R21, a novel particle based vaccine for a multi-component approach to malaria vaccination

Thesis submitted for the degree of Doctor of Philosophy

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Katharine Collins

St Cross College

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R21, a novel particle based vaccine for a multi-component approach to malaria vaccination

Katharine Collins, St Cross College, University of Oxford

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Abstract

Malaria is a major cause of mortality and morbidity and an effective vaccine is an urgent global health priority. This thesis evaluates the liver stage viral vector regimen ChAd63 – MVA ME.TRAP in humans and demonstrates the induction of partial protective efficacy (21%) against sporozoite challenge, mediated by high levels of antigen-specific T cells. Although this efficacy is suboptimal, the only subunit vaccine regimen found to elicit greater efficacy has been the particle vaccine, RTS,S/AS01B. This vaccine has been evaluated in large phase 3 trials in Africa and elicits between 30-50% efficacy, mediated predominantly by antibodies that target the circumsporozoite (CS) protein at the sporozoite stage of infection. The aim of this thesis is to enhance pre-erythrocytic stage subunit vaccine efficacy using two approaches. Firstly, by increasing the immunogenicity of a CS-based particle vaccine and secondly, by combining the particle vaccine with ChAd63 – MVA ME.TRAP. To enhance the immunogenicity of the CS-based particle an improved RTS,S like vaccine called R21 was developed. The major improvement is that the R21 particle is formed from a single CS-HBsAg fusion protein and contains a much higher percentage of CS antigen than RTS,S, which may lead to enhanced immunogenicity and efficacy. R21 was found to be immunogenic at a very low dose, used alone or in a range of adjuvants and elicited sterile efficacy when administered with ISCOM adjuvants. Concurrent induction of both cellular and humoral immunity was achieved by combining R21 with ChAd63 – MVA ME.TRAP and no immunological interference was seen. Furthermore, sterile efficacy against sporozoite challenge was significantly enhanced when R21 was administered with TRAP based viral vectors. These pre-clinical studies have provided important information for the selection of a suitable adjuvant and proof of concept that these vaccines can be combined. R21 is now being taken forward for evaluation in Phase 1/2a clinical trials with the future aim of combining it with the ChAd63 - MVA ME.TRAP regimen in humans.

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Abbreviations

aa	Amino acid
ACK	Ammonium chloride potassium
ACT	Artemisinin-based combination therapy
Adhu	Human adenovirus
AMA1	Apical membrane antigen 1
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen-presenting cells
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BCR	B cell receptor
BSA	Bovine serum albumin
C+1	Challenge plus one day
CBF	Clinical bio-manufacturing facility
CelTOS	Cell-traversal protein for ookinetes and sporozoites
ChAd	Chimpanzee adenovirus
CHMI	Controlled human malaria infection
CMV	Cytomegalovirus
CPS	Immunisation under chloroquine prophylaxis with live sporozoites
CSA	Chondroitin sulphate A
CS	Circumsporozoite protein
CTL	Cytotoxic lymphocyte
D	Day
DCs	Dendritic cells
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
D-PBS	Dulbecco’s phosphate buffered saline
DTT	Dithiolthreitol
EBA 175	Erythrocyte binding antigen 175
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
EMP	Erythrocyte membrane protein
ER	Endoplasmic reticulum
EXP1	Exported protein 1
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FGF	Functional genetics facility
FITC	Fluorescein isothiocyanate
FP9	Fowlpox strain virus FP9
g	The acceleration due to gravity
GAP	Genetically attenuated parasites
GFP	Green fluorescent protein
GIA	Growth inhibitory assay

GLA-SE	Glucopyranosyl lipid adjuvant-stable emulsion
GMP	Good manufacturing practice
GPI	Glycosylphosphatidylinositol
GSK	GlaxoSmithKline
HA	Hyaluronic acid
HBsAg	Hepatitis B surface antigen
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSPGs	Heparin sulfate proteoglycans
kb	Kilobase
kDa	Kilodalton
Kupffer cell	KC
ICAM1	Intracellular adhesion molecule 1
ICS	Intracellular cytokine staining
i.d.	intradermal
IFAT	Immunofluorescence antibody test
IFNγ	Interferon gamma
ifu	Infectious units
IgG	Immunoglobulin G
IL2	Interleukin 2
ILSDA	Inhibition of liver stage development assay
i.m.	Intramuscular
IRS	Indoor residual spraying
i.p.	intraperitoneal
IPT	Intermittent preventative therapy
ISCOM	Immunostimulatory complex
ISI	Inhibition of sporozoite invasion
ITN	Insecticide treated bed nets
i.v.	intravenous
LB	Luria Bertani
LLIN	Long lasting insecticide treated bed nets
LN	Liquid nitrogen
LPR-1	Low-density lipoprotein receptor-related protein
LSA1	Liver stage antigen
mAb	Monoclonal antibody
malERA	Malaria eradication research agenda
MBL	Mannose binding lectin
MDG	Millennium Development Goal
ME	Multi-epitope string
MEM	Minimal essential medium eagle
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
MPL	Monophosphoryl lipid A
MSP	Merozoite surface protein
MVA	Modified vaccinia virus Ankara
mw	Molecular weight

NALP3	NACHT, LRR and PYD domains-containing protein 3
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NYVAC	New York vaccinia viryus
o/w	Oil-in-water
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular patterns
Pb	*Plasmodium berghei*
PbPfCS	*Plasmodium berghei* containing *Plasmodium falciparum* CS
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with 0.05%Tween 20
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pf	*Plasmodium falciparum*
pfu	Plaque forming units
PHA/SEB	Phytohemagglutinin/Staphylococcal enterotoxin B
Phz	Phenylhydrazine
pNPP	Para-Nitrophenylphosphate
pRBC	Parasitized red blood cells
PRRs	Pattern recognition receptors
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
Py	*Plasmodium yoelii*
RAS	Radiation attenuated sporozoites
RBC	Red blood cell
RH	Reticulocyte-binding homologue
RH5	Reticulocyte binding protein homologue 5
RIFIN	Repetitive interspersed family
RBM	Roll Back Malaria
RPM	Revolutions per minute
RT	Room temperature
s.c.	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SEM	Standard error of the mean
SFC	Spot forming cells
SOC	Super optimal broth
Spz	Sporozoites
SPATR	Secreted protein with altered thrombospondin repeat
SPECT	Sporozoite microneme protein essential for traversal
STARP	Sporozoite threonine and asparagine-rich protein
STEVOR	Sub-telomeric variable open reading frame
SURFIN	Surface-associated interspersed gene family
TAP	Transporter associated with antigen processing

TAE	Tris-acetate-EDTA
Tcm	Central memory T cells
TCR	T cell receptor
Tem	Effector memory T cells
Tfh	T-follicular helper cell
Tg	Transgenic
Th	T-helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAP	Thrombospondin-related adhesion protein
Treg	Regulatory T cells
T 1%	Time to 1% parasitaemia
UIS4	Upregulated in sporozoites 4
vp	Viral particles
VLP	Virus like particle
WHO	World Health Organisation

Papers and patents published from these studies

Papers:

O'Hara GA, Duncan CJ, Ewer KJ, **Collins KA**, Elias SC, Halstead FD, Goodman AL, Edwards NJ, Reyes-Sandoval A, Bird P, et al.: ***Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector***. *J Infect Dis* 2012, 205:772-781.

Ewer KJ, O'Hara GA, Duncan CJ, **Collins KA**, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD, et al.: ***Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation***. *Nat Commun* 2013, 4:2836.

Patent:

Collins KA, Hill AVS and Gilbert SC

R21 Malaria Vaccine

UK Patent Application No. 1301022.8

1 Introduction

1.1 Malaria

Malaria still remains a significant global health problem and a major cause of death in young African children (Figure 1.1). Promising reports from the World Health Organisation (WHO) indicate that cases of malaria reduced globally by 25% between 2000 and 2012. However malaria is still endemic in 104 countries putting over 3 billion people at risk of infection [1]. The WHO estimated that in 2012 around 627,000 people died from malaria and a further 207 million were infected, resulting in devastating socio-economic costs [1-3]. The greatest burden remains in Africa where approximately 80% of cases and 90% of deaths occur, with the majority of deaths (77%) being in children under the age of 5 [1]. Malaria is caused by the protozoan parasite *Plasmodium* and there are five species known to infect humans, *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* [4, 5]. Transmission of the parasite is by the female *Anopheles* mosquitoes of which there are over 400 different species, 70 that are known to transmit human malaria, and 41 are considered efficient vectors that can transmit malaria at a level of major public health concern [6]. *P. falciparum* and *P. vivax* are responsible for the majority of infections and both can cause severe disease with *P. falciparum* being the most deadly and also the most prevalent in Africa. These two species are biologically different and differences between the life-cycles permit *P. vivax* to have a wider geographical distribution than *P. falciparum*. *P. vivax* is able to grow in a broader range of temperatures and can have a dormant liver stage. This enables the parasite to survive periods of cooler temperatures such as winter, when *Anopheles* vectors may not be present and it is therefore able to persist in more temperate climates. *P. vivax* is also present in Africa but the risk of infection is significantly reduced largely due to the high prevalence of the Duffy-negative trait which renders red blood cells (RBCs) resistant to infection by

P. vivax. Due to the significantly greater mortality rates the majority of funding and research has been directed towards the elimination and control of *P. falciparum,* however the importance of also developing effective measures to combat *P. vivax* should not be over looked [7-9].

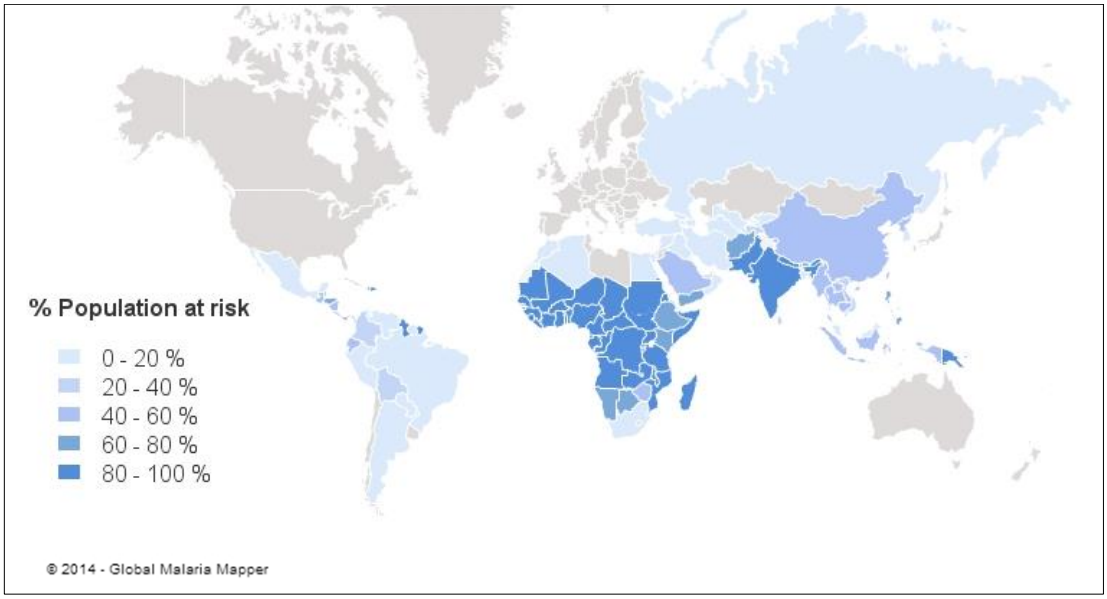


Figure 1.1 Percentage of population at risk from Malaria

Map generated using the Global Malaria Mapper tool with data from the WHO World Malaria report 2013, showing the percentage of population at risk from Malaria © 2014 – Global Malaria Mapper.

1.1.1 Life-cycle of *Plasmodium falciparum*

The *Plasmodium* parasite life-cycle is extremely complex involving multiple stages in both the mosquito and vertebrate host, and the process can vary among species. Detailed here is the life-cycle for *P. falciparum* as it is the focus of the research within this thesis (Figure 1.2).

1.1.1.1 Pre-erythrocytic stage

Infection in humans begins with the pre-erythrocytic stage when an infected female *Anopheles* mosquito deposits tens to hundreds of sporozoites into the skin of the human host when taking a blood meal [10-13]. Some sporozoites remain local in the skin or drain to lymph nodes where they stimulate the host's immune response, but it is likely the majority of the sporozoites instead enter blood vessels and migrate to the liver [14, 15]. In the liver sinusoids the sporozoites traverse the endothelial cell layer and liver-resident macrophages called Kupffer cells and enter the space of Disse. Here they typically traverse a number of hepatocytes before invading and settling in one for the next stage of development [16, 17]. During invasion of the final hepatocyte the sporozoite produces a parasitophorous vacuole (PV) within which it undergoes liver stage development and replicates to give rise to tens of thousands of merozoites. This phase lasts for around 7 days; the merozoites are then packaged into vesicles called merosomes and are released from the hepatocyte into the circulation. The merosomes eventually burst to release the merozoites and the erythrocytic stage of infection begins [18-22]. *P. vivax* and *P. ovale* differ from *P. falciparum* at this stage as some of the intra-hepatic parasites are able to form hypnozoites which can remain dormant in the liver for weeks or years and therefore result in relapses [23].

1.1.1.2 Blood stage

The blood stage or erythrocytic stage begins when merozoites invade RBCs and undergo asexual replication [24]. The intra-erythrocytic parasite progresses through

the immature trophozoite ring-stage, then into the mature trophozoite and finally into the schizont stage when replication occurs. The RBC eventually ruptures releasing an average of 20 daughter merozoites back into the blood which are then able to invade new RBCs. This invasion and replication cycle repeats and is typically around 48 hours for *P.falciparum*, *P. vivax* and *P. ovale*; 72 hours for *P*. *malariae* and 24 hours for *P*. *knowlesi*. It is during this stage that infected individuals become symptomatic [25, 26].

1.1.1.3 Sexual stage

During the asexual cycle some merozoites inside RBCs develop into sexual stage male and female gametocytes, a process termed gametocytogenesis. These gametocytes appear in peripheral circulation about 7-15 days after the start of blood stage infection but it is unclear what triggers the gametocytogenesis [27]. There are five stages to the formation and maturation of the gametocytes within the human host. The early stage gametocytes (stage 1-4) are sequestered in survival niches and only mature stage 5 forms circulate in the peripheral blood and are able to be taken up in the blood meal of a female *Anopheles* mosquito [28-30]. Once ingested the male and female gametocytes become activated gametes and fuse to produce a zygote. The zygote then develops into a motile ookinete that penetrates the mid-gut wall where it forms the oocyst. Sporozoites are formed within the oocyst and are released when the mature oocyst ruptures, and the sporozoites migrate to and invade the salivary glands where they can be injected into the next host [26].

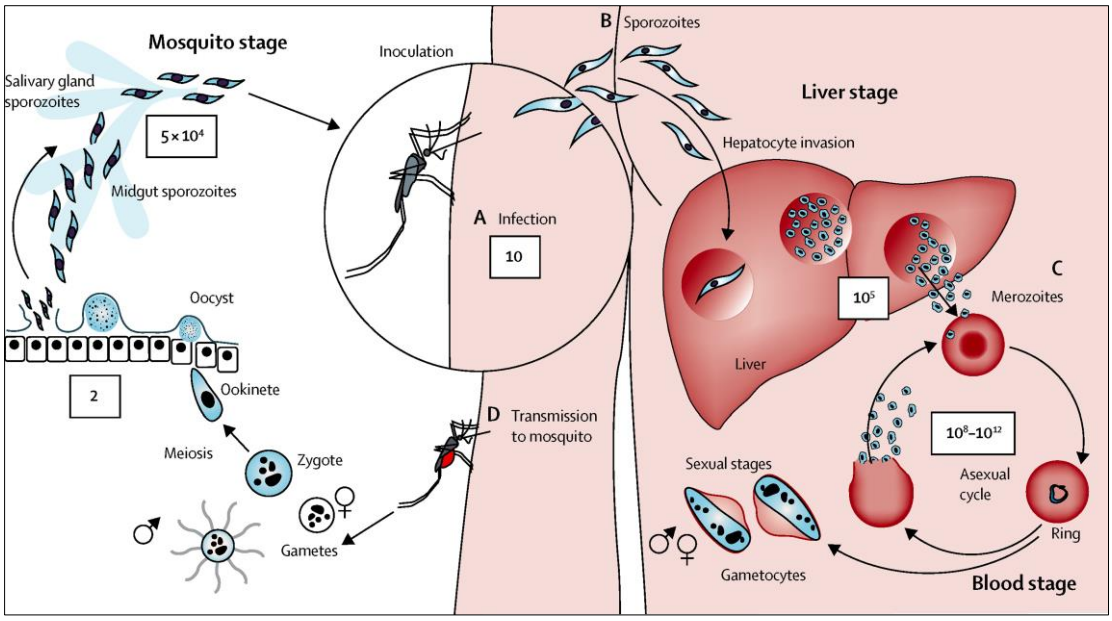


Figure 1.2 Life-cycle of *Plasmodium falciparum*

Image taken from Malaria, Lancet, 2014 [25] Showing the stages of the *Plasmodium falciparum* life-cycle in the mosquito and human host.

1.1.2 Malaria pathogenesis

The pre-erythrocytic stage of infection is clinically silent and it is only during the blood stage that symptoms appear. The severity of symptoms that develop during the blood stage can vary greatly between individuals from asymptomatic or mild, to severe and fatal. The variation is largely dependent on the parasite species and strain; and the individual's age, pre-existing immunity and genetics. *P. falciparum and P. vivax* are the major cause of severe malaria whereas *P. vivax* is rarely fatal; and *P. ovale, P. malariae* and *P. knowlesi* often only cause mild to moderate symptoms. The large majority of deaths occur in children under the age of 5 whereas adults or older children who have had repeated exposure to malaria infections can develop partial immunity. This immunity does not prevent infection but it can limit the severity of the disease [31-33]. In addition certain populations have developed different genetic variants which protect against malaria, such as the variants which cause sickle-cell disease, thalassemia, glucose-6-phosphate dehydrogenase

deficiency and the Duffy-negative phenotype [34, 35]. The initial symptoms of malaria are relatively non-specific and can include headache, fatigue, muscle aches, nausea, abdominal pain and fever. These symptoms coincide with the cyclic rupture of infected erythrocytes and the release of merozoites and metabolic waste by-products into the blood stream. At this stage many infections can be successfully treated with anti-malarial drugs or cleared by the host's immune response. However, some infections progress to severe disease which can manifest in various ways largely dependent on age [36]. Severe malaria is a complex pathogenic process affecting multiple tissues and organs, and is thought to be largely due to impaired oxygen delivery. Cerebral malaria (coma), metabolic acidosis and respiratory distress are common in all age groups, whereas severe malarial anaemia and hypoglycaemia are more common in children; and pulmonary oedema, kidney failure and jaundice are more common in adults [25, 37]. In addition pregnant women, particularly primigravidae, are at increased risk of developing placental malaria which can lead to premature labour, low birth weight and intra-uterine growth retardation all of which increase the risk of infant mortality [25, 38, 39]. Although the precise mechanisms leading to the development of these severe malaria-associated pathologies are not fully understood, they are thought to be caused by both host-parasite interactions and the result of the host's immune response to infection.

1.1.2.1 Host-parasite interactions

In *P. falciparum malaria* the main host-parasite interaction thought to contribute to the broad range of symptoms is the ability of the parasite infected RBCs to adhere to host molecules. The molecules are expressed on the surface of the endothelial cells lining the small blood vessels, and also placental cells [37]. This adhesion effectively sequesters the infected RBCs to a particular location causing a blockage in the microvasculature, thereby impairing the blood flow and the delivery of oxygen to the tissue or organ. The parasites successfully sequester themselves in many organs

including the heart, liver, brain, lung, kidney and placenta. This is due to the parasites ability to bind to the variety of host receptors that are differentially expressed within these organs. Sequestration has been well-studied and a range of molecules have been identified as the receptors to which the infected RBCs bind, including CD36, intracellular adhesion molecule 1 (ICAM-1), E-selectin, chondroitin sulphate A (CSA), CD31 and hyaluronic acid (HA) [37]. Some receptors have been shown to be more important than others in certain organs and syndromes, such as ICAM-1 in the brain associated with cerebral malaria [40] and CSA and HA associated with placental malaria [41, 42]. Infected RBCs are also able to adhere to one another or to uninfected RBCs. This process is known as platelet-mediated agglutination and rosetting, and this feature is also thought to contribute to the blockage of vessels [43-45]. The binding is predominantly mediated by a single parasite adhesive protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is exposed on the RBC surface around 16-20 hours after invasion and can bind to all host receptors [46]. PfEMP1 is encoded by the large *var* multi-gene family, which can undergo antigenic switching in response to immune pressure enabling the parasite to evade specific host immune responses [47]. It is thought that if sequestration is responsible for the severity of disease then receptors and adhesion molecules involved may make attractive targets for vaccination [48]. Although widely accepted, some debate whether there is a simple direct link between reduced microvasculature flow caused by sequestration of infected RBCs and the pathologies seen in severe malaria [49]. Regardless, sequestration clearly enables the parasites in the infected RBCs to successfully evade immune clearance by the spleen. In addition, the significant destruction of RBCs by the invading parasites will likely contribute to malarial anaemia [50] and thereby also reduce oxygen delivery to tissues and organs [37, 51].

1.1.2.2 Immunopathology

Although the host response to infection is not completely understood it is thought that the principal protective mechanisms against the blood stage parasites involve both antibodies and a strong pro-inflammatory immune response [52]. This pro-inflammatory response is also thought to contribute to the development of severe malaria [53]. It includes the production of a range of cytokines such as tumour necrosis factor (TNF), migration inhibitory factor (MIF) and interferon gamma (IFNγ) and the release of mediators such as oxygen free radicals and nitric oxide. These factors have been implicated in cerebral malaria, placental and hepatic dysfunction, the cause of fever, and the inhibition of erythropoiesis thereby contributing to anaemia [49, 53-55]. Many of the same factors are also thought to be important mediators of the immune response against the parasite and parasite killing, as they activate leucocytes and other cells to release toxic oxygen species and nitric oxide. It is likely that the timing and balance of the pro-inflammatory and anti-inflammatory cytokine response as well as the presence of a regulatory T cell response will determine disease progression and parasite clearance [56].

1.1.3 Malaria control and elimination

In 1955 the WHO implemented the Global Malaria Eradication Program which successfully eliminated malaria from many regions worldwide. Unfortunately it soon became apparent that elimination could not be achieved with the current available control measures, and due to financial, technical and administrative issues this program was abandoned in 1969 [57, 58]. In the 1970s and 1980s the international and political interest for malaria control declined rapidly and the lack of financial and strategic support led to a substantial increase in malaria incidence and mortality worldwide [59, 60]. By the 1990s the failure to control malaria was seen as a global disaster and interest in malaria elimination again received international political and

financial support. In 1998 the Roll Back Malaria (RBM) partnership was launched which was established by the WHO, the World Bank, the United Nations and UNICEF; this has since expanded to include national governments, non-governmental organisations and private sector groups [61]. For the first time, a malaria elimination campaign had a specific focus on helping countries with the highest burden of malaria, such as those in Africa. In 2000, reversing the incidence of malaria became a key part of one of the eight United Nations Millennium Development Goals (MDGs) and The Global Fund to Fight AIDS Tuberculosis and Malaria was established in 2002 to help provide funding to meet these goals. The RBM and MDG agendas support a fairly consistent set of objectives and provide considerable funding to support widespread use of the most effective current control measures [62, 63].

1.1.3.1 Current control measures

Currently available control measures involve both treating the disease in humans and attacking the vector, and these have been effective at reducing the burden of malaria over the years [1, 64]. These measures include insecticide treated bed nets (ITNs), indoor residual insecticide spraying (IRS) and rapid drug treatment [65-67].

In addition, non-immune travellers receive continuous chemoprophylaxis whilst in endemic areas and intermittent preventative therapy (IPT) has also been used for high-risk groups, such as pregnant women and children living in certain regions [68, 69]. ITNs which are treated with a pyrethroid insecticide have been distributed by the different control programmes. The insecticide is able to kill the mosquitoes as well as the net preventing contact with the individual, this benefits the whole community not just the user and these original nets would remain active for 6 months in the field. Long lasting insecticide treated bed nets (LLIN) have since been developed that remain active for five years, further increasing the effectiveness of this intervention. IRS uses a broader range of insecticides than ITNs and results in the death of

mosquitoes as they make contact with the treated surfaces, often whilst resting after feeding. The use of both these measures has been effective and studies have shown a significant reduction in mortality and disease [1, 70]. However, despite such promise there have been difficulties with achieving mass coverage and encouraging proper use of the nets. Consequently, there has been a spread of insecticide-resistant mosquitoes, particularly to the pyrethroids, the main insecticides used to treat the nets [71, 72]. Anti-malarials such as quinine, chloroquine and primaquine have traditionally been used to treat malaria, however due to the emergence of drug-resistant strains, artemisinin-based combination therapy (ACT) is now recommended as the first-line treatment [73, 74]. This uses artemisinin in combination with other drugs appropriate for the geographical location and presence of drug-resistant parasites, and has been very effective. Unfortunately, due to the high cost of treatment, fake or substandard anti-malarials are produced and their widespread, uncontrolled use contributes to the increase in parasite resistance [75, 76]. There have now, worryingly, been a number of reports of resistance to arteminisin, which poses a considerable threat as there are no major drugs available to replace it at present [77, 78]. As well as treating malaria, chemotherapy is also used for intermittent preventative therapy. IPT involves the periodic administration of a defined course of anti-malarials irrespective of infection status to clear current infection and also prevent new ones [79]. It was initially used in pregnant women and successfully reduced infections and improved pregnancy outcomes, and has been extended to children and infants with success [68, 69, 80, 81]. IPT also faces the same problems of drug-resistance and of concern for all these measures that temporarily prevent exposure to parasites is that they may interfere with the development of naturally acquired immunity which could have detrimental effects, although this has not been found in many studies [79, 82, 83].

1.1.3.2 Need for a malaria vaccine

Due to the increased prevalence of drug-resistant parasites and insecticide-resistant mosquitoes, the current control methods are now only partially effective and will eventually be obsolete. It is therefore increasingly important to develop new tools to control malaria. These could include the development of new drugs that can target all stages of the parasite life-cycle, the identification of new insecticides, and the more experimental approaches of engineering mosquitoes to reduce survival or transmission of disease [84, 85]. Probably the most useful approach would be the development of an effective malaria vaccine, as historically vaccination has been one of the most effective control measures against infectious disease and many believe eradication will not possible without it [86]. The Malaria Vaccine Technology Roadmap which provides strategic guidance for vaccine development was initially developed in 2006 and has recently updated its ambitious goals. The aims are now stated as the development of a vaccine by 2030 that is over 75% effective against clinical malaria and is also able to reduce transmission [87]. In addition to the Roadmap, the general consensus that elimination would not be achieved with the currently available control measures led to the establishment by the WHO of the Malaria Eradication Research Agenda (malERA) in 2007. This involved a process of extensive scientific consultation to identify knowledge gaps, and novel intervention strategies required for the eventual eradication of malaria. In agreement with the goals of the Roadmap, the agenda highlights the potential utility of an effective malaria vaccine for elimination [63, 88].

1.2 The Human immune system

1.2.1 Innate immunity

The innate immune system is the first line of defence against invading pathogens and once the skin barrier is breached, a rapid, broad, non-specific response can act to clear or control the pathogens and activate the adaptive immune response [89]. Innate immune cells can recognise invading pathogens through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) which recognise pathogen associated molecular patterns (PAMPs). Recognition leads the activation of the immune cells and initiation of a cytokine response, which can be dictated by the type of PRRs activated [90]. This cytokine response is also tailored to initiate the correct type of adaptive immune response required to eliminate the particular pathogen [91, 92]. Innate immunity includes the complement system and a range of innate immune cells. The complement system consists of serum and membrane proteins that interact in a proteolytic cascade via three main pathways to trigger various effector functions. The pathways include: 1) the classical pathway initiated by binding antigen-antibody complexes, 2) the mannose binding lectin (MBL) pathway initiated by binding of MBL to mannose residues on the pathogen surface, and 3) the alternative pathway initiated by direct binding to the pathogen surface. Activation of the cascade can promote phagocytosis by opsonisation, triggering an inflammatory response, inducing the formation of the membrane attack complex, and induction of antibodies [93, 94]. Innate immune cells consist of phagocytes such as neutrophils, macrophages and dendritic cells (DCs) which recognise, internalise and clear pathogens. Macrophages can also become activated and secrete pro-inflammatory cytokines and chemokines and recruit other immune cells. DCs are specifically adapted for antigen presentation to lymphocytes and therefore activate the adaptive immune response. In addition to phagocytes, innate immune cells include natural

killer (NK) cells and γδ T cells, which have cytotoxic functions involving perforin, granzyme and IFNγ; and basophils, mast cells and eosinophils which can destroy pathogens and contribute to the inflammatory response through the release of histidine [95, 96].

1.2.2 Adaptive immune system

Innate immune cells can activate the adaptive immune system to generate a long-lasting immune response capable of immunological memory which is composed of both cellular and humoral components. The main adaptive immune cells are the T and B lymphocytes which in contrast to the innate immune cells respond to unique antigens with their T cell and B cell receptor (TCR and BCR). Once the specific antigen is recognised, the naïve lymphocytes are activated and undergo rapid clonal expansion and differentiate into effector cells. A subset of these antigen-specific cells can persist long after antigen has been eliminated and are thought to be precursors of antigen-specific long-lived memory cells, which are able to respond rapidly upon re-infection [96, 97].

1.2.2.1 Cellular immune response

The cellular response is comprised of T lymphocytes (T cells) that recognise peptides displayed on antigen presenting cells (APCs) by major histocompatibility complex (MHC) molecules in the presence of co-stimulatory molecules. T cells are composed of two classes based on the presence of the surface markers CD4 and CD8, the expression of which is related broadly to function. CD4+ T cells mainly recognise exogenous antigen presented by MHC class II molecules on APCs. These peptides are generated within the endosomes from proteins internalised by the endocytic pathways. The MHC class II molecules are synthesised in the endoplasmic reticulum (ER), delivered to the endo-lysosomal compartment where peptide is loaded onto the MHC class II, and the MHC-peptide complex is formed

and transported to the cell surface [98]. MHC class II expression is thought to be limited to professional APCs including DCs, macrophages and B cells, and autophagy and cross presentation also enables endogenous proteins to be presented via MHC class II molecules [99]. Once naïve CD4+ T cells are activated they act primarily by recruiting and activating other cell types and releasing cytokines, and are therefore termed 'helper' cells, although they can also act as effectors themselves. They can be divided in subclasses defined by their phenotype and functional characteristics which include Th1, Th2, Th17, regulatory T cells (Treg) and T follicular helper cells (Tfh). Differentiation into the different CD4+ T cell types and the effector function is influenced by the cytokines and transcription factors present during clonal expansion [100]. In contrast, CD8+ T cells recognise mainly endogenous antigen processed and presented by APCs on the MHC class I molecules which are expressed in all nucleated cells. Following direct infection of an APC by a pathogen such as a virus, foreign protein is identified, tagged for destruction by ubiquitination, degraded by the proteasome into peptide fragments, and transported to the ER via the TAP transporter where it meets the MHC class I molecules. The MHC-peptide complex is formed in the ER in the presence of chaperone proteins, and once complete it is transported via the Golgi complex to the cell surface [98]. CD8+ T cells can also recognise exogenous antigens through cross-presentation onto MHC class I molecules [101]. The main action of activated CD8+ T cells is the direct killing of infected cells by the induction of apoptosis with either cytotoxic granules containing granzyme and perforin, or the Fas-FasL interaction. CD8+ T cells also mediate killing though the release of cytokines such as TNF and IFNγ as well as chemokines. Secretion of IFNγ contributes to the defence against pathogens in a large number of ways. These include direct inhibition of viral replication, upregulation of MHC antigen presentation and co-receptor molecules, recruitment and activation of other effector cells, further induction of other cytokines, and stimulation of nitric oxide production [102, 103]. Due to the importance of IFNγ in

effector mechanisms, assays that can measure antigen-specific CD8+ T cells that secrete IFNγ have become widely used for assessment of candidate vaccines. In addition to the effector functions of antigen-specific CD4+ and CD8+ T cells, once antigen has been cleared a small proportion of antigen-specific T cells remain that constitute long-lived memory. These were traditionally divided into central memory (Tcm) and effector memory (Tem) subsets based largely on the location and phenotype of these cells [97, 104, 105]. However, further analysis of these populations is revealing a much more complicated picture with dozens of subsets being identified that express unique combinations of surface and intracellular markers and have a variety of functions [106].

1.2.2.2 Humoral immune response

Humoral immunity is generated by B cells that produce antibodies which are essentially the secreted form of the BCR. B cells can recognise conformational as well as linear epitopes and they bind and internalise the circulating antigen as an APC. They process the antigen via the exogenous pathway and present it on the cell surface in association with MHC class II molecules. Once recognised by a CD4+ T helper cell the B cell is activated, undergoes clonal expansion and secretes immunoglobulin (Ig). Activated B cells initially secrete IgM and then undergo isotype class switching to IgG, IgE or IgA. This process is influenced by the cytokine environment and the CD4+ T cell help, and the various resulting isotypes have different effector functions. Proliferating B cells may differentiate into short-lived plasma cells which migrate to the periphery to secrete antigen-specific antibodies. They may also remain in the B cell follicles and germinal centres and undergo affinity maturation and somatic hypermutation to generate high affinity antibodies. Affinity matured B cells may then differentiate into long-lived plasma cells which migrate to the periphery and secrete high-affinity antibody over many years. They can also differentiate into memory B cells which provide immunological memory and can

respond rapidly upon re-exposure to the same antigen [107, 108]. Antibodies can act in a number of ways including neutralisation, activation of the complement cascade as well as mediating effector functions through Fc receptors (FcRs). Binding of the FcRs to the FC region of an antibody can initiate opsonisation by neutrophils and macrophages, and can lead to the induction of either antibody-dependant cellular cytotoxicity through NK cells, antibody-dependant cellular inhibition via monocytes or an antibody-dependant respiratory burst from neutrophils [96].

1.3 Natural Immunity to malaria

The complex immune response to malaria infection is not completely understood, though it is evident that naturally acquired immunity (NAI) develops over time in people living in endemic areas. How this protection works, what determines the rate of acquisition, and what immunological mechanisms are protective is still unclear [109]. The incomplete understanding of what constitutes immunity is one reason why development of an effective malaria vaccine has been so difficult to date. Epidemiological studies indicate that individuals in malaria endemic areas develop NAI slowly only after repeated infections to multiple parasites. This slowness is possibly due to the gradual development of a sufficiently broad repertoire of strain-specific responses that are antigenically diverse [31, 110, 111]. In contrast, immunity to severe malaria and death is thought to be acquired relatively quickly after only one or two infections [32]. Immunity therefore develops first against severe disease, then against mild and clinical episodes and eventually infected individuals are completely asymptomatic, despite sometimes remaining parasitaemic for extended periods of time (premunition) (Figure 1.3). Evidence suggests that this acquired immunity is never sterilising and it wanes quickly once repeated exposure is reduced. Adding to the complexity of NAI, women who have developed pre-existing immunity become highly susceptible to disease again during pregnancy, particularly primigavidae. In addition some studies have linked the onset of puberty, rather than the culmination of repeated exposure, as a dominant factor in the rate of acquisition of NAI [112]. NAI can be both anti-parasitic and anti-disease. Anti-parasitic immunity acts to reduce the burden of parasitaemia and therefore the development and severity of infection. In contrast, anti-disease immunity is thought to involve an anti-inflammatory and regulatory immune response to control and limit the severity of symptoms [113]. Though these key features of NAI have been defined the mechanisms have not. Moreover, evident from the way they are acquired, the

mechanisms that develop quickly to protect against severe malaria are most likely different to those that protect against mild malaria.

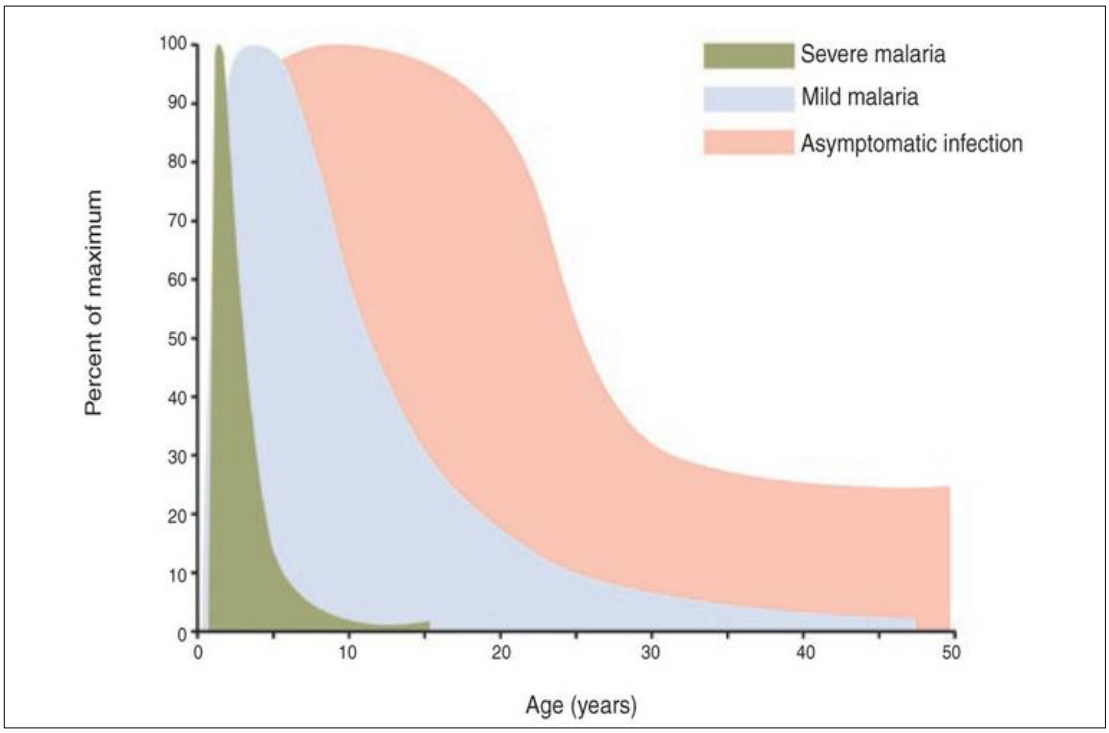


Figure 1.3 Population indices of immunity to malaria in an endemic area
Image taken from [52], the figure shows data combined from a number of studies indicating the prevalence of asymptomatic infection, mild malaria and severe malaria by age.

The naturally acquired protective response could potentially be directed against any stage of the parasite life-cycle. However, studies in naturally exposed individuals have shown limited and conflicting evidence for NAI directed against the pre-erythrocytic stage and evidence for NAI against the blood stage is more conclusive and likely more important. In agreement with this, when blood stage parasites are injected into previously exposed adults, they are able to control parasitaemia [52, 53]. This suggests immunity can develop to the blood-stage alone as this infection method essentially bypasses the pre-erythrocytic stage. Conversely in naïve individuals direct immunisation with whole sporozoites in various vaccination approaches is able to confer complete sterile protection. Studies suggest that this

protection is predominantly a cellular response with CD8+ T cells producing IFNγ that kill parasite infected hepatocytes. [114-116]. However, evidence of this mechanism in natural exposure is limited and therefore not thought to be an important component of NAI. Immunity induced by experimental infection will be discussed in section 1.5.1. Many studies have analysed the immune responses to natural infection in individuals from endemic areas attempting to understand how the infections interact with the immune response. However, studies linking specific immune parameters or profiles with NAI have revealed that no single immune correlate or protective mechanism is consistently and strongly associated with protection.

