PRIME BOOST VACCINATION WITH VIRAL VECTORS TARGETING APICAL MEMBRANE ANTIGEN 1

Thesis submitted for the degree of Doctor of Philosophy
Trinity Term 2009

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ABSTRACT

Apical membrane antigen 1 (AMA1) is a leading candidate vaccine antigen against blood stage malaria and several clinical trials using mostly protein-in-adjuvant vaccines have shown limited success. This thesis describes the development of recombinant adenoviral (AdHu5) and poxviral (MVA) vectors encoding AMA1 from Plasmodium chabaudi murine parasites. In this murine malaria model, AdHu5 and MVA encoding AMA1 when used in a heterologous prime boost regime showed excellent immunogenicity, both humoral and cellular. The vaccination regime was protective against blood stage challenge and both antibodies and CD4+ T cells found to be important for vaccine induced blood stage protection. In parallel to this novel P. falciparum vaccines encoding AMA1 were also developed and administered in a similar prime boost regime to mice and rabbits. The vaccination regime induced cellular immune response and high titre antibodies against AMA1 and these antibodies showed growth inhibitory activity against the homologous parasite strain. In an effort to overcome the issue of antigenic polymorphism and to circumvent pre-existing immunity to human adenovirus, biallelic simian and human adenoviral vectors and MVA encoding AMA1 vaccines were also developed and administered to mice and macaques. These vectors also induced high titre antibodies and the serum from macaques was found to have growth inhibitory activity. These vaccine candidates are now being taken forward to Phase I/II clinical trials in Oxford. This work also described the attempt to improve MVA as a antibody inducing vector to allow better antibody mediated immunity to blood stage malaria.

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TABLE OF CONTENTS

ABBREVIATIONS

aa	Amino acid
ACT	Artemisinin-based combination therapy
ADCI	Antibody-dependant cellular inhibition
AdHu5/Ad	Human adenovirus 5
AIDS	Acquired immunodeficiency syndrome
AMA1	Apical membrane antigen 1
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
CAR	Coxsackie adenovirus receptor
CEF	Chicken embryo fibroblast
CPE	Cytopathic effect
CD	Cluster of differentiation
CMV	Cytomegalovirus
CSP	Circumsporozoite protein
CTL	Cytotoxic lymphocytes
DALYs	Disability-adjusted life years
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBA175	Erythrocyte-binding antigen 175
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunosorbant spot
FoxP3	Forkhead transcription factor 3
FP	Fowlpox
$\gamma\delta$	Gamma delta
GFP	Green fluorescent protein
GIA	Growth inhibitory activity
GLURP	Glutamate-rich protein
GM-CSF	Granulocyte macrophage colony stimulating factor

GNP	Gross national product
HA	Hemagglutinin
HAP2	Hook-associated protein 2
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
ICAM1	Inter-cellular adhesion molecule 1
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
i.d.	Intradermal
IFA	Immunofluorescence assay
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IPT	Intermittent preventive therapy
ITNs	Insecticide-treated nets
i.v.	Intravenous
mAb	monoclonal antibody
MDG	Millennium development goals
ME	Multiple epitope string
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
MVA	Modified vaccinia Ankara
NK	Natural killer
NYVAC	New York vaccinia virus
P.	*Plasmodium*
*Pcc*AS	*Plasmodium chabaudi chabaudi* AS
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfSUB2	P. falciparum subtilisin-like protease 2
Pfs25	*P. falciparum* P25
pfu	Plaque forming units
PPR	Pattern-recognition receptors

pRBC	parasitized red blood cell
RAG	Recombination activating gene
RBC	Red blood cell
RFP	Red fluorescent protein
RTS,S	**R**epeat NANP B cell epitope and **T** cell epitope (from CSP), hepatitis B **s**urface antigen (RTS), co-administered with HB**s**Ag polypeptide (S) (GSK *P. falciparum* vaccine)
SEM	Standard error of the mean
SERA	Serine repeat antigen
SCID	Severe combined immunodefiency
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
TB	Tuberculosis
TGFβ	Transforming growth factor beta
Th	T helper cell
TIM	T Cell Ig- and mucin-domain-containing molecule
TLR	Toll-like receptors
TNFα	Tumour necrosis factor alpha
tPA	Tissue plasminogen activator
TRAP	Thrombospondin –related adhesion protein
T reg	T regulatory cell
VV	Vaccinia virus
WHO	World health organisation
vp	Adenoviral particles
°C	Degree Celsius

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Malaria

1.1.1 The global disease burden and economic impact

The Millennium Development Goals (MDG) were laid down at the Millennium Summit in September 2000 in the largest ever gathering of world leaders. Goal 6 was to Combat Human immunodeficiency virus/Acquired immune deficiency syndrome (HIV/AIDS), Malaria and other diseases. Malaria, HIV/AIDS and Tuberculosis (TB) account for half a billion cases and over six million deaths worldwide per annum. We are more than half way towards the 2015 target of halting the number of deaths caused by malaria and reversing its incidence. The 2008 Millennium Goals Development Report stated that malaria prevention is expanding and in Africa 16 out of 20 countries have at least tripled the use of insecticide-treated bednets but we are far from reaching the target.

Malaria is caused by parasite species of the genus *Plasmodium* and two of these species, *Plasmodium falciparum* and *Plasmodium vivax,* account for the majority of infections in humans. *Plasmodium falciparum* is responsible for most of the deaths and the majority of the morbidity caused by malaria. The symptoms of the disease are non-specific and thus the epidemiology of malaria is poorly understood. It is estimated that in Africa alone there are 200-

450 million cases of malaria in children per year and the annual mortality due to malaria is about 1 million (1-3). More than a third of the world's population live in malaria endemic regions and these countries have an annual income that is about a third of the non-endemic countries (Figure 1-1). It is estimated that the economic burden of malaria in terms of economic growth ranges from 0.25% to 1.30% of a country's per person Gross National Product (GNP). A 10% reduction in malaria was associated with a 0.3% higher economic growth in countries with intensive malaria (4). This is not surprising as the associated costs of treatment, high infant mortality, loss of income due to decrease in productivity, malnutrition, and reduced population growth all contribute to the burden of the disease. Malaria was estimated to be the cause of 45 million disability-adjusted life years (DALYs) in 2000 (5).

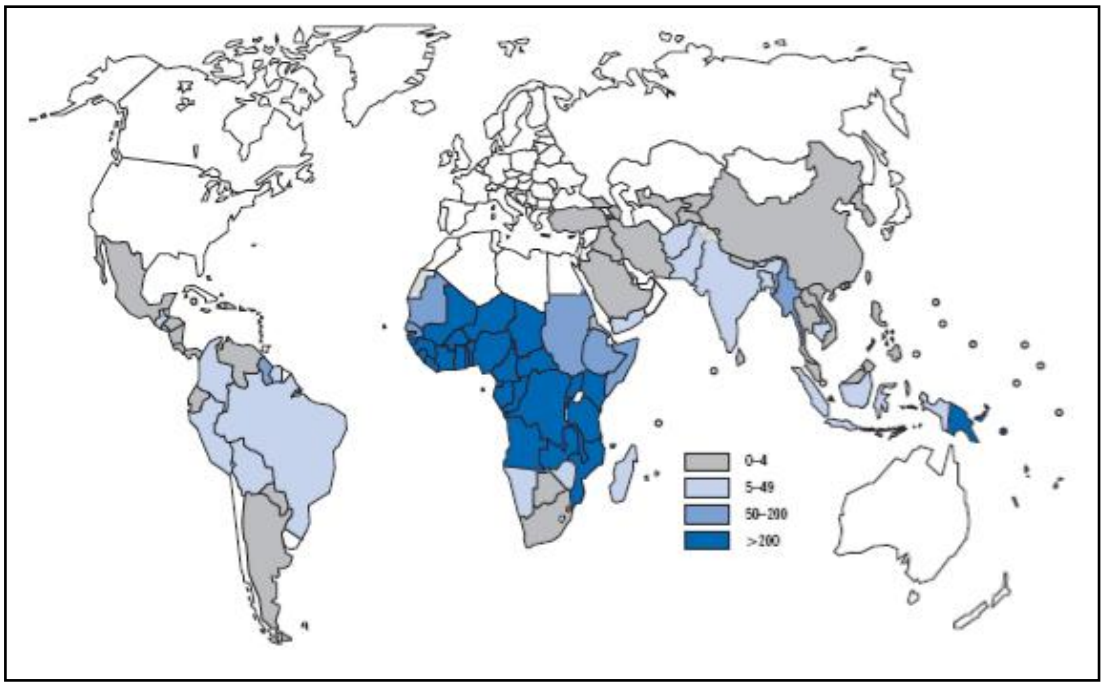


Figure 1-1: Estimated incidence of malaria in 2008

This picture is taken from the World Malaria Report 2008 and shows the estimated number of malaria cases per 1000 population in 2008. There were 247 million cases worldwide in 2008, of which 91% were due to *P. falciparum*.

1.1.2 Control Strategies

The Global Malaria Eradication Program in 1955 succeeded in eliminating malaria from Europe, North America, the Caribbean and parts of Asia and South-Central America but not in Sub-Saharan Africa that accounts for 80% of the disease burden (6, 7). The aspiration of global eradication is renewed after a lapse of more than 40 years and is back on the global health agenda (8). The World Health Organisation (WHO) launched the Roll Back Malaria

Initiative in 1998 to accelerate the control of malaria. The two control strategies that have been widely used to control malaria are i) avoiding contact with the vector by the use of insecticide treated bed nets and ii) use of chemotherapeutic treatment before and after infection. These are the two interventions that were used after World War II to eliminate malaria in sub-tropical countries. Sadly, in countries where malaria is still endemic there are logistical and political problems in implementing these public health interventions and these remain a huge barrier to controlling this disease. In the “eradication era” the environmental control of mosquitoes by draining of breeding grounds and the use of DDT helped in the elimination of malaria but these have not been able to be implemented in tropical countries at the population level.

Insecticide-treated nets (ITNs) have been an important component of the global malaria control strategy since the mid-1990s. There are as many as 81 trials conducted in endemic areas showing the benefits of ITNs in children and adults in Africa, Latin America, Thailand, Pakistan and Iran (9). ITNs have been shown to reduce clinical episodes by 50% in a range of transmission intensities (10). Studies of ITN use by pregnant women have shown significantly reduced parasitaemia and maternal anaemia, and increased birth weight (11-15). They have also been shown to reduce child mortality and it is estimated that approximately 370,000 deaths could be avoided annually if every child in Africa slept under an ITN (9). The inherent difficulties with the use of ITNs are the mass coverage and the growing resistance to pyrethroid insecticides (16, 17). The ITN coverage and individual behaviour affects the

efficacy of the use of ITNs as a control strategy. According to the MDG report for 2008, sub-Saharan African countries showed an increase in ITN use among children under five, but overall insecticide-treated net use still falls short of the global targets. Recently it has been shown that the use of ITNs results in a decrease of naturally acquired immunity and this could be detrimental in the medium term and shift the burden of mortality to older children (18).

Anti-malarial chemotherapy is the most cost effective means of malaria control available to date (19). Chloroquine has been the main prophylaxis for malaria but the failure of chloroquine universally led to the search for new replacement drugs, but the parasite has also successfully gained resistance to quite a few of these drugs. The discovery of artemisinin and the development of artemisinin-based combination therapy (ACTs) have provided some hope (20, 21). ACTs provide mutual protection against resistance and also reduce transmission but there is limited implementation due to the high cost associated with them (20, 22, 23). Due to the cost, counterfeit drugs are in use and uncontrolled use of these drugs contributes to the further development of resistance and leads to increased mortality (24-27). Intermittent preventive therapy (IPT) has been one of the main strategies in the past few years in the management of malaria. This involves the administration of a course of anti-malarial drug regardless of infection status to clear current infection and also to prevent new ones. This was initially designed for pregnant women (IPTp) but later on extended for use in infants

(IPTi) and children (IPTc), as the rate of clinical disease is higher in young children (28-33).

There are several success stories to show that the expansion of prevention programmes and improved access to effective anti-malarial drugs can reduce malaria cases and deaths associated with the disease, but in general achieving this remains a major public health challenge. The national distribution of ITNs in Vietnam dramatically decreased the number of malaria cases in 1991. After the introduction of ACTs and vector control in South Africa the number of cases and deaths due to malaria fell by 80%.

1.2 Vaccines as a control strategy for infectious diseases

Vaccines remain one of the most effective public health interventions to reduce morbidity and mortality. The worldwide eradication of smallpox was due to vaccination and to date no other public health intervention has achieved similar success. In 1796 Edward Jenner was the first to demonstrate that the intradermal inoculation with cowpox could confer protection against smallpox and this is where “vaccination” gets its name. “Vacca” in Latin means cow and the first vaccine was derived from a virus affecting cows (34). Later on in the late nineteenth century Louis Pasteur’s insight into pathogen attenuation and inactivation built the pillars of vaccine development, which still

stand strong today (35). In 1952, polio paralysed more than 20,000 people and after successful vaccination in 2002 there were no further cases of polio in the United States. The other diseases against which vaccination has been an effective control strategy are *Haemophilus* Influenza type b (Hib), Rubella, Measles and Pertussis to name a few. The US Food and Drug Administration (FDA) license a number of new vaccines each year with major potential for controlling infectious diseases such as RotaRix by GlaxoSmithKline against rotavirus diarrhoea, the conjugate vaccine Prevnar developed by Wyeth Vaccines against seven strains of pnuemococcus (36), Gardasil by Merck against Human Papillomavirus and many others. Although there are many success stories there are diseases for which vaccine development has still not succeeded, despite extensive efforts (37).

The WHO uses DALYs as a way to quantify and compare the cost-effectiveness of various public health interventions. Childhood immunization consistently ranks as the most cost-effective intervention available, costing between USD $1-5 per DALY averted in Sub-Saharan Africa. This compares to USD $377 per DALY averted for HIV and AIDS treatment. Inspite of having effective vaccines for some diseases, there are other public health hurdles faced due to the expensive technology used in manufacture, mass production, distribution and funding issues which can all limit vaccine coverage. International organisations like the Global Alliance for Vaccines and Immunisation (GAVI) are working to prevent millions of deaths worldwide by increasing vaccination coverage, and since its establishment in 2000 it has averted an estimated 3.4 million deaths. The WHO estimated that 1.4 million

children died in 2002 of vaccine-preventable diseases (World Health Organisation. 2006. (http://www.who.int/). In order for these vaccines to reach every individual in a developing country, huge amounts of funding and international cooperation are required.

In the case of malaria a vaccine is urgently needed as vaccine-induced immunity in an endemic area could significantly reduce overall transmission, by inducing herd immunity together with other available interventions like ITNs and ACTs. The vaccine would have to be cheap, easy to manufacture and administer and requires international initiatives to be deployed in developing countries after analysing the cost-benefit ratio when an effective vaccine is available.

1.3 Malaria and the human immune system

1.3.1 Life cycle of the parasite

The term malaria in medieval Italian means “bad air” and the disease was formerly called marsh fever due to its association with swamps and marshland. A French army doctor Charles Louis Alphonse Laveran in 1880 was the first to observe parasites in the red blood cells of patients suffering from malaria and thus discovered that malaria is caused by the protozoan parasite *Plasmodium* (38). It was almost two decades later in 1898 when Sir Ronald Ross (who was then working at the Presidency General Hospital in Calcutta) discovered that mosquitoes transmit malaria (39).

In humans malaria is caused five species of *Plasmodium - P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi. P. falciparum* accounts for most of the infection worldwide and is responsible for 80% of the cases and 90% of the deaths (40).

The life cycle of *P. falciparum* is very complex, involving several stages in female *Anopheles* mosquitoes and humans (Figure 1-2). Different antigens are expressed during the different stages of the life cycle thus making it a very complex process. Recently, after the completion of the malaria genome project, some antigens have been found to be expressed at multiple stages of infection (41). The life cycle can be divided into three main stages:

The **Pre-erythrocytic stage** is initiated by the bite of an infected mosquito. The sporozoites in the mosquito's salivary gland are injected during a blood meal into the circulation of the host. Within minutes of infection the sporozoites migrate to the liver. These sporozoites infect the liver cells, multiply asexually for 6-7 days and develop into liver-stage trophozoites and schizonts containing merozoites. During all this development within the liver cells there are no symptoms seen in the human host. The host cell then ruptures to release thousands of merozoites into the systemic circulation, thus beginning the erythrocytic stage of the infection.

The **Blood-stage** or erythrocytic stage of the life cycle begins once the merozoites start infecting the red blood cells. These merozoites follow a cycle of repeated infection of red blood cells, asexual multiplication and rupture of

red blood cells to continue the cycle. All the symptoms associated with the disease are caused by this stage, hence the cyclical fever that corresponds to RBC lysis as schizonts rupture to release new merozoites. This occurs every 48 hours in *P. falciparum* infection. This can be then controlled by the host immune system or drug cure, but if not controlled can lead to serious complications and death.

The **Sexual stage** of the life cycle occurs in the mosquito. Some of the blood-stage merozoites differentiate into male and female gametocytes. These gametocytes differentiate into male and female gametes only once taken up by mosquitoes where they fuse in the mid-gut. The fusion leads to the formation of the ookinete that develops into an oocyst in the gut wall. The oocyst ruptures to release sporozoites that migrate to the salivary glands of the mosquito ready to be transmitted to another human host.

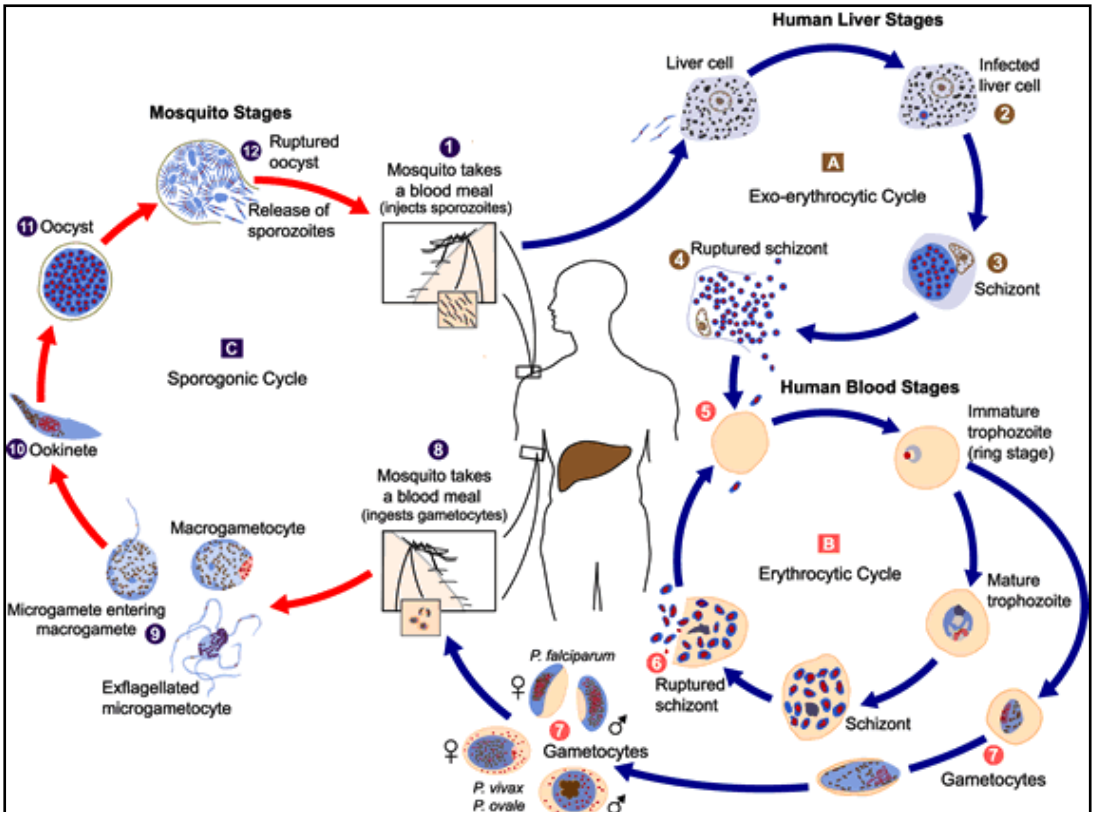


Figure 1-2: Life cycle of *Plasmodium falciparum*.

This picture has been taken from the Centre for Disease Control and Prevention website. It shows the life cycle of the malarial parasite, which involves two hosts: human and mosquito. Stages 1-7 takes place in the human host (liver and blood stage) and stages 8-12 in the mosquito (sexual cycle).

1.3.2 Pathogenesis

The asexual erythrocytic cycle of *Plasmodium* leads to the rapid onset of symptoms and is responsible for all of the morbidity associated with malaria. The symptoms associated with mild-moderate clinical malaria are fever, headache and nausea, which coincide with the rupture of erythrocytic

schizonts and release of merozoites that then invade other red blood cells. *P. falciparum* and *P. vivax* are the major cause of clinical malaria but *P. vivax* is rarely fatal. Both *P. falciparum* and *P. vivax* cause severe anaemia. *P. vivax* only invades Duffy blood group positive RBCs and so in West Africa and Papua New Guinea where most people are Duffy blood group negative there is no *P. vivax* malaria. *P. falciparum* has other redundant pathways to overcome this and can bind to Duffy blood group negative RBCs. *P. falciparum* causes severe disease like cerebral malaria, hypoglycaemia, respiratory distress and metabolic acidosis, which causes fatalities (42-44).

The *P. falciparum* specific *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules are thought to be responsible for the aggregation and adhesion of *P. falciparum* infected RBCs. This PfEMP1 molecule is encoded by polymorphic *var* genes, whose transcriptional changes lead to antigenic variation (45). This single molecule mediates parasite binding to a diverse range of host receptors expressed in various organs like the heart, lung, brain, liver, kidney, endothelium and placenta, which is associated with respiratory distress, cerebral and pregnancy malaria (46-48). Adhesion also protects the parasite from being cleared away rapidly by the spleen (49).

The pro-inflammatory cytokine response, which has a role in blood-stage protection, is also thought to be responsible for severe malaria. Pro-inflammatory cytokines like TNFα have been shown to increase the expression of ICAM1 in the brain endothelial cells in *in vitro* studies (50). Also, in cerebral malaria vessels in the brain exhibit increased expression of ICAM1

(51). The glycosylphosphatidylinositol (GPI) anchor from parasite proteins MSP1 and MSP2 has been shown to induce a pro-inflammatory cytokines response (52) and antibodies to the GPI anchor are associated with a lack of disease in adults (53). Mediators like nitric oxide have also been proposed as contributors to cerebral malaria (54).

1.3.3 The human immune system

1.3.3.1 Innate immune response

The innate non-specific immune mechanisms provide highly effective first-line defence against invading pathogens and also promote adaptive immune response. Professional APC's possess a wealth of pattern-recognition receptors (PRRs) that have evolved to recognise evolutionarily conserved pathogen-associated molecular patterns (PAMPs) (55). These include the TLRs and C-type lectins as PRRs on the cell surface (56, 57), and cytosolic PRRs such as nucleotide-binding oligomerisation domain (NOD) proteins and the caspase recruitment domain (CARD)-helicases (58). Due to this interaction the APC is activated and a cytokine response is generated. The ensuing cytokine profile is tailored to instruct the adaptive system in the nature of the response required to eliminate a particular class of pathogen (59). The activated DCs migrate from the periphery to the secondary lymphoid system bearing peptide antigen in the context of relevant MHC molecules, and present the processed antigen to naïve T lymphocytes. The activated T cell undergo rapid clonal expansion into an armed effector T cell population

that migrates into the periphery. Following expansion, this population contracts to form circulating effector memory (TEM) and central memory (TCM) T cell populations (60).

1.3.3.2 CD4+ T cells

T-helper CD4+ cells recognise antigens presented by antigen-presenting cells (APCs) like macrophages, dendritic cells (DCs) and B cells on Major Histocompatibility Complex (MHC) class II molecules. These antigens are exogenous antigens that are taken up by the APCs from outside the cell. When macrophages engulf material they are contained in a membrane-bound phagosome. The proteins contained in the phagosome are then degraded to produce short peptides that can be loaded into the groove of an MHC class II molecule. Effector CD4+ T cells can be divided into four subsets i) T helper type-1 (Th1), ii) T helper type-2 (Th2) iii) regulatory T cells (T reg) and iv) Th17 cells depending on the markers these cells express and their functional role in terms of the cytokines that they produce.

Naive CD4 T cells differentiae into Th1 cells in the presence of IL-2 and IFNγ secreted by dendritic cells (DCs) and natural killer (NK) cells. The main action of Th1 cells is activation of macrophages displaying peptides to CD4+ T cells. They secrete IFNγ, the main macrophage activating cytokine and TNFα, which also activates macrophages and is directly cytotoxic to some cells. These cytokines mediate the pro-inflammatory responses, induce proliferation

of effector T cells, promote isotype class switching of B cells and stimulate NK cells.

Naive CD4+ T cells activated in the presence of IL-4 differentiate into Th2 cells. Th2 cells secrete IL-4, IL-5, IL-9 and IL-13. They also express the CD40 ligand on their surface. These activate B cells and inhibit macrophage activation.

T regulatory cells as the name suggests produce anti-inflammatory cytokines such as IL-10 and TGFβ thus suppressing and regulating the pro-inflammatory immune response. Mice lacking either cytokine are prone to autoimmune disease. They also express high levels of the α chain of the IL-2 receptor (CD25) and forkhead transcription factor3 (FoxP3) (61).

Th17 cells are often the first subset of effector T cells generated in response to infection. In the absence of IL-4, IFNγ or IL-12, IL-6 from dendritic cells along with TGFβ can induce differentiation of naive CD4 T cells into Th17 cells. These cells migrate to distant sites of infection and release IL-17. The IL-17 receptor is expressed ubiquitously on fibroblasts, epithelial cells and keratinocytes which then secrete a range of chemokines, which directly recruit neutrophils and also increase the production of neutrophils and macrophages in the bone marrow by the action of granulocyte macrophage colony-stimulating factor (GM-CSF) (62).

1.3.3.3 CD8+ T cells

CD8+ cytotoxic lymphocytes (CTL) recognise peptides presented on MHC class I molecules. These are peptides derived from endogenous proteins that are degraded by all nucleated cells in their cytoplasm. Mature dendritic cells that have various co-stimulatory molecules activate naive CD8+ T cells. These CTLs then produce IL-2, which drives their proliferation and differentiation. This occurs in a limited number of viral infections but in most cases CD4 T cell help is required to up-regulate B7 expression on dendritic cells which acts as a co-stimulatory molecule to activate CD8 T cells. Effector CD8+ T cells induce apoptosis or programmed cell death in “self” cells by the production of cytotoxic proteins like perforin, granyme and granulysin, or via the cell-surface receptor-ligand interactions, such as those of the TNF family, particularly Fas and Fas ligand. As well as killing the host cell the apoptotic mechanism can destroy cellular and viral DNA by the action of nucleases. They also secrete cytokines like IFNγ, TGFβ and TNFα that recruit and activate macrophages and induce the production of IL-12, which feeds into the positive feedback loop by up-regulating IFNγ production by T cells. IFNγ can also induce increased expression of MHC class I molecules in infected cells.

1.3.3.4 B cells

B cells are highly efficient APCs and bind to antigens via their B cell receptor (BCR) and internalise the antigen by receptor-mediated endocytosis. The

internalised protein is processed to peptide fragments and presented as a peptide: MHC class II complex. This pathway of antigen presentation allows B cells to be targeted by antigen-specific CD4+ T cells that drive the differentiation of B cells and initiate the process of clonal expansion. Activated B cells either become short-lived plasma cell or undergo somatic hypermutation and affinity maturation to become long lived plasma cells. Plasma cells secrete antibody and constitute the primary response on first exposure to a pathogen. Activated B cells secrete an initial burst of IgM, before undergoing isotype switching to IgG, IgE or IgA – dependent on the instructive cytokine profile and CD4+ T cell help. Class switching does not affect antibody specificity but alters the effector function of the antibody. Some of the B cells that have undergone class switching from memory B cells which persist in the body for years and mount an effective secondary response if they encounters the same antigen.

1.3.4 Targets for vaccination

Malaria subunit vaccine development has focussed mainly on targeting a single or limited number of antigens to induce immunity. Vaccination aims to prevent disease, to control proliferation and survival of the parasite and to prevent transmission.

1.3.4.1 Pre-erythrocytic vaccines

A pre-erythrocytic vaccine is the most attractive strategy and was the main focus of malaria vaccine development for a long time. These vaccines can induce responses that target antigens expressed on the surface of the sporozoites thus preventing them from invading hepatocytes, and/or destroy parasites in infected hepatocytes. The two leading candidate antigens for a pre-erythrocytic vaccines have been the circumsporozoite protein (CSP) and thrombospondin-related adhesion protein (TRAP). CSP is probably the better characterised of the two and is expressed on the extracellular sporozoite and also the intracellular hepatic stages (63). TRAP has been shown to be important for gliding motility of the parasite and invasion of liver cells (64). Early experimental vaccination studies have shown that immunisation with irradiated sporozoites multiple times can lead to protection of 90% of human volunteers (65), but this is possibly not a feasible approach and so most of the subunit vaccines target single antigens expressed on the sporozoite. Vaccines targeting CSP and TRAP have generated potent humoral and cellular immunity both in animal models and humans but failed to achieve the same level of efficacy as irradiated sporozoites (66-75). RTS/S, the leading malaria vaccine candidate in terms of its stage in clinical development is a hybrid of the middle and carboxy terminus of CSP fused to hepatitis B surface antigen (HBsAg) and is expressed together with unfused HBsAg in yeast and is administered with an adjuvant (68). In a recent clinical trial in Kilifi, Kenya, and Korogwe, Tanzania, RTS/S administered with AS01E adjuvant showed an efficacy of 53% in children (76).

1.3.4.2 Blood stage vaccines

Blood stage vaccines aim to mimic immunity that is naturally acquired in endemic areas. Individuals living in malaria endemic countries develop immunity by repeated exposure to the parasite and a significant proportion of this immunity is directed to antigens expressed on the blood stage parasites or infected erythrocytes. Blood stage immunity is mostly antibody mediated (77) though recently the importance of cellular immunity to blood stage immunity has been realised (78). Proteins on the surface of merozoites and in the apical organelles that play a role in the invasion of red blood cell are candidate antigens for the development of a blood stage vaccine (Figure 1-3).

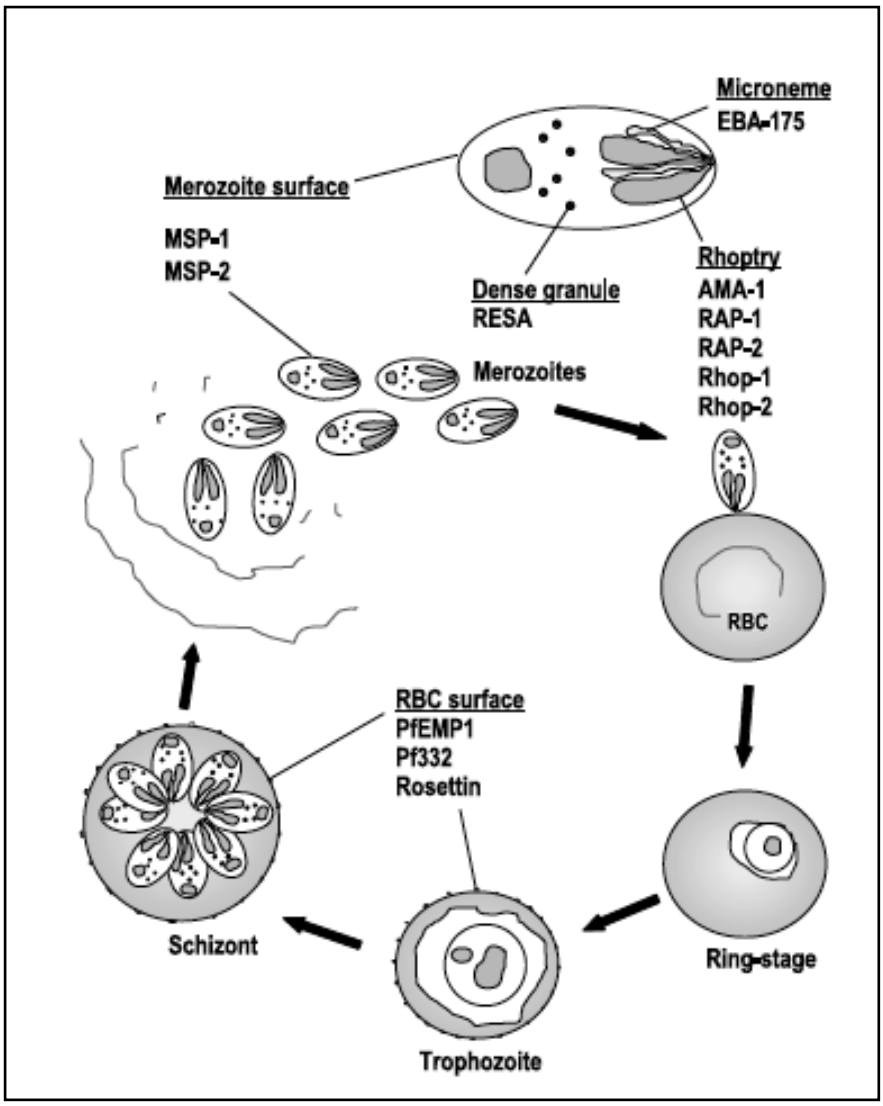


Figure 1-3: Location of target antigens for blood stage vaccines.

This picture has been taken from a review article (79). This shows the location of the target antigens of protective immune response to blood stage malaria. Erythrocyte binding antigen (EBA); Merozoite surface protein (MSP); *P. falciparum* erythrocyte membrane protein1 (PfEMP1); Rhoptry associated protein (RAP); Ring-infected erythrocyte surface antigen (RESA).

1.3.4.3 Transmission blocking vaccine

Transmission blocking vaccines target the sexual stage antigens and prevent mosquitoes (that have fed on an infected individual) from transmitting the parasite to another individual. This type of vaccination does not protect the vaccinated individual directly but instead contributes to protection of the community by achieving herd immunity. This vaccination strategy has been a low priority but in recent years the renewed interests in eradication of malaria makes it very significant. The leading candidate for a transmission-blocking vaccine is Pfs25, which is expressed on the surface of ookinetes and antibodies against Pfs25 block oocyst formation in mosquitoes (80). The others include Pfs230 and Pfs48 expressed by gametocytes and zygotes (81, 82). A male specific sterility gene, HAP2 that is essential for the fusion of gamete surface membranes and antibodies to HAP2 have also shown transmission blocking activity (83). Antibodies against the *Anopheles gambiae* aminopeptidase N (AgAPN1), on the mosquito midgut luminal surface strongly inhibit both *Plasmodium berghei* and *Plasmodium falciparum* development in different mosquito species (84). There are thus several identified transmission blocking vaccine targets, which are currently being tested further.

1.4 Current status of Malaria Vaccine Development

The first gene encoding the malaria vaccine antigen CSP was cloned 25 years ago (85), closely followed by the first blood-stage vaccine antigen MSP1 (86) and to this day these still remain the focus of vaccine development. The very first report of protection was by exposure to radiation-attenuated *P. falciparum* sporozoites, which formed the foundation of vaccine development against malaria and showed that an effective malaria vaccine was a realistic aim (87). Since then several new approaches to vaccination have evolved including subunit vaccines targeting single antigens and also combining antigens from different stages. Several antigens from a single stage of the parasite's life cycle have also been combined to try and increase the potency of the vaccine.

Whole organism approaches have some advantages over subunit vaccines, as they do not require determination and selection of antigens and provide a vast array of antigens in the correct conformation. However the irradiated-sporozoite vaccination regime requires thousands of mosquitoes (65) and with whole blood-stage parasites there are safety concerns, vaccine delivery route issues and significant difficulties in large-scale production and cryopreservation.

The most advanced malaria vaccine in terms of clinical development is RTS, S which is a subunit based on the CSP antigen and recently it has shown 56% efficacy against malaria in children when administered with AS01E as the adjuvant (76). Other vaccine strategies against pre-erythrocytic antigens have been prime-boost vaccination with DNA and viral vectors encoding ME-TRAP that is based on a multiple epitope string (ME) taken from six pre-erythrocytic antigens and TRAP (75, 88, 89). In a recent clinical trial in Oxford, a heterologous prime-boost regime with the simian adenoviral vector AdCh63 and Modified Vaccinia Ankara (MVA) expressing ME-TRAP induced partial protection against malaria (O'Hara et al. unpublished).

Blood-stage malaria vaccine development has mainly focussed on protein-in-adjuvant formulation targeting antigens mostly on the merozoite surface with known important functions. The leading candidates are MSP1 and AMA1 followed by several others like merozoite surface protein 2 (MSP2), merozoite surface protein 3 (MSP3), erythrocyte-binding antigen 175 (EBA175), glutamate-rich protein (GLURP) and serine repeat antigen 5 (SERA5). There have been several clinical trials with these antigens (90). The limited success of such candidates indicates the need for a multivalent vaccine against blood stage comprised of several different antigens or several alleles of the same antigen or to evaluate new antigens. There have been several combination vaccines developed and tested composed of two or more blood stage antigens in order to overcome antigenic diversity (91-94).

In addition to this, multistage malaria vaccines have also been tested in humans that target different stages of the parasite's life cycle. These include SPf66, which showed no evidence of protection in Africa though the initial field trials were promising. NYVAC Pf7, which was a recombinant vaccinia virus expressing seven antigens (CSP, Sporozoite surface protein 2, Liver stage antigen 1, MSP1, AMA1, SERA and Pfs25) was able to protect 1 of 35 volunteers with a time to delay in parasitaemia seen in the others (95).

RTS,S is now entering phase III studies but the challenges ahead are to develop vaccines that have better efficacy than RTS,S. These could be used in combination with RTS,S or alone, to further improve protection and to finally work towards the eradication of malaria.

1.5 Blood stage Immunity to Malaria

Naturally acquired immunity to malaria does develop in individuals living in malaria-endemic countries over a period of time and protects against clinical disease (96). This requires repeated exposure to the parasite but mostly this immunity does not provide sterile protection and immune memory like that seen with other diseases such as yellow fever, diphtheria or other bacterial diseases where a single infection confers life-long protection from subsequent re-infection. When these individuals are drug cured or move away from an endemic country they become re-infected (97). Individuals living in endemic

countries also have asymptomatic parasitaemia. The immune response generated against malaria is also short-lived and species, strain and variant specific (79). More than a century ago Robert Koch reported that repeated life-long exposure to malaria is required to maintain protective immunity against infection. This is in contrast to naturally acquired protection to severe disease, which persists for a long time without parasite exposure (98-102).

1.5.1 Innate immune response

Innate immune responses play an important role in immunity to blood stage malaria. In rodent models it has been shown that bone marrow derived DCs and macrophages isolated from immune mice can present malaria antigens to T cells (103). In the *P. yoelii* model there is also an upregulation of MHC class II molecules on splenic DCs, macrophages and B cells which can then process and present antigens to T cells (104). It has also been suggested that mononuclear macrophages phagocytose infected erythrocytes in the absence of cytophilic or opsonising malaria-specific antibodies (105). $\gamma\delta$ T cell expansion has been reported in acute infection with *P. falciparum* (106) and *P. falciparum*-activated $\gamma\delta$ T cells produce a lot of IFNγ and show anti-parasite function (107). In acute blood stage infection with *P. chabaudi adami* CD4+ T cell dependent expansion of $\gamma\delta$ T cells has been found, but these cells do not seem to be crucial for blood stage immunity (108-110). Natural killer (NK) cells mediate cytotoxicity and IFNγ production and are essential for blood stage immunity (111). Depletion of NK cells results in higher parasitaemia and less efficient resolution of infection in the non-lethal *P. chabaudi chabaudi* AS

infection in C57Bl/6 mice (112). Thus the innate and adaptive immune response to malaria are inextricably linked as the cytokines produced by cells of the innate system modify the outcome of the adaptive response (Figure 1-4).

1.5.2 Humoral immune responses

B cells and antibodies play a crucial role in immunity to blood stage malaria. Naturally acquired immunity is largely dependent on the acquisition of specific and protective antibodies against the polymorphic antigen PfEMP1 (77). Purified IgG transfer from immune African adults to naive Thai patients reduces parasite load and clinical symptoms (113). The antibody level and specific isotypes against blood stage antigens have been shown to correlate with protection in mice, monkeys and humans (114-119). In humans cytophilic antibodies of IgG1 and IgG3 isotypes are important antibodies for protection against *P. falciparum* (120). These antibodies bind to the antigen and activate the complement pathway or killer cells, resulting in cell lysis. In endemic areas individuals with increased levels of IgG1 and IgG3 have lower parasitaemia and reduced risk of malaria pathology (121, 122). Antibodies directed against surface proteins of merozoites either block RBC invasion or make them susceptible to phagocytosis via Antibody-dependant cellular inhibition (ADCI). These antibodies bind to phagocytes via their Fc receptors and lead to inhibition of parasite growth (113, 123-125). Antibodies also initiate parasite clearance by opsonization or complement-mediated damage (126). Malaria-specific serum antibody can also bind to merozoites and form an immune

complex leading to agglutination and inhibition of merozoite dispersal (127). Not all antibodies to blood stage antigens however are protective. Some mAbs to MSP1 have been shown to block action of protective antibodies to MSP_{19}, which inhibit erythrocyte invasion and prevent MSP1 secondary processing (128).

1.5.3 Cellular immune responses

The role of T cells in mediating protection to blood stage malaria has been recently of increasing interest, but is still not well understood in humans. In mice there are several studies showing the importance of T cells in blood stage immunity. Severe combined immunodeficient (SCID) mice reconstituted with T cells from immune donors suppress the growth of *P. chabaudi* parasites suggesting that T cells contribute to immunity (129). Mice challenged with sporozoites and drug cured develop blood stage immunity characterised by IFNγ producing CD4+ T cells (130).

Recently a similar study in humans where volunteers were exposed to bites of mosquitoes once a month for 3 months and drug cured showed that protection was associated with multifunctional effector memory T cells to blood stage parasites. These multifunctional T cells produced IFNγ, TNFα and IL-2 when stimulated with pRBCs (131). In another study in humans, repeated low dose exposure of malaria-naive volunteers to blood-stage *P. falciparum* parasites followed by drug cure induced sterile immunity against a blood-stage challenge. There were no detectable antibodies against the parasite,

and instead the protective immune response was associated with a proliferative CD4+ and CD8+ T cell response and induction of Th1-type cytokines (78).

CD4+ and CD8+ T cells from non-exposed donors have been shown to inhibit *P. falciparum* growth *in vitro* (132). T cell epitopes in AMA1 induce a higher magnitude of T-cell proliferative response in Kenyan residents who are exposed to malaria, but fail to do so in malaria-naive volunteers (133). The degree of *in vitro* proliferation of PBMCs isolated from malaria-exposed individuals to blood stage antigens like MSP1 correlates with the number of previous malaria episodes (134). Thus, cellular immunity plays an important role in immunity to malaria.

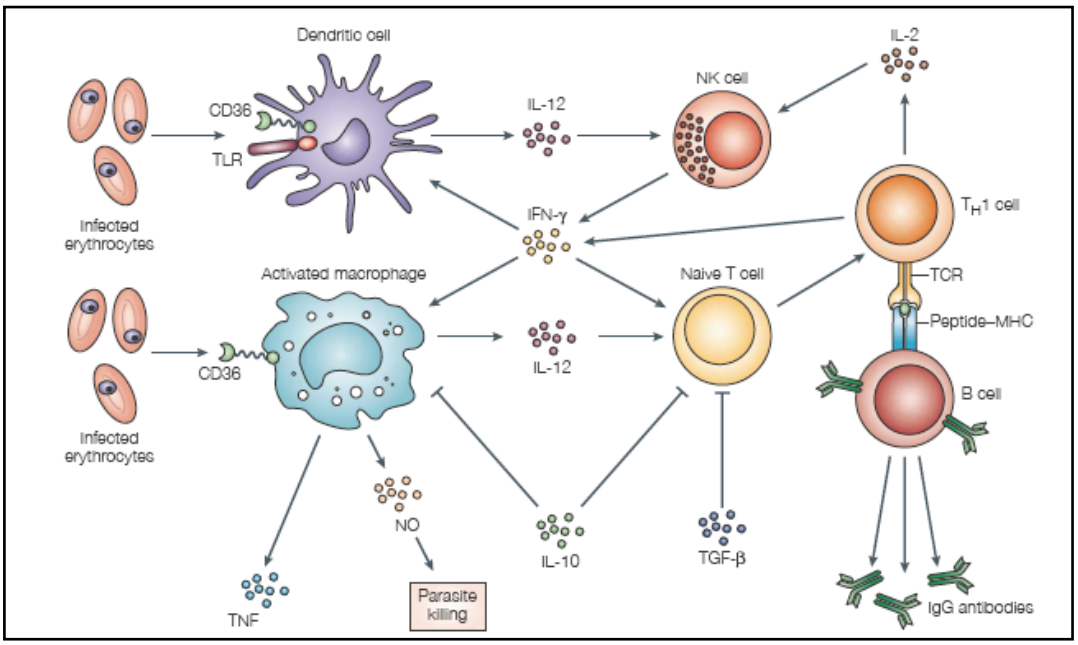


Figure 1-4: Linking the innate and adaptive immune systems.

This picture has been taken from Innate Immunity to Malaria, Nature Reviews Immunology (102). It shows the link between the innate and adaptive immune response to malaria. Parasite ligands are recognised by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, and dendritic cells mature and migrate to the spleen. Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, costimulatory molecules and the production of cytokines, which induce the differentiation of T helper 1 (TH1) cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli. Cytokines such as IL-10 and transforming growth factor-β (TGF-β) negatively regulate both innate and adaptive responses.

1.6 Mouse models for blood stage malaria

Mouse models of malaria serve as an important tool to dissect the mechanisms of blood-stage immunity. These models have not only been used to study the immune responses generated, but have also been used to test vaccine candidates and find correlates of protection. Human malaria parasites cannot infect mice and rodent malarias lack many important antigens and properties of *P. falciparum* so though these models cannot be used to directly assess vaccine efficacy, they can be used to select vaccine candidates for further development.

The four models that have been used are *P. chabaudi, P. yoelii, P. berghei* and *P. vinckei*. The *P. chabaudi* model will be discussed in section 1.7. In the *P. yoelii* model antibodies have been shown to be important in blood stage immunity. Passive transfer of mAbs to blood stage antigens like AMA1 and MSP1 can confer protection against lethal blood stage challenge (135-137). Protective efficacy induced by recombinant $MSP1_{19}$ vaccines with Alum correlates with IgG titre (115). CD8+ T cells do not transfer blood-stage immunity in this model (138). CD4+ T cell help is important for antibody production and effector CD4+ T cell lines, that recognise epitopes within $MSP1_{33}$ are capable of controlling growth of lethal *P. yoelii* YM following adoptive transfer into nude and SCID mice, in the absence of detectable antibodies (139).

The mechanisms of blood-stage immunity to *P. berghei* infection are less well understood but from the literature available it appears that both CD4+ T cell responses and antibodies are required for protection. CD4+ Th1 cell lines raised against *P. berghei* pRBC can control parasite growth but ultimately do not prevent lethal infection in naive mice (140). Serum transfer from mice immunised with formalin-killed whole pRBC antigen and then challenged also confers protection in naive mice (141).

In the *P. vinckei* model CD4+ Th1 responses are required for the development of immunity against lethal infection. B cells are not important as B-cell deficient mice develop immunity in the same way as naive mice (142, 143).

1.6.1 *P. chabaudi*- Immunity to blood stage malaria

The *P. chabaudi* model has been the most extensively dissected in terms of immune responses to blood stage malaria. It is a useful model to study *P. falciparum* malaria as there are many analogous antigens in this model and in most mouse strains the infection is not lethal thus giving the scope to study natural immunity. PccAS causes a non-lethal infection in resistant mouse strains and a lethal infection in susceptible mouse strains. In this model both CD4+ T cells and antibodies are required for protective immunity to blood stage malaria (Figure 1-5). There is a sequential activation of Th1 and Th2 cells and B cells are required for this switch (144, 145). Mice lacking B cells are able to control the acute infection and develop a chronic parasitaemia and

transfer of B cells can resolve this (144, 146). The proinflammatory cytokines IFNγ, TNFα and IL-12 are crucial for the control of early parasitaemia and IFNγ has been shown to decrease and delay the course of infection (147-149). Infection of IFNγ knockout mice with PccAS results in increased morbidity and mortality. The level of IFNγ decreases as the parasitaemia declines and is replaced by Th2 cytokines like IL-4 and IL-10 (145). Administration of anti-TNFα mAb to resistant mice annuls immunity and TNFα mRNA expression in the spleen correlate with resistance to PccAS infection (150).

Th1-type cytophilic IgG2a is predominant during the acute phase of the infection followed by a Th2-type IgG1 response during the chronic phase of the infection (145). Thus in this model both CD4+ T cells and antibodies play an important role in blood stage immunity to natural infection.

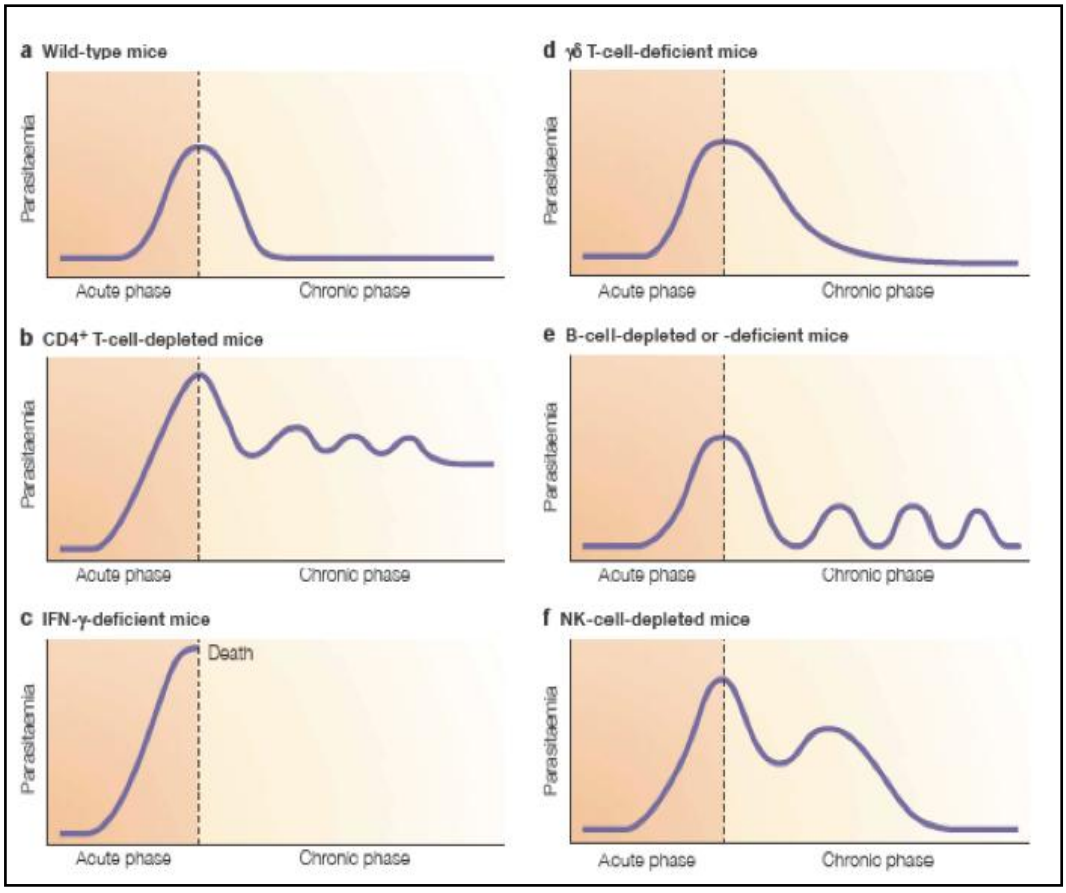


Figure 1-5: Representation of the course of PccAS infection.

This figure has been taken from Innate Immunity to Malaria, Nature Reviews Immunology (102). It shows the course of infection in wild type and mice deficient or depleted of components of the immune system. CD4+ T cells and IFNγ-dependant mechanisms control the initial acute phase of the infection (b and c) whereas the parasites are eliminated by mechanism involving both CD4+ T cells and B cells (b and e). γδ T cells do not play a role in controlling parasitaemia (d). Depletion of NK cells alters the course of infection during both the acute and chronic phases (f). The acute primary parasitaemia is followed by one or more short lasting recrudescence in CD4+ T cell depleted (b), B cell depleted (e) and NK cell-depleted mice (f).

1.7 Vaccine development against the blood stage

1.7.1 AMA1 as a vaccine candidate

AMA1 (also previously known as Pf83 or Pk66) has been one of the leading candidates for a blood stage malaria subunit vaccine. AMA1 is unique to apicomplexan parasites and is a single essential gene found in all *Plasmodium* species. In *Plasmodium* parasites AMA1 was shown to be a merozoite protein whose function could be blocked by antibodies that inhibit blood stage parasite multiplication *in vitro* (151). AMA1 is a type I integral membrane protein the bulk of which forms the ectodomain within which there are 16 conserved cysteine residues which form three disulfide bond constrained domains I, II and III. There is also a N-terminal prosequence and a cytoplasmic region that comprises 50 amino acids (aa). *P. falciparum* AMA1 is 622 aa long (152). During late schizogony AMA1 is expressed and targeted to the micronemes. The prosequence is then proteolytically removed, converting the precursor 83kDa PfAMA1 is converted to a 66kDa protein. PfSUB2 sheds the bulk of the ectodomain in the form of $PfAMA1_{48}$ just at or around the point of invasion (153). The C-terminal region is highly conserved and a Tyr residue is thought to be involved in phosphorylation and signal transduction.

AMA1 is involved in RBC invasion by merozoites and is thought to be involved in the moving junction formation which the merozoite uses to invade RBCs. Polymorphism in PfAMA1 has been one of the main hurdles in vaccine development as it hampers the induction of vaccine-induced cross-strain protective responses. PfAMA1 has 64 polymorphisms, nine in domain I, thirty-two in domain II and eleven in domain III (154). Most of these are dimorphic or trimorphic (155) and are believed to facilitate evasion of inhibitory antibodies (156). Antibodies against AMA1 are thought to either inhibit translocation of AMA1 across the merozoite surface and shedding or by binding to a functionally important region of AMA1 (157). mAb4 G2 binds to a loop in domain II of AMA1 and inhibits erythrocyte invasion by all strains of *P. falciparum* (158, 159). The polymorphic residues of AMA1 are concentrated in a relatively small hypervariable region on domain 1 (160, 161) and polymorphisms cluster in three dimensional space and are located on one side of the AMA1 crystal structure (162).

