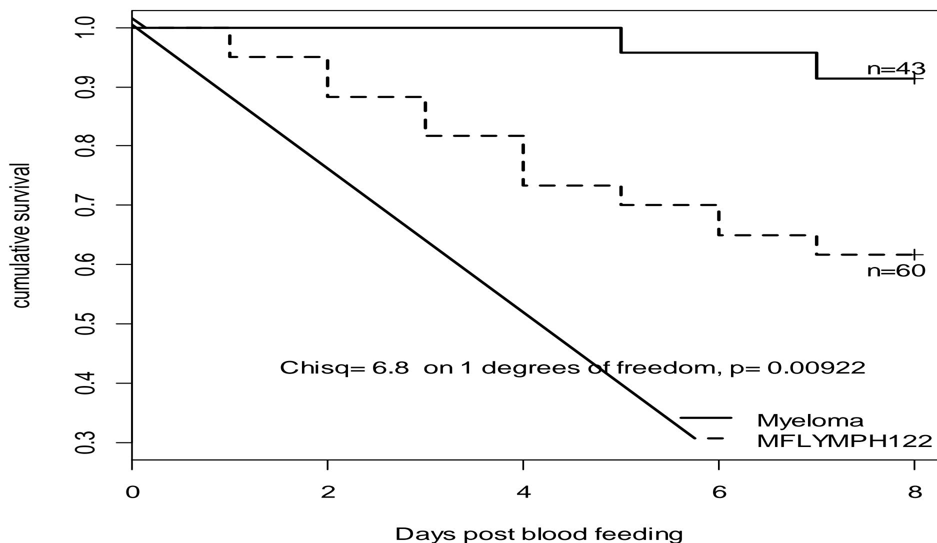

Figure 4.21: Comparison of the daily cumulative survival of the *An. gambiae* mosquitoes surviving to day 7 after feeding on MF/LYMPH/122 clone 10D6 monoclonal IgM anti-mucin secreting hybridoma supernatant and the myeloma cell line supernatant. Constructed from life tables and compared by Kaplan Meier log rank analysis.

Table 4.11: The Log Rank statistics and significance between the daily cumulative survival of *An. gambiae* mosquitoes surviving to day 7 after feeding on anti-mucin antibody containing hybridoma supernatants and myeloma cell line supernatant all mixed with fresh mice blood

Myeloma cell line	MF1.1.B3 (IgG1)	MF1.2.H12 (IgG1,IgG2a and IgG3)	MF1/LYMPH/122 (10D6) (IgM)
Log rank statistic	Chisq=6.7 on 1 degree of freedom; p=0.0098	Chisq=5.3 on 1 degree of freedom; p=0.0216	Chisq=6.8 on 1 degree of freedom; p=0.00922

4.5.5 Determination of complement effect on mosquito killing from anti-mucin secreting supernatants

The polyclonal IgM, IgG1, IgG2a and IgG3 anti-mucin antibody secreting hybridoma cell line MF1/LYMPH/122 supernatant was further subjected to determination of complement effect in its mosquitocidal activity (3.12.3). When the cumulative survival of the mosquitoes fed on supernatants from MF/LYMPH/122 and myeloma cell line in the presence and absence of complement was compared, there were significant differences in the cumulative survival between those fed on MF/LYMPH/122 and those fed on myeloma cell line supernatants. When the cumulative survival of mosquitoes fed on the supernatant from MF/LYMPH/122 either in the presence or absence of complement was compared, no significant differences were observed (Figure 4.22) showing that complement has no role in the antibody mediated mosquito death.

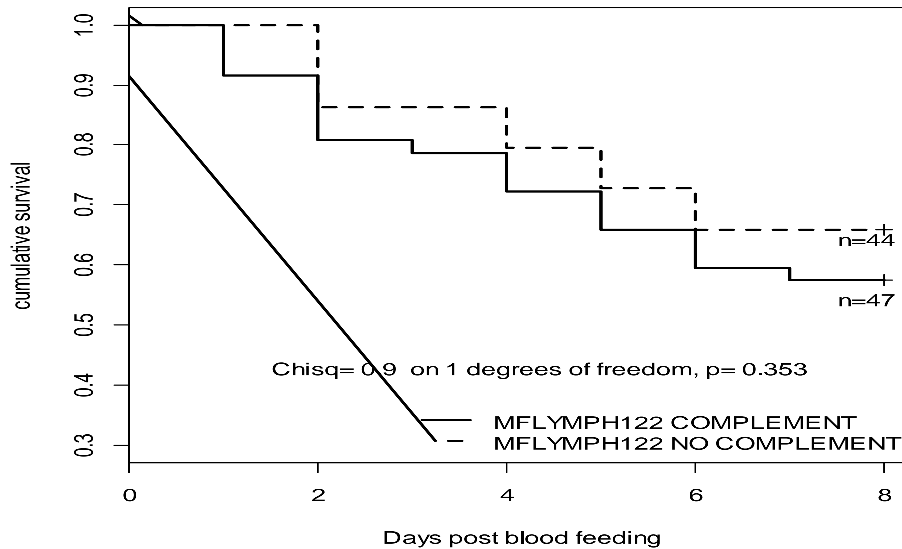

Figure 4.22: Comparison of the daily cumulative survival of the *An. gambiae* mosquitoes surviving to day 7 after feeding on anti-mucin antibody secreting MF/LYMPH/122 hybridoma supernatant mixed with fresh mouse blood either in the presence or absence of complement. Constructed from life tables and compared by Kaplan Meier log rank analysis.

4.5.6 Cross-species killing effects on *An. arabiensis*

Because MF1/LYMPH/122 supernatant contained all the anti-mucin antibody immunoglobulins IgM, IgG1, IgG2a and IgG3 (Table 4.10), this supernatant was further bioassayed to determine whether its mosquitocidal antibodies against *An. gambiae* could effect cross species killing activity against *An. arabensis* mosquitoes (4.12.4).

No significant differences were observed between the cumulative survival of the *An. arabiensis* mosquitoes fed on the mosquitocidal MF1/LYMPH/122 hybridoma supernatant and the control myeloma cell line supernatant (p=0.369; Figure 4.23).