1.3.1 Immunity to the pre-erythrocytic stage

The reasons the pre-erythrocytic stage is poorly targeted by a naturally acquired protective immune responses are unknown. It could however be due to the inoculation of only very low numbers of sporozoites by a mosquito bite (10-100) and also the intrinsic regulatory environments of the skin [117, 118] and liver [119]. Despite this, anti-sporozoite antibodies have been detected in the sera of naturally exposed individuals; the most studied and immunodominant antigen being the circumsporozoite protein (CS). Levels and prevalence have been associated with malaria transmission intensity, and acquisition appears to be age dependant, suggesting a potential role in NAI [120]. Conversely, some studies investigating an association with protective immunity have indicated that the antibodies to CS correlate with exposure rather than protection [121]. Other antigens expressed at the pre-erythrocytic stage have also been identified and evaluated, such as thrombospondin-related adhesion protein (TRAP, also called SSP2), liver stage antigen 1 (LSA1), sporozoite threonine-asparganine-rich protein (STARP) and apical membrane antigen 1 (AMA1) [122-124]. Although antibodies also exist to these and

other antigens after natural exposure, a response to just one antigen has not been consistently associated with protection. However, some studies have associated humoral responses against multiple antigens expressed during the pre-erythrocytic stage with protection in NAI [125-127]. Sporozoite antigen-specific T cell responses have also been detected in naturally exposed individuals to antigens such as TRAP, CS and LSA1. However the responses are very low, they are not consistently associated with protection and can vary greatly between individuals and populations [128]. Therefore, whether humoral and cellular responses to the pre-erythrocytic stages are a component of NAI is still unclear and further investigation using newer approaches of immune profiling and functional assays may help clarify this.

1.3.2 Immunity to the blood stage

During the blood stage of infection a protective immune response can target both the free merozoites and the infected RBCs. These are present in much greater numbers than the pre-erythrocytic parasites, and are thought to be the primary targets of NAI. The protective response is thought to be largely mediated by antibodies, most likely because MHC molecules are not present in red blood cells; though T cells have also been associated with protection in some studies. Clear evidence for antibody mediated immunity to the blood stage was initially seen in studies carried out in the 1960s and repeated in 1991, where naturally infected children were able to control parasitaemia after transfer of serum IgG from immune adults [129-131]. The slow development of NAI is likely due to the ability of the blood stage parasite to evade the immune system with antigenic variation. As such, the parasite-encoded variant surface antigens (VSA) present on the infected RBC surface are thought to be key antibody targets. The most extensively studied VSA is PfEMP1, which has been repeatedly identified as important in NAI. Other families of VSAs have also been described including the repetitive interspersed family (RIFIN) proteins, sub-telomeric

variable open reading frame (STEVOR) proteins, and surface-associated interspersed gene family (SURFIN) proteins, although their contribution to NAI has been less well studied [132]. These antibodies are thought to function to prevent adhesion and sequestration, inhibit schizont rupture and to enhance clearance of infected RBCs by opsonisation, phagocytosis and complement mediated lysis. In addition to targeting the surface of infected erythrocytes, NAI is also directed against the free merozoites and targets merozoite surface antigens and erythrocyte invasion ligands which are secreted upon contact with RBCs [133]. Merozoite surface antigens are highly polymorphic and the most studied include AMA1, merozoite surface proteins 1-9 (MSP1-9) and rhoptry-associated protein 1 (RAP1), among others. Many studies have associated levels of antibodies to these proteins with protection, although this has not always been consistent. These inconsistencies may be due to the different antigen variants and different epitopes used in these studies which could influence the ability to detect a protective responses [134]. Moreover, such responses may be more accurately detected with functional assays. Recently, studies have assessed the breadth of the response to merozoite proteins and have shown that broad antibody responses are more strongly associated with protection [135-137]. The erythrocyte binding antigen (EBA) and *P. falciparum* reticulocyte-binding homologue (PfRh) invasion ligands have been shown to be important because parasites can vary expression and use of these proteins as immune evasion mechanisms [138, 139]. High levels of both EBA and PfRh antibodies have also been associated with protection [140, 141] and of these PfRh5 is particularly interesting. PfRh5 has been shown to be strongly associated with protection, is highly conserved and is also a relatively poor immunogen in natural infection. Therefore it is unlikely to be under immune pressure making it an attractive vaccine target [142]. Antibodies to merozoite surface antigens and erythrocyte invasion ligands may function to prevent invasion of RBCs by directly inhibiting invasion, opsonising and aiding phagocytosis and inducing complement mediated lysis. There

is very little evidence for a role of T cells in blood stage NAI, though it is recognised that CD4+ T cell help is essential for the previously described antibody responses [143], as well as activation of Th1 responses which can mediate other cellular effectors. Some studies have associated MSP1 specific T cells in naturally exposed individuals with protection [144-146]; and in a study in malaria naïve individuals who were repeatedly infected with ultra-low doses of infected RBCs, they developed a protective response characterised by CD4+ and CD8+ T cells and a Th1 cytokine response [147]. In addition it has been suggested that natural regulatory T cells may have a role in modulating the response, both allowing parasites to exist in premunition and preventing severe pathogenesis [148]. A cellular response may therefore be a component of NAI, but likely accompanying a humoral response.

Despite this large body of immuno-epidemiological research and the identification of multiple important antigens in NAI, no single antigen is consistently associated with protection. Furthermore, how all these components of the natural immune response to malaria interact to control parasitaemia and prevent (or promote) pathogenesis is still unclear and a more complete understanding will be necessary for rational design of treatments and vaccines. However, it should also be considered when designing interventions that aim to mimic NAI, that malaria is estimated to be more than 100,000 years old and has likely co-evolved with the human immune system [149]. Therefore the malaria parasite will have become specifically adapted to evade and exist alongside the host immune response long enough to be transmitted to the mosquito to complete its life-cycle. Hence modulation of the host's immune response and the development of non-sterilising NAI may be heavily influenced by the parasite in order for it to persist until taken up by a mosquito [150]. Therefore a vaccine that mimics NAI would indeed benefit the host as acute disease could be avoided, but it could also favour transmission and may also increase the host's susceptibility to co-infections [151].

1.4 Malaria vaccine development

Vaccination has been one of the most effective public health interventions for successfully controlling many infectious diseases including polio, measles, diphtheria, tetanus, meningitis, pneumonia, rabies and small pox. Where vaccination has been successful the pathogens tend to have a low degree of antigen variability and natural infection leads to immunity against re-infection. It was realised that instead of relying on natural infection to generate protection, immunity could be conferred with administration of relatively non-toxic or non-pathogenic organisms. These traditional vaccines were developed with little or incomplete understanding of protective mechanisms. They attempted to mimic natural immunity using either whole live-attenuated or killed organisms, or purified pathogen components such as toxins, proteins and polysaccharides. Most of these licensed vaccines are thought to protect predominantly with either neutralising or opsonising antibodies generally targeting extracellular pathogens [152]. Many of the more complex mycobacteria, virus and parasite infections for which there are no licensed vaccines, may however require a combination of both cellular and humoral immune responses to be eliminated [153, 154]. Eukaryotic parasites particularly, are inherently difficult to vaccinate against as they are genetically and biologically complex organisms. They typically have elaborate life-cycles and well adapted evasion mechanisms and there are currently no licensed human vaccines against any parasites [155]. Therefore there are many challenges for the development of a malaria vaccine as the *Plasmodium* parasite exists as multiple species and strains. It is also highly variable; it induces only non-sterilising natural immunity and can cause persistent chronic or latent infections. In addition it has a complex life-cycle involving rapidly developing, antigenically variable, intracellular and extracellular forms that are successfully adapted for immune evasion. However, despite all these factors, significant protection against malaria in humans was demonstrated in the 1970s through whole

sporozoite vaccination delivered by mosquito bite (discussed fully in section 1.5.1) [156-158]. Although this approach is logistically challenging as a vaccine itself, the result supports the feasibility of developing an effective malaria vaccine.

The general consensus in the malaria vaccine research community at present and also highlighted by the Malaria Vaccine Technology Roadmap [87] is that the ideal vaccine should induce an immune response that can both prevent clinical disease and also interrupt transmission. It has also become increasingly evident that an effective vaccine may need to target multiple stages of the life-cycle (sporozoite, liver stage, blood stage and sexual stage). It may also need to induce more than one type of immune response (humoral and cellular) and it may need to include multiple epitopes to overcome genetic diversity, antigenic variation and immune evasion.

1.4.1 Experimental human infection model

As outlined in section 1.3, identification of protective immunological mechanisms and potential targets for vaccination has involved studying natural infection in humans in endemic areas. Although important, the utility of this method for understanding the host's response is limited. Since in these studies only peripheral blood samples can be taken and individuals will likely have different stages of ongoing malaria infection, various levels of pre-existing immunity and various co-infections. These factors may therefore confound the results and make interpretation difficult. However, studying the efficacy of interventions in these populations is vital, as it can account for this natural variability of infection which is impossible to duplicate in an experimental setting. Another important model for studying the host's response to infection has been experimental human malaria infection with live sporozoites or infected RBCs [159]. The advantages of this method include having the knowledge of the individuals pre-existing immunity and of the precise timing of infection. This allows

peripheral blood samples to be taken for analysis at defined time points post infection. Such controlled conditions may have a greater capacity for identification of immune components or mechanisms that correlate with protection. In addition, sporozoite infection enables multiple stages of the life-cycle to be studied in one infection, from inoculation of sporozoites in the skin by the mosquito through liver stage development and the early events of blood stage infection before treatment is initiated. Experimental sporozoite infection has recently become standardised and known as controlled human malaria infection (CHMI) [160, 161] and along with whole sporozoite immunisation, has been used to study vaccine efficacy and responses to infection. Infection with parasitized RBCs is also a valuable model as it allows the more accurate study of the blood stage of infection. The major advantage is the ability to give a controlled low dose of blood stage inoculum, which enables prolonged monitoring of the host's response before treatment has to be initiated [162, 163]. However limitations of all human models for dissecting the protective immune mechanisms include the inability to sample important organs and tissues where parasites sequester such as the liver, brain and spleen, and the inability to manipulate the immune response for mechanistic studies. Animal models are useful for this; though one main difficulty with the assessment of malaria vaccines pre-clinically is that *P. falciparum* does not infect small animal models or old world macaques. *P. falciparum* does infect new world non-human primates (NHPs), though it does not naturally infect these primates. Consequently the parasite will not have co-evolved with the primate immune system and the parasite biology will not reflect selection pressure and the resulting complex adaptations seen in natural infection. Simian models of malaria with parasites that naturally infect primates do also exist but all of these NHP models are expensive and difficult to perform because there are only limited facilities available worldwide that can carry out this research [159, 164, 165].

1.4.2 Murine models of malaria

Although *P. falciparum* does not infect small animal models a number of malaria parasites have been isolated from wild rodent hosts that are able to infect various strains of laboratory mice. These include *P. berghei, P. yoelii, P. chabaudi and P. vinckei* each consisting of several strains. The life-cycle of these parasites is similar to that of *P. falciparum* with the major difference being that the duration of liver stage development is typically only 48-52 hours. Importantly, these parasites do not naturally infect laboratory mice. So as highlighted previously with the NHP *P. falciparum* model, the parasites will not have co-evolved with the murine immune system, and will not be specifically adapted to the same degree as parasites in natural infection. Consequently none of these parasites are able to mimic all aspects of infection and pathogenesis observed with *P. falciparum* in humans. However various mouse and parasite strain combinations instead lead to different disease progressions and outcomes [166]. Although this has been criticised as a limitation of these models it may also be seen as a strength. This is because the variety of murine malaria presentations may reflect to some degree the diversity seen in human disease and the appropriate model should therefore be chosen for a particular research question [167].

Controlled infection in mice can be initiated by either mosquito bite or the injection of sporozoites dissected from mosquito salivary glands, usually intravenously. These methods can enable the analysis of the transmission of sporozoites from the mosquito to the mammalian host, the liver and blood stages of infection, and the transmission back to the mosquito, as well as disease outcomes. Infection can also be initiated by injection of infected RBCs to bypass the skin and liver stages for assessment of blood stage immunity. The use of a consistent and reproducible *P. berghei* infection model involving intravenous (i.v.) injection of parasites dissected

from *Anopheles* mosquito salivary glands was first described in the 1960s [168]. Since then murine models have been used extensively to analyse protective mechanisms and assess vaccine efficacy. Despite their limitations these models have contributed significantly to the basic understanding of the biology of parasites and the host immune responses. Moreover, new techniques have expanded the utility of these models further. Advances in technology have enabled the efficient and targeted incorporation of DNA into the parasite genome [169-171] and have resulted in the generation of transgenic parasites. Transgenic fluorescent and chemiluminescent parasites allow the monitoring of infection and analysis of host parasite interactions *in vivo* in real time [172-174]. The same technology has also generated transgenic *P. berghei or P. yoelii* parasites that can express *P. falciparum* (or *P. vivax*) antigens enabling the assessment of clinical vaccines in mice [175-179]. Mouse models are also particularly useful because mechanisms of immunity or pathogenesis can be studied in detail by manipulating aspects of the immune system. Immunological or physiological changes can be induced either by depleting lymphocytes populations with monoclonal antibodies or removing splenic T cells with a splenectomy. Antibodies and lymphocytes can also be transferred from immunised / experimental mice into naïve mice to assess their role in protection. In addition the availability of gene-deficient, transgenic or chimeric mice enables detailed dissection of mechanisms. Specifically, the development of liver-humanised mouse models has advantages for the *in vivo* study of the human liver stage of infection [180, 181]. These mouse models can be extremely informative but due to the differences with human malaria infections the results and observations should always be interpreted with caution and followed up with human *P. falciparum* studies [167, 182].

1.4.3 Identifying protective mechanisms and targets for vaccination

Vaccination can be targeted towards all three stages of the malaria life-cycle; however blocking infections at the pre-erythrocytic stage is an attractive strategy that has the potential to prevent clinical disease and also transmission. This stage represents a bottleneck in the parasite's life-cycle because in comparison to the blood stage there are only relatively low numbers of sporozoites or infected hepatocytes that need to be targeted. The focus of this thesis is on development of pre-erythrocytic stage vaccines and therefore the identification of sporozoite and liver stage protective mechanisms and potential vaccine targets are considered in detail below. The development of vaccines towards the other stages will not be discussed in this thesis but the importance of these vaccine strategies for elimination of malaria should not be overlooked.

Induction of pre-erythrocytic immunity was first demonstrated in domestic fowl in the 1940s [183] and repeated in 1966 [184]. In these studies radiation-inactivated *P. gallinaceum* sporozoites were used for immunisation resulting in partial protection against live bite challenge. The first evidence in mice was in 1967 where immunisation with radiation attenuated *P. berghei* sporozoites (RAS) prevented development of blood stage infection after i.v. challenge with 1000 live sporozoites [185]. Induction of sterile immunity by RAS was also demonstrated in humans a few years later [156-158, 186-190]. In these studies volunteers received repeated bites from a large number of heavily infectious irradiated mosquitoes at multiple time points followed by a live bite challenge with non-irradiated mosquitoes. These early results did not identify protective mechanisms but instead fuelled research into the pre-erythrocytic stages and the development of vaccines that may replicate this level of protective efficacy (described in the next section 1.5). Subsequent studies

attempted to understand the protective mechanisms involved and it became clear that a protective pre-erythrocytic vaccine would need to induce an immune response to prevent the sporozoites from invading hepatocytes or to target intra-hepatic parasites.

1.4.3.1 Antibody mediated protection

Sporozoite invasion could be blocked by antibodies that bind to and neutralise or opsonise the sporozoites. In addition antibodies could inhibit interactions with hepatocyte molecules required for invasion or molecules on other cells required for cell traversal and crossing of endothelial layers. Furthermore, anti-sporozoite antibodies could bind to and interfere with the intra-hepatic development of the liver stage parasites [191]. This immune response would likely need to consist of long lasting, high titre, high avidity antibodies with a memory response to confer long term protection. Evidence that anti-sporozoite antibodies can elicit protection has been described in many early studies, and antibodies to CS, one of the first identified and most abundant sporozoite surface antigens, have been implicated in protection and well-studied. In mice immunised with RAS, pre-challenge anti-sporozoite antibody titres have correlated with efficacy [192] and passive transfer of CS-specific serum or monoclonal antibodies is able to protect mice against *P. berghei* or *P. yoelii* challenge [193-195]. Primary hepatocytes and the human hepatoma cell line, HepG2-A16 (and more recently HC04 [196]) have been used to study sporozoite invasion *in vitro.* These studies have shown that monoclonal antibodies to the repeat region of the CS protein can completely block sporozoite attachment and invasion of hepatic cells [191, 197]. In addition antibodies to the CS protein conserved region, N-terminal of the repeat region, can block *P. falciparum* invasion of HepG2-A16 cells [198] and primary hepatocytes [199]. Serum from humans immunised with RAS and protected from challenge is also able to inhibit sporozoite invasion of HepG2-A16 cells [197], whereas serum from volunteers immunised with an anti-CS vaccine

blocked invasion of *P. falciparum* in HepG2-A16 cells, but not primary hepatocytes [200]. This suggested that other interactions or sporozoite invasion mechanisms in addition to the CS-mediated process may be able to overcome CS-specific antibody blocking. How CS blocks invasion is unclear, but anti-CS antibodies have been shown to inhibit motility resulting in no invasion, suggesting that the motility of sporozoites is a function required for invasion [201]. CS has also been shown to bind to HSPGs (heparin sulphate proteoglycans) on the surface of hepatocytes during invasion [202] and upon binding the CS protein is proteolytically cleaved by a cysteine protease. Inhibition of CS cleavage with a cysteine protease inhibitor completely prevents cell invasion *in vitro* [203]. CS has been the most characterised sporozoite antigen involved in motility and invasion, but the microneme proteins TRAP and AMA1 have also been well-studied. They are both type 1 transmembrane proteins that contain adhesive motifs and secretion of these proteins is upregulated after hepatocyte contact. TRAP is a transmembrane protein that links the actin-myosin motor with the extracellular substrate and is required to generate motility [204-206]. Deletion of TRAP from transgenic *P. berghei* sporozoites demonstrated it is essential for motility and invasion [207]. Antibodies to TRAP have been shown to inhibit motility and invasion *in vitro* [208-210] but do not prevent invasion *in vivo* [211], possibly because TRAP is stored in micronemes until host cell contact and therefore relatively inaccessible to antibodies [212]. Conversely, levels of TRAP specific antibodies in naturally exposed individuals have been shown to correlate with protection in the field [122, 125, 126], though this might simply be a marker of exposure. AMA1 was originally identified as a protein involved in erythrocyte invasion, but was recently discovered to be expressed on sporozoites as well. Antibodies to AMA1 have also been shown to inhibit sporozoite invasion of hepatocytes [124], though this has been less well studied. Other proteins thought to be involved in hepatocyte invasion have also been identified by transcription profiling or antibody inhibition studies such as P36p, P36 [213], SPATR (secreted protein with

altered thrombospondin repeat) [214], EBA 175 [215], STARP [123] and PfEMP3 [216], but further studies are needed to determine their role in invasion.

To migrate to the liver the sporozoites must traverse the capillary endothelial layer in the dermis to enter the blood vessels and also the endothelial layer of the liver sinusoid and this process involves both cell traversal mechanisms and motility. The motility and cell traversal during migration to the liver appears to be a sporozoite phenotype, distinct from motility and invasion of hepatocytes described above [217]. These different phenotypes may be mediated by the interaction of CS with HSPGs on the surface of cells. When CS binds to low-sulphate HSPGs present on cells such as those in the skin and epithelium, the sporozoite migrates through the cell; whereas when CS binds to highly-sulphated HSPGs on hepatocytes, sporozoites are activated for invasion [218]. Studies of murine malaria using various microscopy techniques have shown that sporozoites actively pass through KCs, endothelial cells and gaps in the sinusoidal lining [219, 220]. *In vitro s*tudies using *P. yoelii* have suggested that anti-sporozoite antibodies may bind to and influence the fate of the sporozoites leading to destruction by macrophages during migration [221]. In agreement, opsonisation and destruction of *P. berghei* sporozoites was enhanced in the presence of CS-specific serum *in vitro* [222]. Moreover, recent studies have investigated sporozoite suppression of Kupffer cell (KC) function during cell traversal. It was found that binding of CS to proteoglycans and LPR-1 (low-density lipoprotein receptor-related protein) on the surface of KCs was able to prevent the formation of reactive oxygen species [223]. In addition sporozoite contact with KCs has been shown to down-regulate inflammatory cytokines and up-regulate anti-inflammatory cytokines as well as inducing KC apoptosis [224]. Cell traversal mechanisms have therefore been proposed as methods to avoid phagocytic degradation during migration and intra-vital laser spinning-disk confocal microscopy has shown that cell traversal deficient sporozoites (SPECT2^{-} (sporozoite microneme

protein essential for traversal)) were rapidly trapped and degraded by KCs [16]. Cell traversal mechanisms are mediated by proteins such as SPECT, SPECT2 [225] and CelTOS (cell-traversal protein for ookinetes and sporozoites) [220] and targeted disruption of these sporozoite genes or antibodies specific for these mediators as well as for CS may inhibit cell traversal and enable destruction by KCs [226-228].

The ability of anti-sporozoite antibodies to affect the development of intra-hepatic parasites has also been shown *in vitro* with *P. falciparum and P. yoelii*. In cases where invasion was not completely blocked, liver stage maturation was altered, abnormal trophozoites were observed and schizonts did not fully develop [191, 229]. More recently the intra-hepatic parasite has been shown to be able to promote liver stage development by influencing the expression of host cell genes to create a favourable environment for parasite growth and to suppress some hepatocyte functions [230]. It has also been shown that CS is cleaved and transported to the cytoplasm and there it can inhibit host cell protein synthesis [231] and suppress NF-κB and therefore inflammation as well as inhibiting apoptosis of the hepatocyte [230, 232, 233].

1.4.3.2 T cell mediated protection

Targeting parasite infected hepatocytes would require a cellular immune response to recognise parasite antigens presented by MHC molecules on the hepatocyte surface. The importance of T cells in pre-erythrocytic immunity was demonstrated in early murine sporozoite infection studies where depletion of CD8+ T cells abolished protection conferred by immunisation with RAS [115, 234]. This was also shown later in studies using genetically attenuated *P. yoelii* sporozoites (GAPs) in BALB/c mice [235] and attenuated *P. knowlesi* sporozoites in primates [236]; where again protection was abolished with the depletion of CD8+ T cells. Further studies attempted to understand the mechanism of protective T cell immunity and a minimal

protective epitope was identified in *P. bergehi* CS and *P. yoelii* CS [237, 238]. Transfer of T cells specific for these epitopes into naïve mice conferred protection against sporozoite challenge. Although CS was found to be the immunodominant target of T cells in mice, it is likely that a protective pre-erythrocytic cellular response would target many antigens. In agreement with this, it was shown that protection can be elicited in the absence of CS-specific T cells in murine studies [239]. Protection induced to non-CS antigens was also demonstrated with transgenic parasites in which the endogenous CS was replaced with CS derived from another parasite species [240-242]. TRAP was the second T cell antigen to be well characterised and TRAP CD8+ T cells have been detected after *P. yoelii* RAS immunisation in BALB/c mice, moreover, transfer of TRAP-specific T cells into naïve mice can elicit protection [243, 244]. In another model with A/J mice, TRAP-specific CD4+ T cells were also shown to protect against *P. yoelii* challenge [245]. Potential T cell protective mechanisms have also been identified in human studies although their importance is still unclear. CD8+ T cells specific for *P. falciparum* CS and also TRAP have been found in the blood of volunteers immunised with RAS and also in natural immunity [246-249]. In addition, CS-specific CD4+ T cells were also detected in RAS immunised volunteers [250].

The protective CD8+ T cell response was further characterised by the development of a TCR transgenic mouse with CD8+ T cells specific for the immunodominant *P. yoelii* CS epitope [251]. Using this model studies have shown that *in vivo* presentation of the CS CD8+T cell epitope and activation of T cells occurs relatively quickly (8 - 48 hours) after inoculation [252], and development of protective CS-specific CD8+ T cells is dependent on CD4+ T cells secreting IL4 [253]. It was also shown that CS-specific IFNγ secreting CD8+ T cells can be primed by DCs that reside in the skin draining lymph nodes adjacent to the site of inoculation. These primed effector cells migrate to the liver and recognise sporozoite antigens

presented on the cell surface on infected hepatocytes and can eliminate the cell [254, 255]. Parasite infected hepatocytes transferred into naïve mice are also able to induce T cell mediated protection in *P. berghei* and *P. yoelii* models. This suggests T cells can be induced by antigen presented by hepatocytes [256], and antigen presentation by hepatocytes was further demonstrated *in vitro* [257]. Other studies have also shown that after RAS and GAP i.v. immunisation, effector T cells can also be induced to antigens expressed by liver resident DCs [258]. Defining the effector mechanisms used by CD8+ and CD4+ T cells has not been simple, and a number of mechanisms are involved that likely vary between models. Many studies have highlighted the importance of CD8+ and CD4+ T cells and IFNγ [259, 260] and mechanistic studies have shown protection against *P. berghei* and *P. yoelii* liver stages to be dependent on IFNγ and TNF, and in some cases perforin [259]. In contrast however, inhibition of IFNγ or TNF in a study where mice were immunised with RAS did not abolish protection against *P. yoelii* challenge [238]. The same was also seen in a study where mice were still protected against sporozoite challenge with adoptive transfer of IFNγ deficient CS-specific CD8+ T cells [261]. Direct cytotoxic function has been demonstrated by CD8+ T cells from mice immunised with GAPs [262] and CD4+ T cells from humans immunised with RAS. These T cells have been shown to induce apoptosis of infected hepatocytes via the perforin/granzyme mechanisms [263]. More recently cytotoxic T cells to infected RBCs have also been implicated in sterile protection in humans induced by inoculation with live sporozoites under chloroquine cover. In this study levels of cytotoxic CD4+ and CD8+ T cells were higher in protected verses unprotected volunteers [264]. Though there has been clear evidence for cell mediated protection and potential protective mechanisms and antigens have been identified, it has been proposed that a defined extremely high threshold of T cells will be required for sterilising immunity [265]. Induction of such high responses may be a challenge for the currently available vaccination approaches. Moreover, most studies have only

focused on protective CS and TRAP cellular responses, though with the identification of many new antigens that are expressed during the liver stage [18, 266-269] other protective mechanisms may be discovered.

In summary, antibodies and T cells have been shown to be important in pre-erythrocytic protection in a number of murine models although the association with protection in human studies is less clearly defined. Antibodies to sporozoite antigens have the ability to interrupt hepatocyte invasion with various mechanisms and also interfere with the intra-hepatic development of the parasites. Sporozoite antigen-specific CD8+ T cells have been shown to act largely through IFNγ and TNF or perforin/granzyme mediated mechanisms. Furthermore, sporozoite-specific CD4+ T cells are proposed to be important for providing help for the development of a protective CD8+ T cell response, can be cytotoxic themselves and can provide help for optimal antibody responses [270]. Being able to elicit the correct type of protective immune response by vaccination may require help from adjuvants.

1.4.4 Enhancing immune responses with adjuvants

Vaccine adjuvants are substances that are able to enhance the immunogenicity of antigens during vaccination. A large number of adjuvants have been assessed in animal models though they have not all been suitable for use in humans due to reactogenicity. Only a few therefore have been licensed for humans including Alum, MF59, AS04, AS03 and virosomes [271]. Adjuvants appear to enhance immunogenicity by mimicking properties of live pathogens that are known to stimulate the immune response, although mechanisms of action are poorly understood. They generally boost or direct the type of innate immune response generated and in turn produce a particular type of adaptive immune response. Broadly, adjuvants can be described as immunostimulants or delivery systems and can act by promoting up-take of antigen by APCs, up-regulating cytokines, up-regulating MHC class II and co-stimulatory molecule expression, and promoting migration of APCs to the lymph nodes. Adjuvants are becoming increasingly important for vaccination as many new vaccines are composed of highly pure recombinant antigens that are poorly immunogenic on their own due to a lack of exogenous immune activating components [272]. Outlined here are the adjuvants that have been used in this study although many other adjuvants are currently under investigation for malaria vaccine development [273, 274].

1.4.4.1 Alhydrogel

Alhydrogel belongs to the alum (insoluble aluminium salt) group which includes adjuvants based on aluminium hydroxide, aluminium phosphate or aluminium potassium sulphate. The adjuvant property of alum was first discovered in the 1920s and they were the first adjuvants approved for use in humans [275]. They are components of many licensed human vaccines including diphtheria-tentanus-pertussis, hepatitis B, human papilloma virus and *Haemophilus influenza* [276, 277] and have been used extensively for clinical evaluation of malaria vaccines [273].

Alhydrogel is able to induce a moderate Th2 type antibody response though its mechanism of action is not fully understood. It has been proposed that alum increases antigen persistence by the slow release of antigen from the insoluble salt particles at the injection site. This is termed the depot effect and enhances uptake and activation of APCs [278]. More recently it has been shown that alum can directly stimulate the immune system and up-regulate cytokines and immune cell function and through activation of the inflammasome complex via the NALP3 pathway, although it is unclear if this action contributes to the adjuvanticity of alum [279].

1.4.4.2 MF59

There are two types of emulsion-based adjuvant, either water in oil (w/o) or oil in water (o/w) emulsions. MF59 is an o/w nano-emulsion composed of squalene drops (approximately 160nm in size) that are stabilised with Tween 80 and Span 85 and MF59 is a component of a licensed influenza vaccine [280]. MF59 elicits a broader more balanced and more durable antibody response and also stimulates a cellular response. The cellular response induced in mice is a CD4+ T helper Th2 type response thought to be important for the improved quality of the antibodies. MF59 has not been shown to induce Th1 cellular responses. MF59 was initially thought to act through a depot effect but a number of studies have shown that this does not appear to be the case [281]. More recently MF59 has been shown to promote rapid recruitment of monocyte, granulocytes and DCs to the injection site [282] increase antigen uptake [283] and promote efficient transport of immune cells from the injection site to the lymph nodes [281, 282, 284]. In addition MF59 up-regulates expression of genes associated with antigen presentation and leucocyte migration and also cytokines and cytokine receptors [285]. The activation and enhancement of all of these factors is thought to increase the likelihood of interactions between antigen and APCs and enhance transport to lymph nodes and T cell priming.

1.4.4.3 ISCOMS and saponin based adjuvants

Immunostimulatory complexes (ISCOMs) are particulate antigen delivery systems composed of cholesterol, phospholipid and saponin. They consist of cage-like structures about 40nm in diameter formed from the strong affinity between the saponin and cholesterol [286, 287]. They can be grouped into two types, either ISCOMs or ISCOMATRIX formulations. The difference being that with traditional ISCOMs the antigen was incorporated into the cage-like structure; whereas it was later found that antigen incorporation was not critical for the ISCOM properties and antigen could simply be mixed with ISCOMs resulting in similar efficacy (the ISCOMATRIX and Matrix M formulations) [288]. All ISCOMS function as delivery systems due to their size and particulate nature, and therefore promote efficient uptake by APCs and are rapidly removed from the injection site [289, 290]. They also contain saponins which have inherent adjuvant properties and may also promote uptake by targeting the DEC-205 receptor in the surface of DCs enhancing T cell priming [291, 292]. Saponins are isolated from the bark of the *Quillaja saponaria* Molina tree and purification yields distinctive fractions with different characteristics and immunostimulatory properties [293, 294]. Matrix M and Abisco-100, used throughout this study are composed of particles formed from two saponin fractions Fraction A and Fraction C which are mixed at defined ratios to create the final adjuvants [295]. Both adjuvants have been shown to induce a balanced very high antibody response as well as multifunctional and cytotoxic CD4+ and CD8+ T cells in mice [289, 295-298]. AS01 is another saponin based adjuvant but it is not an ISCOM; it contains liposomes, monophosphoryl lipid A (MPL) and QS21. QS21 is a specific purified fraction of saponin which is also a component of Fraction C of Matrix M. MPL is non-toxic derivative of lipopolysaccharide endotoxin and a strong TLR4 agonist and therefore leads to induction of Th1 type responses including the induction of pro-inflammatory cytokines, antibodies and in mice cytotoxic T cells [299]. The AS01 formulation is being used for the development of malaria,

tuberculosis and HIV vaccines [273] and during development of the malaria vaccine RTS,S, it has been shown to induce superior antibody responses, multifunctional T CD4+ cells and efficacy compared to other proprietary GSK adjuvants [300].

1.4.4.4 Carbopol

Carbopol is a synthetic acrylic acid polymer, also known as a polyanionic carbomer. Carbomers have been used in pharmaceutical products for a number of years for controlled release drug formulations and bio-adhesives for topical or trans-mucosal drug delivery [301]. The products within the carbopol polymer family are chemically similar but vary mainly in there crosslinking ability. They are not water soluble but absorb water and swell into hydrated spheres. The crosslinking directly determines the structure of the polymer and its viscosity and flexibility, and this is thought to influence its ability to have a depot effect and therefore its adjuvanticity. Their use as vaccine adjuvants has been described in a number of animal vaccines [302-305] and they have been shown to enhance antibody responses. More recently in small animals for pre-clinical vaccine assessment they have been shown to induce Th1 and Th2 type T cells responses. Furthermore, when carbopol-971P is used in combination with MF59 it has induced a more protective immune response, characterised by an increase in high avidity antibodies and an increase in virus neutralising antibodies [306, 307]. The mechanism of action is not understood, but it is thought that the properties of carbopol will increase depot formation and prolong antigen exposure to APCs and it may extend the activity of MF59 at the injection site [307].

1.5 Current status of pre-erythrocytic malaria vaccine development

Development of malaria vaccines has accelerated in the last decade with the increased funding and support for malaria elimination tools. It has also been aided by the advent of new technologies allowing the rapid discovery of new antigens as well as advances in technologies for the efficient production of candidate vaccines and novel vaccine adjuvants. However there are still only a few vaccine candidates to have reached clinical trials and only one to have entered phase 3 testing. Pre-erythrocytic vaccines have been the most successful vaccines developed and can be broadly divided into groups by the vaccination strategy employed. They can be either whole sporozoite vaccines, or subunit vaccines which include recombinant proteins or particles, plasmid DNA and recombinant viral vectored vaccines. The following sections detail the pre-erythrocytic malaria vaccines that are currently under clinical evaluation.

1.5.1 Whole sporozoite vaccines

As introduced in the earlier sections, whole sporozoite vaccination approaches have been able to elicit high levels of sterile protection at the pre-erythrocytic stage [114, 116, 308-310]. This was achieved initially with radiation attenuated sporozoites but using the same principle, sporozoites have also been attenuated by other means. This includes inoculating humans whilst under chemoprophylaxis to prevent the development of the sporozoites beyond the pre-erythrocytic stage and genetic manipulation that results in the arrest of parasite development at the liver stage.

1.5.1.1 Radiation attenuated sporozoite vaccine

Radiation attenuation disrupts the DNA of the sporozoites and initial studies aimed to identify the level of radiation required to reproducibly generate viable sporozoites

that do not progress to a blood stage infection. These sporozoites are infectious, invade hepatocytes and begin a liver stage development but do not replicate. They do however need to be viable as heat killed sporozoites or over-irradiated sporozoites are not protective and do not induce CD8+ T cell responses. The RAS model has been the most extensively studied whole sporozoite vaccine approach and it was established early on in a number of studies that humans could be protected against CHMI by bites from irradiated mosquitoes [156-158, 186-190]. This research continued in the late 1980s and 1990s and these RAS studies were analysed and published in a review in 2002 [114]. This review highlighted that consistent protection was only achieved after a minimum of 1000 bites from sporozoite infected irradiated mosquitoes and that this efficacy was only short lived (up to 42 weeks) [114]. These studies and the further analysis of protective mechanisms that accompanied them were extremely encouraging. But it was evident that very large numbers of mosquito bites were required for protection and the impossibility of using irradiated mosquitoes as a deployable vaccine could not be ignored. Impressively, the ability to manufacture cryopreserved radiation attenuated sporozoites (PfSPZ) that are aseptic, metabolically active and meet regulatory standards was achieved by Sanaria in 2009 [311]. A first-in-man dose escalation clinical trial was performed with this vaccine using repeated inoculation of large numbers of RAS by either subcutaneous (s.c.) or intradermal (i.d.) immunisation [312]. The vaccine was shown to be safe and there was no evidence of breakthrough infections, however the immunogenicity was poor and only two of 16 volunteers in one of the groups were protected. It was hypothesised that the route of immunisation was responsible for the low level of protection and subsequent studies in NHPs showed that only the i.v. route, not s.c. could induce detectable parasite-specific T cell responses. In addition, anti-sporozoite antibody responses were also higher with i.v. immunisation [312]. Similar studies in mice confirmed these results and also showed that 7-10 times more *P. yoelii* RAS were required with s.c. or i.d.

immunisation compared to i.v. to achieve high levels of protection. PfSPZ given i.v. was therefore assessed in humans in a dose escalation trial and complete sterile protection 6/6 was seen after five doses of 1.35 x 10^5 PfSPZ. Protection and also immunogenicity were shown to be dose dependant [313]. Reducing the number of doses of PfSPZ to four decreased protection to 66% (6/9), lowering the dose to 3 x 10^4 PfSPZ reduced sterile protection to 11% (1/9) and there was no protection with the dose of 7.5 x 10^3 PfSPZ. This ability to induce sterile protection in humans against homologous live bite CHMI with needle and syringe administration of PfSPZ is very exciting. However further studies will need to assess the longevity of protection, and protection against heterologous challenge in both non-immune and semi-immune populations. Essential questions need to be answered about the importance of the size of the initial immunisation dose for development of protective T cell responses, and the benefit of multiple immunisations [314, 315]. Importantly also, the logistics of deploying a vaccine which requires long term liquid nitrogen storage as well as the proposed high cost of this vaccine will need to be addressed.

1.5.1.2 Live sporozoite immunisation under chemoprophylaxis

Induction of protective immunity by immunisation under chloroquine prophylaxis with live sporozoites delivered by mosquito bite (CPS) was first demonstrated in mice in 1977 [316]. This study indicated that protection was directed towards the pre-erythrocytic stage as mice were only protected from challenge with sporozoites and not parasite infected erythrocytes. There was very little research using this vaccination strategy again until 2004 when it was shown that this protection was mediated by CD4+ and CD8+ T cells through IFNγ and nitric oxide mechanisms [317]. The CPS vaccine was then successfully tested in a small number of human volunteers in 2009 [308]. Ten volunteers and 5 controls received a standard chloroquine prophylactic regimen for 13 weeks. During this time the 10 vaccine group volunteers received exposure to bites from *P. falciparum* infected mosquitoes

on 3 occasions with a total of 36-45 bites. The control volunteers also received bites from un-infected mosquitoes at the same time. Eight weeks after the last immunisation and four weeks after the last chloroquine dose, all volunteers received standard sporozoite challenge (CHMI). Complete sterile protection was seen in all vaccinated volunteers, and all controls developed blood-stage parasitaemia during the normal time frame after challenge (7-11 days). Interestingly this immunity is also relatively long lived; 28 months later four out of six re-challenged volunteers were completely protected against CHMI with a delay to blood stage parasitaemia detected in the remaining two volunteers [309]. Vaccination by CPS appears to elicit more effective protection against homologous challenge than RAS vaccination, as fewer bites are required to achieve complete protection. This method, unlike RAS, allows the parasites to complete liver stage development and emerge from the liver and it is only then that the chloroquine is able to eliminate the parasites. This may be an important difference between these two strategies and it may be that exposure to this broader range of liver stage and early blood stage antigens aids in the more easily acquired development of immunity. It will now also be interesting to see if similar levels of protection can be achieved by inoculation of the viable cryopreserved sporozoites (non-irradiated) via needle and syringe (PfSPZ C-VAC) [318]. A further study also demonstrated that this protective immunity in humans was directed against the pre-erythrocytic stage. In this study 100% sterile efficacy against sporozoite CHMI was again seen in five CPS vaccinated volunteers, however none of the nine CPS vaccinated volunteers who were challenged with blood stage parasites were protected [319]. Analysis of protective immune responses following CPS has shown that CD4+ T cells expressing the degranulation marker CD107a and CD8+ T cells producing granzyme B that recognise infected RBCs were significantly higher in completely protected volunteers, suggesting a role for cytotoxic T cells in protection [320]. In addition, IgG from CPS immunised volunteers has been shown to reduce sporozoite motility and liver stage infection *in vitro* in sporozoite gliding

assays, and *in vivo* in a human liver chimeric mouse model [264]. Although CPS appears to elicit more efficient longer lasting protection than RAS, the same questions and challenges remain for the deployment of such a vaccine.