AMA1 antibody levels in individuals living in malaria endemic countries are higher than to other blood-stage antigens like MSP1, MSP2 and GLURP (glutamate rich protein) (163). Antibodies are present in most people and these antibodies affinity purified on the 3D7 allele from immune adults inhibit parasite growth *in vitro* (164). The antibody positivity increases with age (163) and is also partly determined genetically as individuals with MHC class II leukocyte antigen DRB1*1201 were more likely to have antibodies to AMA1 (165). T cell responses to AMA1 were more prevalent in parasitaemic subjects and individuals with a proliferative response to AMA1 peptide

corresponding to aa 259-271 were less likely to be parasitaemic (166). T cell epitopes in AMA1 have been localised to conserved regions (133). These data support the development of subunit vaccines targeting AMA1.

1.7.1.1 Preclinical AMA1 vaccine trials

Immunisation with AMA1 can protect against blood stage infection and this has been shown in several animal models. Recombinant AMA1 has shown varying degrees of protection in mice (167-169) and monkeys (170). Both antibodies and T cells mediate the protective immunity. Protection of mice and monkeys correlates with antibody titre and in the *P. yoelii* model passive immunization of mice with anti-AMA1 mAb confers protection against lethal blood stage challenge (137). Passively transferred antibodies inhibit parasite growth better in mice with an intact immune system thus implying the role of cellular immunity (169). The antibody-mediated protection against AMA1 is strain-specific (167). Antibodies against PfAMA1 induced by vaccination in mice, rabbits and monkeys inhibit parasite growth *in vitro*. In the *P. chabaudi* model CD4+ T cell depletion results in loss of vaccine-induced protection and CD4 T cells specific to cryptic epitopes have been shown to transfer protection to T cell deficient mice (168, 169).

1.7.1.2 Clinical AMA1 vaccine trials

AMA1 has been tested in a few Phase I trials to date. Most of these are using recombinant AMA1 with adjuvants like Alhydrogel, Montanide ISA 720, AS01 and a poxvirus vectored multi-antigen vaccine NYVAC Pf7 (95, 171, 172).

When recombinant 3D7 AMA1 (produced in *E. coli*) was administered with Montanide ISA 720 only six out of 29 subjects seroconverted after three immunisations (171). With the Combination 1 vaccine, which contained both the 3D7 and FVO allele of AMA1, 92% of the individuals were seroconverted but there was detectable GIA in only four out of 22 subjects (172). The poxvirus vectored NYVAC Pf7 expressed seven malarial antigens from all the three stages of the life cycle. The immunogenicity of this vaccine was poor and there was complete protection in 1 out of 35 volunteers and in the others there was a significant delay to parasitaemia (95). In another recent Phase 1 study in the US, the AMA1-C1 vaccines (a mixture of both 3D7 and FVO allelic forms of AMA1) formulated with Alhydrogel plus CPG 7909 elicited good titres of anti-AMA1 antibodies and the IgG purified from the serum showed 96% GIA against 3D7 parasites. The same vaccination regime encoding $MSP1_{42}$ ($MSP1_{42}$-C1) showed only 36% GIA, thus showing the biological importance of anti-AMA1 antibodies (173).

1.8 Vaccine development strategies

1.8.1 Heterologous Prime-boost vaccination strategy

In most vaccination strategies multiple immunisations are required to achieve a magnitude of response sufficient to protect against difficult diseases in humans. DNA and protein vaccines can be given multiple times to try to

achieve this level of response but with viral vectored vaccines the “anti-vector” responses hinder re-administration of the vector to induce maximum immune response to the insert. In order to overcome this problem the heterologous prime-boost vaccination regime was developed (174). The heterologous prime boost vaccination regime involves the administration of two or more vaccine vectors expressing the same transgene sequentially. Multiple approaches have now been used using different vector combinations like DNA-MVA, fowlpox-MVA, influenza-vaccinia, heterologous adenovirus-adenovirus (Ad-Ad) and adenovirus-MVA (Ad-MVA) targeting a wide range of diseases like HIV-1, malaria, tuberculosis, Hepatitis B and even cancer (75, 88, 175, 176). All these vectors used in the prime boost vaccination regimes have differential ability to prime and boost responses and some better than others in generating either a humoral or T cell response or both. Recently, the Ad-MVA or heterologous Ad-Ad regime has been shown to induce good B cells as well as T cell response (177, 178). Adenovirus vectors are good at both priming and boosting antibody responses whereas often MVA fails to prime an antibody response but is able to boost them. This Ad-M regime is ideal for blood stage vaccines as both humoral and cell mediated immunity appears to be important for blood stage protection. The prime boost interval is crucial for this vaccination regime and studies in the *P. yoelii* model encoding the blood-stage antigen $MSP1_{42}$ have shown that an eight week interval is optimal for induction of antibody and T cell response (177). Prime-boost vaccination using the FP9-MVA regime expressing the pre-erythrocytic construct ME-TRAP induced T cell memory, which correlated with protection against malaria in humans (179).

1.8.2 Adenovirus as a vaccine vector

Adenoviruses belong to the family *Adenoviridae* and were first isolated in 1953 from human adenoid tissue removed during tonsillectomy (180). They are double-stranded DNA viruses with a genome of 34-43 kilobases and are easy to manipulate (181). Adenoviral vectors have been an attractive candidate for transfer of foreign genes because the genome is well characterised, they are easily rendered replication incompetent and most wild-type adenoviruses only cause mild disease in immunocompetent individuals.

Adenoviruses show a broad tropism determined by their ability to bind to host cell receptors, which involves the distal knob domain of the fibre protein. Most human serotype adenoviruses like AdHu5 and AdHu2 bind to the coxsackie adenovirus receptor (CAR) that is expressed on a variety of cell types (182). Adenoviruses of the subgroup B like AdHu35 do not use CAR receptor. They associate with CD46, a complement regulatory protein expressed on multiple cell types including dendritic cells (183). Subgroup B1 viruses like AdHu3 bind to the costimulatory molecules CD80 and CD86 expressed on antigen presenting cells (184).

Adenoviruses have been modified in several ways to make them safe to be used in humans as vaccine vectors and also to increase immunogenicity of the virus vectors. The first generation viruses were the E1-deleted adenoviral vectors, which had the crucial regions of the viral genome deleted and this rendered the virus replication-defective and thus safe to use. In order to

accommodate an additional 3.5 kb of foreign sequence, the E3 region was also deleted which encoded nonessential gene products (185).

Recombinant AdHu5 vectors have been the most widely used among the human serotypes as vaccine vectors and have been shown to induce unsurpassed B and T cell responses to the transgene in animal models (186-191). In rhesus macaques an AdHu5 vector expressing the Gag protein of HIV-1 provided protection against challenge with a pathogenic chimeric simian human immunodeficiency virus by preventing CD4+ T cell loss and containing viral load (189). In spite of the strong immunogenicity of these vectors the use of these vectors in humans has been limited because neutralizing antibodies to the vector decreases the expression of the transgene and results in a decrease in immunogenicity (192). The neutralising antibody prevalence to the common vaccine vector, AdHu5 is between 30% and 50% in the USA and Western Europe and up to 98% in African countries (193, 194). In the recent STEP trial human volunteers with neutralising antibodies to AdHu5 had significantly lower CD8+ T cell responses to the HIV-1 antigen when vaccinated with the AdHu5 vector-based HIV-1 vaccine (195). In order to overcome this problem of pre-existing immunity, non-human adenoviral serotypes like chimpanzee adenoviruses have been developed as vaccine carriers as these adenoviruses are normally not neutralised by human sera (196) and the prevalence of neutralising antibodies is very low in target populations (197, 198). These chimpanzee adenovirus vectors like Chimpanzee adenovirus 63 (AdCh63) have been shown to generate CD8+ T cell immunity to transgene peptides. The first Phase I/IIa trial of the

chimpanzee adenovirus AdCh63 ME-TRAP showed promising results. This was used in a heterologous prime-boost with MVA as a pre-erythrocytic malaria vaccine and induced partial protection against malaria (*O'Hara et al*, unpublished).

1.8.3 Poxviruses as a vaccine vector

In 1796 Edward Jenner demonstrated that the intradermal inoculation of cowpox could confer protection against smallpox. This work led to the mass vaccination against smallpox using vaccinia virus, as there is cross-reactivity between Orthopoxviruses. Ultimately this resulted in eradication of smallpox in May 1980. The vaccinia virus used for immunization was a live replication competent virus and so there were safety concerns regarding the use of this virus. Dryvax (NYCBH-Wyeth strain) caused serious side effects in up to 1 in 800 primary vaccinee and death in one per million. This led to the development of attenuated strains of VV like replication-defective NYVAC and MVA. MVA was developed through attenuation of wild-type chorioallantois vaccinia virus Ankara (CVA) by serial passage in primary chicken embryo cells to serve as a safer vaccine for smallpox. As a result of this serial passage 15% of the 208kbp double-stranded DNA genome was lost and this resulted in its inability to grow in many cells of mammalian origin. MVA was used to vaccinate more than 100,000 humans against smallpox and this was the foundation of the safety profile of MVA and its use as a versatile vaccine delivery system. Unlike adenovirus, pre-existing anti-vector immunity to MVA or vaccinia virus (VV) may not affect the immunogenicity of the target antigen

after subsequent immunizations of recombinant MVA as seen with MVA expressing a cytomegalovirus glycoprotein gB antigen (199). Administration of recombinant MVA expressing ME-TRAP 12 months after a primary immunisation expanded the effector T cell pool to a level similar or higher than the primary immunisation in a DNA-MVA vaccination regime (200). Recombinant MVA producing HIV antigens were among the first vector viruses to be evaluated as candidate vaccines in humans (201, 202). The use of heterologous prime-boost strategies using MVA to deliver multiple HIV antigens elicit antibody and cytotoxic T-cell responses in macaques, which effectively control a mucosal challenge with SHIV 89.6P and SIVmac251-derived viruses (203). MVA expressing *Mycobacterium tuberculosis* antigen 85A used in a prime-boost regime with bacilli Calmette-Guerin (BCG) vaccine has shown protection in mice (204). In the case of malaria vaccine development, heterologous prime-boost immunization using AdHu5 and MVA elicited strong CD8+ T-cell immunity and this regime has shown protection against *P. berghei* (205) and P. yoelii (206) challenge in mice. These vaccines have been shown to be safe in human volunteers and also in individuals naturally exposed to malaria (75).

In addition to this MVA could be an effective poxvirus vaccine in the emerging threat of bioterrorism. Although the WHO declared global eradication of smallpox virus, there are still stockpiles of the virus in several countries and reintroduction of variola virus could lead to substantial mortality of up to 30% of exposed non-immune people (207).

1.9 Thesis aims and outline

1.9.1 Aims

- To develop viral vectored vaccines encoding PccAS AMA1 and assess immunogenicity of these vaccines.
- To assess protection against blood-stage challenge and study the mechanism of vaccine-induced blood stage immunity in the PccAS mouse model. This will allow assessment of whether vectored vaccines induce T cell mediated blood stage immunity. Protection in this model is defined as a reduction in peak parasitaemia as *P. chabaudi* infection is non lethal in Balb/c mice.
- To assess the immunogenicity and *in vitro* protection of viral vectored *P. falciparum* AMA1 vaccines. This will help us to address the role of vaccine induced immunity in humans against *P. falciparum* malaria.
- To improve MVA as an antibody inducing vector to allow better antibody mediated immunity to blood stage malaria to be induced.

1.9.2 Thesis hypotheses and outline

This hypothesis behind this work was that viral vectored vaccines targeting AMA1 would induce both humoral and cell mediated immune response which could confer protection against P. chabaudi challenge. This works describes the development of viral vectored blood stage malaria vaccines targeting

AMA1. Prime-boost vaccination using adenovirus and MVA were tested in animal models and as a result of the promising data from this thesis these vaccines are currently due to enter Phase I/IIa human clinical trials.

Chapter II describes the materials and methods used in this project

Chapter III details the design and construction of the vaccines used in the following chapters.

Chapter IV describes the humoral and cell mediated immunogenicity of prime-boost vaccination targeting AMA1 in the PccAS mouse model and also shows protective efficacy against blood stage challenge in this model.

Chapter V dissects the mechanism of vaccine-induced blood stage immunity in this model.

Chapter VI describes the immunogenicity of this vaccination approach targeting *P. falciparum* malaria and also assesses the protective efficacy of the immune response generated by using *in vitro* assays.

Chapter VII describes the attempts to improve MVA as an antibody-inducing vector and also explores direct and cross-presentation by MVA.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Reagents

Material	Supplier
Expand High Fidelity PCR Kit	Roche Diagnostics (Lewes, East Sussex, UK)
pGEM-T Easy Vector System	Promega (Southampton, UK)
Restriction Enzymes	New England Biolabs (Hitchin, Herts, UK)
DNA Molecular Weight Markers	New England Biolabs (Hitchin, Herts, UK)
Lipofectamine™ 2000 transfection reagent	Invitrogen (Paisley, UK)
Adenopure® Kit	Puresyn Inc.
Phusion High-Fidelity PCR Kit	New England Biolabs (Hitchin, Herts, UK)
One Shot Max Efficiency DH5α *E. coli* competent cells	Invitrogen (Paisley, UK)
Rapid DNA Ligation Kit	Roche Diagnostics (Lewes, East Sussex, UK)
pENTR4 vector	Invitrogen (Paisley, UK)
Gateway LR ®Clonase® II enzyme mix	Invitrogen (Paisley, UK)
293A cell line	Invitrogen (Paisley, UK)
S.O.C. medium	Invitrogen (Paisley, UK)
Smart ladder DNA marker	Eurogentec
Qiagen Kits	Qiagen (Crawley, West Sussex, UK)

Mouse IFNγ ELISPOT kit	Mabtech (Nacka Strand, Sweden)
MAIP ELISPOT Plates	Millipore (Watford, UK)
70μm cell strainer	VWR (Lutterworth, Leics., UK)
AP conjugate substrate kit (colour development solution)	Bio-Rad (Hemel Hempstead, Herts., UK)
Microvette serum collection tubes	Sarstedt (Leicester, UK)
Nunc-Immuno maxisorp plates (442404)	Fisher Scientific (Wohlen, Germany)
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma-Aldrich (Poole, Dorset, UK)
Biotinylated rat anti-mouse IgG1 and IgG2a mAbs	BD Biosciences (Oxford, UK)
Anti-rabbit whole IgG alkaline phosphatase conjugate produced in goat	Sigma-Aldrich (Poole, Dorset, UK)
Anti-monkey whole IgG alkaline phosphatase conjugate produced in rabbit	Sigma-Aldrich (Poole, Dorset, UK)
ExtrAvidin alkaline phosphatase conjugate	Sigma-Aldrich (Poole, Dorset, UK)
Alkaline phosphatase labelled human anti-mouse IgG (H+L)	Insight Labs, UK
Alexa Fluor® 488 goat anti-mouse IgG (H+L)	Invitrogen (Paisley, UK)
p-Nitrophenyl phosphate disodium hexahydrate	Sigma-Aldrich (Poole, Dorset, UK)
Diethanolamine buffer	Pierce (Thermo Scientific, UK)
FITC anti-mouse tumour necrosis factor α (TNFα), clone MP6-XT22	Ebiosciences (San Diego, California, USA)
PE anti-mouse interleukin-2 (IL-2), clone JES6-5H4	Ebiosciences (San Diego, California, USA)
APC anti-mouse IFNγ, clone XMG1.2	Ebiosciences (San Diego, California, USA)

Pacific Blue® anti-mouse CD4, clone L3T4	Ebiosciences (San Diego, California, USA)
Anti-CD8-PerCP-Cy5.5, clone 53-6.7	Ebiosciences (San Diego, California, USA)
FITC anti-mouse CD3, clone 145-2C11	Ebiosciences (San Diego, California, USA)
BD Cytofix/Cytoperm™ fixation/permeabilization solution kit with BD GolgiPlug	Ebiosciences (San Diego, California, USA)
Ketaset™	BD Biosciences (Oxford, UK)
Domitor™	Fort Dodge Animal Health
Antisedan™	Pfizer Animal Health
Ultraclear centrifuge tubes 15.0 ml	Pierce (Thermo Scientific, UK)
DMEM without phenol red	Beckman Coulter
Mouse Th1/Th2 Cytokine Kit	BD Biosciences (Oxford, UK)
Luciferase Assay Kit	Promega UK Ltd (Hampshire, UK)

2.2 Solutions

ACK Lysis Buffer: 8.29g NH_4Cl (0.15M), 1g $KHCO_3$ (1mM), 37.2mg Na_2EDTA in 800ml dH_2O. The pH was adjusted to 7.2-7.4 with HCl before making a final solution up to 1l with dH_2O.

Blood-Stage Culture Medium: 11ml FCS, 4.2ml 5% $NaHCO_3$ solution, and 5.5mg neomycin added to 96ml RPMI-1640.

Coating Buffer: 15mM sodium carbonate and 35mM sodium bicarbonate capsules (Sigma C-3041) dissolved in dH_20.

Complete Medium (M10): Minimal Essential Media (MEM) α-modification was supplemented with 5ml L-glutamine (2mM) (Sigma G-7513), 5ml pen/strep (100U penicillin, 100μg strep) (Sigma P-0781), 500μl 2-Mercaptoethanol (50μm) (Gibco BRL31350) and 50ml batch-tested heat inactivated foetal calf serum (FCS) (10%) (Sigma F-2442).

Diethanolamine Buffer: 1 M diethanolamine buffer, pH 9.8, 0.5 mM $MgCl_2$: 97ml diethanolamine (Sigma D8885), 0.2g NaN_3 and 100mg $MgCl_2.6H_2O$ were added to 800 ml of dH_2O, adjusted pH to 9.8 with HCl and made up to 1 litre with dH_2O. Alternatively a 5x stock was purchased (Pierce 34064) and diluted with dH_2O prior to use.

10% DMEM: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5ml L-glutamine (2mM) (Sigma G-7513), 5ml pen/strep (100U penicillin, 100μg strep) (Sigma P-0781), 500μl 2-Mercaptoethanol (50μm) (Gibco BRL31350) and 50ml batch-tested heat inactivated foetal calf serum (FCS) (10%) (Sigma F-2442).

2% DMEM: DMEM supplemented with 5ml L-glutamine (2mM) (Sigma G-7513), 5ml pen/strep (100U penicillin, 100μg strep) (Sigma P-0781), 500μl 2-Mercaptoethanol (50μm) (Gibco BRL31350) and 10ml batch-tested heat inactivated foetal calf serum (FCS) (2%) (Sigma F-2442).

4% PFA: 10ml of 16% paraformaldehyde, 4ml of 10X PBS and 26ml distilled water.

IgG purification elution buffer (EB): 7.5mg of 0.1M glycine was dissolved in 800ml dH_20 and pH adjusted to 2.7 with concentrated HCl: made up to 1l with dH_20.

IgG purification-binding buffer (BB): 0.144M Na_2HPO_4, 0.056M NaH_2PO_4, pH 7.2 in dH_20.

Magnetic cell sorting buffer: 100ml 10X PBS pH 7.4, and 4 ml of 0.5 M EDTA pH 8.0 was added to 800ml purified water in a 1-L beaker. 5g of Bovine Serum Albumin (BSA Fraction V) (Sigma-Aldrich) was added and allowed to dissolve by gravity. The solution was sterilised through a 0.2μm Surfactant-Free Cellulose Acetate (SFCA) filter unit (Nalgene).

Phusion™ High-Fidelity PCR Master Mix: 2x Phusion PCR Master Mix contains 0.04 U/µl Phusion High-Fidelity DNA Polymerase, 2x Phusion HF Buffer and 400 µM of each dNTP. Thermostable Phusion DNA Polymerase is isolated and purified from an *E. coli* strain carrying a plasmid with the cloned Phusion DNA Polymerase gene. Phusion DNA Polymerase is purified free of contaminating endo- and exonucleases.

R10: RPMI (Sigma, R0883) was supplemented with 5ml L-glutamine (2mM) (Gibco, 25030-24), 5ml pen/strep (100U penicillin, 100μg strep) (Gibco, 15140-122) and batch-tested heat inactivated FCS (10%) (Biotest, S1810).

Red Blood Cell Counting Solution: 10ml formalin, and 32g trisodium citrate: made up to 1l in dH_2O.

TE Buffer: 10mM Tris-HCl (pH 8.0), 1mM ethylenediaminetetraacetic acid (EDTA).

2.3 Cloning and Recombinant Virus production.

2.3.1 Agarose Gel Electrophoresis.

DNA reaction products were separated by 1% agarose gel electrophoresis (100V for 40minutes) in 1x Tris-EDTA (TAE) buffer, stained using SYBR safe and visualised on a transilluminator. Size estimates were determined by comparison to ΦX174 DNA – Hae III Digest, λ DNA – Hind III Digest DNA and smart ladder DNA marker (Eurogentec, MW1700).

2.3.2 Phusion High-Fidelity PCR.

Reaction Mix:

	10μl	2x Phusion Mix
	2μl	5μM Forward primer
	2μl	5μM Reverse primer
	1μl	Template DNA
	5μl	Water
Σ=	20μL	

The Phusion Master Mix provides 1.5mM $MgCl_2$ and 200μM of each dNTP in final reaction concentration. The reaction mix was put in 96 well plates and sealed with mineral oil and PCR reactions were performed on a MJ Tetrad using an appropriate programme.

2.3.3 TOPO Blunt Cloning.

	1μl	Salt Solution
	1μl	pCRII-Blunt-TOPO vector
	2μl	PCR product
	2μl	Sterile distilled water
Σ=	6μL	

The TOPO vector has a variety of restriction sites available around the insertion site to facilitate cloning into the final vector. The PCR products were purified using Qiagen MinElute Gel Extraction Kit and eluted in 10μL water. Ligation reactions were performed at RT for ≥ 1h, before transformation into

One Shot® MAX Efficiency™ DH5α-T1R *E. coli*. Clones were selected by ampicillin resistance, and screened by restriction endonuclease digests.

2.3.4 Restriction Endonuclease Digests.

Digests were incubated at 37ºC for ≥ 2h. Subsequent addition of 1µL Antarctic Phosphatase (15min, 37ºC) or CIP Phosphatase (1h, 37ºC) minimized vector religation when digestion generated complimentary “sticky ends”.

	1µl	NEB Restriction Enzyme(s) (10,000U/ml)*
	2µl	NEB restriction buffer (10x)
	2µl	10x bovine serum albumin (BSA) – as required
	xµl	Template DNA (1µg or 10µL miniprep DNA)
	xµl	Water
Σ=	20µL	

* Double digests were done with 0.5 µl of each enzyme.

2.3.5 Ligation Reactions and Transformation.

Digested DNA fragments were excised from agarose gels, and purified using Qiagen MinElute Gel Extraction Kit before ligation using the protocol below and then transformation.

Rapid Ligation Kit for ≥ 1h at RT:

	5μl	Plasmid backbone
	2μl	Insert
	10μl	2x T4 Rapid DNA ligation buffer
	2μl	5x DNA dilution buffer
	1μl	T4 Rapid DNA ligase
Σ=	20μL	

Ligated vector was transformed into One Shot® MAX Efficiency™ DH5α-T1R *E. coli* by adding 1μl of the ligation mix to 50μl of the cells. This mixture was incubated on ice for 5 minutes, before heat-shock at 42ºC for 30s and back on ice for a further 5 minutes. This was then plated onto LB agar plates containing the appropriate antibiotic for selection and incubated at 37ºC overnight.

2.3.6 Plasmid DNA Preparations.

Day1: Bacterial colonies were seen on the plates the day after transformation and single colonies (5-6) were picked and cultured individually over-night in 2ml LB medium containing the same selection antibiotic.

Day2: The next day the plasmid constructs were purified using QIAprep Spin Miniprep Kits and screened using restriction enzyme digests. If a bacterial miniprep culture containing a correct construct was found, it was amplified over-night in 150ml culture as on day1.

Day3: The plasmid constructs from the 150ml culture were purified by Qiagen Plasmid Midi Kits and stored at -20ºC. Bacterial stabilates formed by mixing the culture with 50% glycerol in water were also stored at -80ºC for future use.

The DNA concentration of the plasmid preparations were measured either using a UV spectrophotometer or by using a Nanodrop. The preparations were diluted 1:40 in water before measuring the concentration. For measurements using the spectrophotometer the formula used for calculation is shown below:

$$\frac{(Abs_{260} \times 50 \times dilutionfactor)}{1000} = DNAconcentration(\mu g \mu l^{-1})$$

2.3.7 Sequencing of the constructs.

1.5µg plasmid DNA in total volume of 20µL water was precipitated by addition of 0.1 volumes 3M-sodium acetate and 0.6-volume isopropanol. DNA was pelleted in a bench centrifuge (16000 x g, 10min) and the supernatant discarded. The pellet was washed by addition of 100µL 70% ethanol, centrifuged (16000 x g, 1min) and the supernatant discarded. Samples were air-dried and sent to MWG Biotech (Ebersberg, Germany) for sequencing with the relevant primers.

2.3.8 Transfection and Generation of Recombinant MVA Viruses.

Primary CEFs were supplied from the Institute for Animal Health (Compton, Berks., UK). Cells were passaged every 3-4 days and maintained at 37°C and 5% CO_2 in a humidified incubator. Cells were cultured in 10% DMEM, and then 2% DMEM after infection with MVA.

Day 1: CEFs were plated at $1x10^6$ cells per well and incubated at 37°C and 5% CO_2 overnight. The MVA shuttle vector (4μg) was linearised using the appropriate restriction enzyme, purified using Qiagen MinElute Gel Extraction Kit and eluted in 45ul water.

Day 2: The cells were ~90% confluent the next day. The cells were washed with PBS and infected with MVA expressing red fluorescent protein (MVA-RFP) at a multiplicity of infection (MOI) of 1 in 1ml 2% DMEM. This was incubated for 90 minutes. During the incubation the transfection mix was prepared by adding 4μg (45μl) of the MVA shuttle vector to 315μl of DMEM (no additives) in a 15 ml falcon tube and then 40μl of Superfect Transfection Reagent was added 10 minutes before the end of the incubation. This was vortexed immediately, incubated at RT for 10 minutes and made upto 4 ml with 2% DMEM. After 90 minutes, the medium was aspirated and replaced with 1ml transfection mix (1μg shuttle vector per well) and incubated at 37°C and 5% CO_2 overnight.

Day 3: The next day the medium was replaced with 2% DMEM.

Day 4: The cells were checked for recombination events (green cells) using a fluorescent microscope and harvested for sorting and plaque purification.

2.3.9 MVA Purification and Titration.

The cells were prepared for cell sorting following Jenner protocol J015. The cells containing recombinants expressing green fluorescent protein (GFP) were separated from the wild type MVA-RFP by using a MoFlo^{TM} XDP cell sorter. Drew Worth, Flow Sorting Facility Manager at the Jenner Institute, kindly did the sorting. The cells enriched for recombinant GFP expressing MVA were freeze-thawed 3 times and plated out on fresh CEF monolayers in a 6-well plate using 10-fold serial dilutions and incubated at 37°C and 5% CO_2 for 2 hours. After 2 hours the medium was aspirated and the cells overlaid with 2% low melting point agarose and 4% MEM After 2-3 days green plaques were excised and collected in 100μl 10mM Tris pH 9.0 and assayed for purity and identity by PCR (J079). If the plaques were not pure and free from MVA-RFP, subsequent rounds of plaque picking were undertaken. The virus concentration was determined by immunostaining titration in duplicate (J111).

2.3.10 Generation of Recombinant AdHu5 Viruses.

293 cells are permissive for the production and amplification of replication-incompetent adenovirus as they provide the E1 gene product *in trans*. Recombinant AdHu5 constructs for the respective viruses were made using the ViraPower Adenoviral expression system as described in Chapter III.

Day1: 293 cells were grown in a 60 mm tissue culture dish and maintained at 37°C and 5% CO_2 in a humidified incubator until they were about 70-80% confluent.

Day2: 5μg of the adenovirus vector (pAd/PL-DEST with the respective insert) was digested with *Pac* I in a 50μl reaction to linearise the vector and expose the 5' and 3' inverted terminal repeats (ITRs). 5μl of the digestion mix was run on a 1% agarose gel to confirm the linearization. The expected bands for a linearised vector were ~2kb and ~36kb. The other 45μl of the digestion mix was used for transfection. 255μl of DMEM was added to 45ul of the digestion mix. 30μL Lipofectamine was combined with 270μL DMEM (no additions) and incubated at RT, 5min. The Lipofectamine mixture was added to the digested DNA and incubated at RT for 45 minutes. During the incubation the 293 cells were washed with PBS and 2 ml DMEM without serum or antibiotics was added to the cells. After the incubation, 2.5ml DMEM (no additions) was added to the Lipofectamine-DNA mix and mixed. This was then added to the 293 cells drop-by-drop and incubated at 37°C and 5% CO_2 in a humidified incubator overnight.

Day 3: The medium was aspirated and replaced with 5ml 10% DMEM. Every 2-3 days 2ml of media was removed and replaced with 2 ml fresh 10% DMEM.

After 11-13 days extensive adenovirus cytopathic effect (CPE) was evident. The infected cells were then harvested, freeze-thawed 3 times to produce

crude viral lysate and stored at -20°C. This crude lysate was used to infect 10 150cm^2 tissue culture flasks containing fresh 293 monolayers. Cells were harvested when complete CPE was evident and adenovirus purified.

2.3.11 Adenovirus Purification and Titration.

Recombinant adenovirus was either purified using the PureSyn Adenopure® Kit as per manufacturer's instructions or by density centrifugation using a caesium chloride ($CsCl_2$) gradient.

Ten 150cm^2 tissue culture flasks containing fresh 293A monolayers, and infected with recombinant adenovirus were used to grow the recombinant virus. Once complete CPE was evident (on day 4-5), cells were harvested and passed through 3 freeze-thaw cycles. The harvested cells were then spun (2000 xg, 5 min) to remove cell debris. Benzonase was added to the supernatant, followed by addition of dilution buffer. Columns were prepared and adenovirus was purified by Adenopump® column purification. Pure virus was eluted, and filtered though a 0.22μm syringe filter. Virus particles were quantified by UV spectrophotometry.

Alternatively, recombinant adenovirus was purified by density centrifugation using a caesium chloride ($CsCl_2$) gradient. This was performed by the Vector Core facility at the Jenner Institute (J005). Briefly, an infected cell suspension was prepared from ten 150cm^2 tissue culture flasks containing fresh 293A (or T-REx™ 293) monolayers, which were previously infected with recombinant

adenovirus. Once complete CPE was evident (on days 4-5), cells were harvested and spun (900 xg, 10 min) to pellet infected cells. Cell pellets were resuspended in lysis buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.8) and incubated for 1 hour at RT with 250iu/ml Benzonase. The cell pellet was passed through 3 freeze-thaw cycles and spun (900 xg, 5 min). Three CsCl solutions were prepared at 1.25g/ml, 1.35g/ml and 1.6g/ml CsCl in 10mM Tris, pH 7.8. Gradients were prepared in 15 ml Beckman Ultraclear tubes by under- laying with 5 ml of 1.35 g/ml CsCl. Virus supernatant was added to each of the gradients and tubes were centrifuged (111,500 xg, 2 hours, 4˚C). The lower virus band was collected and virus was diluted in 1.35 g/ml CsCl and carefully underlaid with a 1.6g/ml CsCl solution. Virus was then centrifuged (111,500 xg, 16-18 hours, 4˚C). The (lower) virus band was then collected and virus inserted into dialysis cassettes (Thermo-Fisher) and dialysed against cold storage buffer (10 mM Tris, 7.5% sucrose, pH 7.8). Virus yield was determined by both UV spectrophotometry (J106) for virus particles and plaque immunostaining for infective virus (J006). Virus was stored in aliquots at -80°C until use. The viral identity and purity was confirmed by PCR.

2.4 Immunology

2.4.1 Animals.

Female Balb/c (H-2d) mice from John Radcliffe Hospital, Oxford, UK or Harlan, UK were used in the experiments. Mice were 5-6 weeks old at the start of the experiment. All procedures were performed according to the terms of the UK Animals (Scientific Procedures) Act Project License (PPL 30/2414) and Personal Licence (PIL 30/7792) and were approved by the University of Oxford Animal Care and Ethical Review Committee.

New Zealand white rabbits were used for the rabbit experiment. The vaccines were shipped from Oxford and the immunisation of rabbits and collection of sera were performed by Biogenes GmbH, Berlin, Germany.

Rhesus macaques were used for the macaque study and the immunisations and collection of blood were performed by veterinary staff at the ENEA facility in Rome, Italy. The weight of the macaques ranged from 7.4kg – 14.9 kg. 10ml of blood were collected at each time point in serum tubes and transported in special MediSorb transport boxes to arrive in Oxford by midday on the day following blood collection.

2.4.2 Immunisations

2.4.2.1 Anaesthetic

Animals were anaesthetised for all immunisation procedures via the intradermal (i.d.) or intramuscular (i.m.) route in order to immobilise the animal. A mixture of 75mg/kg Ketaset^{TM} (ketamine hydrochloride) plus 1mg/kg Domitor^{TM} (medetomidine hydrochloride) diluted in endotoxin-free water was administered i.p. as a systemic anaesthetic. Mice were recovered from anaesthesia with 100ul i.p administration of 1:10 solution of Antisedan^{TM} (atipamezole hydrochloride) and endotoxin-free water.

2.4.2.2 Dose and route of vaccine administration

The vaccines were diluted in endotoxin-free DPBS prior to immunization. Vaccines were administered i.d. bilaterally into the ears in a total volume of 50μl for both recombinant adenovirus and MVA vaccines for all *P. chabaudi* and *P. falciparum* AMA1 studies. In mice, the dose of recombinant adenovirus vaccines used was $5x10^{10}$ viral particles (vp); $1x10^{10}$ vp or $1x10^{9}$ vp and the dose of recombinant MVA vaccine used was always $1x10^{7}$ plaque forming units (pfu). For all other routes the volume and site of injection is shown in the Table 2-1.

Route	Site of injection	Volume
Intradermal (i.d.)	Bilaterally into the ears	50μl (25μl in each ear)
Intraperitoneal (i.p.)	Lower left or right quadrant of abdomen	100-200μl
Intramuscular (i.m.)	Caudal thigh muscle	50μl
Subcutaneous (s.c.)	Scruff of neck	100μl
Intravenous (i.v.)	Lateral tail vein	100μl

Table 2-1: Routes of mouse immunisation

The table shows the site and volume of immunization for the different routes used in mice during this project. For rabbits and macaques, the routes and volumes are described in the relevant results section.

2.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was collected from tail veins into Microvette tubes during the course of an experiment or by cardiac puncture for the terminal bleed following cervical dislocation. The blood collected was then allowed to clot overnight at 4°C and centrifuged at 13000 rpm for 4 minutes to pellet the clot. Sera were removed, transferred into clean eppendorfs and stored at -20°C.

2.4.3.1 Total IgG ELISA

Day 1: The recombinant proteins against which the antibody titres were being measured were adsorbed to 96-well Nunc-Immuno Maxisorp plates overnight at room temperature (RT). The concentration of the recombinant protein used was 2μg/ml in PBS unless otherwise stated.

Day 2: Plates were washed 6 times with PBS/0.05% Tween 20 (PBS/T) and blocked with 10% skimmed milk powder in PBS/T for 1 hour at RT. Serum was typically diluted to 1:100 for pre-boost samples and 1:1000 for post boot samples in PBS/T. The sera was added in duplicate and serially diluted 3-fold. Serum from naïve non-immunized mice was diluted 1:100 and added in duplicate for each ELISA to calculate the background. In the case of rabbits and macaques, each animal was bled prior to immunisation and this was used as to calculate the background. Following 2 hour incubation at room temperature, plates were washed 6 times with PBS/T and the bound antibodies were detected using 50μl/well of alkaline phosphatase-conjugated goat anti-mouse (Sigma A3562) or anti-rabbit (Sigma A8025) or anti-monkey (Sigma 1929) total IgG. The secondary antibody was diluted 1:5000 in PBS/T and incubated for 1 hour at RT. During the incubation the development buffer was prepared by dissolving *p*-Nitrophenyl phosphate disodium hexahydrate tablets (Sigma N2765) in diethanolamine buffer at a concentration of 1mg/ml. After 1 hour incubation and a final 6x wash in PBS/T, 100μl of the development buffer was added as the substrate and optical density was read at 405nm using a Model 550 Microplate Reader (Bio-Rad, Hemel Hempstead,

Herts., UK). A reference high-titre positive serum sample was included in all assays and the plates were developed till the positive serum gave the expected OD_{405}. Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value 2x standard deviations (S.D.) greater than the OD_{405} for naive mouse serum (typical cut-off OD_{405} for positive sera = 0.15).

2.4.3.2 Antibody Isotypes.

Mouse isotype ELISA assays were prepared exactly as for total IgG, except antibodies were detected by incubation for 1h at RT with biotinylated rat anti-mouse IgG1 or IgG2a mAbs diluted to 1µg/mL in PBS/T. Plates were washed 6x in PBS/T and then incubated for 30min at RT with ExtrAvidin conjugated to alkaline phosphatase diluted 1/5000 in PBS/T. After the incubation the plates were developed the same way as for total IgG.

2.4.3.3 IgG purification

An equal volume of serum and IgG purification binding buffer was mixed and centrifuged at 3095 xg for 20 minutes to allow the fibrin to pellet. The supernatant was passed through a 0.22µm syringe filter. The protein G column was prepared by passing 10ml of binding buffer five times through the column. The column was then washed with 5 column volumes of binding buffer. The serum binding buffer mixture was then loaded onto the column. The column was washed with 5 column volumes of binding buffer. Pure IgG was eluted in 5 column volumes of IgG purification elution buffer and collected

into a 15 ml conical tube containing 300μl of 1M Tris pH 9.0. The eluted sample was dialysed in PBS using a slide-a-lyzer dialysis cassette (Pierce, UK). The purified IgG in PBS was passed through a 0.22μm syringe filter to sterilise. The sample was then concentrated by centrifugation in a centrifugal filter unit (Amicon Ultra 15, Millipore, 2000 xg, 15min). The final protein IgG concentration was measured by a Nanodrop (Labtech International Ltd., UK).

2.4.4 Cellular Assays

2.4.4.1 Peptides

All peptides used for the cellular assays were commercially synthesised by Protein Peptide Research or Sigma-Aldrich Company Ltd, UK. The AMA1 peptides were crude peptides of >50% purity and the CSP peptides were >80% pure. The peptides were dissolved in DMSO at a concentration of 20mg/ml and stored at -20°C. Working stocks were further diluted in complete medium at a final concentration of 1mg/ml. For *P. falciparum* and *P. chabaudi* AMA1 overlapping peptides were synthesised at crude concentrations to the whole of AMA1 and the ectodomain of AMA1 respectively. The peptides were 15 mers overlapping by 10 amino acids. The individual CSP peptides are shown in Table 2-2.

2.4.4.2 Splenocyte Preparation for Cellular Assays.

Mice were sacrificed by cervical dislocation and spleens removed by dissection. Spleens were crushed in PBS, passed through a sterile 70μm cell strainer and centrifuged at 500 xg for 5min. Supernatants were discarded and pellets resuspended in ACK lysis buffer for 5 min before addition of 25ml PBS. This was immediately centrifuged again (500 xg, 5min) and resuspended in 3ml of complete medium.

2.4.4.3 Intracellular Cytokine Staining (ICS).

Day 1: Splenocytes were prepared as described in section 2.4.4.2 and 100 μl/well of the splenocytes were added to a 96-well U-bottom plate. 50μl of Brefeldin A (GolgiPlug) at 1 μg/ml and 50μl of peptide or peptide pools diluted in complete medium at 4x final concentration were added to the test wells. In the control wells GolgiPlug and medium alone were added. Cells was incubated at 37^{o}C 5% CO_2 for 5 hours. After incubation the cells were left at 4^{o}C overnight.

Day 2: The cells were transferred to a V-bottom plate and centrifuged at 1250 rpm for 5 minutes. 50μl of Fcγ receptor block diluted 1:100 in PBS/BSA was added to the test wells and PBS/BSA was added to the control unstained cells and these were incubated for 15 minutes. After the incubation cells were washed with PBS/BSA, centrifuged and the supernatant discarded. Cells were surface stained for 30min at 4ºC with Peridinin-chlorophyll proteins (PerCP-

Cy5.5)-conjugated anti-mouse CD8α and pacific blue (PB)-labelled anti-mouse CD4 diluted in PBS/BSA at a dilution individually assessed by titration for each antibody. Cells were washed 2x with PBS, and permeabilised in 100µL 1x Cytofix/Cytoperm solution for 20min at 4ºC. Cells were washed 2x with 1x Perm/Wash solution and then intracellularly stained for 30min at 4ºC with allophycocyanin (APC)-conjugated anti-mouse IFN-γ, fluorescein isothiocyanate (FITC)-conjugated anti-mouse TNFα and phycoerythrin (PE)-conjugated anti-mouse IL-2. After the incubation, cells were washed three times in 1x Perm/Wash solution and resuspended in 200µL PBS containing 1% formalin.

Stained cells were acquired using a LSR II flow cytometer (BD Biosciences) and data analysed using FlowJo (version 9), Pestle and SPICE (Mario Roederer) software. Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis.

2.4.4.4 *Ex-vivo* IFN-γ Blood ELISPOT.

Day 1: Anti-mouse-IFNγ-mAb AN18 (Mabtech) was diluted to 5µg/ml in carbonate-bicarbonate coating buffer. MAIP ELISPOT plates (Millipore, MAIPS4510) were coated with 50µl/well of the anti-mouse-IFNγ-mAb and stored overnight at 4°C.

Day 2: Plates were blocked with 100μl/well M10 media for 1 hour at 37°C. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected from tail veins into 200μl 10mM EDTA. The red blood cells were lysed using Puregene RBC Lysis Solution and centrifuged at 1500 xg for 4 minutes to harvest the PBMCs. The PBMCs were resuspended in complete medium and counted using a CASY counter (Schärfe Systems, Germany). 50μl of the cell suspension was plated in duplicate into the coated wells. 50μl of peptide diluted in complete medium (2x final concentration) plus 0.25 x 10^6 naïve splenocytes were added to test wells, and medium alone plus 0.25 x 10^6 naïve splenocytes were added to control wells. Plates were incubated at 37°C, 5% CO_2 in a humidified incubator for ~18 hours.

Day 3: After the incubation plates were washed 6x with PBS and incubated for 2 hours with biotinylated anti-mouse-IFNγ mAb R46A2 diluted to 1μg/ml in PBS. Plates were washed again 6x with PBS and incubated for 1 hour with streptavidin alkaline phosphate polymer diluted to 1μg/ml in PBS. After a final 6x PBS wash, the colour development buffer was added to develop the spots. Once dry, plates were counted using an ELISPOT counter (Autoimmune Diagnostika (AID), Straβberg, Germany). Cell counts were used to back-calculate responses and expressed as spot forming cells (SFC) per million PBMCs. Background responses in media only wells were subtracted from those measured in peptide-stimulated wells.

2.4.4.5 In vivo CD4+ T cell depletion.

CD4+ T cells were depleted using anti-CD4 GK1.5 (rat IgG2a) monoclonal antibody (mAb). The mAbs were purified using protein G affinity chromatography from hybridoma culture supernatants. Mice were injected intraperitoneally with 200µg of the mAb diluted in PBS on days -2 and -1 and on the day of challenge and the control mice were given normal rat polyclonal IgG (Sigma) purified in the same way. The degree of *in vivo* CD4+ T cell depletion was assessed by flow cytometry in the PBMCs of depleted and control mice.

2.4.4.6 Isolation and Adoptive transfer of CD4+ T cells from Spleens.

Splenocytes were prepared as described in section 2.4.4.2 and CD4+ T cells were isolated using the CD4 T Cell Isolation Kit (MACS; Miltenyi Biotec) by depletion of non CD4+ T cells (negative selection). Briefly, splenocytes were resuspended in 40ml of MACS buffer and the cells were counted using a CASY counter. The cells were then centrifuged at 300 xg for 10 minutes and 40µl of MACS buffer per $1x10^7$ cells were added. 10µl per $1x10^7$ cells of "Biotin conjugated antibody cocktail" containing biotin-conjugated monoclonal antibodies against CD8α(Ly-2), CD45R (B220), DX5, CD11b (Mac-1) and Ter-119 were added to this, mixed well and incubated for 10 minutes at 4°C. After the incubation 30µl of MACS buffer and 20µl of Anti-Biotin Microbeads per $1x10^7$ cells were added and incubated for 15 minutes at 4°C. The cells were then washed with buffer by adding 10 times the labelling volume and

centrifuged at 300 xg for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 500µl of buffer per $1x10^8$ total cells.

The magnetic separation of the cells was done using a MACS separator and LS column (MACS; Miltenyi Biotec). The columns were attached to the magnets in the MACS separator. The columns were prepared by rinsing it with 3ml of MACS buffer and the cell suspension was added to the column 10ml at a time and allowed to pass through. The effluent was collected which contained the unlabelled cells, i.e. the CD4+ T cell enriched fraction. The columns were then washed 4 times with 3ml of MACS buffer and flow through collected into the same tube as the effluent. The cells were spun down and resuspended in medium and the cell count was determined. The column was taken off the magnetic field and the plunger was used to wash the CD4-negative fraction (labelled cells) into a separate tube. After counting the cells, they were centrifuged at 1250 rpm for 5 minutes and the pellet was dissolved at a suitable concentration of PBS for injection. The cells were injected intravenously in 200µl volume two days before pRBC challenge.

The purity of the CD4+ T cell fraction and the negative fraction containing the labelled cells was analysed by flow cytometry. 50µl aliquots of the crude sample before separation and the positive and negative fraction after separation were stained and analysed.

2.4.5 Growth Inhibition Activity (GIA) Assay

A GIA assay is an *in vitro* assay used to measure the functional activity of antibodies induced by vaccination. Serum from immunized animals is used to test their ability to inhibit parasite growth. The mouse GIA assays were performed using a standardised method (172) at the GIA Reference Centre (Laboratory of Malaria and Vector Research, NIH) against both 3D7 and FVO strains of *P. falciparum* parasites. The rabbit and macaque GIA assay was performed at Biomedical Primate Research Centre, Netherlands against the 3D7 and FCR3 parasite strains following a similar protocol (208). IgG was purified from serum using a protein G column and the purified polyclonal IgG samples were dialysed and concentrated. Concentrated IgG was pre-absorbed with uninfected human O+ RBCs for 1 hour. *P. falciparum* late trophozoites and schizonts were mixed with test or control samples and culture medium in 96-well culture plates. The final concentration of the culture was 0.3 ± 0.1% parasitaemia, 1% haematocrit in growth medium. Each sample was tested in triplicate. Cultures were maintained for one cycle (40-42 h). Measurement of parasite lactate dehydrogenase (pLDH) was used to quantify relative parasitaemia (172). Inhibition of growth was determined using the formula: -

$$\%GIA = \frac{[1 - (A_{650}sampleIgG - A_{650}unexposedRBCs)]}{A_{650}unexposedRBCs} \times 100$$

2.4.6 Immunoflourescence Assay (IFA)

Day1: Hela cells were grown on 22mm coverslips in 6-well plates to about 50% confluency. 20μl Lipofectamine 2K and 500 μl Optimem (for each well) were mixed and incubated for 5 minutes at room temperature. 4μg plasmid DNA of the individual constructs were mixed with 500 μl of Optimem (per well) gently. The DNA-Optimem and the Lipofectamine-Optimem solutions were combined and incubated for 20 minutes at RT. These were then used to transfect the cells grown on the coverslips by adding 1ml of the solution to each well. These were incubated for 3-4 hours at 37°C and 5% CO_2 in a humidified incubator and then 1ml of Optimem was added to each well and left overnight.

Day2: The plates were kept on ice and the cells were gently washed twice with ice-cold PBS. The cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes on ice and then 20 minutes at RT. The cells were then washed with PBS three times over 10 minutes and quenched with 50mM ammonium chloride for 5 minutes. After this the cells were permeabilised (if required) with 0.2% Tween-100 in PBS for 5 minutes and washed 3 times with PBS. If the cells are not permeabilised only the cell surface expression of the protein can be detected. The cells were then blocked with 0.5% BSA in PBS for 30 minutes and then incubated with polyclonal mouse serum diluted 1:100 in PBS. After 30 minutes Alexa-conjugated anti-mouse IgG (1:200 dilution) was added (Invitrogen, UK) and incubated for another 30 minutes. After the incubation the cells were washed with PBS and the cover slip was mounted

using 30μl Mowiol-DAPI (4', 6'-diamino 2-phenylindole, 500 ng/ml), which stains the DNA and can be observed under a fluorescence microscope using the appropriate filters.

The parasite immunoflourescence assay was performed at BPRC, Netherlands according to a published protocol (209). Cold methanol-fixed smears prepared from a synchronised culture of *P. falciparum* with fully mature schizonts were used for the immunoflourescence. The slides were fixed with acetone and incubated with serial dilution of the post immunisation serum and control serum in PBS containing 1% BSA and 0.01% sodium azide for 30 minutes. After incubation these were washed and incubated with a 1:80 dilution of flourescein isothiocyanate-conjugated goat anti-rabbit IgG (Dako, United Kingdom) for 30 minutes. The slides were immersed in a solution of 0.1% Evans blue and 0.001% DAPI and mounted on coverslips and examined microscopically using the appropriate filters.

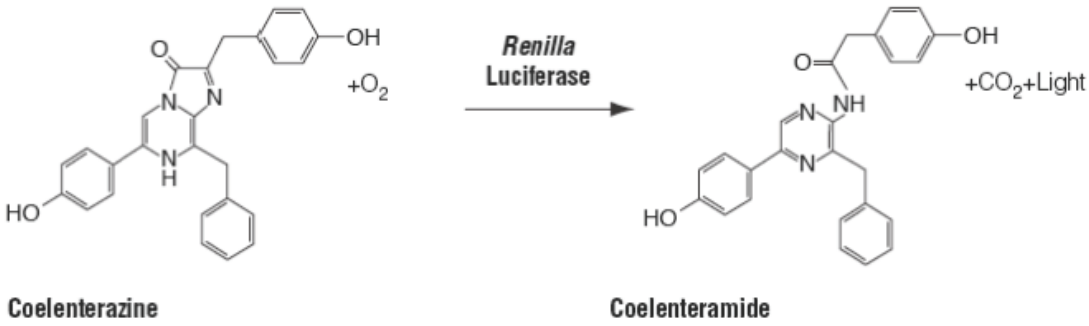
2.4.7 Luciferase Assay.

Day 1: Hela cells were grown in a 96-well plate and incubated at 37°C and 5% CO_2 in an incubator.

Day 2: The cells were infected with MVA expressing LacZ (2ml Optimem plus 5μl virus) at an MOI of 1, and then incubated for 2 hours. During the incubation the Optimem and Lipofectamine 2K were mixed together and incubated for 5 minutes at RT. 0.5μg of each of the plasmid constructs

expressing *Renilla* Luciferase was diluted in Optimem. The diluted DNA was then added to the Optimem-Lipofectamine 2K mix and incubated for 20 minutes at RT. After 2 hours the medium from the wells were removed and 50 μl of the transfection mix was added to the wells. This was then incubated overnight.

Day 3: The Luciferase assay was performed using the *Renilla* Luciferase Assay kit (Promega, UK). The *Renilla* Luciferase Assay system provides a fast and sensitive method of detecting sea pansy (*Renilla reniformis*) luciferase. Renilla luciferase catalyzes coelenterazine (substrate) oxidation to produce light (picture taken from Renilla Luciferase Assay System instructions, Promega).



The assay lysis buffer (5X concentrate) was diluted 1:4 in distilled water and mixed well. The cell supernatant from the wells were removed and stored in eppendorf tubes. The cells were gently washed with 100μl PBS, and 20μl lysis buffer was added per well and incubated for 15 minutes. After 15

minutes the cells were scraped off using a pipette and 10μl of cell lysate was added to a 96-well plate. 5μl of the lysis buffer was added to 20μl of the supernatant and 10μl of the supernatant was added to the plate. The luciferase substrate was then prepared by adding 10μl of 100x *Renilla* Luciferase Assay Substrate to 1ml of *Renilla* Luciferase Buffer in a glass vial. 50μl of the substrate was added to each well and the plate was read using a Varioskan Flash Electron luminometer (Thermo Corporation).

2.4.8 Mouse Th1/Th2 Serum Cytokine Analysis.

Vaccinated and control mice were bled pre- and post-challenge from the tail vein. The blood was stored overnight at 4°C, centrifuged to obtain the serum and frozen until further use. The cytokine levels in the serum (IL-2, IL-4, IL-5, IFNγ and TNF) were assessed using a Cytometric Bead Array (Mouse Th1/Th2 Cytokine Kit, BD Biosciences) according to the manufacturer's instruction.

Briefly, the lyophilized cytokine standards were reconstituted in assay diluent and serially diluted. The capture beads were pooled together (10μl for each assay tube) and mixed thoroughly. The serum was diluted 1:2 in the assay diluent. For each sample 50μl of the mixed capture beads, 50μl of the diluted serum sample and 50μl of the PE detection reagent was added and mixed well. For the control assay tubes, 50μl of the appropriate mouse Th1/Th2 cytokine standard dilutions were added to the serum and detection reagent. The assay tubes were incubated for 2 hours at RT in the dark. During the

incubation the cytometer setup beads were washed and the BD FACSCalibur flow cytometer was setup using the manufacturer's instructions. After the 2 hour incubation, 1ml of Wash buffer was added to each assay and control tube and centrifuged at 200 xg for 5 minutes. The supernatant was carefully discarded and the pellet was resuspended in 300μl wash buffer. The samples were run on the instruments settings from the cytometer set up beads. The samples were analysed using the BD CBA software.

2.5 Parasitology

2.5.1 P. chabaudi parasites

P. chabaudi chabaudi AS (PccAS) parasites which cause a non-lethal infection in Balb/c mice were kindly provided by Dr Jean Langhorne, National Institute for Medical Research, London. UK.

2.5.2 Cryopreservation of PccAS blood-stage parasites.

PccAS infected blood was thawed and injected immediately i.p. into a donor mouse. The parasitaemia was monitored until it reached 15-20% and then the mouse was bled by cardiac puncture into 10x heparin following cardiac puncture. This was then injected i.p into 10 mice in order to generate large stocks of PccAS-infected blood. Blood stage parasitaemia was monitored and the mice were bled as before. For long-term storage the blood was mixed with an equal volume of ice-cold 20% DMSO in blood-stage culture medium and

divided into 100μl aliquots and snap frozen in liquid nitrogen. This was stored in liquid nitrogen tanks at -196°C for later use.