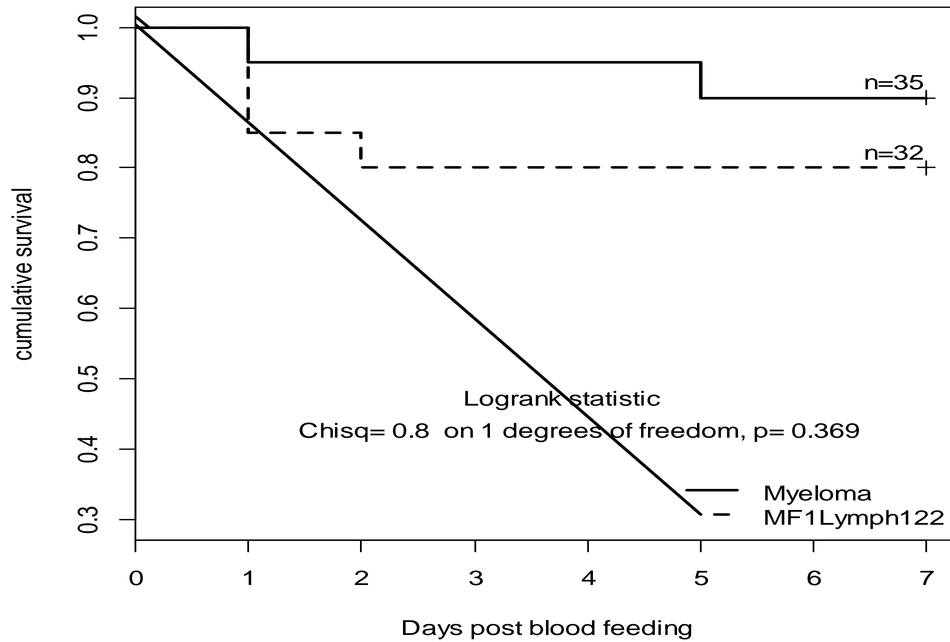

Figure 4.23: Comparison of the daily cumulative survival of the *An. arabiensis* mosquitoes surviving to day 7 after feeding on anti-mucin antibody containing supernatant secreted by hybridoma cell line MF/LYMPH/122 mixed with fresh mouse blood. Constructed from life tables and compared by Kaplan Meier log rank analysis.

CHAPTER 5: DISCUSSION

5.1 Optimal AgMUC1 cDNA immunization schedule that produces mosquito killing immune response

In this immunization strategy up to four injections with AgMUC1 cDNA was the optimal immunization schedule that elicited significant mosquitocidal immune response. The schedule involving one or two injections with AgMUC1 cDNA appeared to prime the mice and although IgG2a anti-mucin antibodies were detected they were not effective in killing the mosquitoes fed on the immunized mice. IgG2a antibodies have two important characteristics that may be important in effecting pathology that may lead to mosquito death. One of them is that IgG2a antibodies fix complement leading to cytolysis. The second property is that IgG2a is a cytophilic antibody. It has been reported to adhere onto the Fcγ receptors on NK cells and macrophages thus directing them to anti-IgG2a specific targets (Abbas *et al*, 1997). The interaction of these cells with these targets can then lead to cell death through antibody dependent cellular cytotoxicity (ADCC) mechanism. These two mechanisms, cytolysis and ADCC, are probably not effective in killing mosquitoes fed on the AgMUC1 cDNA immunized mice because of the low levels of antibodies observed.

After three injections with AgMUC1 cDNA, there appeared to be an antibody response switch to IgG1 antibody production. This antibody isotype switch coincided with the significant increase in mosquito killing observed in the group of mosquitoes fed on the AgMUC1 cDNA immunized mice. After the third AgMUC1 cDNA injection no further

demonstrable increase in the anti-mucin antibody titres was observed. IgG1 antibody subtype effector mechanisms include complement activation and sensitization for binding by NK cells. Since the target mucin protein is membrane bound, a perfect situation for the activities involving complement mediated cytolysis or ADCC may exist. Thus the increased mortality observed in mosquitoes fed on these mice could be mediated by either of the two mechanisms in the presence of adequate levels of anti-mucin IgG1 antibodies.

5.2 Effects of the immunostimulatory cytokines GM-CSF and IL-12 on AgMUC1 cDNA immunization

The cytokines used in this study were selected in order to modify the immune response towards enhancing either the cell-mediated immune response or the antibody mediated immune response or both. This was done in order to determine the immune mechanisms responsible for mosquito killing following AgMUC1 cDNA immunization. In this study, GM-CSF co-immunization did not enhance the antibody response as expected (Table 4.6). The antibody titers generated following co-immunization with GM-CSF were only two-fold higher than those generated following AgMUC1 cDNA immunization alone. In mice, it has been observed that after intramuscular immunization with plasmids, antibody production can vary widely from moderate titers to undetectable levels depending on the encoded antigen (Gardner *et al*., 1996). Thus, the moderate titers obtained after AgMUC1 cDNA immunization with and without GM-CSF are not surprising. Other reports have shown that when plasmid encoding murine GM-CSF is mixed with *Plasmodium yoelli* circumsporozoite plasmid and the mixture used to immunize mice,

specific antibodies increased 8-fold (Weiss *et al*., 1998), tremendously improving vaccine efficacy. Highly significant differences were also observed between the protective capacity of *Plasmodium yoelli* circumsporozoite protein administered alone and when co administered with plasmid GM-CSF (Weiss *et al.,* 1998).

In this study, following co-administration of AgMUCI cDNA and plasmid GM-CSF, antibody levels increased only two-fold (Table 4.6). This could be attributed to the fact that AgMUC1 protein is naturally membrane based in the mosquito midgut and is probably presented differently from the circumsporozoite protein in the malaria parasite. The membrane based AgMUC1 antigen in this study is being presented as a secreted protein from the transfected cells and this may affect the uptake and processing of this protein by antigen presenting cells and ultimately influence the type of antibody produced. This same phenomenon was observed when different components of the merozoite specific protein 1 were administered separately (Cavanagh *et al*., 2001) and also when a plasmid encoding a rabies protein was administered mixed with GM-CSF plasmid with resultant increase in anti-rabies antibody production (Xiang and Ertl, 1995). When GM-CSF plasmid mixed with a plasmid encoding influenza nucleoprotein were co-administered, enhanced CTL responses as opposed to antibody responses were observed (Iwasaki *et al.* 1997) while when GM-CSF plasmid mixed with plasmids encoding proteins from HIV-1 were co-administered increased antibody production as well as T cell proliferation were observed (Kim *et al*. 1997). In this study, plasmid GM-CSF administered mixed with AgMUC1 cDNA did not result into a striking increase in anti-mucin antibody production as was anticipated (Table 4.6).