1.5.1.3 Genetically attenuated parasites

Genetically attenuated parasites were developed as an alternative method for attenuating sporozoites for whole parasite vaccination, with the aim of achieving high levels of protective efficacy similar to RAS. Parasites are attenuated through the targeted disruption of different genes that are essential for development during or beyond the liver stage and this was first demonstrated in murine models in 2005 [321-323]. A large number of different GAPs have been generated by the disruption of different genes that enable arrest of parasite development at defined points during the liver stage [310]. The recent focus has been on development of GAPs that arrest late in liver stage, which allows expression of a broader range of pre-erythrocytic antigens, considered to be an advantage of this method over RAS [324, 325]. The difficulty in producing such late arresting parasites has been breakthrough infections, however, studies have indicated that disrupting multiple genes from potentially independent pathways may overcome this [326]. The first PfGAP which entered a first-in-man clinical trial in 2010 was generated by the deletion of the P52 and P36 genes in the NF54 wild-type stain of *P. falciparum* (Pfp52^{-}/p36^{-}GAP). Immunisation was safe and well tolerated but exposure to >200 bites from Pf52^{-}/p36^{-}GAP infected mosquitoes lead to a breakthrough infection in one of six volunteers, indicating incomplete attenuation [327]. However, encouragingly, all volunteers developed antibodies to CS that were able to inhibit sporozoite invasion of hepatocytes [328] as well as cytokine producing sporozoite-specific T cells [327]. Challenges of generating GAPs suitable for human vaccination are the same as those for the development of RAS or CPS. But in addition they need to obtain complete attenuation of the parasite to prevent breakthrough infections, by deleting

genes that are essential only for liver stage development and also ensure reversion to wild-type is not possible [310].

Whole sporozoite vaccines are the most protective malaria vaccination approaches assessed to date and can elicit high levels of sterile efficacy in mice, NHPs and humans. This has been demonstrated most successfully with CPS immunisation where complete protection is induced in humans by <45 sporozoite infected mosquito bites. This is significantly less than is required for RAS induced immunity, and complete protection remained high at 66%, 28 months later. Although encouraging, these studies so far have only assessed protection against an homologous strain in non-immune adults, whereas the target would be cross-strain protection induced in semi-immune adults and pre-exposed infants. In these endemic populations it is possible that pre-existing immunity may limit the development of a protective immune response [329]. One significant hurdle for the development of these vaccine strategies has been overcome by Sanaria who have developed a method for cryopreserving sporozoites that remain viable for use as a vaccine. However, studies also indicate that the route of administration, either mosquito bite or i.v., has been essential for the development of protective immunity. Thus the induction of protection by administering the vaccines as cryopreserved sporozoites by needle and syringe via a more deployable route needs to be fully assessed. In addition, the logistics of deploying a vaccine requiring liquid nitrogen storage will also need to be overcome if it is to be used for malaria elimination campaigns.

1.5.2 Subunit vaccines

Development of pre-erythrocytic subunit vaccines has largely been targeted towards the CS antigen due to the large amount of research indicating it can mediate protective immunity as described in section 1.4.3, and also toward the second most studied pre-erythrocytic antigen, TRAP. A list of the current vaccines in clinical development is provided by the WHO [330] and the two most advanced vaccines are the CS-based particle vaccine RTS,S, and the viral vector regimen, ChAd63 - MVA ME.TRAP. Relatively few other pre-erythrocytic antigens have been assessed clinically as vaccine candidates; they include EXP1, CelTOS, LSA1, LSA3 and STARP either as vaccines themselves or as components of a multi-antigen vaccine.

1.5.2.1 Recombinant protein and particle vaccines

Recombinant proteins are generally poor immunogens and are normally delivered in adjuvant to improve their immunogenicity. A number of recombinant protein or particle pre-erythrocytic vaccine candidates have been assessed in clinical trials over the years but very few have been successful. For example in a recent phase 1 trial a Hepatitis B Core virus-like particle expressing CS (ICC-1132) was found to be poorly immunogenic [331]; and in phase 1/2a trials the CS long synthetic peptide, PfCS102 [332] and the LSA1 recombinant protein [333] elicited no protection at all. Another vaccine candidate only recently entering clinical trials is the PfCelTOS recombinant protein vaccine which is being assessed in GLA-SE adjuvant. It induced sterile protection in pre-clinical models, but lack of efficacy in humans was recently reported in an oral presentation at the American Society of Tropical Medicine and Hygiene meeting in 2013. The most successful recombinant protein or particle vaccine has been RTS,S, which was initially developed by GlaxoSmithKline (GSK) in 1987 and is still in clinical development today [334, 335]. RTS,S is composed of a CS fusion protein that is co-expressed in yeast with the hepatitis B surface antigen (HBsAg) and these two proteins form a 22nm lipid-protein particle [336]. The CS fusion

protein is formed from the C-terminal region of CS which contains 19 NANP repeats (“R”) (the predominant B cell epitope in CS) [337, 338] and some T cell epitopes in the C-terminal flanking region (“T”) [246, 339], and this is all fused to the HBsAg (“S”) (Figure 1.4).

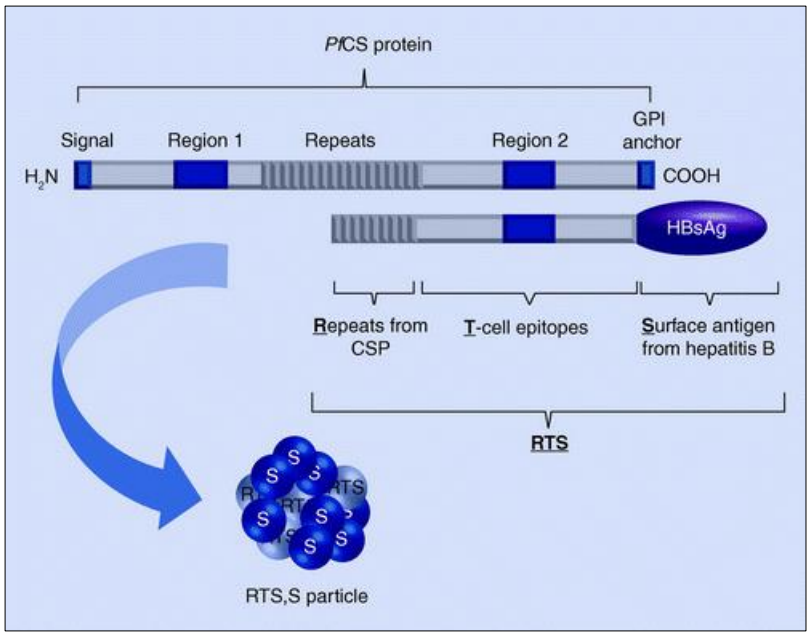


Figure 1.4 RTS,S particle composition

Image taken from [336] that illustrates the composition of the RTS fusion protein and the formation of the RTS,S particle.

RTS,S is formulated with adjuvant and the current clinical formulation is with AS01, which is composed of monophosphoryl lipid A (MPL), the saponin QS21 and lysosomes. However, when RTS,S was first assessed in humans in 1995 it was formulated with either alum or alum plus MPL. In this trial none of the volunteers who received RTS,S with alum were protected from CHMI, and only 25% (2/8) were protected with the RTS,S alum-MPL formulation [335]. This indicated that the adjuvant may be critical for the induction of a protective immune response. Trials went on to assess RTS,S in various adjuvant formulations and the highest protective efficacy was reported in 1997. These volunteers received RTS,S formulated in an

o/w emulsion with MPL and QS21, now known as AS02. In this trial 86% (6/7) of volunteers were completely protected against CHMI, but surprisingly this level of efficacy has never been repeated in a clinical trial since [340]. Interestingly, volunteers in this trial received a 50µg dose of RTS,S on day 0 and again at one month. They were scheduled to receive the same dose at six months, but due to reactogenicity the dose was reduced to 10µg of RTS,S. This is a key difference between this trial and all subsequent trials, so it is now being investigated if this reduction in the final dose of RTS,S had an effect on improving protective efficacy [330]. A number of phase 1 and 2 trials followed, using RTS,S in AS02 in malaria-naïve volunteers investigating various doses and immunisation schedules, as well as liquid verses lyophilised formulations [341-343], and efficacy was around 30-50%. A head to head trial was then carried out with RTS,S/AS02 compared to the newer RTS,S/AS01 formulation and resulted in 32% and 50% efficacy, respectively. The AS01 formulation was thus taken forward thereafter. In addition to this high level of sterile protection, around 20% of the volunteers showed non-sterilising efficacy [300]. This is seen as a delay to the development of blood stage parasitaemia after challenge compared to unvaccinated controls. RTS,S has also been assessed in a number of phase 1 and 2 trials in African adults and children, and in these trials efficacy is generally determined by time-to-event analyses within a set time period. These trials showed that in endemic populations RTS,S could reduce malaria episodes by between 30 - 50% [344, 345] and RTS,S/AS01E (AS01E being the paediatric formulation) is now the first malaria vaccine to enter a phase 3 trial [346, 347]. This trial involves 15,460 children at 11 sites in seven African countries and the initial reports indicate that during the first 12 month follow up, RTS,S/AS01 conferred 50% protection in 5-17 month old children, and 30% in 6-12 month old infants [348, 349]. This trial is ongoing and the effect of a booster vaccine at 18 months will be assessed as well as the duration of efficacy. Although a four year follow up study looking at long term efficacy in a previous trial has shown that efficacy declines over

time [350], and a pooled analysis has shown that efficacy reduces to zero, 3 years after vaccination [344]. So although achieving efficacy in the target population is encouraging, it is still well below the goals set by the Malaria Vaccine Technology Roadmap and development of a more effective vaccine is still a major priority.

1.5.2.2 DNA and viral-vectored vaccines

DNA and viral vectored vaccines both work by delivering genes into the host cells that are subsequently transcribed and translated, and the antigen is expressed within the host. These vaccines are therefore very effective at inducing T cell responses as the endogenous protein can be processed and presented on MHC molecules. In addition the virus itself also acts as an adjuvant and enhances the immune response [20]. Both DNA and viral vectors appear to be most effective when used in heterologous prime-boost regimens, not as single vectors [351]. With this approach the same antigen or antigenic insert is delivered using two different vectors in a distinct order. A number of different vectors have been clinically assessed for use in the prime-boost regimens including poxviruses such as NYVAC, the fowlpox FP9, and modified vaccinia virus Ankara (MVA) [352]; and adenoviruses including human adenovirus 5 (Ad5) and 35 (Ad35), and the simian adenovirus 63 (ChAd63) [351, 353]. The majority of the research with the prime-boost approach has been with the TRAP antigen and the most advanced viral vector regimen in development is ChAd63 - MVA prime-boost expressing the ME.TRAP insert. This combination and order of viral vectors encoding the same antigenic insert elicited superior immunogenicity when compared to other combinations. The ME.TRAP insert encodes the TRAP antigen fused to a multi-epitope string which includes a number of T cell epitopes from the pre-erythrocytic antigens LSA1, CS, STARP, LSA3 and EXP1 [354, 355]. At the Jenner Institute, Phase 1 and 2a trials of this vaccine regimen have been recently completed and it has been shown to be safe and immunogenic. Furthermore, it was shown to be partially protective and elicited sterile

efficacy in 21% (3/14) of volunteer and it delayed the time to blood stage parasitaemia in 36% (5/14). These results will be reported fully in Chapter 3 and have also been recently published [356, 357]. This vaccine regimen has now also entered clinical trials in Africa, it has been shown to be safe and immunogenic in adults [358, 359] and is now being assessed in children and infants. This same prime-boost regimen has also recently been used by our group to assess the viral vectors expressing the CS antigen, ChAd63 - MVA CS, in a phase 1 and 2a trials (unpublished). It has been shown to be safe and immunogenic but only protected one out of 15 vaccinated volunteers. CS is also currently under investigation in phase 1/2a trials encoded by the human adenovirus 35 vector, (Ad35 CS), and is being used in a prime-boost regimen with RTS,S/AS01. The regimen, Ad35CS followed by two immunisations of RTS,S/AS01 at monthly intervals (A R R) is being compared to three immunisations of RTS,S/AS01 (R R R), and although currently unpublished it was recently reported that efficacy was 44% and 52.4% respectively [360].

1.6 Thesis aims and outline

Whilst there has been considerable progress in the development of a malaria vaccine, there is still no deployable highly efficacious vaccine available. The most effective vaccine strategies are the use of whole sporozoites and data emerging from these studies in humans indicates a broad immune response targeting the pre-erythrocytic stage is required for protection. Although promising, these vaccine strategies face many logistical challenges which some feel may be insurmountable in the near future. Pre-erythrocytic stage subunit vaccines are therefore being developed to induce broader immune responses. The pre-erythrocytic stage of malaria infection involves both intra-cellular and extracellular stages and hence both humoral and cellular immune responses are able to mediate protection in various models. However despite success in pre-clinical studies, vaccination targeted towards one pre-erythrocytic antigen has yet to yield high levels of protective efficacy in humans. The two leading vaccines in clinical development, RTS,S and ChAd63 – MVA ME.TRAP, each target a different pre-erythrocytic stage antigen. They also induce different arms of the immune response and they both currently only elicit suboptimal protection. Therefore, one approach to improve vaccine efficacy would be to combine these vaccines together. This would enable induction of high titres of antibodies and high frequencies of T cells targeting two pre-erythrocytic antigens. At the time this study began there was no prospect of accessing RTS,S/AS01B from GSK to undertake such combination studies, hence the major aim of this project was to produce and evaluate an improved version of RTS,S that can be assessed in combination with the ChAd63 - MVA ME.TRAP prime-boost regimen.

1.6.1 Aims

1) To assess in humans the immunogenicity and efficacy of ChAd63 - MVA ME.TRAP, a T cell inducing viral vector vaccine regimen that targets the liver stage of infection.

2) To design and produce an improved RTS,S like particle vaccine, here called R21, for use in a multi-component malaria vaccine strategy with viral vector vaccines with the aim of increasing efficacy.

3) To assess the immunogenicity of R21, used alone and in a range of adjuvants to determine which adjuvants enhance the induction of superior cellular and humoral immune responses.

4) To determine if the particle vaccine, R21 can be successfully combined with a viral vector vaccine regimen without immunological interference.

5) To assess the protective efficacy of CS-based particle vaccines (R21 and RTS,S) and the viral vector vaccine regimen in mice using transgenic parasites. Moreover, to determine if these vaccine regimens combined together enhance protective efficacy.

1.6.2 Outline

Chapter 2 describes the materials and methods employed throughout this study.

Chapter 3 investigates the immunogenicity and efficacy of a liver stage ME.TRAP based viral vector vaccine regimen in humans.

Chapter 4 describes the design, generation and effective purification of a CS-based particle vaccine, R21, for use in a multi-component malaria vaccine strategy with the viral vector vaccine regimen assessed in Chapter 3.

Chapter 5 investigates the immunogenicity of R21 administered alone and with a range of adjuvants in BALB/c mice. It also investigates the immunogenicity of the ME.TRAP viral vector vaccine regimen in BALB/c mice in a range of adjuvants and assesses whether it can be combined with R21 without affecting the immunogenicity of either vaccine regimen.

Chapter 6 investigates the protective efficacy of CS-based particles (R21 and RTS,S) and TRAP based viral vector vaccine regimens using a transgenic parasites in mice. It also investigates whether protective efficacy can be enhanced with combining vaccine regimens.

Chapter 7 describes and summarises the results of this study and discusses future directions.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Table 2.1 Details of the commercially available reagents used in this study.

Material	**Supplier**	**Catalogue Number**
0.1mm electroporation cuvettes	Bio-Rad	165-2083
0.2µm syringe filter	Sartorius	16534
12-well tissue culture plates	Appleton Woods	BC011
15mL centrifuge tubes	Fisher Scientific	FB55950
2-β Mercaptoethanol, 50µm	Gibco	31350-010
2ml syringes	N-Care Products	M0185
50mL syringes	N-Care Products	M0865
50mL centrifuge tubes	Greiner	227261
5ml polystyrene tubes	VWR international	734-0001
5ml syringes	N-Care Products	M2187
60mm petri dishes	Corning	430166
6-well tissue culture plates	Fisher Scientific	TKT-520-030T
70µm cell strainers	BD Biosciences	352350
8-well microscope slides	VWR International	631-0451
96-well U bottom plates	VWR International	734-0027
Agar	Sigma Aldrich	A6686
Agarose	Sigma Aldrich	A9539
Ampicillin	Sigma Aldrich	A5354
Antarctic Phosphatase	New England Biolabs	M0289L
Ammonium chloride (NH_4Cl) 0.15M	Sigma Aldrich	A4514
Alexa Flour-488 goat anti-mouse IgG	Life Technologies	A11008
Alexa Flour-488 goat anti-human IgG	Life Technologies	A11013
Alkaline Phosphatase-AffiniPure Donkey Anti-Mouse IgG (H+L)	Stratech Scientific Ltd	715-055-150
Anti-CD28	BD Biosciences	340975
Anti-CD49d	BD Biosciences	340976
Anti-human CCR7 APC	eBioscience	17-1979-41
Anti-human CD14 Pacific blue	Invitrogen	MHCD1428

Anti-human CD19 Pacific blue	Invitrogen	MHCD1928
Anti-human CD27 QD605	Invitrogen	Q10065
Anti-human CD3 Alexa Fluor 700	eBioscience	56-0038-82
Anti-human CD3 PE Cy5	eBioscience	15-0038-73
Anti-human CD4 APC	eBioscience	17-0049-73
Anti-human CD4 QD655	Invitrogen	Q10007
Anti-human CD45RO ECD	Beckman Coulter	IM2712U
Anti-human CD57 QD565	Custom	N/A
Anti-human CD8 APC-AF700	eBioscience	56-0038-82
Anti-human IFNγ FITC	eBioscience	11-7319-82
Anti-human IL2 PE	eBioscience	12-7029-82
Anti-human TNF PE Cy7	eBioscience	25-7349-82
Anti-mouse CD8α PerCP-Cy5.5	BD Biosciences	551162
Anti-mouse CD8α eFluor450	eBioscience	48-0081-80
Anti-mouse IFNγ APC	eBioscience	17-7311-82
Anti-mouse interleukin-2 (IL2)	eBioscience	25-7021-80
Anti-mouse TNF FITC	eBioscience	48-7321-80
Benzonase® Nuclease	Novagen	70664-3
Brefeldin A	eBioscience	00-4506-51
Bovine Serum Albumin (BSA)	PAA Laboratories	K41-001
Carbonate-bicarbonate buffer	Sigma Aldrich	C3041
Caesium Chloride	Sigma Aldrich	D8537
Cluster tubes	Corning	4401
Cryogenic vials	Star Lab	E3110-6122
CD16/32 (Fc block)	eBioscience	14-0161-82
Cytofix/Cytoperm™ fixation/permeabilization kit	BD Biosciences	555028
ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa)	New England Biolabs	P7711S
DH5α sub-cloning efficiency cells	Invitrogen	18265-017
Diethanolamine buffer	Pierce	34064
Dimethyl sulphoxide (DMSO)	Sigma Aldrich	D2650
Dried skimmed milk powder	Waitrose	N/A
Dulbecco's PBS	Sigma Aldrich	D8537
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich	D6546
Dulbecco's Phosphate Buffered Saline	Sigma Aldrich	D8537
ELISpot colour development kit	Bio-Rad	170-6432
ELISpot developer – BCIP/NBT (plus) solution	Europa Bioproducts	M0711A
Endotoxin Free water	Sigma Aldrich	W3500
Ethanol	Sigma Aldrich	32221

Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E7889
Fetal Calf Serum (FCS)	Sigma Aldrich	F2442
Flask vented baffled bottom 125mL	Fisher Scientific	TKV-137-300C
Flask vented baffled bottom 1000mL	Fisher Scientific	TKV-137-330Q
Flask vented baffled bottom 2000mL	Fisher Scientific	TKV-137-340N
Fructose	Sigma Aldrich	F0127
Giemsa	Sigma Aldrich	GS1L
Glass beads (acid washed 0.425-600µm)	Sigma Aldrich	G8772
Glycerol	Sigma Aldrich	G5516-1L
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich	A3562
Goat anti-human whole IgG alkaline phosphatase conjugate	Sigma Aldrich	A3187
Golgi Plug	BD Biosciences	555028
Halt Protease Inhibitor	Fisher Scientific	PN78430
Heparin	Sigma Aldrich	H4784
HEPES	Sigma Aldrich	H3375
HiPrep 16/60 Sephacryl S-500 HR	GE Healthcare	28-9356-06
Human IFNγ ELISpot kit	Mabtech	3420-2A
Hydrochloric Acid (HCl) [1M]	Sigma Aldrich	S7653
Imperial protein Stain Thermo Scientific Pierce	Fisher Scientific	PN24615
Immersion Oil	Sigma-Aldrich	10890
Isofluorane	Oxford University Veterinary Services	N/A
Kanamycin	Sigma Aldrich	K0254
Ketaset™	Oxford University Veterinary Services	N/A
Klenow DNA Polymerase	Fermentas	EP0421
LB agar tablet	Sigma Aldrich	L7025-500TAB
LB broth tablet	Sigma Aldrich	L7275-500TAB
Leucosep tubes	VWR International	GRE1227290UK
L-glutamine [4mM]	Sigma Aldrich	G7513
LIVE/DEAD® Fixable Violet Dead Cell Stain Kit	Invitrogen	L34955
Low phosphate PBS	Gibco	10010-031
Lymphoprep	Aiere	NYC-1114547
Magnesium Chloride ($MgCl_2$)	Sigma Aldrich	M2393
MAIP ELISpot Plates	Millipore	MAIPS4510
MEM α-modification	Sigma Aldrich	M4526
Methanol	Sigma Aldrich	M/3900/17

Micro-fine Insulin Syringe with needle 0.5mL 29g x 12.7mm	Nu-care products	M4892
Micro-fine Insulin Syringe with needle 1mL 29g x 12.7mm	Nu-care products	M4891
Microlance needle 26g x 5/8 inch	Nu-care products	M4300
Microscope slides	Fisher Scientific	MNJ-200-010H
Microvette tubes	Sarstedt	16.444
Mini-PROTEAN TGX Gels, 12%, 12-well	Bio-Rad	456-1045
Minimum Essential Medium Eagle (MEM) α-modification	Sigma Aldrich	M4526
Monensin	eBioscience	00-4505-51
Monolisa® HBsAg Ultra, (96 Tests)	Bio-Rad	72346
Mouse anti-hepatitis B surface antigen monoclonal antibody	Serotec Ltd	MCA4658
Mouse anti-NANP monoclonal antibody (2A10)	MR4	MRA-183
Mouse IFNγ ELISpot kit (ALP)	Mabtech	3321-2A
Mr Frosty cryocontainer	Fisher Scientific	CRY-120-010T
$CaNa_2EDTA$	Sigma Aldrich	ED2SC
Na_2EDTA	Sigma Aldrich	ED2SS
Neomycin sulfate	Invitrogen	21810-031
Neutral buffered formalin	Sigma-Aldrich	HT501128
Non-reducing sample buffer (5X)	Fisher Scientific	PN39001
NUNC Maxisorp 96-well plates	Fisher Scientific	DIS-971-030J
PBS/Tween Sachets	Sigma Aldrich	P3563
PCR primers	Sigma Aldrich	N/A
Empty PD-10 columns	Sigma Aldrich	54806
P-amino benzoic acid (PABA)	Sigma Aldrich	A9878
Penicillin/streptomycin [100U pen/ 100µg strep]	Sigma Aldrich	P0781
Percoll	Sigma Aldrich	P1644
Phusion High-Fidelity Master Mix with buffer	New England Biolabs	F-531L
Phytohemagglutinin (PHA)	Sigma Aldrich	L1668
PichiaPink™ Expression system	Invitrogen	A11150
PichiaPink™ Media Kit	Invitrogen	A11156
pNPP (20mg tablets)	Sigma Aldrich	N-2765
Polycarbonate centrifuge tube, 3.2 ml	Beckman Coulter	362305
Polypropylene columns	Pierce	29924
Potassium bicarbonate ($KHCO_3$) 1mM	Sigma Aldrich	P9144
Potassium phosphate monobasic	Sigma Aldrich	P8416
Propan-2-ol	Fisher Scientific	P/7490/17

Protein G agarose	Pierce	22852
Qiagen MinElute DNA purificationi kit	Qiagen	28604
Qiagen plasmid midi kit	Qiagen	12143
Qiaprep spin miniprep kit	Qiagen	27106
Recombinant human IL2	AbD Serotec	PHP042A
Reddymix PCR Mastermix	Sigma Aldrich	PCR-300-600G
Restriction and Ligation Enzymes	New England Biolabs	Various
RPMI-1640	Sigma Aldrich	R0883
Scalpel (surgical blade)	Swann Morton	0302
Sephadex® G-100 Medium	Sigma Aldrich	G100120
SIGMAFAST™ BCIP®/NBT	Sigma Aldrich	B5655-25TAB
Silica gel desiccant	Sigma Aldrich	13767
Smart Ladder DNA marker	EurogenTec	MW-1700-02
S.O.C. medium	Invitrogen	15544-034
Sodium Azide (NaN_3)	Fluka Analytical	08591
Sodium Chloride (NaCl)	Sigma Aldrich	S5886
Sodium dodecyl sulfate (SDS)	Sigma Aldrich	L4390
Sodium phosphate monobasic solution	Sigma Aldrich	74092
Staphylococcal enterotoxin B (SEB)	Sigma Aldrich	S4881
SYBR Safe	Invitrogen	S33102
Transblot turbo Transfer Mini	Bio-Rad	170-4158
Transfer pipettes	Sigma Aldrich	FB55348
Tris-acetate-EDTA (TAE)	Fisher Scientific	BP1332-1
Tris/Glycine/SDS 10x	Bio-Rad	161-0732
Triton™ X-100	Sigma Aldrich	X100
Trizma® HCL buffer solution (Ph7.8)	Sigma Aldrich	T-2569
Ultraclear centrifuge tubes 13.2 ml	Beckman Coulter	344059
Xanthurenic acid	Sigma Aldrich	D102804
Yeast nitrogen base with ammonium sulphate without amino acids	Invitrogen	Q300-09

2.1.2 Solutions

1.1g/ml density CsCl (0.79M): 26.7g CsCl and 2ml 1M Tris pH 7.8 dissolved in dH_2O to a final volume of 200ml and filter sterilised.

1.25g/ml density CsCl (1.58M): 66.6g CsCl and 2ml 1M Tris pH 7.8 dissolved in dH_2O to a final volume of 200ml and filter sterilised.

1.3g/ml density CsCl (2.38M): 80.1g CsCl and 2ml 1M Tris pH 7.8 dissolved in dH_2O to a final volume of 200ml and filter sterilised.

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

BMGY (buffered glycerol complex medium): 1% yeast extract, 3% peptone, 100mM potassium phosphate, pH6.0, 1.34% YNB, 0.0004% biotin and 1% glycerol

BMMY (buffered methanol complex medium): 1% yeast extract, 3% peptone, 100mM potassium phosphate, pH6.0, 1.34% YNB, 0.0004% biotin and 0.5% methanol.

Coating Buffer: 15mM sodium carbonate and 35mM sodium bicarbonate capsules were dissolved in dH_2O and autoclaved.

Complete α-MEM Medium: 500ml Minimal Essential Media (MEM) α-modification was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin), 500µl 2-Mercaptoethanol (50µm) and 50mL of heat inactivated foetal calf serum (FCS) (10%).

Dextrose 20%: Contents of dextrose sachet from PichiaPink™ media kit dissolved in 1000ml dH_2O and autoclaved.

Diethanolamine Buffer: A 5x stock was diluted with dH_2O before use.

Exflagellation Medium: RPMI-1640 was supplemented with 25mM HEPES, 20% FCS, 10mM sodium bicarbonate and 50µm xanthurenic acid, pH adjusted to 7.6.

FACS buffer: 0.5% Bovine Serum Albumin (BSA) and 0.05% sodium azide in PBS.

Giemsa: 5% Giemsa in dH_2O

LB Agar/Broth: Tablets were dissolved in dH_2O. Antibiotics added to 100µg/ml for ampicillin and 25µg/ml for kanamycin.

Loading Dye: 0.4% orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 8.0), 50mM EDTA (pH 8.0) in dH_2O.

Lysis buffer: 0.1% Triton x-100, 1mM EDTA, 1mM $MgCl_2$, 10mM Tris pH 7.8.

Mowiol: 6g glycerol and 2.4g polyvinyl alcohol 4-88 were dissolved in 6ml dH_2O for 2 hours at 50°C with agitation. 12ml Tris pH 8.5 (0.2M) was added and the solution was dissolved for a further three hours at 50°C. The solution was centrifuged at 2500rpm for five minutes to remove any undissolved solids. DAPI was then added at a final concentration 0.1µg/ml.

PAD agar: Contents of PAD sachet from PichiaPink™ media kit dissolved in 900ml dH_2O, autoclaved and then 100ml 20% dextrose added.

Perm/Wash: 10X Perm/Wash buffer was diluted to 1X in dH_2O prior to use.

Phosphate buffered saline (PBS) (0.1M): NaCl 0.138M, KCl 0.0027M, pH 7.4: made by dissolving tablets or sachets in dH_2O.

PBS/Tween (PBS/T) (0.1M): NaCl 0.138M, KCl 0.0027M, pH 7.4 Tween-20 0.05%. Sachets dissolved in dH_2O.

Reducing laemmli lysis buffer: 4% SDS , 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl .

R0 Medium: 500ml RPMI-1640 was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin).

R10 Medium: 500ml RPMI-1640 was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin) and 50ml of heat inactivated FCS (10%).

Tris-acetate-EDTA (TAE) buffer: 50X stock solution was diluted to 1X working concentration in dH_2O.

Tris buffer 10mM: A stock solution of 1M Trizma-HCl pH 7.8 was diluted in dH_2O to a working concentration of 10mM and filter sterilised prior to use.

YPD agar: Contents of YP agar sachet from PichiaPink™ media kit was dissolved in 900ml dH_2O, autoclaved and then 100ml 20% dextrose added.

YPD broth: Contents of YP media sachet from PichiaPink™ media kit was dissolved in 900ml dH_2O, autoclave and then add 100ml 20% dextrose.

YPDS medium: Dissolve contents of YPS agar sachet from PichiaPink™ media kit in 180ml dH_2O, autoclaved and then 20ml 20% dextrose added.

2.1.3 Viral vector antigen inserts

***P. falciparum* ME.TRAP:** The ME.TRAP construct was generated as described previously in [354, 355]. The TRAP sequence is from the *P. falciparum* T9/96 strain [361] with 15 amino acids deleted (5 PNP repeats) and it contains its own signal sequence. The ME string was codon optimised for expression in human cells; it contains 14 CD8+ T cell epitopes and 3 CD4+ T cell epitopes from pre-erythrocytic *P. falciparum* antigens, tetanus toxoid and Bacille Calmette-Guerin and also two B cell epitopes from *Pf* CS and the TRAP adhesion motif (TRAP-AM). The ME string also includes the H-2K^d Pb9 epitope for pre-clinical evaluation in BALB/c mice.

***P. berghei* TRAP:** The PbTRAP DNA sequence (NCBI AAB63302.1) was modified with the addition of the tPA leader sequence and the removal of the transmembrane domain (Dr Karolis Bauza, DPhil Thesis) and synthesised by GeneArt (Life Technologies).

2.1.4 Vaccines

The R21 vaccine was produced by Katharine Collins as described in section 2.2, 2.3 and 2.4. Staff at the Clinical Bio-manufacturing Facility (CBF, Jenner Institute), cloned the ME.TRAP construct into the ChAd63 and MVA viral vectors and produced the vaccines to GMP for clinical trials. The preclinical versions were produced by the Jenner Institute Vector Core Facility. The PbTRAP construct was cloned into the ChAd63 and MVA vectors by Dr Karolis Bauza (DPhil Thesis) and vaccines were produced by the Jenner Institute Vector Core Facility. All vaccines were formulated with adjuvants as detailed in section 2.1.5 using endotoxin free low phosphate PBS and kept on ice until administration. For co-administration of VLP (R21 or RTS,S) and viral vectors the vaccines were formulated separately and injected into separate hind limbs (i.m.). For VLP (R21 or RTS,S) and viral vector mixture vaccinations the vaccines were formulated together and given in the same syringe (i.m.) split between both hind limbs.

2.1.5 Adjuvants

The adjuvants used throughout this study were dosed and formulated as below and were obtained by the Jenner Institute adjuvant bank, either commercially available or through material transfer agreements with the relevant companies.

Table 2.2 Adjuvant dose and formulation details.

Adjuvant	Type	Single dose formulation	Source
Alhydrogel	Aluminium hydroxide	85µg/dose in 100µl total injection volume - mix with vaccine and low phosphate PBS, rotate for 30 minutes at 4°C	Brentag Biosector
Abisco-100	Saponin, phospholipid and cholesterol complex	12µg/dose in 100µl total injection volume - mix with vaccine and low phosphate PBS by shaking	Isconova (now Novavax)
Matrix M	Saponin, phospholipid and cholesterol complex	12µg/dose in 100µl total injection volume - mix with vaccine and low phosphate PBS by shaking	Isconova (now Novavax)
MF59	squalene-based oil-in-water nano-emulsion	(1:1 MF59:antigen) 50µl/dose in 100µl total injection volume - mix equal volume of vaccine and low phosphate PBS with MF59	Novartis
AddaVax	squalene-based oil-in-water nano-emulsion	(1:1 AddaVax:antigen) 50µl/dose in 100µl total injection volume - mix equal volume of vaccine and low phosphate PBS with MF59	InvivoGen
LMQ	Liposomes, monophosphoryl lipid A and QS21	30µl/dose in a total injection volume of 100µl - mix with vaccine and low phosphate PBS by shaking	Vaccine Formulation Laboratory (VFL) at the University of Lausanne
AS01B	Liposomes, monophosphoryl lipid A and QS21	50µl total injection volume formulated with antigen	GSK

2.2 Molecular Biology

Design and details of the R21 and RS fusion proteins are described in Chapter 4 and the DNA sequences were synthesised by GeneArt.

2.2.1 Bacterial transformations

GeneArt plasmids p2483 and p2485 containing R21 and RS sequences respectively were reconstituted to 100ng/µl in EB buffer (Qiagen). Two vials of DH5α *Escherichia coli* cells were thawed on ice and 1µl of plasmid added to each vial. Cells and plasmid were left on ice for 30 minutes, then heat shocked for 30 seconds at 42°C and placed immediately back on ice for 5 minutes. 900µl of SOC media was added and cells placed at 37°C in a shaking incubator (250rpm) for 1 hour. 100µl of cells from each transformation was then plated onto LB agar plates containing kanamycin and incubated at 37°C overnight (Jenner protocol J062).

2.2.2 Plasmid DNA preparation

Plasmid DNA was prepared by inoculating 100ml LB media containing 25µg/ml kanamycin with a single colony from each transformation. Flasks were incubated overnight at 37°C in a shaking incubator (250rpm). The culture was then centrifuged at 6000rpm for 10 minutes at 4°C, the supernatant discarded and DNA purified using the Qiagen MIDI prep plasmid plus kit following the standard manufacturer's instructions. Purified DNA was collected in 200µl of EB buffer and stored at -20°C until further use.

2.2.3 Restriction endonuclease digest

R21 and RS genes were cloned from the GeneArt plasmids (p2483 and p2485) into the pPINK HC and pPINK LC PichiaPink expression vectors (p2581 and p2582) using *EcoR* I *and Kpn* I restriction enzymes. GeneArt and pPINK individual plasmid

restriction digest reactions were prepared as below in a final volume of 50µl DNase/RNase free water and incubated at 37°C for 2 hours (Jenner protocol J056).

Plasmid DNA	5µg	
NEB 10X buffer 1	5µl	
10U *Kpn* I	1µl	(=20,000U/ml)
10U *EcoR* I	0.5µl	(=10,000U/ml)
100X BSA	0.5µl	

Reactions were then purified using the Qiaquick PCR purification kit as per the manufacturer's instructions and eluted in 30µl of water. The DNA was then alkaline phosphatase treated to prevent re-ligation with Arctic Phosphatase following the manufacturer's instructions (Jenner protocol J090v2). The whole restriction digest reactions were then run on a 1% agarose gel as detailed in 2.2.4. DNA bands of digested plasmids were then excised from the agarose gel and the DNA extracted using the Qiaquick gel extraction kit following the manufacturer's instructions (Jenner protocol J085). Products were run on 1% agarose gel to confirm product prior to ligation reaction.

2.2.4 Agarose gels

DNA restriction digests or PCR products were separated by 1% agarose gel electrophoresis (100V, 40-60min) in 1X Tris-acetate-EDTA (TAE) buffer stained using SYBR Safe. A relevant amount of 6X loading dye was added to each sample immediately before loading of DNA onto the gel. DNA was visualised with the transilluminator and the size of bands was estimated by comparison to the DNA marker smart ladder (Jenner protocol J065).

2.2.5 DNA ligation reactions

Ligation of the R21 and RS genes into the pPINK HC and LC expression plasmids was performed using Invitrogen T4 DNA ligase with a vector to insert ratio of 1:3

(Jenner protocol J098v4). Reactions were prepared as below and incubated for 1 hour at room temperature. DH5α cells were then transformed with each ligation reaction as per 2.2.1.

Ligation	**Plasmids**	**Ligase (µl)**	**5X ligation buffer (µl)**	**Insert (µl)**	**Backbone (µl)**	**ddH_2O (µl)**
R21+pPINK HC	2483+2581	0.2	4	1.4	2.0	12.4
R21+pPINK LC	2483+2582	0.2	4	1.4	2.3	12.1
RS+pPINK HC	2485+2581	0.2	4	1.5	2.0	12.3
RS+pPINK LC	2485+2582	0.2	4	1.5	2.3	12.0

2.2.6 Polymerase chain reaction (PCR)

The ligation reactions were screened by PCR using the KAPA 2G Robust method (Jenner protocol J115v2). PCR reaction mixture was prepared as below with each colony to be screened using the 5' *AOX1* forward primer and the 3' *CYC1* reverse primer supplied in the Invitrogen PichiaPink™ expression kit.

	Final Concentration
Reaction buffer B 5X	1X
Primer 1 (10µM)	0.5µM
Primer 2 (10µM)	0.5µM
Equal mix dNTP's (10mM each)	200µM
KAPA 2G Robust polymerase (5U/µl)	0.1U/µl
ddH_2O	

PCR reactions were performed as per the program below and the products visualised on a 1% agarose gel.

Primary denature	95°C	5 minutes	
Denature	95°C	30 seconds	X29
Anneal	54°C	30 seconds	
Extension	72°C	30 second/kb	

2.2.7 Linearising DNA for transformation into yeast

The DNA was linearised for incorporation into the PichiPink™ yeast by using the restriction enzyme *Afl* II which is a unique cutter within the *TRP2* region of the pPINK HC and pPINK LC vectors. Cutting the vector in this region means the linearised DNA can only be incorporated into the *TRP2* locus of the PichiaPink™ yeast strains. The restriction endonuclease digest reactions were set up as below in DNase/RNase free water and incubated at 37°C for 2 hours (Jenner protocol J056). The enzyme was then deactivated by incubating the reaction for 20 minutes at 65°C followed phenol/chloroform extraction of the DNA and ethanol precipitation of the reaction using 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, eluting in 45µl of sterile dH_2O (Jenner protocol J274). The linearised DNA was analysed on 1% agarose gel to confirm the digest and stored at -20°C until use.