2.5.3 Blood-stage challenge.

Five days prior to challenge, a frozen aliquot of PccAS-infected blood was thawed and injected i.p. into a donor mouse. On the day of challenge, the number of RBCs per μl of blood was counted and the % parasitaemia was measured by methods described in 2.5.4 and 2.5.5 respectively. This was used to calculate the number of parasitized red blood cells (pRBCs) per μl of blood. Blood from the donor mouse was diluted accordingly in KGS (PBS with 0.2% glucose) supplemented with 10% mouse serum to give $1x10^7$ pRBCs/ml. Mice were infected with 100μl of this solution ($1x10^6$ pRBCs) i.v. via the tail vein. The mice were ear-tagged to enable identification. From day 4 post-challenge the parasitaemia was monitored daily by Giemsa-stained thin-blood smear and the health status was monitored twice daily during acute parasitaemia. PccAS non-lethal infection in naïve Balb/c mice reaches a maximum parasitaemia of 50-60% and then is controlled. PccAS infection in mice led to mild symptoms including anaemia. In exceptional circumstances, some mice become very anaemic and do not seem to recover – at this point they are sacrificed. In general, mice are monitored until they clear their patent parasitaemia.

2.5.4 Red Blood Cell (RBC) Counts

5μl of blood from the donor mouse was diluted in 1ml of RBC counting solution on the day of challenge. The number of cells in five small central squares on the Improved Neubaurer Haemocytometer was counted and the number of RBC/μl of blood was calculated according to the equation shown below as published (210) previously:

$$\left(\frac{No\ of RBC counted \times 200}{0.02}\right) = RBC\mu l^{-1}$$

2.5.5 Monitoring Blood-Stage Parasitaemia.

Blood-stage parasitaemia was monitored from day 4 post challenge. A drop of blood obtained by a tail snip was placed on a glass slide and a thin-smear was prepared. The slide was then air-dried, fixed in methanol for 5 minutes and then stained in 5% Giemsa diluted in water for 1 hour. After staining the slide was air-dried and examined using a light microscope under oil immersion at 100x magnification. The number of total RBC and the number of parasitized RBCs were counted and the % of pRBCs was calculated. The number of fields of view counted was dependent on the % parasitaemia.

The field of view counted had approximately 500 RBCs. If the parasitaemia was around 1% one field of view was counted. If the parasitaemia was < 1% > 0.1% five fields of view were counted, and if the parasitaemia was < 0.1%, 50

fields were counted. If the mice were anaemic and a field of 500 parasites could not be found, then several fields were counted.

2.5.6 Blood-Stage PccAS Parasite Lysate.

Mice were infected with PccAS-infected pRBC and the parasitaemia was monitored until it reached ~20-30%. The mice were then sacrificed and bled by cardiac puncture into 10x heparin (final heparin concentration 30U/ml blood) and the blood was pooled and stored on ice in a 15ml falcon tube. The blood was centrifuged at 2500 rpm for 5 minutes. The supernatant was removed carefully using a pipette. The pellet was then resuspended in 5ml of PBS and mixed by inverting the tube 5-6 times. A Plasmodipur filter (Euro-Diagnostika) was prepared with PBS and the sample was passed through the filter using a 20ml syringe into a 10ml falcon tube. The original falcon tube was washed with PBS and filtered twice. The cells were washed after filtration with 10ml of PBS and resuspended in 8ml PBS + 0.06% saponin, mixed and incubated at RT for 10 minutes. After the incubation the sample was centrifuged at 2500 rpm for 5 minutes and the pellet was washed twice in 5ml of PBS and resuspended in 1ml PBS. This was then freeze-thawed three times to lyse the cells, and centrifuged at 2500 rpm for 5 minutes before the supernatant was transferred to an eppendorf tube. The tube was centrifuged at 12000rpm for 30 minutes to remove any remaining insoluble materials. The protein concentration of the lysate was measured using a Nanodrop.

2.6 Statistical analysis

Data were analysed for statistical significance using GraphPad Prism version 5 for windows (GraphPad Software, San Diego, CA). The normality of the data set (parametric or non-parametric) was determined using the Kolmogorov-Smirnov one-sample test. For non-parametric data a Mann-Whitney U test was used to compare two groups and a Kruskal-Wallis test was used to compare more than two groups. Parametric data were compared either using t-test (for comparing two groups) or one-way ANOVA (for more than two groups). A post-hoc Dunnet's correction was used to compare groups to one control group whereas a Bonferroni correction was used when all the groups were compared to each other.

ELISA titres for mice, rabbits and macaques were logarithmically (log10) transformed in order to normalise the data and enable a more stringent parametric analysis. Correlations were tested using Pearson rank correlation for parametric data (log-transformed data) or Spearman's rank correlation for non-parametric data. The area under the curve (AUC) was calculated to analyse the challenge data in section 5.2.5 and the different groups were compared by ANOVA. $P \leq 0.05$ was considered significant in all cases ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$).

CHAPTER 3

VACCINE DESIGN AND CONSTRUCTION

3 Vaccine design and construction

3.1 Introduction

This chapter describes the design and generation of poxviral and adenoviral vectored vaccines for the delivery of *P. chabaudi* and *P. falciparum* AMA1 and also *P. yoelii* CSP. Animal and human prime-boost studies using these vectors have shown these to be effective vectors in the generation of protective immune response against diseases such as TB (204, 211-214) and pre-erythrocytic malaria (75, 88, 175, 205, 215-218). In this project the adenoviral and poxviral vectors encoding *P. chabaudi* and *P. falciparum* AMA1 were used in a prime-boost vaccination regime to assess the immunogenicity and protection of these vectors against blood stage malaria. Two MVA promoters P7.5 and I1L were also compared in an attempt to improve MVA as an antibody-inducing vector. These two promoters were used separately to drive the expression of the pre-erythrocytic antigen CSP from *P. yoelii* to compare the activity of the two promoters.

3.1.1 Construction of Adenovirus Vaccines

The utility of adenoviruses as a heterologous gene delivery system was first realised when it was discovered that the early gene E1 could be deleted from the viral genome and supplied *in trans* in the producer cell line. The E1-deleted replication-incompetent adenovirus vector can be generated from

molecular clones in which the entire genome is carried within a bacterial plasmid (219). The adenoviral genome can allow an increase of an extra 1.8kbp in the genome without disrupting viral stability. The vectors deleted of the E1 and the non-essential E3 region can accommodate up to 7.5kbp of foreign DNA and thus are very suited as subunit vaccine vectors (185).

Recombinant AdHu5 vaccines were made using the ViraPower Adenoviral expression system (Invitrogen, Paisley, UK) based on a 36kbp E1 and E3 deleted pAd genome vector. This system uses the “Gateway technology” based on site-specific recombination system from bacteriophage lambda (λ) – a system that normally facilitates the integration of the bacteriophage λ into the *E. coli* chromosome. In the adenoviral system, the antigen is inserted into the E1 site. Bases 1-458 include the 5’ L-inverted terminal repeats (ITR) and packaging signal, and bases 3513-35935 with E3 region deleted include the 3’ R-ITR. The recombinant proteins BP and LR encoded by λ and *E. coli* mediate the site-specific recombination and this is conservative and maintains the orientation of the insert and the reading frame. After recombination and ligation of the construct into the E1 deleted site the bacterial sequences can be removed by restriction enzyme digests which expose the ITRs. When this plasmid is then transfected into a packaging cell line that supplies the essential E1 gene products *in trans* (deleted from the pAd vector) pure recombinants are formed. The HEK 293 cell line is used as the packaging cell line. This cell line was generated by transformation of cultures of human embryonic kidney cells with sheared adenovirus 5 DNA, which led to the incorporation of about 4.5 kb of the left arm of the viral genome into

chromosome 19 (220, 221). This cell line expresses the missing proteins required for the propagation of the E1 and E3 deleted vectors. There is a small risk of recombination between the homologous flanking sequences of the E1 gene and the pAd genome vector, which can lead to the generation of replication-competent wild-type AdHu5 (220).

The vaccine construct is first cloned into the polylinker of the entry vector pENTR4.CMV.BGH. This entry clone has the 1.9kbp CMV promoter (with regulatory element, enhancer and intron A) and the polylinker and BGH poly (A) transcription termination sequence. This is then recombined using the LR clonase enzyme to insert it into the promoter-less pAd/PL-DEST vector and the recombination is confirmed using restriction enzyme digest. This pAd vector expressing the vaccine construct is then used to transfect 293A cells to generate the recombinant adenovirus expressing the antigen. The purity and identity of the recombinant adenovirus is confirmed by PCR.

3.1.2 Construction of Modified Vaccinia Ankara Virus Vaccines

Modified Vaccinia Ankara (MVA) has a relatively large genome size of 178 kbp. It is a double stranded DNA virus and can replicate entirely in the cytosol. Unlike other DNA viruses, the DNA alone of poxviruses is not infectious upon transfection. The vaccine construct is cloned into a plasmid shuttle vector (MVA-GFP) under the control of a 5’ viral promoter and this is flanked by viral genome sequence. The promoter used in most vaccine constructs is the early P7.5 promoter that allows a continuous level of gene expression. This vaccine

construct can then be inserted into the genome of the virus by homologous recombination. In the case of MVA, the classical TK gene locus often serves as the insertion site. The cytosolic homologous recombination events occur with a modest frequency of ~0.1% upon transfection of the shuttle vector into chick embryo fibroblasts (CEFs) infected with the parental virus. Clonal selection and plaque purification of the MVA recombinants are done using different fluorescent proteins. The two fluorescent proteins, red fluorescent protein (RFP) and green fluorescent protein (GFP), under the control of the late fowlpox promoter 4b are used to identify the parental strain (MVA-RFP) and the recombinant (MVA-GFP) using cell sorting techniques which are very quick and efficient.

The MVA-GFP shuttle vector drives the expression of the insert under the P7.5 early/late promoter and the GFP marker gene from the fowlpox late promoter, FP4b. The shuttle vector is linearised and used to transfect CEFs infected with MVA-RFP. *In vitro* recombination replaces the RFP at the TK locus with the GFP-antigen construct cassette. There are single crossover events that resolve in a 50:50 fashion to MVA-RFP or the recombinant expressing the GFP-antigen construct. The desired recombinant expressing the vaccine construct and GFP is sorted using a fluorescence-activated cell sorter, which helps to enrich it from the parental MVA-RFP. Once enriched the desired recombinant expressing the antigen is purified by repeated plaque picking using the GFP marker until pure green plaques can be isolated. The purity and identity of the recombinant MVA is then checked using a PCR assay before bulking up the amount of virus. Once it is grown the MVA is

purified by centrifugation through a sucrose gradient and titred by plaque assay.

3.2 Results

3.2.1 *P. falciparum* AMA1 vaccines

3.2.1.1 Assembly of *P. falciparum* 3D7 AMA1 construct

The vaccine construct was deigned to contain the ectodomain of *P. falciparum* 3D7 AMA1. This was based on the construct used in another study, which used AMA1 sequence encoding amino acids 25 to 546 (GenBank Accession No U65407) (222). The N-terminal signal sequence, pro-sequence and the C-terminal transmembrane domain and cytoplasmic tail were not included in this construct. The N-glycosylation sites were removed as a loss of vaccine efficacy due to N-glycosylation has been observed previously with a malaria vaccine candidate (223). There were five putative N-glycosylation sites in the amino acid sequence of the ectodomain of AMA1 and these were removed by changing the asparagine residues to glutamine. The construct was sent to Geneart, Germany to get it codon-optimised for expression in humans. The codon optimised construct was cloned into the human tissue plasminogen activator vector (177) to create an in-frame fusion with the N-terminal tPA leader sequence, thus containing the entire construct between two *Bam*H I sites**.** The tPA leader sequence has been shown to assist the efficacy of MSP1-based DNA vaccines (224), and is required for antigen secretion, given the absence of the natural AMA1 leader sequence in the vaccine construct.

3.2.1.2 Construction of AdHu5-PfAMA1

The antigen construct was cloned into the unique *Bam*H I site of the adenovirus shuttle vector pENTRV.CMV.BGH (225). This construct (flanked by the *att*L sites) was recombined into the pAd/PL-DEST vector (based on 36kbp E1- and E3-deleted pAd genome vectors) using the LR Clonase reaction kit (Invitrogen, Paisley, UK) (Figure 3-1). 5µg of the recombinant AdHu5 vector was digested with *Pac* I to expose the 5' and 3' inverted terminal repeats (ITRs) and then used to transfect 293A cells. Extensive adenovirus cytopathic effect (CPE) was evident after 10 days. The cells were harvested and this was used as the crude lysate to grow the virus. Recombinant adenovirus was purified using PureSyn Adenopure Kit and stored at -80°C. Adenoviral DNA was extracted from 293A cells and viral identity and purity assayed by PCR (Figure 3-2).

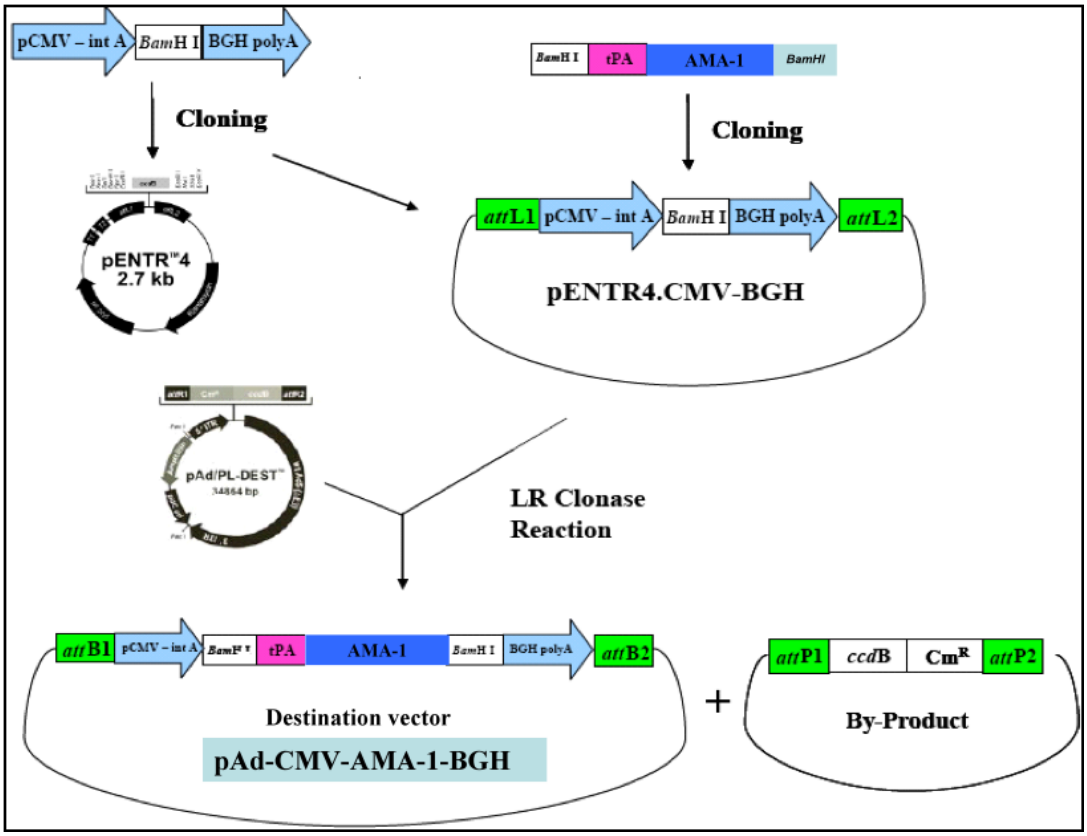


Figure 3-1: Illustration of the steps involved in generation of recombinant AdHu5-AMA1 vaccines.

The AMA1 vaccine constructs are cloned into the unique *Bam*H I site of pENTR4.CMV.BGH. Presence of the insert is confirmed by restriction digests as shown in Figure 3-2. This construct flanked by the *att*L sites was recombined into pAd/ PL-DEST vector replacing the ccdB and chloramphenicol genes with the two *att*R sites. The destination vector was used to transfect 293A cells to grow the virus and later purified using Adenopure columns.

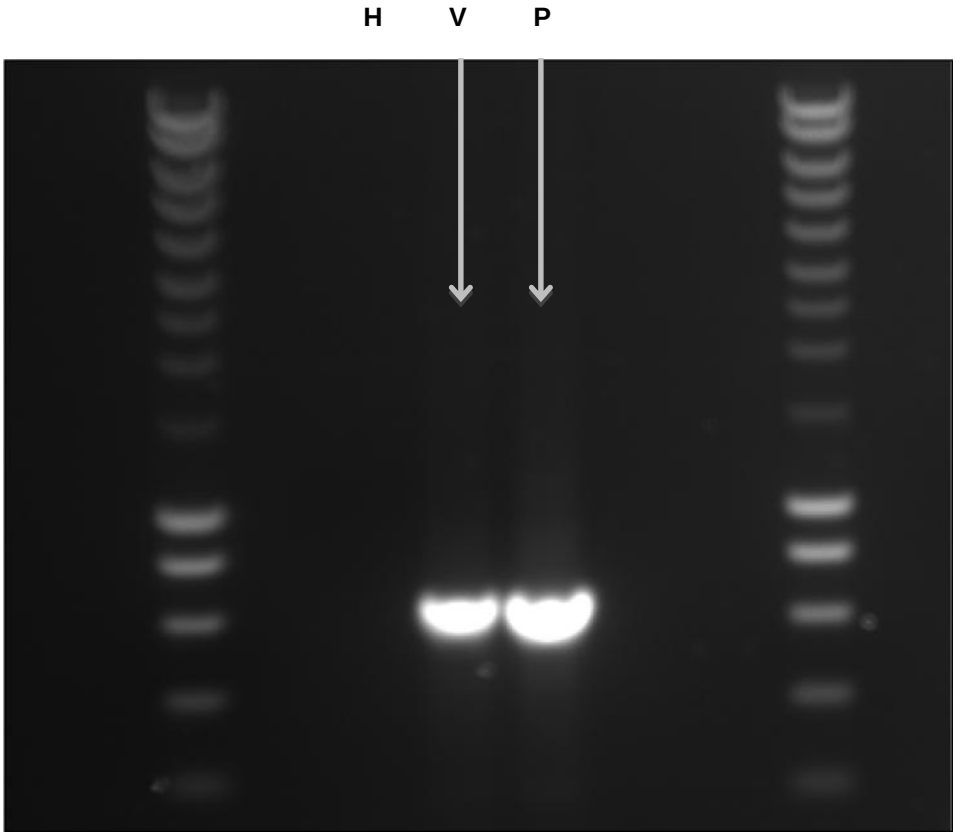


Figure 3-2: Viral identity and purity PCR for AdHu5-PfAMA1 vaccines.

The viral identity and purity was determined using PCR reactions. Two PCR reactions were run in parallel, each containing the lower primer AdL which is specific for the insertion site and the upper primer M=PfAMA1 specific primer. The test sample was DNA extracted from final virus preparation AdHu5-PfAMA1: **V** and water was used as the negative control **H**. The pAd-PfAMA1 plasmid was used as the postitive control **P**. The PCR product of around 620bp when using the M upper primer shows the presence of the AMA1 construct that is also seen in the plasmid but not in the negative control.

3.2.1.3 Construction of MVA-PfAMA1

The tPA-PfAMA1 construct was excised by *Bam*H I digest and cloned into the unique *Bgl* II site of the MVA shuttle vector, MVA-GFP. The MVA shuttle vector was then linearised and transfected into Chicken embryo fibroblasts (CEFs) infected with MVA-RFP; i*n vitro* recombination replaces RFP at the TK locus in the MVA genome, with the GFP-AMA-1 cassette (Figure 3-3). CEFs infected with recombinants expressing GFP were enriched from those infected with MVA-RFP using a fluorescence-activated cell sorter. Pure recombinant MVA-AMA1 was subsequently isolated by repeated plaque picking in CEFs using the GFP marker and PCR assays for identity and purity (Figure 3-4). Once isolated, a pure MVA vaccine preparation was grown, purified and titred and stored at -80ºC.

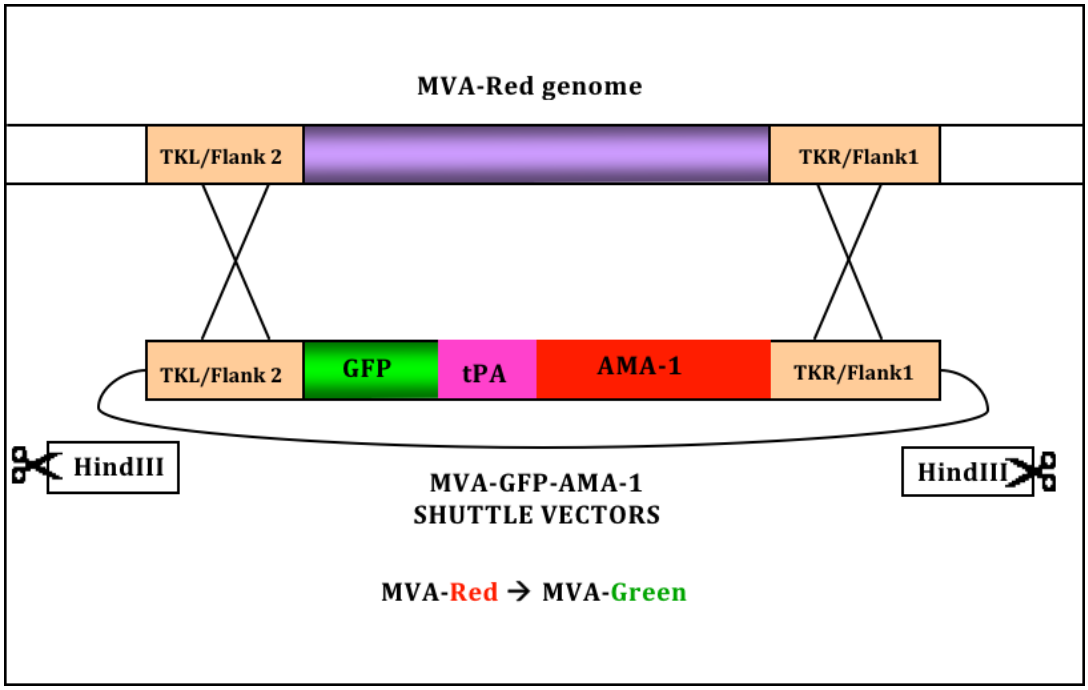


Figure 3-3: Illustration of the steps involved in the generation of recombinant MVA-PfAMA1 vaccines.

AMA-1 antigens are brought under control of the early/late poxviral promoter P7.5. Chicken embryo fibroblast cells (CEFs) infected with MVA expressing red fluorescent protein (RFP) are transfected with linearised shuttle vector, where sequences flanking the constructs allow insertion, by recombination, into the viral genomes. The GFP and AMA-1 cassette replaces RFP. Cells containing stable GFP-expressing recombinants were sorted by flow cytometry following transfection. Sorted cells were re-plated. Following two further rounds of plaque-picking, pure recombinant MVA-AMA-1 was isolated.

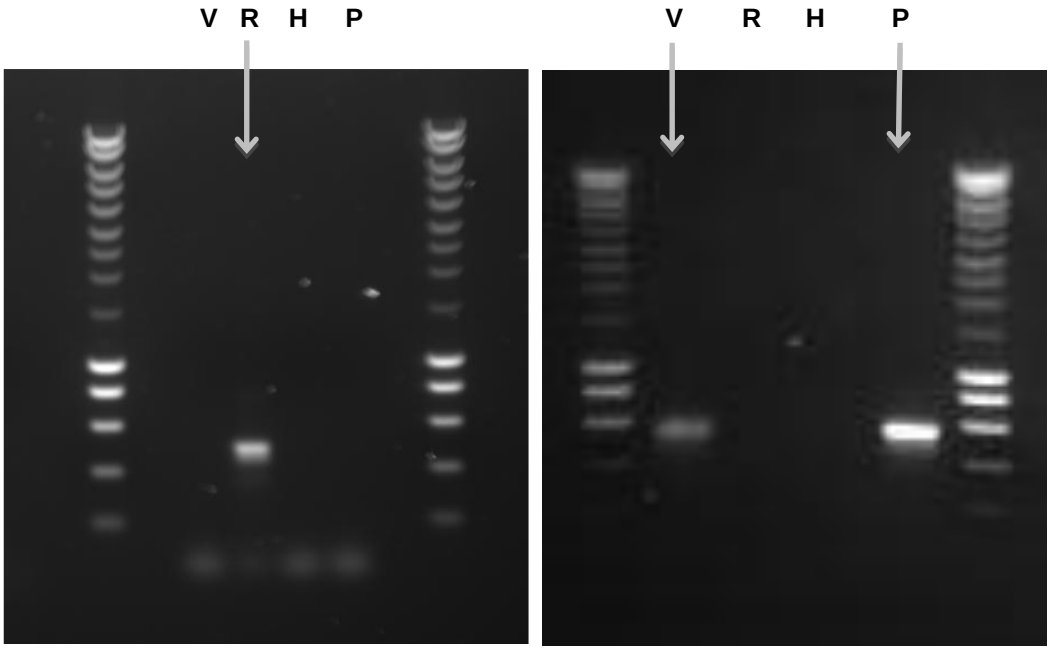


Figure 3-4: Viral identity and purity PCR for MVA-PfAMA1 vaccines.

The viral identity and purity was determined using PCR reactions. Two PCR reactions were run in parallel, each containing the same lower primer, TKL, which is specific for the insertion site and a different upper primer, either A=PfAMA1 specific primer (A) or R=RFP primer (wild-type MVA) (B). The test sample was DNA extracted from final virus preparation MVA-PfAMA1: **V** and water: **H** was used as the negative control. The DNA from MVA-RFP was also used as a control: **R**. The PCR product of 562bp shows the presence of the construct (B) both in the virus and the plasmid and the absence of PCR product when using the Red F upper primer shows the absence of parental MVA-RFP (A). The plasmid MVA-GFP PfAMA1 was also included as the positive control: **P**.

3.2.2 *P. chabaudi chabaudi* AS AMA1 vaccines

3.2.2.1 Assembly of *P. chabaudi* AMA1 construct

The complete sequence of PccAS AMA1 was not available so it was sequenced at the Wellcome Trust Centre for Human Genetics, Oxford from

parasite genomic DNA to obtain the complete amino acid sequence. The vaccine construct i.e. the ectodomain of AMA-1 (amino acids 21- 479) after removal of the four N- glycosylation sites was sent to Geneart, Germany to get it codon optimised for expression in mice. The N-terminal signal sequence and C-terminal transmembrane domain and cytoplasmic tail were not included in the construct.

3.2.2.2 Construction of AdHu5-PccAMA1

The tPA-PccAMA1 construct was cloned into the unique *Bam*H I site of the adenovirus shuttle vector pENTRV.CMV.BGH. It was then used to make recombinant adenovirus as described earlier in 3.2.1.2.

3.2.2.3 Construction of MVA-PccAMA1

The tPA-PccAMA1 construct was excised by *Bam*H I digest and cloned into the unique *Bgl* II site of the MVA shuttle vector, MVA-GFP. Pure recombinant MVA-PccAMA1 was made using methods previously described in 3.2.1.3.

3.2.3 *P. yoelii* CSP vaccines

3.2.3.1 Assembly of *P. yoelii* CSP construct

P. yoelii CSP (aa 1-356) was expanded by Phusion PCR (New England Biolabs, Ipswich UK) using the PyCSP F forward primer 5'-GTC GAC ATG AAG AAG TGT ACC ATT TTA GTT GTA GCG-3' and PyCSP R reverse primer 5'-GCA TGC GGA TCC TCA GTC TAG ACC TAG CAA AGG GTT

AGG AAT TCC TAA TGA ATT GCT TAC AAT ATT AAA TAT ACT TGA-3' from template DNA (217) (a kind gift from M. Tsuji, New York University, USA). The forward primer encoded a *Sal* I restriction enzyme site and the reverse primer encoded a C-terminal monoclonal antibody recognition tag followed by *Bam*H I and *Sph* I restriction enzyme sites. The monoclonal antibody epitope tag (aa sequence IPNPLLGLD) enables the detection of antigen expression using the anti-PK mAb. The C-terminal GPI anchor sequence of CSP was not included in the construct. The sequence was verified using methods described earlier. This construct (SalI-PyCSP-PK-BamHI-SphI) was cloned in-frame into a vector containing the N-terminal tPA leader sequence.

A set of primers was designed to replace the P7.5 promoter with the I1L promoter in the MVA-GFP shuttle vector. The minimal sequence required for full activity of the I1L promoter is TAT TTA AAA GTT GTT TGG TGA ACT TAA ATG G [12]. The I1L-tPA forward primer also contained the *Pst* I enzyme site followed by the I1L sequence. The I1L-tPA reverse primer contained the *Xho* I restriction enzyme site. This was then used to replace P7.5-tPA in the MVA-GFP vector with I1L-tPA by restriction enzyme digest.

3.2.3.2 Construction of MVA-P7.5PyCSP and MVA-I1LPyCSP

The tPA-PyCSP constructs were excised by *Bam*H I digest and cloned into the unique *Bgl* II site of the MVA shuttle vector, MVA-GFP. This shuttle vector drives expression of the insert using the vaccinia P7.5 early/late promoter. The I1L-tPA sequence was cloned into *Pst* I and *Bam*H I sites of the MVA-

GFP shuttle vector, thus removing the P7.5 promoter and leaving the antigen to be expressed under the control of I1L promoter.

Pure recombinant MVA-P7.5PyCSP and MVA-I1LPyCSP were subsequently isolated using methods previously described. The purity, identity and orientation of the construct were checked using PCR assays. Once isolated, pure MVA vaccine preparations were grown, purified and titred and stored at -80°C.

3.2.3.3 Construction of AdHu5-PyCSP

The tPA-PyCSP construct was cloned into the entry vector pENTR4.CMV.BGH using the BamH I restriction enzyme sites and then recombined into the pAD/PL-DEST vector. Recombinant AdHu5-PyCSP was generated as previously described, purified and the purity and identity of the virus was assayed using PCR.

3.3 Discussion

The vaccines were made successfully using the methods described in this chapter. Both recombinant AdHu5 and MVA vaccines expressing *P. falciparum* AMA1, *P. chabaudi* AMA1 and *P. yoelii* CSP were made. These vaccines were pure and had no contamination with either wild type adenovirus or MVA-RFP as assessed by PCR assays.

3.4 Vaccine summary

	Vaccine construct	Abbreviated name
1	AdHu5 expressing *P. falciparum* 3D7 AMA1 ectodomain	**AdHu5-PfAMA1 (3D7)**
2	MVA expressing *P. falciparum* 3D7 AMA1 ectodomain	**MVA-PfAMA1 (3D7)**
3	AdHu5 expressing *P. chabaudi chabaudi* AS ectodomain	**AdHu5-PccAMA1**
4	MVA expressing *P. chabaudi chabaudi* AS ectodomain	**MVA-PccAMA1**
5	AdHu5 expressing *P. yoelii* CSP	**AdHu5-PyCSP**
6	MVA expressing *P. yoelii* CSP under P7.5 promoter	**MVA-P7.5PyCSP**
7	MVA expressing *P. yoelii* CSP under I1L promoter	**MVA-I1LPyCSP**

CHAPTER 4

IMMUNOGENICITY AND PROTECTIVE EFFICACY OF Ad_M *Pcc*AMA1 VACCINATION REGIME

4 Immunogenicity and protection of Ad_M PccAMA1 vaccination regime.

4.1 Introduction

Blood-stage malaria vaccine development generally targets antigens associated with the merozoite surface or located in the apical organelles of the merozoite. AMA1 is one of the leading candidates for subunit vaccine design against the asexual stage of the parasite that appears on the surface of merozoites after its release from the micronemes. The malaria vaccine world has classically focused on recombinant protein-in-adjuvant vaccines that require multiple immunizations in animal models to induce antibody responses of a protective magnitude, and clinical trials of such candidate blood-stage vaccines remain disappointing. Recombinant protein vaccines are hampered by the inherent difficulties of reliably purifying large amounts of correctly folded protein, and formulation in effective human-compatible adjuvants. Viral vaccine vectors, deployed in heterologous prime-boost regimes, have been developed to induce CD8+ and CD4+ T cell responses targeting intracellular pathogens and with specific regimes also induce high levels of antibody (226). They circumvent the above difficulties and can express large antigen constructs. Replication-defective poxviruses, such as MVA and adenoviruses, such as AdCh63, have displayed a suitable safety

profile for use in humans as prophylactic vaccines, and have shown excellent efficacy against pre-erythrocytic malaria in mouse models.

There have been several studies suggesting that cellular immunity in conjunction with antibody responses may increase the efficacy of a blood stage malaria vaccine (78, 131, 227). Previous work by Draper et al has shown that recombinant AdHu5_MVA prime-boost encoding the blood stage antigen $MSP1_{42}$ can induce complete protection against challenge with *P. yoelii* blood-stage parasites and partial protection against the liver-stage (228). Vaccine-induced blood-stage immunity correlates with $MSP1_{19}$-specific antibodies, whilst $MSP1_{33}$-specific T cell responses protect against the liver-stage parasite (226). Similarly viral vectored vaccines targeting *P. falciparum* MSP1 have shown efficacy in inducing antibodies with growth inhibition activity (GIA) comparable to protein adjuvant vaccines (A. Goodman et al, unpublished).

The PccAS variably lethal murine model has provided an informative model for the study of blood stage immunity. In this model both CD4+ T cells and antibodies have been shown to be important for blood stage immunity. Immunoglobulin (Ig) μ chain deficient mice are unable to clear parasites from PccAS infection and develop chronic parasitaemia. (144, 146). When immunised with recombinant AMA1 it has been shown that CD4+ T cells (acting independently of antibodies) play an important role in vaccine induced protection in the *P. chabaudi adami* murine model (169). Peptide immunisation with highly immunogenic synthetic peptides from the putative

domain I of AMA1 was unable to protect against challenge with *P. chabaudi adami* (229). So both humoral and cellular immunity is required for protection in this model.

This chapter investigates the potential to induce blood stage immunity in this model with a prime boost vaccination regime using AdHu5 and MVA vectored vaccines encoding AMA1. It also studies the nature of the immune responses generated by these vectors, in terms of both humoral and cell mediated immunity.

4.2 Results

4.2.1 Sequencing of *P. chabaudi chabaudi* AS AMA1

The complete sequence of PccAS AMA-1 was not available, so primers were designed against conserved regions by comparing the sequence from closely related strains to amplify this region (168). The primers were then used to PCR the antigen from PccAS genomic DNA, obtained from Dr Jean Langhorne, NIMR, London, U.K. DNA sequencing of these amplified regions was then completed to obtain the amino acid sequence for PccAS AMA-1. The sequencing was done at the WTCHG, Oxford and MWG Biotech (Ebensburg, Germany). The sequence of PccAS differed from the other closely related *P. chabaudi adami DS* strain by 34 amino acids (Figure 4-1).

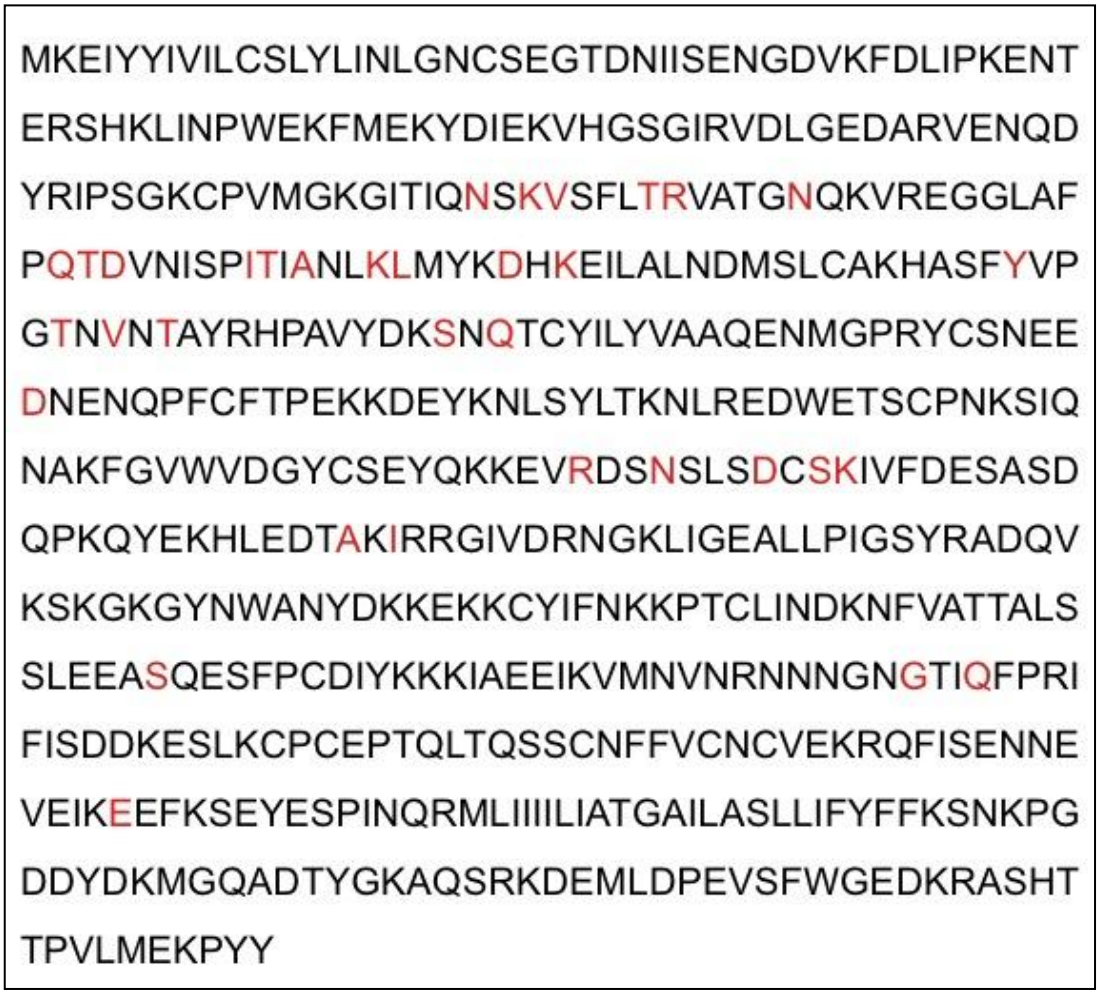


Figure 4-1: Complete sequence of *P. chabaudi chabaudi* AS AMA1

The complete amino acid sequence of P*. chabaudi chabaudi AS* AMA1 is shown in the above figure. The polymorphic amino acids in comparison to the other closely related strains was compared and are depicted in red (168). The sequence was different from closely related *P. chabaudi adami DS* strain by 34 amino acids among a total of 558 aa.

4.2.2 Vaccine design

The vaccine construct contains the ectodomain of AMA1 (amino acids 21-479) with four N- glycosylation sites removed, and is codon optimised for expression in mice. This construct was used to make recombinant AdHu5 and MVA expressing AMA1. The AdHu5-PccAMA1 and MVA-PccAMA1 were used in an 8-week prime boost vaccination regime to assess immunogenicity and protection in the P. chabaudi rodent malaria model (Figure 4-2).

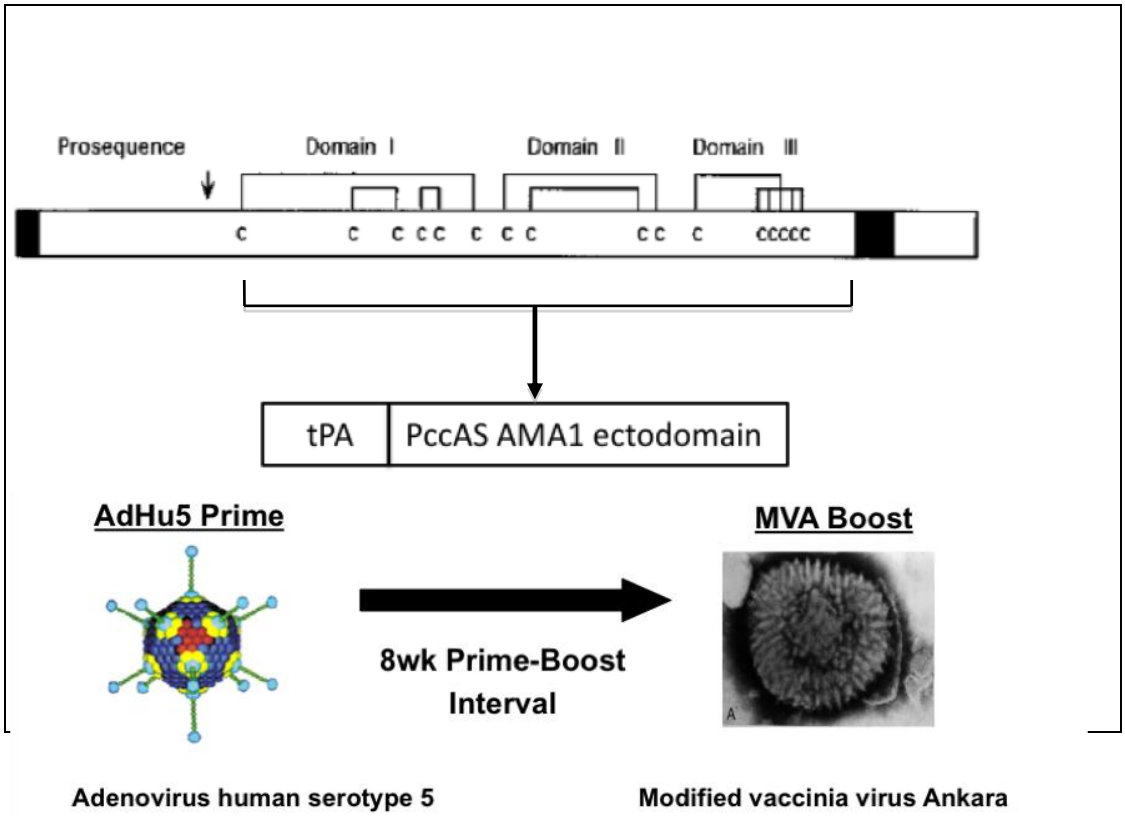


Figure 4-2: A schematic representation of the design of the vaccine construct.

The vaccine construct contains the tissue plasminogen activator (tPA) leader sequence followed by the ectodomain of AMA1 of *P. chabaudi chabaudi AS.* The constructs were used to make recombinant AdHu5 and MVA expressing AMA1 that were subsequently used for immunogenicity and protection studies in mice. The vaccines were administered in a prime boost vaccination regime. The mice were primed with $5X10^{10}$ viral particles (vp) of AdHu5-PccAMA1 and boosted 8 weeks later with $1X10^{7}$ plaque forming units (pfu) of MVA-PccAMA1.

4.2.3 Immunogenicity of the prime-boost vaccination regime

4.2.3.1 Humoral immune response to recombinant PccAS AMA1 protein.

The total IgG response against the ectodomain of PccAS AMA1 was measured by Enzyme Linked Immuno Sorbent Assay (ELISA). The recombinant protein was a kind gift from Dr Jean Langhorne, NIMR, Mill Hill, London, U.K. 5-6 week old BALB/c mice (n=5) were immunized intradermally (i.d.) with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted eight weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. This 8 week prime boost interval has been shown to be optimal for both antibody and T cell responses using these two viral vectors in prime boost vaccination regime (177, 230). Serum was obtained 2 weeks post prime (Day 14), pre boost (Day 55) and 2 weeks post boost (Day70) and ELISA performed to assess the total IgG levels. There was detectable IgG response 2 weeks after prime, which increased over time and was significantly higher at day 55 (pre boost). The MVA boost further increased the antibody level significantly as measured on day 70 (Figure 4-3A).

The IgG isotype profile was also measured by an isotype ELISA to assess if the Ad_M PccAMA1 vaccination regime induced a Th1-type IgG2a or Th2-type IgG1 response. Two weeks post boost the IgG isotype profile in the serum showed a balanced IgG1 and IgG2a response (Figure 4-3B).

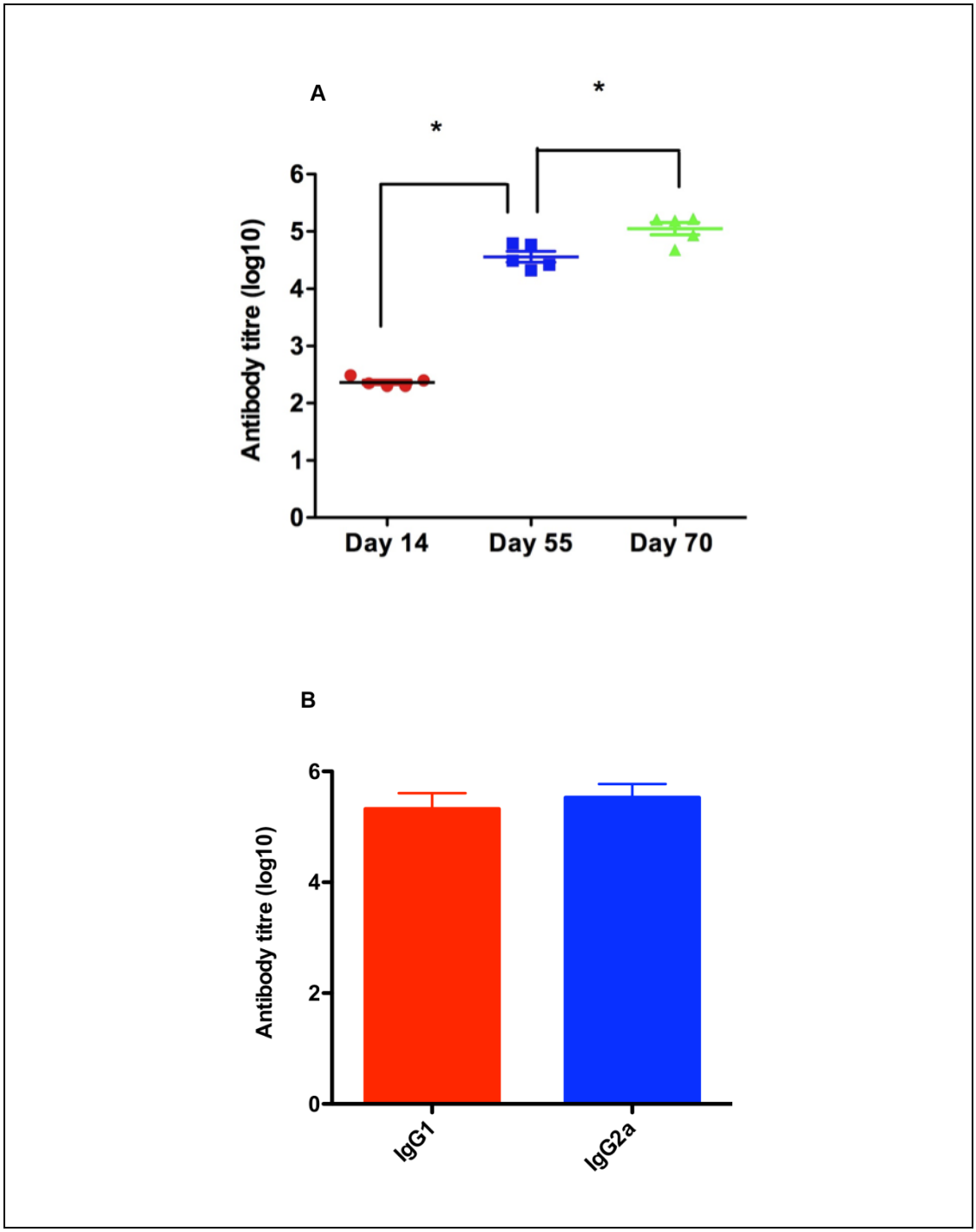

A
*
*
Antibody titre (log10)
6
5
4
3
2
1
0
Day 14
Day 55
Day 70
B
Antibody titre (log10)
6
4
2
0
IgG1
IgG2a

Figure 4-3: Antibody responses induced by the prime boost vaccination regime targeting AMA1

Female 5-6 week old Balb/c mice (n=5) were primed with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA-1. The AMA1 specific total IgG response against recombinant PccAMA1 was measured in the serum at day 14 (post prime), 55 (pre boost) and 70 (post boost) (A). Each point indicates the response from individual mice and the lines represent the mean and SEM. The asterisk indicates a significant difference between the different time points as analysed by Mann Whitney test (* $P \leq 0.05$). The IgG isotype profile (IgG1 and IgG2a) was assessed at day 70 in the serum against recombinant Pcc AS AMA1 (B).

4.2.3.2 T cell response to AMA1 peptide pools and epitope mapping within the pools.

The T cell response against a range of published CD4+ and predicted CD8+ epitopes was measured by intracellular cytokine staining (ICS). Six peptide pools containing 15 mer peptides overlapping by 10 amino acids (aa) were tested. The peptides were divided into pools according to whether they were published to contain CD4+ (168) or predicted CD8+ T cell epitopes, and also according to the position of the peptide (Table 4-1). Pool 1 had 10 peptides (Pcc7 to Pcc16) and contained published CD4+ eptiopes. Pool 2 (Pcc25 and Pcc26), Pool 3 (Pcc29 and Pcc30) and Pool 4 (Pcc74 and Pcc75) each had 2 peptides and were predicted to be CD8+ epitopes from known H-2 class I binding motifs using the epitope prediction section of the SYFPEITH website (http://www.syfpeithi.de.). Pool 5 (Pcc79 and Pcc80) and Pool 6 (Pcc84 and

Pcc85) also contained two peptides each, which were published to be CD4+ T cell epitopes. Splenocytes from Balb/c mice two weeks after final immunisation with AdHu5_MVA PccAMA1 were stimulated with the peptide pools for 5 hours, surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2. Pool 1 and pool 5 were identified as responding peptide pools to this vaccination regime. These were CD4+ T cell peptide pools (Figure 4-4A-C). There was no CD8+ T cell response detected to any of the peptide pool tested (Figure 4-4D-F). The individual epitopes within the responding peptide pool was then identified in a separate experiment.

Two CD4+ T cell epitopes in Balb/c mice P16 (within Pool1) and P79 (within Pool5) were identified in the ectodomain of Pcc AS AMA1. These cells produced all the three cytokines but the levels of IFNγ and TNFα were higher than the levels of IL-2 production. There were no CD8+ T cell epitopes identified in Balb/c mice within the pools tested (Figure 4-5).

POOL	PEPTIDE	SEQUENCE	CD4 or CD8 Pool
POOL 1	P7	LINPWEKFMEKYDIE	CD4
	P8	EKFMEKYDIEKVHGS	
	P9	KYDIEKVHGSGIRVD	
	P10	KVHGSGIRVDLGEDA	
	P11	GIRVDLGEDARVENQ	
	P12	LGEDARVENQDYRIP	
	P13	RVENQDYRIPSGKCP	
	P14	DYRIPSGKCPVMGKG	
	P15	SGKCPVMGKGITIQN	
	P16	VMGKGITIQNSKVSF	
POOL 2	P25	ANLKLMYKDHKEILA	CD8
	P26	MYKDHKEILALNDMS	
POOL 3	P29	LCAKHASFYVPGTNV	CD8
	P30	ASFYVPGTNVNTAYR	
POOL 4	P74	SFPCDIYKKKIAEEI	CD8
	P75	IYKKKIAEEIKVMNV	
POOL 5	P79	GNGTIQFPRIFISDD	CD4
	P80	QFPRIFISDDKESLK	
POOL 6	P85	SSCNFFVCNCVEKRQ	CD4
	P86	FVCNCVEKRQFISEN	

Table 4-1: CD4+ and CD8+ peptide pools tested by ICS.

The table shows the peptide pools tested by ICS. Six peptide pools with the aa sequence of each peptide is shown. The far right column indicates if the peptide pools were published (168) to be CD4+ T cell epitopes or predicted to be CD8+ T cell epitopes.