The daily cumulative survival of the blood fed mosquitoes surviving to day 7 after feeding on AgMUC1 cDNA immunized mice compared to that of mosquitoes fed on AgMUC1/GM-CSF cDNA immunized mice in this study was also found to be significant. This shows that GM-CSF indeed increased the mosquito killing activity following co-administration with AgMUC1 cDNA. However, it is the unacceptably high mortality in mosquitoes fed on the control empty vector/GM-CSF cDNA immunized mice group (Figure 4.7) that invalidates this observation. GM-CSF is known to promote the activation of the inflammatory cells of the immune system (Grabstein *et al.,* 1986; Ruef and Coleman, 1990; Gasson, 1991). In this system therefore, it can be inferred that GM-CSF might be activating the inflammatory cells of the mouse immune system to produce TNF-α, superoxides, peroxidases, and lysozymes that could be injurious to the epithelial cells lining the mosquito midgut.

On *in vitro* stimulation of spleen cells from AgMUC1/GM-CSF immunized mice, the levels of TNF-α detected were not that high compared to that detected in the control mice spleen cells (Table 4.4). This shows that probably TNF-α has no role in mosquito death. If mosquito death is to be attributed to the activated inflammatory cells both in the experimental and control immunization groups then it could be the production of superoxides, peroxidases, and lysozymes by the activated inflammatory cells that are involved. This is reflected in the fact that no significant differences were observed in the daily cumulative survival of the mosquitoes surviving to day 7 post blood feeding between the mosquitoes fed on the AgMUC1/GM-CSF and those fed on the Empty vector/GM-CSF immunized mice (Figure 4.7). In this case, the mechanisms involved in

the death of the mosquitoes fed on mice immunized with AgMUCI/ GM-CSF cDNA are not attributable to any specific anti-mucin immune responses in the immunized mice.

Following co-administration of AgMUC1 cDNA with IL-12 cDNA, highly significant differences were observed between the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding when fed on AgMUC1/IL-12 cDNA immunized mice compared to those fed on empty vector/IL-12 cDNA immunized mice (Figure 4.8). IL-12 is a cytokine that promotes cell mediated immune responses by stimulating the proliferation of activated T lymphocytes, enhancing IFN-γ secretion and the lytic activity by NK cells and CD8+ T-cells (Stern *et al.*, 1996). In the AgMUC1/IL-12 cDNA immunized mice, it is unlikely that the cytotoxic mechanism that promotes the mosquitocidal effect observed could be due to CD8+ T cell cytotoxicity as there are no reports that indicate that these molecules are expressed on the epithelial cells of the blood fed mosquito midgut. Thus, the anti-mucin antibodies present in the blood meal from the AgMUC1/IL-12 cDNA immunized mice probably adhere onto the epithelial cells of the blood fed mosquito midgut and attracts other cytotoxic cells in the blood that have Fc receptors such as the NK cells and the macrophages, both of which are activated by IFN-γ (Janeway and Travers, 2001). Since the cytotoxic effects of activated macrophages are effective after phagocytosis, it is unlikely that these cells would play a role in inflicting injury to the epithelial cells lining the midgut. The only injurious effect they can cause would be to adhere onto the midgut epithelium and cause blockage of the channels through which nutrients are absorbed into the mosquito body system.

Thus, following AgMUCI /IL-12 cDNA immunization, the immune mediated pathologic mechanisms on the epithelium of the mosquito midgut that would result into mosquito killing are most likely to be mediated by NK cells, a mechanism that could be dependent on the presence of IFN-γ, IL-12 and nitric oxide. NK cells therefore may constitute part of an antigen specific adaptive immune response, as opposed to the innate nonspecific immunity, initiated by anti-mucin antibodies and dependent on IL-12 representing a novel mechanism of killing mosquitoes feeding on AgMUCI/IL-12 cDNA immunized mice.

Such a killing mechanism most likely would involve IFN-γ and IL-12 activated NK cells adhering onto the antibody molecules that are adhering onto the epithelial cells through their Fc receptors. While attached their cytolytic mechanisms could be activated and oriented towards the attachment site. Then, at the attachment site perforins would be released, making pores in the epithelial cell membranes. Through these pores would flow the granzymes that are released together with perforin. When granzymes enter the cell they could then program the cell to die through apoptosis. In apoptosis the cell nucleus is known to undergo fragmentation followed by the shrinking of the affected cell and death (Ferluga and Allison, 1974). The midgut contents could also enter the epithelial cell wall through these pores and cause the cell to swell and eventually die. The bursting of these cells could lead to necrotic processes along the mosquito midgut lining and cause death (Plate 4.8).

The mechanisms that mediate mosquito death observed in AgMUC1 cDNA immunized mice therefore are enhanced through co-immunization with IL-12 cDNA. Since the mechanisms most likely require the presence of both antibody and activated NK cells, these mechanisms are probably mediated through antibody dependent cellular cytotoxicity (ADCC). Mechanisms involving ADCC do not require the presence of high antibody levels and in the AgMUC1/IL12 cDNA immunized mice, antibody levels were moderate; only two-fold higher than the levels detected in the group that was immunized with AgMUC1 cDNA only. The results of this study demonstrated that high antibody levels are probably not effective in ADCC mechanisms. Following boosting of AgMUC1 cDNA immunized mice with recombinant mucin protein, very high anti-mucin antibody levels were attained (>1:100,000) without a corresponding increase in mosquito mortality (Table 4.6). This shows that if mosquito death was solely antibody mediated there would have been a highly significant increase in mosquito death following the recombinant mucin protein boost

When GM-CSF and IL-12 cDNA were administered together with AgMUC1 cDNA the same trend in the immune response as that observed when AgMUC1 cDNA was co-administered with IL-12 alone was observed. This shows that probabaly there is neither antagonism nor synergism in the mosquitocidal immune response between the two cytokines. It is probably only the activation of NK cells by IL-12 that is required to effect significant mosquito death. IL-12 activates NK cells to produce IFN-γ, which further activates the cytotoxic mechanisms of the NK cell (Janeway *et al.*, 2001). Macrophages and neutrophils are activated through the effect of GM-CSF. All the cells that are capable

of being activated by IL-12 and GM-CSF are capable of mediating ADCC. The ADCC that could be effective in mosquito killing can not be exercised by phagocytic macrophages and neutrophils as the target mucin protein is membrane bound. Thus, only the activated NK cell mediated ADCC can be effective in mediating the mosquitocidal activities observed in the AgMUC1/GM-CSF/IL-12 cDNA immunized mice. This explains why there are no significant differences in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between groups of mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice compared to those fed on AgMUC1/GM-CSF/IL-12 cDNA immunized mice.