Plasmid DNA (40µg)	30µl
10X buffer 4	80µl
Afl II	20µl
100X BSA	8µl
ddH_2O	662µl

2.2.8 Transforming yeast with linearised DNA

Growing and preparing yeast

The 4 PichiaPink™ yeast strains supplied in the kit were streaked onto YPD agar plates and grown at 30°C for 3-5 days until colonies appear. A single colony from each of the 4 yeast strains was then used to inoculate 4, 125ml baffled flasks containing 10ml of YPD media. The flasks were incubated at 30°C shaking at 300rpm overnight. These starter cultures were used to inoculate 4, 100ml YPD media cultures in 1L flasks. Flasks were incubated at 30°C shaking at 300rpm overnight.

PichiaPink™ yeast was prepared for electroporation by transferring the cultures to 250ml centrifuge bottles and centrifuging the yeast at 1500xg at 4°C for 5 minutes. The supernatant was discarded and the yeast pellet was resuspended in 250ml ice cold sterile water. The yeast was centrifuged again as above, the supernatant was discarded and the pellet resuspended in 50ml ice cold sterile water. The centrifugation was repeated and the supernatant again discarded and the pellet resuspended in 10ml of 1M ice cold sorbitol. The centrifugation was repeated again and the pellet finally resuspended in 1ml of ice cold sorbitol.

Electroporation

The electroporation was performed by aliquotting 80µl of yeast into a 0.2cm cuvette and adding 10µg of linearised DNA. The yeast and DNA were incubated on ice for 5 minutes and then pulsed using the electroporator (GenePulser Xcell™ Bio-Rad) and the manufacturer's instructions. Immediately after pulsing 1ml of ice cold YPDS was added to the cuvette, mixed with the yeast and DNA, and incubated at 30°C for 2 hours. After incubation 200µl of electroporated yeast was plated onto PAD (adenine deficient) selection plates and incubated at 30°C for 3-10 days. When colonies appeared, 4 white colonies were selected and re-streaked into fresh PAD plates.

2.2.9 Genomic DNA isolation for sequencing

Genomic DNA was isolated from the transformed yeast strains by aliquotting 75µl of TE buffer into PCR tubes and adding a single yeast colony. The PCR tubes were sealed and microwaved on full power for 2.5 minutes followed by vortexing. This was repeated by microwaving for 2 minutes followed by vortexing, then microwaving for 1 minute and vortexing, then 30 seconds and vortexing followed by freezing at -80°C overnight. The tubes were then thawed at 95°C for 2 minutes and then centrifuged at 2500rpm for 10mins. PCR reactions were set up with 5µl of DNA and 50µl of the reaction detailed below using Phusion High-Fidelity Master Mix with buffer.

Phusion HF mastermix	25µl
5’ forward primer (100uM)	0.5µl
3’ reverse primer (100uM)	0.5µl
ddH_2O	24µl

PCR reactions were performed on the thermocycler as per the program below and the products visualised on a 1% agarose gel.

Primary denature	98°C	2 minutes	
Denature	98°C	1 minute	X35
Anneal	62°C	1 minute	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	

The PCR reactions were cleaned up using MiniElute PCR purification kit as per the manufacturer’s instructions before being sent for sequencing to confirm the correct insert in the yeast.

2.3 Particle production

2.3.1 Growing transformed yeast strains

Transformed yeast strains were grown by inoculating 25ml BMGY media with a stab of glycerol stock in a 250ml baffled flask. Yeast was grown at 30°C shaking at 250-300rpm until the culture reached an optical density (OD) of between 2-6 OD_{600}. This 25ml of yeast culture was then used to inoculate 1L of BMGY in two, 2L baffled flasks. This was grown at 30°C shaking at 250-300rpm until culture reached log phase, between 2-6 OD_{600}. (2-3 days). Expression was induced by centrifuging yeast in 4 sterile 250ml centrifuge bottles at 1500xg for 5 minutes at RT, the supernatant was discarded and the pellet resuspended in 400ml of methanol containing media (BMMY). This was grown in a 2L baffled flask at 30°C, shaking (250-300rpm) and 100% methanol was added to 0.5% every 24 hours for 3 days. Yeast was harvested by centrifuging in two 250ml centrifuge bottles for 5 min at 1500xg, 4°C to pellet and the supernatant was discarded. The pellet was then washed by adding 100ml of cold sterile water to each bottle followed by centrifugation as above and the supernatant removed. The pellets were then frozen at -20°C until further use or processed as below.

2.3.2 Yeast Cell disruption

Yeast pellets were thawed on ice and resuspended in a lysis buffer containing 10mM Tris (pH 7.8), 0.1%Triton X-100, 1mM EDTA and 250U/ml benzonase. Acid washed glass beads (0.425-600µm) were added and the sample was disrupted by 10 cycles of vortexing for one minute then placing on ice for one minute.

2.3.3 Clarification

After yeast cell disruption and prior to particle purification the yeast debris was removed by centrifugation for 5 minutes at 1500xg and the supernatant was then clarified by ultracentrifugation at 13000xg for 20 minutes.

2.3.4 CsCl discontinuous gradient centrifugation

The clarified yeast lysate was layered onto a discontinuous CsCl gradient containing equal parts of 1.1g/ml CsCl layered on top of 1.3g/ml CsCl. After ultracentrifugation for 2 hours at 40000rpm in a SW41 Ti rotor (Beckman Coulter Optima L-100 XP) the particle containing fraction was collected.

2.3.5 Gel filtration

The particle containing fraction collected from the discontinuous gradient was applied to a PD10 column containing Sephadex G100. The sample was eluted in 10mM Tris (pH 7.8) and the particle containing fractions were pooled.

2.3.6 CsCl Isopycnic gradient centrifugation

The pooled sample eluted from the gel filtration column was added to an Isopycnic CsCl gradient containing 1.2g/ml CsCl. After ultracentrifugation for 20 hours at 40000rpm in a SW41 Ti rotor, (Beckman Coulter Optima L-100 XP) the particle containing fraction was collected.

2.3.7 Size exclusion chromatography on Sephacryl 500

Sample was applied to a Hiprep 16/60 Sephacryl S-500 HR gel filtration column to exchange the buffer and remove any remaining lower molecular weight contaminants. The sample was eluted in 10mM Tris buffer (pH 7.8) and pure particle fractions were pooled.

2.4 Particle characterisation

2.4.1 Transmission electron microscopy

The presence and size of the particles were visualised using the FEI Tecnai 12 Transmission Electron Microscope by negative staining the samples with 2% uranyl acetate.

2.4.2 SDS-PAGE

Samples from various stages in the purification process and the final purified R21 particle prep were analysed by reducing and non-reducing Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were silver stained to assess the purity and analysed by western blotting with a monoclonal anti-NANP antibody and an anti-HBsAg monoclonal antibody to assess the immunoreactivity of the product. In brief, samples were prepared in 1X reducing or non-reducing laemmli lysis buffer, vortexed and heated at 95°C for 10 minutes. Samples were loaded onto 12% Tris-glycine, Mini-PROTEAN precast gels alongside pre-stained and unstained molecular weight markers and run at 100V for 10 minutes followed by 250V for 25 minutes using the Bio-Rad mini-PROTEAN 3 system. Gels were either stained with the Pierce Silver Stain Kit according to the manufacturer's standard procedure, or the proteins were electrophoretically transferred to a nitrocellulose membrane for western blotting analysis using the Trans-Blot Turbo transfer system (Bio-Rad). After transfer, nitrocellulose membranes were washed with PBS/T and blocked with 5% semi-skimmed milk in PBS for 1 hour at room temperature. They were then washed again and incubated for 1 hour at room temperature with either monoclonal anti-NANP antibody 1/20,000 in 3% BSA/PBS, or anti-HBsAg monoclonal antibody 1/200 in 3% BSA/PBS. For detection, membranes were incubated for 1 hour at room temperature with Alkaline Phosphatase-AffiniPure Donkey Anti-Mouse IgG (H+L)

diluted 1/3000 in 3% BSA/PBS. Colour was developed with the addition of SIGMAFAST™ BCIP®/NBT substrate dissolved in water.

2.4.3 ELISA to detect the CS repeat region

The presence and accessibility of the NANP repeat region on the surface of the particle was assessed by ELISA. Nunc-Immuno Maxisorp 96 well plates were coated with purified R21 in carbonate-bicarbonate coating buffer for 1 hour at room temperature. Plates were washed in PBS/T and then incubated with mouse monoclonal antibody to the NANP repeat region for 1 hour at room temperature. Plates were washed again and goat anti-mouse whole IgG conjugated to alkaline phosphatase was added for 1 hour at room temperature. Following a final wash, plates were developed by adding p-nitrophenylphosphate at 1mg/mL in diethanolamine buffer and OD was read at 405nm.

2.4.4 ELISA to detect HBsAg

The presence and accessibility of the HBsAg portion of fusion protein in the particle was assessed by sandwich ELISA using the Monolisa ULTRA HBsAg ELISA kit following the manufacturer's instructions. In brief, plates were coated with a monoclonal antibody to the HBsAg overnight at 4°C, then washed with PBS/T and incubated with R21 particle for 2 hours at room temperature. Plates were washed again and then incubated with a cocktail of antibodies to the HBsAg. Development was carried out according to the Monolisa ULTRA HBsAg ELISA kit standard procedure.

2.4.5 R21 Quantification by Absorbance at 280nm

The purified particle prep was quantified by measuring absorbance at 280nm and converting this to mg/ml using the extinction coefficient for the R21 of 2.09 (calculated by inputting the R21 sequence into the ExPASy - ProtParam web tool).

2.5 Human Immunology

2.5.1 Peptides

Crude 20-mer peptides overlapping by 10 amino acids spanning the length of the *P. falciparum* T9/96 sequence contained in the ME.TRAP vaccine insert were synthesised by Neo Group Inc., Mimotopes, or Thermo Fisher Scientific. Peptides were reconstituted in DMSO at a concentration of 50-100mg/ml depending on solubility and stored at -80°C until use. For *ex vivo* and cultured ELISpot assays peptides were pooled into 6 pools TRAP pools (7-10 peptides per pool) and 1 ME pool (20 peptides) as detailed in Appendix 1, and for ICS and affinity ELISpot a total pool containing all 57 TRAP peptides was used.

2.5.2 Isolation of human PBMCs, plasma and serum

Blood was collected in lithium heparin tubes and Peripheral blood mononuclear cells (PBMCs) were separated by density gradient using Lymphoprep. Lymphoprep (15ml) was added to each leucosep tube and centrifuged at 1000xg for 1 minute. Whole blood was then added to the tube (between 15-30ml) and centrifuged for 13 minutes at 1000xg without the break at room temperature. Plasma (1ml) was collected from each tube and stored at -80°C and PBMCs were collected from the interface and transferred to fresh falcon tubes. PBMCs were washed in 40ml R0, by centrifugation for 5 minutes at 750xg. The supernatant was discarded and the PBMCs were resuspended and washed again in 30ml R0. The final pellet was resuspended in 10ml R10 and PBMCs were counted using a CASY counter (Innovatis, Roche Diagnostics). Serum was collected after centrifuging blood at 750xg for 5 minutes; it was placed in cryogenic vials and stored at -80°C.

2.5.3 Freezing and thawing PBMC

PBMCs were frozen using Mr Frosty cryocontainers containing propan-2-ol, which were pre-chilled for a minimum of 30 minutes before use. Between 5-10 x 10^6 cells were frozen per vial by resuspension in 0.5ml FCS per vial to be frozen, and placing at 4°C for 30 minutes. An equal volume of 20% DMSO in FCS was then added to the tube and cells aliquotted into cryogenic vials and placed in the Mr Frosty. Mr Frosty cryocontainers were stored at -80°C overnight and vials transferred to LN_2 the following day. To thaw, PBMCs were immersed in a 37°C water bath until 50% thawed. Cells were then added drop-wise to 15ml warm R10 in a falcon tube. Cells were centrifuged at 750xg for 5 minutes and the pellet resuspended in 2ml R10 with 2µl benzonase per ml of cells. Cells were incubated for a minimum of 2 hours before use at 37°C 5% CO_2 in a humidified incubator.

2.5.4 *Ex vivo* IFNγ PBMC ELISpot

Ex vivo IFNγ ELISpot assays were performed as described in [356, 357].Briefly, MultiScreen-IP 0.45µm sterile plates were coated with 10µg/ml anti-mouse IFNγ monoclonal antibody 1-DIK diluted in carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were washed twice with 100µl PBS per well and then blocked for at least one hour at 37°C with 100µl R10 medium per well. Cells were prepared as described in section 2.5.2 or 2.5.3. For Vac33 400,000 cells per well were plated per well and for Mal34 250,000 cells were plated per well, and stimulated for 18-20 hours 37°C 5% CO_2 in a humidified incubator. Cells were stimulated with positive control (10µg/ml PHA/0.02µg/ml SEB), negative control (R10 only) or pools of peptides (20mers overlapping by 10) at 10µg/ml final concentration. Following incubation plates were then washed six times with PBS/T and incubated with 1µg/ml biotinylated anti-human-IFNγ mAb 7-B6-1 diluted in PBS for two hours at room temperature. Plates were washed again and incubated with 1µg/ml streptavidin alkaline phosphatase diluted in PBS for one hour at room temperature. Spots were

developed by addition of 50µl per well of colour development buffer (BCIP/NBT) and counted using ELISpot software (AID). Results are expressed as spot forming cells (SFC) per million PBMCs after background responses in unstimulated wells were subtracted. *Ex vivo* IFN ELISpot assays assessing T cell affinity were performed as above, except were the cells were restimulated (250,000 PBMC/well) with a total pool of 57 TRAP peptides at a range of concentrations (10, 2, 0.4 and 0.08µg/ml).

2.5.5 Cultured IFNγ ELISpot assay

For cultured ELISpots cryopreserved PBMCs were thawed as in 2.5.3 and cultured in 7 wells of a 24 well plate at 1 x 10^6 PBMC per well in a total volume of 1ml of R10. Each well was stimulated with one of 6 TRAP peptide pools or the ME peptide pool at a final concentration of 10ug/ml. After 3 days incubation at 37°C 5% CO_2, 500µl of cell culture supernatant was removed and replaced with R10 supplemented with IL2 for a final concentration of 25IU IL2 per ml. This was repeated on day 7. On day 9 cells were washed 3 times and left to rest overnight at 37°C 5% CO_2 in R10. On day 10 the cells were washed again and resuspended in exactly 500µl R10. 50µl of cell suspension containing 1 x 10^5 original cells were plated in an ELISpot as per 2.5.4. Each sample was restimulated with a positive control (10µg/ml PHA/0.02µg/ml SEB), a negative control (R10) and the 7 pools of ME.TRAP peptides. The ELISpot was carried out as for the *ex vivo* assay above (2.5.4). The values reported are the sum of the response (after background subtracted) to the 6 TRAP pools and 1 ME pool and expressed as the number of spot forming cells (SFC) per million originally cultured PBMCs.

2.5.6 Blood *ex vivo* intracellular cytokine staining (ICS)

T cell responses were assessed for Vac33 from frozen PBMCs thawing the cells as in 2.5.3 and using a 7 colour flow cytometry staining panel as described in [356]. Responses were assessed for Mal34 by either a 7-colour (part A) or 12-colour (part

B) staining panel, performed on freshly isolated PBMC (2.5.2) as described in [357]. In brief, 1 x 10^6 PBMC were incubated for 18 hours with either a total pool of 57 TRAP peptides at 2µg/ml, R10 medium alone or a positive control (10µg/ml PHA/0.02µg/ml SEB). All samples also contained anti-CD28 and anti-CD49d at 1µg/ml and for the last 16 hours of incubation Brefeldin A and monensin, both at 1µg/ml, were added. Following incubation cells were dead cell dye and then surface stained (as per the table below) at 4°C for 30 minutes. Cells were permeablised with Cytofix/Cytoperm and then stained with the intracellular stain for 30 minutes at room temperature (as indicated below). Cells were then washed and re-suspended in 250µl FACS buffer for acquisition on the LSRII flow cytometer (BD Biosciences) and data analysed in FlowJo (Tree Star Inc.). Results reported as the percentage of parent population (CD4+ or CD8+) secreting cytokine (TNF, IL2 or IFNγ) after unstimulated response is subtracted from the stimulated sample.

7 colour panel - Surface stain

CD14 pacific blue	2µl per test
CD19 pacific blue	2µl per test
CD4 APC	5µl per test
Total volume per test	9µl

7 colour panel - Intracellular stain

CD3 PE-Cy5	5µl per test
CD8 APC-AF750	5µl per test
IFNγ FITC	2µl per test
IL2 PE	1µl per test
TNF PE-Cy7	2µl per test
Total volume per test	15µl

12 colour panel - Surface stain

CD14 pacific blue	2µl per test
CD19 pacific blue	2µl per test
CD4 QD655	0.5µl per test
CD27 QD605	0.5µl per test
CD45RO ECD	10µl per test
CD57 QD 565	1µl per test
CCR7 APC	5µl per test
FACS buffer	24µl per test
Total volume per test	45µl

12 colour panel - Intracellular stain

CD3 Alexa Fluor 700	1µl per test
CD8 APC-AF780	5µl per test
IFNγ FITC	2µl per test
IL2 PE	1µl per test
TNF PE-Cy7	2µl per test
FACs buffer	39µl per test
Total volume per test	50µl

2.5.7 Whole IgG Enzyme linked Immunosorbent Assay (ELISA)

Total IgG ELISAs were carried out on serum as detailed in section 2.5.3. Nunc-Immuno Maxisorp 96 well plates were coated with 1µg/ml of PfTRAP protein in carbonate-bicarbonate coating buffer overnight at 4°C. Plates were washed with PBS/T and blocked with 1% BSA in PBS. Sera were diluted at a starting concentration of 1:100, added in duplicate, and serially diluted 3-fold. Plates were incubated for 2 hours at room temperature and then washed in PBS/T. Goat anti-human whole IgG conjugated to alkaline phosphatase was added for 1 hour at room temperature. Plates were washed in PBS/T and developed by adding p-nitrophenylphosphate at 1mg/mL in diethanolamine buffer. OD was read at 405nm. Serum antibody endpoint titres were taken as the x-axis intercept of the dilution

curve at an absorbance value three standard deviations greater than the OD405 for a pool of serum from unvaccinated volunteers. A standard positive serum sample was included in each assay as a reference control.

2.5.8 Immunofluoresence antibody test (IFAT)

Sporozoites were isolated from the salivary glands of infected mosquitoes in PBS containing azide and 3000 sporozoites were added in 10µl PBS to each well on an 8-well microscope slide. Slides were air-dried, wrapped in foil and stored with desiccant at -20ºC until use. For the IFAT, all incubation steps were carried out at RT in a dark humidified chamber and slides were washed three times in PBS between each step. Wells were blocked for 2 hours using 1% BSA in PBS and then washed using PBS. Serum samples were then added at a dilution of 1:100 in PBS for 1 hour. Slides were washed again and anti-human IgG conjugated to Alexa Fluor-488 was added at a dilution of 1:200 (in 1% BSA, PBS). Slides were incubated for 30 minutes, washed again in PBS and mounted with Mowiol® and a coverslip. Slides were dried at RT overnight in the dark and viewed under a Leica DMI3000 microscope.

2.6 Murine Immunology

2.6.1 Animals and immunisations

For all immunogenicity and efficacy studies 6-10 week old female inbred BALB/c (H-2^d) mice (Harlan, UK) were anethaesthised using vaporised Isoflurane and immunised intramuscularly (i.m) with a total volume of 100µl divided equally in the tibialis muscles of both hind limbs of each animal. All mice were housed under Specific Pathogen Free (SPF) conditions in the Wellcome Trust Centre for Human Genetics Functional Genetics Facility (FGF). All procedures were performed by Rebecca Hillson research technician at the Jenner Institute in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Oxford Animal Care and Ethical Review Committee for use under the Project License PPL 30/2414 or 30/2889.

2.6.2 Peptides

Crude 20-mer peptides overlapping by 10 amino acids spanning the length of the PbTRAP vaccine insert sequence, or 15-mer peptides overlapping by 11 amino acids spanning the *P. falciparum* CS sequence present in R21 and RTS,S, were synthesised by Mimotopes, UK. Peptides were reconstituted in DMSO at a concentration of 50-100mg/ml depending on solubility and stored at -80°C until use. Peptides were pooled into a single PbTRAP pool and a single CS pool for ICS and *ex vivo* ELISpot assays. To assess immune responses to the ME.TRAP vaccine insert in mice the Pb9 peptide (SYIPSAEKI) was used which is an immunodominant CD8+ epitope from *Plasmodium berghei* circumsporozoite protein.

2.6.3 Collection of mouse sera

Serum was obtained from mice by collecting 5 to 6 drops of blood from the lateral tail vein in a microcuvette tube. Alternatively it was obtained by cardiac puncture with a

26 gauge needle to withdraw blood from the heart under anaesthetic (3.5% isoflurane, 2L/minute oxygen). Blood was allowed to clot by storing it at 4°C overnight before centrifuging it at 13000rpm for 4 minutes to separate the sera. Sera was removed and stored at -20°C until use.

2.6.4 Whole IgG ELISA

Total IgG ELISAs were carried out on serum collected as described above. Nunc-Immuno Maxisorp 96 well plates were coated with either antigen (2µg/ml $NANP_6C$ peptide for CS ELISAs, 1µg/ml of PfTRAP protein or 1µg/ml of PbTRAP protein) in carbonate-bicarbonate coating buffer overnight at 4°C. Plates were washed with PBS/T and blocked with 10% skimmed milk powder in PBS. Sera were diluted at a starting concentration of 1:100 for post-prime samples or 1:1000 for post-boost samples, added in duplicate, and serially diluted 3-fold. Plates were incubated for 2 hours at room temperature and then washed as before. Goat anti-mouse whole IgG conjugated to alkaline phosphatase was added for 1 hour at room temperature. Following a final wash, plates were developed by adding p-nitrophenylphosphate at 1mg/mL in diethanolamine buffer and OD was read at 405nm. Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD405 for serum from naïve mice. A standard positive serum sample was included in each assay as a reference control and a naïve serum sample was negative for antigen-specific responses to all antigens.

2.6.5 Splenocyte preparation

Single cell suspensions were prepared by homogenising and straining the spleens followed by brief incubation in ACK lysis buffer to remove erythrocytes. Cells were then washed with PBS, pelleted, resuspended in complete α-MEM and counted using the CASY cell counter.

2.6.6 *Ex-vivo* IFNγ Spleen ELISpot

Three weeks after the final immunisation mice were culled and spleens removed and processed as in 2.6.5. Briefly, MultiScreen-IP 0.45µm sterile plates were coated with 5µg/ml rat anti-mouse interferon gamma monoclonal antibody AN18 diluted in carbonate-bicarbonate buffer overnight at 4°C. Plates were then blocked for at least one hour at 37°C with complete α-MEM. Cells were plated, serially diluted 2 fold and stimulated for 18-20 hours with either 1µg/ml of Pb9 peptide (CD8+ epitope from *Plasmodium berghei* circumsporozoite protein, SYIPSAEKI) for viral vector immunisations or 2µg/ml of a pool of overlapping 15mer peptides spanning the CS sequence present in R21. Cells were added at a starting concentration of 125,000 cells/well for Pb9 stimulation and 500,000 cells/well for CS stimulation. Plates were then washed and incubated with 1µg/ml biotinylated anti-mouse-IFNγ mAb R46A2, followed by incubation with 1µg/ml streptavidin alkaline phosphatase. Spots were developed by addition of 50µl per well of colour development buffer and counted using ELISpot software (AID). Results are expressed as spot forming cells (SFC) per million splenocytes after background responses in unstimulated wells were subtracted.

2.6.7 Blood *ex vivo* ICS

Blood was collected in 10mM EDTA/PBS and incubated briefly with ACK to lyse erythrocytes. PBMCs were then pelleted and resuspended in complete α-MEM media and incubated 96 well U bottom plates for 6 hours at 37ºC with either GolgiPlug and complete α-MEM or GolgiPlug and peptide (1µg/ml for Pb9 peptide or 5µg/ml for CS and PbTRAP pools). PBMC's were then washed and stained for 30 minutes on ice with 50µl of surface stain mixture containing 1/50 Fc Block (CD16/CD32), 1/200 CD4 e450 and 1/200 CD8 Per CP Cy5.5 in PBS 0.5% BSA. Cells were then washed, fixed with 4% paraformaldehyde and permeabilised with Perm/Wash buffer followed by staining for 30 minutes on ice with 50µl of the

intracellular stain mixture containing 1/100 TNF FITC, 1/100 IL2 PE and 1/200 IFNγ APC in Perm/Wash. Cells were then washed and re-suspended in 100µl FACS buffer for acquisition on the LSRII flow cytometer (BD Biosciences) and data were analysed in FlowJo (Tree Star Inc.). Results reported as the percentage of parent population (CD4+ or CD8+) secreting cytokine (TNF, IL2 or IFNγ) after unstimulated response is subtracted from the stimulated sample.

2.6.8 Whole IgG purification and passive transfer

Serum was collected from mice as previously described 2.6.3 and whole IgG was purified using a Pierce polypropylene column pre-packed with 2ml of protein G resin following the manufacturer's instructions (Jenner protocol J135). Purified IgG was then injected i.v. into mice that were challenged approximately 6 hours after IgG transfer.

2.7 Parasitology

2.7.1 Parasite strains

Plasmodium berghei transgenic parasites were kindly provided by Ahmed Salman a DPhil student at the Jenner Institute from a collaboration with Dr Shahid Khan and Dr Chris Jansen of Leiden University. The transgenic *P. berghei* parasite contained an additional copy of the *Plasmodium falciparum* CS gene inserted at the 230p locus under the control of the *P. berghei* UIS4 promoter. Generation of the parasites used the 'gene insertion/marker out' technology previously described [362].

2.7.2 Sporozoite production and preparation of inoculum

100-300µl of frozen parasite stock was injected i.p. into a naïve donor mouse and after 4 days the parasitaemia was determined by thin film blood smear (2.7.4). The donor mouse was cardiac bled using a syringe lined with 300U/ml of heparin and 1 x

10^7 parasitised RBCs were injected i.p. into recipient mice. Three days later parasitaemia of the recipient mice was determined and the mouse was anaesthetised and fed to a pot of starved female *Anopheles stephensi* mosquitoes for approximately 10 minutes. Exflagellation was measured by adding one drop of blood to one drop of room temperature exflagellation media and viewing under a light microscope at 40x. The mosquitoes were fed on Fructose/PABA solution and maintained at 19-21°C in a humidified incubator on twelve-hour day-night cycle.

2.7.3 Sporozoite challenge

Challenge experiments were performed in BALB/c mice with the transgenic parasite described in section 2.7.1. Approximately 21 days after feeding on parasite infected mice, mosquitoes were dissected and the salivary glands were removed and placed in RPMI-1640. The salivary glands were gently disrupted to release the sporozoites with a tissue homogeniser and the sporozoites were then counted using a haemocytometer. 1000 sporozoites were injected i.v. in a total volume of 100µl into the lateral tail vein of each mouse. Mice were monitored from day 5 post challenge by thin film blood smear as below. After three consecutive parasite positive blood films the mice were sacrificed by cervical dislocation. The time taken to develop 1% parasitaemia was calculated using linear regression analysis for the parasite positive mice and if no parasites were detected on day 14 after challenge the mice were considered protected.

2.7.4 Preparation of thin film blood smears

Parasitaemia of infected mice was monitored from day 5 after challenge. Thin film blood smears were prepared by snipping the end of the tail and collecting 1 drop of blood onto a glass slide. After air drying the smear was fixed in 100% methanol for 1 minute and then stained for 1 hour in 5% Giemsa in dH_2O. The slides were viewed using a light microscope with 100x oil immersion and the percentage of parasitised

red blood cells (RBC) was counted in a region where the cells formed a monolayer. A single field of monolayer contained approximately 500 RBCs. The number of parasitized RBCs was counted in between 5 - 60 fields (number of fields counted determined by parasitaemia to obtain an accurate count) and percentage parasitaemia calculated.

2.8 Statistical analysis

Statistical analysis was performed using Prism version 5 (Graphpad). Where necessary the D'Agostino-Pearson normality test was used to determine if the data were normally distributed and all ELISA data were $\log_{10}$ transformed prior to analysis. When comparing two groups the Mann Whitney test was used for non-parametric data and the Unpaired t test was used for parametric data. Two or more groups of parametric data were compared by One-way ANOVA with Bonferroni's multiple comparison test (comparing all pairs of groups) or with Dunnett's multiple comparison test (comparing all groups to a one group). Two or more groups of non-parametric data were compared with Kruskal-Wallis with Dunn's multiple comparison test and correlations were assessed using Spearman's rank correlation. Challenge results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. Significance was indicated when value of $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3 Immunogenicity and protective efficacy of the liver stage ChAd63 - MVA ME.TRAP prime-boost regimen in humans

3.1 Introduction

Thrombospondin related adhesion protein (TRAP), also known as SSP2 was one of the first pre-erythrocytic malaria antigens to be identified and it has been well-studied. It is a sporozoite microneme protein that links the actin-myosin motor to the substrate and has been shown to be important for gliding motility of the sporozoite [204, 205, 207] and invasion of hepatocytes [207, 363]. In a number of models, immune responses directed against TRAP can be protective against sporozoite challenge [206, 364]. However, evidence *in vivo* suggests that antibodies to TRAP may not be able to prevent invasion of hepatocytes [211] and a number of murine studies have shown that a cellular response directed towards TRAP can be protective [234, 243-245]. Therefore induction of TRAP specific T cells has been an active area of investigation.

Induction of cellular immune responses by subunit vaccination has been most successful with DNA and viral vector vaccines including poxviruses and adenoviruses. The use of poxviruses in vaccination began in 1796 when Edward Jenner used cowpox virus to confer protection against a closely related orthopox, variola virus, the causative agent of smallpox. Though this was not the first example of the use of poxvirus vaccination, Edward Jenner was the first to publicise his findings and promote active vaccination to prevent disease [352, 365]. The World Health Organisation later used another orthopox, Vaccinia, in the smallpox eradication campaign in 1966. This campaign successfully eradicated smallpox

globally by 1980 [366]. Although successful, Vaccinia vaccination was particularly reactogenic and the need for a safer alternative led to the development of attenuated forms of Vaccinia. Modified Vaccinia virus Ankara (MVA) was produced by serial passage of the chorioallantois vaccinia virus Ankara over 570 times in chicken embryo fibroblasts [367]. This resulted in deletion of approximately 15% of the genome and a virus that could no longer replicate in mammalian cells, despite remaining infective and very immunogenic [368]. The virus was therefore able to accommodate up to 25kb of foreign DNA making it an excellent gene delivery vector. It has since been used to create experimental vaccines for a number of diseases including malaria, HIV, tuberculosis, hepatitis C, influenza and cancer [351, 369-376].

The first malaria vaccine study in humans using viral vectors was with the attenuated Vaccinia virus vaccine NYVAC-Pf7 that encoded seven malaria antigens [377, 378]. This vaccine was variably immunogenic, induced only low levels of antibodies but was able to induce cellular responses in >90% of volunteers to either TRAP, CS or MSP1. Sterile efficacy was also seen in 1 out of 35 vaccinated volunteers after sporozoite challenge (CHMI) [379]. Studies at the Jenner Institute began exploring the immunogenicity and protective efficacy of the TRAP antigen fused to a multi-epitope string (ME.TRAP) [354] using DNA and MVA vaccines both pre-clinically and clinically [380]. MVA ME.TRAP was shown to be more immunogenic in humans than DNA, but greater immunogenicity was elicited when used together in a prime-boost regimen. DNA – MVA prime-boost was also more protective and sterilely protected 1 out of 8 volunteers, whereas single vector vaccination only delayed time to development of blood stage parasitaemia [355, 381-384]. The attenuated fowlpox vector FP9 was also tested in the prime-boost regimen and was found to be a better a priming vaccine than DNA. Two out of 16 volunteers receiving two immunisations of FP9 followed by one immunisation of MVA both expressing ME.TRAP were

sterilely protected [385]. T cell responses measured in this trial by either *ex vivo* or cultured ELISPOT were associated with protection and it was estimated that a high threshold of cellular response would need to be reached for protection [385-387]. This efficacy seen in malaria naïve adults did not translate into efficacy in the field and this was likely due to lower immunogenicity in children [388-392]. This led to a move towards the use of replication deficient adenoviral vectors that are potentially more immunogenic with a greater ability to induce both antibodies and CD8+ T cells [393, 394].

Adenoviruses were rendered replication incompetent by the deletion of the E1 region and further deletion of the E3 region resulted in an increase in space for transgene incorporation making them useful as vaccine vectors. Adenoviruses exist as many different serotypes (viruses that are not neutralised by antibodies against another serotype) and are species specific [393]. Human adenovirus serotype 5 (Ad5) has been the most extensively studied as a vaccine vector for many diseases including malaria [394-399]. However concerns regarding the prevalence of pre-existing immunity in humans to common adenovirus serotypes such as Ad5, which may reduce the potency of the adenovirus led to development of vaccine vectors from rarer human serotypes such as Ad26 and Ad35 [400-403] and simian derived adenoviruses such as chimpanzee serotype 68 and 63, among others [404-406]. A number of simian adenoviruses were assessed in mice, macaques and humans by researchers at the Jenner Institute and it was found that these vectors were safe and immunogenic [351, 407, 408]. They were also shown to be more immunogenic than the FP9 and MVA and induced single vector protection against *P. berghei* challenge in mice [407, 408]. In addition using ChAd63 in a prime-boost regimen with MVA induced even greater efficacy that was also longer lasting [409]. The ChAd63 – MVA ME.TRAP regimen was shown to be very immunogenic in NHPs and induced high levels of multifunctional CD4+ and CD8+ T cells and TRAP-specific antibodies. In

addition intramuscular immunisation was shown to be more immunogenic than intradermal and the initial prime boost response could be re-boosted by further MVA immunisation [410]. Further studies in mice have indicated that effector CD8+ T cells induced by these simian adenovirus prime - MVA boost regimens are important for protection [411].

Due to the encouraging results obtained in pre-clinical studies and the successful manufacture of ChAd63 ME.TRAP, the aim of this chapter was to assess the immunogenicity of ChAd63 ME.TRAP and the ChAd63 – MVA ME.TRAP 8 week prime-boost regimen in malaria naïve adults. In addition, the ability to re-boost the vaccine induced response with either ChAd63 or MVA was also assessed as well as the ability to induce an immune response by mixing and co-administering both ChAd63 and MVA together [412]. Since CD8+ T cells have been implicated in protection in pre-clinical models [409, 411, 413, 414] the main immunological readout from this clinical trial was measurement of IFNγ secreting T cells by *ex vivo* ELISpot performed on all samples. Additional immunological assays carried out on various sample subsets included analysis of TRAP-specific T cell functionality by multi-parameter flow cytometry with intracellular cytokine staining, assessment of vaccine induced memory responses by cultured IFNγ ELISpot and measurement of TRAP specific antibodies by ELISA. Protective efficacy of these vaccines was also determined using the standard CHMI model where volunteers are challenged with five bites from *P. falciparum* infected mosquitoes. The results presented are from two separate studies carried out at the Jenner Institute, Oxford University.

3.2 Results – Vac33 Phase 1 clinical trial

3.2.1 Study Design

The simian adenovirus ChAd63 was assessed as a new vector for the induction of TRAP-specific T cells in a Phase 1 dose escalation and route finding study called Vac33 (NCT number NCT00890019). In groups 1-4 the dose of ChAd63 was initially escalated from 1 x 10^8 viral particles (vp) to 5 x 10^{10} vp by intradermal (i.d.) immunisation. In groups 5-7 dose escalation was then performed by intramuscular (i.m.) immunisation from 1 x 10^{10} vp to 2 x 10^{11} vp. Subgroups A received only the ChAd63 ME.TRAP vaccine, and subgroups B received ChAd63 ME.TRAP followed by an intradermal immunisation of 2 x 10^8 plaque forming units (pfu) of MVA ME.TRAP 8 weeks later (day 56). In group 7, there was a subgroup C in which four volunteers received the MVA ME.TRAP by intramuscular immunisation (Table 3.1) Blood was taken for immunology analysis as indicated in Figure 3.1. Katharine Collins and Dr Katie Ewer performed the majority of the immunological assays and analysis with some assistance from other Research Assistants at the Jenner Institute. Specifically, they processed the majority of the blood samples and performed the *ex vivo* ELISpot assays. Katharine Collins carried out cultured ELISpots and ELISA assays. Clinical staff at the Jenner Institute, Centre for Clinical Vaccinology and Tropical Medicine performed all vaccinations, and collection of blood samples.

Table 3.1 Vac33 study design – vaccine dose and route

Malaria naïve adults between 18 and 50 years of age were vaccinated with ChAd63 ME.TRAP in a range of doses. All groups (A, B and C) received a prime vaccination with ChAd63 ME.TRAP and only groups B and C received the boost vaccination with MVA.ME TRAP.

Group	ChAd63 dose and route	MVA dose and route	No. vols
1 (A+B)	1 x 10^8 vp i.d.	2 x 10^8 pfu i.d.	8
2 (A+B)	1 x 10^9 vp i.d.	2 x 10^8 pfu i.d.	8
3 (A+B)	1 x 10^{10} vp i.d.	2 x 10^8 pfu i.d.	8
4 (A+B)	5 x 10^{10} vp i.d.	2 x 10^8 pfu i.d.	8
5 (A)	1 x 10^{10} vp i.m.	2 x 10^8 pfu i.d.	4
6 (A+B)	5 x 10^{10} vp i.m.	2 x 10^8 pfu i.d.	8
7 (A+B)	2 x 10^{11} vp i.m.	2 x 10^8 pfu i.d.	6
7 (C)	2 x 10^{11} vp i.m.	2 x 10^8 pfu i.m.	4

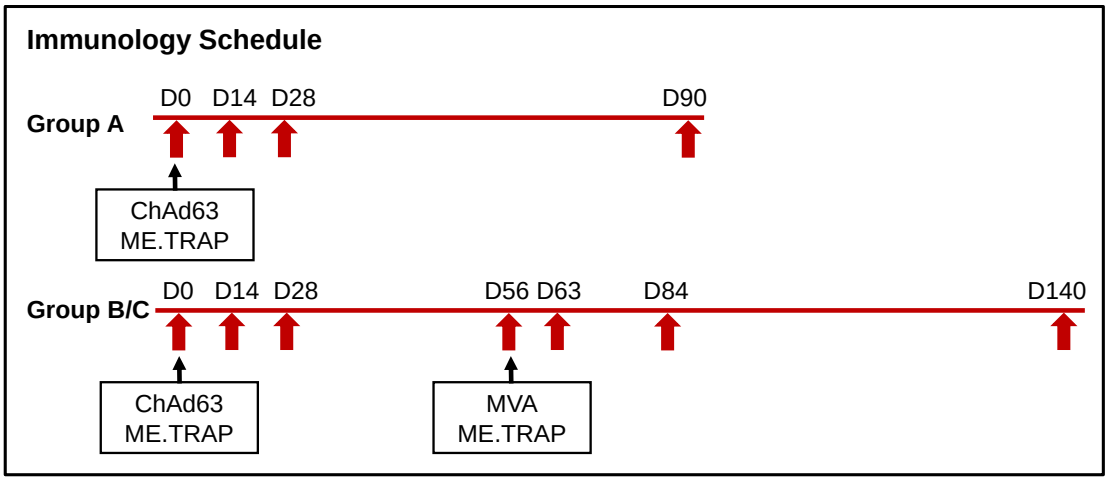


Figure 3.1 Vac33 study design – immunology schedule

In all groups (A, B and C) samples were taken for immunology prior to vaccination (D 0), two weeks, four weeks and eight weeks after ChAd63 immunisation (D 14, 28 and 56). In subgroup A only, a sample was also taken at three months after ChAd63 immunisation (D 90). In subgroups B and C only, samples were taken one week, four weeks and three months after the MVA immunisation (D 63, 84 and 140).