CD4 CD8

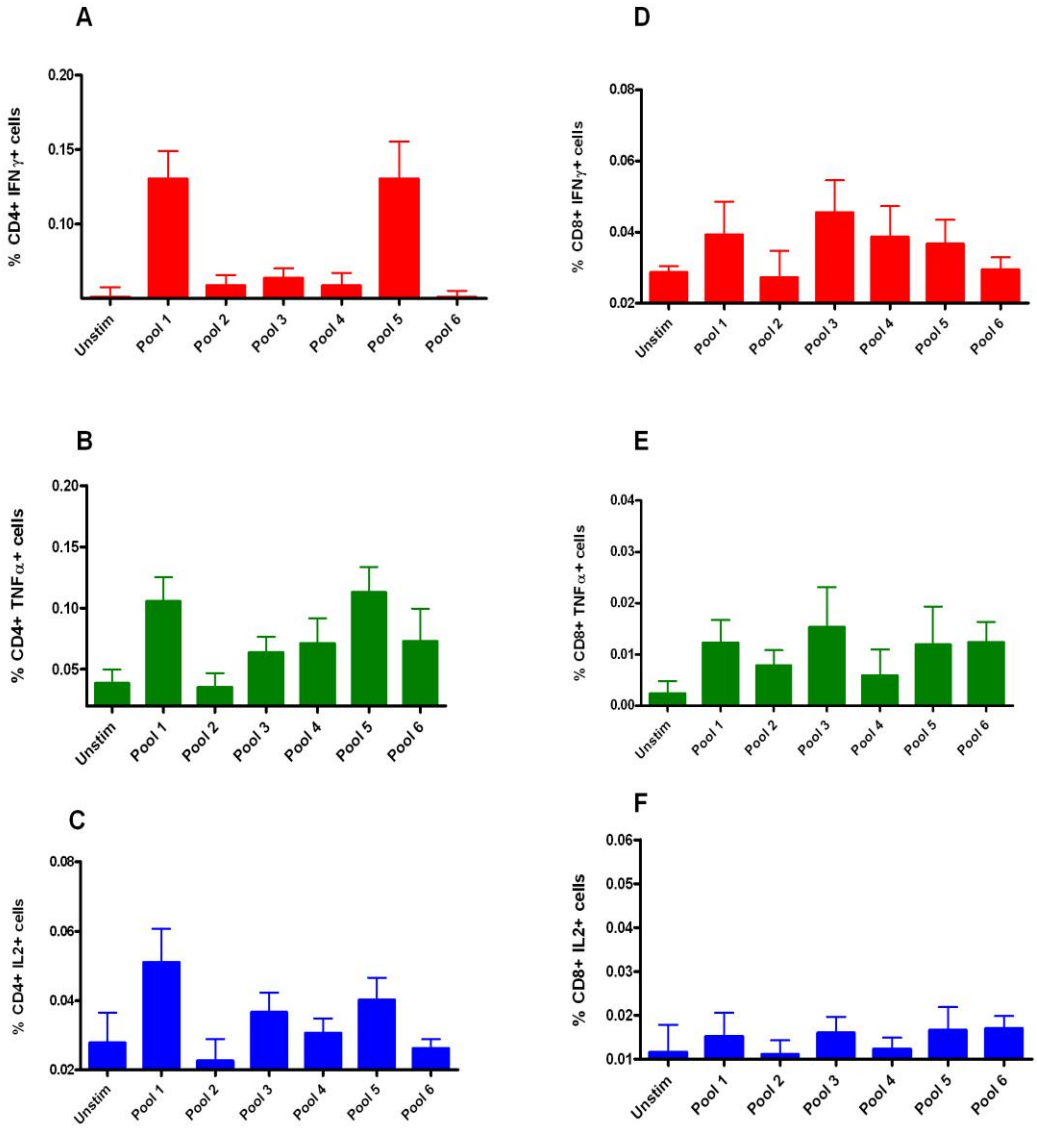
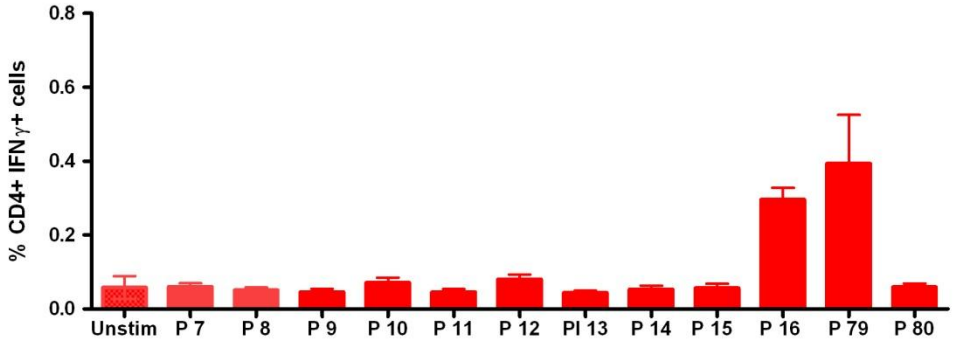
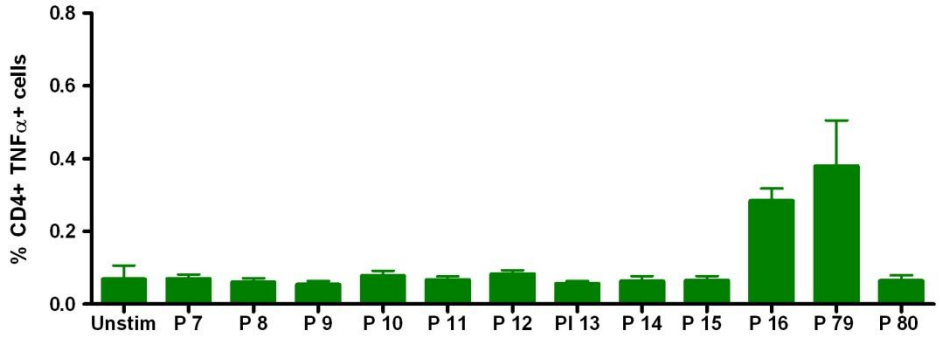

A
% CD4+ IFNγ+ cells
0.20
0.15
0.10
Unstim
Pool 1
Pool 2
Pool 3
Pool 4
Pool 5
Pool 6
D
% CD8+ IFNγ+ cells
0.08
0.06
0.04
0.02
B
% CD4+ TNFα+ cells
0.20
0.15
0.10
0.05
E
% CD8+ TNFα+ cells
0.04
0.03
0.02
0.01
0.00
C
% CD4+ IL2+ cells
0.08
0.06
0.04
0.02
F
% CD8+ IL2+ cells
0.06
0.05
0.04
0.03
0.02
0.01

Figure 4-4: T cell responses assessed in the spleen to AMA1 peptide pools after AdHu5_MVA PccAMA1 vaccination regime.

Female 5-6 week old Balb/c mice (n=5) were primed with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost splenocytes were isolated and stimulated with pools of over-lapping peptides (Pool 1 to Pool 6) or no peptide (Unstim) for 5h. Cells were surface-stained for CD4 (left panels) and CD8 (right panels), and intracellularly stained for IFNγ (A, D), TNFα (B, E) and IL-2 (C, F), and assessed by flow cytometry to determine the % of responding cells per total CD8+ or CD4+ T cell subset. The bars represent the mean responses with standard error of mean (SEM).


% CD4+ IFNγ+ cells
0.8
0.6
0.4
0.2
0.0
Unstim
P 7
P 8
P 9
P 10
P 11
P 12
Pl 13
P 14
P 15
P 16
P 79
P 80


% CD4+ TNFα+ cells
0.8
0.6
0.4
0.2
0.0
Unstim
P 7
P 8
P 9
P 10
P 11
P 12
Pl 13
P 14
P 15
P 16
P 79
P 80

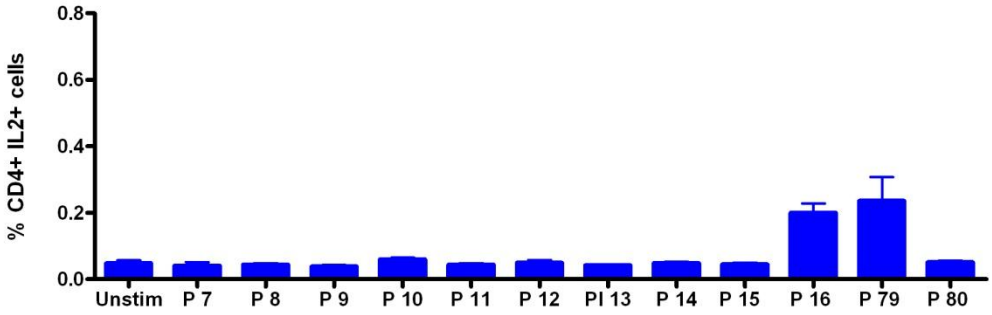

% CD4+ IL2+ cells
0.8
0.6
0.4
0.2
0.0
Unstim
P 7
P 8
P 9
P 10
P 11
P 12
Pl 13
P 14
P 15
P 16
P 79
P 80

Figure 4-5: T cell epitopes within the responding overlapping peptide pools within the ectodomain of PccAS AMA1.

Female 5-6 week old Balb/c mice (n=5) were primed with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost splenocytes were isolated and stimulated with individual peptides from Pool1 (P7 to P16), Pool 5 (P79 and P80) or no peptide (Unstim) for 5h. Cells were surface-stained for CD4 and intracellularly stained for IFNγ (A), TNFα (B) and IL-2 (C) and assessed by flow cytometry to determine % of responding cells per total CD4+ T cell subset. The bars represent mean response and SEM.

4.2.3.3 Epitope mapping for the ectodomain of PccAS AMA1

Two CD4+ T cell epitopes were identified in the peptide pools previously tested. These peptides were the ones that were previously identified after immunisation with recombinant AMA1 protein (168). In this study using a viral vectored vaccination regime it could be possible that viral vectored vaccines induce T cell responses to other epitopes as well as to the ones identified after protein immunisation. In order to test this T cell epitopes in BALB/c were determined using overlapping peptide pools to the whole ectodomain of AMA1 by ICS. The peptides were 15 mers, overlapping by 10 aa covering the entire ectodomain of PccAS AMA1. Splenocytes were isolated 2 weeks post boost and stimulated with 7 peptide pools (10 peptide in each pool) or no peptide (Unstim) for 5 hours. After 5 hours the cells were surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2. The CD4 T cell epitopes P16 and P79 confirmed in the previous set of experiments (Figure 4-5) were included as positive controls. There was no response to any of the peptide pools except a very weak CD8+ IFNγ+ response in Pool 5 (Figure 4-6).

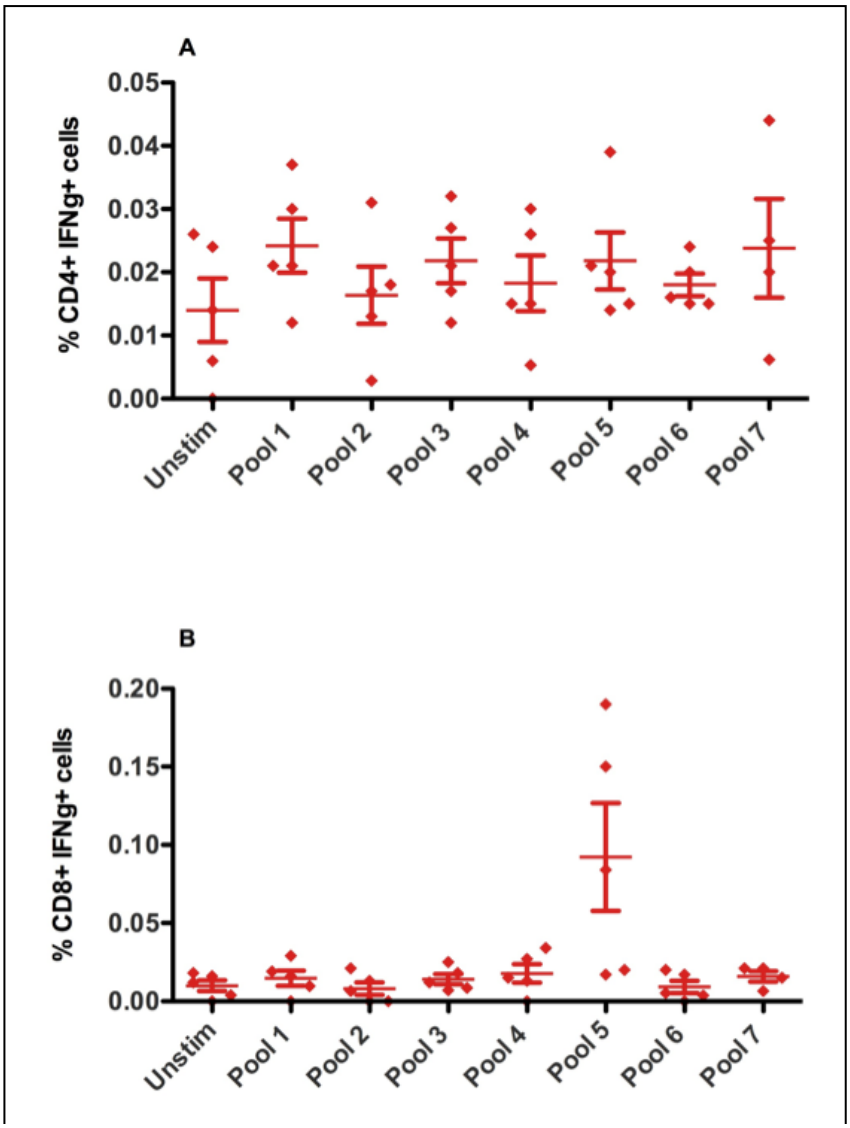


Figure 4-6: Epitope mapping within the ectodomain of PccAS AMA1.

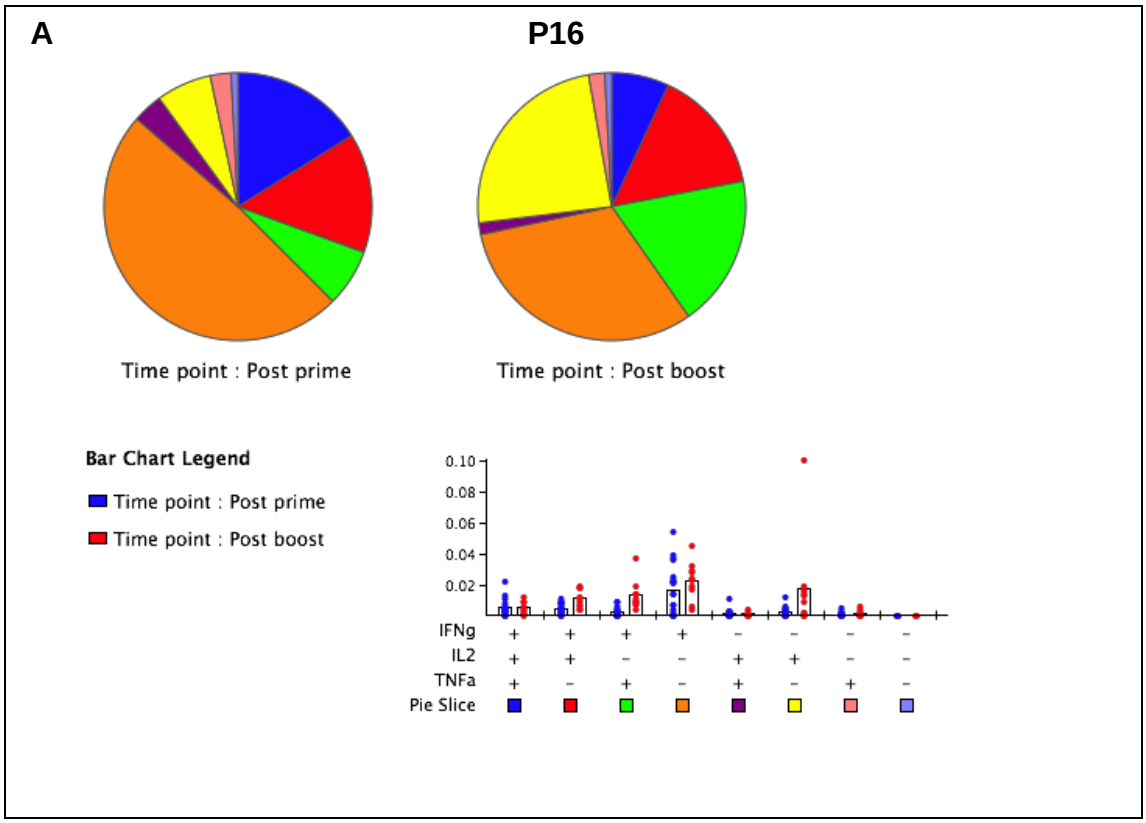
Female 5-6 week old Balb/c mice (n=5 per group) were primed with $5x10^{10}$ AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu MVA-PccAMA1. Spleens were harvested two weeks post boost and splenocytes isolated. The splenocytes were stimulated with 7 pools containing 10 peptide (Pool1 to Pool 7) or no peptide (Unstim) for 5 hours. After 5 hours the cells were surface stained for CD4 or CD8 and then intracellularly for IFNγ, TNFα and IL-2. The figure shows the % of CD4+ IFNγ+ cells (A) or CD8+ IFNγ+ cells (B). The dots represent responses from individual mice and the lines represent mean and SEM.

4.2.3.4 Multifunctionality of the AMA1-specific CD4+ T cell response after prime and boost vaccination.

The multifunctionality of the CD4+ T cells was determined both after prime and boost in order to see if there was a change in the functionality of the CD4+ T cells induced. Splenocytes were isolated two weeks post prime and two weeks post boost and stimulated for 5 hours with the two peptides containing the CD4+ T cell epitopes identified (P16 and P79), surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2. Triple positive cells were defined as cells which simultaneously produce all the three cytokines, double positive cells produce any two of the three cytokines and single positive cells are those which produce only a single cytokine. It has been previously shown that the frequency of CD4+ T cells producing all the three cytokines correlates with protection against *Leishmania major* in mice (231). The multifunctionality or "quality" of CD4+ T cells was analysed using the SPICE software (M Roederer, NIH, Bethesda, USA).

For the epitope P16, the number of triple positive cells producing IFNγ, TNFα and IL-2 simultaneously does not change after the boost. There is a trend towards increased number of double positive cells producing IFNγ and IL-2 or IFNγ and TNFα. Cells producing only TNFα and IL-2 were not detectable. There is a significant increase in the number of cells producing IL-2 after boost and also a trend for an increase in the number of cells producing only IFNγ after the boost (Figure 4-7A).

For P79 the multifunctionality of the CD4+ T cells was similar to that of P16 and there is no significant difference in the number of triple positive cells after the MVA-PccAMA1 boost (Figure 4-7B). As the MVA boost does not increase the number of multifunctional cells it could be that a single dose of AdHu5-PccAMA1, could lead to reduction in blood stage parasitaemia if these cells are important and sufficient on their own to protect but one has to consider that the antibody levels will be lower after prime only than prime-boost. The role of multifunctional cells in immunity to malaria has not been demonstrated yet and it could be that the monofunctional cells are as protective as multifunctional cells against blood stage malaria.


A
P16
Time point : Post prime
Time point : Post boost
Bar Chart Legend
Time point : Post prime
Time point : Post boost
0.10
0.08
0.06
0.04
0.02
IFNg
IL2
TNFa
Pie Slice

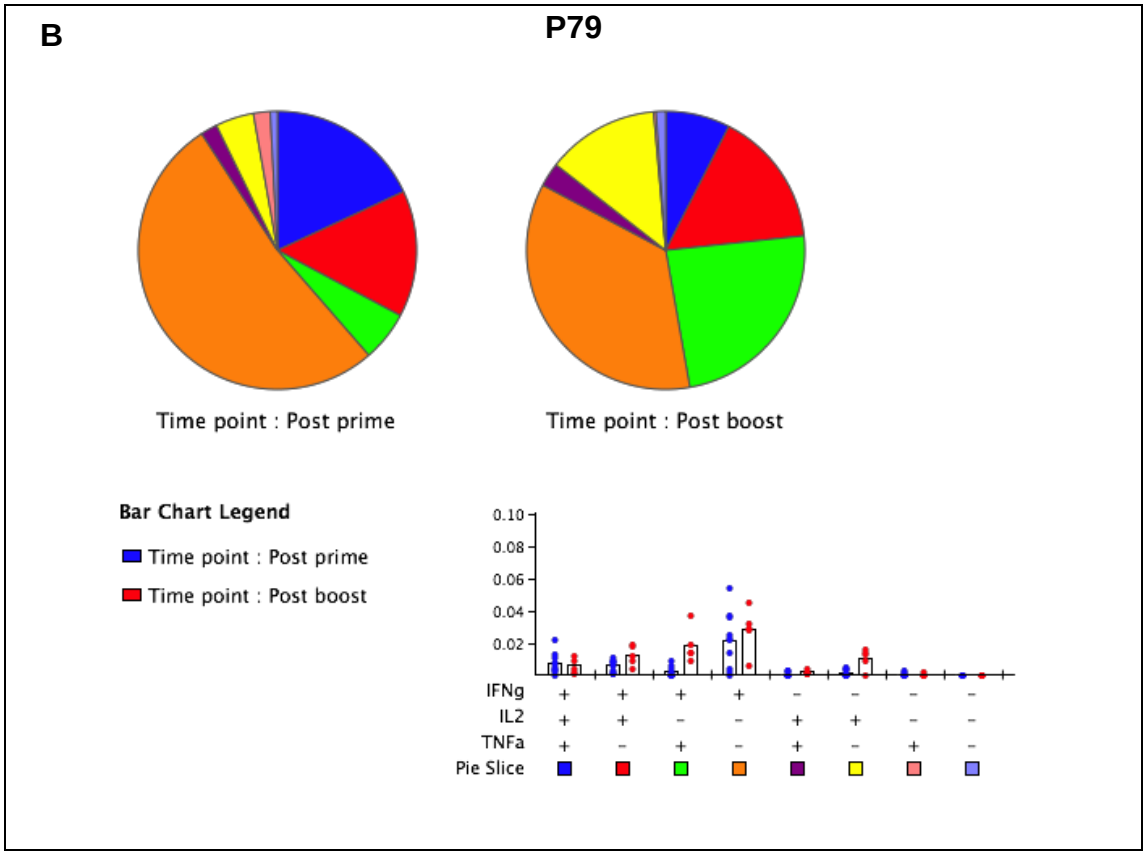

B
P79
Time point : Post prime
Time point : Post boost
Bar Chart Legend
Time point : Post prime
Time point : Post boost
0.10
0.08
0.06
0.04
0.02
IFNg
IL2
TNFa
Pie Slice

Figure 4-7: Multifunctionality of CD4+ T cells after prime and boost.

Female 5-6 week old Balb/c mice (n=5 per group) were primed with $1x10^{10}$ vp of AdHu5-PccAMA1 only or primed and then boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post prime (AdHu5-PccAMA1) and two weeks post boost (AdHu5_MVA PccAMA1) splenocytes were isolated and stimulated with the peptides P16 (A) and P79 (B) respectively (CD4+ T cell epitopes mapped previously) or no peptide (Unstim) for 5h. Cells were surface-stained for CD4+ and intracellularly stained for IFNγ, TNFα and IL-2, and assessed by flow cytometry to determine the % of responding cells per total CD4+ T cell subset. The multifunctionality or "quality" of CD4+ T cells was analysed using the SPICE software.

4.2.3.5 Antibody response to native AMA1 protein

In section 4.2.3.1 the sera from vaccinated mice recognise His_6-tagged recombinant AMA1 protein. Though His_6-tagged proteins have been previously shown to correctly fold (232), an ELISA was done to demonstrate that the vaccine induced antibodies recognise native AMA1 protein from parasite lysate. Balb/c mice were vaccinated with the Ad_M PccAMA1 vaccination regime and two weeks post boost the serum was obtained. Naive mice were infected with PccAS parasites and the parasite lysate was extracted from the infected blood at peak parasitaemia. This PccAS parasite lysate was used in an ELISA to assess if the antibodies induced by vaccination bound to the native AMA1 protein in the lysate. The sera recognised the native protein though the titres were not as high as the responses measured against recombinant PccAS AMA1 but this could be because of the difference in the coating antigens used in the ELISA (Figure 4-8). This could be due to less schizonts in the lysate and lower expression of AMA1.

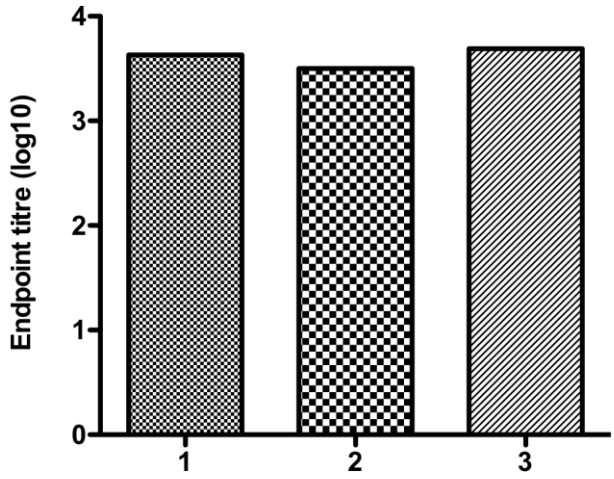


Figure 4-8: Antibody response to native AMA1 protein

Female 5-6 week old Balb/c mice (n=3) were primed with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA-1. Total IgG responses were measured against PccAS parasite lysate two weeks after the final vaccination. The parasite lysate was extracted from the blood of mice infected with PccAS parasites. Columns represent responses from individual mice.

4.2.4 Protective efficacy of Ad_M PccAMA1 vaccination regime against blood stage challenge

The protective efficacy of the above mentioned prime boost vaccination regime was assessed by blood stage challenge with Pcc AS parasites. 5-6 week old female Balb/c mice (n=6) were vaccinated with the Ad_M PccAMA1 vaccination regime and challenged two weeks post boost intravenously with 10^{5} non-lethal Pcc AS parasitized red blood cells (pRBC). Naive Balb/c mice (n=6) were also challenged at the same time. Parasitaemia was monitored from day 4 post challenge by Giemsa-stained thin blood smears and results

were expressed as the % infected RBCs. As Pcc AS infection in naive Balb/c mice is non-lethal the protective efficacy of this vaccination regime was measured in terms of significant difference in parasitaemia post challenge between the naïve and vaccinated groups. The mean percentage of parasitaemia +/- SEM was calculated (Figure 4-9). Significantly lower parasitaemia was observed in the Ad_M PccAMA1 vaccinated mice than the naive mice as early as from day 5 and lasted until day 7. At peak parasitaemia (day 7) the vaccinated mice had a parasitaemia of 24.78 +/- 1.07 whereas the naive mice had a significantly higher parasitaemia of 43.69 +/- 2.29. Thus the vaccination regime leads to a significant reduction in parasitaemia after blood stage challenge.

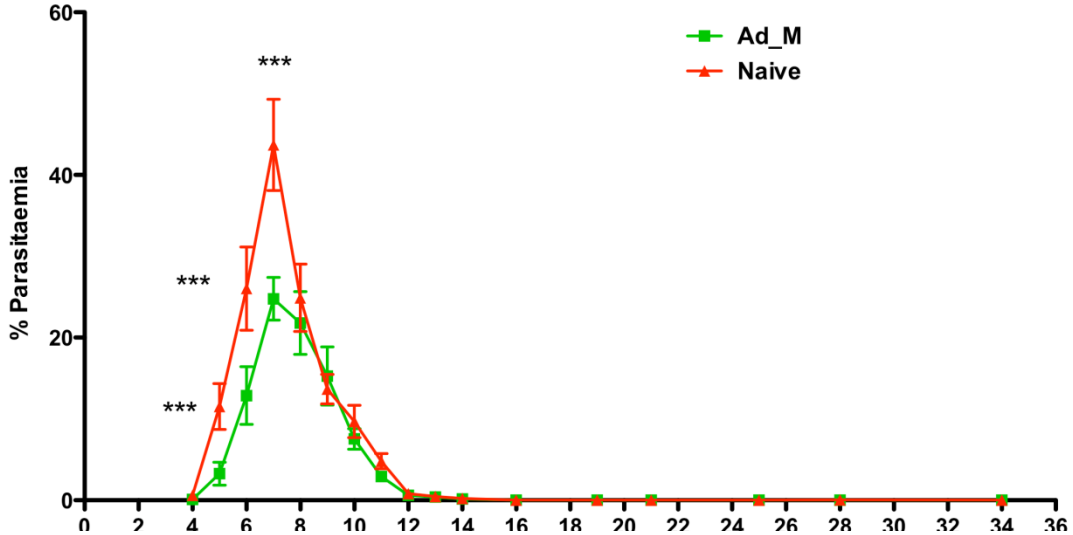


Figure 4-9: Efficacy of the Ad_M PccAMA1 regime against blood stage challenge.

Six 5-6 week old Balb/c mice were vaccinated with $5x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA-1. These mice were challenged intravenously 14 days post-boost with 10^{5} parasitized red blood cells (pRBCs). Parasitaemia was monitored by Giemsa-stained thin blood smears and results are expressed as the % infected RBCs. Six naive mice were also challenged simultaneously and the reduction in parasitaemia was measured as the vaccine efficacy. The mean percentage of parasitaemia +/- SEM was calculated. The asterisk indicates a significant difference (Mann Whitney test ** $P \leq 0.01$, and *** $P \leq 0.001$) in the % parasitaemia between the vaccinated and the naive mice.

4.3 Discussion

This study explores the immunogenicity and protection induced by the prime boost vaccination regime using viral vectors encoding *P. chabaudi* AMA1. Before the construction of the vaccine the complete sequence of Pcc AS AMA1 was determined as it was not available. The ectodomain has been previously shown to be sufficient to induce protective humoral and cellular immune responses (168, 233). Both humoral and cellular immune responses were measured after the prime boost vaccination regime using AdHu5 and MVA expressing PccAS AMA1 to the ectodomain of AMA1.

The antibody response measured was comparable to those obtained by multiple immunisations with classical protein-in-adjuvant immunisation though it is probably not easy to directly compare the two vaccination regimes (233). The AdHu5-PccAMA1 vaccine primed a detectable antibody response that increased over time and was boosted significantly after the administration of MVA-PccAMA1. This is in accordance with previous findings in the *P. yoelii* model using viral vectors expressing MSP1 where an 8-week interval between prime and boost is shown to be optimal for the induction of both antibodies and T cells (226). The kinetics of the antibody response are similar to those seen by vaccination with AdHu5 expressing other antigens like avian influenza H5N1 HA (234) and Ebola virus glycoprotein (191), where the antibody response plateaus by six weeks post-vaccination, and increased by a similar order of magnitude. Following antigen exposure, short-lived plasma B cells will develop, with CD4+ T cell help that are isotype-switched but not

affinity-driven by the germinal centre reaction. Long-lived plasma B cells, that produce high-affinity antibody following their development in the germinal centre and somatic hypermutation of the genes encoding the BCR, typically follow by the second week of the primary response and maintain significant levels of antibody, often for the lifetime of the individual (235, 236). This is likely to account for the continued increase in antibody levels for the 6-8 week period following recombinant AdHu5 immunisation. Antibody levels have been shown to correlate with protection in the *P. chabaudi* model when mice are immunised with recombinant protein AMA1 (168). It has been argued that high levels of IgG are necessary for vaccines targeting the antigens on the merozoite surface because of the limited time of exposure of these antigens on the merozoite surface between schizont rupture and merozoite reinvasion and thus it will be interesting to see if the Ad_M vaccination regime can achieve this level in clinical trials (237). The isotype profile of the IgG response induced by the Ad_M PccAMA1 vaccination regime was of a balanced Th1-type IgG2a and Th2-type IgG1 response. There was no change in the IgG antibody isotypes after the boost though in other studies it has been reported that AdHu5 vectors induce a dominating Th1-type IgG2a response (238). After boosting with MVA-PccAMA1 this IgG2a dominance was not seen in this study. This could be dependent on the antigen and the mouse model. In mice, the cytophilic IgG2a antibodies have been shown to correlate with protection against *Plasmodium* infection (239, 240).

Two CD4+ T cell epitopes were identified in the spleen of immunised Balb/c mice using flow cytometry and peptides to the ectodomain of PccAS AMA1.

These peptides contained epitopes that were previously described after immunisation with recombinant AMA1 (168). There was a very weak CD8+ T cell response to one of the peptide pools tested for epitope mapping within the whole ectodomain of AMA1 but no response to the peptides predicted by the H-2^d class I epitope motif. There was no CD4+ T cell epitope identified in Balb/c mice other than the ones previously described. The prime-boost vaccination regime induced multifunctional CD4+ T cell responses to epitopes in the ectodomain of AMA1. In the mouse model for *Leishmania major*, the quality of the CD4+ T cell response has been shown to be a crucial determinant in vaccine efficacy and an immune correlate of protection. In that study CD4 T cells which produce IFNγ, TNFα and IL-2 simultaneously were shown to be the best correlate of protection (231). The frequency of IFNγ producing cells is classically measured as the primary correlate of vaccine induced protection (241) but in several studies it has been shown that IFNγ alone is not always a reliable correlate of protection in diseases like tuberculosis and leishmaniasis (242-245). In tuberculosis multifunctional memory CD4+ T cells have been shown to correlate with long term vaccine induced protective immunity (246). IFNγ and TNFα together are known to mediate killing of pathogens and IL-2 enhances the expansion of CD4+ and CD8+ T cells thus it is plausible that cells which produce these three cytokines simultaneously are better correlates of protection. Indeed, peripheral blood mononuclear cells from children with mild *P. falciparum* infection produce high levels of IFNγ when stimulated *in vitro* with merozoite antigens (247).

Adenovirus vectors have been shown previously to induce significantly higher frequencies of 1+ and 2+ and slightly higher 3+ CD8+ T cells than pox viral vectors against a malaria fusion protein ME.TRAP (225). In this study it was investigated if boosting the response generated by AdHu5-PccAMA1 with MVAPccAMA1 could increase the number of 1+, 2+ and 3+ CD4+ T cells. The quantity of 3+ CD4+ T cell was the same after the boost though there was a trend towards an increase in the number of 2+ cells. The role of these cells in malaria needs to be investigated further.

It has been recently shown that it is important to direct antibodies by vaccination to conformation-dependant epitopes (248). Achieving high titre antibody response is not enough to ensure vaccine efficacy as antibodies directed against non-conformational epitopes is not a good correlate of protection and also limits the effectiveness of antibodies to conformation-dependant epitopes (248). The antibodies against AMA1 induced by the vaccination regime bound to native AMA1 protein in parasite lysate in an ELISA, so one could assume that some of the antibodies generated were to conformation-dependant epitopes. However this does not prove the conformation of the antibodies generated. A better proof would be to reduce and alkylate the recombinant protein used for ELISA and see if the vaccine induced antibodies still bound to the protein. If the antibodies still bound to the protein it would indicate that a proportion of the antibodies recognised linear epitopes in AMA1. It has been shown that antibodies against linear epitopes of PcAMA1 are partially effective against nonlethal P. chabaudi malaria but that the most effective protective response involves disulfide-dependent B-cell

epitopes (248). It was suggested that high titre antibodies directed against conformation-independent epitopes might limit the effectiveness of protective antibodies that recognize conformation-dependent epitopes. Thus it may well be important to generate conformation-dependent antibodies by vaccination.

This work has also demonstrated the ability of a recombinant AdHu5-MVA PccAMA1 prime-boost vaccine regime to afford protective efficacy against a P. chabaudi blood stage challenge. The protection was measured as a reduction in peak parasitaemia between the vaccinated and the naive group. As early as day 5 there is a significant difference in parasitaemia between the vaccinated and naive mice. This early reduction in parasitaemia could be due to pro-inflammatory cytokines secreted by the CD4+ T cells or antibodies generated by vaccination (Figure 4-9) or both. A meaningful CD8+ T cell response was not measured after vaccination so the role of these cells in vaccine induced protection was not assessed. The mechanism of vaccine induced protection is further dissected in the chapter 5.

CHAPTER 5

MECHANISM OF VACCINE INDUCED BLOOD STAGE PROTECTION

5 Mechanism of vaccine induced blood stage protection

5.1 Introduction

In the *P. chabaudi* murine malaria model both CD4+ T cells and antibodies have been shown to be important for blood stage immunity. Immunologically competent mice generate a protective immune response that involves both Th1 and Th2-type CD4+ T cells (249). The importance of CD4+ T cells has been shown in several depletion and adoptive transfer studies in immunodeficient mice (250-254). In the acute stage of the infection there is a Th1 type response, which is antibody independent, and this Th1 response controls the initial peak parasitaemia and is mediated by production of type1 effector molecules such as IFNγ and TNFα (112, 129, 146, 148, 255-258). A Th2 CD4+ T cell response predominates during the later phases of infection and these cells produce IL-4, IL-5, IL-6 and IL-10 which also provide help for antibody production by B cells (259, 260). B cells are important for the clearance of the parasite and are not required for early control. B cell depletion affects the clearance of the parasite and the resolution of the chronic phase of infection (129, 146, 148, 261). B cells have been shown to play a dual role in blood stage immunity to Pcc AS infection. They help in the clearance of the parasite by producing antibodies but studies in B cell depleted mice have also shown that these cells are essential for the switch

from a Th1 to Th2 response which is characteristic of the CD4+ T cell response during *P. chabaudi* blood stage infection (145). It has been suggested that the persistence of the Th1 response to *P. chabaudi* in B-cell-depleted mice is due to a relative lack of IL-10 production by B cells (145). Other experiments have also shown that in B cell depleted mice the Th1 response is maintained as late as day 44, unlike control mice in which a Th2 response dominates from day 20 post infection (262).

Vaccine studies with recombinant AMA1 in the *P. chabaudi adami* model have also shown the importance of antibodies and CD4+ T cells acting independently of antibodies in vaccine induced protection. AMA1 antibodies correlate with protection in mice and passive immunization of mice with anti-AMA1 monoclonal antibodies confers protection against lethal *P. yoelii* YM challenge, another species of rodent malaria (137). A significant decrease in antiparasite control is also seen after CD4+ T cell depletion in immunised mice though there is no decrease in the levels of anti-AMA1 antibodies (169). The evidence for the role of CD4+ T cells and antibodies in protection against blood stage infection is contradictory and will be assessed using viral vectors in this chapter.

Viral vectors are potent inducers of both T cell and antibody responses. This chapter investigates the mechanism of vaccine induced protection in the *P. chabaudi* model, given these protective vectors provide an ideal system with which to dissect the role of both the cellular and humoral arm of the immune system. The role of vaccine induced CD4+ T cells and antibodies has been

studied by depletion and adoptive transfer studies in both naive and immunocompromised mice.

5.2 Results

5.2.1 *In vivo* depletion of CD4+ T cells

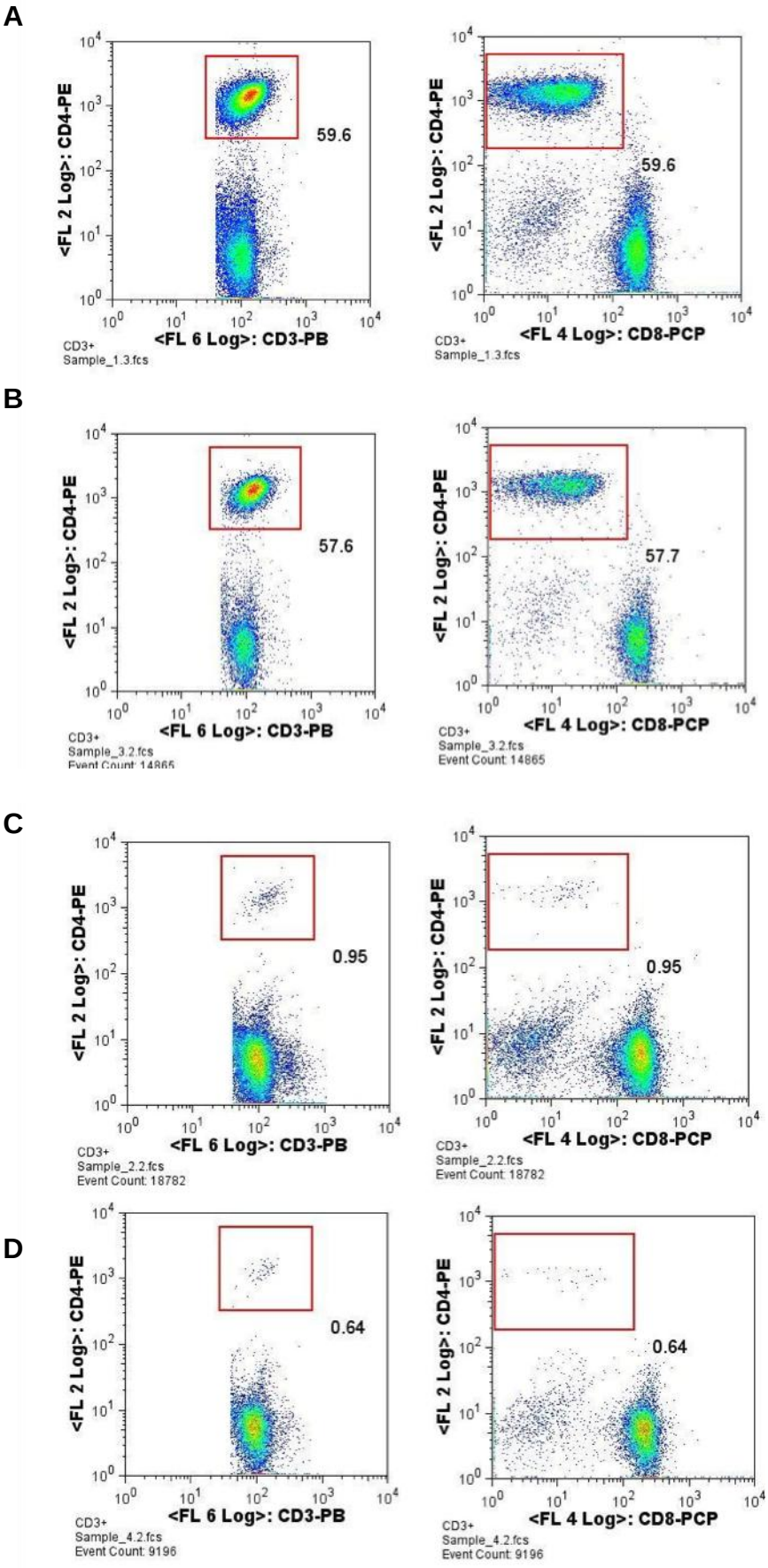
The contribution of CD4+ T cells in vaccine-induced protection against blood stage challenge in this model was assessed by *in vivo* CD4+ T cell depletion using monoclonal antibodies (mAbs). The anti-CD4 GK 1.5 (rat IgG2a) mAb has been reported to deplete CD4+ T cells for up to 10 days, after which the CD4+ T cells are replenished to about 50% of the original level by 4 weeks (263). Two groups of Balb/c mice (n=6) were immunised using the Ad_M PccAMA1 vaccine regime. CD4+ T cells were depleted in one group of naive and one group of vaccinated mice with anti-CD4 GK1.5 mAb. Four groups of mice: i) vaccinated CD4+ T cell depleted; ii) vaccinated non CD4+ T cell depleted; iii) naive CD4+ T cell depleted and iv) naive non CD4+ T cell depleted were challenged intravenously with 10^6 Pcc AS pRBCs. Mice were injected intraperitoneally with 200µg of the depleting mAb on days -2 and -1 and on the day of challenge (day 0). The degree of *in vivo* CD4+ T cell depletion was assessed by flow cytometry in the peripheral blood mononuclear cells (PBMCs) of depleted and control mice and there was more than 98% depletion in the depleted groups. A different anti-CD4 clone (LT34) was used to stain to ensue that the CD4+ T cells were depleted rather than

the binding being blocked by GK1.5 antibodies. The control mice were given normal rat polyclonal IgG (Figure 5-1).

The protective efficacy of the Ad_M PccAMA1 vaccination regime was maintained even when challenged with 10^6 pRBC as compared to a challenge with 10^5 pBRC (Figure 4-9). As seen before, there was a significant difference in parasitaemia between the vaccinated and the naïve groups at days 6 and 7 (Fig 5-2A).

The CD4+ T cell depletion resulted in the loss of vaccine-induced protection as seen by a loss of reduction in blood-stage parasitaemia (Fig 5-2B) suggesting a possible role for CD4+ T cells in the mechanism that mediates control of initial parasitaemia. The vaccinated depleted mice did not clear the parasites as early as the non-depleted mice and have a second peak of parasitaemia.

It is interesting that the vaccinated depleted mice clear their parasitaemia faster than the naive-depleted mice (Fig 5-2C). This could be because of the presence of AMA1 specific antibodies in the vaccinated mice and needs to be investigated further. The naive CD4 depleted mice do not clear their parasitaemia even after one month post challenge (Fig 5-2D).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59.6
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Event Count: 9196
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0.64
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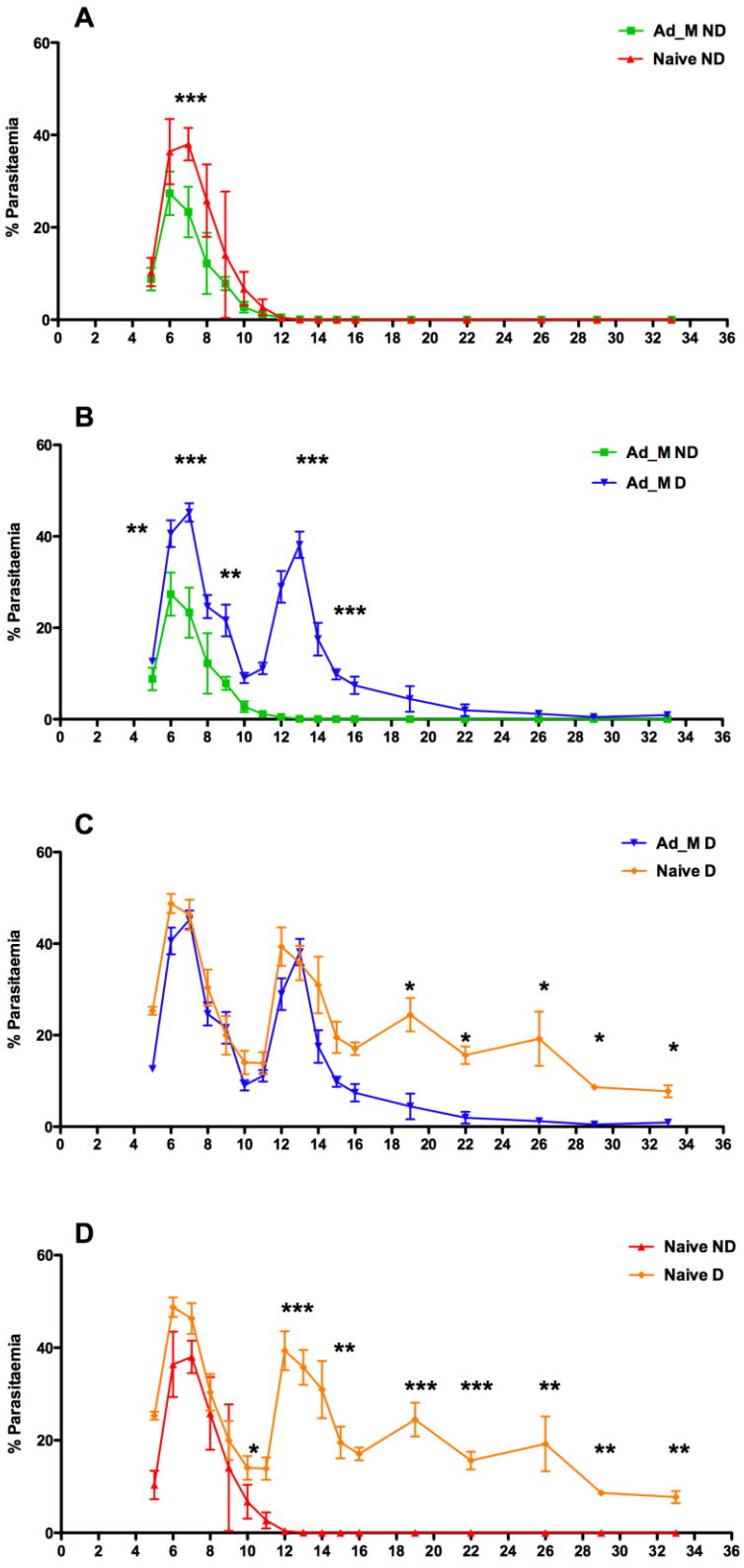
CD3+
Sample_4.2.fcs
Event Count: 9196

Figure 5-1: *In vivo* CD4+ T cell depletion using anti-CD4 GK1.5 mAb.

Two groups of Balb/c mice (n=6) were immunised with $5X10^{10}$ vp of AdHuPccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVAPccAMA1. CD4+ T cells were depleted in one group of naive and one group of vaccinated mice with anti-CD4 GK1.5 mAb. The mice received 200ug of depleting anti-CD4 mAbs intraperitoneally (i.p.) on day -2, -1 and day of challenge (day 0). Seven days after challenge the PMBCs were isolated from a blood sample and surface stained for CD3 and CD4. The anti-CD4 LT34 clone was used to stain the cells. Cells were analysed by flow cytometry for % CD3+ CD4+ T cells in the depleted and non-depleted samples. The plots show representative flow plots for vaccinated non-depleted (A), naive non-depleted (B), vaccinated depleted (C) and naive depleted (D). There was still more than 98% depletion at day 7 post challenge.


A
Ad_M ND
Naive ND
% Parasitaemia
***
B
Ad_M ND
Ad_M D
% Parasitaemia
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C
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Naive D
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D
Naive ND
Naive D
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Figure 5-2: Effect of In vivo CD4+ T cell depletion on vaccine efficacy

Two groups of six Balb/c mice were vaccinated with $5X10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA-1. These mice were challenged intravenously 14 days post-boost with 10^{6} pRBC. Two groups of naive mice were also challenged at the same time. One group each of vaccinated and naive mice were depleted of CD4+ T cells as shown in Figure 5-1. Parasitaemia was monitored by Giemsa-stained thin blood smears and results are expressed as the % infected RBCs. The reduction in parasitaemia was measured as the vaccine efficacy. The figure shows the difference in mean parasitaemia +/_ SEM between the different groups: vaccinated non depleted (Ad_M ND) and naive non depleted (Naive ND) (Figure 5-2A), vaccinated depleted (Ad_M D) and non depleted (Ad_M ND) (Figure 5-2B), vaccinated depleted (Ad_M D) and naive depleted (Naive D) (Figure 5-2C) and naive depleted (Naive D) and non depleted (Naive ND) (Figure 5-2D). The asterisk indicates a significant difference in the % parasitaemia between the different groups (Mann Whitney test * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

5.2.2 Role of AMA1 specific and whole parasite antibodies in clearance

Two groups of Balb/c mice (n=6) were immunised using the Ad_M PccAMA1 vaccine regime. CD4+ T cells were depleted in one group of naive and one group of vaccinated mice with anti-CD4 GK1.5 mAb. Four groups of mice i) vaccinated CD4+ T cell depleted, ii) vaccinated non CD4+ T cell depleted, iii) naive CD4+ T cell depleted and iv) naive non CD4+T cell depleted were challenged intravenously with 10^{6} Pcc AS parasitized RBC. Antibody titres were measured one-month post challenge in the serum of CD4+ T cell depleted and non-depleted vaccinated and naive mice. The total IgG

response to recombinant PccAS AMA1 protein and whole parasite lysate was assessed by ELISA.

There was no difference in AMA1 specific antibodies in the vaccinated depleted and non-depleted mice. An AMA1 specific antibody response was detected in non-CD4+ T cell depleted naive mice but the levels were significantly lower than the vaccinated mice. There was no detectable AMA1 specific response in the naive-depleted mice suggesting CD4+ T cell help is necessary for antibody production post challenge (Figure 5-3A)

The response to the whole parasite lysate was similar in the ND vaccinated and the ND naive groups. There was a significantly lower antibody response to the whole parasite post challenge in the CD4+ T cell depleted groups as compared to the non-depleted groups (vaccinated and naive) (Figure 5-3B). This is probably due to the lack of CD4+ T cell help in the antibody production to other antigens in the lysate. So, CD4+ T cells help is required for antibody production for both AMA1 and other parasite antigens in the lysate.

The group of mice that was still parasitaemic one month post challenge was the naive CD4+ T cell depleted mice (Figure 5-3D) and it is interesting to note that these mice have no AMA1 specific antibodies but similar lysate specific antibody levels when compared to vaccinated depleted mice. At the same time both the vaccinated and the naïve non-depleted mice clear the parasite at the same time though the naives have significantly lower AMA1 specific antibodies (Figure 5-3C). This would suggest both AMA1 specific antibodies

and antibodies to several other antigens (lysate) may contribute to parasite clearance.

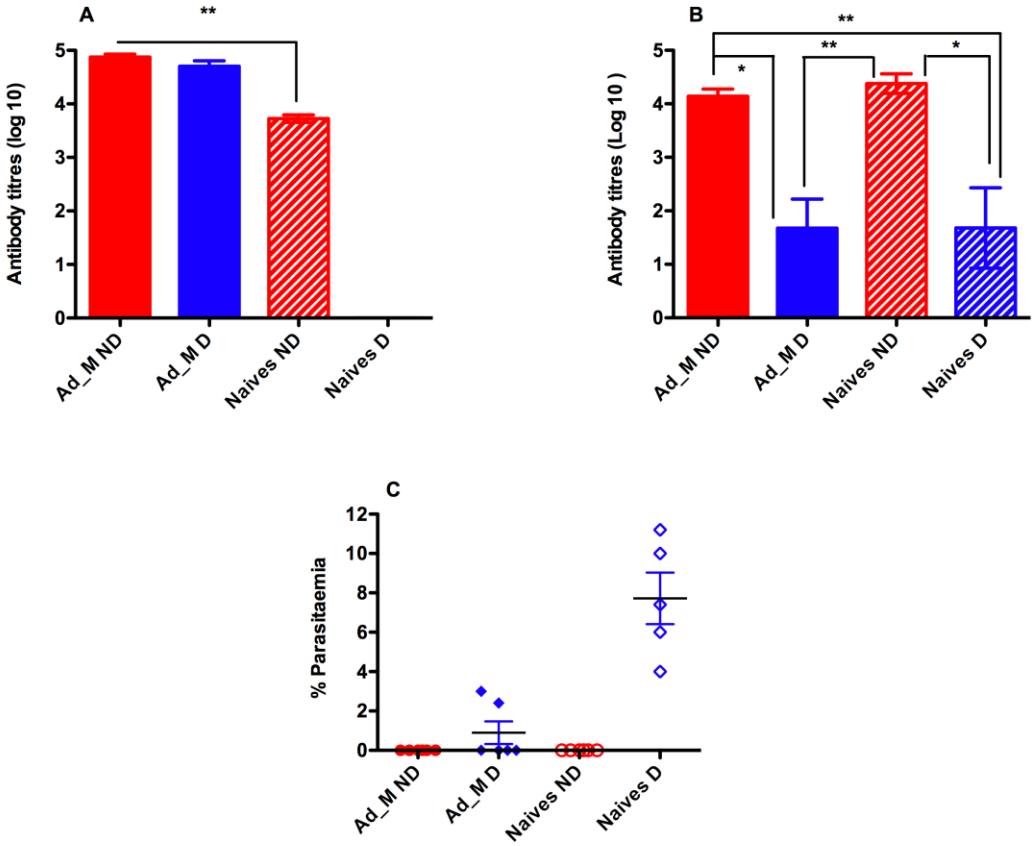


Figure 5-3: Role of AMA1 specific and parasite lysate antibodies in clearance

Two groups of Balb/c mice (n=6) were immunised using the Ad_MPccAMA1 vaccine regime. CD4+ T cells were depleted in one group of naive and one group of vaccinated mice with anti-CD4 GK1.5 mAb. Four groups of mice i) vaccinated CD4+ T cell depleted (Ad_M D), ii) vaccinated non CD4+ T cell depleted (Ad_M ND), iii) naive CD4+ T cell depleted (Naive D) and iv) naive non CD4+ T cell depleted (Naive ND) were challenged intravenously with 10^6 Pcc AS parasitized RBCs. Antibody levels in the serum of the mice were analysed one-month post challenge (Day 33) in both depleted and non-depleted mice (vaccinated and naives). Antibodies were measured against PccAS AMA1 ectodomain (A) and also whole PccAS parasite lysate (B). The asterisk indicates a significant difference in antibody levels between the different groups (ANOVA * $P \leq 0.05$, ** $P \leq 0.01$). The % parasitaemia in the different groups on day 33 is shown (C).