5.3. Characterization of the type of mosquito killing immune response responsible for mosquito death

5.3.1 Anti-mucin antibody killing mechanism

In correlating anti-mucin antibody levels to mosquito survival, mosquitocidal activity is achieved when a negative correlation greater than 0.5 is observed between the daily cumulative survival of the blood fed mosquitoes surviving to day 7 and the anti-mucin antibody levels in the immunized mouse. This was observed in the mosquitoes fed on the mice immunized with AgMUC1/GM-CSF cDNA ($r=-0.759$), AgMUC1/IL-12 cDNA ($r=–0.782$) and AgMUC1/IL-12/GM-CSF ($r=–0.832$). Positive correlations greater than 0.5 means no correlation as this is interpreted that the presence of antibody promotes mosquito survival, a situation that is not realistic. Correlation values below -0.5 are also

interpreted as there being no correlation because only high correlations are required to demonstrate a biologic activity.

Based on this criteria therefore, no significant correlations were observed between the daily cumulative survival of the mosquitoes fed on the AgMUC1 / mucin protein immunized mice and the antibody levels in these mice as all correlations were <0.5 with the excepetion of IgG1 (*r=–0.671)*. This is despite the fact that antibody levels were greatest in this group of mice. Elsewhere, high antibody levels following immunization of rabbits with extracts of *An. stephensi* midgut also showed no statistically significant effect on mortality and longevity of blood feeding mosquitoes (Suneja *et al.,* 2003). In another study, anti-mosquito antibodies produced in mice, inoculated with mosquito homogenates showed significant increase in mortality which correlated to both the titer and specificity of the anti-mosquito antibodies detected (Hatfield, 1988). In these studies however, the antibodies responsible for these effects were not determined. When anti-mucin IgG1 antibodies are specifically correlated with the daily cumulative survival of the blood fed mosquitoes surviving to day 7, a negative correlation was observed in all the immunization groups (*r*>-0.5; Table 10) showing that it could be the anti-mucin IgG1 antibody subtype that is effective in killing mosquitoes fed on AgMUC1 cDNA immunized mice.

The effector mechanism of an IgG1 antibody subtype for a membrane associated protein like AgMUC1 could involve complement mediated cytolysis and/or ADCC. When pooled mice sera containing anti-mucin IgG1 antibody was fed on groups of mosquitoes

in an *in vitro* membrane feeding system in presence and absence of complement, no significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between the groups of mosquitoes fed to these antibodies in presence and absence of complement (Figure 4.11). This probably rules out the participation of complement in mosquito killing. Complement independent killing has also been reported following immunization of New Zealand rabbits with *An. gambiae* midguts, which elicited high antibody titers (Noden *et al.,* 1995).

5.3.2 Correlations between Cytokine and antibody profiles in mice given one, two, three or four AgMUC1 cDNA injections

Cytokine functions are complex, but there is evidence to suggest that the analysis of relatively simple regulatory networks *in vitro* may be highly relevant to genuine physiological functions *in vivo* (Mosmann, 1991). IL-2 stimulates the growth of NK cells, antibody synthesis and enhances the antibody's cytolytic function (Morris, *et al.,* 1986). Despite the fact that there were no appreciable differences in the levels of IFN- γ and IL-12 production, at two AgMUC1 cDNA injections there appeared to be decreased levels of these two cytokines compared to the controls (Table 4.5). Whether this can be associated with increased anti-mucin antibody levels and mosquito mortality in the third and subsequent fourth injections is difficult to explain. It has been reported that IFN-γ acts on B cells to promote switching to IgG2a and IgG3 subclasses in mice (Janeway, *et al*., 2001). These two antibody isotypes are the most potent complement activating IgG subclass. In mice, IFN-γ inhibits the proliferation of Th2 cells involved in antibody production (Gajewski *et al,* 1988). The levels of IFN-γ appeared to increase from the

third injection moving to the fourth injection without any appreciable change in anti-mucin antibody levels. Thus IFN-γ levels in these groups of mice do not seem to play a role in the immune response.

5.3.3 Correlation between Cytokine and antibody profiles in mice immunized with AgMUC1 cDNA mixed with GM-CSF or IL-12 cDNA

Following *in vitro* stimulation of spleen cells from AgMUC1 cDNA immunized mice, IL-4 which is important for the humoral immune responses was undetectable while increased levels of IFN-γ and IL-5 were observed (Table 4.4). In the murine system, IL-4 preferentially induces switching to IgG1 antibody subtype production while inhibiting the production of IgG2a and IgG3 antibody subtypes (Banchereau *et al.,* 1991)**.** IFN- γ induces switching to IgG2a and IgG3 antibody production while inhibiting IgG1 antibody production (Schijns *et al.,* 1994). The IgG antibody subtype produced in the AgMUC1 cDNA immunized mice was IgG1 with a mean titer of 1:3,000 and a negative correlation with the daily cumulative survival of the blood fed mosquitoes surviving to day 7 ($r=-0.582$), and IgG2a with a titer of about 1:640 and no correlation ($r=0.444$) with the blood fed mosquito's daily cumulative survival to day 7 (Table 4.6).

Thus the IgG antibody subtype responsible for the observed mosquito mortality in this group appears to be IgG1 mediated as its negative correlation shows that mortality increases with increasing IgG1 antibody levels. The increasing IgG1 anti-mucin antibody levels could be only effective to a certain level beyond which their efficacy could be reduced as observed with the group of mosquitoes fed on AgMUC1 cDNA immunized / recombinant mucin protein boosted mice group.

When AgMUC1 cDNA is co-administered with IL-12 cDNA, increased levels of IL-4 (1.1 pg/ml) and IFN-γ (4633 pg/ml) and lower levels of IL-5 (1.5 pg/ml) are observed compared to the other immunization groups (Table 4.4). IL-2 and TNF-α levels remain within the range as seen in the other immunization groups. IL-12 is known to enhance IFN- γ production while down regulating IL-5 secretion (Chan *et al.,* 1991). The predominant IgG antibody subtype produced in the AgMUC1/IL-12 cDNA immunized group was IgG1 with a titer of 1:6000. *In vitro* experiments have demonstrated a co-operative effect between IL-5 and IL-2 in an IL-4 promoted IgG1 production system by B-cells (Purkerson *et al*., 1988). This phenomenon appears to be the same *in vivo* hence the bias towards IgG1 antibody subtype production in the AgMUC1/IL-12 cDNA immunized mice.