3.2.2 Immunogenicity

3.2.2.1 Effect of route of immunisation on cellular immunogenicity

To assess the effect of the route of immunisation on the induction of a cellular immune response by ChAd63 ME.TRAP, four groups were immunised with two different doses (1 x 10^{10} vp or 5 x 10^{10} vp) administered either i.d. or i.m. Volunteers were bled two weeks after vaccination and the peripheral blood mononuclear cells (PBMCs) were isolated. Antigen specific IFNγ secreting T cells were assayed in an *ex vivo* ELISpot by overnight re-stimulation with 7 pools of overlapping peptides spanning the ME.TRAP insert. Responses are displayed as the sum of the response to the 7 pools after background subtracted. IFNγ secreting T cells were detected in all groups and were not significantly different between the groups receiving the same dose by different routes, either after prime (Figure 3.2-A) or after a boost immunisation with 2 x 10^{8} pfu of MVA ME.TRAP 8 weeks later (Figure 3.2-B). Route of MVA ME.TRAP immunisation was also assessed in group 7 where two of six volunteers primed with 2 x 10^{11} vp ChAd63 ME.TRAP received the MVA ME.TRAP immunisation via the intradermal route (subgroup 7B) and four received it intramuscularly (subgroup 7C). There was no significant difference between the T cell responses at day 63 or day 140 in the two subgroups as detected in the ELISpot (Figure 3.2-C). The route of administration therefore had no effect on immunogenicity, and in addition it had no effect of the safety of the vaccines [356]. Consequently, the data from immunisation by different routes was pooled for the volunteers that received the same dose and further analysis of dose escalation of ChAd63 ME.TRAP was carried out.

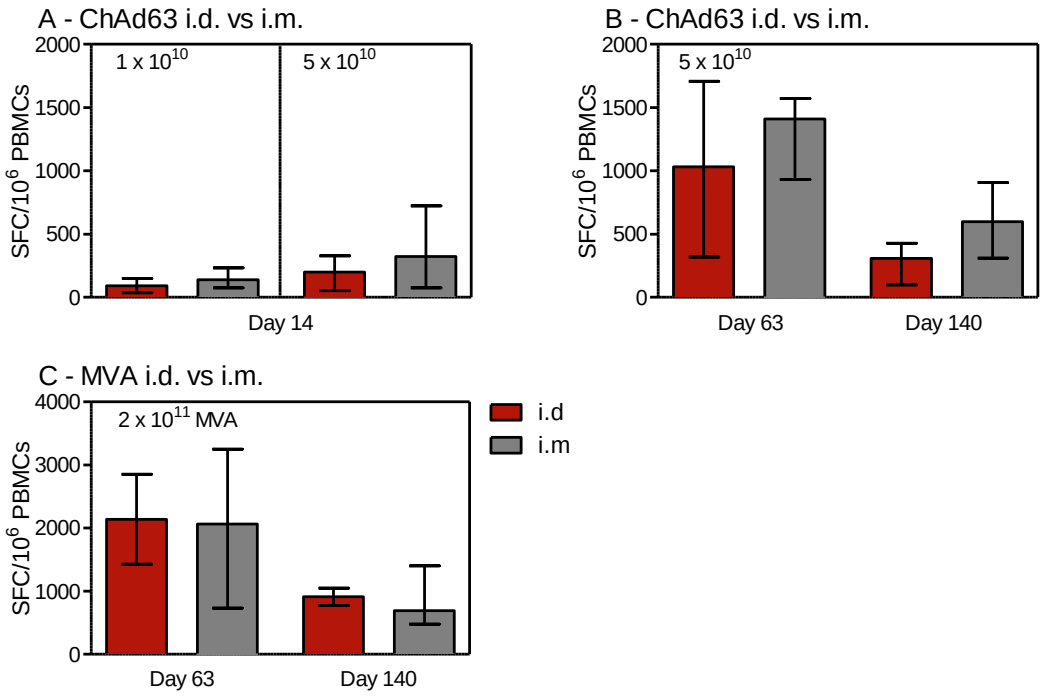


Figure 3.2 Effect of route on the cellular immunogenicity of ChAd63 ME.TRAP and MVA ME.TRAP vaccination

To assess route of ChAd63 ME.TRAP immunisation, groups of volunteers were immunised either i.d. or i.m. at two different doses (1 x 10^{10} vp or 5 x 10^{10} vp) and T cell responses measured by IFNγ ELISpot to pools of overlapping peptides, two weeks after vaccination **(A)**. Volunteers primed with 5 x 10^{10} vp ChAd63 ME.TRAP were also boosted 8 weeks later with 2 x 10^{8} pfu MVA ME.TRAP i.d. and T cell responses were measured one week and three months after vaccination **(B)**. To assess route of MVA immunisation two groups of volunteers were primed with 2 x 10^{11} vp ChAd63 ME.TRAP and boosted 8 weeks later with 2 x 10^{8} pfu MVA ME.TRAP either i.d. or i.m. **(C)**. Median responses (with interquartile range) are shown and groups compared by Mann-Whitney test.

3.2.2.2 Assessment of effector T cell responses by ex vivo IFNγ ELISpot

To assess the magnitude of the T cell response induced using escalating doses of ChAd63 ME.TRAP, volunteers were immunised with a range of doses from 1 x 10^8 vp to 2 x 10^{11} vp as detailed in Table 3.1. ME.TRAP specific T cell responses measured in an *ex vivo* IFNγ ELISpot were detected in all groups after ChAd63 ME.TRAP vaccination and peaked at either day 14 or day 28 for the individual volunteers (Figure 3.3). At day 14 the median T cell response was highest in the group that received the highest dose (2 x 10^{11} vp), approximately three fold higher than the response in the lower dose groups, and this was significantly higher than the two lowest dose groups 1 x 10^8 vp and 1 x 10^9 vp (Kruskal-Wallis with Dunn's multiple comparison test *p<0.05) (Figure 3.4-A). Volunteers were given a boost vaccination of MVA ME.TRAP 8 weeks later and the T cell responses peaked one week after boost (day 63) (Figure 3.3). The T cell levels were boosted significantly above the prime response ($p < 0.0001$) and a response was detected in all volunteers. At day 63 there was no significant difference in the magnitude of the response between the four dose groups, and there did not appear to be a dose dependant effect (Figure 3.4-B). However three months after boost (day 140) there does appear to be a trend in increased level of response with increased dose, and the T cell responses were better maintained in the highest dose group, over two fold higher than the other groups (median responses of 733 SFC/10^6 PBMC compared to (122-340) SFC/10^6 PBMC) (Figure 3.4-C). So although the peak of the response does not differ by dose, the response may be more durable for the higher doses, although this is not statistically significant.

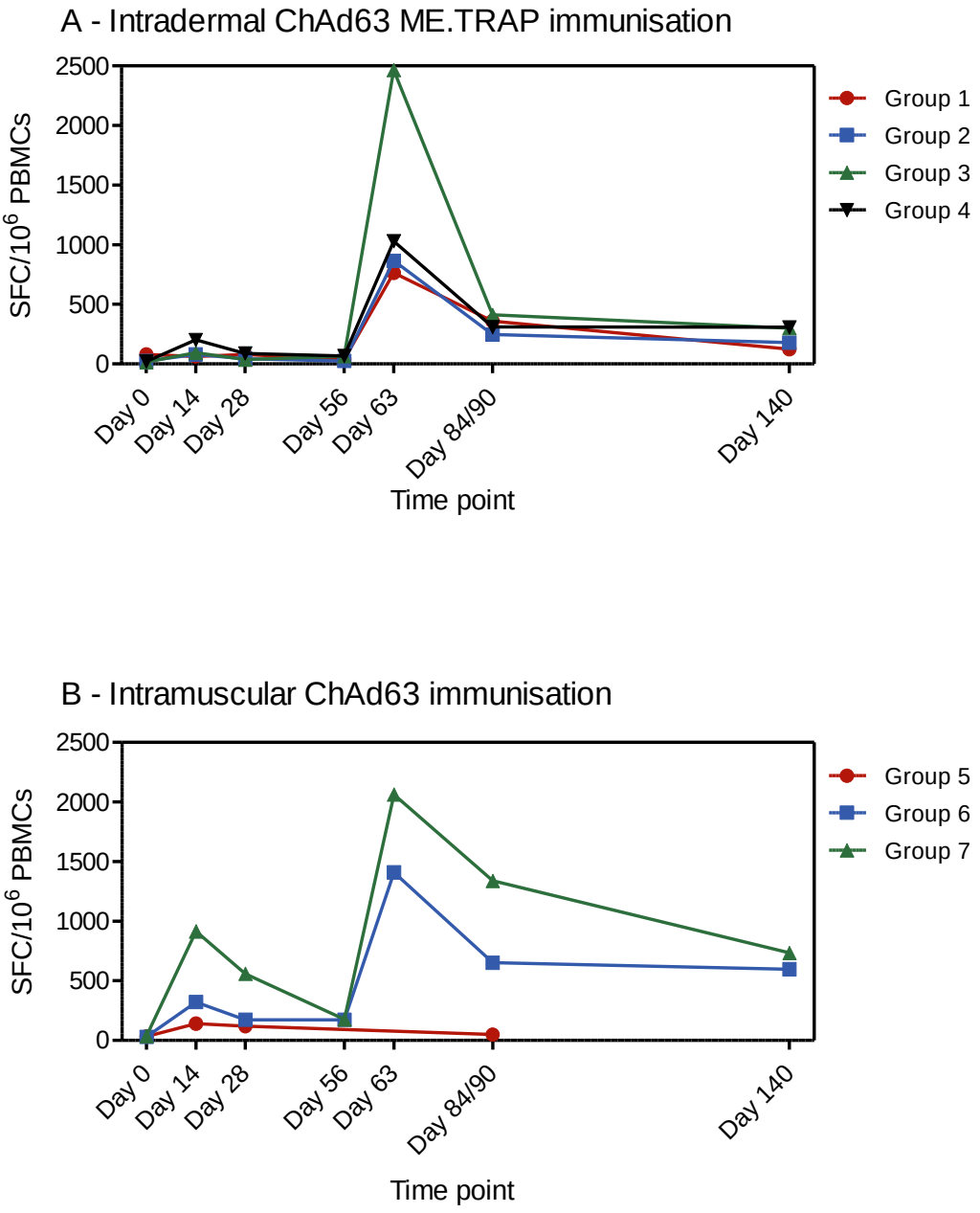


Figure 3.3 Kinetics of the T cell response induced by ChAd63 ME.TRAP – MVA ME.TRAP immunisation

Groups of volunteers were immunised either i.d. **(A)** or i.m. **(B)** with ChAd63 ME.TRAP at range of different doses (1 x 10^8 vp - 2 x 10^{11} vp) and boosted 8 weeks later with 2 x 10^8 pfu MVA ME.TRAP. ME.TRAP specific T cell responses were measured by IFNγ ELISpot after vaccination. Median group responses are shown.

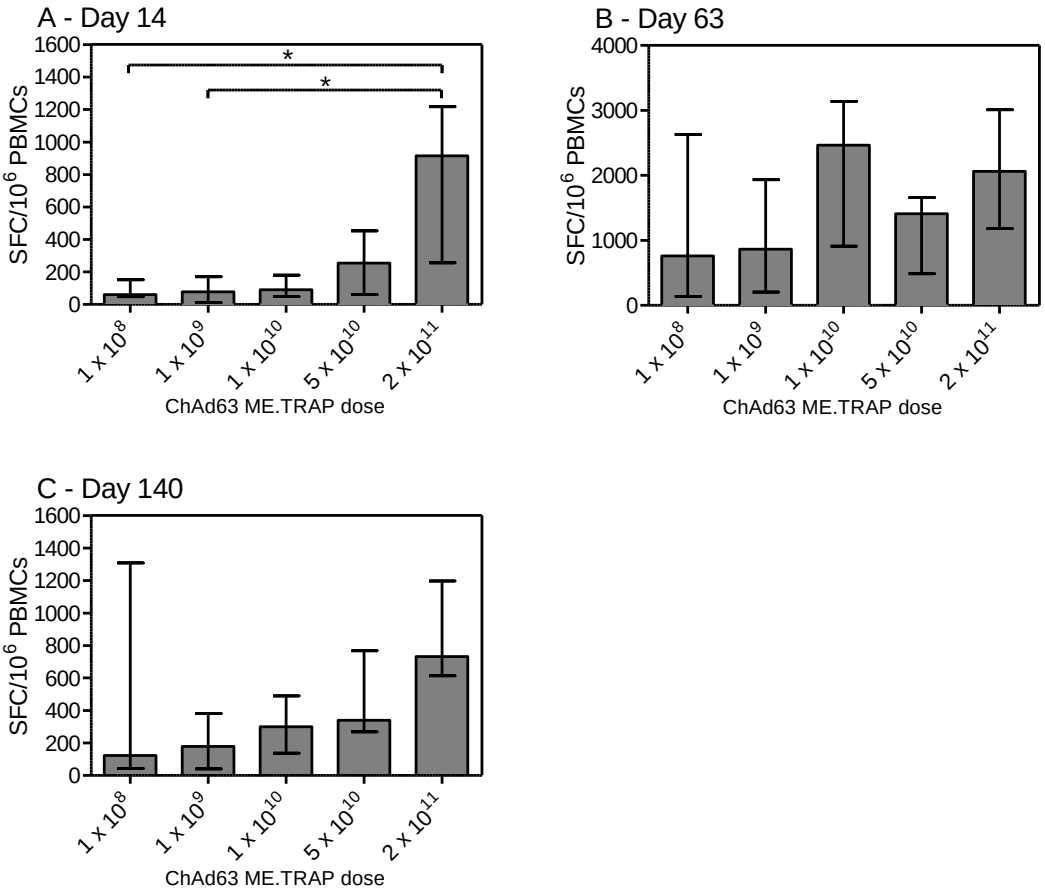
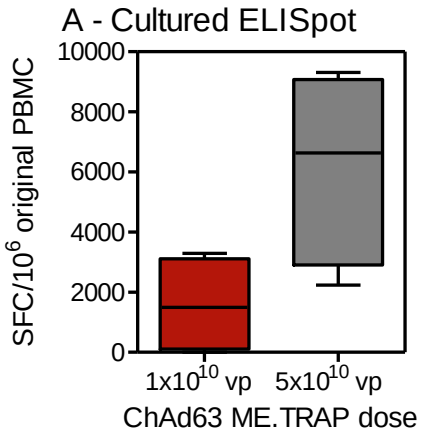


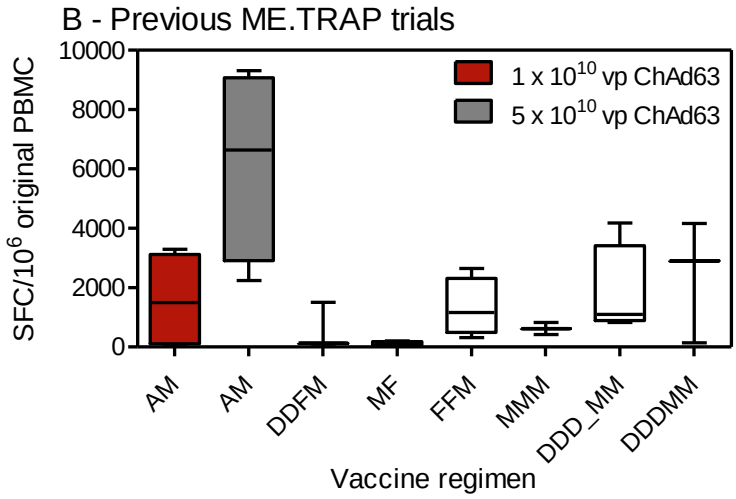
Figure 3.4 Dose escalation of ChAd63 ME.TRAP

Groups of volunteers were immunised with ChAd63 ME.TRAP at a range of different doses (1 x 10^8 vp - 2 x 10^{11} vp) and boosted 8 weeks later with 2 x 10^8 pfu MVA ME.TRAP. ME.TRAP specific T cell responses were measured by IFNγ ELISpot after prime vaccination on day 14 **(A)** and after boost at day 63 **(B)** and day 140 **(C)**. Median group responses with interquartile range are shown and are compared by Kruskal-Wallis with Dunn's multiple comparison test *p<0.05.

3.2.2.3 Assessment of memory T cell responses by cultured IFNγ ELISpot

Previous studies of TRAP based vectored vaccines, have employed cultured ELISpot assays to quantify the memory T cell responses. Notably, one study in malaria naïve adults identified a relationship between memory T cells measured in vaccinated volunteers on the day of sporozoite challenge and protection against malaria in CHMI [386]. Another study assessing natural immunity in malaria exposed individuals, showed that the cultured memory TRAP specific T cell response were associated with a reduced incidence of developing clinical malaria infection [415]. This assay was therefore used to assess the induction of memory T cells by the ChAd63 – MVA ME.TRAP regimen. Responses were measured three months after the boost vaccination, in groups 3 and 4, to the ME.TRAP insert in an IFNγ ELISpot after 10 days of culture with antigen. Memory T cells were successfully detected in 7 out of the 8 volunteers assessed. The median response was approximately 4 fold higher in group 4, which received the greater i.d. dose of ChAd63 ME.TRAP (5 x 10^{10} vp), although this was not significantly higher likely due to the small sample size per group (Mann-Whitney test) (Figure 3.5-A). Interestingly, the median response in this group (6638 SFC/10^6 original PBMC) was also double the response measured for the most immunogenic viral vector regimen previously tested at the Jenner Institute (2900 SFC/10^6 original PBMC) (Figure 3.5-B). This previous regimen was multiple immunisations of DNA and MVA vectors expressing ME.TRAP (D-D-D-M-M) [386]. In previous studies they also reported a relationship between the magnitude of the peak *ex vivo* ELISpot response and the cultured ELISpot response once the effector T cell population had contracted but this was not seen in these volunteers, but sample size is small (Figure 3.5-C) [386].


A - Cultured ELISpot
SFC/10^6 original PBMC
10000
8000
6000
4000
2000
0
1x10^10 vp
5x10^10 vp
ChAd63 ME.TRAP dose


B - Previous ME.TRAP trials
SFC/10^6 original PBMC
10000
8000
6000
4000
2000
0
1 x 10^10 vp ChAd63
5 x 10^10 vp ChAd63
AM
AM
DDFM
MF
FFM
MMM
DDD_MM
DDDMM
Vaccine regimen

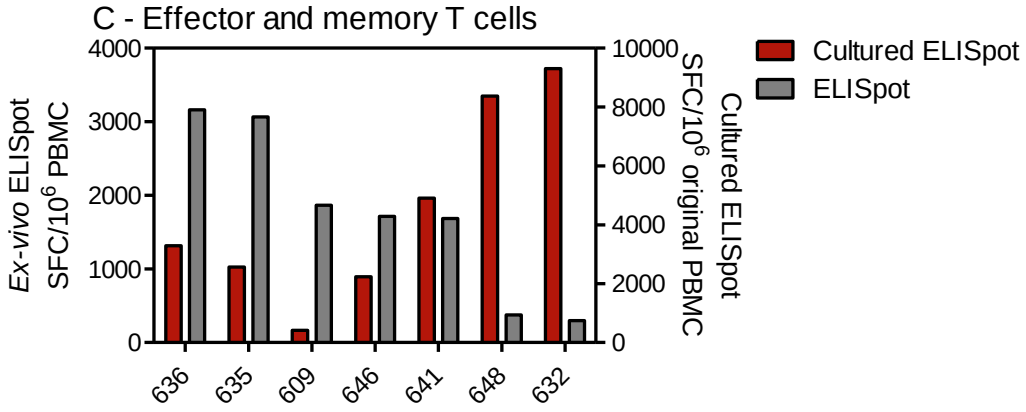

C - Effector and memory T cells
Ex-vivo ELISpot SFC/10^6 PBMC
4000
3000
2000
1000
0
SFC/10^6 original PBMC
Cultured ELISpot
10000
8000
6000
4000
2000
0
Cultured ELISpot
ELISpot
636
635
609
646
641
648
632

Figure 3.5 Memory T cell responses induced by vaccination with ChAd63 – MVA ME.TRAP

Two groups of volunteers were immunised with either 1 x 10^{10} vp or 5 x 10^{10}vp of ChAd63 ME.TRAP and boosted 8 weeks later with 2 x 10^{8} pfu MVA ME.TRAP. ME.TRAP specific memory T cells were measured by IFNγ ELISpot after 10 days of culture with ME.TRAP peptides **(A).** Comparison of cultured ELISpot responses measured in Vac33 and previous ME.TRAP trials [386]. *A=ChAd63 ME.TRAP, M=MVA ME.TRAP, F=FP9 ME.TRAP D=DNA ME.TRAP*. Box plots displaying the median with whiskers showing the minimum and maximum responses are shown **(C)** Comparison of the *ex vivo* IFNγ ELISpot response measured on day 63 and the cultured IFNγ ELISpot responses measured on day 140, Individual responses are shown.

3.2.2.4 Humoral immunogenicity

TRAP specific IgG induced by immunisation with ChAd63 – MVA ME.TRAP in groups 1-4 was assayed at every time point by ELISA using TRAP recombinant protein. After the ChAd63 prime, responses peaked at either day 14 or 28 for individuals, and TRAP specific IgG was detected in 10 out of 16 volunteers. After the MVA boost vaccination all volunteers had seroconverted and responses peaked at either day 63 or day 84 (Figure 3.6-A-D). There was a trend for increased response with increased ChAd63 ME.TRAP dose and the median peak group response was highest in the highest dose group 5 x 10^{10} vp although not significant (Figure 3.6-E).

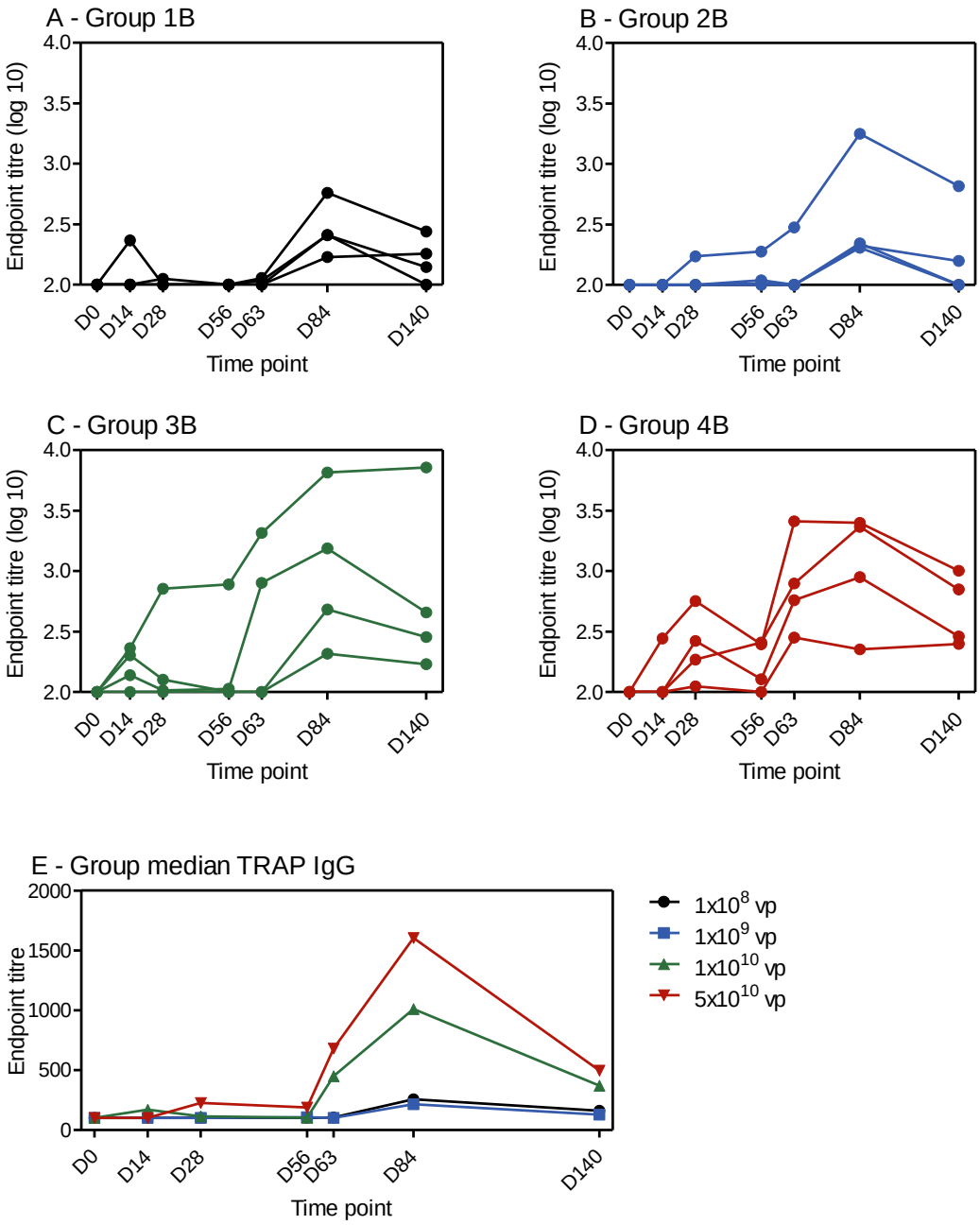


Figure 3.6 TRAP specific IgG induced by vaccination with ChAd63 – MVA ME.TRAP

Groups of volunteers (n=4/gp) were immunised with ChAd63 ME.TRAP at a range of different doses and boosted 8 weeks later with 2 x 10^8 pfu MVA ME.TRAP. TRAP specific IgG was measured after vaccination by ELISA using the TRAP recombinant protein. **(A)** 1 x 10^8 vp, **(B)** 1 x 10^9 vp, **(C)** 1 x 10^{10} vp, **(D)** 5 x 10^{10} vp. Individual responses are shown. **(E)** Group median response shown, peak response compared by Kruskal-Wallis with Dunn's multiple comparison test.

3.2.3 Cellular immunogenicity can be re-boosted with an additional viral vector immunisation

In addition to the dose escalation and route finding study a selection of volunteers who had received the ChAd63 – MVA ME.TRAP regimen were assessed approximately 8-9 month after MVA vaccination to see if the ME.TRAP specific T cell responses could be re-boosted. Volunteers were divided into two groups and received a third immunisation of either ChAd63 ME.TRAP or MVA ME.TRAP (Ad-M-Ad or Ad-M-M) and the groups were stratified to ensure equivalent median time since last vaccination (Figure 3.7-A). Blood was taken on day 7 and 28 after vaccination for the group receiving MVA, and on day 14 and 28 after vaccination for the group receiving ChAd63 to compare the peak of the immune responses. The ME.TRAP specific ELISpot responses on the day of vaccination were not significantly different between the two groups and both vaccines were able to boost responses significantly above pre-boost levels. The peak responses (day 7 or 14) were slightly but not significantly higher in the Ad-M-Ad group than the Ad-M-M group, but by day 28 the responses were not different (Figure 3.7-B+C). The Ad-M-Ad regimen may be more effective at re-boosting since the median response after re-boost was 109% of the original boost response, whereas the median re-boost response in the Ad-M-M group was only 76% of the original boost, although there was no significant difference between boost and re-boost for the two groups (Figure 3.7-D). Importantly both vectors were able to significantly re-boost the ME.TRAP IFNγ T cell response 8-9 months after standard ChAd63 – MVA prime-boost immunisation, suggesting anti-vector immunity does not interfere and this could have important implications if viral vector vaccines become widely used.

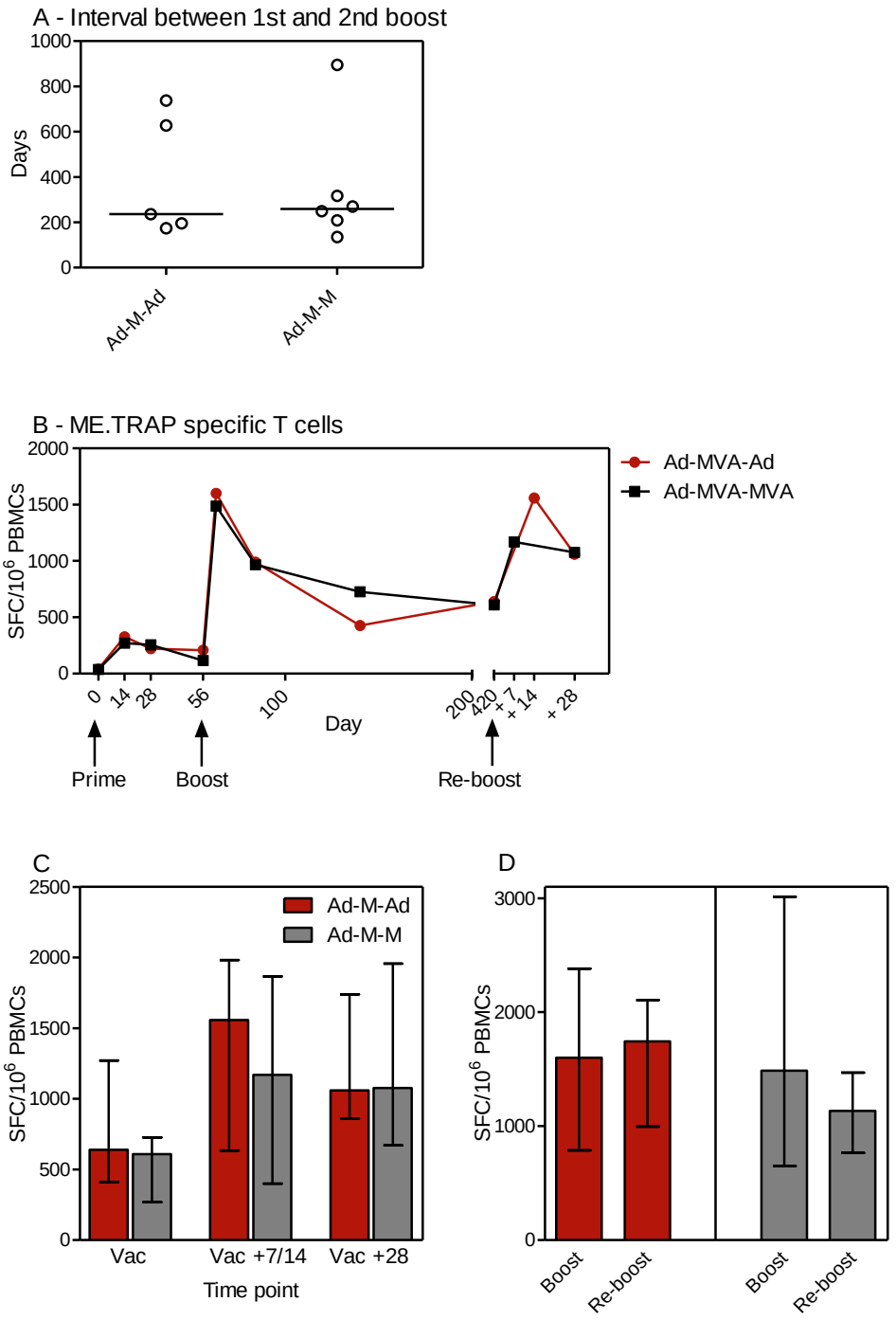


Figure 3.7 Cellular immune responses after a third viral vector vaccination

Two groups of volunteers previously vaccinated with ChAd63 – MVA ME.TRAP were given a third vaccination 8-9 months later with either the ChAd63 or the MVA vaccine. **(A)** Volunteers were stratified by time since MVA vaccination (median time is shown). **(B)** ME.TRAP specific T cells were measured by IFNγ ELISpot after vaccination (group median shown). *Ad=ChAd63 ME.TRAP, M/MVA=MVA ME.TRAP*. **(C)** Comparison of responses after a third vaccination with either ChAd63 or MVA. **(D)** Comparison of the peak response after the first and second boost vaccination (median with interquartile range shown).

3.3 Results – Mal34 Phase 1/2a clinical trial

3.3.1 Study design

The efficacy of the simian adenovirus ChAd63 was assessed in the Phase 1/2a clinical trial, Mal34 (NCT00890760) and this was conducted in two parts. In part A, the efficacy of a single vaccination of ChAd63 was compared to the ChAd63 – MVA prime-boost regimen. Therefore group 1 received 5 x 10^{10} vp of ChAd63 ME.TRAP i.m. followed 8 weeks later by 2 x 10^{8} pfu of MVA ME.TRAP i.d., group 2 received 5 x 10^{10} vp ChAd63 ME.TRAP i.m. alone and group 3 were the no vaccination controls.

In part B, efficacy of the prime-boost regimen was confirmed by repeating the comparison to unvaccinated volunteers and in addition the duration of the efficacy of the prime-boost regimen was assessed with a longer interval between vaccination and challenge. Therefore, group 4 and 5 received 5 x 10^{10} vp of ChAd63 ME.TRAP i.m. followed 8 weeks later by 2 x 10^{8} pfu of MVA ME.TRAP i.d. and group 7 were the no vaccination controls. The immunogenicity and efficacy of mixed vector immunisation was also assessed in groups 8, 9 and 10. These groups received 5 x 10^{10} vp of ChAd63 ME.TRAP and 2 x 10^{8} pfu of MVA ME.TRAP mixed and co-administered i.m. in varying regimens detailed in Table 3.2. Volunteers were challenged with five bites from *P.falciparum* infected mosquitoes at two - three weeks after the MVA boost (except group 4 which were challenged 12 weeks after MVA boost), and the ChAd63 alone group were challenged three - four weeks after vaccination. The difference in interval between vaccination and challenge for the prime-boost and ChAd63 only groups was to ensure comparison of the peak of the immune response as identified in section 3.2.2. Blood was taken for immunology analysis as indicated in Figure 3.8, and volunteers were monitored from day six post challenge by thick film blood smear and PCR to detect parasitaemia and treatment was initiated after parasite detection in the blood. The development of parasitaemia

in the control volunteers in the two challenge studies, part A and part B was not significantly different therefore data from both challenge studies was combined and analysed together. The Senior Immunologist was Dr Katie Ewer and a team of Research Assistants at the Jenner Institute, including Katharine Collins, Sean Elias and Fenella Halstead, processed the blood samples, performed the *ex vivo* ELISpot assays and assisted Dr Katie Ewer with the ICS. Katharine Collins carried out the cultured ELISpots, T cell affinity assays, ELISA and IFA assays. Dr Katie Ewer performed all ICS data analysis. All other data presented in this section were analysed by Katharine Collins. A team of Research Assistants performed the PCR and a team of clinical staff at the Jenner Institute, Centre for Clinical Vaccinology and Tropical Medicine performed all vaccinations, the sporozoite challenge and collection of blood samples.

Table 3.2 Mal34 study design – vaccination and challenge regimens

Healthy malaria naïve volunteers aged between 18 and 50 were vaccinated and challenged as detailed below. Part A was carried out prior to part B. *ChAd63 = ChAd63 ME.TRAP, MVA = MVA ME.TRAP and Ad+M = both vaccines mixed prior to administration.*

Group	Part	Vaccine regimen	Interval between vaccinations	Interval between vaccination and challenge (weeks)
1	A	ChAd63 – MVA	8 week	2-3
2	A	ChAd63		3-4
3	A	No vaccine control		
4	B	ChAd63 – MVA	8 week	12
5	B	ChAd63 – MVA	8 week	2-3
6	B	Re-challenge		
7	B	No vaccine control		
8	B	Ad+M – Ad+M – Ad+M	8 week	2-3
9	B	Ad+M – Ad+M	8 week	2-3
10	B	Ad+M – Ad+M – Ad+M	4 week	2-3

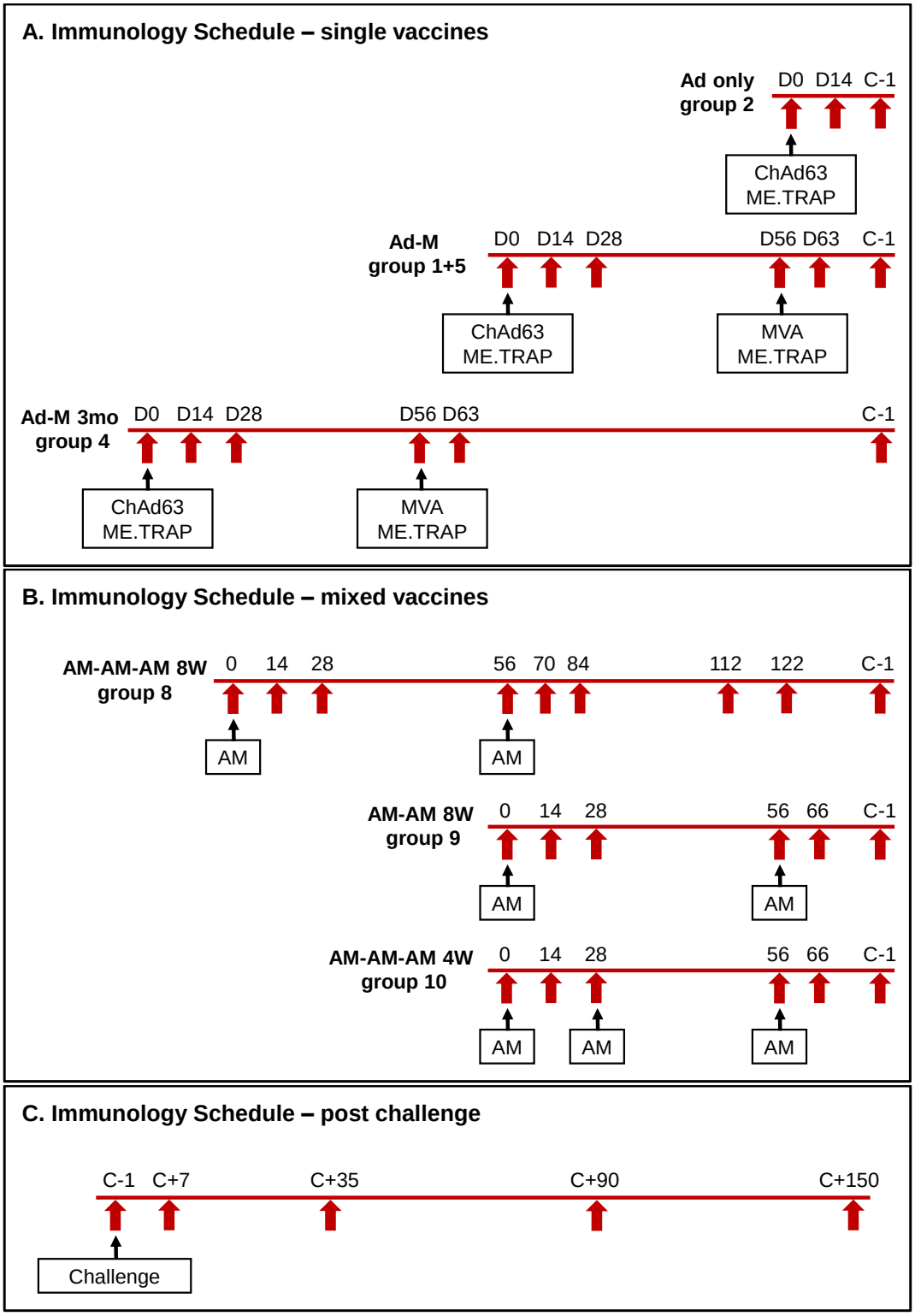


Figure 3.8 Mal34 study design – immunology schedule

In all groups samples were taken for immunology prior to vaccination (D 0), and at various time points post vaccination dependant on immunisation regimen, indicated by the red arrows **(A+B)**. For all groups samples were also taken after challenge as indicated in **(C)**.