5.2.3 Adoptive transfer of CD4+ T cells and IgG from vaccinated mice into naive mice.

Female Balb/c mice, 5-6 week old (n=24) were vaccinated with $5x10^{10}$ vp of AdHuPccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVAPccAMA1. Two weeks post boost the spleens and serum were harvested. The CD4+ T cells were isolated from the spleens using the CD4+ T Cell Isolation Kit (Miltenyi Biotec). After isolation the purity of the CD4+ T cells was analysed by flow cytometry. Samples were taken pre-isolation (Figure 5-4A) and from the CD4+ T cell positive (Figure 5-4B) and CD4+ T cell negative fraction (Figure 5-4C) after isolation. The cells were surface stained for CD3, CD4 and the percentage of CD3+ CD4+ cells was analysed. The gating strategy is shown in figure 5-7. The CD4+ T cells isolated were 91% pure (Fig 5-4A-C). $7x10^{7}$ CD4+ T cells were injected intravenously into each naive mouse (n=6). One day after transfer the response to the P79 peptide was measured in the blood by ELISPOT in the CD4+ T cell transferred mice and compared to vaccinated mice (n=3). One out of three mice in the CD4+ T cell transferred group responded to the peptide whereas all three in the vaccinated group responded (Fig 5-4D). Two possible explanations for this could be either there were too few CD4 T cells transferred or the CD4+ T cells transferred were spleen homing (as they were isolated from the spleen) and therefore not detectable in the blood.

The IgG was also purified from the serum using a Protein G column. The purified IgG was injected i.v. into naive mice (n=3) on day -2 and day -1 of challenge (2.5 mg total into each mouse). These mice were then challenged with 10^6 PccAS pRBC. Groups of vaccinated and naive mice were also challenged as before.

Both the CD4+ T cell transferred and the vaccinated mice controlled initial parasitaemia better than the naive mice, suggesting the CD4+ T cells transferred from the vaccinated mice help in initial control of parasitaemia. The vaccinated mice clear the parasites faster than the CD4+ T cell transferred mice (Fig 5-5A). This delayed clearance could be due to the absence of AMA1 antibodies. Alternatively, it could also be that the presence of AMA1 specific CD4 T cells skews the antibody response towards AMA1, which are not enough for clearance of the parasites.

On day 4 and 5 the IgG transferred group control initial parasitaemia better than the naives but after day 6 the peak parasitaemia is comparable to naïve controls. The mice begin to control the parasites but there is a second peak at day 15. One of the mice died on day 10 and the other on day 24 (Fig 5-5B). The effect of IgG transfer was inconclusive as there were only three mice and two of them died during the course of the infection. This experiment is repeated later (Section 5.2.4, Figure 5-7C).

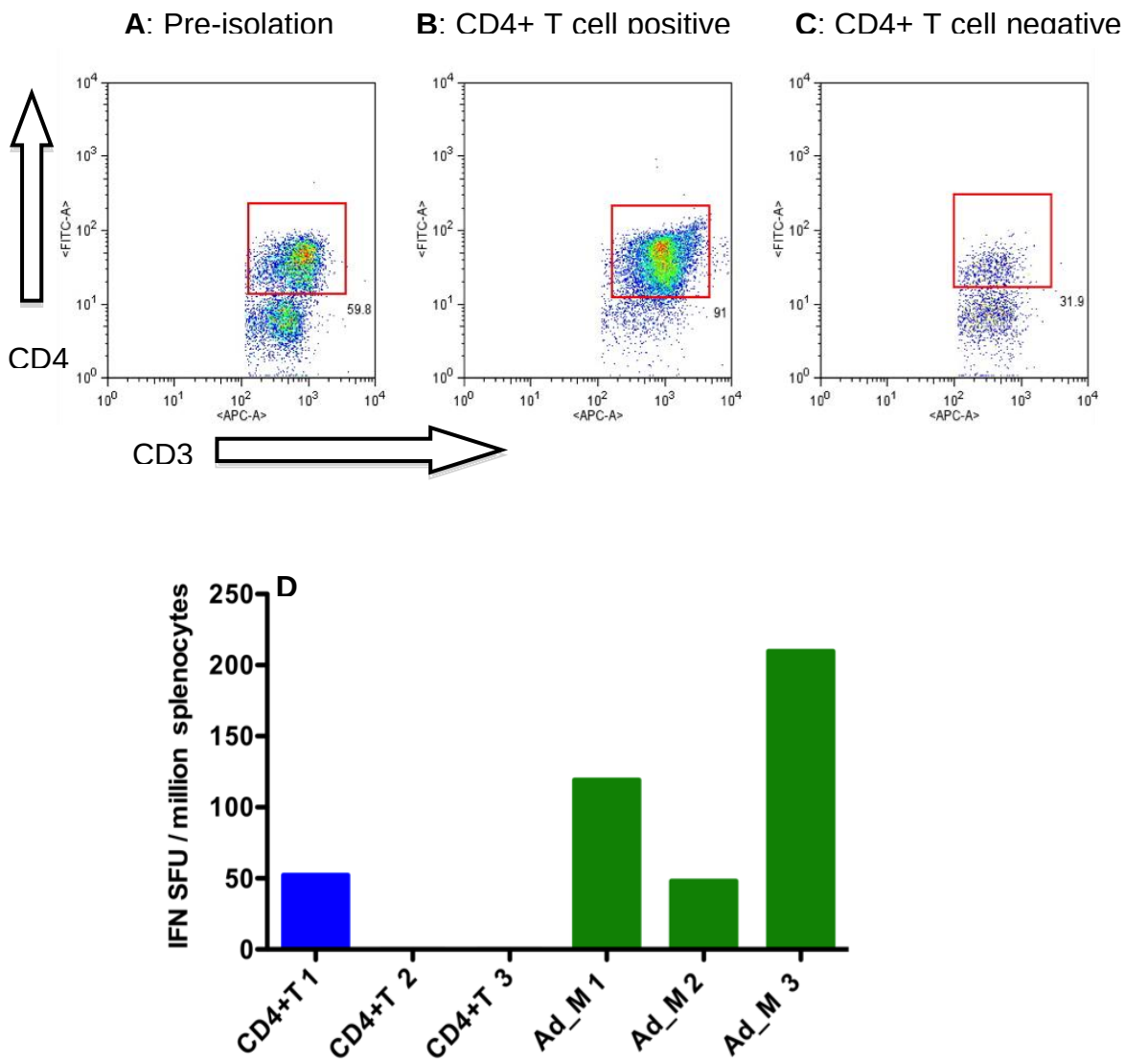


Figure 5-4: Purity of the CD4+ T cells transferred and blood responses to AMA1 in adoptively transferred mice.

Female Balb/c mice (5-6 weeks old) were vaccinated with $5x10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost the spleens were harvested and the splenocytes isolated. The CD4+ T cells were isolated from the spleen using the CD4+ T Cell Isolation Kit (Miltenyi Biotec). After isolation the purity of the CD4+ T cells was analysed by flow cytometry in samples taken before isolation (A) and in the CD4+ T cell positive (B) and CD4+ T cell negative fraction (C). The cells were surface stained for

CD3, CD4 and CD8 and the percentage of CD3+ CD4+ cells analysed to assess the purity of the CD4+ T cells isolated. After transfer into naive mice the response to P79 peptide was measured in the blood by ELISPOT in the CD4+ T cell transferred mice and compared to vaccinated mice (n=3) (D).

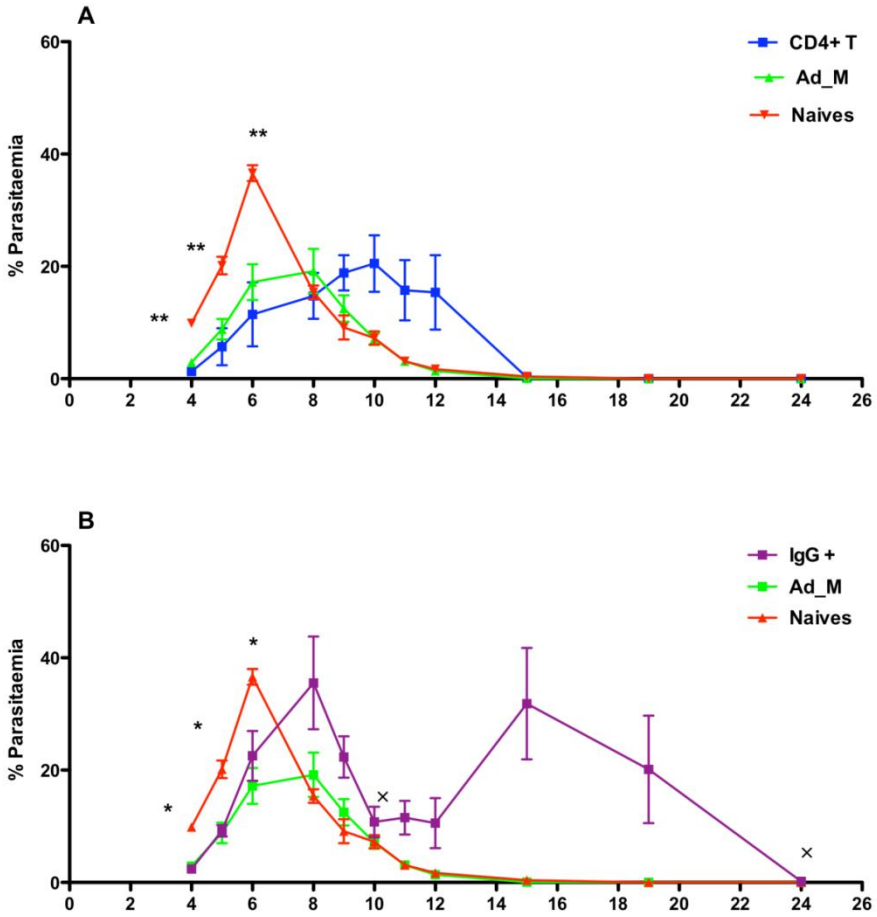


Figure 5-5: Adoptive transfer of CD4+ T cells and IgG from vaccinated mice to naive mice.

Female Balb/c mice (5-6 weeks old) were vaccinated with $5x10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost the spleens were harvested and the splenocytes isolated. The CD4+ T cells were isolated from the splenocytes and $7x10^{7}$ CD4+ T cells were injected intravenously into each naive mouse (n=6) (CD4+ T). The IgG was also purified from the serum and injected into another group of naïve mice (n=3) (IgG+). These mice were then challenged with 10^{6} pRBC. At the same time groups of vaccinated (Ad_M) and naive (Naive) mice were also challenged. Parasitaemia was monitored by Giemsa-stained thin blood smears and results were expressed as the % infected RBCs. The reduction in parasitaemia was measured as the vaccine efficacy. The figure shows the difference in % parasitaemia between the groups. The asterisk indicates a significant difference (ANOVA * $P \leq 0.05$ and ** $P \leq 0.01$) in the % parasitaemia between the

groups. In 5A the asterisks indicate significant differences between the naives and the CD4+ T cell transferred. In figure 5B the asterisks indicate significant differences between the naives and IgG transferred mice. “X” represents the death of animals at that time point in the IgG transferred group.

5.2.4 T cell response to heterologous peptides from other *P. chabaudi* strains

Unlike other blood stage malaria antigens such as MSP1 and MSP2, AMA1 lacks sequence repeats and very marked polymorphism but there is sequence variability among the different alleles of AMA1. These polymorphic residues are a result of single point mutations in *P. falciparum* AMA1 coding sequence (264). Despite relatively few aa changes, polymorphism in AMA1 is still a major hurdle for vaccines encoding this antigen. The protective antibody response to AMA-1 is allele specific and vaccination with one strain does not confer cross strain protection. There are 36 amino acid substitutions between *P. chabaudi chabaudi* DS and *P. chabaudi chabaudi* DK. Immunisation with refolded ectodomain protein of DS AMA1 in Montanide ISA 720 confers protection against homologous DS challenge but not against heterologous DK challenge (167). Viral vectored vaccines induce cellular immunity and these CD4 epitopes are important in protection as shown and so if they are conserved they could confer cross strain protection better than IgG, which is strain-specific.

The aa sequence of the two CD4 epitopes P16 and P79 was compared amongst various strains: *P. chabaudi chabaudi* AS (vaccine strain), *P.*

chabaudi adami 556/DK, *P. chabaudi adami* CB and *P. chabaudi adami* DS (heterologous strain). The mapped CD4 epitopes were not highly conserved between these four *P. chabaudi* strains. P16 was conserved in all but *P. chabaudi adami* DS whereas the sequence of P79 was different to the vaccine strain in all the other three strains compared (Table 5-1). The heterologous peptides P16B and P79B were used to stimulate splenocytes from Balb/c mice 2 weeks after prime (AdHu5-PccAMA1 only) and boost (Ad_M PccAMA1) immunisation. The splenocytes were stimulated for 5 hours with the heterologous peptides or no peptide (Unstim). The homologous peptides P16 and P79 were also included as positive controls. After stimulating the cells for 5 hours the cells were surface stained for CD4 and CD8 and then intracellularly stained for IFNγ.

Unfortunately, there was no response to the heterologous peptide for either P16B or P79B but there was a response seen to the homologous peptides as described before (Figure 5-6). So the response to the CD4+ T cell epitope generated by the vaccination regime was strain-specific. The P16 epitope is conserved in *P. chabaudi adami* DK and *P. chabaudi adami* CB so it will be interesting to see if the response to this epitope could confer cross-strain protection by challenging with these heterologous parasites.

PEPTIDE	PARASITE STRAIN	SEQUENCE	VACCINE STRAIN
P16	*P. chabaudi chabaudi* AS	VMGKGITIQNSKVSF	Yes
P16	*P. chabaudi adami* 556/DK	VMGKGITIQNSKVSF	Yes
P16	*P. chabaudi adami* CB	VMGKGITIQNSKVSF	Yes
P16B	*P. chabaudi adami* DS	VMGKGITIQ**K**S**TK**SF	No
P79	*P. chabaudi chabaudi* AS	GNGTIQFPRIFISDD	Yes
P79B	*P. chabaudi adami* 556/DK	GN**D**TI**K**FPRIFISDD	No
P79B	*P. chabaudi adami* CB	GN**D**TI**K**FPRIFISDD	No
P79B	*P. chabaudi adami* DS	GN**D**TI**K**FPRIFISDD	No

Table 5-1: Comparison of the sequence of the CD4+ T cell epitopes in AMA1 from various strains of *P. chabaudi*.

The table shows the sequence comparison of the two CD4+ T cell epitopes identified. P16 and P79 are the sequence in the homologous strain and P16B and P79B represent the sequence from the heterologous strains. The aa shown in bold letters differ in the heterologous strain.

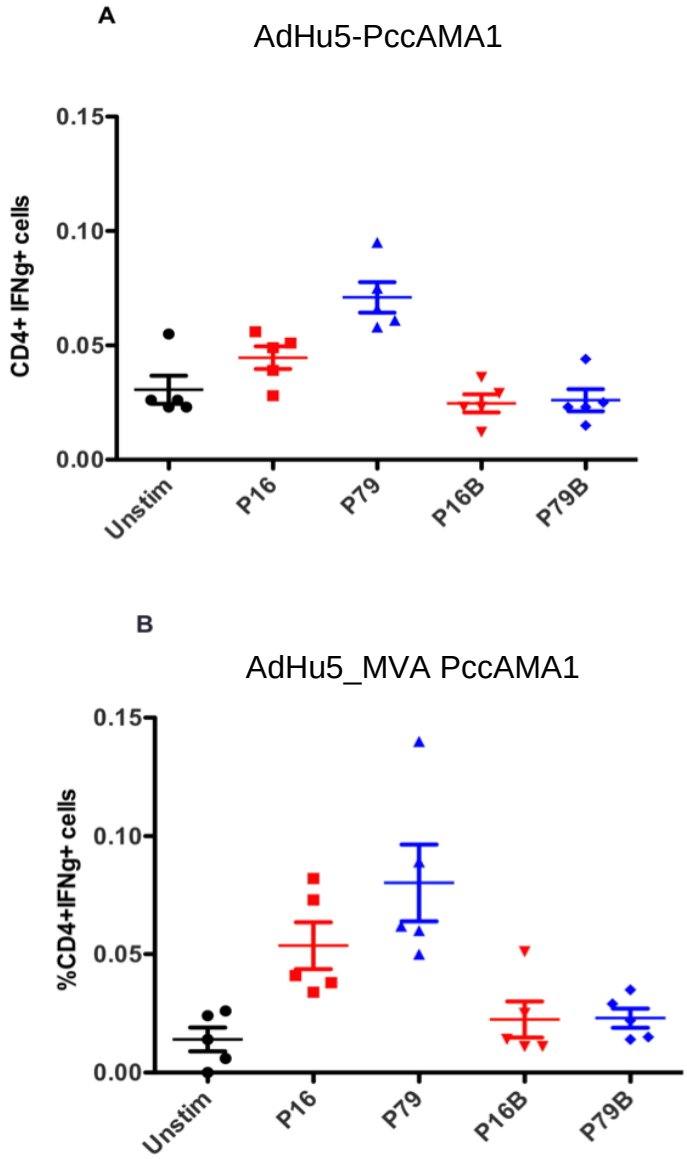


Figure 5-6: Response to heterologous peptides from other *P. chaubaudi* strains.

Female 5-6 week old Balb/c mice (n=10) were primed with $1x10^{10}$ vp of AdHu5-PccAMA1 and boosted 8 week later with $1x10^{7}$ pfu of MVA-PccAMA-1. Two weeks post prime (n=5) and two weeks post boost (n=5) splenocytes were isolated and stimulated with the peptides P16, P79 (CD4 T cell epitopes described before), P16B, P79B (heterologous peptides to P16 and P79 respectively) or no peptide (Unstim) for 5h. Cells were surface-stained for CD4+ and intracellularly stained for IFNγ, TNFα and IL-2, assessed by flow cytometry to determine % of responding cells per total CD4+ T cell subset. The figure shows the CD4+ IFNγ+ response to the peptides after prime (A) and after boost (B).

5.2.5 Adoptive transfer of CD4+ T cell from vaccinated mice to immunocompromised mice.

Female 5-6 week old Balb/c mice were primed with $5x10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost the spleens and serum were harvested from the mice. The CD4+ T cells were isolated as described previously. In addition to this, spleens and sera were also harvested from mice that were immunised with the vector control vaccination regime (Ad_M C). These mice were primed with AdHu5.CMV.BGH and boosted 8 weeks later with MVA.GFP at the same doses as the Ad_M PccAMA1 vaccination regime. These vector controls did not express AMA1, thus enabling an assessment of the role of AMA1 specific CD4+ T cells and to confirm that the effect was antigen specific and not vector specific. The purity of the CD4+ T cells was analysed using flow cytometry (Figure 5-7). An aliquot of sample was taken pre-isolation (Figure 5-7B), and the CD4+ T cell positive (Figure 5-7C) and negative fraction (Figure 5-7D) after sorting. Cells were surface stained for CD3, CD4 and CD8 and percentage of CD3+CD4+ cells analysed to assess the purity of the CD4+ T cells isolated.

In the previous experiment (Figure 5-5), the CD4+ T cells from vaccinated mice were transferred into naive mice (which have an intact immune system), so in order to see if the vaccine induced CD4+ T cells were sufficient on their

own to control the infection, CD4+ T cells and serum from vaccinated mice were transferred into immunocompromised mice. CD4+ T cells were transferred into rag knockout mice (Balb/c RAG1/2$^{-/-}$) and nude mice (Balb/c nu/nu).

RAG1/2 knockout mice lack both T and B cells, but the mice used in this adoptive transfer experiment also have the common gamma chain knocked out. Adoptive transfer of CD4+ T cells from vaccinated mice into these rag knockout mice would therefore answer whether the CD4+ T cells transferred are sufficient to control initial parasitaemia in the absence of other naive T cells and also if they can clear the parasites without B cell help.

In contrast, nude mice lack T cells but have normal B cells and NK cells. CD4+ T cell transfer from vaccinated mice to nude mice will address the role of AMA1-specific CD4+ T cell help in antibody production, and asks whether CD4+ T cell help is crucial in antibody dependent parasite clearance. It will also address if these CD4+ T cells can control initial parasitaemia when B and NK cells are present (in contrast to RAG1/2 knockout mice).

Eight groups of 5 mice each were challenged with 10^6 Pcc AS pRBC (Table 5-2). Parasitaemia was monitored from day 4 post challenge by Giemsa-stained thin blood smears and results were expressed as the % infected RBCs. As Pcc AS infection in naive Balb/c mice is non-lethal the protective efficacy of this vaccination regime was measured in terms of significant difference in parasitaemia post challenge between the naives and the respective groups.

The area under the curve was analysed for groups 1-4 and 7 as all the mice in these groups were alive for the duration of the experiment.

There was a significantly lower parasitaemia as measured by area under the curve for both the vaccinated and CD4+ T cell transferred mice as seen before (Figure 5-8A). The mice in which the CD4+ T cells from Ad_M C were transferred have lower parasitaemia than the naives on day 4 and 5 but there is no significant difference in the area under the curve or the peak parasitaemia (Figure 5-8B).

Serum transfers from vaccinated mice also show a similar effect to CD4+ T cell transfer. These mice have a significantly lower area under the curve analysis than the naive mice. The area under the curve analysis for the mice in which serum was transferred from Ad_M C mice was not included because one of the mice died on day 9 post challenge. However, the serum transfer from vector control mice gave no difference in parasitaemia as compared to the naive mice (Figure 5-8C). This suggests that both CD4+ T cell and serum play a role in control of initial parasitaemia.

Two out of five RAG1/2 knockout mice survived whereas 4 out of 5 mice nude mice survived. The nude mice have B cells, which the RAG1/2 knockout mice lack and these trended to do better in terms of survival, which implies that B cells are important for survival. The RAG knockout mice that survived the infection did not clear the infection whereas the nude mice showed better survival and also cleared the parasites by day 30. This shows that though

AMA1 specific CD4+ T cells play an important role in protection, they are not enough on their own to control initial parasitaemia in an immunocompromised host.

1	Naive Balb/c mice	Naive
2	Ad_M PccAMA1 vaccinated Balb/c mice	Ad_M
3	CD4+ T cells transferred from vaccinated into naive Balb/c mice	CD4+T
4	Serum transferred from vaccinated into naive Balb/c mice	Serum
5	CD4+ T cells transferred from vaccinated into RAG1/2 knockout Balb/c mice	CD4+TRag
6	CD4+ T cells transferred from vaccinated into nude Balb/c mice	CD4+TNude
7	CD4+ T cells transferred from vector control into naive Balb/c mice	CD4+TVector
8	Serum transferred from vector control into naive Balb/c mice	SerumV

Table 5-2: Groups of mice challenged after adoptive transfer.

The table shows the eight groups of mice that were challenged after adoptive transfer of CD4+ T cells and serum from both vaccinated and vector control mice (Section 5.2.5). Groups of vaccinated and naive mice were also transferred.

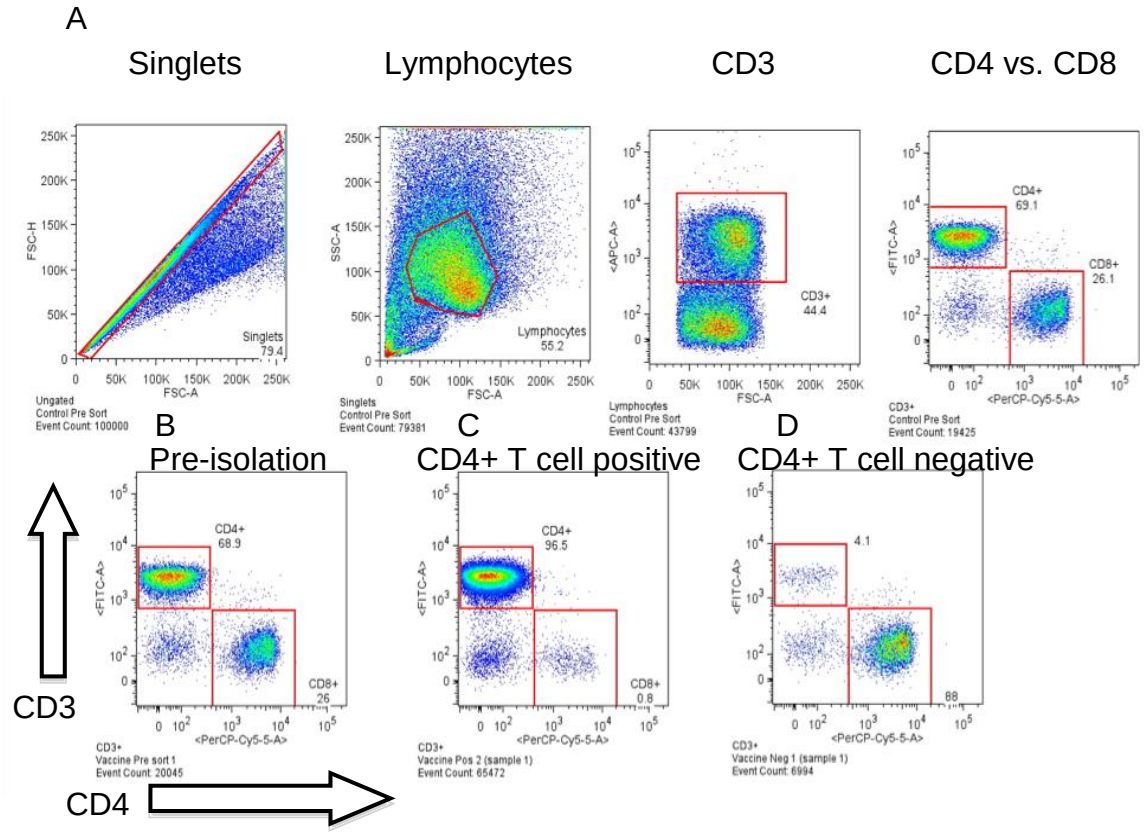


Figure 5-7: Purity of the transferred CD4+ T cells.

The CD4+ T cells were isolated from spleens using the CD4+ T Cell Isolation Kit. After isolation the purity of the CD4+ T cells was analysed by flow cytometry. The cells were surface stained for CD3, CD4 and CD8 and the percentage of CD3+CD4+ cells analysed to assess the purity of the CD4+ T cells. The figure shows the gating strategy used to assess the % of CD3+CD4+ cells. The CD3+CD4+ cells in sample pre-isolation (B), positive (C) and negative fraction (D) is shown. Singlet cells were selected using forward scatter area versus height. Lymphocytes were selected using forward versus side scatter.

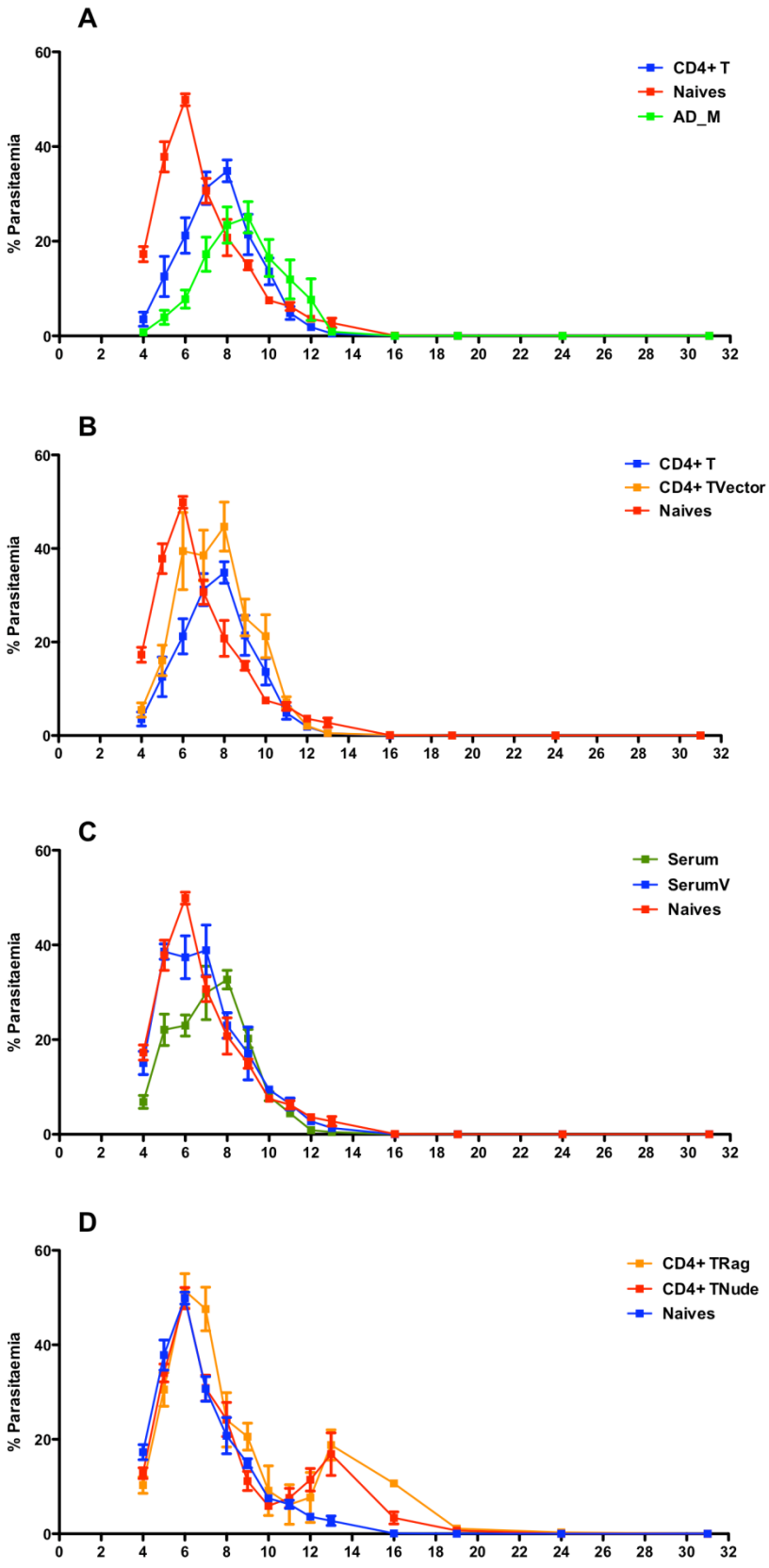

A
% Parasitaemia
CD4+ T
Naives
AD_M
B
% Parasitaemia
CD4+ T
CD4+ TVector
Naives
C
% Parasitaemia
Serum
SerumV
Naives
D
% Parasitaemia
CD4+ TRag
CD4+ TNude
Naives

Figure 5-8: Adoptive transfer of CD4+ T cells from vaccinated mice to immunocompromised mice

Female Balb/c mice, 5-6 week old were vaccinated with $5x10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. Two weeks post boost the spleens were harvested and the splenocytes isolated. The CD4+ T cells were isolated from the splenocytes and $9x10^{7}$ CD4+ T cells from vaccinated mice were injected intravenously into RAG1/2$^{-/-}$ mice, Balb/c nu/nu mice or naive mice. CD4+ T cells from vector control mice were also isolated and transferred into naive mice. Serum was transferred from vaccinated and vector control mice into two groups of naive mice. 500ul of serum was injected i.p on day -1 and day of challenge (total 1ml into each mouse). There were 5 mice in each group except for the serum transfer groups that had 4 mice per group. These mice were then challenged with 10^{6} PccAS pRBC. At the same time one group of vaccinated and naive control mice were also challenged. Parasitaemia was monitored by Giemsa-stained thin blood smears and results were expressed as the % infected RBCs. The figure shows the difference in % parasitaemia between the groups. The area under the curve was analysed for the different groups. In the CD4+TRag group 3 mice died on day 8, 9 and 11 respectively and in the CD4+TNude group one mouse died on day 10.

5.2.6 Cytokine profile pre and post challenge in vaccinated and naïve mice.

The cytokine profile of the serum from vaccinated and naive mice was assayed using a Cytometric Bead Array (Mouse Th1/Th2 Cytokine Kit, CBA, BD Biosciences, San Diego, CA). Serum was collected from naive and vaccinated mice (Ad_M PccAMA1) pre challenge and on days 11 and 25 post challenge. The levels of IFNγ, TNFα, IL-2, IL-4 and IL-5 were measured in the pooled serum. The serum samples were diluted 1:2 and used in the assay as

per manufacturer's instructions. The bead array employs a series of particles with discrete fluorescence in intensities to simultaneously detect multiple soluble analytes in the samples. Two colour cytometric analysis was performed using a FACScalibur flow cytometer (BD) and data were acquired and analysed using BD Cytometric Bead Array software.

The levels of cytokine measured in the serum samples were low and the Th1 to Th2 switch, which is characteristic of *P. chabaudi* infection (256), was not clear in either the naive or vaccinated mice (Figure 5-9). In the pre challenge and day 11 post challenge serum samples all five cytokines were detected in both the naives and the vaccinated mice, but on day 25 post challenge none of the cytokines were detected in the serum samples from AdHu5_M PccAMA1 vaccinated mice. In other studies the cytokines levels have been measured by sandwich ELISA in the cell culture supernatants of splenic CD4+ T cells (145). Thus, the Th1 to Th2 switch was not seen using this assay and need to be further investigated using other methods.

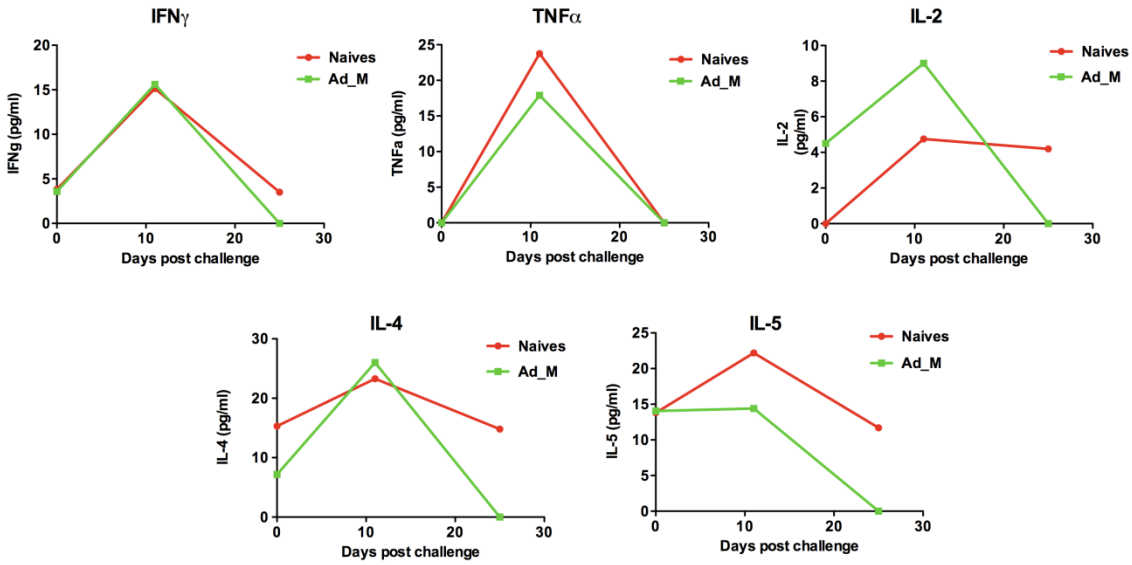


Figure 5-9: Serum cytokine response pre and post challenge in vaccinated and naive mice.

5-6 week old female Balb/c mice were vaccinated with $5x10^{10}$ vp of AdHu-PccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PccAMA1. These mice were challenged with 10^{6} PccAS pRBC. One group of naive mice were also challenged. Serum was collected from naive and vaccinated mice (Ad_M PccAMA1) pre challenge and day 11 and day 25 post challenge. Serum cytokine levels were assayed by flow cytometry using a cytometric bead array assay.

5.2.7 IgG isotype in vaccinated and naïve mice pre and post challenge.

The IgG isotype profile of serum taken from vaccinated and naive mice was assayed by isotype ELISA using recombinant AMA1. Serum was collected from naive and vaccinated mice pre challenge and on days 11 and 25 post challenge. This was used to examine the IgG isotype profile after challenge to

see if there was a switch in the IgG 1:2a ratio. This was also assessed in naive mice to see if there was a switch in IgG isotype between the acute and chronic phase of infection.

In the vaccinated mice the Ad_M Pcc AMA1 vaccination regime induced a balanced Th2-type IgG1 and Th1-type IgG2a response pre challenge. Post challenge there was no switch in the IgG 1:2a isotype ratio either on day 11 or day 25 (Figure 5-10).

In the naive mice there was no IgG1 or IgG2a detected to AMA1 pre challenge as one would expect but post challenge the isotype profile was similar to the vaccinated mice and there was no switch in the IgG 1:2a isotype ratio to recombinant AMA1 between day 11 and Day 25 post challenge (Figure 5-10).

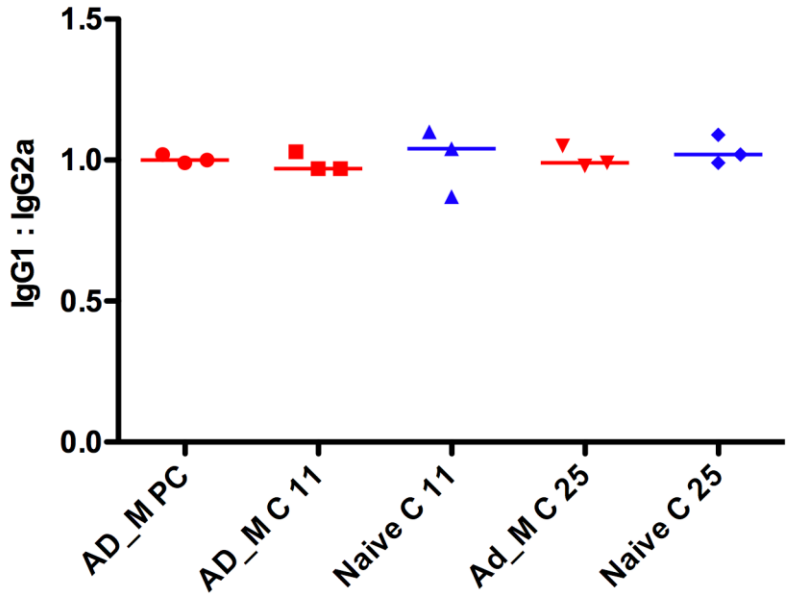


Figure 5-10: IgG isotype profile pre and post challenge in vaccinated and naive mice

Female Balb/c mice, 5-6 week old were vaccinated with $5x10^{10}$ vp of AdHuPccAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu of MVAPccAMA1. Two weeks post boost mice were challenged with 10^{6} pRBC. One group of naive mice was also challenged at the same time. The IgG isotype profile was measured by an Isotype ELISA in the serum at three time points: pre-challenge (PC), day 11 post challenge (C 11) and day 25 post challenge (C 25) in vaccinated (Ad_M) and naive mice (Naive). The symbols represent the IgG1 :IgG2a ratio in individual mice (n=3) and the line represents the median.

5.3 Discussion

This work has explored the mechanism of vaccine-induced protection in the *P. chabaudi* model. In other mouse models, the Ad_M prime boost vaccination regime has been shown to enhance CD8+ T cell immunogenicity to protective levels against the pre-erythrocytic antigen circumsporozoite protein from *P.*

berghei (216). Classical protein-in-adjuvant vaccines generate a good humoral immune response but fail to generate a strong T cell response, which is important for protection in the *P. chabaudi* model (265). Previous work in the lab has shown in the *P. yoelii* model the Ad_M vaccine regime encoding the blood stage antigen $MSP1_{42}$ induces potent antibody and T cell responses. Antibodies against the blood stage and CD8+ T cells against the liver stages mediated the protection. CD4+ T cells were also essential to provide help for priming B cells but depletion of CD4+ T cells did not affect vaccine efficacy (226).

In this study the importance of vaccine induced CD4+ T cells in initial control of parasitaemia is shown. *In vivo* depletion of CD4+ T cells before challenge leads to loss of vaccine induced initial control of parasitaemia. These CD4+ T cells seem to work independently of antibodies as in both the vaccinated CD4 depleted and non depleted groups, the level of AMA1 antibodies after challenge is the same (Figure 5-3A). AMA1-specific antibodies were not detected in naive CD4+ T cell depleted mice suggesting that CD4+ T cell help is required for production of AMA1 specific antibodies (Figure 5-3A). CD4+ T cell help is also required for the production of antibodies to the whole parasites as both the vaccinated and naive CD4+ T cell depleted mice have significantly lower antibodies to the whole parasite than non depleted mice (Figure 5-3B). Previously this has been shown using recombinant protein vaccines that CD4+ T cell depletion results in loss of immunity but does not result in a lower AMA1 specific antibody response after infection (169). T cells

for a cryptic epitope in AMA1 have also been shown to be able to confer partial resistance to infection in *P. chabaudi* (168).

This study also shows that vaccine induced CD4+ T cells can be adoptively transferred into naive mice and these mice control the initial parasitaemia as well as the vaccinated mice, but are unable however to clear the parasites as fast as the vaccinated mice. This could be due to the absence of AMA1 antibodies in the CD4+ T cell transferred mice (Figure 5-5A). This emphasizes the fact that although in many previous studies it has been shown that cell mediated immunity is important for initial control of parasitaemia against P. chabaudi, antibodies are crucial for clearance. This implies that vaccines against the blood stage should be able to generate both humoral and cell mediated immune response and this is achievable by the viral vectored vaccine regime as shown here. On the contrary it could also be hypothesized that the presence of AMA1 specific CD4+ T cells skews the antibody response towards AMA1, which is not enough for clearance of the parasites (Figure 5-3).

The failure to detect any response to the heterologous peptide shows that even slight variation (2 aa) in the sequence of AMA1 has a consequence in the immune response generated and can affect vaccine efficacy against heterologous strains. When mice are immunised with recombinant AMA1 it has previously been shown that there is no cross strain protection between *P. chabaudi chabaudi* DS and *P. chabaudi adami* 556KA that differ by only 36 amino acids. The viral vector vaccination regime induced responses to two

CD4+ T cell epitopes but these epitopes were not conserved. There was no response to the heterologous peptide thus one would speculate that if these CD4+ T cell epitopes were protective they would not confer cross strain protection. This has important implications when designing vaccines targeting AMA1. One could either succeed in generating an immune response to the conserved regions of the molecule, which is more likely in case of *P. falciparum* as none of the *P. falciparum* AMA1 sequence are as diverse as the two *P. chabaudi* strains, or possibly target a conserved region of *P. falciparum* AMA1 in the vaccine.

Multifunctional CD4+ T cells were measured in the spleen after both the prime and prime-boost vaccination regime. (Section 4.2.3.4). It will be interesting to see the effect of adoptively transferring the cells which produce all three cytokines at the same time (IFNγ, TNFα and IL-2) into naive mice as these cells have been shown to correlate with protection in the mouse model for *Leishmania major* (231). In order to adoptively transfer these cells they would need to be separated from the other cells producing a single cytokine or two cytokines at the same time. It is difficult to isolate these cells and it was not possible to investigate this further within the scope of this project.

Studies in immunodeficient mice show that the nude mice fare better in terms of survival than the RAG1/2 knockout mice consistent with the fact that B cells are crucial for clearance and survival. In other studies it has been shown that adoptive transfer of B cells from immune mice at a later stage of infection can restore the ability of immune deficient mice to clear blood stage parasites

(146). It appears that CD4+ T cells are an important component of vaccine-induced protection but the protection is better in an immunocompetent host. AMA1 specific CD4+ T cells are thus important in vaccine-induced protection but not sufficient by themselves to control initial parasitaemia. The CD4+ T cells transferred from vector control mice seemed to suppress the parasitaemia very early in the infection though later on it peaked to similar levels as the naive. This very early initial control could be because of the non-specific effect of IFNγ production from these cells. It has been previously reported that potent activators of macrophages like *Mycobacterium bovis* BCG, *Corynebacterium parvum* and IFNγ, as well as products of activated macrophages, can lead to death of blood-stage parasites (266). The role of CD4+ T cells immunity to human malaria is not well understood. In children living in highly endemic areas, antigen-specific proliferative response correlates with protection against *P. falciparum* (267). Another study from humans showed that repeated ultra-low dose exposure of malaria-naïve human volunteers to blood-stage P. falciparum parasites, followed by drug-cure, induces sterile immunity against a subsequent blood-stage challenge in the absence of detectable antibodies against the parasite or pRBCs. Responses to erythrocytic-stage infection were characteristic of cellular immunity – a proliferative T cell response (CD4+ and CD8+) and induction of Th1-type cytokines (78).

The level of cytokine measured in the serum was very low and a Th1 to Th2 switch was not clear (Figure 5-9). This switch has been shown using purified splenic CD4+ T cells recovered from Pcc infected mice (145). At various time

points after infection, purified CD4+ T cells were cultured in vitro with either mitogenic or antigenic stimulus for 48 hours and the culture supernatant was tested for the presence of IFNγ, IL-2, IL-4 and IL-10 by cytokine ELISA. During acute parasitaemia (day 7 after challenge) a high level of IFNγ and IL-2 was measured whereas later on (day 20 after challenge), there was production of IL-4 and IL-10. This switch was not seen here but this could be because the assay was not sensitive enough or because it was measured in the serum and not using CD4+ T cells from spleen. In *P. falciparum* infection a shift from Th2 response to a more pronounced Th1 response is associated with the resolution of infection (268).

The Th1 to Th2 switch in this model could be assessed using the TIM cell surface marker by flow cytometry. The TIM gene family has been shown to have a role in the regulation of Th1 and Th2 effector T cell responses (269). Tim-3 is a Th1 specific cell surface protein (270) where Tim-2 is expressed on Th2 cells (271). These specific Th1 and Th2 cell surface markers could be used to differentiate CD4+ Th1 and Th2 cells pre and post challenge.

The IgG isotype measured against recombinant AMA1 did not change over time. There was a balanced Th1-type IgG1 and Th2-type IgG2a response measured pre and post challenge in both vaccinated and naive mice at the different time points measured (Figure 5-10). This is in contrast to antibodies measured in mice infected with Pcc AS where there is a predominant cytophilic IgG2a response during the primary ascending parasitaemia followed by an IgG1 response during the chronic phase of infection, which

coincides with a Th1 to Th2 CD4+ T cell switching (145). This could be due to the fact that these studies measure the response to whole parasite lysate whereas the isotype profile in this study is measured only against recombinant AMA1 and not whole parasite lysate.

It will be interesting to assess the efficacy of the vaccination regime against Pcc AS sporozoite challenge perhaps in a mouse strain with CD8+ T cell epitope. AMA1 is also expressed during the sporozoite stage, lost after invasion of hepatocytes and re-expressed in liver merozoites (272). Anti-AMA1 antibodies have been shown to neutralize circulating sporozoites (272), whilst cellular mechanisms could target the infected hepatocytes. A similar effect was seen using the same viral vectored vaccination regime targeting *P. yoelii* $MSP1_{42}$, where levels of protection were increased following sporozoite rather than blood-stage pRBC challenge. In this model MSP-1-specific CD4+ T cells were shown to provide essential help for protective B cell responses important for blood stage protection, and CD8+ T cells mediated significant antiparasitic activity against liver-stage parasites (273). A similar effect could be seen with a vaccine targeting AMA1, which will possibly improve the efficacy of this vaccination regime and also induce multistage protection against malaria.

CHAPTER 6

AMA1 BASED VACCINE AGAINST *P. falciparum* MALARIA

6 AMA1 based vaccine against *P. falciparum* malaria

6.1 Introduction

AMA1 has been one of the leading blood stage vaccine candidate antigens for a considerable amount of time and there have been several vaccine studies both pre-clinical and clinical using AMA1. There have also been several field studies in endemic areas addressing the importance of both antibodies and T cell responses to AMA1 in naturally exposed people living in malaria endemic areas. Most studies have concentrated on antibodies and these show that naturally exposed individuals have antibodies to AMA1 and the prevalence increases with age. The IgG antibodies have been associated with reduced risk of clinical malaria in individuals parasitaemic before the transmission season (163, 274-276). In other studies in The Gambia it has been shown that antibodies to a combination of AMA1 and MSP2 were significantly associated with protection from clinical malaria (277). The studies of T cell mediated immunity against AMA1 have been limited. In one study seven naturally immunogenic T cell epitopes in AMA1 were found. A cross sectional study showed that some T cell epitopes localise to highly conserved areas of AMA1 and this could be important in terms of vaccination strategy against this polymorphic antigen (133). Unlike CSP, AMA1 peptides induced a higher magnitude of T cell proliferative response in Kenyan residents than individuals

not previously exposed to malaria (133). Individuals showing proliferative response to one AMA1 peptide corresponding to aa 259-271 were found to be less likely to be parasitaemic on follow up (166)

In vitro studies with AMA1 have shown that antibodies that inhibit parasite multiplication blocked AMA1 function in *Plasmodium* (151, 278). There have been several vaccine studies of *P. falciparum* AMA1 homologues in murine malaria models, mainly *P. chabaudi* and *P. yoelii*, and they have shown varying degrees of protection and have helped to elucidate the mechanisms of protection involved in blood stage immunity (137, 167-169, 229, 233, 279, 280). Studies in macaques have shown that immunisation with recombinant *P. knowlesi* 66kDa AMA1 with saponin as the adjuvant can protect these animals against blood stage challenge (281). A study in *Aotus vociferans* monkeys with recombinant *P. falciparum* AMA1 with Freund's adjuvant has shown partial protection (282). Most of the studies listed above have used recombinant protein-in-adjuvant formulations that are very potent at inducing antibody responses but not cellular immune response. A study in humans has shown that repeated ultra-low dose exposure of malaria-naïve human volunteers to blood-stage parasites and drug cure can induce sterile protection against a blood stage challenge. In this study there was no detectable antibody response against the parasite. A cell-mediated immune response was measured – mainly characterised by a proliferative CD4+ and CD8+ T cell response and Th1-type cytokine induction (78). In another recent study, volunteers were protected against malaria challenge with infected mosquitoes after sporozoite inoculation and drug cure and parasite-specific

pluripotent effector memory T cells were found to be a promising immunologic marker of protection (131). In the same study the T cell response to blood-stage parasites before challenge was a distinctive marker of exposure and protection. With the increasing evidence of the role for T cells in immunity to malaria, vaccine development should focus on vaccines capable of generating both antibodies and T cells. This strategy could induce a broader repertoire of immune responses to target such polymorphic malarial proteins. Recently a study in the *P. chabaudi* AS model showed that the failure to maintain long-term protective responses was due to a gradual decline in the malaria-specific memory T cell response which was mainly CD4+ T cells. There was persistent B cell memory and circulating antibody in this study (283). This study provides an important insight into T and B cell memory to malaria and encourages vaccination strategies that induce memory T cells. Despite these murine studies, there have been very few studies trying to address why people in endemic areas have difficulty in generating and maintaining an immune response against malaria (284, 285).

In this chapter novel viral vectored vaccines targeting *P. falciparum* AMA1 have been developed and tested in mice, rabbits and macaques to assess the immunogenicity of these vaccines in a prime-boost vaccination regime. The protective value of the immune response generated was assessed using both traditional methods of measuring antibody levels such as ELISA and IFA, and also the functional activities of these antibodies were tested *in vitro* using an assay for growth inhibition activity (GIA). These vectors are currently being developed for Phase I/IIa human clinical trials and thus it will be essential to

follow-up the work described here, to get a better understanding of the relevance of these models and assays used for pre-clinical studies to predicting clinical outcome. These studies will also help in the search for a surrogate marker of protection if there is a correlation found between a functional assay and protection in humans.

6.2 Results

6.2.1 Design of the first generation AMA1 vaccines

The first generation monoallelic AMA1 construct was based on the recombinant protein *P. falciparum* strain 3D7 AMA1 vaccine developed at the National Institute of Health, USA (222). The vaccine encoded the ectodomain of *P. falciparum* 3D7 AMA1 (aa 25-546). The native N-terminal signal sequence (N), transmembrane domain (TM) and the C-terminal cytoplasmic domain (C) were not included in the construct**.** The tPA leader sequence replaced the native AMA1 signal sequence. This construct was used to make recombinant AdHu5 and MVA expressing PfAMA1 3D7 (Chapter 2).

6.2.2 Humoral immune responses to *P. falciparum* AMA1 recombinant viral vaccines

6.2.2.1 Mice

Seven 5-6 week old female Balb/c mice were immunized with $5x10^{10}$ vp of AdHu5-PfAMA1 (3D7) and boosted 8 weeks later with $1x10^{7}$ pfu of MVA-PfAMA1 (3D7). Blood was obtained by tail bleed on day 14 and day 55 post prime and by terminal cardiac bleed two weeks post boost (day 70). The total IgG response was measured by ELISA in the serum from a blood sample at day 14 (two weeks post prime), day 55 (pre boost) and day 70 (two weeks post boost). The ELISA was performed using recombinant protein against both the homologous 3D7 allele (vaccine strain) (Figure 6-1A) and the heterologous FVO allele (Figure 6-1B) to assess if there were any cross-reactive antibodies induced by the Ad_M-PfAMA1 (3D7) vaccine regime. The recombinant 3D7 and FVO AMA1 proteins were kind gifts from Dr Chetan E. Chitnis, ICGEB, India and Dr Mike Blackman, NIMR, Mill Hill, London, respectively. The IgG responses that were measured two weeks after the prime significantly increased by day 56, and the MVA boost further increased the antibody response significantly. There were cross-reactive antibodies measured to the heterologous FVO allele. There was no significant correlation between the antibodies to 3D7 and FVO PfAMA1 two weeks post boost. (Figure 6-1C).

The IgG isotypes were also measured in the sera two weeks post boost by isotype ELISA. There was a balanced Th1-type IgG2a and Th2-type IgG1 response in mice at this time point (Figure 6-1D).