When AgMUC1 cDNA is co-administered with plasmid GM-CSF lower levels of IFN-γ (2020 pg/ml) compared to the other groups are observed. All the other cytokine levels remain within the range of production as in the other groups. Most of the antibody produced in this group is IgG1 subtype with a mean titer of 1:6,000 and a significant negative correlation ($r=-0.914; p=0.001$) with the daily cumulative survival of the blood fed mosquitoes surviving to day 7. IFN- γ has been shown to up regulate IgG2a antibody subtype production (Schijns *et al.,* 1994) and since lower levels of it were secreted by stimulated spleen cells from these mice, undetectable levels of this antibody subtype were observed. Thus IFN-γ could not drive IgG2a anti-mucin antibody production in these mice. Unlike in this study, the inclusion of plasmid GM-CSF in *Plasmodium yoelli*

circumsporozoite immunization was shown to stimulate an increase in the number of spleen cells capable of secreting antigen specific IL-2 and IFN- γ, showing an enhancement in Th1 type cytokines with serum IgGI, IgG2a and IgG2b isotypes equally increased (Weiss *et al.,* 1998). In this study also, secretory cytokines were measured as opposed to measuring the number of cells secreting these cytokines as was done by Weiss *et al.,* (1998). The measurement of the number of cells secreting cytokines is a more definitive measure of cytokine activity compared to the measurement of secreted cytokines.

In the AgMUC1 / mucin protein boost group, comparatively lower levels of IL-2 and IFN-γ with undetectable levels of IL-5 were oberved in the mucin protein stimulated spleen cells. However, very high mean antibody titers were detected (IgG1 1:66600, IgG2a 1:82000 and IgG3 1:1800). Of the three IgG antibody subtypes, only IgG1 had a negative correlation ($r=-0.671$) with the blood fed mosquito's daily cumulative survival to day 7. The high antibody titers despite reduced cytokine levels is not surprising as the influence of cytokines on antibody production is concentration dependent and very little of it is required to effect a high magnitude of effect (Hughes *et al.,* 1992

5.3.4 Duration of mosquito killing immune response in AgMUC1/IL-12 cDNA immunized mice

Of all the co-immunization strategies employed in these studies, it is the AgMUC1/IL-12 cDNA co-immunization that showed a highly significant killing effect on blood feeding mosquitoes. The differences in the daily cumulative survival of the blood fed mosquitoes

surviving to day 7 after feeding on AgMUC1/IL-12 cDNA immunized mice compared to AgMUC1 cDNA immunized mice was also significant. With such significant difference in mosquito survival it was envisaged that the mosquitocidal immunity would be long lasting. However, the mosquitocidal immune response elicited following AgMUC1/IL-12 cDNA immunization appeared to be short-lived despite the fact that significant differences were observed between the daily cumulative survival of the blood fed mosquitoes surviving to day 7 after feeding on the AgMUC1/IL-12 cDNA immunized mice and the empty vector/IL-12 cDNA immunized mice at 5.5, 15 and 36 weeks. When the mosquitocidal effects were compared between the feeding intervals, a significant reduction in the mosquitocidal effects of the AgMUC1/IL-12 cDNA immunized mice was observed (Figure 4.18).

Lowered immune responses following cDNA immunizations have been observed elsewhere. Following immunization with 100 ug of *Plasmodium yoelli* circumsporozoite plus 30 ug of plasmid GM-CSF and challenge with 50 *p. yoelii* sporozoites, protection two weeks after immunization was 70% - 100% which declined to 30-40% by the 28th week (Sedegah *et al.,* 2002). In the strict principles of cDNA immunization this is not supposed to be the case as the immune response is supposed to be akin to that elicited by a virus infected cell hence long lived. Genetic immunization by the very nature of its mechanism is meant to provide a long lasting immune response from only one or two immunizations akin to the use of attenuated live viral vaccines (Danko and Wolff, 1994).This is the principle behind the long-lived immunity elicited through vaccination with live viral vaccines.

The fast waning of the mosquitocidal immune response could be due to the mechanisms that are responsible for mosquito killing. ADCC is a mechanism whose specificity is induced through antibody/antigen interaction. There will be no action if the Fc portions of the antigen/antibody complexes or the complement receptors are not enough to be recognized by the NK cells. In this system, adequate levels of IFN-γ are required by the IL-12 activated NK cells for the activated NK cells to persist longer in the mosquito midgut and effect its functions. NK cell mediated cytolysis is a multi-stage process consisting of binding of the NK cell to the target cell, delivering a lethal hit and recycling of this cell which requires constant reactivation with IFN-γ and IL-12. Thus the mosquitocidal effects in this system are a function of anti-mucin antibody reaction with mucin and IFN- γ activated NK cells. As seen with anti-mucin IgG1 secreting hybridoma supernatant, when fed to mosquitoes mixed with naive mouse blood in which the NK cells are not as activated as in the immunized mice, mosquito killing is not comparable to that seen in the mosquitoes fed directly on the AgMUC1/IL-12 cDNA immunized mice.

5.4 Immune pathological features in the mosquito midgut that lead to the death of mosquitoes when fed on AgMUC1/I-12 cDNA immunized mice

The adherence of white blood cells onto the surface of the mosquito midgut epithelial lining is due to the fact that the cells lining the midgut epithelium express AgMUC1 protein after a blood meal. Antibodies to this protein present in the blood meal from AgMUC1/IL-12 cDNA immunized mice react with this protein forming antigen-antibody complexes on the epithelial surface. The antigen – antibody complexes formed may

attract the Fc receptor and complement receptor bearing white blood cells (Plates 4.1, 4.2 and 4.3) through the recognition of either the Fc portion of the adherent antibody or the C3 portion of the attaching complement. These events must happen before the formation of the peritrophic membrane which confines the immune components to within the food bolus.

The adherent antibodies, complement and cellular immune components react with their specific receptors turning their targets into a focus of immune activity that may lead to the formation of nodules (Plate 4.6). The sequence of events could be such that the first to react with their specific targets would be the anti-mucin antibodies followed by complement, the Fc receptor bearing white blood cells and finally the C3 receptor bearing white blood cells. The consequence of the reaction of anti-mucin antibodies with the mucin protein and the subsequent activation of complement is the concentration of Fc and complement components in the focus of this reaction. The cells of the immune system that have the Fc and C3 receptors are known to be involved in mediating the phenomenon of antibody dependent cellular cytotoxicity (ADCC).