3.3.2 Efficacy against sporozoite challenge

ChAd63 – MVA ME.TRAP was shown in the previous section 3.2.2, to be a highly immunogenic regimen that induces high levels of IFNγ T cells and moderate levels of TRAP specific IgG. Here the ability of these vaccines to induce an immune response that can protect against sporozoite infection was assessed. ChAd63 ME.TRAP and MVA ME.TRAP were administered in various regimens (Table 3.2) followed by exposure to five bites from *P. falciparum* infected mosquitoes and volunteers were monitored from day six after challenge for the development of blood stage parasitaemia. Volunteers were considered sterilely protected if they were slide negative at day 21 after challenge and partial vaccine efficacy was assessed as a significant delay in the development of parasitaemia in the blood compared to control volunteers, determined by comparison of Kaplan-Meier survival curves by Log-rank (Mantel-Cox) Test.

The prime-boost regimen followed by challenge two - three weeks after vaccination, elicited significant protective efficacy ($p = 0.0079$) when comparing vaccinated volunteers to unvaccinated controls by Log-rank (Mantel-Cox) Test (Figure 3.9-A). This regimen sterilely protected 21% of the volunteers (3/14) and also delayed the development of parasitaemia in the blood by >2 days in 5 other volunteers, compared to the controls. This level of sterile protection is greater than that achieved by other vectored vaccine regimens previously assessed using the ME.TRAP insert (0-12.5%) [355, 384, 385] and it is estimated that the delay in parasitaemia corresponds to a 96% reduction in liver parasite burden [357, 383]. No volunteers were protected when the interval between vaccination and challenge was increased to three months (Figure 3.9-C) and immunisation with ChAd63 ME.TRAP administered alone or mixed and administered with MVA in various regimens did not

confer any protective efficacy (Figure 3.9-B+D). The prime-boost regimen therefore appears important for the induction of the protective immune response.

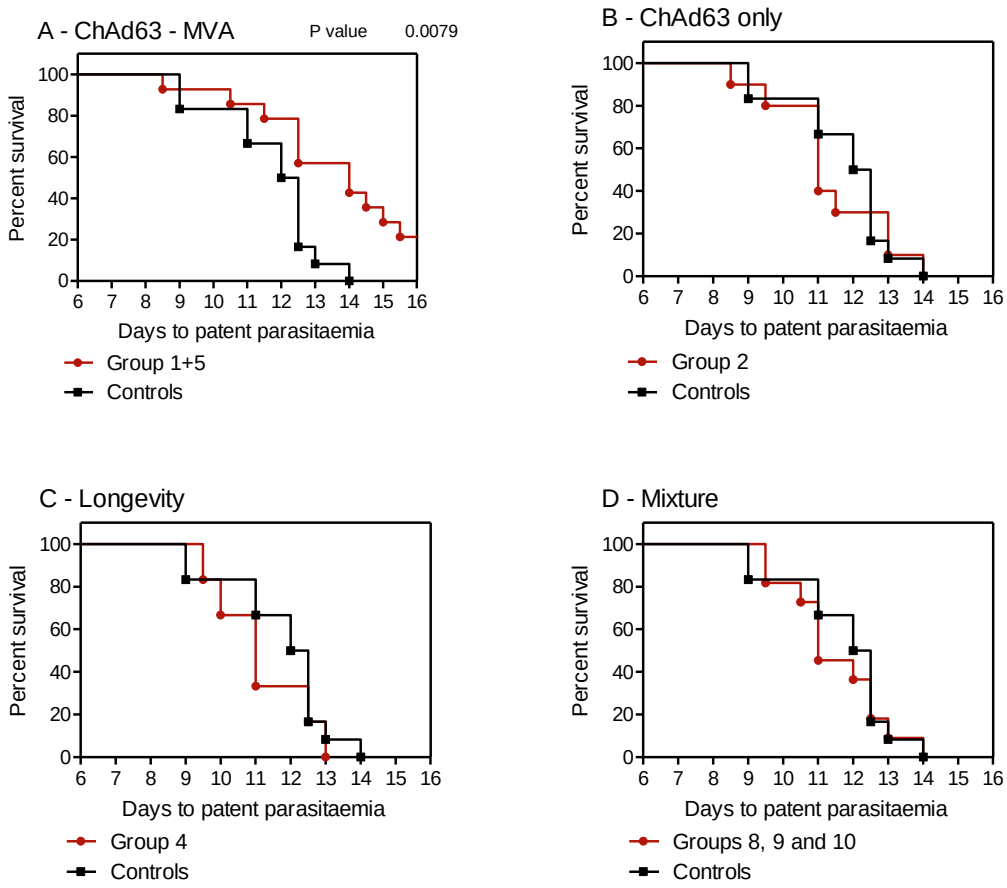


Figure 3.9 Protective efficacy elicited by ChAd63 ME.TRAP and MVA ME.TRAP vaccines in a controlled human malaria infection model

Groups of volunteers were vaccinated with ChAd63 ME.TRAP and MVA ME.TRAP as detailed in Table 3.2 followed by exposure to five bites from *P. falciparum* infected mosquitoes along with unvaccinated controls (n=12). Volunteers were monitored from day 6 post challenge for the development of blood stage parasitaemia until slide positive or considered sterilely protected if slide negative at day 21 post challenge. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** Group 1+5, ChAd63 – MVA, n=14 (P = 0.0079), **(B)** Group 2, ChAd63 only, (n=10) **(C)** group 4, ChAd63 – MVA delayed challenge at 3 months, n=6, **(D)** Groups 8,9 and 10, ChAd63+MVA mixture regimens (n=11).

3.3.3 Cellular immunogenicity

3.3.3.1 Assessment of effector T cell responses by ex vivo IFNγ ELISpot

Vaccination induced T cell responses were measured by *ex vivo* IFNγ ELISpot after vaccination and challenge as indicated in Figure 3.8 and ME.TRAP specific responses were detected in all vaccinated volunteers. T cell responses were detected in volunteers receiving the same regimen in part A and part B (groups 1 and 5) were not significantly different at any time point and hence the data were pooled from these groups for analysis (Figure 3.10-A). ChAd63 – MVA ME.TRAP administered as an eight week heterologous prime-boost regimen (Ad-M) was more immunogenic than these two vaccines mixed and administered together in the same eight week prime-boost regimen (AM-AM 8W) (Figure 3.10-B). After the prime vaccination ME.TRAP specific T cell responses were equivalent for these two groups (median 384 and 364 SFC/10^6 PBMC); however after the second vaccination, the mixed vaccine was only able to boost the responses to a median of 656 SFC/10^6 PBMC whereas the median response after the MVA boost in the Ad-M regimen was almost four times this, 2456 SFC/10^6 PBMC. ChAd63 ME.TRAP administered alone was also able to induce a robust T cell response with the peak median response being 864 SFC/10^6 PBMC (Figure 3.10-C). At the time of sporozoite challenge (C-1) all groups had reasonable levels of ME.TRAP specific T cells detected in the ELISpot and the median group response was highest in the Ad-M vaccinated groups, with the lowest responses in the mixed vaccine regimen groups, although despite the differences in the median group responses, there was a large spread in each group and therefore no significant difference (Figure 3.10-D-E).

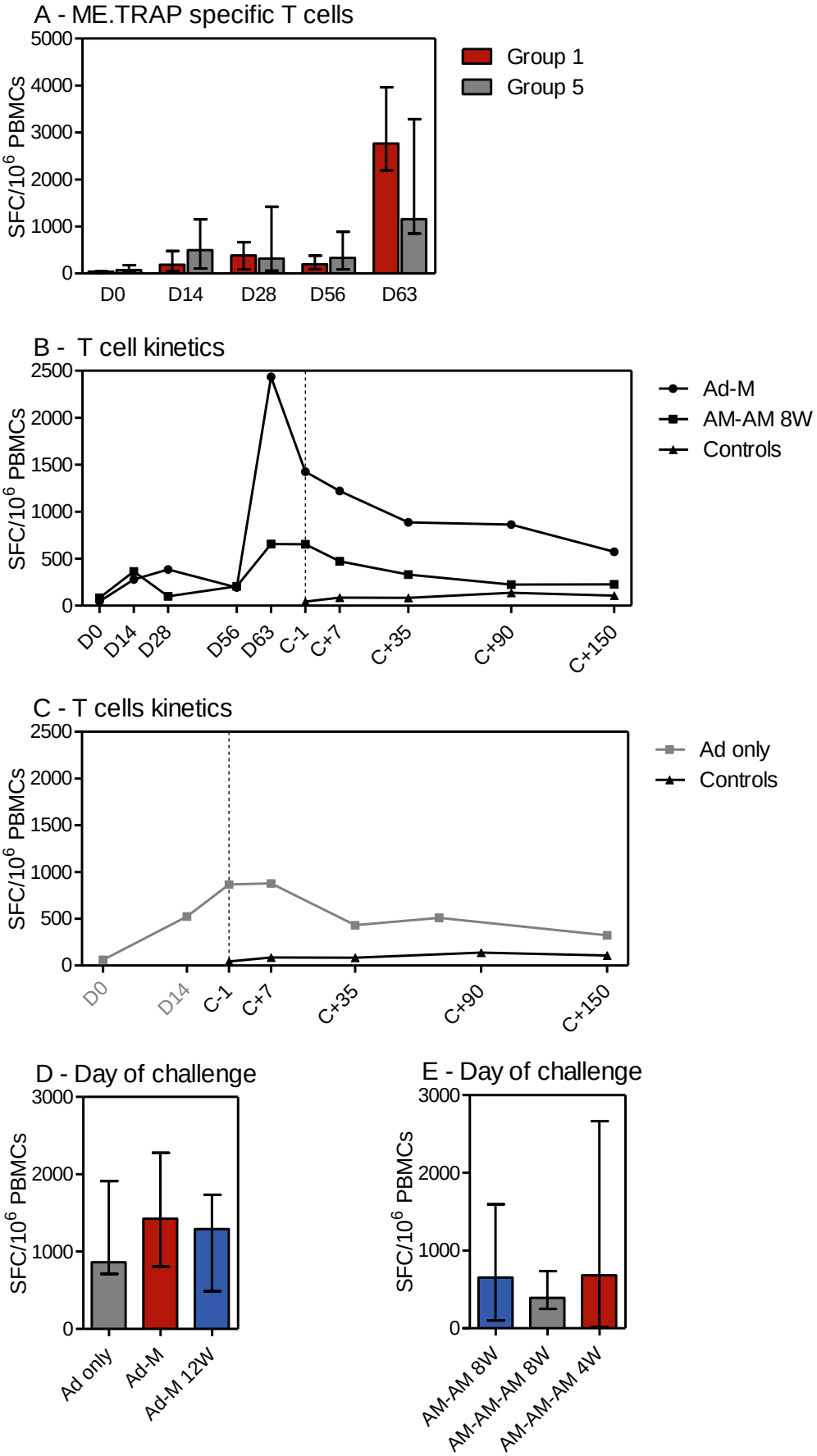
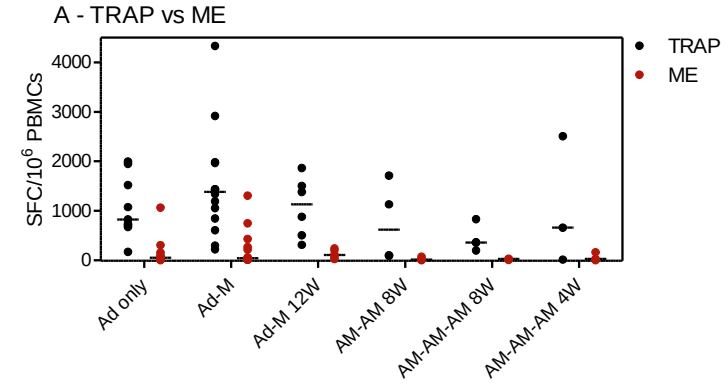
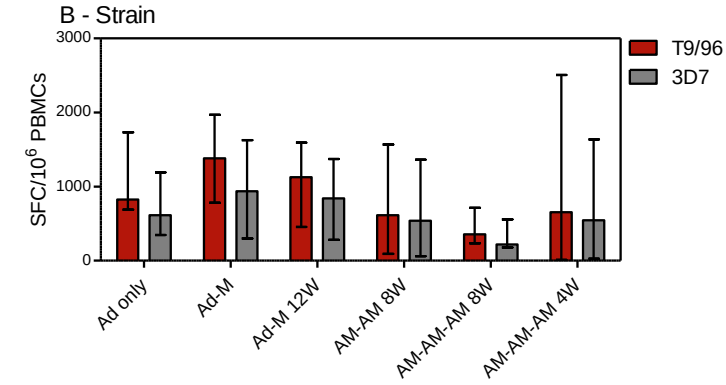

A - ME.TRAP specific T cells
SFC/10^6 PBMCs
5000
4000
3000
2000
1000
0
D0
D14
D28
D56
D63
Group 1
Group 5
B - T cell kinetics
SFC/10^6 PBMCs
2500
2000
1500
1000
500
0
D0
D14
D28
D56
D63
C-1
C+7
C+35
C+90
C+150
Ad-M
AM-AM 8W
Controls
C - T cells kinetics
SFC/10^6 PBMCs
2500
2000
1500
1000
500
0
D0
D14
C-1
C+7
C+35
C+90
C+150
Ad only
Controls
D - Day of challenge
SFC/10^6 PBMCs
3000
2000
1000
0
Ad only
Ad-M
Ad-M 12W
E - Day of challenge
SFC/10^6 PBMCs
3000
2000
1000
0
AM-AM 8W
AM-AM-AM 8W
AM-AM-AM 4W

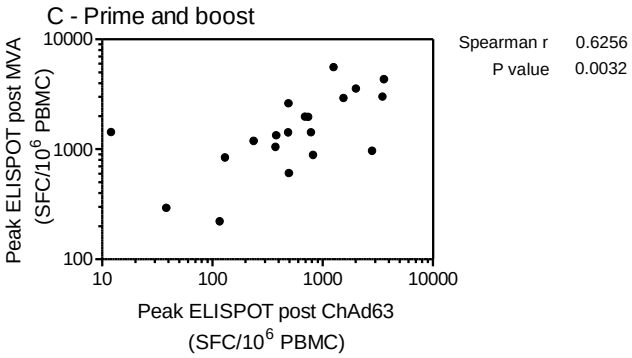
Figure 3.10 ME.TRAP specific T cell responses during vaccination and challenge

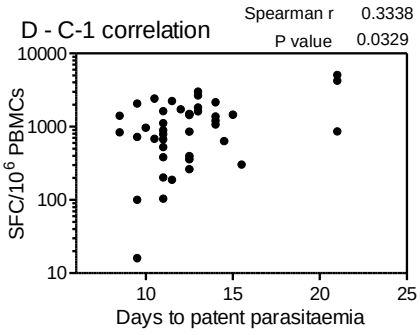
Groups of volunteers were vaccinated with ChAd63 ME.TRAP and MVA ME.TRAP in the regimens detailed in Table 3.2. ME.TRAP specific T cell responses were measured in an IFNγ ELISpot after vaccination and challenge. **(A)** Comparison of responses from group 1 in part A and group 5 in part B which received the same prime-boost regimen. **(B)** Kinetics of the T cell response for volunteers receiving ChAd63 – MVA or mixed vaccination. **(C)** Kinetics of the T cell response for volunteers receiving ChAd63 only. **(D)** Comparison of T cell responses the day before sporozoite challenge (C-1). Median responses (with interquartile range) are shown. *Ad = ChAd63 ME.TRAP. Ad-M = ChAd63 – MVA ME.TRAP 8 week prime boost regimen. AM = ChAd63 ME.TRAP and MVA ME.TRAP mixed and administered together. 8W and 4W = interval between vaccinations. 12W = interval between vaccination and sporozoite challenge. D0 etc. = day 0 and C+7 etc. = Challenge plus 7 days.*

The majority of the T cell response detected in the IFNγ ELISpot was generated towards the TRAP antigen in the vector insert as opposed to the ME string (Figure 3.11-A). The TRAP response was measured to both the strain used in the vaccine (T9/96) and the strain used at challenge (3D7). These sequences vary by 37 amino acids and are approximately 6.5% different; the challenge therefore is considered heterologous (Figure 3.11-B). The responses detected to the 3D7 strain were on average 73% (60-90) of the response to the T9/96 strain and these responses strongly correlated with each other. For the prime-boost groups (gp1, 4 and 5), the responses to the ChAd63 prime were strongly associated with the magnitude of the response after the MVA boost (Spearman's correlation $p = 0.0032$, $r = 0.6256$) (Figure 3.11-C), indicating that a response primed by the ChAd63 vector is important and may impact the peak effector T cell response generated after the MVA boost. Interestingly the magnitude of the *ex vivo* ELISpot response detected in the vaccinated volunteers on the day before challenge (C-1) correlated with protective efficacy as measured by days taken to develop parasitaemia in the blood (Spearman's correlation $p = 0.0329$, $r = 0.3338$) (Figure 3.11-D). Furthermore, the magnitude of the response measured on day 7 after challenge was even more strongly associated with efficacy (Spearman's correlation $p = 0.0089$, $r = 0.4032$) (Figure 3.11-E).


A - TRAP vs ME
TRAP
ME
SFC/10^6 PBMCs
4000
3000
2000
1000
0
Ad only
Ad-M
Ad-M 12W
AM-AM 8W
AM-AM-AM 8W
AM-AM-AM 4W


B - Strain
T9/96
3D7
SFC/10^6 PBMCs
3000
2000
1000
0
Ad only
Ad-M
Ad-M 12W
AM-AM 8W
AM-AM-AM 8W
AM-AM-AM 4W


C - Prime and boost
Spearman r 0.6256
P value 0.0032
Peak ELISPOT post MVA (SFC/10^6 PBMC)
10000
1000
100
10
100
1000
10000
Peak ELISPOT post ChAd63 (SFC/10^6 PBMC)


D - C-1 correlation
Spearman r 0.3338
P value 0.0329
SFC/10^6 PBMCs
10000
1000
100
10
10
15
20
25
Days to patent parasitaemia

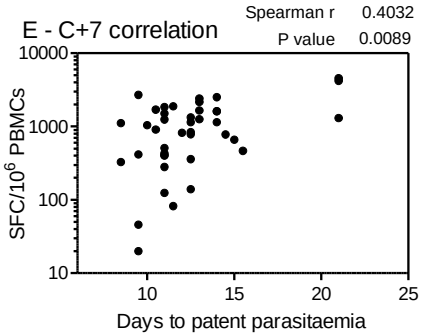

E - C+7 correlation
Spearman r 0.4032
P value 0.0089
SFC/10^6 PBMCs
10000
1000
100
10
10
15
20
25
Days to patent parasitaemia

Figure 3.11 Analysis of T cell responses measured by *ex vivo* IFNγ ELISpot

Groups of volunteers were vaccinated with ChAd63 ME.TRAP and MVA ME.TRAP in the regimens detailed in Table 3.2. ME.TRAP specific T cell responses were measured in an IFNγ ELISpot to pools of overlapping peptides. **(A)** Responses measured to either the TRAP or ME portion of the insert **(B)** Responses measured to TRAP sequence in the vaccine (parasite strain T9/96) and challenge strain (3D7) **(C)** Correlation of peak response after prime and peak response after boost to the ME.TRAP insert in groups 1, 4 and 5 (n=20). **(D)** Correlation of the IFNγ ELISpot response to ME.TRAP on the day before challenge and efficacy (all vaccinated volunteers n=41). **(E)** Correlation of the IFNγ ELISpot response to ME.TRAP 7 days after challenge and efficacy (all vaccinated volunteers n=41) Median responses with interquartile range are shown. Spearman's rank correlation used for C, D and E.

3.3.3.2 Assessment of effector T cell responses by flow cytometry

TRAP specific T cells were also measured by flow cytometry with intracellular cytokine staining (ICS) to assess the frequencies of T cells populations expressing the cytokines IFNγ, TNF, IL2. In the groups receiving the ChAd63 – MVA ME.TRAP prime-boost (Gps 1, 4 and 5), T cells expressing all three cytokines were detected and the levels of CD8+ T and CD4+ T cell subsets were similar (Figure 3.12-A). When these parameters were correlated with protective efficacy, the strongest association was seen with CD8+ T cells that express IFNγ (Spearman rank correlation, $p = 0.0219$ and $r = 0.5089$) (Figure 3.12-B). The multi-functionality of the T cells was also assessed looking at how many of the three cytokines, and which combination of cytokines the individual cells expressed (data not shown) [357]. The association with protective efficacy was even stronger in the population that were not polyfunctional and expressed IFNγ only and not TNF or IL2 (Spearman rank correlation, $p = 0.0003$, $r = 0.7268$) (Figure 3.12-C). This correlation reinforces the association seen between the ELISpot data and protective efficacy and highlights the importance of examining the subsets of T cells that contribute to the response detected in the ELISpot in order to understand what may be contributing to a protective vaccine induced immune response.

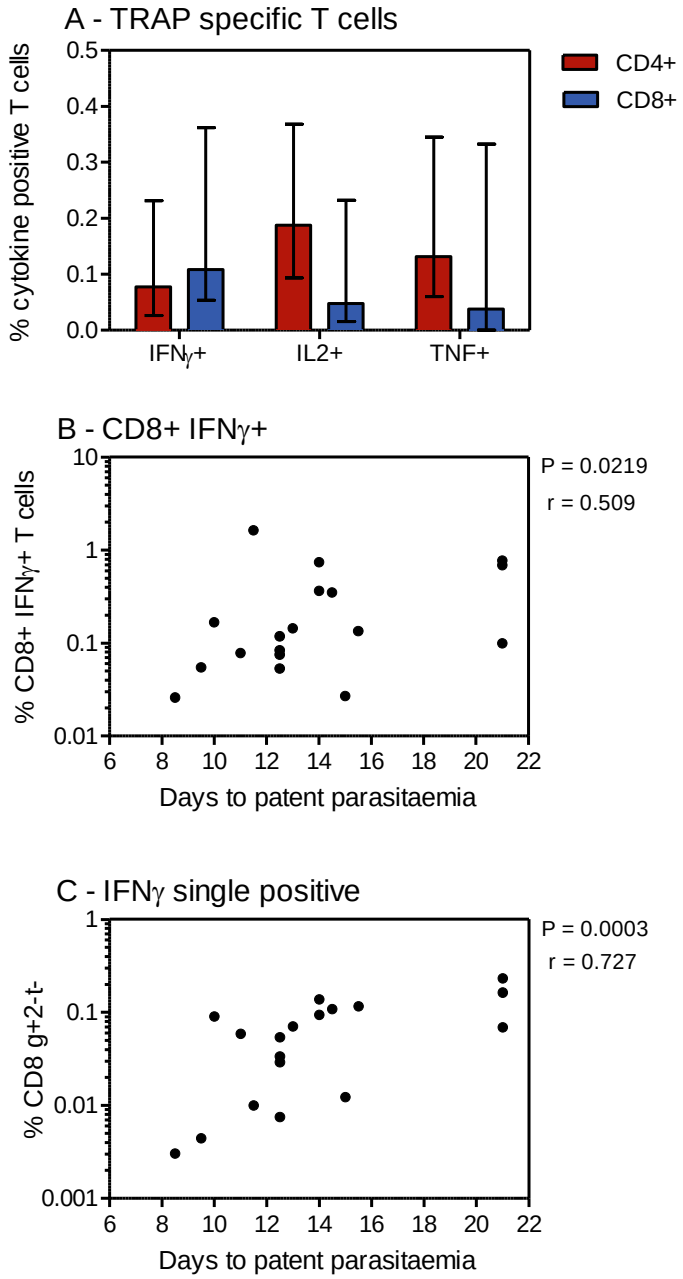


Figure 3.12 TRAP specific T cell responses measured by flow cytometry with intracellular cytokine staining

Volunteers were vaccinated with ChAd63 - MVA ME.TRAP 8 week prime-boost regimen (groups 1, 4 and 5) and CD4+ and CD8+ cytokine secreting T cell frequencies were assessed on the day before challenge by ICS and flow cytometry. Cells were stimulated for 18 hours with a single pool of overlapping TRAP peptides and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD4+ or CD8+ T cells expressing the cytokine (Group medians with interquartile range are shown) **(A)**. Correlation CD8+ IFNγ+ T cells and efficacy **(B).** Correlation of single positive IFNγ+ CD8+ T cells and efficacy **(C)**. Spearman's rank correlation used for B and C.

3.3.3.3 T cells with a higher affinity for antigen are more protective

In part B of the study the concentration of peptide used in an ELISpot to re-stimulate the T cells was evaluated. This was to determine if there was a difference between the volunteers in the affinity/avidity of their T cells generated by vaccination and if the T cell response measured by a lower concentration of peptide is more relevant to or more strongly associated with protection. Are the T cells that have a higher affinity for their antigen i.e. respond to lower concentrations of peptide more protective? [416] In this study ELISpots were performed on all volunteers the day before challenge to a single pool of TRAP peptides using a range of concentrations (10, 2, 0.4, 0.08µg/ml). In the analysis volunteers are grouped by efficacy, those that were either protected or delayed (developed parasitaemia later than day 13) and those that were unprotected and there is an increasing difference between the magnitudes of the responses in the protected vs unprotected volunteers as the concentration of peptide is reduced (Figure 3.13-A-D). The statistical significance between these groups also increases accordingly, P = 0.1195 at 10µg/ml whereas p = 0.0117 at 0.08µg/ml peptide. The TRAP specific T cell responses also correlate with protective efficacy when measured using 0.08ug/ml but not 10µg/ml (Figure 3.13-E). Therefore when measured using 0.08µg/ml, the TRAP specific T cells responses are higher in protected volunteers than unprotected on the day before challenge. As an alternative way of interpreting the data, affinity could be represented as the response detected to 0.08µg/ml as a percentage of the max response (response to 10µg/ml). Again, T cell affinity is higher in the protected vs the unprotected volunteers and this measure also correlates strongly with protective efficacy (Figure 3.13-F+G). Therefore the robust and cost effective ELISpot assay may be more effective at assessing vaccine efficacy by simply using a lower concentration of peptide.

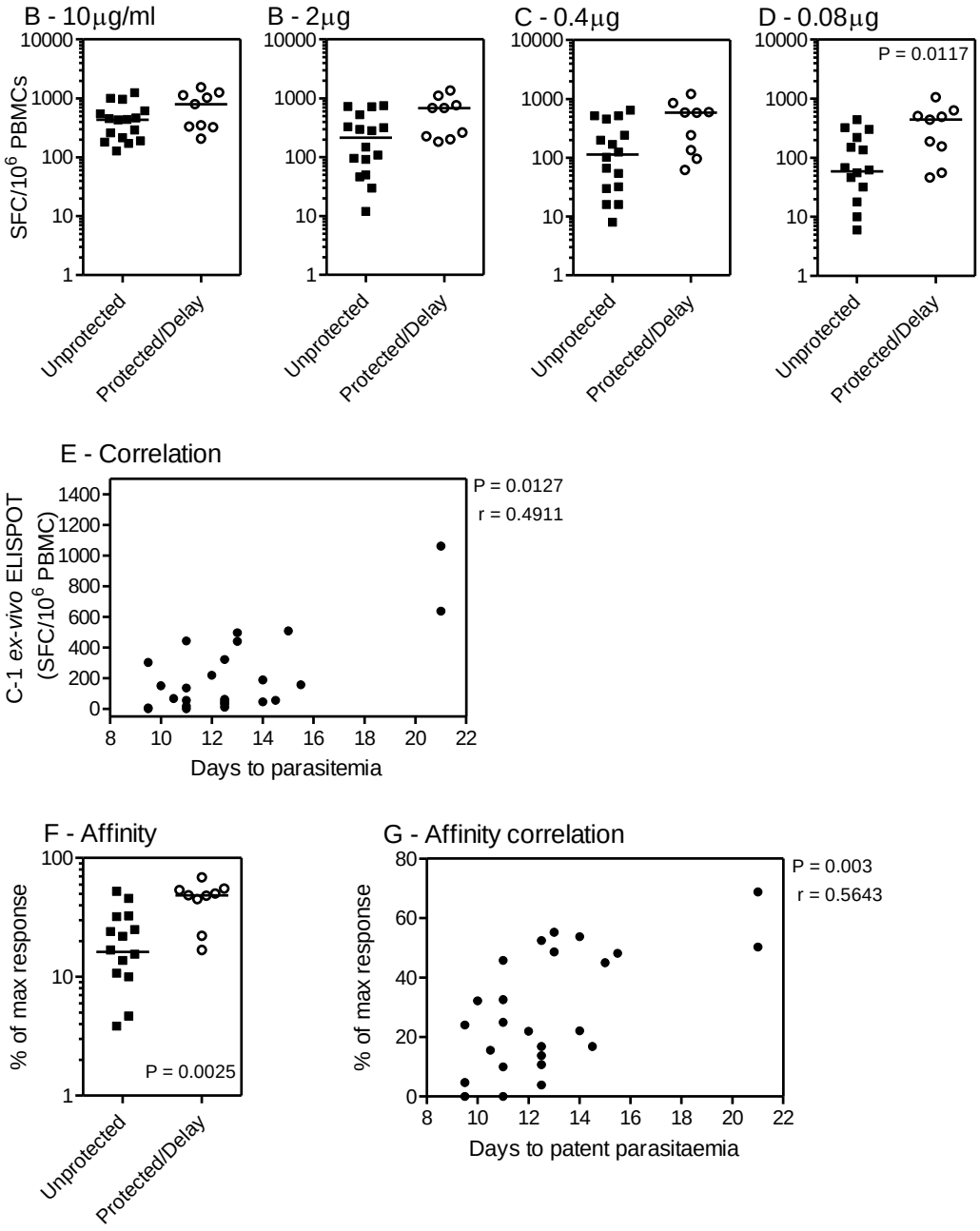


Figure 3.13 Assessment of T cell affinity using peptide titration in an *ex vivo* IFNγ ELISpot

TRAP specific T cell responses from volunteers in part B of the study (n=25) were assayed in an IFNγ *ex vivo* ELISpot using a single pool of TRAP peptides at a range of concentrations (10, 2, 0.4, 0.08μg/ml) **(A-D)**. Responses measured at the lowest peptide concentration 0.08μg/ml correlated with efficacy **(E)**. Affinity, defined as the response at 0.08μg/ml as a percentage of the response at 10μg/ml **(F)**. Affinity correlated with efficacy **(G)**. Median responses shown and compared by (Mann-Whitney test) and Spearman's rank correlation used for E and G.

3.3.3.4 Assessment of T cell memory responses by IFNγ cultured ELISpot

Memory T cell responses were induced by the ChAd63 – MVA ME.TRAP regimen in the dose escalation study described in the previous section (3.2.2.3) and were higher than those detected in previous studies of TRAP based vectored vaccines. In these previous studies in malaria naïve adults a relationship between memory T cells measured on the day of sporozoite challenge and protection against malaria in CHMI was identified [386]. The cultured ELISpot assays was therefore used in this trial to quantify the memory T cell responses induced by the ChAd63 - MVA ME.TRAP vaccines used in the prime-boost regimen or ChAd63 ME.TRAP alone to assess any relationship with protective efficacy. Responses were measured the day before sporozoite challenge (C-1), in groups 1 and 2, to the ME.TRAP insert in an IFNγ ELISpot after 10 days of culture with antigen (Figure 3.14-A). Memory T cells were successfully detected in all volunteers and responses were similar in the two groups suggesting that the ChAd63 vaccine may be important in the induction of memory T cells and in accordance with this, the peak *ex vivo* ELISpot response after ChAd63 correlates with the cultured ELISpot response on the day before challenge (Figure 3.14-B). No association was seen however between the level of memory T cells detected in the cultured ELISpot assay and protective efficacy, as seen in previous trials of ME.TRAP based vectors (Figure 3.14-C).

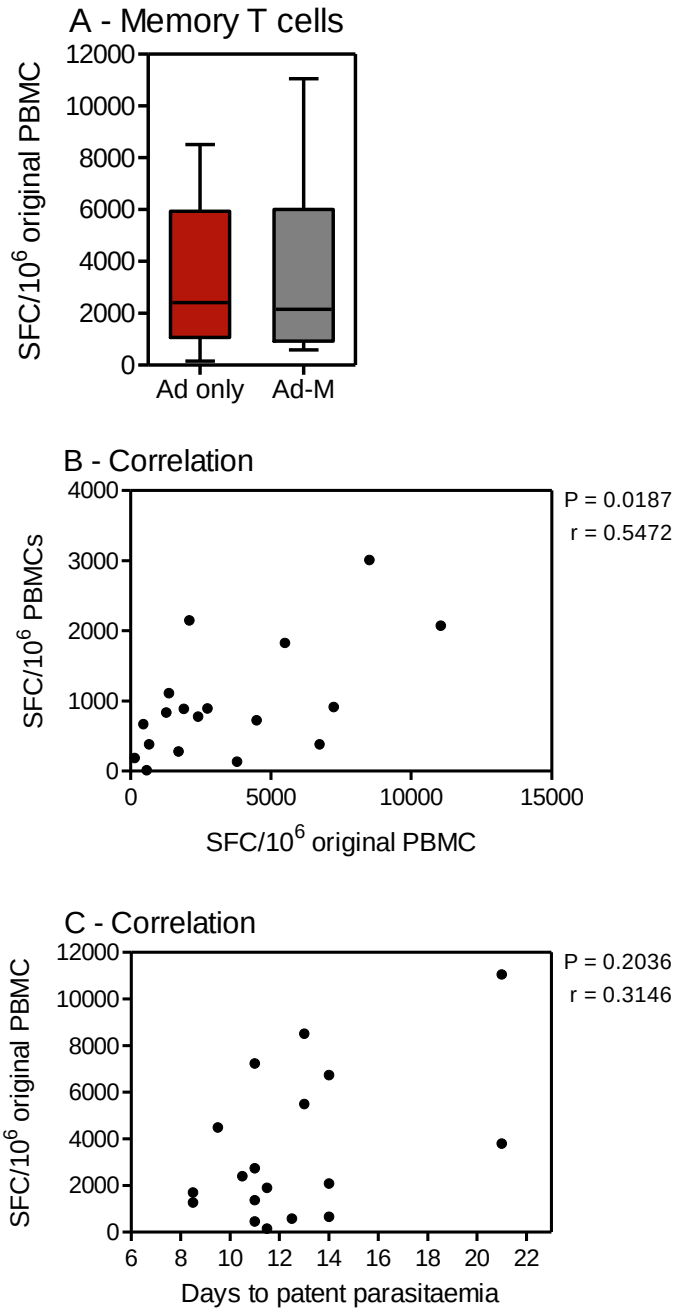


Figure 3.14 Memory T cell responses induced by vaccination with ChAd63 ME.TRAP and MVA ME.TRAP before challenge

ME.TRAP specific memory T cells were measured in part A of the study the day before challenge by IFNγ ELISpot after 10 days of culture with ME.TRAP peptides. **(A)** Comparison of responses measured in the prime only (Ad only) and prime-boost (Ad-M) groups. Box plots displaying the median with whiskers showing the minimum and maximum responses are shown **(B)** Correlation of *ex vivo* IFNγ ELISpot response after prime and cultured IFNγ ELISpot responses the day before challenge. **(C)** Correlation of memory T cells and efficacy. Individual responses are shown. Spearman's rank correlation analysis used for B and C.

3.3.4 Humoral immunogenicity

3.3.4.1 Assessment of TRAP specific IgG responses by ELISA

TRAP specific IgG measured by ELISA was detected in 17/18 volunteers after vaccination with either ChAd63 – MVA ME.TRAP or ChAd63 alone and the remaining negative volunteer seroconverted after challenge. The median peak endpoint titre for the prime-boost regimen was 760 EU and for the ChAd63 alone group the median titre was 383 EU (Figure 3.15-A). On the day before challenge there was no significant difference between TRAP specific IgG levels detected in the two groups and there was no association between the TRAP specific IgG and protective efficacy (Figure 3.15-B+C). This suggests that either the TRAP specific IgG does not have a significant role in protection against sporozoite challenge or that the IgG detected in this assay is not functional and does not bind to native TRAP on the surface of the sporozoites.

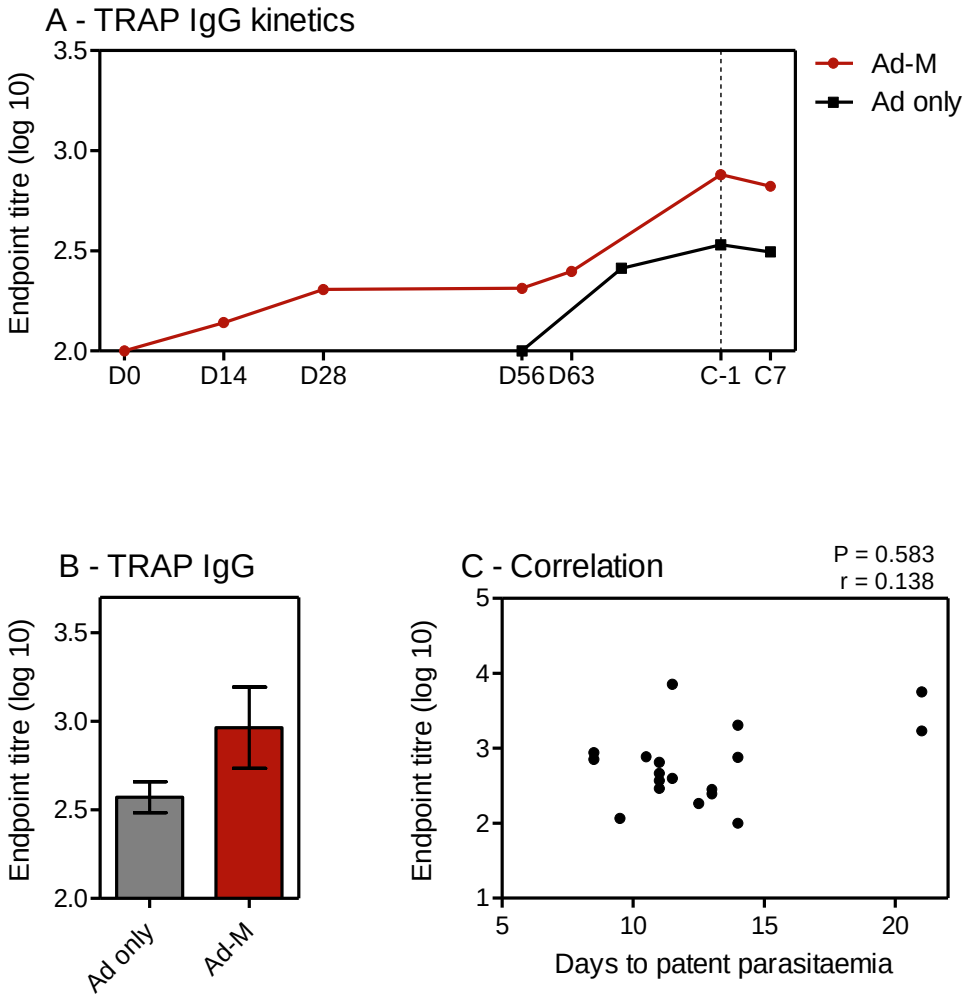


Figure 3.15 TRAP specific IgG measured prior to challenge

TRAP specific IgG was measured by ELISA in volunteers in part A of the study (Ad-M n=8) (Ad only n=10) **(A)**. Responses measured the day before challenge **(B)**. TRAP IgG measured the day before challenge compared to efficacy **(C)**. Median responses (with interquartile range) shown and compared by (Mann Whitney-test) and Spearman's rank correlation for C.