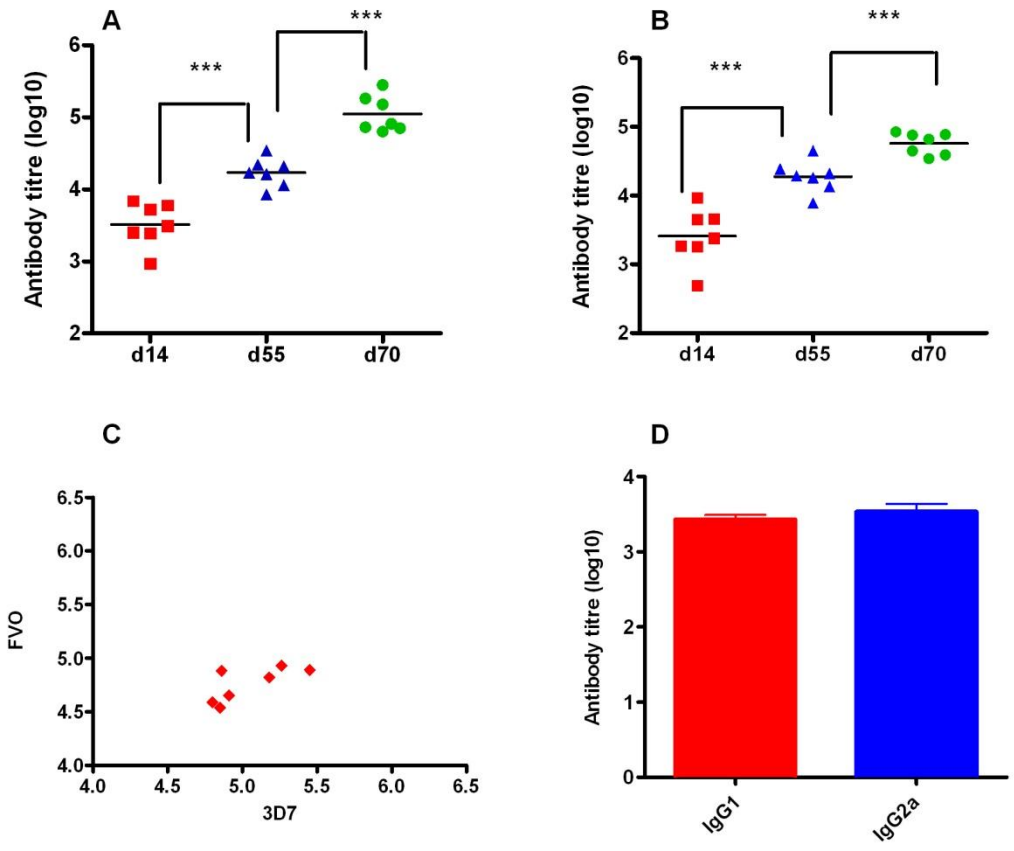


Figure 6-1: Murine antibody responses generated by Ad_M-PfAMA1 (3D7) vaccination

5-6 week old female Balb/c mice (n=7) were immunised with $5x10^{10}$ vp of AdHu5-PfAMA1 and boosted 8 weeks later with $1x10^{7}$ pfu MVA-PfAMA1. The AMA1 specific antibody response against both homologous 3D7 (A) and heterologous FVO (B) PfAMA1 recombinant protein was measured in the serum at day 14 (post prime), 55 (pre boost) and 70 (post boost). Each point indicates the response from an individual mouse and the lines represent geometric mean. The asterisk indicates a significant difference between the different time points as analysed by Mann Whitney test (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$). The correlation between the 3D7 and FVO antibodies at day 70 was analysed. (R^2=0.52, P=0.06) (C). The IgG isotypes were also assessed at day 70 in the serum against recombinant FVO PfAMA1 (D).

6.2.2.2 Rabbits

Eight New Zealand White rabbits approximately 12 weeks old were immunized with $5x10^{10}$ vp of AdHu5-PfAMA-1 and boosted 8 weeks later with $5x10^{7}$ pfu of MVA-PfAMA-1. Blood was obtained by tail bleed on day 14 and day 55 post prime and by terminal cardiac bleed two weeks post boost (day 70). The total IgG response was measured by ELISA in the serum at day 14, 55 and 70 against both 3D7 (Figure 6-2A) and FVO (Figure 6-2B) PfAMA1 recombinant protein. The antibody response measured at day 56 trended to be lower than day 14. After the MVA boost the antibody levels did not increase. There were cross-reactive antibodies measured against FVO recombinant protein. There was a strong correlation between the 3D7 and FVO antibodies two weeks post boost in rabbits (R^2= 0.96, P< 0.0001) which was not seen in mice (Figure 6-2C). This was also seen with a *P. falciparum* $MSP1_{42}$ based vaccine (Anna Goodman personal communication), suggesting differences in antibody cross-reactivity in different mammalian species.

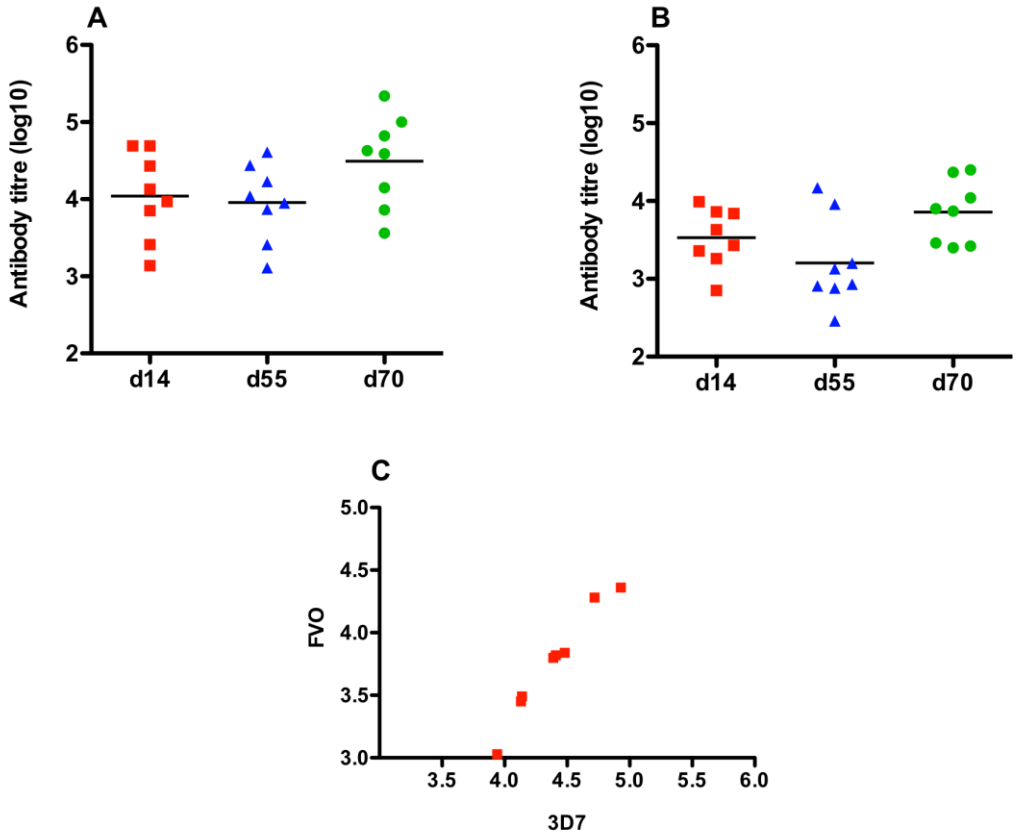


Figure 6-2: *P. falciparum* AMA1 specific total IgG responses induced by immunisation in rabbits

New Zealand rabbits (n=8) were immunised with $5x10^{10}$ vp of AdHu5-PfAMA1 and boosted 8 weeks later with $5x10^{7}$ pfu of MVA-PfAMA1. The AMA1 specific antibody response against both homologous 3D7 (A) and heterologous FVO (B) PfAMA1 recombinant protein was measured in the serum at day 14 (post prime), 55 (pre boost) and 70 (post boost). Each point indicates the response from individual rabbits and the lines represent geometric mean. The correlation between the 3D7 and FVO antibodies at day 70 was analysed using Pearson's correlation. There was a highly significant positive correlation (R^2= 0.96, *P*< 0.0001).

6.2.3 Humoral responses to PfAMA1 native protein

In order to confirm that the antibodies induced by vaccination recognised native AMA1 protein, an immunofluorescence assay (IFA) was performed. IFA was done using slides of *P. falciparum* schizont infected RBCs which were incubated with sera from immunised rabbits (day 70). The sera bound to the *P. falciparum* schizont stages as shown by the punctate apical staining of merozoites that is typical of that seen for AMA1. The IFA titres were between 1:1600 and 1:6400.

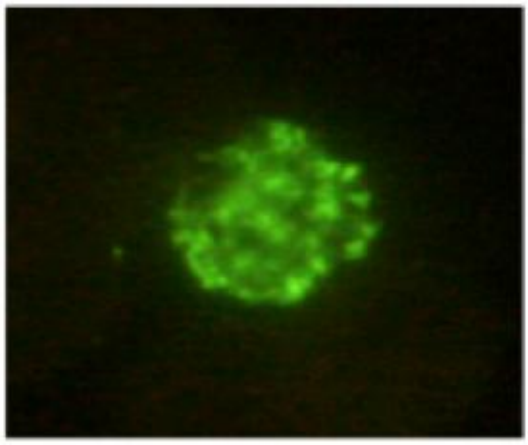


Figure 6-3: Binding of sera from immunised mice to *P. falciparum* schizonts.

The binding of the antibody induced by Ad_M-PfAMA1 (3D7) to native AMA1 protein is shown. Serum from immunised rabbits was incubated with a *P. falciparum* schizont slide and typical AMA1-specific punctate apical staining of merozoites was seen. The IFA titres were between 1:1600 and 1:6400.

6.2.4 Functional activity of antibodies generated in mice and rabbits

The functional activity of antibodies induced by the Ad_M-PfAMA1 (3D7) vaccination regime was determined using growth inhibition activity (GIA) assays. This is a standardized *in vitro* assay to measure growth inhibition of blood-stage *P. falciparum* parasites by purified IgG from immunised animals or humans. The purified IgG is mixed with *P. falciparum* infected human red blood cells and the growth inhibition activity of the serum is determined by measuring parasite lactate dehydrogenase (pLDH) release after a 40-48 hour incubation period.

GIA was determined for the pooled mouse serum obtained two weeks after the final immunisation. The concentration of purified IgG used for the GIA assay was 5 mg/ml. The assay was performed for both the homologous 3D7 vaccine strain and the hetrologous FVO strain. The serum had a GIA of 98% against the homologous 3D7 strain, and was higher than that seen against FVO parasites (54%) (Figure 6-4).

The GIA assays for individual rabbit (n=8) serum samples taken two weeks after the final immunisation were performed using homologous 3D7 and heterologous FCR3 strains. The concentration of purified IgG used in the assay was 6, 3, 1 and 0.75 mg/ml. In rabbits there was moderate growth

inhibition against the homologous 3D7 strain but little or no GIA against the FCR3 strain (Figure 6-5A). There was no correlation between 3D7 and FCR3 GIA titres (Figure 6-5B). There was a strong correlation between the 3D7 ELISA titres and 3D7 GIA (R^2=0.68, *P<0.05) and also with FVO GIA (R^2 =0.51, *P<0.05) (Figure 6-5C).

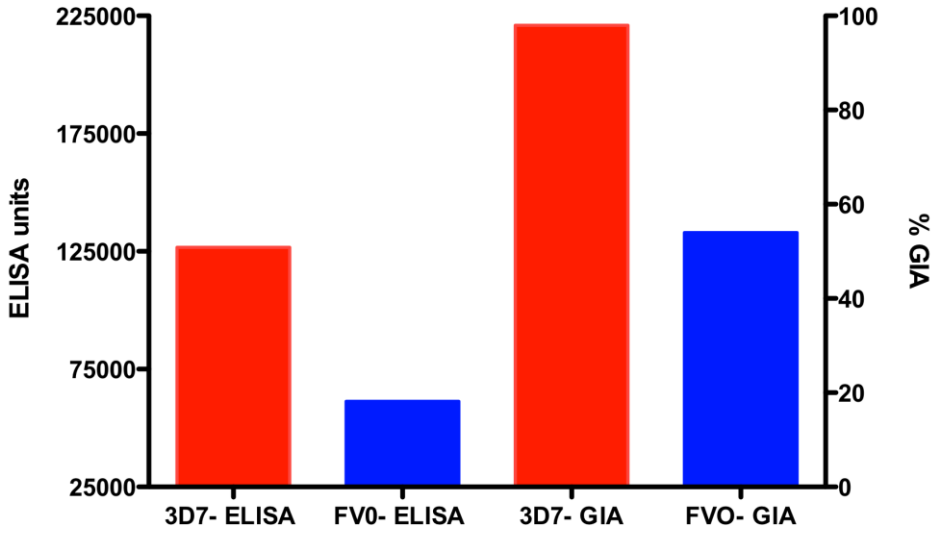


Figure 6-4: Growth inhibitory activity of sera from immunised mice.

5-6 week old female Balb/c mice (n=7) were immunised with 5x10^{10} vp of AdHu5-PfAMA1 and boosted 8 weeks later with 1x10^7 pfu of MVA-PfAMA1. Sera was obtained 2 weeks post boost (day 70) by terminal bleed and the IgG was purified from the pooled serum (n=7). The GIA activity of the serum was assessed by incubating the IgG at a final concentration of 5mg/ml with *P. falciparum* cultures. The % growth inhibition of the pooled serum is shown against both the homologous 3D7 and heterologous FVO strains of PfAMA1. The ELISA titres for the pooled samples are also shown against 3D7 and FVO protein.

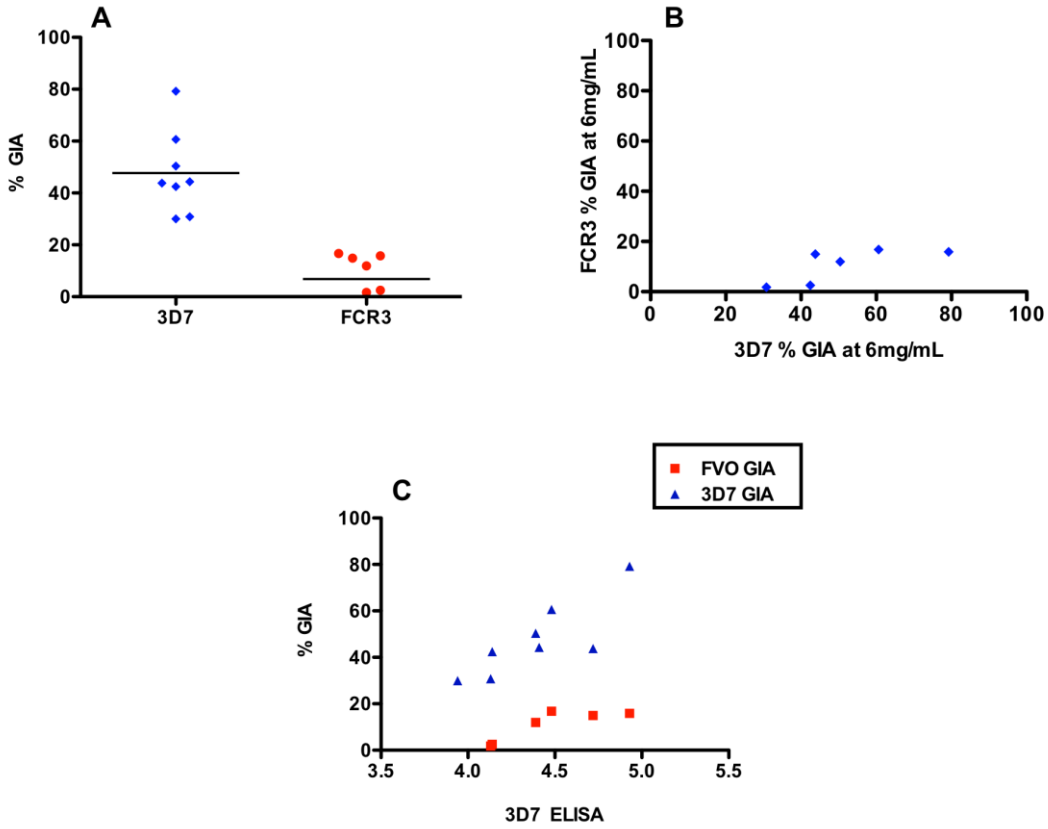


Figure 6-5: Growth inhibitory activity of sera from immunised rabbits.

New Zealand white rabbits (n=8) were immunised with $5x10^{10}$ vp of AdHu5-PfAMA1 and boosted 8 weeks later with $5x10^{7}$ pfu of MVA-PfAMA1. Two weeks after the final vaccination the sera was obtained from the rabbits and the IgG was purified using Protein A. The effect of the purified IgG on parasite growth from each individual rabbit was evaluated by incubating the IgG at a concentration of 6, 3, 1.5 and 0.75 mg/ml with mature *P. falciparum* schizonts. The % GIA values for the 8 rabbits are shown against both the 3D7 and FCR3 strains at 6mg/ml IgG concentration (A). The dots represent the individual GIA titres and the lines are the mean. There was no correlation between 3D7 and FCR3 GIA titres (B). There was a significant positive correlation between the 3D7 ELISA titres and 3D7 GIA (R^2 =0.68, **P*<0.05) and with FVO GIA (R^2 =0.51, **P*<0.05) (C) using Pearson's correlation.

6.2.5 Design of second generation PfAMA1 vaccines

The original vaccine construct used for the previous studies in this chapter had the tissue plasminogen activator (tPA) leader sequence instead of the native N-terminus (N) AMA1 signal sequence. The transmembrane domain (TM) and the cytoplasmic tail (C) of PfAMA1 were not included in the first generation monoallelic construct. As part of the process to develop a biallelic clinical construct based on PfAMA1 the effect of addition of the native N and C terminus was tested. The biallelic constructs were designed to address the issue of polymorphism in AMA1 as it has been shown that human sera against the 3D7 strain show little or no inhibition of the heterologous FVO strain (286). The 3D7 and FVO alleles were included in the biallelic construct. The 3D7 and FVO alleles are the most divergent in terms of polymorphism and differ by 24 amino acids (287). Most of the AMA1 vaccines in development are based on these two alleles (222, 288, 289). Three new constructs were designed and these were cloned into AdHu5 to compare the immunogenicity of the constructs based on the level of antibody response generated. A schematic representation of the different constructs is shown in Figure 6-6.

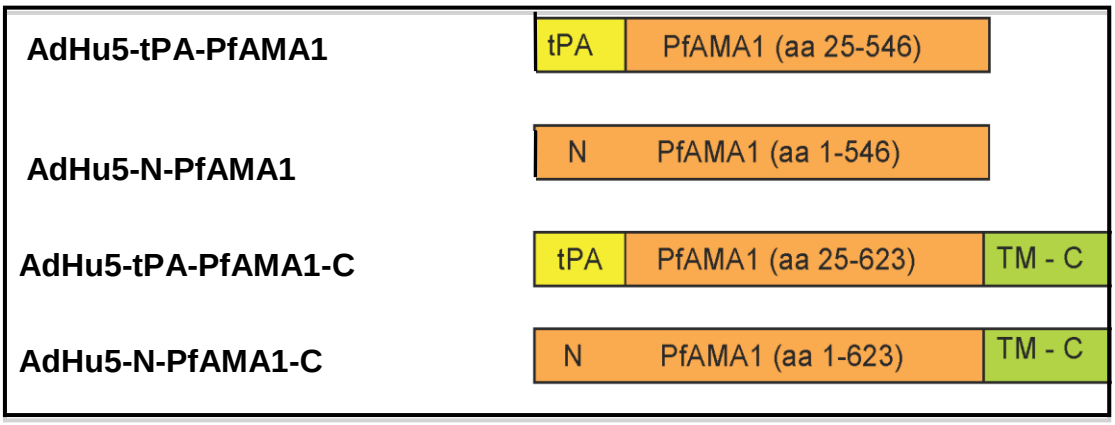


Figure 6-6: Schematic representation of the AdHu5-PfAMA1 (3D7) constructs including native N and C-termini of AMA1.

The figure shows the different AdHu5 constructs generated to test the effect of replacing the human tPA leader sequence (yellow) with the native AMA1 N-terminus (orange aa 1-24) and also the effect of addition of the native transmembrane domain and C-terminus (green aa 547-623)). PfAMA1 in this figure represents the ectodomain of *P. falciparum* 3D7 AMA1 (orange aa 25-546).

6.2.6 Comparison of humoral immune response generated by the different PfAMA1 3D7 constructs in the AdHu5 vector

The immunogenicity of the four constructs described in Figure 6-6 was compared in terms of the antibody response generated. Four groups of 5-6 week old female Balb/c mice were immunised with the four different AdHu5 constructs i) AdHu5-tPA-PfAMA1, ii) AdHu5-tPA-PfAMA1-C, iii) AdHu5-N-PfAMA1 and iv) AdHu5-N-PfAMA1-C. The total IgG response to the ectodomain of PfAMA1 was measured by ELISA in the serum 12 days post vaccination. The IgG response was measured against recombinant PfAMA1 (3D7) protein (Figure 6-7). Inclusion of the human tPA leader sequence in the vaccine led to significantly higher antibody titres than when the native parasite N terminus was used. In another study, a DNA-based vaccine tagetting the conserved nucleoprotein protein of influenza virus showed improved immunogenicity when fused with the tPA signal sequence (290). The addition of the C terminus led to significantly increased immunogenicity when used with the native N terminus but not with tPA.

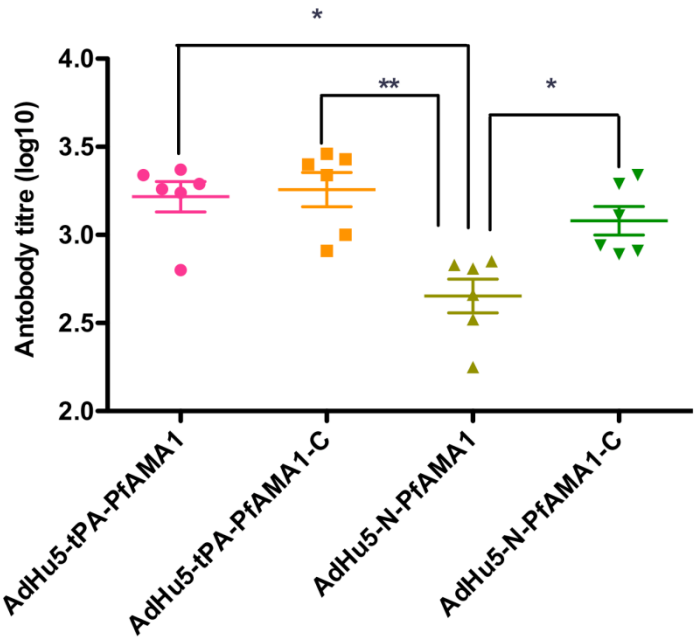


Figure 6-7: Comparison of antibody responses to recombinant PfAMA1 (3D7) protein induced by the different PfAMA1 3D7 constructs.

Four groups of 5-6 week old female Balb/c mice were immunized with one of the four constructs i) AdHu5-tPA-PfAMA1 or ii) AdHu5-tPA-PfAMA1-C or iii) AdHu5-N-PfAMA1 or iv) AdHu5-N-PfAMA1-C at a dose of $1x10^{10}$ vp. The total IgG response was measured by ELISA in the serum 12 days after immunisation against *P. falciparum* 3D7 recombinant protein. The endpoint titres (log 10) for individual mice are shown and the lines and error bars are the mean and SEM. The asterisk indicates a significant difference in endpoint titre between the groups by ANOVA (* $P \leq 0.05$, ** $P \leq 0.01$).

6.2.7 Effect of the addition of the C terminus on cellular localisation of AMA1

In order to further investigate the increased immunogenicity upon addition of the C terminus, an immunofluorescence assay was performed to see if there was any difference in the cellular localisation of PfAMA1 when using the vaccine construct with or without the C terminus. Hela cells were grown on cover slips and infected with the constructs AdHu5-tPA-PfAMA1 or AdHu5-tPA-PfAMA1-C. After an overnight incubation polyclonal serum was used to detect the localisation of AMA1. Alexa-conjugated anti-mouse antibody was used as the secondary antibody and the nucleus was stained with DAPI. The construct that has the C-terminus showed a cell surface localisation of AMA1 in both the permeabilised and non-permeabilised cells (Figure 6-8C and D). In contrast, when using AdHu5 virus that expressed the construct without the C terminus, AMA1 expression was only detectable in the permeabilised cells, where it localised in the endoplasmic reticulum as seen by the classical ER staining pattern (Figure 6-8A). Thus the addition of the C-terminus lead to the cell surface expression of AMA1.

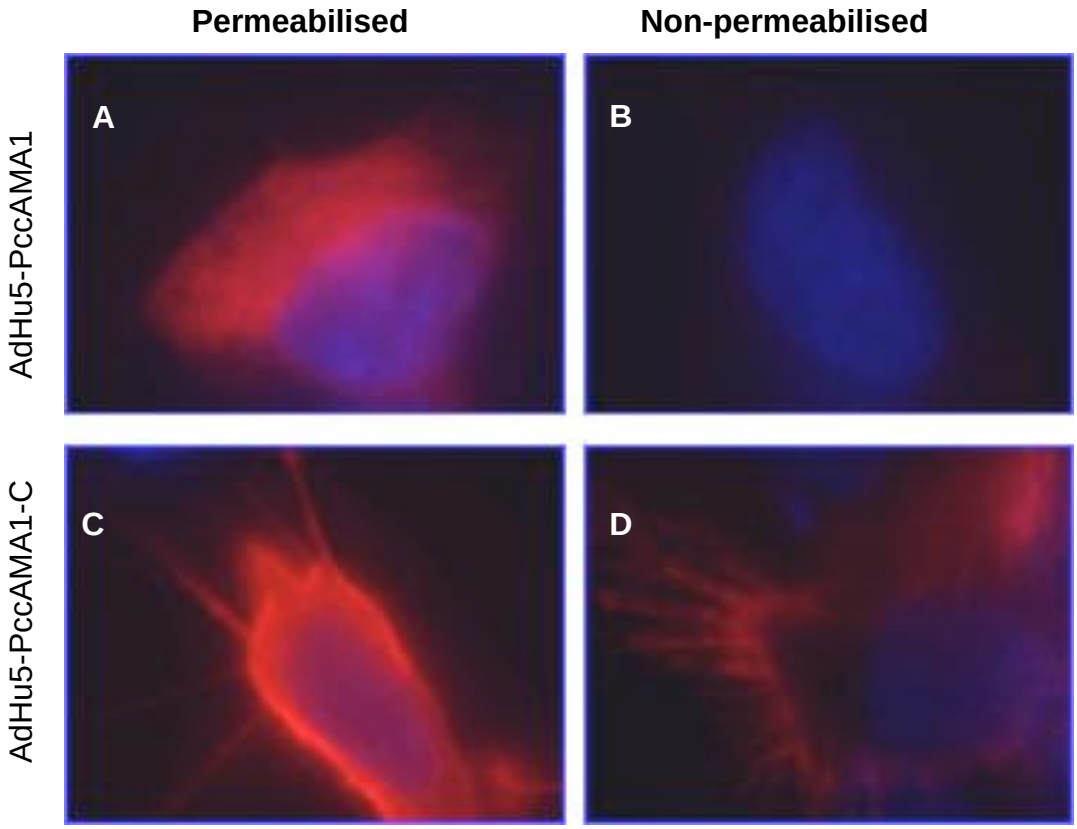


Figure 6-8: Cellular localisation of PfAMA1 after addition of the C-terminus to the vaccine insert

Hela cells were grown on cover slips and infected with the constructs AdHu5-tPA-PfAMA1 or AdHu5-tPA-PfAMA1-C. After overnight incubation polyclonal serum was used to detect the localisation of AMA1. Alexa-conjugated anti-mouse antibody was used as the secondary antibody and the nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). Cell surface localisation of AMA1 is seen in the construct that has the C terminus in both the permeabilised (C) and non-permeabilised samples (D). There is no cell surface associated AMA1 in the construct without the C terminus (A and B).

6.2.8 Design of a biallelic AMA1 clinical construct

The final clinical construct was a fusion construct of two alleles of AMA1 (3D7 and FVO) linked by a Glycine-Proline (Gly-Pro) linker. It also has the human tPA leader sequence and the TM and C terminus of FVO (Figure 6-9). This construct was cloned into AdCh63, a simian adenovirus in order to overcome anti vector immunity to AdHu5. Natural immunity to AdHu5 has been shown to interfere with the immunogenicity to AdHu5 based vaccines in clinical trials (195). It was also cloned into AdHu5 and MVA for pre-clinical studies. The immunogenicity of the biallelic construct was compared to that of the monoallelic 3D7 construct.

Figure 6-9: A schematic representation of the biallelic PfAMA1 construct.

The biallelic construct has the tPA leader sequence followed by the ectodomain of both 3D7 and FVO PfAMA1. A Gly-Pro linker (GGGPGGG) was used to join the two. The ectodomain of FVO was followed by the TM and C terminus of FVO. This construct was cloned into AdHu5, AdCh63 and MVA and these are referred to as AdHu5-biallelic, AdCh63-biallelic and MVA-biallelic respectively in this chapter from now.

6.2.9 Antibody responses in mice to recombinant AMA1

5-6 week old female Balb/c mice were primed with $1x10^9$ vp of adenovirus expressing different AMA1 constructs: i) AdCh63-biallelic, ii) AdHu5-biallelic and iii) AdHu5-PfAMA1 (3D7). Groups i) and ii) were boosted with $1x10^7$ pfu of MVA-biallelic and group iii) was boosted with the monoallelic MVA-PfAMA1 (3D7) vaccine. Total IgG was measured in the serum on days 14, 55 and 70 by ELISA against recombinant 3D7 and FVO PfAMA1. AMA1 specific IgG responses were detected in all the groups to both 3D7 and FVO recombinant proteins. Two weeks post prime there was a significantly lower response to recombinant 3D7 PfAMA1 in the AdCh63-biallelic group as compared to the monoallelic 3D7 vaccine (Figure 6-10A). Responses to FVO at day 14 post prime were the same for the biallelic and monoallelic AdHu5 groups but the AdCh63-bialleic vaccine gave a significantly lower response than the AdHu5 equivalent (Figure 6-10B). However, by day 55 there were no differences in the antibody responses seen in the different groups (Figure 6-10C and D). This could be that after priming with AdCh63, the antibodies take longer to reach similar levels as AdHu5. The MVA boost further increased the antibody response in all the groups to similar levels at day 70 (Figure 6-10E and F).

AdCh63 and AdHu5 vectors expressing the biallelic construct induced similar IgG response after an Ad_M-PfAMA1 vaccination regime. The monoallelic and biallelic constructs were indistinguishable based on the IgG titres as the

ratio of the 3D7 to FVO ELISA titres were the same for the monoallelic and biallelic vaccines (data not shown), so to address this a functional GIA assay needs to be done.

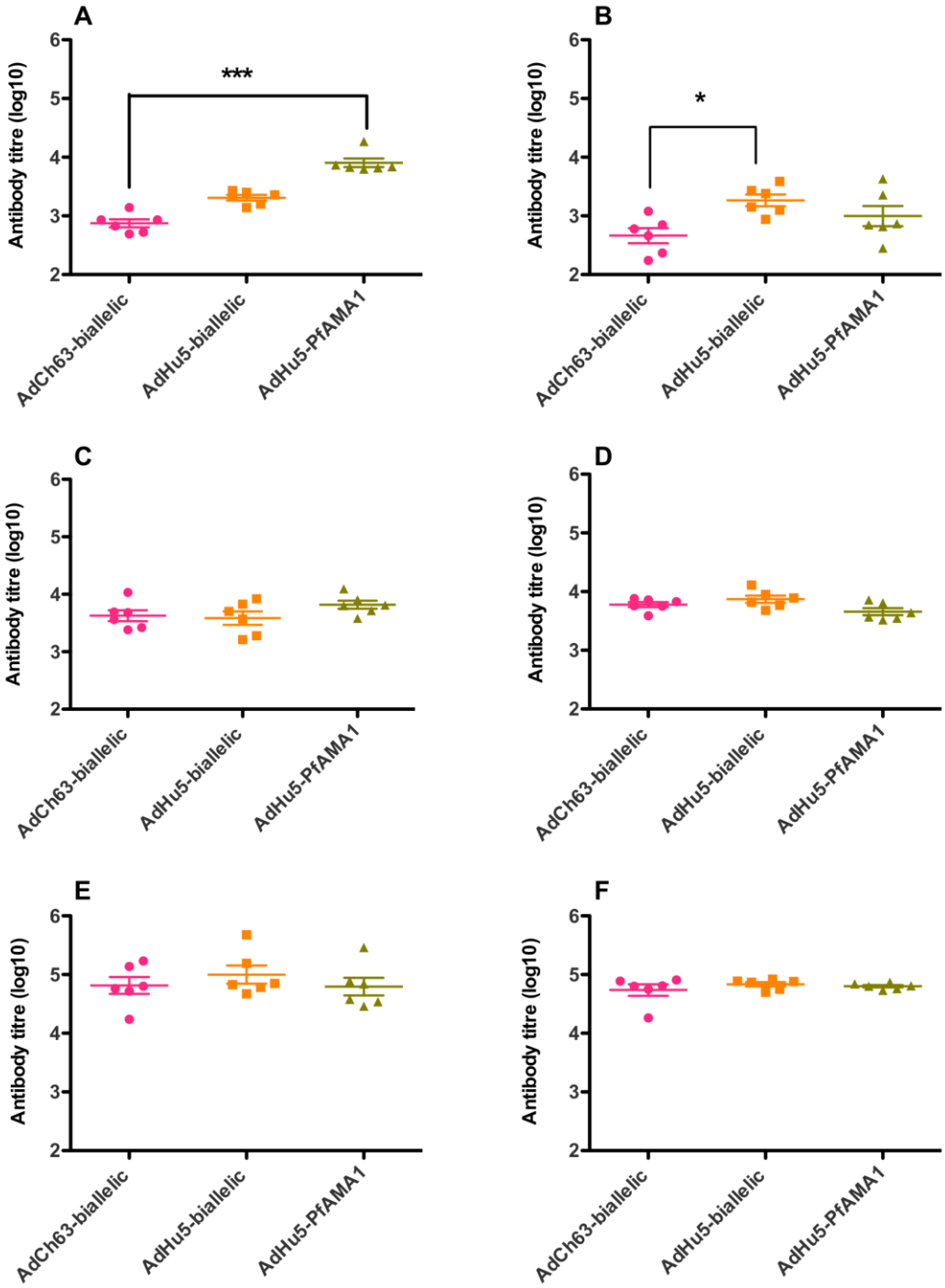

A
B
C
D
E
F
Antibody titre (log10)
2
3
4
5
6
***
*
AdCh63-biallelic
AdHu5-biallelic
AdHu5-PfAMA1

Figure 6-10: Antibody responses in mice induced by the monoallelic and biallelic PfAMA1 vaccine constructs.

5-6 week old female Balb/c mice (n=6) were primed with $1x10^9$ vp of each of the adenovirus constructs i) AdCh63-biallelic, ii) AdHu5-biallelic and iii) AdHu5-PfAMA1 (3D7). Groups i) and ii) were boosted with $1x10^7$ pfu of MVA-biallelic and group iii) was boosted with the monoallelic MVA-PfAMA1 (3D7). The total IgG response was measured by ELISA in the serum two weeks after prime (day14) (A, B), pre-boost (day 55) (C, D) and two weeks post boost (day70) (E, F). The ELISA was done against both 3D7 (A, C, E) and FVO (B, D, F) PfAMA1 recombinant protein. The endpoint titre (log 10) is shown in the figure above for individual mice and the lines are the mean and SEM. The asterisk indicates a significant difference in the endpoint titre between the groups by ANOVA (* $P \leq 0.05$, *** $P \leq 0.001$).

6.2.10 Murine T cell responses to *P. falciparum* AMA1

5-6 week old female Balb/c mice were primed with $1x10^9$ vp of each of the adenovirus constructs i) AdCh63-biallelic, ii) AdHu5-biallelic and iii) AdHu5-PfAMA1 (3D7). Groups i) and ii) were boosted with the biallelic MVA construct and group iii) was boosted with the monoallelic MVA-PfAMA1 (3D7). T cell responses were measured by ICS using four overlapping peptide pools corresponding to PfAMA1 3D7 and FVO. The peptides were 15 mers, overlapping by 10 aa covering both 3D7 and FVO AMA1.

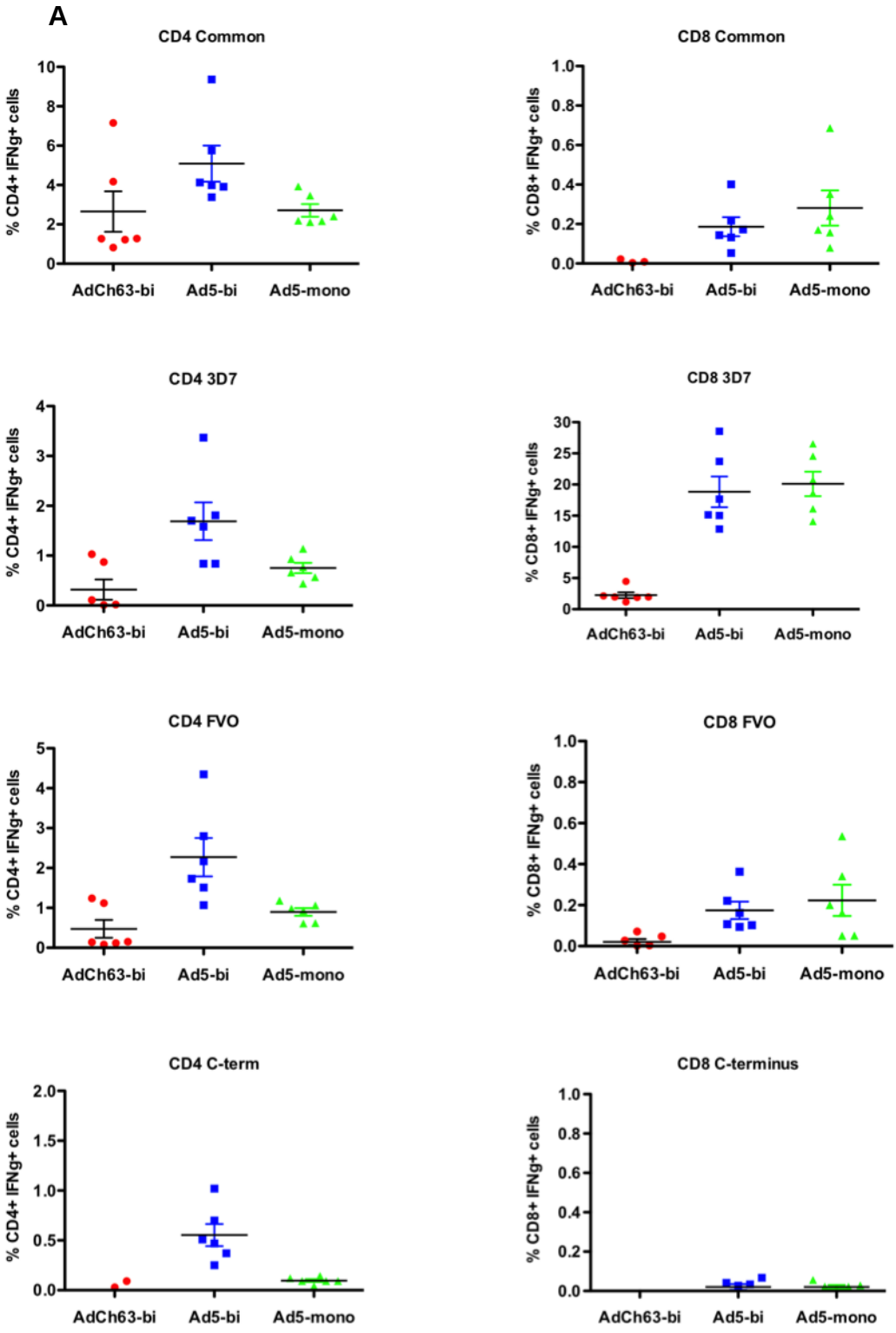
Pool 1 contained 64 overlapping peptides common to both alleles of AMA1. Pool 2 and 3 contained peptides unique to 3D7 and FVO respectively. Pool 2 and 3 had 47 peptides each. Pool 4 had only 12 peptides corresponding to

the C-terminus of the FVO allele. Splenocytes were isolated 2 weeks post boost and stimulated with the four peptide pools or no peptide (Unstim) for 5 hours. After this time the cells were surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2 by flow cytometry for % of responding cells per total CD4+ or CD8+ T cell subset (Figure 6-11A).

CD8+ T cell responses were detected to the common, 3D7 and FVO pools. In all the three pools the AdCh63-biallelic construct primed a lower CD8 T cell response than the AdHu5-biallelic construct. There were cross-reactive CD8 T cell responses detected to the FVO pool when mice were immunised with Ad_MPfAMA1 (3D7) regime. The dominant CD8+ T cell response was to the unique 3D7 pool. There was no response detected to the C-terminus.

CD4+ T cell responses were detected to all four peptide pools, although the overall magnitude of the response was lower than the CD8+ T cell response. Consistent with the CD8+ T cell data, the AdCh63-biallelic construct primed a weaker T cell response. It is encouraging that cross-reactive T cell responses were detected to the FVO pool of peptides when mice were immunised with the monoallelic 3D7 vaccine. This could be relevant to the issue of AMA1 polymorphism if these epitopes are important for protection. As one would expect there was no response detected to the C-terminus when mice were immunised with the monoallelic 3D7 construct (as it did not contain the C-terminus), however the biallelic construct which does contain this sequence induced an immune response to this peptide pool. The multifunctionality of the CD4+ T cell response to the common pool was assessed as CD4+ T cells

producing IFNγ, TNFα and IL-2 simultaneously have been shown to correlate with protection against Leishmania major in mice (231). There was no significant difference in the quantity of multifunctional cells among the different groups tested (Figure 6-11B)


A
CD4 Common
% CD4+ IFNg+ cells
AdCh63-bi
Ad5-bi
Ad5-mono
CD8 Common
% CD8+ IFNg+ cells
CD4 3D7
CD8 3D7
CD4 FVO
CD8 FVO
CD4 C-term
CD8 C-terminus

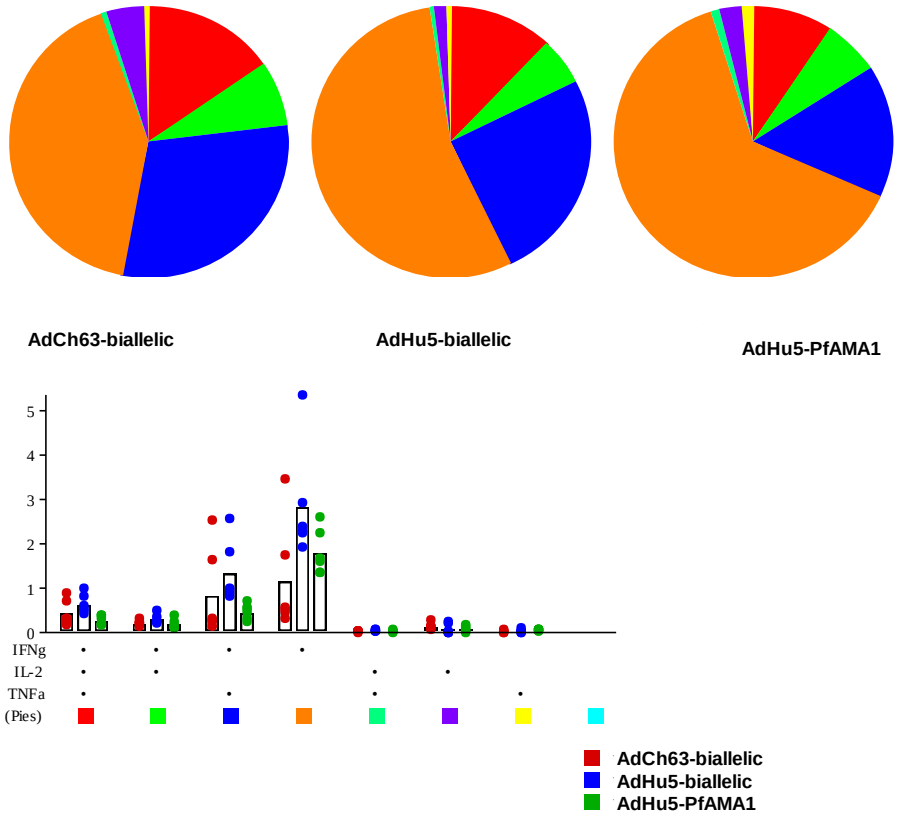


Figure 6-11: T cell responses induced in mice by vaccination with the biallelic constructs.

5-6 week old female Balb/c mice (n=6) were primed with $1x10^9$ vp of each of the adenovirus constructs i) AdCh63-biallelic, ii) AdHu5-biallelic and iii) AdHu5-PfAMA1 (3D7). Groups i) and ii) were boosted with $1x10^7$ pfu of the biallelic MVA construct and group iii) was boosted with the monoallelic MVA-PfAMA1 (3D7). Splenocytes were isolated two weeks after the final immunisation and stimulated with 4 peptide pools (Common, 3D7, FVO and C-terminus) or no peptide (Unstim) for 5 hours. After this time the cells were surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2 and analysed by flow cytometry for % of responding cells per total CD4+ or CD8+ T cell subset (A). The multifunctionality or "quality" of CD4 T cells was analysed using the SPICE software (B).

6.2.11 AdCh63_MVA induced humoral immune responses in rhesus macaques

Eleven rhesus macaques (*Macaca mulatta*) were primed with $5x10^{10}$ vp of AdCh63-biallelic PfAMA1 intramuscularly (i.m.). Nine of these macaques were boosted i.m. 8 weeks later with $5x10^{8}$ pfu of MVA-biallelic AMA1. Blood samples were obtained at weeks 0,4, 8, 9, 10, 12, 14 and 16. The total IgG response was measured by ELISA in the serum against both recombinant 3D7 and FVO PfAMA1 at the different time points (Figure 6-13). All monkeys seroconverted after the AdCh63-biallelicPfAMA1 prime immunisation as measured at weeks 4 and 8. The nine macaques that were boosted with MVA-biallelic AMA1 had significantly higher IgG responses one week post boost. This response was maintained even at week 16 and the response trended to be higher than pre boost (Figure 6-12A and B). In the two macaques, which were not boosted, there was no increase in the IgG response after week 4 post prime and the responses were maintained over the 16 week follow up period (Figure 6-12C and D).

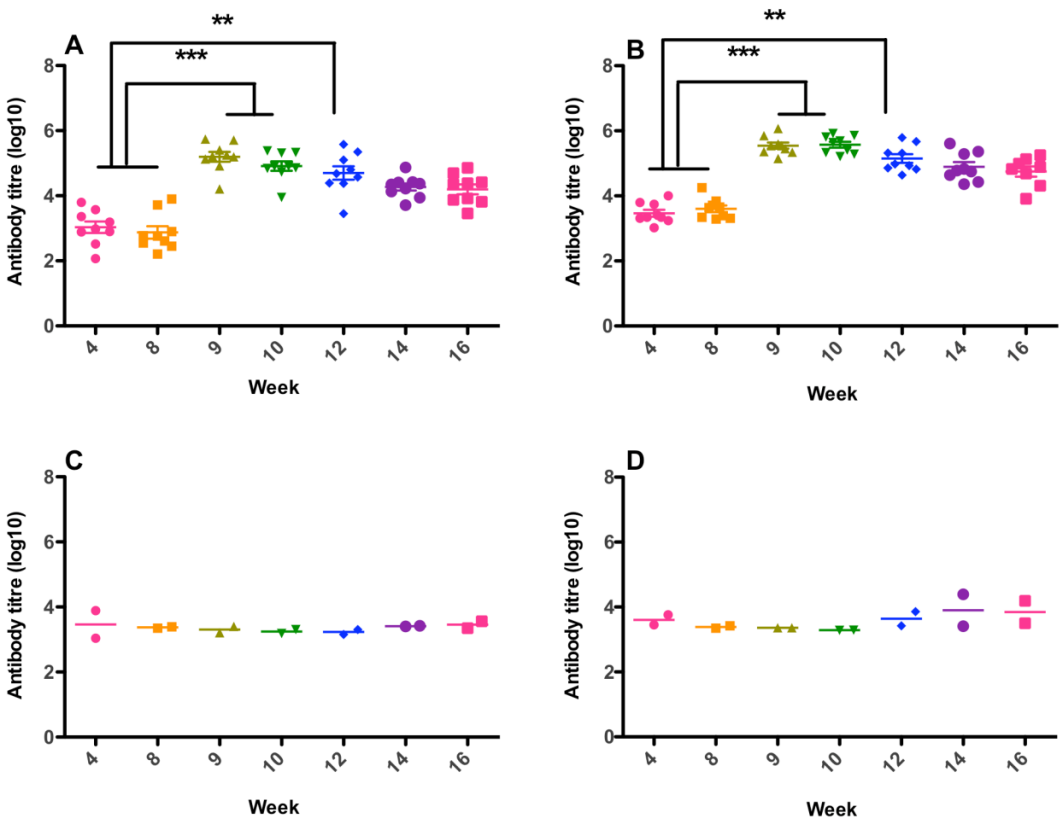


Figure 6-12: Humoral immune responses induced by AdCh63_MVA PfAMA1 biallelic vaccination in rhesus macaques.

Eleven rhesus macaques were primed with $5x10^{10}$ vp AdCh63-biallelic PfAMA1. Nine of these macaques were boosted 8 weeks later with $5x10^{8}$ pfu of MVA-biallelic AMA1. Blood samples were obtained at weeks 0, 4, 8, 9, 10, 12, 14 and 16 and the total IgG response was measured in the serum against both 3D7 and FVO recombinant PfAMA1 proteins. The endpoint titres against both 3D7 (A and C) and FVO (B and D) alleles at the different time points are shown for the AdCh63_M (A and B) and AdCh63 biallelic groups (C and D). The symbols represent responses from individual macaques and the lines represent mean and SEM for A and B. In C and D the line represent the median response (n=2). The asterisk indicates a significant difference in the endpoint titre between the different time points by ANOVA (** $P \leq 0.01$ and *** $P \leq 0.001$).

6.2.12 Growth inhibitory activity of sera from immunised macaques

The functional activity of antibodies induced by the AdCh63-biallelic PfAMA1 vaccine alone and when boosted with MVA-biallelic PfAMA1 was determined using the growth inhibition activity (GIA) assay. Eleven macaques were primed with $5x10^{10}$ vp AdCh63-biallelic PfAMA1. Nine of these macaques were boosted 8 weeks later with $5x10^{8}$ pfu of MVA-biallelic PfAMA1. The GIA of the serum samples obtained at the different time points was determined by purifying IgG. The purified IgG was incubated with *in vitro P. falciparum* schizonts and the growth inhibition was measured by pLDH assay. The GIA assay was done using both 5 mg/ml and 10mg/ml of purified IgG. This was done to assess the relationship between %GIA and IgG concentration used in the assay. The GIA assays were performed against both the 3D7 and FCR3 strains of P*. falciparum*. The FCR3 allele of AMA1 differs from the FVO allele by 1 aa. The antibodies inhibited the growth of both the 3D7 (Figure 6-13A) and FCR3 parasites (Figure 6-13C). There was a significant correlation between the 3D7 ELISA and 3D7 GIA, R^2= 0.63, *P<0.01 (Figure 6-13B) and FVO ELISA and FCR3 GIA, R^2=0.89, ***P< 0.001 (Figure 6-13D). There was also a significant correlation between GIA against the 3D7 and FCR3 parasite strain, R^2= 0.61, ***P<0.0001 (Figure 6-13E).

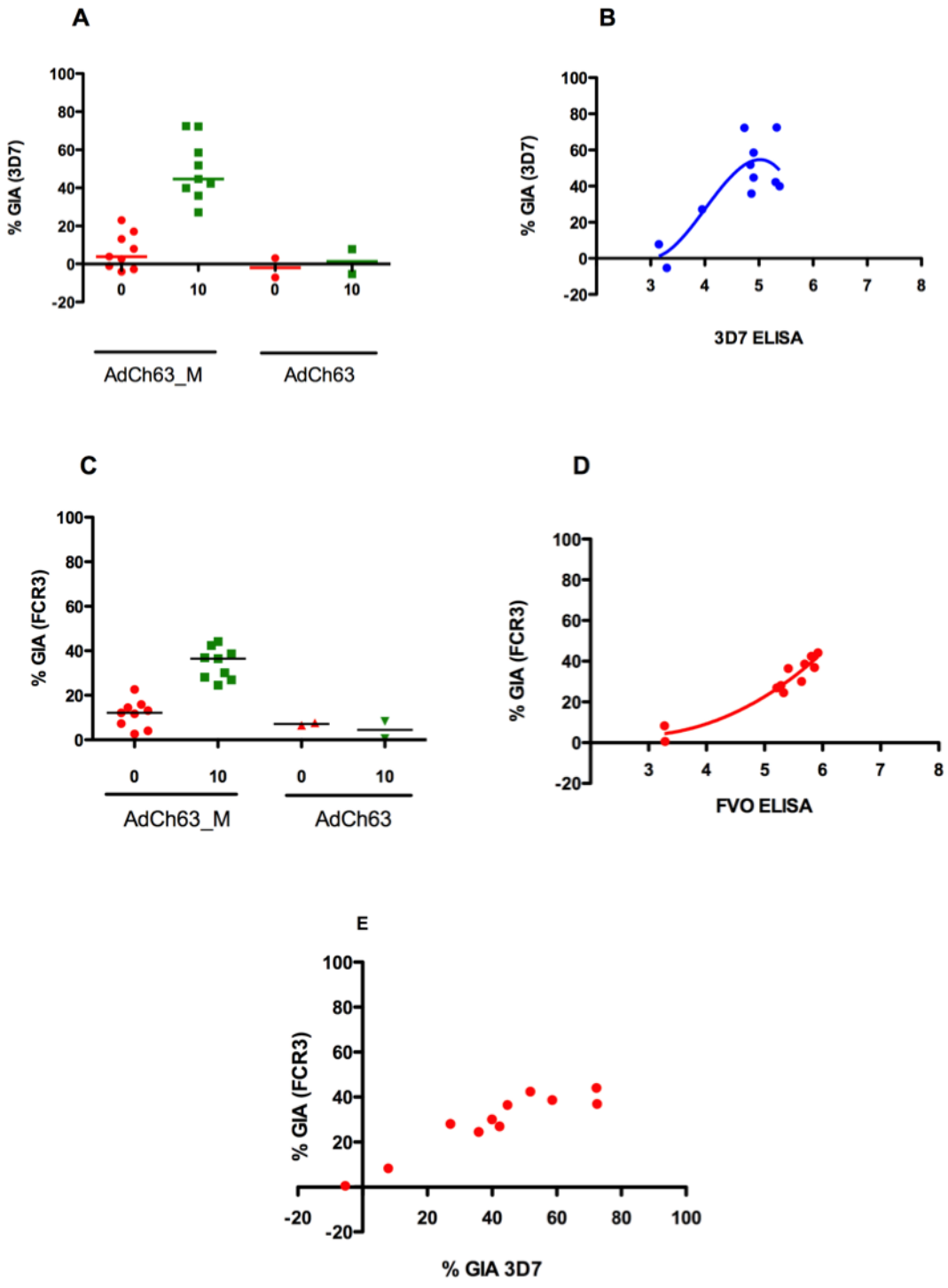

A
% GIA (3D7)
100
80
60
40
20
0
-20
0
10
0
10
AdCh63_M
AdCh63
B
% GIA (3D7)
100
80
60
40
20
0
-20
3
4
5
6
7
8
3D7 ELISA
C
% GIA (FCR3)
100
80
60
40
20
0
0
10
0
10
AdCh63_M
AdCh63
D
% GIA (FCR3)
100
80
60
40
20
0
-20
3
4
5
6
7
8
FVO ELISA
E
% GIA (FCR3)
100
80
60
40
20
-20
-20
20
40
60
80
100
% GIA 3D7

Figure 6-13: Growth inhibitory activity of sera from macaques

Eleven macaques were primed with $5x10^{10}$ vp AdCh63-biallelic PfAMA1. Nine of these macaques were boosted 8 weeks later with $5x10^{8}$ pfu of MVA-biallelic AMA1. The GIA of the serum at week 0 and 10 was determined for the macaques. IgG was purified from the serum and incubated with *in vitro P. falciparum* schizonts and the % inhibition against 3D7 (A) and FCR3 (C) strains of *P. falciparum* is shown. The correlation between the ELISA titres and the GIA at week 10 for the macaques that were boosted is shown for both 3D7, R^2= 0.63, **P<0.01 (B) and FVO, R^2=0.89, ***P< 0.001(D). The correlation between GIA against the 3D7 and FCR3 is shown, R^2= 0.61, ***P<0.0001 (E).