The Fc receptor and C3 receptor bearing cells that are involved in ADCC include macrophages, NK cells and Neutrophils (Abbas *et al.,* 1997). The Fc receptors are membrane glycoproteins that show specificity for various IgG subclasses. They are also signal transducing molecules generating super oxide ions and prostaglandins. In the mouse, the Fcγ receptors preferentially show specificity for IgG2a and IgG2b over IgG1 and IgG3 (Ravetch and Kinet, 1991). The Fcγ receptor is a high affinity receptor whose

expression in monocytes, macrophages and neutrophils is enhanced by IFN-γ. In this study more IFN-γ could be generated through activation of NK cells by IL-12. Thus the most likely white blood cells observed adhering on the epithelium of the *An. gambiae* midgut fed on AgMUC1/IL-12 cDNA immunized mice could be the NK cells (Plates 4.1, 4.2 and 4.3; Plates 4.15 and 4.16). Although NK cells are also activated in the empty vector/IL-12 cDNA immunized mice, lack of specific anti-mucin antibodies in these mice blood meals makes them not attach onto the midgut epithelium (Plates 4.4 and 4.5). It appears that some white blood cells are trapped onto or within the epithelial cells (Plate 4.6) where they appear like a nodule. In the event that this nodule breaks there will be chance that it could cause necrotic processes along the epithelial lining. Through such processes along the epithelial lining, the midgut and hemocel contents could mix leading to mosquito death.

The other mechanism that could lead to mosquito death is where the NK cells attach onto the epithelial cells lining the midgut and delivers a lethal hit that result in the dissolution of the affected epithelial cell. Two such epithelial cells whose nuclei appear to be beginning to under go dissolution were observed (Plate 4.7). The cell nucleus defragmentation can be seen in the two cells compared to the adjacent unaffected cells. The NK cell lethal hit involves NK cell granule exocytosis and pore formation in the target cell that then starts to undergo lysis (Ferluga *et al.,* 1974). As the target cell undergoes lysis, the cells shrink (Plate 4.7), creating intercellular spaces (Plates 4.9, 4.10 and 4.11) through which there could be sippage of the midgut contents into the hemocell. The mixing of the midgut contents and the hemocel through these intercellular spaces is

the other mechanism that could lead to the death of the affected mosquito. Where the apoptotic cells do not give way to intercellular spaces, there might most likely be repair whereby the adjacent non-affected epithelial cells come together closing the space left behind by the damaged cell, a mechanism that would be a kin to that observed with epithelial cells invaded by plasmodium ookinetes (Gupta *et al*., 2005).

The other mechanism that could lead to mosquito death is the pores inflicted in the epithelial cells through ADCC. The pores made by ADCC are reported to be larger than those made through complement action (Dourmashkin, 1980). Since the ADCC pores inflicted in the epithelial cells are large enough, the midgut contents would pass into the epithelial cell which will swell (Plate 4.8) and burst. Once the epithelial cell bursts, there will be mixing of the midgut contents and the hemocel, leading to the death of the affected mosquito.

The midgut epithelium has been reported to be an immune competent organ that produces defense components (Dimopoulos *et al.,* 2003). Any immune reactions occurring on the epithelium will trigger off the serine protease cascade that in turn will activate defense reactions in the mosquito hemocel (Hoffman and Reichhart, 2002). Such immune defense mechanisms will involve the activation of hemocytes. The hemolymph contains large quantities of immune components and hemocytes that circulate freely or are attached to different organs like the basement membrane. Hemocytes secrete immune components and are also capable of phagocytosis and encapsulation of foreign particles such as apoptotic cells.

In mosquitoes fed on AgMUC1/IL-12 cDNA immunized mice, activated hemocytes were seen in amoeboid forms along the basement membrane probably as a result of the heightened immune activity along the enlarged basement membrane (Plates 4.12, 4.13 and 4.14). Hemocytes are a defense mechanism of the mosquito immune system (Dimopoulos *et al.,* 2003). The documented innate mosquito immune responses to malaria infection may partly be as a result of the injury that is caused by the parasite invasion of the epithelial tissue as well as the microbial components of the midgut that may be present at the site of invasion (Dimopoulos *et al.,* 2003). Such could be similarly occurring following injury to the epithelial cell resulting from the pores made by the NK cell lytic activity. It is not known what will be the consequence of the activation of the mosquito innate immune mechanism in this case.

5.5 Preparation of hybridomas secreting mosquito killing antibodies

Anti-midgut mAbs have been produced that reduced mosquito survivorship and fecundity (Lal *et al*., 2001). In this study hybridomas were generated that secreted anti-mosquito antibodies of subtype IgG1, IgM and a mixture of IgG1, IgG2a and IgG3 subtypes that demonstrated significant killing of mosquitoes fed to these antibodies mixed with fresh mouse blood. These results reveal that mosquito midgut-based antibodies have the potential to reduce vector abundance, by decreasing mosquito survivorship. How antibodies alone kill mosquitoes remains to be determined.

5.6 Mechanisms by which anti-mucin antibodies secreted by hybridoma cells kill mosquitoes

5.6.1 The role of complement in antibody mediated mosquito killing

Anti-mosquito antibodies secreted by hybridoma cell lines mixed with blood and fed to mosquitoes have been reported to reduce survivorship of the blood fed mosquitoes (Lai *et al.,* 2001). It is however doubtful that antibodies alone would mediate mosquito killing especially when targeted against a cell-membrane-associated protein like AgMUC1 (Foy *et al.,* 2003). The killing probably requires the synergistic effects between anti-mosquito antibodies, complement, lectins and immune effector cells. These conditions are only available when the mosquitoes are fed directly on the immunized animal. Antibodies on their own only bind to their target protein molecules and hinder enzyme activity or block the protein ion channels (Tellam *et al.,* 1996). Antibodies in conjunction with complement will direct membrane attack complexes to the epithelial surface or direct cytotoxic cells to bind to the membrane surface bound molecules (Janeway *et al*., 2001). Complement and the cellular immune effectors have not been tested for their effects against vector tissues in the presence of specific tissue antibodies although both complement and cytotoxic cells have been reported to be active in the mosquito midgut during the first few hours following a blood meal (Noden *et al.,* 1995; Almeida *et al*., 1998; Lai *et al*., 2001,).