3.3.4.2 Functional analysis of vaccine induced IgG by IFAT

To determine if the vaccine induced TRAP specific antibodies detected in the ELISA were able to bind to TRAP exposed on the surface of sporozoites, Immunofluorescence antibody test (IFAT) were performed. Serum was tested from samples collected before vaccination (day 0) and the day before challenge (C-1). After vaccination the IgG in serum samples, presumably TRAP specific, bound to the sporozoites and was detected in the IFAT, whereas day 0 samples did not shown any binding (Figure 3.16). This indicates that the TRAP specific IgG may be functional and may have a role in protection against sporozoite challenge, although other aspects of antibody type or quality may be important and these are not measured in this assay.

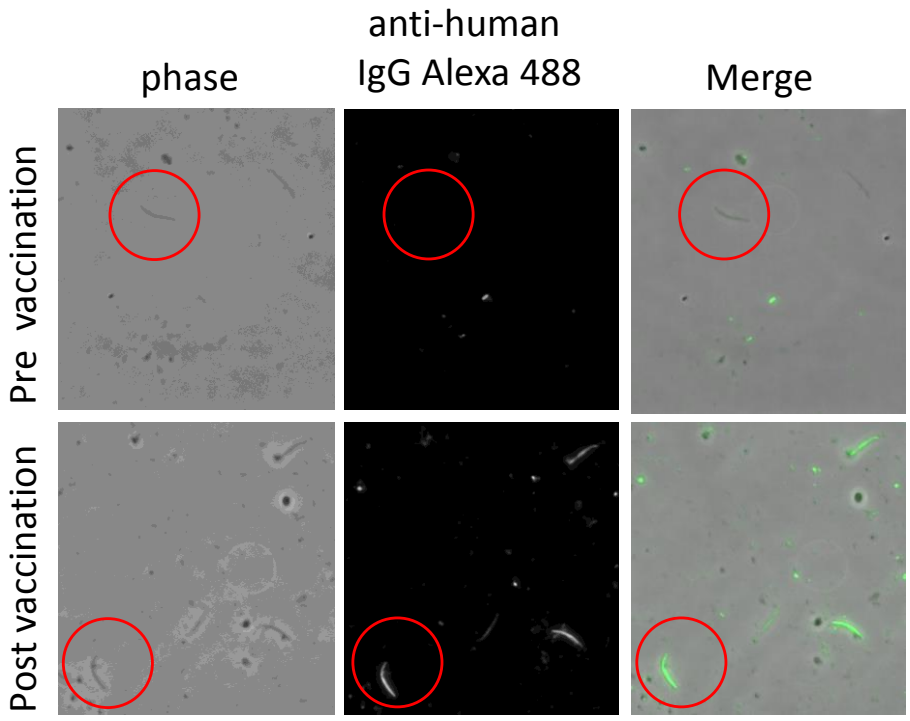


Figure 3.16 Immunofluorescence antibody test

Serum collected from volunteers pre and post vaccination was incubated with sporozoites to assess IgG binding. Phase contrast and fluorescence images were taken to detect bound IgG with anti-human IgG conjugated to Alexa Fluor-488.

3.4 Discussion

The initial aim of this chapter was to assess the simian adenovirus ChAd63 as a new vector for the induction of ME.TRAP-specific T cells in a Phase 1 dose escalation and route finding study. Immunisation with ChAd63 ME.TRAP was safe and well tolerated when given alone using either intradermal or intramuscular administration and when used in the ChAd63 – MVA ME.TRAP regimen [356]. ChAd63 ME.TRAP induced T cell responses that were detected in an *ex vivo* ELISpot in 100% of vaccinated volunteers and the highest responses were seen with the highest vaccine dose (2×10^{11} vp). These responses were much higher than those induced in previous studies using DNA, FP9 and MVA vectors expressing the same ME.TRAP insert, either used alone or in prime-boost regimens [355, 382, 384, 385, 387]. In these previous trials in malaria naïve adults, the most immunogenic regimens employed multiple vectors and induced an average of 450 SFC/10^6 PBMC [355, 384, 385]. However, ChAd63 appears to be much more potent than these vectors, and the same level of cellular response can be reached by only a single high dose immunisation with ChAd63 ME.TRAP (2×10^{11} vp) on its own (915 SFC/10^6 PBMC). The MVA immunisation significantly boosted the response and the ChAd63 – MVA ME.TRAP prime-boost induced extraordinarily high levels of antigen specific T cells with the group median response exceeding 2000 SFC/10^6 PBMC in two of the dose groups (1×10^{10} vp and 2×10^{11} vp). There was a trend to increased response with dose, seen most clearly after prime and three months after boost. Though assessing a clear effect of dose on immunogenicity is difficult with such a large spread in the magnitude of the responses and only 4 – 8 volunteers per group. Since there is a large body of evidence that cellular responses towards TRAP can be protective in various models, induction of such high levels of T cells may mean TRAP mediated efficacy is enhanced by this regimen. In addition to assessing the magnitude in effector T cells after prime-boost immunisation a subset of volunteers were also

given a third vaccination to assess the ability of these vectors to safely re-boost the cellular response. Volunteers received an additional ChAd63 ME.TRAP or MVA ME.TRAP (Ad-M-Ad or Ad-M-M) and both vaccines were able to effectively re-boost the response back to the level of the original boost. The Ad-M-Ad regimen may be slightly more effective as the re-boost response was 109% of the original boost response, compared to 76% for Ad-M-M group. Both vectors however were safe and effective suggesting anti-vector immunity does not impair immunogenicity and this could have important implications if viral vector vaccines become widely used.

In previous clinical trials with ME.TRAP vectors, in addition to the effector T cell response the memory T cells were also assessed with a cultured ELISpot assay. The principle of this being that after immunisation T cells recognise antigen presented on APCs and are triggered to proliferate and differentiate resulting in rapid expansion of an effector T cell population. Within a relatively short period of time the effector response peaks and then contracts and a large percentage of the effector cells die [417, 418]. The remaining T cells are thought to primarily be central memory T cells capable of responding to re-infection and it is thought that these populations will be crucial for long term protection [260, 385, 418, 419]. Following prime-boost immunisation with ChAd63 – MVA the effector T cell response expands rapidly and peaks approximately 7 days after the MVA and then begins to contract. Memory T cells are therefore likely to respond to infection once the effector population has contracted and the cultured ELISpot assay can be used to assess the induction of this population after vaccination [386, 420]. The assay uses the production of IFNγ from T cells after overnight re-stimulation with peptide as a readout in the same manner as the *ex vivo* ELISpot assay. The difference between the assays is that the PBMCs are cultured prior to the ELISpot allowing the resting memory T cells to become activated and to divide, enabling the central memory cells to differentiate into effector cells. ChAd63 – MVA ME.TRAP was shown to be very effective at

inducing memory T cells and they were detected in 7 out of 8 samples that were assayed three months after the MVA boost. This regimen induced higher levels of memory T cells than previous viral vector regimens [386] and at this time point the effector T cell response was very low in these groups suggesting any T cell mediated protection would be elicited by these memory cells. A number of studies have indicated that memory T cell responses are associated with protection [260, 385, 415, 419-422], so this greater ability of this regimen to induce memory cells may result in enhanced efficacy. The prime-boost regimen was also able to induce good levels of TRAP-specific IgG, which is a significant improvement upon previous viral vector regimens which have been unable to induce antibodies to TRAP [385]. What impact these antibodies might have on protection is unclear. Inhibition of TRAP has been shown to be protective [364], correlations of TRAP specific antibodies in naturally exposed individuals with protection have been observed [122, 125, 126] and the ability of antibodies to TRAP to bind to and inhibit invasion has been shown *in vitro* [208-210] but not *in vivo* [211]. One study has suggested that antibodies to three pre-erythrocytic proteins TRAP, CSP and LSA1 were more protective than antibodies to a single protein, so it may be that the TRAP antibodies induced here could contribute to protection in a multi-component vaccine, if not protective on their own [125, 423].

The second aim of this chapter was to assess the efficacy of ChAd63 and MVA ME.TRAP. The prime-boost regimen followed by challenge two - three weeks after vaccination sterilely protected 21% of the volunteers (3/14) and also delayed the development of parasitaemia in the blood in a further 36% (5/14). This level of sterile efficacy is greater than that achieved by previously assessed vectored vaccine regimens using the ME.TRAP insert (0-12.5%) [355, 384, 385]. Although this regimen did not induce sterile protection in all volunteers, these vaccine-induced responses appear very effective at eliminating the liver stage parasites, as it is

estimated that the delay in parasitaemia corresponds to a 96% reduction in liver parasite burden [357, 383]. No volunteers were protected when the interval between vaccination and challenge was increased to three months, though numbers in this group were small. This suggests that it will be important to assess more fully the durability of efficacy in future studies. Notably, the three protected volunteers from this study were re-challenged 8 months after immunisation and first challenge and one showed sterile protection and the other two were significantly delayed [68].

Immunisation with ChAd63 ME.TRAP alone or mixed and administered with MVA in various regimens did not confer any protective efficacy in contrast to that seen with ME.TRAP vectors in mice [412]. The heterologous prime-boost regimen may therefore be important for the induction of the protective immune response in humans, suggesting the magnitude or type or response may differ between the groups. Indeed, the ChAd63 – MVA ME.TRAP heterologous prime-boost was the most immunogenic regimen and induced the highest levels of ME.TRAP specific T cells. However at the time of sporozoite challenge although the median group response was highest in the ChAd63 – MVA ME.TRAP vaccinated groups there was a large spread in the magnitude of the responses and therefore no significant difference between the groups. Interestingly though, the magnitude of the *ex vivo* ELISpot response detected the day before challenge correlated loosely with protective efficacy (Spearman's correlation $p = 0.0329$, $r = 0.3338$) and this association was even stronger 7 days after challenge suggesting some of the T cells measured in the IFNγ ELISpot may be populations that contribute to protection. The standard *ex vivo* ELISpot assay however measures T cell responses after re-stimulation with a very high concentration of peptide. This is potentially useful as it is able to identify all antigen specific T cells with both low or high affinity and avidity for their antigen. Yet this level of peptide would be far greater than that encountered at a physiological level. Therefore the assay may be identifying T cells that would not

normally have a role *in vivo* as they would not be able to respond to the low level of peptide. Thus by performing the *ex vivo* ELISpot with lower concentrations of peptide, only T cells that have a higher affinity for their antigen would be detected [416]. This therefore determines if there was a difference between the volunteers in the affinity of their T cells generated by vaccination and it was found that higher affinity T cells were more strongly associated with protection (Spearman's correlation $p = 0.003$, $r = 0.5643$). This supports the correlation seen after the standard *ex vivo* ELISpot and suggests that by simply using a lower concentration of peptide the robust ELISpot assay may be more effective at assessing vaccine efficacy, as this may allow the detection of a more functionally relevant population T cells. T cell affinity and avidity studies have been used to assess the quality and type of protective T cell responses to viral infection and tumours [416] and a number of studies have associated high avidity CD8+ T cells with greater efficacy [424-426]. In addition to looking at the magnitude and affinity of T cell responses the type and quality was also assessed using intra-cellular cytokine staining and flow cytometry in the groups receiving the ChAd63 – MVA ME.TRAP prime-boost. T cells expressing all three cytokines were detected and the levels of both CD8+ and CD4+ T cell subsets were similar, this is in contrast to prime-boost immunisation with previous viral vector regimens where DNA induced predominantly CD4+ T cells and FP9 induced CD4+ dependant CD8+ T cells [427]. It may therefore be the greater proportion of CD8+ T cells induced by ChAd63 – MVA ME.TRAP which contributes to the increased efficacy. As discussed in Chapter 1, CD8+ T cells have been associated with protection against malaria in a large number of studies [413, 414] and specifically after immunisation with TRAP based viral vectors in mice [409]. In agreement with this, when correlated with protective efficacy the strongest association was seen with CD8+ T cells that express IFNγ and interestingly the association was even stronger in the population that were not polyfunctional and expressed IFNγ only and not TNF or IL2. This correlation supports the association

seen between vaccine induced T cells measured in the *ex vivo* ELISpot assays and protective efficacy and highlights the importance of also assessing the functionality of T cells in order to determine what may constitute the protective mechanism.

The majority of the T cell response detected in the IFNγ ELISpot was generated towards the TRAP antigen in the vector insert as opposed to the ME string and the TRAP response was heterologous and measured to both the strain used in the vaccine (T9/96) and the strain used at challenge (3D7). In the prime-boost groups, the responses to the ChAd63 prime were strongly associated with the magnitude of the response after the MVA boost indicating that a response primed by the ChAd63 vector is essential and may impact the peak effector T cell response generated after the MVA boost. Accordingly, vaccination with the same insert in other heterologous prime-boost regimens using different priming vectors did not result in such high T cell responses after boost [385]. Memory T cells were also detected in all volunteers and responses were similar in prime only and prime-boost groups suggesting that the ChAd63 vaccine may be important in the induction of memory T cells. In accordance with this, the peak *ex vivo* ELISpot response after ChAd63 correlates with the cultured ELISpot response on the day before challenge. No association was seen however between the level of memory T cells detected in the cultured ELISpot assay and protective efficacy, in contrast to that seen in previous trials of ME.TRAP based vectors [386]. Serum taken from vaccinated volunteers on the day before challenge was shown to bind to sporozoites; however there was no association between the TRAP specific IgG and protective efficacy. This suggests that either the TRAP specific IgG does not have a significant role in protection as seen in the *in vivo* studies [211] or that this assay is not able to detect protective type of IgG. This assay measures total IgG and does not look at the isotype or the avidity of the IgG, whereas the type or quality of the antibodies may be important for their function. An assay that assesses the ability of IgG to inhibit sporozoite invasion of hepatocytes, or

to inhibit liver stage development [428, 429] could be used to evaluate vaccine induced antibodies. In addition measuring avidity and different isotypes may help dissect an antibody response that is associated with protection.

In this chapter ChAd63 – MVA ME.TRAP prime-boost has been shown to be safe and immunogenic in malaria naïve adults and elicits sterile efficacy in 21% of vaccinated volunteers, mediated by vaccine induced T cells. This is the most immunogenic and protective viral vector regimen assessed to date and it has now also entered safety and immunogenicity studies in African adults and children. Although this result is extremely promising as only one other subunit vaccine has resulted in greater protective efficacy (RTS,S/AS01B), the level of efficacy of both of these regimens is sub-optimal. Efficacy is well below the goals of the malaria vaccine technology roadmap for a vaccine that can contribute significantly to malaria elimination, so ChAd63 – MVA ME.TRAP is unlikely to be deployed in its current form. Further research and development could therefore look to dissect the precise nature of the protective immune response and potentially fine tune and enhance the induction of a more specific type or quality of response by these vectors [430]. Alternatively this vaccine could be improved upon by using it in a multi-component or multi-stage approach and this strategy will be addressed in the following chapters.

4 Generation of R21, a novel CS-based particle vaccine

4.1 Introduction

Virus-like particles (VLPs) are formed from virus capsid or envelope proteins that have the ability to self-assemble into particulate structures resembling native virus or subviral particles. Generally, they present proteins in their natural confirmation and consist of highly ordered structures mimicking the native antigenic properties of a virus. They lack the viral genome and are therefore non-infectious but their size and structure means they can trigger strong cellular and humoral immune responses making them attractive vaccine candidates against viral infections [431-433]. The first VLP to be used as a vaccine was the hepatitis B surface antigen subviral particle (HBsAg) which is approximately 22nm in size (originally termed the Australian antigen). The particle is composed of around 30% hepatitis B surface envelope protein and 70% host lipids, and these particles were the main component of HEPTAVAX, the first hepatitis B vaccine licensed in 1981 [434]. The development of this vaccine was based on studies in the 1970s which had shown heat-inactivated serum from hepatitis B infected individuals was immunogenic and non-infectious in naïve adults, and HBsAg particles were found to be the immunogenic component. Furthermore, studies also identified high levels of anti-HBsAg antibodies in the serum of individuals who had recovered from hepatitis B infection. The vaccine therefore consisted of these inactivated particles that had been extensively purified from the serum of chronically infected individuals and it induced good levels of anti-HBsAg antibodies [434, 435]. Despite the success of this vaccine there were concerns with the availability of donor serum, as well as obvious concerns with the safety of blood derived products. This was particularly pertinent during the early

1980s when HIV infection was emerging. However, with the advent of recombinant DNA technology the possibility of producing recombinant HBsAg was explored. HBsAg was successfully expressed in the yeast *Saccharomyces cerevisiae* and it was found that with sufficient levels of expression, disruption of the yeast cell membrane resulted in the spontaneous formation of HBsAg particles [436]. These particles were similar in size and buoyant density to those found in serum of infected patients, and also induced a good immune response. These particles therefore comprised RECOMBIVAX, the first licensed recombinant protein vaccine for hepatitis B [436-439]. HBsAg has since been successfully produced in yeast in a number of different strains including *S. cerevisiae, Pichia pastoris and Hansenula polymorpha* and also in mammalian cells using Chinese hamster ovary cells [436, 440-442], and a number of different licensed vaccine products exist.

VLPs can also be used as vaccine platforms to display foreign epitopes on the surface of the particles by genetically fusing or chemically conjugating them to the VLP. This technology was used to generate the leading malaria vaccine RTS,S. RTS,S exploits the intrinsic property of HBsAg to spontaneously form particles when expressed in yeast, by fusing a C-terminal portion of the CS malaria antigen to the HBsAg. Thus due to the orientation of the HBsAg in the lipid layer, when the particle is formed with this fusion protein the CS epitopes are exposed on the particle surface. The RTS fusion protein is expressed in *S. cerevisiae* yeast that has also been transformed with the unfused HBsAg (S) expression plasmid [334]. The resulting particles are therefore hybrid particles formed from a mixture of these two proteins, RTS and S. The ratio of the RTS fusion protein to the HBsAg in the particle has been reported as 1:4, and since only approximately 50% of the fusion protein is composed of CS the resulting protein content of the particle is only 10% CS antigen [335, 336, 443]. CS is therefore not displayed on the surface of RTS,S at very high densities and this vaccine does not take full advantage of the intrinsic

immunogenicity that can be achieved by repetitive antigen display on a VLP [433, 444]. As introduced in Chapter 1, RTS,S administered in AS01 adjuvant is the most advanced malaria vaccine in development. It has now been comprehensively tested in both adults and children and results in only short-lived [344] partial efficacy [348, 349], and it is therefore unlikely to be widely deployed in its current form. This vaccine however was developed around 30 years ago shortly after the success of the recombinant hepatitis B vaccine and the design has not been improved or altered since, except for the use of increasingly potent adjuvants [300]. It has been indicated in RTS,S publications and also from communication with authors [443] that using the yeast technology available for recombinant protein production when RTS,S was initially generated, it was not possible to form the particle without a considerable excess of HBsAg. Therefore a disadvantage of this vaccine is that a large amount of HBsAg is required for the formation of the particle and consequently a large proportion of the antibody response is induced towards the HBsAg instead of the malaria antigen [445]. This may hinder the induction of protective anti-CS immunity. It has been hypothesised, that a greater response to the CS antigen can be induced by increasing the proportion of CS in the vaccine compared to HBsAg, and also increasing the density of CS antigen on the surface of the VLP. This may prevent the diversion of the immune response toward the HBsAg, improve efficacy and may also allow for a dose sparing effect. Newer technologies such as the use of the high expressing yeast strain *Pichia pastoris* [446] may enable expression of a CS-HBsAg fusion protein at high enough concentrations for particles to form in the absence of HBsAg. The *P. pastoris* yeast strains used in this thesis have advantages over *S. cerevisiae* in that they are able to grow to very high densities and they use the strong, tightly regulated, inducible alcohol oxidase (*AOX1*) promoter. This enables large amounts of biomass to be generated before protein expression is induced, thereby reducing any negative effect accumulation of recombinant protein may have on biomass production. The high densities of biomass coupled with the strong *AOX1*

promoter therefore permits expression of large amounts of recombinant protein. In addition the yeast strains are also protease deficient, which may improve yield by reducing the amount of recombinant protein degradation without the need for protease inhibitors [447].

The aim of this chapter was to design and generate a potentially improved RTS,S like particle vaccine that is composed of a single CS-HBsAg fusion protein, using *P. pastoris* yeast. Therefore ~ 100% of the protein content comprising the particle will be the CS fusion protein and CS will be displayed at high density on the particle surface. This could result in a particle that is able to induce higher CS-specific immune responses and elicit greater protective efficacy. Importantly, this particle vaccine will also be available for evaluation in a multi-component vaccination strategy with the ChAd63 – MVA ME.TRAP regimen.

4.2 Results

4.2.1 Vaccine design

The R21 particle vaccine was designed to utilise the inherent properties of hepatitis B surface antigen (HBsAg) to facilitate the spontaneous formation of a particle. A single fusion protein was generated, termed R21, which consists of a C-terminal portion of CS from *P. falciparum* strain NF54 fused to the N terminus of the HBsAg, *adw* serotype. The sequence comprises 410 amino acids; from N to C terminus it includes a methionine start codon, 74 amino acids of the CS repeat, 105 amino acids of the CS protein C-terminus, 4 amino acids from the pre-S2 region of the hepatitis B virus, and 226 amino acids of the HBsAg (Figure 4.1)

74 amino acids of the CS repeat

DPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANP

NANPNANPNANPNANPNANP

105 amino acids of the CS protein C-terminus

NKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNS

LSTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKCSSV

4 amino acids from the pre-S2 region of the hepatitis B virus

PVTN

226 amino acids of the HBsAg

MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSPT

SNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSTTTN

TGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYLWEWASVRFSW

LSLLVPFVQWFVGLSPTVWLSAIWMMWYWGPSLYSIVSPFIPLLPIFFCLWVYI

Figure 4.1 R21 fusion protein sequence

The R21 fusion protein consists of 410 amino acids comprising a C-terminal portion of CS fused to the HBsAg.

The C-terminal portion of CS used in this vaccine contains 19 NANP repeats, which have been shown to be a protective B cell epitope, and also some important T cell epitopes [246, 337-339]. The design of R21 differs from RTS in that it contains 14 fewer amino acids; 10 fewer amino acids at the C-terminal end of CS and 4 fewer amino acids that were only included in RTS as a result of previous cloning site requirements, and hence are not necessary in R21. The R21 particle differs from the RTS,S particle in that it will be formed from a single fusion protein and will not be co-expressed with unfused HBsAg (Figure 4.2).

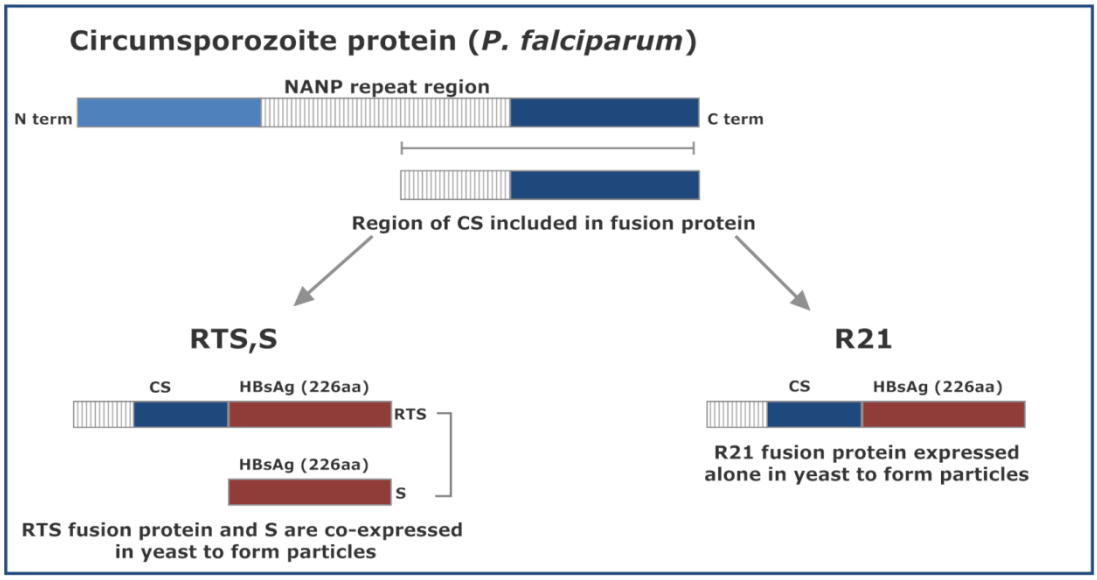


Figure 4.2 Comparison of R21 and RTS,S particle vaccines.

R21 is formed from a single CS-HBsAg fusion protein, without the co-expression of unfused HBsAg.

The aim is to express this fusion protein in the *P. pastoris* yeast strain; and following expression to disrupt the yeast membranes to release the fusion protein. Disruption of the yeast membrane is required as it has been shown that particles do not form in the yeast, they form after lysis and during purification. The hydrophobic segments of

the HBsAg monomers are inserted into the endoplasmic reticulum (ER) [448, 449] and remain there until detergent mediated disassembly of these membranes. Therefore lysis should enable the protein monomers to self-assemble into particles incorporating lipids from the yeast ER membrane. This particle can then be purified away from the yeast debris with techniques that utilise its unique size and buoyant density [433].

4.2.2 Generation of R21 expressing yeast and selection of expression plasmid and clone

4.2.2.1 Cloning and transformation

The R21 DNA sequence was synthesised by GeneArt and codon optimised for optimal expression in yeast. The R21 expression plasmids were constructed by inserting the R21 DNA sequence from the GeneArt plasmid into the pPink-HC (high copy) and the pPINK-LC (low copy) expression plasmids from the PichiaPink™ Expression System (Invitrogen). This was done by restriction cloning using *EcoR1* and *Kpn1,* and the presence of the insert in the plasmids was confirmed by PCR. Both the high copy number and low copy number expression plasmids were constructed in order to assess which plasmid induced highest levels of R21 expression. The R21 expressing yeast was then generated by linearising the R21.pPink-HC and R21.pPink-LC expression plasmids with *AflII* restriction digest. The linerarised plasmids could then be integrated into the genome of the four strains of PichiaPink™ yeast (Invitrogen), using electroporation. Strain 1 is wild type, strain 2 is a single knock-out for proteinase A, (*pep4*), strain 3 is a single knock out for proteinase B (*prb1*) and strain 4 is a double knock-out for both proteinases A and B. Positive transformants were selected by growth on adenine deficient media since the expression plasmid contains the *Ade2* gene and hence only yeast clones successfully transformed would be able to grow. During this study, in addition to R21 a second fusion protein was generated as described above, which was used for optimisation of the protein expression and particle purification process. The protein was termed RS and differed from R21 in that it does not contain the T cell epitopes from the C-terminal of CS and is therefore 105 amino acids smaller. This was generated because it was hypothesised that if R21 could not form a particle as a single fusion protein, this may be due to the size of the C-terminal region fused to HBsAg and therefore RS may be better able to form particles.

4.2.2.2 Selection of yeast strain and clone

Four clones were selected for each yeast strain/plasmid combination (Strain 1-4 / pPINK-HC or LC) and relative levels of fusion protein expression were assessed to in order to select a clone with high levels of expression and minimal protein degradation. This was achieved by growing small 1 mL pilot cultures of the positively transformed colonies, and inducing protein expression by the addition of methanol. Initially a time course experiment was performed to assess the optimal duration for induction of protein expression. Samples were taken prior to the induction of protein expression and every 24 hours after induction for 4 days and levels of protein were assessed by western blot using a monoclonal antibody to the NANP repeat (MR4 2A10) present in the CS portion of the fusion protein. The western blots indicate that the levels of protein present in the yeast lysate did not increase further after 3 days of induced expression Figure 4.3-A. Expression from each of the four clones from the multiple yeast/strain combinations were therefore analysed after 3 days of induced protein expression. The western blots suggest that the slightly higher levels of protein expression are present with the use of the high copy number expression plasmid but that the level of expression was also influenced notably by individual clone Figure 4.3-B-C. Hence it is important to screen multiple clones in order to select a suitable R21 expressing clone before progressing, and as such, clone 2, from yeast strain 4 transformed with R21.pPINK-HC was selected (Figure 4.3-D). This clone was chosen firstly because it was one of the clones that produced the highest levels of recombinant protein with minimal levels of degradation products, and secondly because it was generated from yeast strain 4. This strain contains the double protease knockout and may therefore result in lower levels of degradation from large batch cultures. Identity of the insert in the selected clone was verified by PCR amplification from genomic DNA followed by sequencing. The selected clone was grown in a 1 litre batch culture and length of expression required for induction of high levels of protein and minimal levels of degradation was assessed again (data

not shown). After induction under optimal conditions the yeast was pelleted by centrifugation, the supernatant removed and the yeast frozen at -80°C.

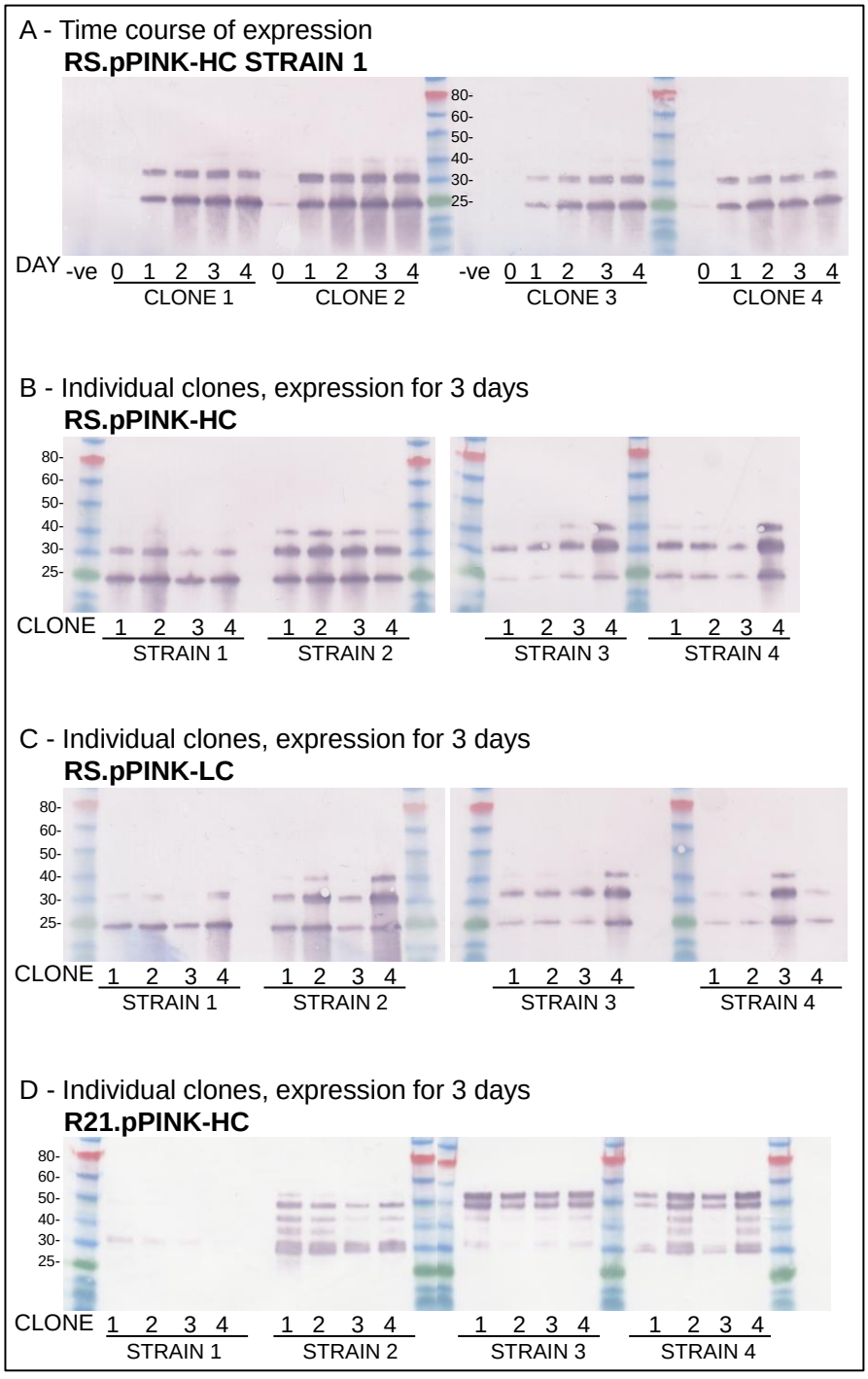


Figure 4.3 Assessment of relative levels of fusion protein expression

Levels of fusion protein expression were evaluated in 1 ml pilot cultures by western blot analysis using the monoclonal antibody to the NANP repeat present in CS. **(A)** Time course of expression for 4 RS.pPINK-HC clones from yeast strain 1. Evaluation of four different clones from **(B)** yeast strains 1-4 transformed with RS.pPINK-HC, **(C)** yeast strains 1-4 transformed with RS.pPINK-LC, **(D)** yeast strains 1-4 transformed with R21.pPINK-HC.

4.2.3 Optimisation of a particle purification strategy for R21

As a VLP R21 can be extracted from the yeast and purified by separation techniques that utilise the unique size and buoyant density of the particles. A simple purification strategy provided initial evidence that the RS fusion protein would form particles alone in the absence of additional HBsAg. Further optimisation resulted in a purification method that also enabled the efficient formation and purification of R21 particles (Figure 4.4-A). The formation of the CS-HBsAg particle from a single recombinant protein was a novel finding since it had previously been found that the ratio of 4:1 of HBsAg (S) and RTS fusion protein was required for formation of RTS,S particles [443]. The purification process optimisation was to reduce aggregation and increase purity and yield of the particle. The process involved lysing the yeast with glass beads and vigorous vortexing in the presence of detergent to disrupt the yeast membranes and liberate the fusion proteins. It was found that performing the lysis and purification steps using a 10mM TRIS pH 7.8 as the base of all the buffers and also the addition of benzonase to the yeast lysis buffer reduced the level of aggregation and increased the yield. The particle was then separated from the yeast debris with ultracentrifugation and the supernatant applied to the discontinuous CsCl gradient. This separates the components in the supernatant based on size and mass using the rate-zonal centrifugation method where larger components will sediment faster. This enabled a crude separation or R21 particles from other yeast components, and R21 forms a band at the interface of the two different density CsCl solutions used, 1.1 and 1.3g/ml. This band was extracted from the gradient and applied to a gravity flow gel filtration column. This broadly separates the components based on size and removes mainly lower molecular weight contaminants and exchanges the buffer. The fractions eluted from the gel filtration column containing R21 were collected and then applied to an isopycnic CsCl density gradient. This separates based on buoyant density, and the R21 particles of the same density,

1.2g/ml, form a distinct band in the gradient. The previous gel filtration step was found to be required for effective banding of R21 on the isopycnic gradient. This is likely due to the removal of contaminants and buffer components which may facilitate the formation of the gradient and hence the particles will be able to band. Furthermore, lowering the volume of yeast used for the initial lysis step also improved the banding of the R21 particle on the CsCl gradient and reduced visible aggregation seen as a second band below the R21 particle. This again was likely due to a reduction in the total protein content being applied to the CsCl gradient, allowing the gradient to form completely. For the final step, the R21 containing band was extracted from the CsCl gradient and applied to a size exclusion chromatography (SEC) column. This separates the particles from all remaining lower and higher molecular weight contaminants, and this step was required as a final polishing step to produce a product sufficiently pure for pre-clinical studies (Figure 4.4-B+C). This optimisation involved analysis of each stage of the process assessing samples visually, by western blot analysis, or using size exclusion chromatography and changes were introduced and conditions adjusted where necessary resulting in the final process detailed in Section 2.3.

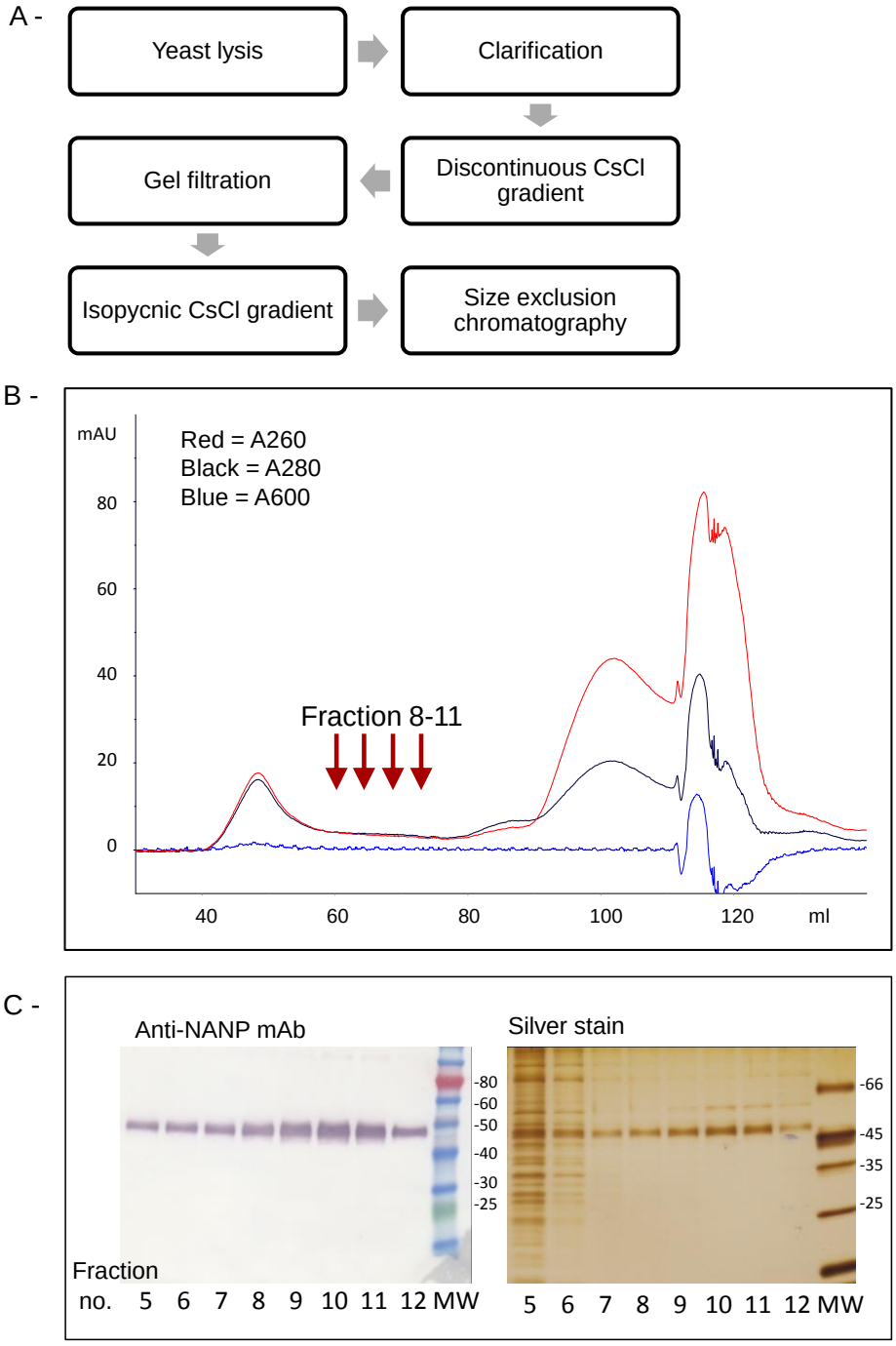


Figure 4.4 R21 purification

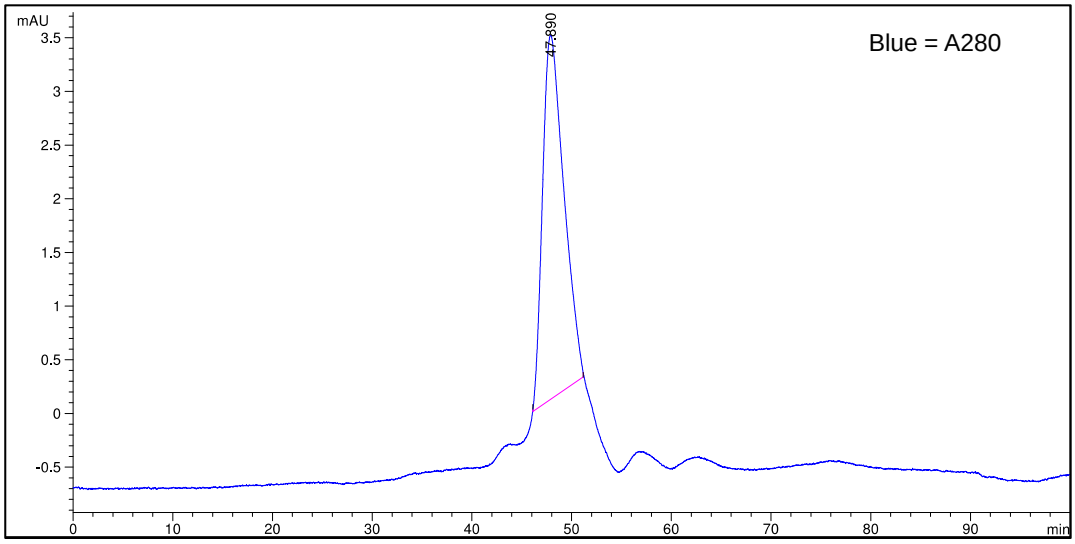
R21 particles are purified from yeast as detailed in section 2.3 and summarised in **(A)**. The final step is separation of the R21 particle from contaminants by size exclusion chromatography on a Hiprep 16/60 Sephacryl S-500 HR column (GE Healthcare). **(B)** The resulting elution from this column is displayed in the micrograph and the red arrows indicate the fractions used in the immunisation studies where R21 elutes without contaminants. **(C)** The eluted fractions are analysed in SDS-PAGE gel with silver staining to show purity and western blot analysis using a monoclonal antibody to the NANP repeat to identify R21.