6.3 Discussion

The work undertaken here shows that viral vectored vaccines encoding PfAMA1 are highly immunogenic and induce strong antibody and T cell responses in mice, rabbits and macaques. These viral vectored vaccines are becoming a cheap, safe and immunogenic alternative to recombinant protein-in-adjuvant vaccines (291).

The monoallelic 3D7 AMA1 vaccination regime induces cross-reactive antibodies to the heterologous FVO allele of AMA1 both in mice and rabbits as measured by ELISA, however, there is a better correlation between the 3D7 and FVO antibodies after the MVA boost in rabbits than in mice. This has been seen before with another AMA1 vaccine and also with a P. falciparum $MSP1_{42}$ based vaccine, suggesting there are differences in antibody cross-reactivity in different mammalian species. The cross reactivity of the antibodies was seen only by ELISA whereas the growth inhibitory activity was

strain-specific. The limitations of ELISA as a predictor for GI activity have been demonstrated recently by comparing antibodies against AMA1 and $MSP1_{42}$ (173). All antibodies measured by ELISA do not have equal effect on parasite growth in vitro. ELISA and GIA are the only tools that are available currently to predict the efficacy of a blood-stage vaccine but the relevance of these assays to predict the outcome of clinical trials is debatable. Recently, a clinical trial with recombinant AMA1 failed to prevent clinical infection though the vaccine induced very high levels of GIA in human volunteers (292).

These antibodies induced by the viral vectored vaccination regime also bound to native AMA1 protein as shown by IFA on schizont-infected erythrocytes. This shows that the vaccination regime induces some conformationally correct antibodies. It has been shown that IgG isolated from rabbits immunised with reduced AMA1 ectodomain do not have any growth inhibitory activity *in vitro* (164) so it is important to induce antibodies against conformational epitopes to measure growth inhibitory activity *in vitro*. In this study in mice there was 94% growth inhibitory activity against the homologous 3D7 strain but only 54% against the heterologous strain. Similarly in rabbits the GI activity was 40% on average against 3D7 and less than 10% against FVO. Polymorphism in AMA1 has been one of the main hurdles in vaccine development targeting this antigen. AMA1 polymorphisms are under balancing selection, especially within domains I and II that are the most polymorphic. It is speculated that these polymorphisms are maintained due to selective pressure by the host immune system (160, 161, 293, 294). In mice there is better cross-strain *in vitro* inhibition than in rabbits, though conversely in rabbits the correlation

between 3D7 and FVO antibodies as measured by ELISA is stronger. This is in accordance with results published from several other groups that show that rabbit antisera are more effective in growth inhibition of homologous strains *in vitro* than heterologous strains (164, 222, 289, 295). In this study there is better cross-strain GIA seen in mice than in rabbits. This is not in agreement with the literature where the pattern of strain specificity was found to be similar in mice and rabbits (296).

The addition of the tPA leader sequence showed significantly better antibody response than the native N-terminus of AMA1. The tPA signal sequence is a specific targeting signal that targets the expressed antigen directly to the endoplasmic reticulum (ER) (297) and influenza virus NP protein when fused to tPA was shown to be expressed at higher levels and was found to be in a partially secreted form in vitro (290). When fused to the tPA signal, viral envelope protein E of Japanese encephalitis virus was expressed at higher levels in transfected cells than the wild-type construct and was mainly localised in the cytosolic and membrane fraction. The tPA fusion constructs elicited stronger humoral and cellular response and better protection against virus challenge in animals (298).

In order to overcome this allele specific response of functional IgG, biallelic AMA1 vaccines have been developed by several groups such as AMA1-C1 that contain equal amounts of 3D7 and FVO AMA1 recombinant proteins. These biallelic vaccines show better cross-reactivity in terms of GIA than monoallelic vaccines.

The AdCh63_MVA biallelic PfAMA1 vaccination regime induced antibodies against both the 3D7 and FVO recombinant protein in mice and macaques. The data also demonstrate that AdCh63 could be used to replace AdHu5 in this prime-boost vaccination regime in order to avoid anti-vector immunity without compromising the humoral and cell-mediated immunity against the transgene. The antibodies induced in macaques after immunising with the biallelic vaccine had growth inhibitory activity against both 3D7 and FVO *P. falciparum* parasites. In contrast to the previous results with the monoallelic vaccine, where the antibodies were cross-reactive in ELISA but not in GIA, the biallelic vaccine induced functional antibodies against both the strains. This is likely to be due to the inclusion of both the alleles of AMA1 in the biallelic vaccine that induces antibodies to strain-conserved regions of both 3D7 and FVO in addition to cross-reactive antibodies. Though this is likely to be the case, previously it had been shown that the allele specificity of antibodies to AMA1 depends on the species immunized (296). Immunisation of rhesus monkeys with recombinant AMA1 showed a completely different strain specificity pattern in terms of GIA. Even when monkeys were immunized with single-allele AMA1 vaccines, the elicited antibodies could bind indistinguishably to both homologous and heterologous AMA1 proteins on ELISA plates, and the antibodies showed cross-reactive biological activities, as judged by GIA and antigen reversal GIA assays (296). Due to this it is difficult to say if the cross-reactive GIA was due to the biallelic vaccine or similar effect would be seen if the monoallelic vaccine was tested in macaques. This needs further investigation and so rabbits have been immunised with both the monoallelic PfAMA1 3D7 vaccine and the biallelic

vaccine to compare the functional activity of the antibodies induced. Unfortunately, the results will not be available until after the submission of this thesis.

Data on human antibody responses to AMA1 show that affinity purified antibodies from individuals living in malaria endemic areas can inhibit *P. falciparum* growth *in vitro* (164). Cortes *et al.* have also examined the allele specificities of plasma samples of adults from Papua New Guinea and found that antibodies against conserved regions of the molecule were much more common than allele-specific antibodies (276). If these conserved regions are important in antibody-mediated vaccine protection and if conserved T cell responses induced by the viral vectored vaccine regime described here are also protective, then these data may indicate that AMA1 polymorphism could be less of a problem in humans than was originally thought after vaccine studies in small mammals. T cell responses to both common and unique epitopes have been measured here by flow cytometry in mice. This is encouraging because if the common epitopes are important in protection then they could confer cross strain protection against heterologous strains. In agreement with this hypothesis, there have been suggestions based on analysis of sequence polymorphism frequencies that the selection pressure on AMA1 domain III is mediated by T-cell immunity (275). Multifunctional CD4+ T cells to the common epitope were also induced by vaccination in this study after both AdCh63 and AdHu5 prime and MVA boost. In another recent study, pluripotent effector memory T cells against infected erythrocytes have been associated with protection in humans after a vaccination with

sporozoites and treatment with choloroquine (131).

Recombinant adenoviruses (E1 deleted) such as AdHu5 were initially developed for gene therapy (299, 300). The advantages of recombinant adenovirus vectors as vaccine carriers are numerous and they are highly immunogenic, however the host generates an immune response not only to the transgene but to the vector as well (301, 302). AdHu5 vectors have been developed for vaccine delivery for several diseases and tested in rodents, primates and recently in humans in a vaccine against human immunodefiency virus 1 (186-189, 191). In the United Sates 40 - 60% of the population carry AdHu5 virus-neutralizing antibodies and the percentage is even higher in developing countries (238, 303-306). Anti-AdHu5 antibodies have been shown in several preclinical and clinical studies to hamper the immunogenicity of recombinant AdHu5 vaccines (192, 304, 307-311). Due to the need to overcome this problem, chimpanzee adenovirus vectors like AdCh63 have been developed. A recent study in a cohort of children from Kenya showed that 23% of the children had high-titre neutralizing antibodies to AdHu5 whereas only 4% had high titre antibodies to simian adenovirus AdCh63 (312). Other studies have shown that in countries with holoendemic malaria incidence antibodies against two chimpanzee adenoviruses were less than 10% (313). It is thus encouraging from this study that the AdCh63 vector is as potent as AdHu5 vectors in generating an antibody response. The CD4+ and CD8+ T cell responses tended to be lower than those induced by AdHu5 expressing the same vaccine construct, but this did not reach statistical significance. The low prevalence of AdCh63 in malaria endemic regions and

the potent immune responses generated by this vector in this study are encouraging for its potential use as a malaria vaccine vector. These data demonstrate that this viral vectored vaccine regime induces responses equal to and better than those induced by recombinant protein immunisation. The regime involves only two immunisations whereas most recombinant protein immunisation schedules involve at least three, and have the inherent problems of finding strong adjuvants, which are safe to use in humans.

CHAPTER 7

COMPARISON OF MVA PROMOTERS: P7.5 AND I1L

7 Comparison of MVA promoters: P7.5 and I1L

7.1 Introduction

MVA is a highly attenuated strain of Vaccinia virus and has lost its ability to replicate in almost all mammalian cells after losing 15% of its genome in the course of more than 500 passages in chick embryo fibroblasts (CEFs) (314). MVA has been shown to preferentially infect professional antigen presenting cells. Upon infection of dendritic cells (DCs) *in vitro* only early genes of the virus are expressed within the infected DCs. The viral life cycle is in fact arrested before late gene expression can occur in both murine (315) and human DCs (316, 317). The induction of CD8+ T cells by antigen presenting cells against a viral infection requires the presentation of peptides derived from viral proteins bound to MHC class I molecules (318). The presentation of rapidly degraded endogenous translation products on MHC class I molecules leads to direct antigen presentation of the viral peptides on the infected DC. The endogenous peptides are transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum. This peptide binding leads to the release of nascent MHC class I molecules from the loading complex and these are then transported to the plasma membrane as peptide-MHC complexes (319). Conversely, infected DCs undergo apoptosis, and the

apoptotic blebs are taken up by uninfected DCs and cross-presented on MHC class I molecules that induce CD8+ T cells via the cross presentation pathway (320). Similarly, long-lived protein substrates from other virus-infected cells like somatic cells can be transferred to professional antigen presenting cells (APCs) and presented via the cross presentation pathway (321). MVA viruses expressing antigens driven by early promoters are thus expressed in dendritic cells and can elicit a strong CD8+ T cell response (by direct presentation). In contrast, late promoter driven antigens are not expressed in DCs. Instead, these late promoter driven antigens are expressed in somatic cells and can potentially be cross-presented by DCs. Late promoters, especially I1L, have been reported to express antigens at a much higher level than early promoters (322) like P7.5, which has been used to drive foreign genes in Vaccinia and MVA vectors based on the fact that these are promoters for genes encoding relatively abundantly expressed proteins (323). So we hypothesised that antigens expressed under I1L which is a late promoter (324) will not be directly presented, instead the antigen will be taken up by the exogenous antigen processing pathway, would lead to similar or reduced CD8+ T cell responses (by cross presentation), and possibly a better CD4+ T cell and antibody response, which is ideal for a blood-stage malaria vaccine.

MVA vectors expressing the antigen under the early/late P7.5 promoter are very good at boosting antibody levels unlike adenovirus vectors which can both prime and boost antibody response (177). Following adenovirus immunization there is a prolonged high-level antigen expression, which might be beneficial for priming an antibody response. MVA infection leads to a short

burst of antigen, which is probably not enough to prime an antibody response but is very efficient at boosting it (325). In an attempt to improve MVA as an antibody-priming and/or boosting vector, recombinant MVAs expressing *P. yoelii* Circumsporozoite Protein (CSP) under a vaccinia virus early/late promoter P7.5 and an immediate/late promoter I1L were made and compared. The antibody responses generated when the CSP antigen is expressed under the two promoters either after a single immunisation with MVA or in a homologous and heterologous prime boost regime were compared. The CD8+ and CD4+ T cell response to published CD8 and CD8 epitopes was also assessed using flow cytometry.

7.2 Results

7.2.1 Vaccine design

The antigen used in this study was *P. yoelii* CSP – an antigen that is naturally secreted by the parasite. It is the most abundant protein on the surface of sporozoites (326).This antigen was chosen because in the *P. yoelii* Balb/c model there are published CD8+ T cell, CD4+ T cell and B cells epitopes (228, 327-329) for this antigen which provided an ideal system with which to compare the two promoters in MVA PyCSP vaccines. The immunodominant CD8+ T cell epitope corresponds to aa 280-288 and the CD8+ T cell clones specific for this epitope induce high levels of cytolysis in vitro upon exposure to peptide-incubated MHC-compatible target cells and adoptive transfer of these clones conferred complete protection against sporozoite challenge in

naive mice (327). The CD4+ T cell epitope (Py1) corresponds to aa 59-79 and CD4+ T cell clones derived from mice immunised with this peptide were able to inhibit *in vitro* hepatic development of the parasites. Passive transfer of these CD4+ T cell clones gave complete protection in mice (328). Within the CD4+ T cell epitope a subdominant cytotoxic CD8+ T cell epitope has been described corresponding to aa 58-67, which can eliminate infected hepatocytes *in vitro (228)*. The B cell epitopes lie within the CSP repeat region (aa 139- 228) and are the main target of antibody responses. The anti-repeat antibodies have been shown to have neutralising activity in several animal models (330-332) (Figure 7-1).

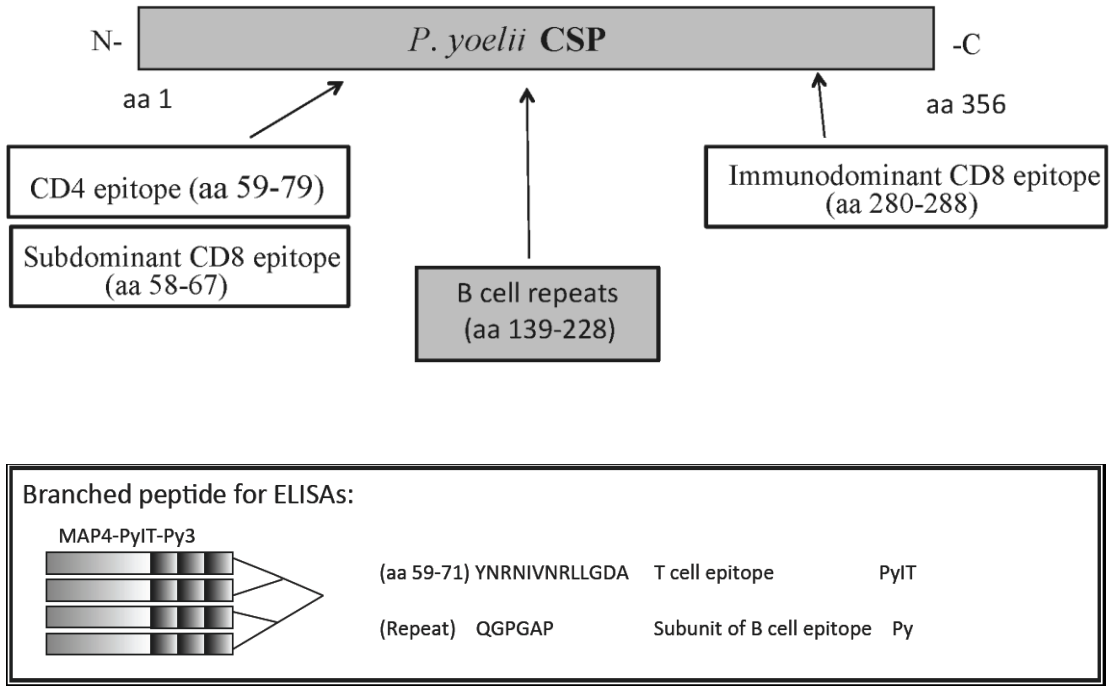


Figure 7-1: T and B cell epitopes in *P. yoelii* CSP.

The figure above shows the T and B cell epitopes described in section 7.2.1. *P. yoelii* CSP is 356 aa long. The aa positions for the different epitopes are shown. The branched peptide used for measuring antibody responses to CSP is also shown. This contains a subunit repeat (QGPGAP) of the B cell epitope (329).

7.2.2 Humoral immune responses generated by the recombinant MVA PyCSP vaccines.

Two groups of Balb/c mice (n=4) were immunised intradermally with $1x10^7$ pfu of MVA-P7.5PyCSP and MVA-I1LPyCSP and the total IgG and IgM response was measured in the serum 7 days post vaccination by ELISA. The antibody response was measured against the branched peptide MAP4-Py1T-Py3 (shown in Figure 7-1). The MAP4-Py1T-Py3 contains the Py1T epitope (CD4+ T cell epitope) cross-linked to three (QGPGAP) repeats of *P. yoelii* CSP (329).

There was no detectable IgG or IgM response in either of the groups after a single MVA immunization. As there was no detectable IgG or IgM after a single MVA immunisation, the vaccines were compared in a heterologous prime boost regime after priming with AdHu5-PyCSP. Two groups of 5-6 week old Balb/c mice were primed i.d. with $1x10^{10}$ vp of AdHu5-PyCSP and boosted eight weeks later either with MVA-P7.5-PyCSP or MVA-I1L-PyCSP. Two weeks post boost the total IgG response in the serum was measured by ELISA. The total IgG response was significantly lower in the group boosted with the MVA-I1LPyCSP vaccine than the MVA-P7.5PyCSP vaccine (Figure 7-2).

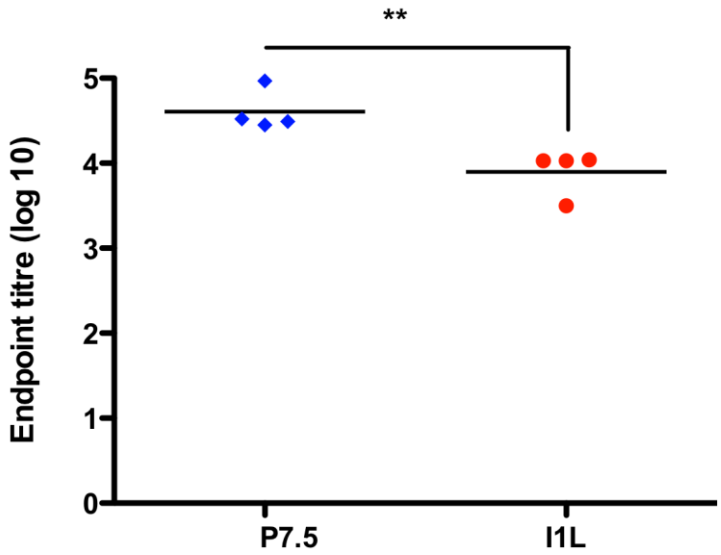


Figure 7-2: Humoral immune responses generated by the recombinant MVA PyCSP vaccines.

Two groups of 5-6 week old Balb/c mice were primed intradermally with $1x10^{10}$ vp of AdHu5-PyCSP and boosted eight weeks later either with $1X10^{7}$ pfu MVA-P7.5-PyCSP or MVA-I1L-PyCSP. The total IgG response in the serum two weeks after the final vaccination was measured by ELISA against the branched peptide. Each point indicates the response from an individual mouse and the lines represent the geometric mean. The asterisk indicates a significant difference in the ELISA titre between the groups as analysed by Mann Whitney test (** $P \leq 0.01$).

7.2.3 T cell responses induced by the recombinant MVA PyCSP vaccines.

The T cell responses generated by the two MVA vaccines were measured after a single immunisation with either of the MVA constructs and also in an AdHu5-MVA prime boost regime. Two groups of Balb/c mice (n=4) were immunised intradermally with $1x10^{7}$ pfu of MVA-P7.5PyCSP and MVA-

I1LPyCSP. Splenocytes were isolated from the spleens seven days after the immunisation and stimulated with the relevant peptide for 5 hours, surface stained for CD4 and CD8 and then intracellularly stained for IFNγ, TNFα and IL-2. The T cell response was assessed against published CD8+ and CD4+ T cell epitopes described in section 7.2.1. There was no CD4+ T cell response detected in either of the groups (data not shown). There was no significant difference in the CD8+ T cell response between the two groups for either the dominant (Figure 7-3A) or subdominant epitopes (Figure 7-3B).

In a separate experiment two groups of 5-6 week old Balb/c mice were primed intradermally with $1x10^{10}$ vp of AdHu5-PyCSP and boosted 8 weeks later with either MVA-P7.5PyCSP or MVA-I1LPyCSP. The T cell immunogenicity was assessed 2 weeks post boost. There was a significant difference in the % CD8+ IFNγ+ cells in the two groups that were primed with AdHu5-PyCSP and boosted with the respective MVA (Figure 7-3C). The CD8+ IFNγ+ T response was the same in both the groups against the subdominant CD8+ T cell epitopes (Figure 7-3D). Unlike after single immunisation with MVA, a weak CD4+IFNγ+ response was detected after the prime boost vaccination regime in both the groups but there was no significant difference between the two (Figure 7-3E).

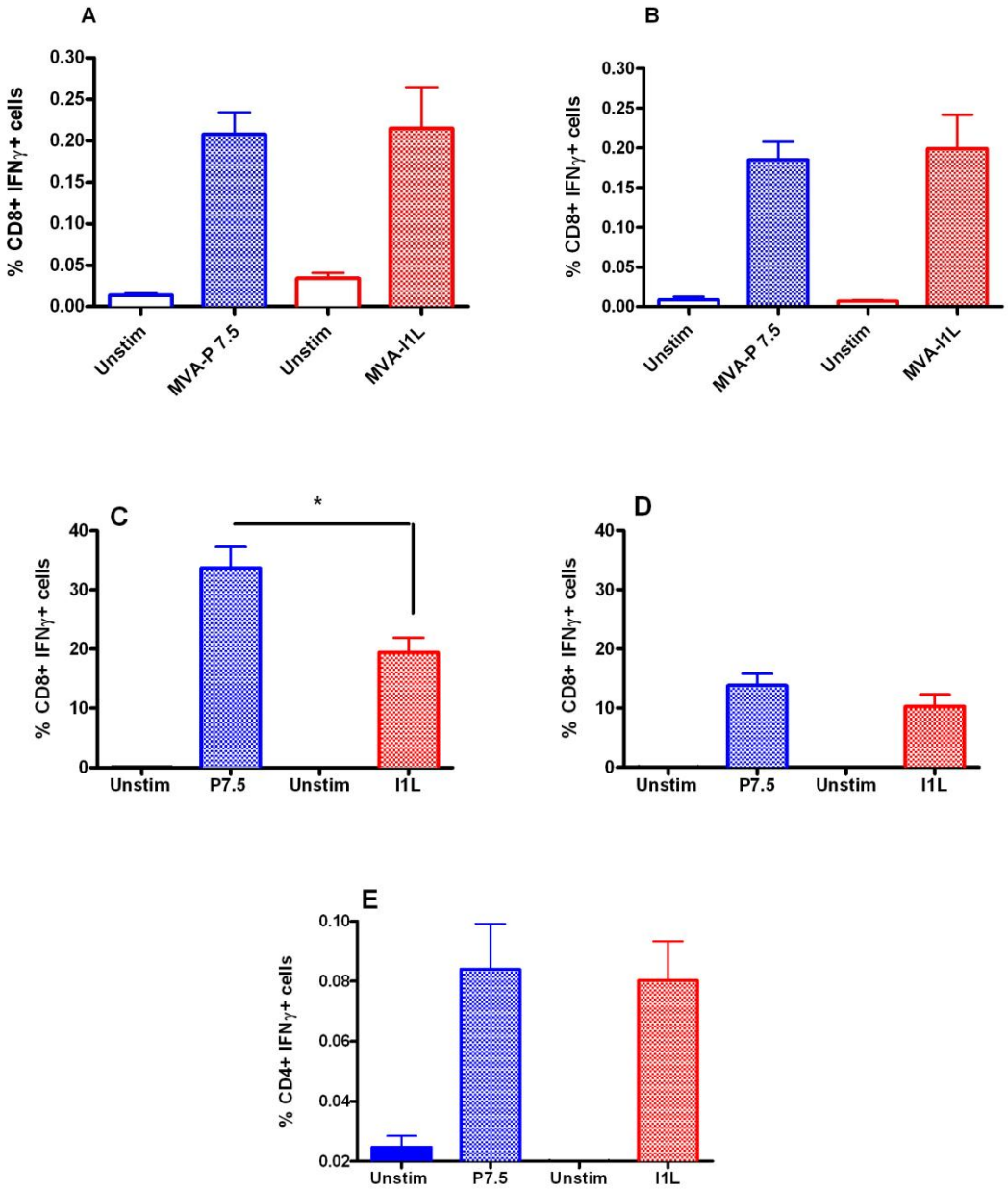

A
% CD8+ IFNγ+ cells
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Unstim
MVA-P 7.5
Unstim
MVA-I1L
B
% CD8+ IFNγ+ cells
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Unstim
MVA-P 7.5
Unstim
MVA-I1L
C
*
% CD8+ IFNγ+ cells
40
30
20
10
0
Unstim
P7.5
Unstim
I1L
D
% CD8+ IFNγ+ cells
40
30
20
10
0
Unstim
P7.5
Unstim
I1L
E
% CD4+ IFNγ+ cells
0.10
0.08
0.06
0.04
0.02
Unstim
P7.5
Unstim
I1L

Figure 7-3: T cell responses induced by the recombinant MVA PyCSP vaccines.

Two groups of Balb/c mice were immunised with MVA-P7.5PyCSP and MVA-I1LPyCSP. In a separate experiment two groups of 5-6 week old Balb/c mice were primed intradermally with $1x10^{10}$ vp of AdHu5-PyCSP and boosted eight weeks later either with $1x10^{7}$ pfu MVA-P7.5-PyCSP or MVA-I1L-PyCSP. One week after vaccination in the case of MVA prime only and two weeks post boost in the prime-boost experiment, splenocytes were isolated and stimulated with CD8 dominant, CD8 subdominant, CD4 peptide or no peptide (Unstim) for 5h. Cells were surface-stained for CD4 and CD8, and intracellularly stained for IFNγ, TNFα and IL-2 and analysed by flow cytometry. The % CD8+ IFNγ+ cells after a single immunization is shown for the dominant (A) and subdominant (B) CD8 epitopes. The % CD8+ IFNγ+ cells after prime-boost is shown for the dominant (C) and subdominant (D) CD8 epitope. The % CD4+ IFNγ+ cells after prime boost is shown in E. There was a significant difference in the % CD8+ IFNγ+ cells in the two groups which were primed with AdHu5-PyCSP and boosted with the respective MVA (C) as analyzed by Mann Whitney test (* $P \leq 0.05$).

7.2.4 CD8+ T cell responses to the dominant CD8+ T cell epitope following different routes of vaccine administration

The US11 protein from human cytomegalovirus has been reported to cause selective degradation of MHC class I molecules and thus inhibit direct presentation within CMV infected cells (333). US11 leads to the dislocation of the class I heavy chain from the lumen of the endoplasmic reticulum, which leads to the degradation of the heavy chain by the proteosome after being deglycolysated by host N-glycanase (334). US11 protein reduced the immune

response to US11-recombinant vaccinia virus (VV) only when injected via intraperitoneal (i.p.), intramuscular (i.m.) and intravenous (i.v.) routes but not when administered intradermally (i.d.) or subcutaneously (s.c.) (335). These data would suggest that direct priming does not occur when VV is given s.c. or i.d.. It has been previously shown that induction of CD8+ T cell immunity with vaccines based on MVA strongly, if not exclusively, depends on cross presentation (321). Infecting TAP-deficient or MHC class I-mismatched non-pAPC donor cells with live MVA has shown to evoke similar CD8+ T cell frequencies and inhibition of cross-presentation by in vivo maturation of DC completely abrogates priming of MVA-specific T cells (321). In the absence of any improvement of CD8+ T cell or antibody responses with MVA-I1LPyCSP, the opportunity was taken to use the same tools to attempt to address the question of the relative contributions of direct and cross presentation in MVA vaccination.

In order to investigate whether the route of administration had an effect on direct and cross-presentation, 10 groups of 5-6 week old Balb/c mice (n=5/group) were immunised with either MVA-P7.5PyCSP or MVA-I1LPyCSP by the five different routes of administration (i.d., i.p., i.m., i.v. and s.c.). The CD8+ IFNγ+ T cell response was measured using ICS against the dominant CD8 epitope (Figure 7-4). The responses induced by the two constructs were similar for all the routes except i.p. where in this case there was a significantly lower response with MVA-I1LPyCSP in one experiment (data not shown separately). However this significant effect was not reproducible and when the data from two experiments were combined there was only a trend towards a

lower response. These data suggest that, unlike for VV where cross priming occurs via the i.d. and s.c. routes, in the case of MVA cross-priming occurs via the i.d., s.c., i.m. and i.v. routes, and that direct priming may contribute to the response via the i.p. route as the CD8+ T cell response trend to be lower when MVA-I1LPyCSP is given i,p. though this is not significant. These conclusions are however reliant on the fact that I1L driven antigens are cross-presented by DCs, whereas P7.5 driven can be directly presented. This remains to be formally proven.

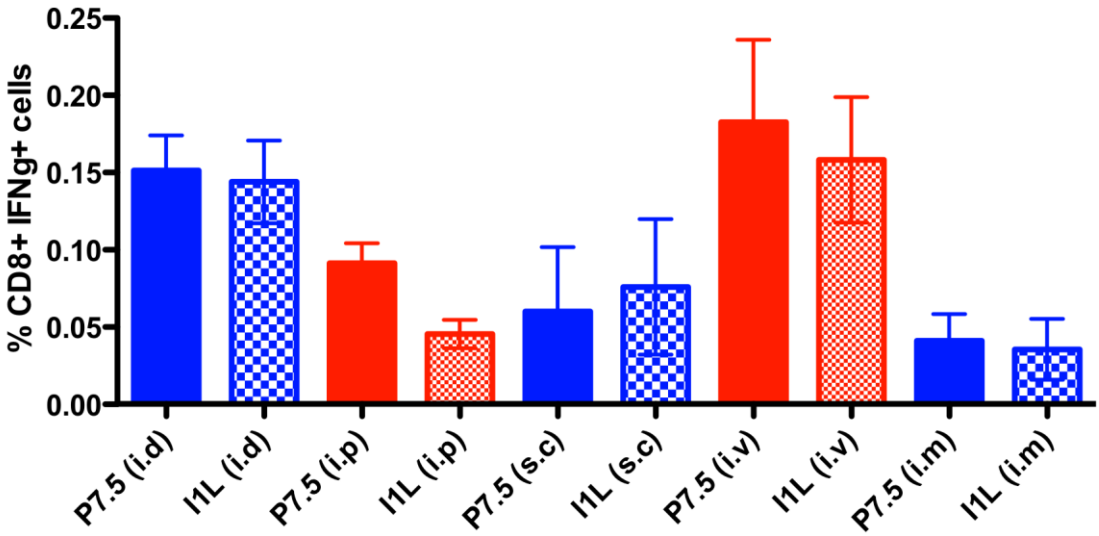


Figure 7-4: Comparison of MVA PyCSP immunogenicity using different routes of vaccine immunisation.

5-6 week old Balb/c mice were immunized with $1x10^7$ pfu of either MVA-P7.5-PyCSP or MVA-I1L-PyCSP. The five routes of immunization used were i.d., i.p., s.c., i.v. and i.m. Splenocytes were isolated from the spleen 7 days after immunisation and the CD8+ IFNγ+ response to the dominant CD8 epitope was measured by ICS. Results from two different experiments for the i.d. and i.p. routes were combined. There was no statistically significant difference between the groups by ANOVA.

7.2.5 Quantification of luciferase expression driven by the two promoters

The level of antigen expression by the two promoters was assessed by using an *in vitro* luciferase assay. Two recombinant MVA plasmid constructs were

made (by M. Dicks) which expressed *Renilla* luciferase under the P7.5 (MVA-P7.5Pb9rLuc) and I1L promoters (MVA-I1LPb9rLuc). These constructs were used to transfect Hela cells *in vitro* to quantify the expression of luciferase from the two promoters. After 24 hours, the level of luciferase activity was assayed in cells (Lysate) and supernatant (Medium) by using the Promega *Renilla* Luciferase Assay system (described in Chapter 2). The MVA-I1LPb9rLuc construct expressed higher amounts of luciferase when compared to MVA-P7.5Pb9rLuc construct both in the cells and supernatant (Figure 7-5). This shows that I1L does produce more luciferase then P7.5 and agrees with the data previously published [11]. MVA-GFP was used as the transfection control and untransfected and uninfected cells were also used as controls.

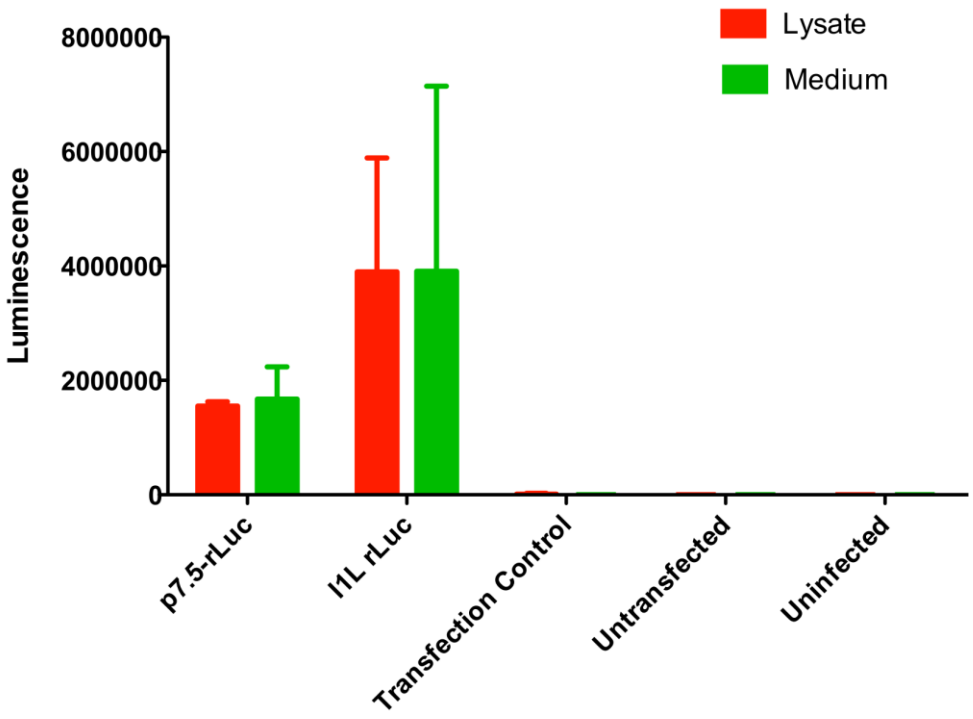


Figure 7-5: Luciferase expression under the P7.5 and I1L promoters.

The figure shows the quantity of luciferase expressed by the two promoters *in vitro*. Hela cells were transfected with either MVA-P7.5Pb9rLuc or MVA-I1LPb9rLuc and the luciferase expression by the two promoters was assessed using the Promega Renilla Luciferase Assay system. The appropriate transfection control and untransfected and uninfected cells were also included. The level of luciferase was assayed in cells and supernatant 24 hours after the transfections.

7.2.6 T cell responses to the *P. berghei* CSP (Pb9) epitope.

The luciferase constructs used to measure the *in vitro* luciferase expression also contained the dominant CD8+ T cell epitope of *P. berghei CSP* (Pb9) in Balb/c mice. This was done to see if there was a difference in immunogenicity

of the vaccines *in vivo*. If direct presentation was important to induce CD8+ T cell response via these routes the P7.5 promoter should induce higher numbers of CD8+ T cells than I1L. Two groups of Balb/c (n=5) were immunised i.d. with MVA-P7.5Pb9rLuc and MVA-I1LPb9rLuc. At the same time two groups of Balb/c mice were immunised i.p. with MVA-P7.5Pb9rLuc and MVA-I1LPb9rLuc. The CD8+ IFNγ+ T cell response was measured using ICS against Pb9 and E3. The splenocytes were isolated 7 days after the immunisation and stimulated with Pb9 or no peptide for 5 hours, surface stained for CD4 and CD8 and intracellularly stained for IFNγ, TNFα and IL-2. There was no significant difference in the CD8+ IFNγ+ response to the Pb9 epitope (Figure 7-6A) and the E3, which is a CD8+ T cell epitope in MVA (336) (Figure 7-6B). Unfortunately, a T5NT early transcription termination sequence was present in the luciferase cDNA, which could affect comparability with the PyCSP experiments, since early (but not late) gene expression may be impaired.

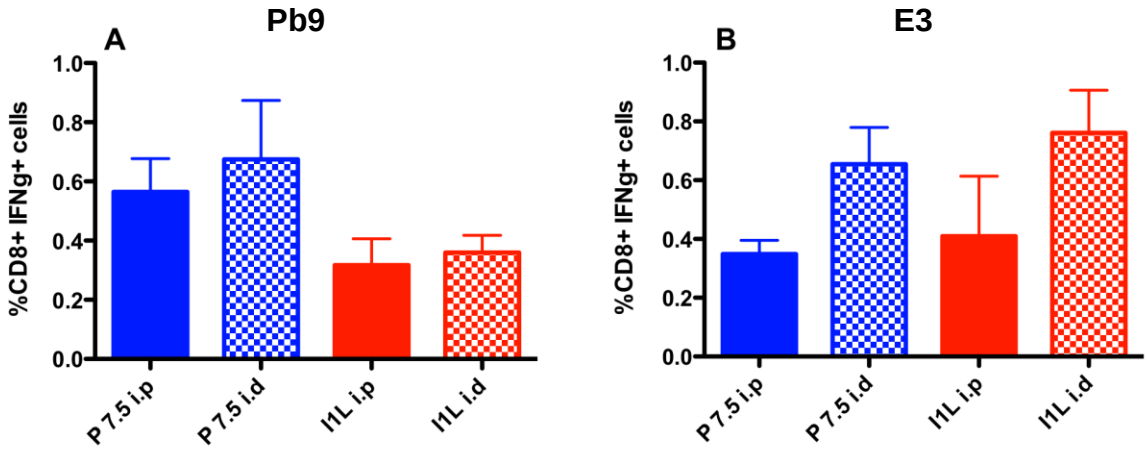


Figure 7-6: T cell responses to the *P. berghei* CSP (Pb9) epitope.

5-6 week old female Balb/c mice (n=5) were immunized with $1x10^7$ pfu of either MVA-P7.5-PyCSP or MVA-I1L-PyCSP via both the i.d. and the i.p. routes. Splenocytes were isolated from the spleen 7 days after immunisation and the CD8+ IFNγ+ response to the Pb9 epitope (A) and the E3 (B) epitope of MVA (described in section 7.2.7) was measured by ICS. There was no significant difference between the different groups by ANOVA.

7.2.7 Effect of US11 protein on MVA immunogenicity.

In order to test the effect of US11 on direct and cross-presentation by MVA, two recombinant MVA expressing the US11 protein were made under the P7.5 or I1L promoters. The US11 cDNA was obtained from Dr Jonathan Yewdell, NIH and PCR was used to put restriction sites on for cloning it into the MVA shuttle vector and the virus was made using methods described in chapter 2. These viruses were made by Dr Matt Cottingham. Because the

expression of US11 inhibits the presentation of peptides on the MHC class I molecule it was hypothesised that if direct priming was the major component of an immune response via any of these immunisation routes there would be a reduction in the response to the MVA virus itself in those vectors expressing US11. Two groups of 5-6 week old Balb/c mice (n=5) were immunised using $1x10^7$ pfu of the control virus MVA-GFP via the i.d. or the i.p. routes. At the same time two groups of Balb/c mice were also immunised with the virus expressing the US11 protein via the i.d. and the i.p. routes. The CD8+ IFNγ+ response to the E3 (VGPSNSPTF) and F2G (SPGAAGYDL) peptides of MVA were measured using ICS. Both E3 and F2G are early gene products and have been recognised as immunodominant CD8+ T cell epitopes in MVA (336). Given they are early gene products, they should be directly presented by infected DCs based on published data. However, there was no significant difference in the CD8+ IFNγ+ response between the virus expressing US11 and the controls either when given via the i.d. or i.p. routes for E3 (Figure 7-7A) or F2G (Figure 7-7B). This would suggest that the majority of the response to MVA when administered i.d. or i.p. is by cross presentation. The expression and bioactivity of US11 in MVA-infected cells needs to be confirmed.

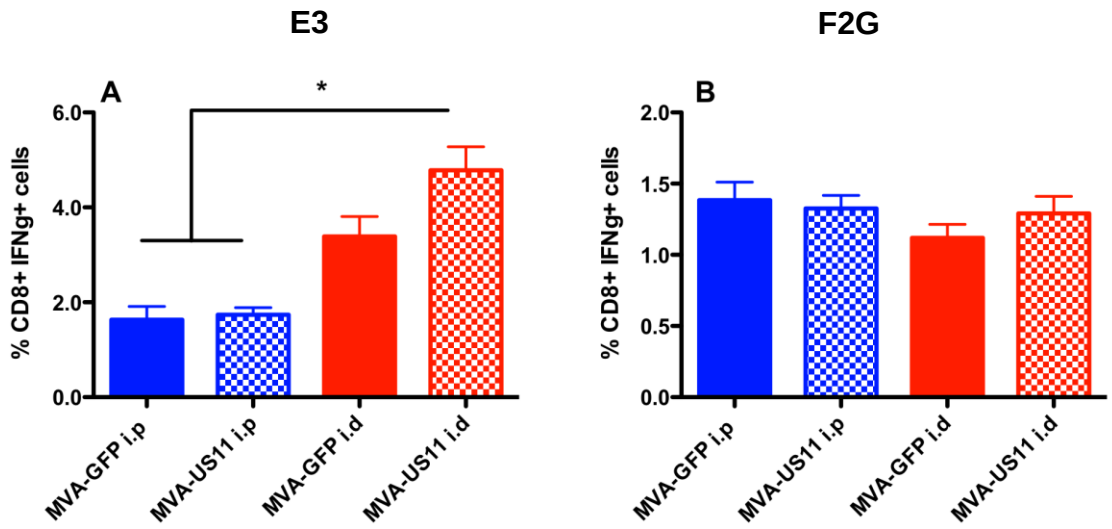


Figure 7-7: Effect of US11 on the CD8+ T cell response against MVA epitopes.

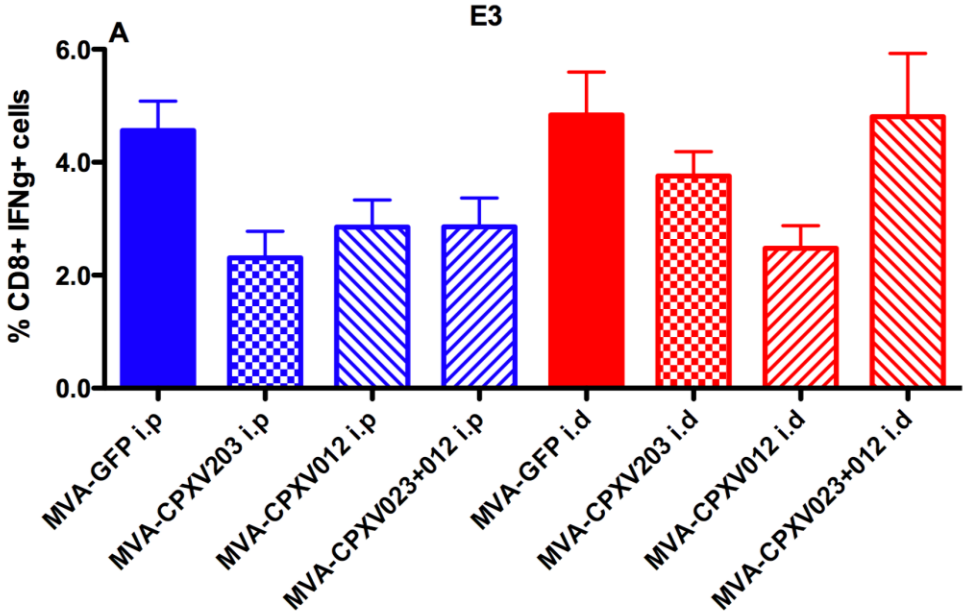
Two groups of Balb/c mice (n=5) were immunised using $1x10^7$ pfu of the control virus MVA-GFP via the i.d. or the i.p. route. At the same time two groups of Balb/c mice were also immunised with the virus expressing the US11 protein via the i.d. or the i.p. routes. The splenocytes were harvested 7 days after immunisation and the CD8+ IFNγ+ response was measured against the E3 (A) and F2G epitopes (B) of MVA by ICS. The splenocytes were stimulated with the relevant peptide or no peptide (Unstim) for 5 hours, surface stained for CD8 and CD4 and intracellularly stained for IFNγ. The CD8+ IFNγ+ response to E3 was significantly higher when administered i.d. than i.p. (A).

7.2.8 Effect of cowpox virus genes on MVA immunogenicity.

The cowpox virus (CPXV) encodes the most complete repertoire of immunomodulatory genes (in order to evade the host immune system) of all orthopoxviruses (337, 338). The CPXV203 protein has been shown to cause selective downregulation of both murine and human MHC class I molecules by using the physiologic KDEL pathway to retain MHC class I molecules in the endoplasmic reticulum (339). The CPXV012 protein has also been shown to inhibit transport of MHC class I molecules to the cell surface but the mechanism is unknown (Byun, M., Wang, X., Pak, M. Pickup, D. J., Hansen, T. H., Yokoyama, W. M., Cowpox virus encodes two genes that downregulate MHC class I, oral presentation at the 17th International Poxvirus and Iridovirus Conference, Grainau, Germany, June 7-12 2008). It has also been shown that mutant viruses with deletions of both CPXV203 and CPXV012 completely lack the ability to downregulate MHC class I molecules.

Recombinant MVAs expressing the CPXV203 and CPXV012 genes were made. The genes were PCR amplified from CPXV genomic DNA kindly provided by Karsten Tischer, Freie Universitat Berlin, cloned into MVA shuttle vector and recombinant MVA was made as described in chapter 2. These viruses were made by Dr Matt Cottingham. In addition to this, a recombinant MVA expressing both CPXV203 and CPXV012 was made. 5-6 week old Balb/c mice (n=5 per group) were immunised with 1x10^6 pfu of MVA-CPXV203, MVA-CPXV012 or MVA-CPXV203+012 i.d. and i.p (total 6 groups). At the same time, two groups of mice were also immunised with the

MVA-GFP control virus i.d. and i.p. The splenocytes were isolated seven days after the immunisation and the cells were stimulated with E3 and F2G peptides or no peptide. After 5 hour incubation, the cells were surface stained for CD4 and CD8 and then intracellularly stained for IFNγ. There was no significant difference in the CD8+ IFNγ+ response between the viruses expressing the cowpox genes and the controls either when given via the i.d. or i.p. routes for E3 (Figure 7-8A) or F2G (Figure 7-8B). This would also suggest that the majority of response to MVA epitopes when administered i.d. and i.p. is by cross-presentation as shown in section 7.2.8 using US11, but the expression and bioactivity of the proteins need to be confirmed as before.


E3
A
% CD8+ IFNg+ cells
6.0
4.0
2.0
0.0
MVA-GFP i.p
MVA-CPXV203 i.p
MVA-CPXV012 i.p
MVA-CPXV023+012 i.p
MVA-GFP i.d
MVA-CPXV203 i.d
MVA-CPXV012 i.d
MVA-CPXV023+012 i.d

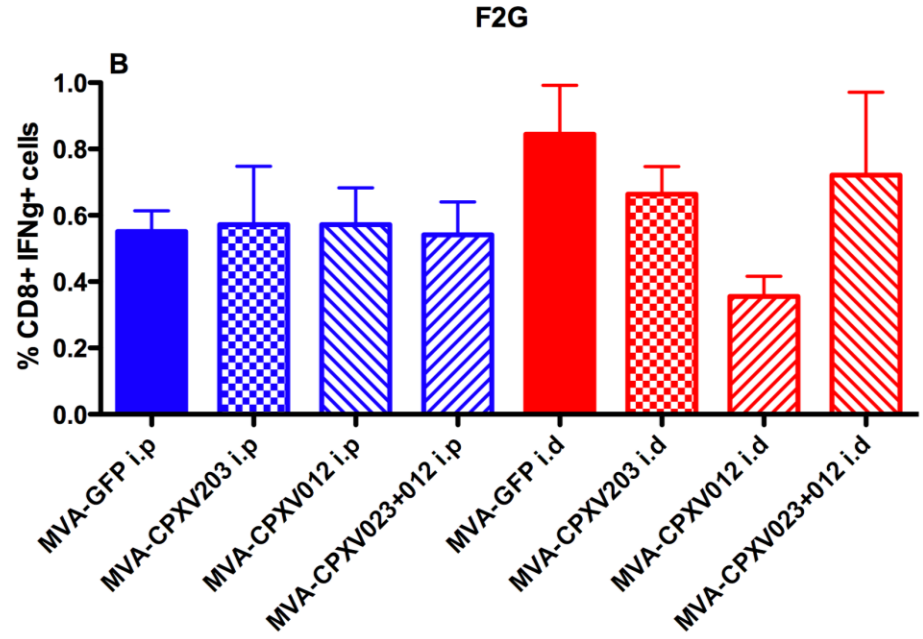

F2G
B
% CD8+ IFNg+ cells
1.0
0.8
0.6
0.4
0.2
0.0
MVA-GFP i.p
MVA-CPXV203 i.p
MVA-CPXV012 i.p
MVA-CPXV023+012 i.p
MVA-GFP i.d
MVA-CPXV203 i.d
MVA-CPXV012 i.d
MVA-CPXV023+012 i.d

Figure 7-8: Effect of cowpox virus genes on the CD8+ T cell response against MVA epitopes.

Eight groups of 5-6 week old Balb/c mice (n=5) were immunised using $1x10^6$ pfu of one of the four constructs (MVA-GFP, MVA-CPXV203, MVA-CPXV012 and MVA-CPXV203+012) via the i.d. or the i.p. routes. The splenocytes were harvested 7 days after immunisation and the CD8+ IFNγ+ response was measured against the E3 and F2G epitopes by ICS. The splenocytes were stimulated with the relevant peptide or no peptide for 5 hours, surface stained for CD8 and CD4 and intracellularly stained for IFNγ, TNFα and IL-2. The figure shows the % CD8+ IFNγ+ response in all the groups for E3 (A) and F2G (B). Data for TNFα and IL-2 showed a similar trend (not shown).

7.3 Discussion

This study was initially started in an attempt to improve MVA as an antibody inducing vector. MVA is able to boost antibody responses but appears unable to prime them. The I1L promoter was reported to have exceptional capacity to drive protein synthesis [11], and therefore it was hypothesised that if IgG boosting is dependent on antigen load, then driving antigen expression by I1L rather than P7.5 could lead to improved boosting and/or priming of B cell responses. Similarly, given antigens expressed under late promoters are reported to not be expressed in DCs, we hypothesised that I1L promoter driven antigen expression in comparison to P7.5 could lead to similar or reduced CD8 T cell responses (by cross presentation), and possibly a better CD4 T cell and antibody response (which is ideal for a blood stage vaccine). After a single immunisation with MVA-P7.5PyCSP or MVA-I1LPyCSP, an IgG

or IgM antibody response was not detected. When the two promoters were tested in the AdHu5-MVA prime boost vaccination regime we did not find this and instead, the P7.5 promoter, which we have used as the promoter in all other vaccines to date, induced better antibody responses (Figure 7-2). There was no significant difference in the CD8+ T cell responses after a single immunisation with MVA-P7.5PyCSP or MVA-I1LPyCSP when administered i.d.(Figure 7-3A and B) but there was a significantly lower CD8+ IFNγ+ response in the group boosted by MVA-I1LPyCSP than MVA-P7.5PyCSP after priming with AdHu5PyCSP (Figure 7-3C). The effect of different promoters during boosting (especially following the very strong CD8+ T cell response after the adenovirus prime) is unclear and needs further investigation. The pre-existing T cells might eliminate the virus quicker, promoting the importance of early gene expression over late.

It has been previously shown with vaccinia virus that antigens driven by late promoters stimulate a poor CD8+ T cell response, in comparison to the same antigen being expressed under an early promoter (340). Vaccinia virus can specifically prevent the presentation of its protein by uninfected cells by sequestration of some antigens in pox virus factories thus evading the cross presentation pathway (340). This may not be the case for foreign antigens often driven by early/late poxvirus promoters because these are not usually targeted to such poxvirus factories. It is important that the consequences of such observations to vaccine-induced T cell responses against the vector and the transgene are better understood.

In the route of administration study we showed that unlike VV, where cross priming occurs via the i.d. and s.c. routes, in the case of MVA the CD8+ T cell response seems to depend on cross-presentation for all the routes tested as there was no significant difference in the CD8+ T cell response to the antigen when driven either by early or late promoters (Figure 7-4). There was a trend towards a lower response for PyCSP when administered i.p. but when this was repeated later using the luciferase Pb9 constructs there was no significant difference in the immune response via the i.p. route between the antigens being driven by early or late promoters suggesting this could be specific to the individual construct or antigen being tested. Cellular localisation and antigen stability has also been previously shown to affect the efficiency of cross presentation (320). The *in vitro* luciferase assay confirms that (at least at 24 hours post infection) I1L drives the expression of more luciferase than P7.5.