Cellular immune effectors responsible for mosquito death would involve antibody mediated mechanisms that act by releasing cytotoxic agents such as perforins and

granzymes that leads to tissue damage during the first few hours. These activities correlate well with the presence of anti-mucin IgG1 antibodies. IgG1 antibodies fix complement and in this study complement effect was found not to be required for mosquito killing because there were no significant differences in the cumulative survival of mosquitoes fed on either pooled anti-mucin IgG1 containing mice sera or the anti-mucin containing IgM, IgG1, IgG2a and IgG3 supernatant secreted from MF/LYMPH/122 hybridoma cell lines in the presence and absence of complement (Figures 4.11, 4.12 and 4.13; Figure 4.22).

In this study, feeding of the mosquitoes on was done through *in vitro* membrane feeders. Such a system does not provide conditions comparable to those attainable following feeding mosquitoes directly on the immunized animal. Thus the effects of cellular elements that would mediate cytotoxicity cannot be attained in the *in vitro* feeding system. An ideal situation would be whereby cell mediated effects are demonstrable in the absence of complement and vice versa. This would require an environment in which the viable cellular mediators like NK cells are enriched and fed to the mosquitoes. In the *in vitro* membrane feeding system, it would be difficult to enrich the blood meal of these cells and guarantee their viability at the same time.

5.6.2 Cross species mosquito killing effects

Cross species killing effects were not demonstrated against *An. arabiensis* from antibodies prepared from a protein isolated from *An. gambiae*. Two studies involving

Anophlene mosquitoes have described cross-species killing with mosquitocidal antibodies (Noden *et al.,* 1995; Lal *et al.,* 2001). Cross species killing has also been reported with rabbit antibodies generated following immunization of rabbits with antigens derived from the midgut of *Anopheles tessellatus* against *culex quinquefasciatus* (Ramasamy *et al*., 1992).

Both *An. gambiae*, *An. funestus* and *An. arabiensis* belong to the *An. gambiae* complex with different climatic distribution patterns (Lindsay *et al., 1998*). Considering that in malaria endemic areas like the lower western Kenya where all the three species have been reported to be abundant (Minakawa *et al.,* 2001), a protein that would produce mosquitocidal effect on all the three species belonging to the *An. gambiae* complex would be very useful in malaria control. This is more so because, two or more species within this complex have been reported to coexist in many areas (Coetzee *et al.,* 2000). The absence of cross-species killing as demonstrated in this study could be a major limitation to vaccine development efforts against the mosquito vector. Malaria deaths are due to the intensity of *Plasmodium falciparum* transmission by the three widespread and efficient mosquito vectors: *An. gambiae, An. arabiensis* and *An. funestus* (Curtis, 1994) hence the need to have a vaccine strategy that can simultaneously affect the vectorial capacity of these mosquitoes at once for an effective mosquito control program.

It appears that the immune response elicited following AgMUC1 cDNA immunization does not recognize the mucins secreted by *An. arabiensis.* This could be attributed to the differences in antigenic configurations between the two mucins or just that the two have

no demonstrable similarity. Furthermore the *An. arabiensis* used in this study was a strain that had been recently colonized at ICIPE and in its third generation. Whether this would affect the response of these mosquitoes to the mosquitocidal immune components generated after AgMUC1 cDNA immunization is a subject that might require further investigation.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The results of this study demonstrate that AgMUC1 cDNA when injected into BALB/c mice elicits an immune response that kills blood feeding female *An. gambiae* mosquitoes. To attain an effective mosquito killing immune response atleast three injections of AgMUC1 cDNA are required.

ii. Mosquitocidal immune response following AgMUC1 cDNA immunization is significantly potentiated through the co-administration of plasmid AgMUC1 mixed with plasmid IL-12. The mechanisms by which IL-12 cDNA potentiates the mosquito killing immune response to AgMUC1 cDNA immunization involve the activation of NK cells by IL-12 to secrete IFN-γ that amplifies the activation of immune NK cells to become the effector cells that destroy antibody coated epithelial cells within the short period the activated NK cells are active in the mosquito midgut.

iii. Mortality in mosquitoes fed on AgMUC1, AgMUC1 / GM-CSF, AgMUC1 / IL-12 and AgMUC1 / mucin protein boosted immunized mice suggest that the mosquito killing immune response could be IgG1 antibody mediated and that the T-cell cytokines IL-2, IL-4, IL-5 and IFN-γ act synergistically to bias the immune response to secrete this antibody subtype. The effector mechanisms of this

antibody subtype is NK cell mediated ADCC that requires the presence of adequate levels of IFN-γ.

iv. The immune pathologic mechanisms responsible for mosquito killing involve a combination of immunological activities along the mosquito midgut epithelium including nodulation, apoptosis and necrosis. Mosquito death could also result from sippage of the midgut contents into the hemocell through intracellular spaces created by the dead apoptotic epithelial cells or pores inflicted by NK cells.

v. Mosquitocidal anti-mucin antibodies are secreted by hybridoma cell lines derived from the fusion of AgMUC1 cDNA immunized mice spleen cells and myeloma cells. These anti-mucin antibodies do not require complement to exert their mosquitocidal effect. Furthermore these mosquitocidal anti-mucin antibodies do not exert cross species killing on *An. arabiensis* despite the fact that both the *An. gambiae* and *An. arabiensis* belong to the *An. gambiae* complex.

vi. The mosquitocidal immune response generated following AgMUC1 cDNA immunization declined over the 36 week period of observation.

6.2 Recommendations

The approach to malaria control targeting mosquito control by immunological attack of critical molecules within the mosquito midgut such as the AgMUC1 could be moved further to the primate model to determine if the same could be replicated there. With malaria being the single most killer of children under the age of five in sub-saharan Africa, this could be an appealing strategy that could be combined with other anti-malaria parasite strategies that are aimed at killing the malaria parasites and the responsible use of insecticides and insecticide treated bed nets.

6.3 Suggestions for further studies

i. Special staining techniques can be used to conclusively identify NK cells, Hemocytes and apoptotic cells. This will conclusively demonstrate the immune mechanisms responsible for mosquito killing.

ii. Strategies to reduce the number of injections and at the same time induce long lived immune responses should be further pursued.

iii. Further studies on whether there are any effects of AgMUC1 cDNA immunization on the malaria parasite development in the mosquito midgut might be useful.

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APPENDICES

APPENDIX I: Preparation of Laboratory reagents

1. Hybridoma Culture Media preparation

1.1 HAT MEDIUM

1.1.1 Hypoxanthine stock solution:

Add 0.68 g hypoxanthine to 500 ml distilled water. Dissolve by heating in a water bath. Cool to room temperature. Filter sterilize using 0.45 um filter. Freeze at -20 ^{o}C in 10 ml aliquots.

1.1.2 Aminopterin stock solution

Add 1.9 mg aminopterin to 99 ml distilled water. Add 0.5 ml 1 N sodium hydroxide so as to dissolve the drug. Neutralize with 0.5 ml 1 N hydrochloric acid. Filter sterilize and freeze (section 1.1.1).

NB: Aminopterin must be stored protected from light and must be used within six months after it is dissolved.

1.1.3 Thymidine stock solution

Dissolve 0.387 g thymidine in 200 ml distilled water by heating to near boiling. Add 0.0225 g glycine and cool to room temp. Filter sterilize and freeze (section 1.1.1).

1.1.4 HAT Medium

Hypoxanthine stock solution – 5.0 ml

Aminopterin stock solution – 5.0 ml

Thymidine stock solution – 1.0 ml

200mM glutamine – 5.0 ml

7.5% sodium bicarbonate – 12.0 ml

Fetal Bovine serum – 100 ml

RPMI 1640 with HEPES, L-Glutamine, Sodium Bicarbonate – 400 ml

Store at 4 ^{0}C. Add more glutamine if storage is longer than 6 weeks.

1.1.5 PEG I

5 g PEG 1550; melt by micro waving for 3 minutes or autoclaving. Add 5.5 ml of plain RPMI 1640 and 1.5 ml DMSO. This forms the fusion medium.

1.1.6 PEG II

5 g PEG 1550, melt by micro waving for 3 minutes or autoclaving. Add 15 ml of plain RPMI 1640. This forms the fusion reaction-stopping medium.

Both PEG I and II are kept protected from light and are warmed to 37 ^{0}C before use.

1.1.7 Growth medium

RPMI 1640 containing 20% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-Glutamine and NaHCO3 (0.85 gms/liter)

1.1.8 Freezing medium

Fetal bovine serum (FBS) – 20 ml

DMSO – 10 ml

Growth medium without FBS – up to 100ml

2. Reagents for ELISA

2.1 Coating buffer (Carbonate buffer pH 9.6)

15mM Na_2CO_3 – 1.59g

35mM $NaHCO_3$ – 2.93g

Dist. H_2O – Up to 1 liter

2.2 Phosphate buffered saline (PBS pH 7.4)

NaCL – 8g

KCL – 0.2g

KH_2PO_4 – 0.2g

Na_2HPO_4 – 1.3g

Dist. H_2O – Up to 1L

2.3 Washing buffer:

0.05% Tween-20 in PBS pH 7.4

2.4 Blocking buffer:

10% BSA in washing buffer

3. Electron Microscopy fixative

0.05M Sodium cacodylate buffer Ph 7.4 containing 0.01M Calcium chloride, 2.5% Glutaldehyde and 5% Sucrose.

APPENDIX II: Manuscripts published

1. **Injera, W.E.; Foy, B. D; Gicheru, M. M.; Kabiru, E. W.; Githure, J. I. & Beier, J. C. (**2013). Mosquitocidal Immune Response in BALB/c Mice Is Enhanced When *Anopheles gambiae* Mucin-1 cDNA Is Co-Administered with Interleukin-12 cDNA *Int. J. Biol* ***5(2)***: 46-56.

Abstract

The midgut of the malaria - transmitting mosquito, *Anopheles gambiae,* can be targeted by vaccine-induced host immune factors that kill the mosquito after it ingests immunized host blood. The *An. gambiae* mucin 1 protein (AgMuc1) is expressed on the mosquito midgut where it likely functions in protecting the midgut epithelium from its own secreted digestive enzymes, toxic substances and pathogenic microbes taken in with the blood meal. Immunization of mice with plasmid containing the AgMuc1 gene has been shown to induce mosquitocidal immune responses and the immunological characteristics of this immunity suggested that it could be cell mediated. In this paper, we co-immunized mice with AgMuc1 cDNA and plasmid containing Murine granulocyte-macrophage stimulating factor (GM-CSF) or Interleukin 12 (IL-12) cytokine cDNA in order to further potentiate the mosquitocidal immune response and better define the nature of this mosquitocidal immunity. While co-immunization with GM-CSF cDNA failed to increase anti-mosquito immunity (Chisq=3.3 on 1 degree of freedom, p=0.068), a significantly enhanced mosquitocidal effect was observed from mice co-immunized with AgMuc1 and IL-12 cDNA (Chisq=39.1 on I degree of freedom, p=4.06e-10). Furthermore, the cumulative survival of the blood fed mosquitoes surviving to day 7 in the AgMuc1/IL-12 co-immunized group highly correlated negatively with the anti-mucin IgG1 antibody subtype levels (Pearson correlation coefficient r = -0.782) suggesting that the mosquitocidal immunity induced by AgMuc1 cDNA immunization could be IgG1 antibody subtype mediated.

2. **Injera, W.E.; Gicheru, M. M.; Kabiru, E. W.; Githure, J. I. & Beier, J. C.** (2013). Immunopathological features developing in the mosquito midgut after feeding on *Anopheles gambiae* Mucin-1 / Interleukin-12 cDNA immunized mice. *Int. J. Morphol.* ***31(1)***:329- 337.

Abstract

The mosquito midgut is the organ into which the blood meal passes and in which, within a peritrophic membrane secreted by the epithelium, the blood is retained during digestion and absorption. The mosquito midgut is lined with an actin filled microvilli that are exposed to the harsh environment of the gut lumen such as food particle abrasion, digestive hydrolases and attack by pathogens and parasites that are taken in by the blood meal. These microvilli are protected them from these effects by the peritrophic matrix, the glycocalyx and the mucin proteins that line their epithelial surfaces. Immunization of BALB/c mice with AgMUC1/IL-12

cDNA has been shown to kill mosquitoes when fed on these mice. Mucin is one of the proteins produced in the mosquito midgut after a blood meal. The fine structure of the mosquito midgut epithelium interacting with immune factors such as antibodies or immune cells is of special significance for interpreting early events in the interaction between the mosquito midgut lining and the specific immune components present in the blood of AgMUC1/IL-12 cDNA immunized BALB/c mice. Following bright light microscopy, scanning electron and transmission electron microscopic observations of the features seen in mosquito midgut sections from *Anopheles gambiae* mosquitoes fed on BALB/c mice immunized with AgMUC1/IL-12 cDNA, the most likely immune mechanisms responsible for mosquito killing could be cell mediated, most likely antibody dependent cellular cutotoxicity (ADCC). Both necrotic and apoptotic processes that could be the cause of mosquito death were seen to take place in the cells lining the midgut epithelium.