In the experiments trying to inhibit MHC class I expression using US11 and the cowpox virus genes there was no difference in the immunogenicity using both the i.d. and the i.p. administration route. This is probably not surprising in light of the data from other studies, where it appears that in the case of MVA, cross presentation is the major pathway driving the CD8+ T cell responses generated by MVA (321). This work shows that induction of CD8+ T cell response is mostly if not exclusively by cross-presentation if the US11 and Cowpox virus genes are expressed correctly and this needs to be investigated. MVA had been reported to impair the capacity of DC to migrate and adequately respond to chemokines (341) and so it has been speculated

that the inability of MVA-infected DC to prime is due to altered plasticity and inability to form immunological synapses (321). In vaccines based on MVA, antigens expressed under the P7.5 early/late promoter is better at inducing both antibody and T cell responses when used in a prime boost vaccination regime than the I1L promoter.

Stable antigen is thought to be the substrate for efficient cross priming whereas direct presentation is enhanced if there is endogenous presentation of peptides or rapidly degradable protein (320, 342-344). So for each vector it is thus important to find out which pathway is important for CD8+ T cell induction and thus modify the antigen accordingly to target the principal pathway for CD8+ T cell induction.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8 Conclusions and Future Directions

8.1 Summary

This thesis describes the development, immunogenicity and efficacy of viral vectored blood stage malaria vaccines targeting AMA1. The blood stage malaria vaccine field has traditionally focussed on the development of protein-in-adjuvant formulations to induce antibody-mediated protection. In this thesis a prime-boost vaccination regime with viral vectors expressing AMA1 has been shown to induce protective immunity against *P. chabaudi* rodent malaria. Previously viral vectors have been shown to induce both antibodies and cellular immunity against MSP-1_{42}, which was protective against *P. yoelii* rodent malaria, but the protection induced against the blood stage was shown to be solely antibody mediated after pRBC challenge (273). In this thesis (using the alternative *P. chabaudi* model), the role of CD4+ T cells in blood stage vaccine-induced immunity has been investigated, and shows a clear contribution of these cells in mediating immunity – quite in contrast to the *P. yoelii* model system. A priming immunisation with recombinant AdHu5, followed by a boost 8 weeks later with recombinant MVA induced potent humoral and cellular immune responses against AMA1, and these responses were protective as measured by a significant reduction in parasitaemia against blood stage challenge two weeks after boost. The role of CD4+ T cells and antibodies in blood stage protection was shown to be important. In vivo depletion of CD4+T cells resulted in loss of vaccine-induced protection and

adoptively transferred CD4+ T cells and serum from vaccinated mice can control parasitaemia in naive mice.

In light of these encouraging data from the rodent malaria model, novel viral vectored vaccines against *P. falciparum* AMA1 (strain 3D7) were also developed. These vaccines were administered in a similar prime boost regime to mice and rabbits. The vaccination regime induced antibody responses in mice and rabbits and the antibodies were able to inhibit parasite growth *in vitro* against the homologous parasite strain (3D7) but not against a heterologous parasite strain (FVO). Polymorphism in AMA1 remains a significant hurdle in the development of vaccines targeting AMA1 (345). In an attempt to overcome this problem recombinant AdHu5 and MVA vectors expressing both 3D7 and FVO alleles of AMA1 (biallelic AMA1) were made and tested in mice and macaques. A simian adenoviral AdCh63 vector expressing the biallelic AMA1 construct was also developed and similarly tested. The AdCh63_MVA AMA1 vaccines induce both high levels of antibody and strong T cell responses in macaques (T cell response data not shown in this thesis, personal communication by Dr Simon Draper). The antibodies showed functional activity against both vaccine strains of *P. falciparum* (3D7 and FCR3) AMA1 unlike the monoallelic 3D7 vaccine but this needs to be confirmed by comparing the growth inhibitory activity of the monoallelic and biallelic vaccines in rabbits as it has been shown previously that in macaques immunisation with a single allele induces cross-reactive antibodies (296). Inclusion of multiple alleles in a vaccine can overcome the strain-specificity of the antibodies induced by monoallelic vaccines but there are practical

limitations to the number of alleles that could be included for efficacy in the field. The simian adenoviral vectors were developed in order to circumvent the effect of pre-existing antibodies to AdHu5 in the human population – these have been shown to interfere with immunogenicity of AdHu5 based vaccines in clinical trials (195). These promising results from this work has led to the manufacture of AdCh63 and MVA vaccines expressing the biallelic AMA1 construct to good manufacturing practice (GMP) and these will enter Phase I/IIa clinical trials in Oxford in January 2010. This thesis has formed the basis of the development of these new vaccines and these preclinical studies in mice, rabbits and macaques will provide an opportunity to understand the relevance of these models and *in vitro* assays in predicting vaccine efficacy in human trials. If these viral vectored vaccines are able to induce protection against blood stage malaria, such viral vector vaccine technologies could be applied to several other diseases where T cells and antibodies are important for protection.

Unlike adenovirus vectors, MVA appears unable to prime an antibody response against most antigens, but can boost responses primed by an adenovirus equally well as a heterologous adenoviral vector (Anna Goodman unpublished work) (177). Work in this thesis also attempted to improve MVA as an antibody-inducing vector by using a very strong late viral promoter (I1L) that was shown to express antigens at a much higher level than early promoters (322). Although I1L did not improve the antibody response, the P7.5 and I1L promoters were used to understand the mechanism of direct and

cross presentation by MVA. The results showed that the majority of the T cell immune response generated by MVA is achieved by cross presentation.

8.2 Conclusions.

8.2.1 Role of vaccine induced humoral and cellular immune responses in blood-stage immunity against *P. chabaudi*.

This thesis describes the development of viral vectors encoding AMA1 and the nature of immunity induced by these vectors in the *P. chabaudi* rodent model using a prime boost vaccination regime. The complete sequence of Pcc AS AMA1 was determined and recombinant AdHu5 and MVA expressing Pcc AS AMA1 were generated successfully. The Ad_M prime boost vaccination regime encoding *P. chabaudi* AMA1 induced detectable antibody and T cell responses in Balb/c mice both after prime (AdHu5-PccAMA1) and prime-boost (Ad_M PccAMA1) vaccination, and the Ad_M vaccination regime was protective against a blood stage challenge. This interval of 8 weeks between the prime and boost vaccination has been previously shown to be optimal for induction of antibody and T cell responses (177). When using high doses of viral vectors, antigen re-exposure within a short time period (2 weeks) can lead to reduced responses after the boost immunisation, probably due to antigen-overload and effector T cell over-stimulation. The prolonged

prime-boost interval is probably essential for the formation of optimal B cell and T helper cell memory populations (177).

The vaccination regime induced multifunctional CD4+ T cells against two CD4+ T cell epitopes previously published (168). Multifunctional CD4+ T cells producing IFNγ, TNFα and IL-2 simultaneously have been shown to correlate with protection in the *Leishmania major* mouse model (231) but their role in immunity to malaria needs further investigation. *In vivo* depletion of CD4+ T cells resulted in loss of vaccine-induced protection and a significant decrease in parasite control, thus showing that CD4+ T cells play an important role in this process. The level of AMA1 antibodies in the CD4+ T cell depleted and non depleted were the same thus showing that these CD4+ T cell act independently of antibodies. Adoptive transfer of these CD4+ T cells into naïve mice also resulted in control of parasite densities further illustrating the importance of CD4+ T cell in blood stage immunity in this model. Although, these mice can naturally control the infection due to antibodies and T cells of other specificities that are induced by infection, AMA1 specific CD4+ T cells were shown to play an important role in vaccine induced protection.

When the CD4+ T cells from the vaccinated mice were transferred to immuno-compromised mice they were unable to control acute parasitaemia. This could be because AMA1-specific CD4+ T cells are not sufficient on their own to control initial parasitaemia and other T and B cells are also required, indicating that responses to other parasite antigens are required for protection.

This study also shows the importance of antibody-mediated protection in this model. Serum transfer from vaccinated mice resulted in significantly lower parasite densities in naïve immuno-competent mice. Serum was not transferred into immuno-compromised mice but there was a better survival in nude mice (lacking T cells) than RAG knockout mice (lacking T and B cells) arguing that B cells are important for survival, a finding that is in agreement with the literature.

Unfortunately, in this mouse model the CD4+ T cell responses generated after Ad_M PccAMA1 vaccination were to strain-specific epitopes and so one would probably expect the protection to be strain-specific as well. The CD4+ T cell epitope P16 is conserved in two other *P. chabaudi* strains and thus it would be interesting to see if this could confer cross-strain protection even if the other P79 epitope is not conserved in these strains. The Th1/Th2 switch, which is reported to be characteristic of *P. chabaudi* infection (145) was not seen in the serum of mice tested here, but the levels of the cytokine responses detected were very low. *P. chabaudi* is considered to be a model where cell mediated immunity is important in blood-stage protection (129, 346, 347), and this study confirms that although CD4+ T cells are important, both antibodies and T cells are required for complete vaccine-induced blood stage protection.

8.2.2 Novel viral vectored vaccine targeting *P. falciparum* AMA1.

Novel *P. falciparum* AMA1 vaccines were constructed and the immunogenicity and *in vitro* efficacy of these vectors were assessed in this project. Initially recombinant AdHu5 and MVA vectors expressing *P. falciparum* 3D7 AMA1 were made. The AdHu5_MVA PfAMA1 (3D7) monoallelic vaccination induced antibodies in mice and rabbits. The antibodies were cross-reactive with the heterologous FVO recombinant protein as measured by ELISA and the IgG isotype profile in mice was a balanced Th1-type IgG2a and Th2-type IgG1 response. There was better cross-reactivity between the 3D7 and FVO ELISA in mice than in rabbits. The antibodies were also functional in *in vitro* GIA assays, but the GIA was strain specific especially in rabbits. This was not surprising because polymorphism in AMA1 has been a major hurdle in vaccine design. In mice there was 54% GIA against the heterologous strain compared to 98% GIA against the homologous strain. The rabbit serum did not show any GIA against the heterologous FCR3. This has been previously reported in other studies where antiserum from rabbits immunised with a single allele of recombinant protein AMA1 (3D7 or FVO) inhibited the growth of homologous parasite better than the heterologous parasites (222). Immunisation of rabbits with AMA1-C1, which is a mixture of recombinant 3D7 and FVO PfAMA1, elicited growth-inhibitory antiserum, which had comparable activities against both the 3D7 and FVO allele of AMA1 (222). It was shown that when rabbits were immunised with a combination of the two alleles the antibodies produced were directed against strain-conserved determinants in contrast to vaccination with a single allele in which approximately half of the

antibodies recognised cross-reactive epitopes and the other half of the antibodies were allele specific (296). This could be assessed using antigen reversal GIA by incubating the IgG induced with either 3D7 or FVO recombinant protein and then measuring the growth inhibitory activity.

In order to try to overcome this issue of polymorphism, a biallelic AMA1 construct was designed and cloned into AdHu5, AdCh63 and MVA. The AdHu5_MVA and AdCh63_MVA biallelic AMA1 vaccines induced similar levels of antibodies in mice. It is encouraging that the simian adenovirus AdCh63 is as potent as AdHu5 in generating an antibody response, given anti-AdHu5 neutralising antibodies have been shown to hamper the immunogenicity of a recombinant AdHu5 HIV-1 vaccine in clinical trials (195). The AdCh63_MVA biallelic AMA1 vaccination regime induced strong antibody responses in macaques and the IgG inhibited *in vitro* growth of both 3D7 and FCR3 parasites. A monoallelic AdCh63_MVA PfAMA1 3D7 group was not included in the study and so the functional activity of the serum induced by vaccination with one allele or both alleles could not be compared. This would be interesting to investigate as previously it has been shown that, unlike mice and rabbits, when macaques were immunised with a single allele of recombinant AMA1 protein, the induced antibodies showed cross reactive biological activities in GIA assays which was confirmed by antigen reversal (296). This could be because macaque B cells preferentially recognise conserved antigenic determinants. This would suggest that if the study described here was repeated with the monoallelic PfAMA1 3D7 vaccines, that the induced antibodies might inhibit growth of heterologous parasites.

Polyfunctional CD4+ and CD8+ T cells responses to both common and unique epitopes in 3D7 and FVO AMA1 were measured in mice. This is encouraging because if the common epitopes are important in protection (as shown in the *P. chabaudi* rodent model) then they could confer cross strain protection against heterologous *P. falciparum* strains in humans. Multifunctional T cell responses to blood-stage antigens are likely to be of importance in protecting against *P. falciparum* malaria as polyfunctional effector memory T cells against infected erythrocytes have been associated with protection in humans after vaccination with sporozoites and treatment with choloroquine (131). There was no significant difference in the T cell responses generated by AdCh63 and AdHu5 after the prime boost vaccination regime. An AdCh63_MVA regime encoding a liver stage construct ME-TRAP has been shown to generate CD8+ T cell immunity to transgene peptides in the *P. berghei* model (Arturo Reyes-Sandoval personal communication). The first Phase I/IIa trial of the chimpanzee adenovirus AdCh63_MVA ME-TRAP regime showed promising results as a pre-erythrocytic malaria vaccine and induced sterile or partial protection against malaria in 4/8 volunteers (O'Hara *et al.*, unpublished). These data support the clinical development of viral vectors expressing the biallelic AMA1 construct and these may become an important component of an efficacious vaccine against *P. falciparum* disease or infection.

8.2.3 MVA as a vaccine vector.

The work on the MVA promoter showed that the P7.5 promoter was better than I1L in boosting antibody responses. There was no antibody detected and no difference in the CD8+ IFNγ+ response after a single immunisation with recombinant MVAs expressing PyCSP under the two promoters. After an adenovirus prime, the MVA-P7.5 boosted the response to the dominant CD8+ T cell epitope better than I1L. These results suggest that the P7.5 promoter is better than I1L for the induction of antibodies and T cells in an adenovirus prime – MVA boost regime.

This work also shows that cross presentation is the principal pathway for induction of CD8+ T cell response after immunisation with recombinant MVA. This was shown to be the case for both viral epitopes (E3 and F2G) using the US11 and cowpox proteins, and also to the transgene by using the MVA expressing PyCSP under P7.5 and I1L. Stable antigen is thought to be the substrate for efficient cross priming whereas direct presentation is enhanced if there is endogenous presentation of peptides or rapidly degradable protein (320, 342-344). Thus, understanding the principal pathways for CD8+ T cell induction can help in the design of vaccines as modifying the antigen to target the principal pathway for CD8+ T cell induction can generate better immune responses when these vectors are used in prime boost regimes.

8.3 Future directions.

The work described in this thesis demonstrates that both antibodies and T cells can be generated using prime-boost vaccination regimes encoding AMA1. Studies in mice using the *P. chabaudi* model show the importance of both CD4+ T cells and antibodies in protection. Adoptive transfer studies in immuno-compromised mice show that antigen-specific CD4+ T cells on their own are not sufficient to control parasitaemia, so it will be of interest to investigate further if the transfer of both antibodies and CD4+ T cells could transfer protection in these mice. It has been previously shown that CD4+ T cells and antibodies can act synergistically. When AMA1 specific CD4+ T cells plus rabbit antiserum are transferred into athymic mice, there is better survival than mice receiving either CD4+ T cells or antiserum alone (169). It will be interesting to further analyse the CD4+ T cells, which can adoptively transfer protection to naive immuno-competent mice in this model. The analysis of the proportion of Th1 or Th2 cells could help in better understanding the role of these cells in blood stage protection. The vaccination regime induced multifunctional CD4+ T cells but the role of these cells in protection was not determined. If it was possible to separate the 3+, 2+ or 1+ cells, equal numbers of these could be transferred separately into naive mice to see if “triple positive” cells confer better protection. The separation of these subsets of cells is a challenge and is not currently possible and requires further investigation. It would also be interesting to see if a single immunisation with AdHu5-PccAMA1 showed any efficacy against

blood stage challenge given there was no difference in the quantity of CD4+ T cells after prime versus prime boost. The response after the boost was measured two weeks after boost but the peak of the response after immunisation with MVA is one week so it could be possible that there is a difference one week post boost. If there was no protection seen in this case, it could be due to lower antibody levels without the MVA boost – again indicating the need for T cells and antibodies to protect in this model. The P16 epitope (but not P79) is conserved in *P. chabaudi adami* DK and *P. chabaudi adami* CB so it will be interesting to see if the responses to this epitope alone could confer cross-strain protection by challenging with these heterologous parasites. It has been shown previously that protective immune response to AMA1 in *P. chabaudi* after immunisation with recombinant protein involves recognition of strain-specific epitopes in mice (167). If the P16 CD4+ T cell epitope (and not P79) is important in protection, T cells against this epitope could possibly confer partial protection against the heterologous strain.

The longevity of the protective effect of vaccination can be furthered investigated by challenging at a later time point, rather than two weeks post boost as used in these studies. It has been shown that CD4+ T cells play a major role in long term protection and a gradual decline in malaria-specific memory T cell responses leads to loss of long term protective immunity to PccAS despite persistence of B cell memory and circulating antibody (283). The memory CD4+ T cell populations can be analysed by flow cytometry and the role of these cells in maintaining long term protective immunity could be assessed. Publication of the malaria parasite genomes, including that of *P.*

falciparum, is leading to better definition of the stage-specificity of malaria antigen expression (348). AMA1 is also expressed by sporozoites and these can be neutralised by anti-AMA-1 antibodies (272). The expression of AMA1 in PccAS sporozoites could be investigated by IFAT's, and a sporozoite challenge after the AdHu5_MVA PccAMA1 vaccination regime could show better protection than blood stage challenge if the anti-AMA1 antibodies are effective at the pre-erythrocytic stage of infection.

In order to address the issue of polymorphism in AMA1, three diversity-covering (DiCo) PfAMA1 sequences have been designed which on average incorporate 97% of amino acid variability observed in 355 PfAMA1 sequences (208). When rabbits were immunised with the three recombinant Dico proteins, the levels of growth inhibition (approximately 70%) induced by the mix of three DiCo proteins were comparable for FVO, 3D7, and HB3 strains, suggesting that a considerable degree of diversity in AMA1 is adequately covered. These Dico sequences could be cloned into viral vectors in order to further address the issue of polymorphism of AMA1 but these sequences could lead to the loss of T cell epitopes.

The clinical trial of the simian adenoviral vector AdCh63 and MVA expressing the biallelic PfAMA1 is due to commence in January 2010. The biological activity of the anti-AMA1 antibodies induced by vaccination in humans and by natural infection in controls or serum from people living in endemic areas can then be compared using the GIA assay to assess if pre-exposure to malaria can affect the efficacy of vaccine induced antibodies. Malaria-specific non-

AMA1 serum IgG from Malians (living in the malaria-endemic country) has been shown to reduce the biological activity in GIA assays of anti-AMA1 antibodies induced by vaccination with recombinant AMA1-C1 formulated in Alhydrogel with or without CPG 7909 (349). This can be further combined with other blood stage antigens, similar to that attempted with recombinant *P. falciparum* MSP-1 and AMA-1 protein vaccines (92, 350) to target multiple antigens on the merozoite surface. The clinical trials of AdCh63 and MVA expressing PfM128 (a biallelic MSP1 construct) are due to commence in October 2009. Consequently the protective efficacy of the combination of these two blood stage viral vectored vaccines can be tested. Viral vectors expressing AMA1 could also be combined with pre-existing pre-erythrocytic stage viral vectored vaccines (291) or leading recombinant protein-in-adjuvant vaccines to try to increase the antibody levels generated in humans (350). It may also be possible to generate multi-cistronic vectors, similar to NYVAC-Pf7 (351), which could express antigens, from several stages of infection: pre-erythrocytic, blood and transmission stages. Thus, this project encourages the development of viral vectored blood stage vaccines which, if efficacious, could be combined with proven pre-erythrocytic and transmission blocking vaccines to control malaria. These vaccines, combined with other public health interventions, will hopefully one day lead to the eradication of malaria.

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APPENDIX:

JENNER PROTOCOLS

J005, J006, J079, J106 and

J111

1.0 Jenner Laboratory Protocol Number: J005

2.0 Version Number 05

3.0 Title: Adenovirus purification using discontinuous and isopycnic CsCl gradients

4.0 Other documents

MSDS reagents:	refer to MSDS for the relevant safety information on the individual \\lmsnw3_jenner_server\jenner\hill_group\Safety\COSHH assessments\Manufacturers material safety data sheets
J008	Infecting 293 cells with adenovirus
J011	Passaging 293 cells
J104	Adenovirus Titre Immunoassay
J005 App I	Annotated images of CsCl centrifugation steps for troubleshooting.

5.0 Definitions

Ad	Adenovirus
CsCl	Caesium chloride
CPE	Cytopathic effect
PBS	Phosphate buffered saline
HF	Hyperflasks

6.0 Objective

The purification of recombinant adenovirus by two different methods of density centrifugation using Caesium chloride solutions as solvents. The first step involves a form of rate zonal centrifugation whereby the component particles are separated in a manner based upon mass (larger particles will sediment faster). The second centrifugation step is isopycnic centrifugation whereby the particles are separated according to their density.

Note concerning incubation time for benzonase treatment of harvested, infected 293 cells:

The optimum incubation temperature for benzonase is 37°C. However, as a compromise between virus stability and enzyme activity, the incubation is performed at RT.

General virus handling:

- Ensure only one virus is handled at any one time in the hood.
- Wipe hood thoroughly with Microsol solution between viruses and change gloves.
- Soak forceps in 10% Microsol solution for 10 mins after use with each virus as these come into very close contact with the virus preparations. Use a fresh pair of forceps if more than one virus is being purified simultaneously. Ensure that the virus is not exposed to Microsol.

This protocol should not be performed without prior training as some of the procedures are complex and demonstration of the techniques is required.

7.0 Reagents

30 x T150 flasks or 3 Hyperflasks containing adenovirus-infected 293 cells.
Cells should be showing a cytopathic effect CPE (round grapelike clusters), but the majority should still be attached to the flask.

18.2 MΩ water

1M Trizma-HCl pH7.8 T-2569-100ml	Sigma
Caesium Chloride 203025	Sigma
PBS containing Ca & Mg H15-001	PAA
250U/μl Benzonase E8263-25Ku	Sigma
1M $MgCl_2$ 63069-100ML	Sigma
Sucrose* 84097	Fluka

*it is important that this material is low in endotoxins, do not substitute this supplier

Solution preparation:

Add solids to a Duran bottle and add some 18.2 MΩ-water, then add liquids. Add a clean magnetic stir bar and place on a stir plate until solids have dissolved. Transfer to a volumetric flask and top up with water.
Filter sterilise the solution and transfer to sterile Duran bottles

For each new solution record lot number of Trizma base and Sucrose used.

1.25 g/ml density CsCl (density = 1.25 g/mL = 1.98 M) in 10 mM Tris pH 7.8.
166.89 g CsCl
5 ml 1 M Tris pH 7.8
Add 18.2 MΩ water to 500 ml using volumetric flask
Filter sterilise.

1.35 g/ml density CsCl (density = 1.35 g/mL = 2.78 M) in 10 mM Tris pH 7.8.

233.65 g CsCl
5 ml 1 M Tris pH 7.8
18.2 MΩ water to 500 ml using volumetric flask.
Filter sterilise.

Lysis buffer (10 mM Tris, 1 mM $MgCl_2$. pH 7.8)

5 ml 1 M Tris-HCl pH 7.8
0.5 ml 1 M $MgCl_2$
Make up to 500 ml using a measuring cylinder
Filter sterilise and transfer to ~100 ml aliquots to sterile Duran bottles.

Storage buffer (10 mM Tris, 7.5% w/v sucrose. pH 7.8)

Weigh 75 g sucrose and transfer to a 1 litre measuring cylinder.
Add 18.2 MΩ water up to ~800 ml.
Add 10 ml 1 M Tris-HCl pH 7.8
Seal top of measuring cylinder with parafilm and invert cylinder repeatedly until sucrose has full dissolved.
Adjust volume to 1000 ml.
Filter sterilise and store at least one bottle in the virus lab at 4°C. Place the remaining bottles in the molecular biology lab fridge.

8.0 Equipment

Class II BioSafety Cabinet Mars	Scanlaf
Benchtop Centrifuge Allegra X-12R	Beckman Coulter
CO_2 incubator Galaxy R	RS Biotech
37°C water bath SUB6	Grant
Microscope DMIL	Leica
IMS M/4400/17	Fisher
Corning conical tubes, 250 ml PP Centrifuge tube, plug seal cap CFT-900-021Y	Fisher
Bottle-top filters TKV-238-050Y	Fisher
Volumetric flasks	
500 ml and 1 l sterile Duran bottles	
5 ml syringes SZR-150-170T19G	Fisher
Needles M1500	nu-care products
Ultraclear centrifuge tubes 14.0 ml	Beckman Coulter

Dialysis cassettes 3-12 ml & 0.5-3ml Thermo-Fisher
66453 & 66455
Slide-A-Lyzer Buoys for 3-12 ml and 0.5-3 ml dialysis cassettes
66432 & 66430
250 ml polypropylene bottles
Dry ice in suitable container
Beckman ultracentrifuge Beckman Coulter
SW40 rotor & buckets Beckman Coulter
Balance
Forceps
Retort stand with clamp
Folder with black surface for viewing virus bands

9.0 Method

Harvest infected 293 cells - <u>The time of harvest is critical for optimum virus yield.</u> Monitor the cells every day for CPE and harvest the cells when the majority of the cells are rounded up but still adhered to the surface of the flask.

9.1 Detach adenovirus-infected 293 cells by gently banging the flasks with the palm of your hand until the cells detach from the flasks.

9.2 Pour the media into 250 ml polypropylene tubes.

If using hyperflasks (HF), after detaching adenovirus-infected 293 cells by gently banging the HF with the palm of your hand until the cells detach from the flasks and pouring the media into 250 ml bottles, wash out the HF with ~250 ml PBS. Tip the HF so that the PBS is evenly distributed across the layers and agitate to remove any remaining adherent cells. Pool the PBS wash with the harvested media.

9.3 Centrifuge for 10 min. at 2000 rpm (~900 x g) and discard supernatant into a dispo-jar containing Microsol or aspirate.

9.4 Resuspend the cell pellets in a total volume of 18 ml lysis buffer (for 30 x T150 or 3 Hyperflasks) and either freeze on dry ice or place at -20°C.

The protocol may be stopped at this point by storing the resuspended cell pellet at -20°C.

9.5 Thaw the cell pellet and add 250 U of Benzonase per ml of cell lysate. We are now using Sigma concentrated Benzonase @ 250 U/μl, so add 18 μl to 18 ml cell lysate and incubate for 1 hour at RT.

9.6 Freeze/ thaw (dry ice/37°C water bath) the cell pellet a further two times. Whilst thawing leave at 37°C for the shortest time possible.

9.7 Centrifuge at 2000 rpm (~900 x g) for 10 minutes at 4°C and transfer the supernatant to a fresh tube.

9.8 Make the total volume of the supernatant up to ~18 ml using lysis buffer.

At this stage, check that the centrifuge buckets are clean, place buckets in the biological safety cabinet and spray with 70% IMS to sterilise. Wipe off excess IMS with blue roll and allow the remaining liquid to evaporate whilst the samples are prepared.

9.9 Set up 3 Caesium Chloride gradients in 14 ml Beckman Ultraclear tubes by underlaying 3 ml 1.25 g/ml CsCl with 3 ml of 1.35 g/ml CsCl. Ensure that the interface between the two solutions is visible.

9.10 Add ~6 ml of virus supernatant to each of the gradients (do not disturb the interface whilst adding the sample. This can be avoided by adding the sample slowly down the side of the tube). Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure.

9.11 Place centrifuge tubes in the buckets for the SW40 rotor and spin for 2 hours at 25,000 rpm @ 4˚C.

NOTE: use all 6 buckets correctly sealed and mounted in the rotor in their numbered positions as this is a balanced set.

9.12 Once the centrifuge run is complete, remove rotor from the ultracentrifuge, transfer the buckets to the holder and move to the biological safety hood.

9.13 Unscrew the bucket lids and remove the tubes using forceps.

9.14 Place the tube in a clamp stand above a beaker of Microsol.

9.15 Two virus bands should be visible close to the centre of the tube; the upper band is incomplete virus and the lower band intact virus. The bands may be easier to visualize by placing a dark surface behind the tube.

9.16 Using a 19G needle and 5 ml syringe pierce the tube approximately 5 mm below the band by gently twisting and pushing the needle through the wall of the tube. *Be careful not to hold your other hand on the other side of the tube whilst doing this for risk of injury.*

9.17 Once the needle is inside the tube gently pull on the syringe with the bevel pointing upwards to extract the lower band and store in a TREFF tube on ice.

9.18 Discard the used centrifuge tube into the beaker of Microsol. Wipe up any spills carefully with Microsol wipes or Microsol dampened tissue. Decontaminate the buckets using Microsol, rinse with water and ensure they are clean and dry.

9.19 Decide how many tubes are required for the second centrifugation step:

This is based on experience. The major concern is that placing too much virus in each tube will lead to aggregation of the virus particles.

Dilute virus in lysis buffer, you need approx 7mls of virus per gradient, this allows space for the tubes to be balanced with lysis buffer

9.20 Set up the second caesium gradients in 14ml Beckman ultraclear tubes:

Add 6 ml of 1.35 g/ml CsCl to each tube
Add ~7 ml of virus supernatant to each of the gradients (do not disturb the interface whilst adding the sample. This can be avoided by adding the sample slowly down the side of the tube). Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure.

9.21 Balance the tubes if required with lysis buffer and place in the buckets for the SW40 rotor and centrifuge at 30 000 rpm for 16-18 hr (i.e. overnight).

9.22 Once the centrifuge run has finished, remove rotor from the centrifuge, transfer the buckets to the holder and move to the biological safety hood.

9.23 Remove the centrifuge tube using forceps and place in the retort clamp. Two bands may be visible. However, the upper band may not be present if the virus added onto the 2nd gradient was very pure. Remove the lower band using a 19G needle and 5 ml syringe – note the volume that the bands are removed in and store in a TREFF tube on ice.

At this stage, mark a dialysis cassette with the name of the virus and pre-wet with pre-chilled storage buffer.

9.24 Discard the used centrifuge tubes into the beaker of Microsol. Wipe up any spills carefully with Microsol wipes or Microsol dampened tissue. Decontaminate the buckets using Microsol, rinse with water and ensure they are clean and dry.

9.25 Add the purified virus band to a dialysis cassette using a needle and syringe:

9.25.1 Transfer the wetted dialysis cassette to the hood.

9.25.2 Insert cassette into the buoy to function as a holder.

9.25.3 Place a needle on a 5 ml syringe and insert the needle so that just the bevel of the needle in inside the cassette. Insert a small amount of air to the cassette so that it is slightly inflated which makes it easier to add the virus without piercing the membrane.

9.25.4 Add virus solution

9.25.5 Tilt cassette and remove air such that the cassette is full with no air bubble.

9.26 Place the cassette back into the beaker of 500 ml of cold storage buffer, cover with foil and place on a magnetic stir plate. Dialyse for 90 minutes changing the buffer every 30 mins.

9.27 Remove virus from dialysis cassette, tilting the cassette ensure all of the solution is removed, and aliquot.

Ensure there are at least 3 x 10 μl aliquots for immunostaining and determination of virus particle number by spec reading.

Check File Maker Pro for the next available number. Label virus clearly with stock number. Store at -80°C.

Create a Production Record
Enter in the File Maker Pro Virus database the details and location of the new virus.

10.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
2	**Amount of Benzonase added**	**Too little being used**	**Nicky Green**
2	**2nd gradient preparation, dilution of band from first gradient and only using heavy CsCl**	**To prevent loss of bands on 2nd gradient**	**Nicky Green**
3	**Amount of Benzonase added**	**Now using concentrated stock from Sigma**	**Nicky Green**
3	**Dialysis step added between bandings** **2 x 2 step gradients**	**To have consistency with Julies method**	**Nicky Green**
4	**Buffer recipes to include ready-made stocks of Tris buffer and $MgCl_2$.** **Removal of dialysis step between bandings.** **Second, optimised, isopycnic centrifugation procedure added instead of step gradient.**	**Protocol optimised**	**Ali turner**
5	**Modified for new ultracentrifuge**	**CsCl densities and spin speeds**	**Nicky Green**

1.0 Jenner Laboratory Protocol Number J006

2.0 Version Number 02

3.0 Plaque forming unit (pfu) assay

4.0 Other documents

J011 Passaging 293 cells

5.0 Definitions

Definitions of abbreviations

6.0 Objective

To calculate the infectious viral titre by visualizing plaque forming units

7.0 Reagents

Dulbecco's Modified Eagles Media (DMEM) - high glucose with 4500 mg/L glucose, sodium pyruvate, and sodium bicarbonate, without L-glutamine (Sigma, product # D6546-500ML)

L-Glutamine (Sigma, product # 8540)

Penicillin/Streptomycin solution (Sigma # 4333)

Foetal Bovine Serum (FBS) (Sigma, product # F6178-500 ml)

2% low melting point agarose (Sigma, product # A9414)

6 well plates containing confluent monolayer of 293 cells (set up the day before infection, see protocol J011 for passaging 293 cells)

8.0 Equipment

List equipment needed and if appropriate specify where it is in the Jenner labs

9.0 Method

ALL work should be carried out in the Biosafety Cabinet. The cabinet should swabbed with Microsol before use. Gloves and a lab coat should be worn at all times.

1. Warm media (DMEM with no additions) to 37°C in a water bath for 10 minutes.

2. Prepare work space in hood by swabbing with microsol.

Before placing bottles in the hood they should also be wiped with microsol taking particular care around the bottle necks.

3. Once warmed aliquot media into a series of 15 ml tubes or 1.5 ml Treff tubes according to the table shown below.

Tube Number	Media (ml)	Virus (μl)	Dilution
1	1	1	-3
2	1	1	-6
3	0.9	100	-7
4	0.9	100	-8
5	0.9	100	-9
6	0.9	100	-10
7	0.9	100	-11

4. Add 1 μl of pure virus to tube 1 containing 1 ml of media. Mix well by pipeting or inverting the tube.

5. Add 1 μl of diluted virus from tube 1 to tube 2 and mix well by pipeting or inverting the tube. . Continue to serially dilute the virus as detailed in the table above.

For example the subsequent dilution (-7) will be created by adding 100 μl of diluted virus from tube 2 to 900 μl of media in tube 3.

6. Aspirate media from 6 well plates prepared the previous day.

Take care not to touch the cell monolayer with the pipette. This can be avoided by tilting the plate so that the media flows to the corner of the well.

7. Add 200μl of each dilution per well, gently tilt plate so that that the media covers the monolayer, Incubate at 37° C for 2 hours rocking the plates every 15 mins to ensure that the cells do not dry out.

Usually a dilution range of -6 to -11 (in duplicate) is sufficient to determine viral titre.

8. Warm 2X complete media in a 37 °C water bath for 30 minutes.

9. Heat 2% agarose in microwave for 3 minutes or until it boils, allow to cool at 37°C water bath for approximately 20 minutes

Ensure that the agarose is not above 40 °C when added as this may cause damage to the cells.

10. Prepare work space in hood by swabbing with microsol.

Before placing bottles in the hood they should also be wiped with microsol taking particular care around the bottle necks.

9. Mix media and agarose together in a 1:1 ratio in a pre-warmed tube, allow 3mls per well

10. Aspirate media from 6 well plates and gently add 3 ml of complete media/agarose mix to each well using a pre-warmed serological pipette. Allow to solidify in laminar flow hood for approx 10 minutes before placing in 37°C incubator.

11. After 3 days overlay with 2 ml of 2 xDMEM/2% agarose

An additional overlay is usually required 9 days post infection if the agarose appears yellow.

12. 10 days post infection stain living cells using MTT (5mg per ml PBS, add 100μl per ml of overlay) and count the number of plaques.

Alternatively if the adenoviral construct contains GFP, plaques can be counted using the inverted microscope.

10.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
02	**Volume of media cells infected in**	**Should be as small volume as possible**	**Nicky Green**
02	**Timing of agarose overlay, after 2 hours NOT overnight**	**Standard protocol**	**Nicky Green**
02	**Use of pre-warmed tubes and pipettes**	**To prevent agarose from setting**	**Nicky Green**

1.0 Jenner Laboratory Protocol Number: J079

2.0 Version Number: 01

3.0 Title: ID and Purity PCR for recombinant MVA

4.0 Other Documents

J065 Agarose Gel Electrophoresis and Gel Imaging
J014 Infection and transfection of CEF with MVA and shuttle vector
J002 DNA extraction from Adenovirus and MVA

5.0 Definitions

PCR	polymerase chain reaction
BSA	Bovine serum albumin
dH_2O	Distilled water
RE	Restriction enzyme
RNAse	Ribonuclease
DNAse	Deoxyribonuclease
MVA	Modified vaccinia Ankara

6.0 Objective

This procedure confirms, using specific primers, the insertion of various antigens into the thymidine kinase locus of MVA, and hence the identity of the MVA virus sample. Using parental virus-specific primers the presence of contaminating parental virus can be determined in the test samples.

7.0 Reagents

DNA extracted from MVA as per J002
Positive control plasmid DNA
AmpliTaq Gold with Geneamp (10x PCR buffer II & $MgCl_2$) Applied Biosystems 4311818
5U/µl AmpliTaq DNA polymerase
10x PCR buffer (100 mM Tris-HCl, pH8.3, 500 mM KCl)
25 mM $MgCl_2$

8 mM equal mix dNTPs
Sterile distilled water (RNAse & DNAse Free) Sigma W4502
10 μM DNA primers (see primers database) MWG

e.g. for MVA.Red primers: Redvirus 3 and TKL
for specific insert: specific primer + TKL

11.0 Equipment

Microfuge
PCR machine MJ Research DNA engine Tetrad
96-well PCR plate and adhesive lid Corning
Pipette tips with hydrophobic filters
Gilson pipettes

12.0 Method

12.1 Prepare two master mixes on a thoroughly clean bench in 1.5 ml microcentrifuge tubes, labeled 'Purity (containing MVA.Red3 and TKL primers)' and 'ID (containing specific primer + TKL)', as appropriate. Prepare sufficient for each virus test sample to be analysed plus four additional samples (MVA.wt, MVA.Red, reference material (plasmid), sterile water and one extra for pipetting error). The amount of each master mix required for a single sample is as follows:

0.5 μg of template DNA is recommended by Applied Biosystems for the Amplitaq enzyme.

Refer to the Amplitaq instruction manual for any further information required.

Prepare the following master mix for the required number of reactions + allow 1 rxn volume for pipetting error:

1 x PCR mix

4 μl	sterile water	
1 μl	10x AmpliTaq buffer	final conc 1x
0.6 μl	25 mM $MgCl_2$	final conc. 1.5 mM
1 μl	10 μM forward primer	final conc 1 μM
1 μl	10 μM reverse primer	final conc 1 μM

1 µl	8 mM equal dNTP mix	final conc 200 µM each
0.4 µl	5U/µl AmpliTaq polymerase	final conc 2 U
1 µl	DNA sample	

	Final Conc.	Vol. per 1 reaction (µl)	Vol. per 2 reactions (µl)	Vol. per 3 reactions (µl)	Vol. per 4 reactions (µl)	Vol. per 5 reactions (µl)	Vol. per 6 reactions (µl)
Sterile H_2O	-	4	8	12	16	20	24
Amplitaq Buffer (10x)	(1x)	1	2	3	4	5	6
25 mM $MgCl_2$	150 µM	0.6	1.2	1.8	2.4	3.0	3.6
Forward primer (10 µM)	1 µM	1	2	3	4	5	6
Reverse primer (10 µM)	1 µM	1	2	3	4	5	6
Equal mix dNTP's (8mM)	*200 µM*	*1*	*2*	*3*	*4*	*5*	*6*
AmpliTaq Polymerase (5U/µl)	0.2 Unit/µl	0.4	0.8	1.2	1.6	2.0	2.4
Total		9	18	27	36	45	54

Aliquot 9 µl of the master mix into wells of the 96-well plate. Add 1 µl of the sample to be tested, plasmid positive control or sterile water to 9 µl of Master Mix.

PCR Conditions

Primary Denature	95°C	1min
Denature	94°C	10sec
Annealing	52°C	30sec
Extension	72°C	1 min
Final Extension	72°C	3 min
Finish & Hold	4°C	forever

Fix the adhesive lid into place on the 96 well plate and place the plate in the PCR machine.
Start the relevant PCR run, using the heated lid option.
Completed reactions are analyzed by standard agarose gel electrophoresis.
Samples may be stored at -20°C until analysis by electrophoresis.

1.0 Jenner Laboratory Protocol Number: J106

2.0 Version Number: 01

3.0 Title: Quantification of virus particles using Beckman Coulter DU 730 spectrophotometer

4.0 Other documents

None

5.0 Definitions

dH_2O distilled water
SDS
Storage buffer as described in J005

6.0 Objective

This protocol describes the method for measuring the concentration of viral particles per ml, using UV spectrophotometry at wavelengths of 260 and 320nm.

The concentration of viral particles may be measured directly by UV-spectroscopy, since proteins have a UV absorbance at 277 nm due to their tryptophan and tyrosine content, and DNA has an absorbance maximum at 260 nm. Intact viral particles have a diameter between 65 to 85 nm. Their high organization and potential aggregation state cause light scattering which can bias the UV-measurement. In this assay, the SDS disassembles the virus into its component proteins and DNA. The UV-absorbance of the lysed virus in SDS at 320 nm provides a measurement of viral aggregation, whilst the readings at 260 nm and 280nm account for viral DNA content and protein content respectively. The viral particle concentration is calculated using a method described by Maizel *et al*. In this method, an absorbance of 1.00 (AU, 1 cm pathlength) at 260 nm corresponds to 1.1 x 1012 viral particles/mL.

The Beckman Coulter DU 730 has an adapter for the 0.1ml cuvette which is screwed in to the base. The cuvette is placed in the adapter and is ‘blanked’ using Jenner storage buffer with 1% SDS. The cuvette should not be moved for duration of the session. Virus is diluted in Jenner storage buffer with 1% SDS. The spectrophotometer programme to ‘Adeno’ can be used to calculate OD260-OD320. Three readings should be taken and the average absorbance reading should be used to calculate VP/ml using the formula below.

Average of 3 readings : (OD260-OD320) x dil factor x 1.12e12

7.0 Reagents

Viral sample
Jenner storage buffer with 0.1% SDS
Sterile distilled water (RNAse & DNAse Free) Sigma W4502

8.0 Equipment

Gilson Pipettes
Pipette tips
Beckman Coulter DU 730 Spectrophotometer with small cuvette holder

0.1 ml curvette

Eppendorf tubes

9.0 Method

Note:

The linear range at which the UV spectrophotometer can provide accurate readings of VP/ml is for absorbance values between 0.1and 1. The virus should be diluted further with Jenner storage buffer with 0.1% SDS until all absorbance readings for a given virus fall within this range.

Sample preparation in virus hood

1) Dilute virus 1:10 in Jenner storage Buffer with 0.1% SDS, 10ul virus and 90ul Buffer

(Get cuvette from rack in virus lab)

Virus quantification

2) Ensure the insert for the 0.1ml cuvette is in place.Clean the cuvette with a kim wipe to dry and remove any fingerprints that may interfere with the passing of light through sample. The cuvette should be positioned so the red dot is positioned in the bottom left corner. Do not remove the cuvette for duration of session.

3) Switch on the spectrophotometer, close sliding lid and allow self checks to run.

4) Select ‘User programmes’ from menu

5) Select ‘Adeno’ programme

6) Using the pipettes from the viral DNA extraction bench in Immunology, put 100ul of buffer (Jenner storage buffer plus 0.1% SDS) in cuvette. Close the sliding door of the spectrophotometer..

7) Press ‘blank’ on the menu screen. (during first use, the machine warms the lamps which takes a few minutes)

8) When blank has been performed, open the door and remove the buffer from the cuvette with a pipette.

   Load sample, ensuring no bubbles in sample and that the volume of sample to be analyzed is greater than 85ul

9) Press ‘Read’ on the menu screen. Record the net 260 reading.

10) Take 2 further readings by pressing ‘Read’ again. Record the readings.

11) If doing more than one virus, remove the liquid from the cuvette, add 100ul buffer, pipette up and down twice and remove. Fill with a fresh 100ul of buffer and read. Check that the reading is 0.

12) Repeat steps 9 to 12.

13) Remove the cuvette, clean it and place in a falcon tube of microsol. Place back in the rack in the virus lab.

14) Remove the insert that holds the cuvette.

15) Turn off the spectrophometer.

16) Record the data in S:\Vector Core Facility\immuno and spec readings in the ‘Adeno Immunostaining and spec readings 2’ file. This works out the VP/ml

1.0 Jenner Laboratory Protocol Number: J111

2.0 Version Number: 02

3.0 Title: MVA Immunostaining titration using CEF in 6-well plates

4.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3_jenner_server\jenner\hill_group\Safety\COSHH assessments\Manufacturers material safety data sheets

R003 Poxvirus Risk Assessment
\\Imsnw3_jenner_server\jenner\hill_group\Safety\GMO RA\R003 poxvirus.doc

J013 CEF culture

J019 Bulking up poxviruses

5.0 Definitions

CEF	Chicken embryo fibroblasts
FCS	foetal calf serum
PBS	Phosphate buffered saline
DMEM	Dulbecco's modified Eagle's media
MVA	Modified vaccinia Ankara
LMP	Low melting point
CMC	Carboxymethylcellulose

6.0 Objective

To titrate a preparation of MVA using CEF in 6-well plates. Method is given for screening by immunostaining for markerless MVA virus. Each virus preparation should be tested in quadruplicate: Two different CEF preps are used at different passage number (i.e. seed 6-well plates from fresh CEF and the previous week's delivery). Two separate serial dilutions are titrated on each cell batch. A single concentration of a reference virus should be titred at the same time. Therefore,

plate one 6-well plate of each cell batch for the virus titration and on a separate plates seed two wells with each cell batch for the reference virus. GFP expressing reference virus can be counted by immunostaining to gauge accuracy of initial GFP plaque counting, but this is not essential.

7.0 Reagents

1 x 6 well plate containing 90% confluent CEF per virus to be titred

DMEM D6546	Sigma
100x Pen/Strep solution (10,000 units penicillin-G and 10 mg strep per ml) P0781	Sigma
200 mM Glutamine G7513	Sigma
FCS F2442	Sigma
PBS (1x) H15-001	PAA
2 x MEM without phenol red 21935	Gibco
2% LMP agarose in dH_20 (heat sterilized) A9414	Sigma
CMC high viscosity 279294T	BDH#
16% Paraformaldehyde (aqueous methanol free) 43368*	Alfa Aesar
0.2% Triton-X 100 in PBS	
ImmPACT DAB (3,3'-Diaminobenzidine) + SK-4105	Vector Labs
Liquid Chromogen Stain & Dilutent	
Pre-Sept tablet SPR25	ASP
Primary antibody: Rabbit anti-Vaccinia Virus 126-1063	ISL
Secondary antibody: Anti-Rabbit HRP Conjugated NA934V ISL 126-1063	Amersham
(from Donkey)	

Complete 2% (10%) FCS DMEM:

500 ml DMEM	
10 (50 ml) ml FCS	(2% (10%) final conc)
5 ml Pen/strep	(100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine	(4 mM final conc)

4% FCS 2xMEM:

250 ml 2xMEM
10 ml FCS (4% final conc)
5 ml Pen/strep (200 U Penicillin, 0.2 mg strep ml^{-1} final conc)
10 ml L-glutamine (8 mM final conc)

PBS + 3% FCS:

Add 3ml FCS per 100ml sterile PBS (1x)

CMC:

1. Weigh out 5.12g high viscosity CMC
2. Add approximately 300mL ultrapure water into a 0.5L Duran bottle.
3. Add approximately 1g CMC to the water and shake the bottle vigorously. The powered CMC will form a small ball. Repeat until all the CMC is in the water.
4. Make up to 400mL.
5. Shake frequently until the CMC has dissolved.
6. Autoclave (121ºC 15 min)
7. Store at room temperature

4% Paraformaldehyde in PBS:

In the fume hood dilute 16% paraformaldehyde solution 1:4 in sterile PBS. Brek the ampoule open with care and dispose of glass in a sharps bin.

0.2% Triton X-100 in PBS:

Disolve 100ul Triton X-100 in 50ml sterile PBS. Cut of end of pipette tip to dispense this viscous detergent.

Do not substitute for alternative supplier. This product has been found to be lower in endotoxins levels than those from other suppliers.

11.0 Equipment

Class II BioSafety Cabinet Mars	Scanlaf
CO_2 incubator Galaxy R	RS Biotech
37°C water bath SUB6	Grant
Microscope DMIL	Leica
6-well tissue culture plates	
Benchtop Centrifuge Allegra X-12R	Beckman Coulter
Disposable haemocytometer	
1.5 ml microcentrifuge tubes (heat sterilised)	
10 μl filter tips	

200 µl filter tips
1000 µl filter tips
Vacuum aspirator
Aspirator pipettes
5, 10 and 25 ml serological pipettes
Vortex
500ml waste bottle and lid (Duran) marked for formaldehyde waste
250ml waste bottle and lid (Duran) marked for DAB waste

12.0 Method

12.1 Prepare a 6 well plate per virus with 1x 10^6 CEF cells per well in 10% FCS DMEM.
12.2 Incubate (37°C, 5% CO_2) overnight.
12.3 Next day aspirate the 10% FCS DMEM and replace with 1 ml of 2% FCS DMEM. Incubate (37°C, 5% CO_2) until required.
12.4 Label sufficient 1.5 ml microcentrifuge tubes for the following dilutions, in duplicate for each virus:

	Virus stock	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}
Virus (µl)	Stock	10	10	10	100	100
2% DMEM (µl)	-	990	990	990	900	900

12.5 Add the required volume of 2% FCS DMEM to each tube.
12.6 Thaw the virus (es) to be titred and add 10 µl of the stock to the first tube of the series. Continue to prepare the dilution series as shown in the table above. **N.B**. **vortex every preparation of the new dilution for 3 seconds and change pipette tips for every dilution.**
12.7 Add 100 µl of the appropriate viral dilutions to the relevant wells of the 6-well plate as shown below:

Series 1: 10^{-6}	Series 1: 10^{-7}	Series 1: 10^{-8}
Series 2:10^{-6}	Series 2:10^{-7}	Series 2: 10^{-8}

Reference at 10^{-2}	Reference at 10^{-4}	Reference at 10^{-6}

Once 100 µl added, mix evenly in well by repeated movements left/right and forward/backwards. **DO NOT** swirl plates, this will lead to plaques concentrated around the sides of the wells.

12.8 Incubate at 37ºC (+/- 2ºC) + 5% CO_2 for 1 hour.
12.9 Proceed with either Agarose or CMC overlay:

Agarose overlay:

12.10 During the incubation, take the bottle of 2% LMP agarose (stored in the tissue culture lab) to melt (2-3 minutes on high power in the microwave in the mol. biol. lab). Transfer required amount of agarose to 50 ml Falcon tubes in the tissue culture lab biological safety cabinet. Pass the Falcon tubes through to the virus lab and place in the water bath at 37°C for at least 15 minutes.

12.11 Place the 4% FCS MEM in 37°C water bath in the virus lab.

12.12 Aspirate the culture medium from the wells (do not allow cells to dry out).

12.13 Pour 4% FCS MEM into an equal volume of 2% low melting point agarose in 50 ml Falcon tube *(note: add FCS MEM to the agarose to prevent agarose setting in the 50 ml Falcon tube as it is poured).*

12.14 Quickly pipette 2 ml per well of this mixture (to prevent the agarose solidifying).

12.15 Incubate for 2-4 days and count the plaques under fluorescent microscope.

CMC overlay:

12.16 Mix sufficient D2 media and CMC in a ratio 2/3 D2:1/3 CMC for 2 ml per well required.

12.17 Aspirate media containing virus.

12.18 Replace with 2 ml D2/CMC overlay per well.

12.19 Incubate for 2-4 days and count the plaques under fluorescent microscope.

13.0 Staining

13.1 Remove the fluid from the wells with the Vacusafe aspirator

13.2 Fix the cells with approximately 1ml per well of paraformaldehyde (4% in PBS) for 5mins at room temperature.

13.3 Remove the fixative **with a pipette to the formaldehyde waste bottle**.

13.4 Wash once with 2 ml per well of PBS. Again remove waste with a pipette to formaldehyde waste bottle.

13.5 Permeabilise cells with 0.2% Triton X-100 in PBS. Add 1ml per well for 5mins at room temperature. Aspirate waste

13.6 Wash cells twice with 2mls per well PBS using Vacusafe aspirator.

13.7 Block with 1ml per well PBS + 3% FCS and incubate for 1hr at room temperature

13.8 Remove PBS + 3% FCS using Vacusafe aspirator.

13.9 Prepare 1ml per well of primary antibody diluted 1:1000 in PBS + 3% FCS

13.10 Add 1ml of the primary antibody per well and incubate for 1hr at room temperature.

13.11 Remove antibody solution and wash twice with approximately 2ml of PBSper well. Remove using Vacusafe aspirator
13.12 Prepare the secondary HRP-conjugated antibody by diluting 1:300 in PBS + 3% FCS.
13.13 Add 1ml of secondary antibody solution per well. Incubate for 45 minutes.

13.14 Remove the antibody solution and wash twice with approximately 2ml of PBSper well. Remove using Vacusafe aspirator
13.15 Make up the DAB Liquid Chromagen developing solution: (allow 1ml/well) add one drop of Chromogen liquid to every 1ml of DAB Liquid buffer solution that is requird and mix well. (Always make up this solution freshly)
13.16 Add 1ml of developing solution per well. Plaques will stain a distinct brown colour. Leave for 10 minutes.
13.17 Discard solution into a glass bottle (marked DAB waste) containing one Pre-Sept tablet
13.18 Wash plates with approximately 2mls tap water per well. Discard water into DAB waste bottle. Bottle left over night and contents discarded with plenty of water down sink.
13.19 Allow plates to dry for half hour in air. Ideally leave upside down on a paper towel.

14.0 Determination of Titre (pfu/ml)

14.1 Count the number of plaques in duplicate wells containing 10 - 100 spots per well.
14.2 Use the following formula to determine the titre in pfu/ml:

"*average number of spots"* x "*serial dilution"* x "*dilution factor"* = "*x"* pfu/ml

$$\text{e.g. } \frac{(36+40)}{2} = 38 \times (1\times10^{7}) \times 10 = 3.8 \times 10^{9} \text{ pfu/ml}$$

15.0 Database maintenance

15.1 Virus storage (s:Reagent storage info/virus storage/virus stock database)
Account name: user
Password: virus
Add new record and fill titre details.
15.2 MVA production chart: (s:Technical group meetings/vector core meetings/MVA production chart).
Add virus titre to relevant entry

16.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First written	N/A	S. Elias
03	Reference dilutions and dilution mixing clarified		S. Elias