4.2.4 R21 characterisation

Analysis of R21 present in the final purified product by transmission electron microscopy (TEM) using negative staining with 2% uranyl acetate reveals that R21 forms particles that are approximately 22nm in size (Figure 4.5-B). This is as expected since both HBsAg and RTS,S particles are reported to be of similar size [334, 436]. The purity of the particle was assessed by silver staining the proteins present in the final product after separation by SDS-PAGE. The presence of only one predominant band indicates that the particle was relatively pure (Figure 4.4-C). This was confirmed by analysing the final product by analytical SEC which would separate the components by size, and the presence of only one peak confirms the purity seen in the silver gel (Figure 4.5-A). Further evidence of the particulate structure was seen in non-reducing gel electrophoresis as the purified product was unable to enter the gel indicating it is a higher oligomer structure containing disulphide bonds that are not denatured by SDS (Figure 4.5-C). The accessibility of the CS NANP repeat region on the surface of the R21 particles was demonstrated in an ELISA adsorbing the purified R21 particle onto a plate and using a monoclonal antibody to the NANP repeat region for detection. The presence of HBsAg portion of the fusion protein was confirmed by western blot analysis with a monoclonal antibody to the HBsAg. However the HBsAg appeared to be relatively in accessible on the surface of R21 particles demonstrated by very weak binding in a Monolisa HBsAg ELISA assay in comparison to the HBsAg particle (Figure 4.5-D+E).

In summary, after optimisation the purification process resulted in CS based particles approximately 22nm in size with CS exposed on the surface which is >90% pure (estimated from silver stained samples on SDS-PAGE) (Figure 4.4) and therefore suitable for immunisation studies in mice.

A -



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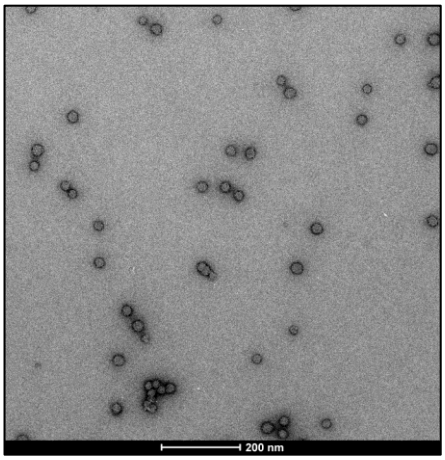

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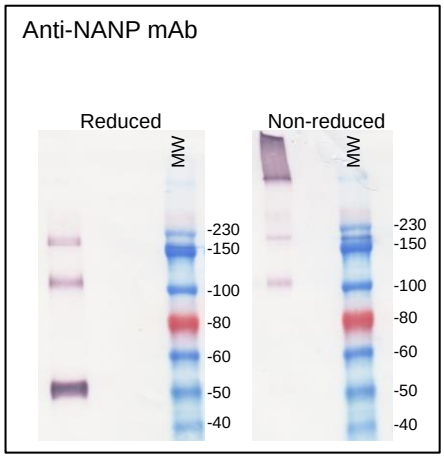
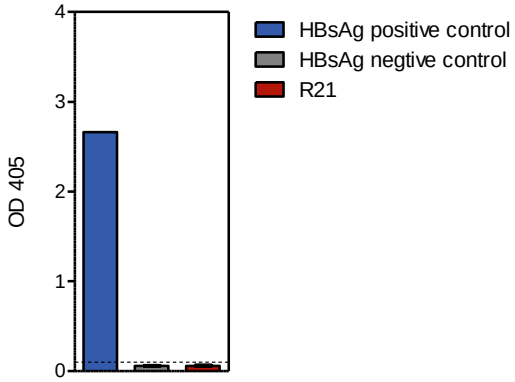


D - HBsAg particle ELISA



E - CS anitgen ELISA

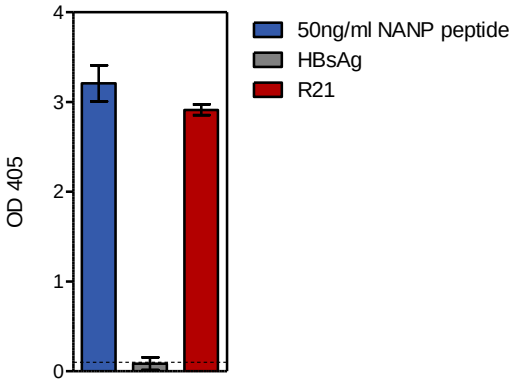


Figure 4.5 R21 Characterisation

(A) Analysis of the components present in the final R21 vaccine product by analytical size exclusion chromatography. **(B)** Analysis of particle size by transmission electron microscopy visualised by negative staining with 2% uranyl acetate. **(C)** Reducing and non-reducing SDS-PAGE electrophoresis with western blot analysis using anti-CS antibody, to demonstrate that R21 exist as a higher oligomer structure that is not denatured in the presence of SDS. **(D)** Assessment of HBsAg accessibility of the surface of the particle using the Monolisa HBsAg ELISA kit. **(E)** Assessment of NANP accessibility on the surface of the R21 particle using and CS antigen ELISA.

4.3 Discussion

The aim of this chapter was to produce a CS-based particle vaccine for use in a multi-component vaccination strategy. The approach was to generate an improved version of the current leading malaria vaccine RTS,S and this was done by expressing a single CS fusion protein (R21) in the yeast *Pichia pastoris.* It was found that the R21 fusion protein formed particles when expressed alone in yeast without the requirement of additional HBsAg (S). This was surprising as it has been previously reported that the formation of the RTS,S particles requires co-expression and purification of RTS and S in a ratio of 1:4 [443]. This single fusion protein particle may be considered an improvement upon RTS,S as a greater amount of the particle will be composed of the CS antigen instead of the HBsAg and therefore a greater proportion of the immune response may be generated towards the malaria antigen. Specifically, R21 in terms of protein composition is approximately 50% malaria antigen and 50% HBsAg, whereas RTS,S is only 10% malaria antigen. Since the efficacy of RTS,S has been directly linked to the level of NANP-specific antibody titres and CS-specific cellular responses [450], an increase in the proportion of response generated towards the malaria antigen may result in increased levels of protection. Unfortunately, since the RTS,S vaccine is currently unavailable for comparison it will be difficult to assess directly whether the improved particle does result in an increased immune response.

In addition to potentially improving the particle by forming it from a single CS fusion protein a further improvement has been the ability to express R21 in the high yielding yeast strain *Pichia pastoris* [446]*.* This yeast strain has been reported as being able to express proteins to a higher yield than the strain *Saccharomyces cerevisiae* which was used to produce RTS,S and may therefore result in higher yields of particle. This may have an important economic consequence as it could lead to an improved

manufacturing process resulting in a lower cost vaccine. This is a consideration which is particularly important for a vaccine aimed at children and infants in low income countries.

In this study the R21 particle was shown to be >90% pure by silver stain analysis of SDS-PAGE and analytical SEC, and the presence of 22nm particles was confirmed by TEM. The accessibility of the CS antigen on the surface of the protein was confirmed in a direct ELISA and in contrast, the relative inaccessibility of the HBsAg was demonstrated in the Monolisa HBsAg ELISA assay, where very little binding was detected. This indicates that as predicted due to the orientation of HBsAg in the particle [451], the majority of the surface is covered in CS. This can be further confirmed with techniques such as immunogold labelling with electron microscopy using antibodies to CS and HBsAg to assess antigen exposure on the surface [452]. Although this study has demonstrated the presence of approximately 22nm particles by TEM, further work could be carried out to fully characterise the size, structure and organisation of the particles. This could be done using atomic-force microscopy, cryo-electron microscopy, and steady-state or time-resolved fluorescence spectroscopy in a similar fashion to the assessment of HBsAg [448, 453-455]. In addition, since HBsAg is composed of approximately 30% lipid and 70% protein, the lipid content and organisation could also be determined [456]. This is of interest because studies have shown that the lipid content directly influences the antigenic activity of the HBsAg particles [442, 457, 458]. This is potentially caused by altering the presentation of antigens on the particle surface or by altering the particle uptake and processing by APCs.

The current purification strategy developed in this study has been sufficient for lab scale production of R21 for pre-clinical studies, however large scale manufacture may require the process to be further optimised. In addition, the stability of the

particle and suitable storage condition of the vaccine would also need to be fully assessed, though preliminary data indicates that the particles remain intact after being frozen at -80°C and thawed. The purification process could potentially be improved by using an epitope tag, such as the system developed by Life Technologies. In this system a small tag consisting of only four amino acids, E-P-E-A, is used to affinity purify recombinant proteins. The advantage of this system over affinity purification using other tags is that binding and elution occur under physiological conditions with high recovery rates [459]. This is ideal for purification of particles as strong elution conditions may denature or alter the particle structure Furthermore, due to its small size, the tag is unlikely to interfere with the formation of the particle or affect immunogenicity. Moreover, due to the inert nature of the tag it may also be suitable for use in clinical vaccine products and provisional regulatory approval has been given to assess EPEA tagged vaccines in clinical trials at the Jenner Institute. Preliminary data have shown that adding the EPEA tag to the C-terminus of the R21 fusion protein greatly improves and simplifies the purification of R21 particles and results in an improved yield (in work with F. Brod). Therefore purification of R21 by this method is currently being fully evaluated at the Jenner Institute.

In summary the method developed in this study for the generation and purification of chimeric HBsAg particles is a simple strategy for the rapid generation of vaccine candidates for pre-clinical assessment. Furthermore, results in the near future may confirm the improved purification process with the EPEA tag that may also be suitable for production of clinical products. Hence these methods can potentially be used for the production of VLPs displaying other malaria antigen, or for vaccines targeting other diseases. Indeed chimeric HBsAg vaccines using similar approaches have already been developed for Hepatitis C, dengue and HIV [460-462].

In this chapter a potentially improved CS-based particle vaccine has been developed and produced using a *Pichia* based system. The particle has been evaluated using TEM, SEC and ELISA all of which have demonstrated the particle to show the desired characteristics. The next step will be to assess the immunogenicity of the vaccine pre-clinically, either used alone or in a multi-component vaccination strategy with viral vectors.

5 Immunogenicity of R21 and the ChAd63–MVA ME.TRAP prime-boost regimen in BALB/c mice and the effect of co-administration

5.1 Introduction

The leading malaria vaccine in clinical development is a pre-erythrocytic subunit vaccine, RTS,S. It was developed by GlaxoSmithKline (GSK) in 1987 and has been extensively tested and is now in large Phase 3 trials in African children [346, 347]. RTS,S as described fully in Chapter 2, it is a lipid-protein particle vaccine that is composed of a fusion protein of the HBsAg fused to a C-terminal portion of CS protein as well as un-fused HBsAg [335, 336]. The C-terminal portion of CS was selected because it contains the NANP repeat region previously shown to be a protective B cell epitope [337, 338] and some important T cell epitopes [246, 339]. Repeated immunisation of this vaccine with a potent adjuvant induces high levels of antibody that bind to the CS protein, a major surface component of the malaria sporozoite, and thereby prevents or reduces parasite entry into the liver and intra-hepatic development of the parasites [450]. RTS,S is most effective when combined with the saponin and MPL based adjuvant AS01 and consistently confers between 30-50% efficacy in CHMI studies and large field trials [300, 345, 348, 349, 463]. The second most successful approach to malaria subunit vaccination has been the development of the viral vector heterologous prime-boost regimens fully described in Chapter 3 [351]. The most immunogenic and protective of these regimens evaluated to date being the simian adenoviral vector ChAd63 as the prime with modified vaccinia virus Ankara (MVA) as the boost [409-411]. In Chapter 3, this regimen using the pre-erythrocytic liver stage antigenic insert ME.TRAP in both vectors was shown

to induce exceptionally high levels of CD8+ T cells, moderate levels of antibodies, and it elicits 21% sterile protection and 36% partial protection in CHMI [356, 357].

Despite being the most successful subunit vaccines tested to date these two regimens still only elicits modest protective efficacy. Therefore it was hypothesised that because they target different antigens from different areas of the pre-erythrocytic stage of infection to improve efficacy it may be possible to combine together these two partially effective vaccine regimens. Potentially, using two different antigens in vaccines that have different mechanisms of action, therefore targeting the sporozoites and the infected hepatocytes with both robust antibodies and T cells may increase the chances of eliminating or neutralising the parasite [464]. Therefore, in order to evaluate this hypothesis, the major aim of this thesis research was to produce an improved version of RTS,S that can be assessed in combination with the ChAd63 – MVA ME.TRAP prime-boost regimen. Chapter 4 described the development and successful purification of the CS-based particle vaccine called R21, and the next step therefore was to assess the immunogenicity of this vaccine pre-clinically. RTS,S is immunogenic with the GSK AS series of adjuvants, however the level of immunogenicity and efficacy elicited in humans has been largely influenced by the adjuvant used [300, 335, 340, 465]. Therefore selection of a suitable adjuvant for use with R21 is important as it may impact not only the magnitude and type of immune response induced but also the level of protection elicited by the vaccine. The exact mechanism of protection induced by RTS,S is currently unclear, it is largely believed to be mediated primarily through high titres of CS antibodies [335, 342, 343, 463, 466-468], but there is also increasing evidence that CD4+ T cells may play a critical role as well [300, 421, 450, 469-474]. Hence an adjuvant that can enhance both the humoral and cellular immunogenicity of R21 may be required. Selecting a suitable adjuvant that can also be used in humans however is not a simple task, many adjuvants have been developed that have shown promise

in pre-clinical studies but they unfortunately have had unacceptable safety profiles in human trials. In addition to these problems with reactogenicity there is also a lack of access to many of the most promising adjuvants, as they are chiefly developed by private companies. There have therefore only been a handful of adjuvants that have been evaluated fully and are licensed for human use [271-274].

Since the GSK adjuvant AS01, the most successful adjuvant used with RTS,S, was unavailable to us, a range of other promising pre-clinical and clinically tested / approved adjuvants were selected (described fully in Chapter 1). They include the aluminium hydroxide based Alhydrogel, which is the most widely used adjuvant and a component of several licensed vaccines. It has an excellent safety profile and predominantly induces a Th2 type antibody response [276]. MF59 and AddaVax are squalene based oil-in-water emulsions that enhance antibody titres and induce a more balanced Th1/Th2 type response. MF59 has been tested in a large number of clinical trials; it has an acceptable safety profile and is licensed for use in the influenza vaccines [280, 475]. Abisco-100 and Matrix M are saponin-based ISCOM adjuvants. Matrix M is currently approved for testing in humans and induces high titre antibody responses as well as CD4+ and CD8+ T cell responses in a range of studies [295, 298]. Carbopol is more experimental, it is a synthetic polymer of cross-linked acrylic acid chains that has previously been used to stabilise, suspend and slow the release of pharmaceutical products, and has only more recently been evaluated as vaccine adjuvant [301, 306]. The final adjuvant used is a bio-similar of the GSK AS01 adjuvant, which has been produced by The Vaccine Formulation Laboratory (VFL) at the University of Lausanne called LMQ and is composed of liposomes, MPL and QS21, with a similar formulation to AS01.

The main aims of this chapter were to assess the immunogenicity of R21 and to compare the potency of several promising adjuvants when administered with R21. A

further aim was to compare the immunogenicity of R21 to the immunogenicity of non-particulate recombinant CS protein, to determine if the CS antigen is more immunogenic when administered in the R21 particle. The overall goal is to combine the R21 and adjuvant regimen with viral vectored vaccines and the most deployable way to achieve this would involve mixing the vaccines together prior to administration. Therefore the effect of the adjuvants on the immunogenicity of the viral vectors was also evaluated followed by assessing R21 in combination with the ChAd63 – MVA ME.TRAP regimen to assess any potential immunological interference.

As discussed previously, CS-based vaccines are thought to act largely through the CS-specific antibodies which bind to the NANP repeat B cell epitope on the surface of sporozoites. Therefore the assay used to primarily assess immunogenicity in RTS,S vaccine trials is an ELISA measuring the NANP-specific IgG titres. Evidence also suggests a role of CD4+ T cells and as such IFNγ ELISpot assays and multiple-parameter flow cytometry with intracellular cytokine staining (ICS) are also used to assess T cells frequencies. Hence these assays will be used here to evaluate the immunogenicity of R21 and viral vectors. All animal handling and immunisations were performed by Rebecca Hillson a research technician at the Jenner Institute. Preparation of all vaccines, processing of all samples, all immunological assays and all data analysis was performed by Katharine Collins.

5.2 Results

5.2.1 Immunogenicity of R21 administered with adjuvants in BALB/c mice

5.2.1.1 Initial assessment of R21 immunogenicity

Following the successful production of R21, it was initially assessed with two adjuvants in a three shot regimen, to determine if the dose of 0.5µg of R21 would be sufficient to induce a reasonable immune response in mice. The adjuvants included the aluminium hydroxide based Alhydrogel and the saponin based ISCOM, Abisco-100, both described fully in Chapter 1.

Groups of BALB/c mice were immunised intramuscularly (i.m.) with R21 alone or formulated with Alhydrogel or Abisco-100. Three immunisations were given three weeks apart and the immunogenicity was assessed by measuring serum antibody titres three weeks after each immunisation and antigen-specific T cell responses in the spleen three weeks after the final immunisation. After the first immunisation, CS-specific IgG responses to the NANP repeat were measured in an ELISA and were detected in all mice immunised with R21 when formulated with adjuvant. In the group that received R21 with no adjuvant only one mouse had developed a detectable CS-specific IgG response at this time (Figure 5.1-A). After the second immunisation all but one mouse in the R21 alone group had seroconverted and the responses in the R21 and adjuvant groups were boosted significantly above the prime response. The responses in all groups were boosted by a third immunisation (Figure 5.1-C) and R21 + Abisco-100 induced the highest titres of NANP-specific IgG and the response for this group was significantly higher than both R21 + Alhydrogel and R21 alone ($p<0.05$, by Kruskal-Wallis with Dunn's multiple comparison test).

CS-specific IFNγ producing T cells were assayed in a spleen ELISpot after the final immunisation and they were detected in all mice receiving R21 with or without adjuvant. The median response in the R21 + Abisco-100 group was 700 SFC/10^6 splenocytes, but in the R21 + Alhydrogel and R21 alone groups, although the IFNγ producing T cells were detected they were only just above background (Figure 5.1-E). This indicates that in the absence of an adjuvant that can stimulate cellular immunity, such as Abisco-100, R21 is not very effective at inducing CS-specific IFNγ producing T cell responses on its own.

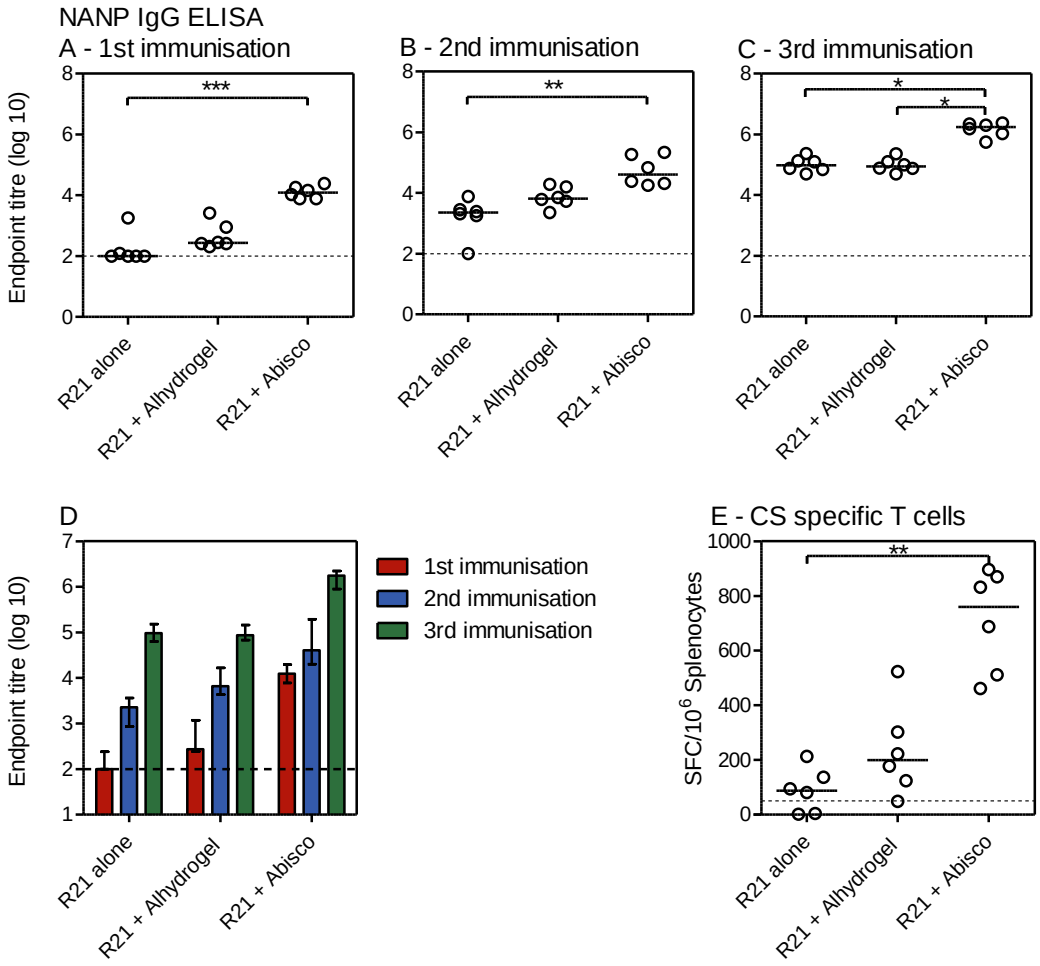


Figure 5.1 Immunogenicity of R21 in a three immunisation regimen

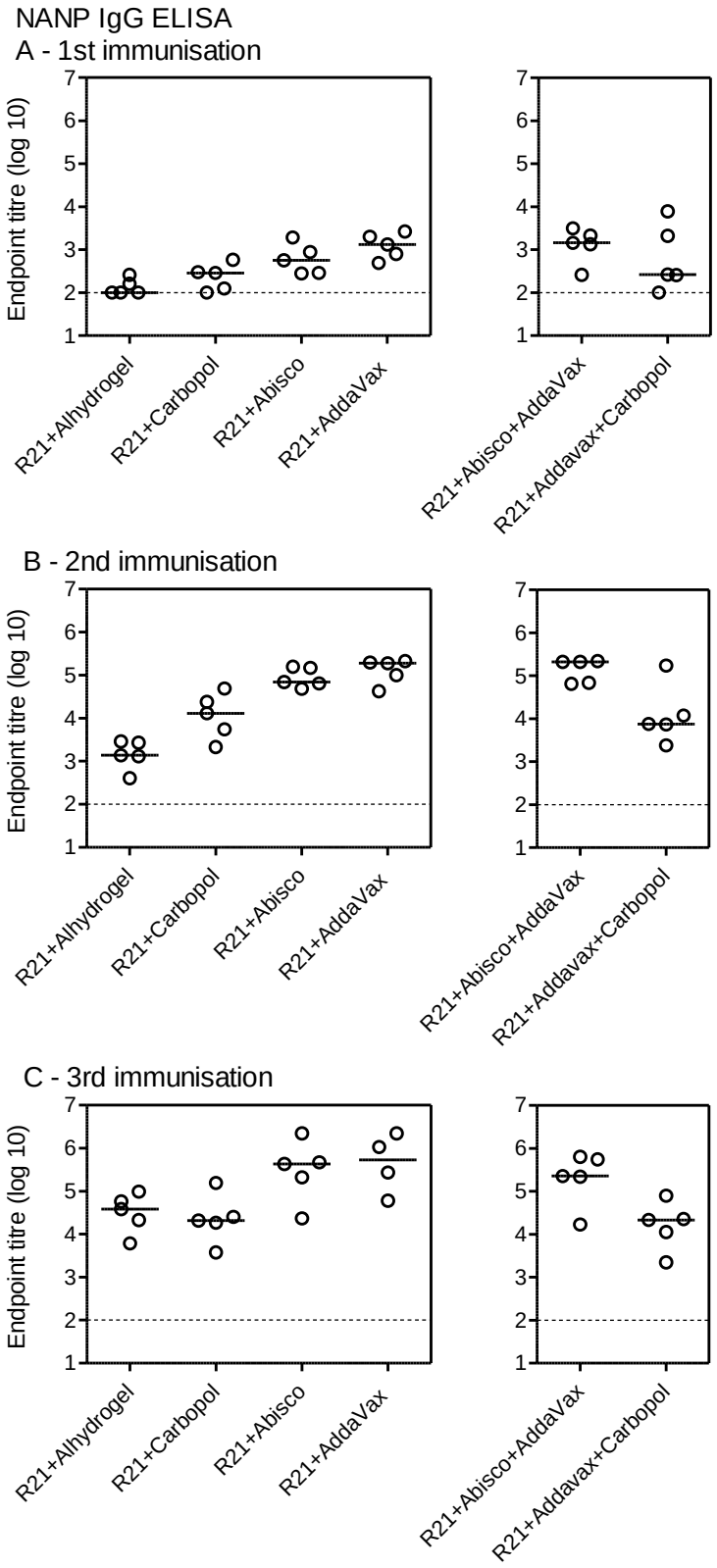
BALB/c mice were immunised i.m. with 0.5µg R21 alone or formulated with adjuvant. Three immunisations were given three weeks apart and blood taken for immunology three weeks after each vaccination. CS-specific IgG was assayed by ELISA to the NANP repeat B cell epitope, after the first **(A)**, second **(B)** and third **(C)** immunisations. All ELISA data are displayed in **(D)** and the dotted lines indicate the limit of detection. Spleens were taken three weeks after final vaccination and antigen-specific IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot using the a pool of overlapping CS peptides **(E).** The dotted line indicates the background response. Group median responses are shown with interquartile range are compared by Kruskal-Wallis with Dunn's multiple comparison test *p<0.05 **p<0.01 ***p<0.001.

5.2.1.2 Humoral and cellular immune responses induced by R21 immunisation can be enhanced by adjuvants

To further investigate the effect of adjuvants on R21 immunogenicity several adjuvants were directly compared head to head. This panel includes the two adjuvants described above, Abisco-100 and Alhydrogel, a squalene based oil-in-water emulsion, Addavax and the polyanionic carbomer, Carbopol. It has also been shown that certain combinations of adjuvants have been successful at enhancing the immune response [306, 307] and hence two groups also received two adjuvants combined together which were both mixed with R21 prior to immunisation.

Groups of BALB/c mice were immunised (i.m.) with 0.5µg of R21 formulated with adjuvant as detailed in Figure 5.2. As previously, three immunisations were given three weeks apart and the immunogenicity was assessed by measuring NANP-specific IgG titres in the serum three weeks after each immunisation and CS-specific T cell responses in the spleen three weeks after the final immunisation. After the first immunisation all but 5 mice had developed detectable NANP-specific IgG titres, with the groups receiving R21 administered with Abisco-100 or AddaVax having the highest responses (Figure 5.2-A). This hierarchy was maintained after the second and third immunisations and there was no significant difference in the responses after mice received R21 with a single adjuvant (Abisco-100 or Addavax) or both Abisco-100 and Addavax together (groups are compared by Kruskal-Wallis with Dunn's multiple comparison test) (Figure 5.2-B+C). IFNγ producing T cells detected in the spleen ELISpot were induced in all groups but as previously seen (5.2.1.1) the responses were very low and only just above background except for the groups receiving R21 + Abisco-100 (Figure 5.2-D). In these experiments Abisco-100 appears to be the superior adjuvant and it is required for the induction of a moderate T cell response to R21 and this response is maintained even when Abisco-100 is co-

administered with Addavax. It is also extremely effective at inducing high titre antibody responses making this the ideal adjuvant for further evaluation.



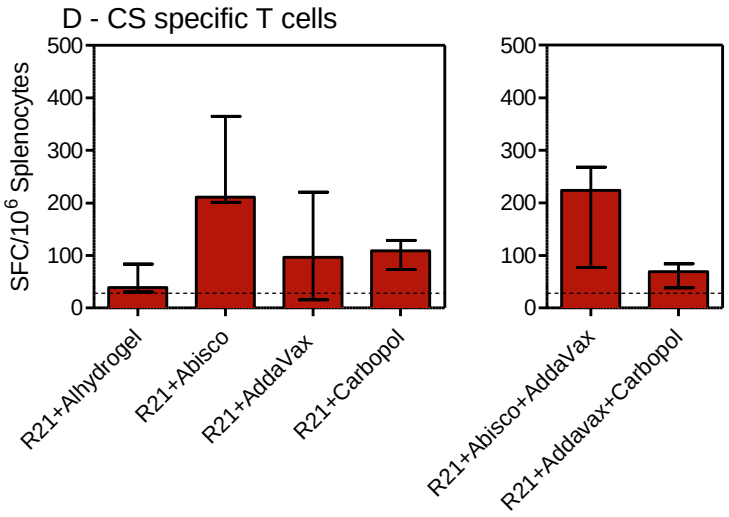


Figure 5.2 R21 immunogenicity in a range of adjuvants

BALB/c mice were immunised i.m. with 0.5µg R21 alone or formulated with adjuvant as detailed in the graph. Three immunisations were given three weeks apart and blood was taken for immunology three weeks after each vaccination. NANP-specific IgG was assayed by ELISA after the first **(A)**, second **(B)** and third **(C)** immunisations and the dotted line indicates the limit of detection. Spleens were taken three weeks after final vaccination and antigen-specific IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot using the a pool of overlapping CS peptides **(D).** The dotted line indicates the background response. Group median responses are shown with interquartile range.

5.2.1.3 R21 can be administered as an eight week prime-boost regimen for optimal immunogenicity.

R21 has been shown to be highly immunogenic when administered with Abisco-100 in a regimen where three shots were given three weeks apart. Since the aim of developing R21 is for use in combination with viral vectors in the eight week prime-boost regimen it is important to determine the effect of immunisation interval on antibody and T cell induction. The aim of this study was to determine if an eight week interval reduces the level of antibodies and T cells induced and if so, can this be improved by the addition of a third immunisation either between the two viral vectors at four weeks or after the viral vector boost immunisation at 16 weeks.

Groups of BALB/c mice were immunised with 0.5µg of R21 + Abisco-100 in four different regimens as described in (Table 5.1). CS-specific antibodies were measured three weeks after each immunisation and CS-specific T cells were measured three weeks after the final immunisation. After the first immunisation, as expected, since all groups had received the same vaccine, there was no difference between the groups in the levels of NANP-specific IgG. After the final immunisations there is statistically no difference in the response if two immunisations are given with an eight week interval compared to the three shots, three week interval regimen (Figure 5.3-A) (groups compared by Kruskal-Wallis with Dunn's multiple comparison test). Interestingly, if an additional immunisation is added to the two shot regimen at four weeks, there is very little increase in antibody titre and also if an additional immunisation is added after the two shot regimen at 16 weeks there is only a slight boost in the NANP-specific IgG titres. When compared statistically there is no significant difference between any group after the final immunisation (groups compared by Kruskal-Wallis with Dunn's multiple comparison test). CS-specific IFNγ producing T cells were also detected in all mice after the final immunisation and again there was no significant difference in the magnitude of the response between

the different regimens (Figure 5.3-B). Overall, there was no improvement if an additional immunisation was added to the two shot eight week interval regimen at either four or 16 weeks. This indicates that the high titre antibodies and moderate levels of T cells generated in the previous experiments can also be generated with the eight week prime-boost regimen and so interval will not affect the level of antibodies induced when R21 is combined with viral vectors.

Table 5.1 Immunisation regimen study

Vaccination regimens used to assess the effect of vaccine interval on immunogenicity

Gp	No. mice	Vaccine	No. shots	Interval
1	6	0.5µg R21 + Abisco IM	3	3 weeks
2	6	0.5µg R21 + Abisco IM	3	4 weeks
3	6	0.5µg R21 + Abisco IM	2	8 weeks
4	6	0.5µg R21 + Abisco IM	3	8 weeks

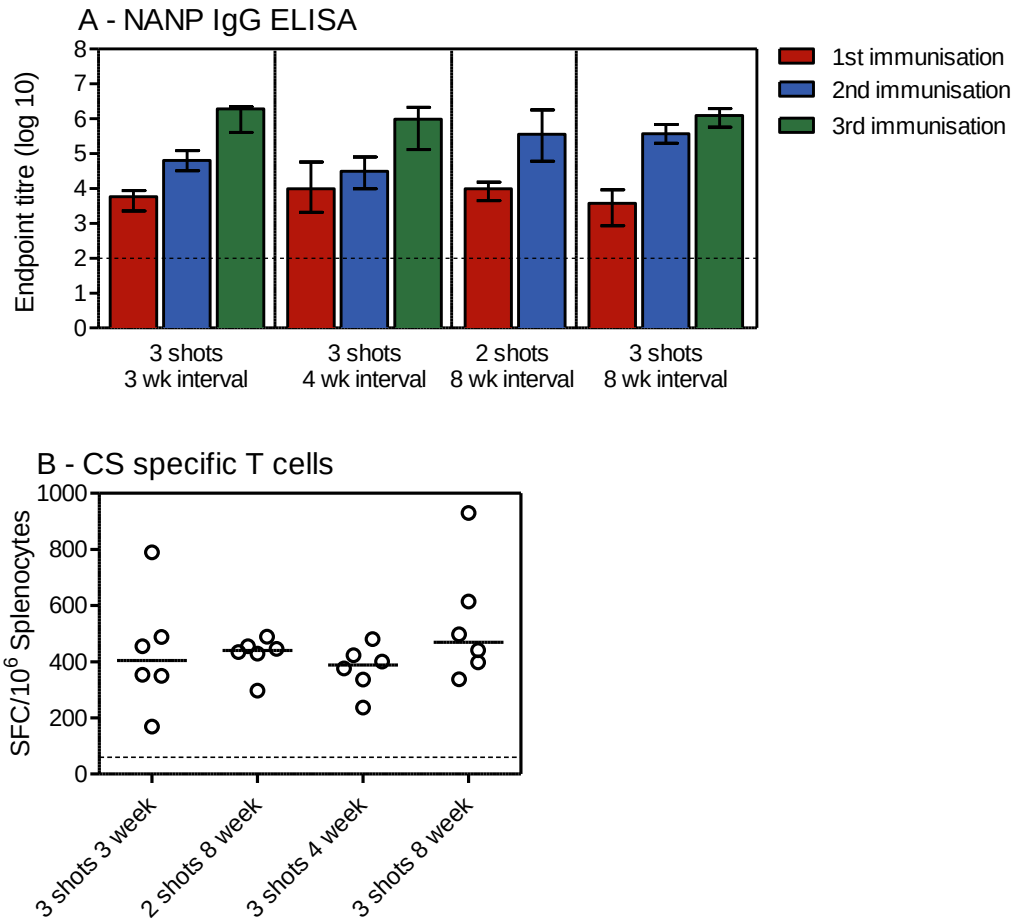


Figure 5.3 Immunogenicity assessment after immunisation with different regimens

BALB/c mice were immunised i.m. with 0.5µg R21 formulated with Abisco-100. Groups received different immunisation regimens as detailed in Table 5.1. Blood was taken for immunology three weeks after each vaccination and NANP-specific IgG was assayed by ELISA **(A)**, dotted line indicates the limit of detection. Spleens were taken three weeks after final vaccination and antigen-specific IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot using the a pool of overlapping CS peptides **(B),** the dotted line indicates the background response. Group median responses are shown with interquartile range and compared by Kruskal-Wallis with Dunn's multiple comparison test.

5.2.1.4 The majority of the antibody response induced by R21 is directed towards the malaria antigen rather than the HBsAg

R21 particles are formed from a fusion protein which contains a portion of the CS antigen fused to the N terminus of the HBsAg and it is predicted due to the orientation of the HBsAg in the particle [451, 455], that the CS epitopes will be exposed on the surface of the particle. This is also evident from the induction of high CS-specific antibody titres following particle immunisation described in the previous sections. Immunisation with RTS,S however has been shown to induce high titres of HBsAg-specific antibodies and induction of this immune response may compete or interfere with the induction of CS-specific antibodies. It is hypothesised therefore that since R21 is formed from a single fusion protein as opposed to RTS,S which is only 20% fusion protein and 80% HBsAg, that a greater proportion of the immune response to R21 will be generated towards CS rather than HBsAg. Since the efficacy of RTS,S has been directly linked to the level NANP antibody titres and CS-specific cellular immune responses [469] an increase in the proportion of the response generated towards the malaria antigen may result in increased levels of protection. Unfortunately, since the RTS,S vaccine was unavailable to us at this time or comparison it will be difficult to assess directly whether the R21 particle does result in an improved CS immune response. However a comparison can be made to immunisation with HBsAg alone to determine if there is a significant reduction in the HBsAg-specific response, indicating whether the surface of the particle is covered in CS antigen as predicted or HBsAg.

To assess the reduction in HBsAg-specific IgG response, groups of BALB/c mice were immunised with either 0.5µg HBsAg or 0.5µg R21 formulated with Alhydrogel. To evaluate whether the HBsAg response induced by R21 was affected by adjuvant, R21 was also given alone, (previously determined least immunogenic, 5.2.1.1) or with Abisco-100 (previously determined to be most immunogenic, 5.2.1.2). HBsAg-

specific antibodies were measured three weeks after each immunisation and when R21 was given alone or with Alhydrogel there was no detectable HBsAg-specific IgG, even after three immunisations. When R21 is given with Abisco-100, which induced the highest levels of CS-specific antibodies, there was some HBsAg-specific IgG detected, but the responses are very low level and barely above background. This can be compared to the HBsAg-specific IgG induced by immunisation with the same dose of HBsAg + Alhydrogel which is almost 2000 times higher (Figure 5.4-A). NANP-specific IgG was also assayed in this experiment three weeks after each immunisation (Figure 5.4-B) and the responses after three immunisations are compared in Figure 5.4-C and it is evident from that the majority of the IgG response to R21 is generated towards CS and not HBsAg, which is a potential improvement upon RTS,S.

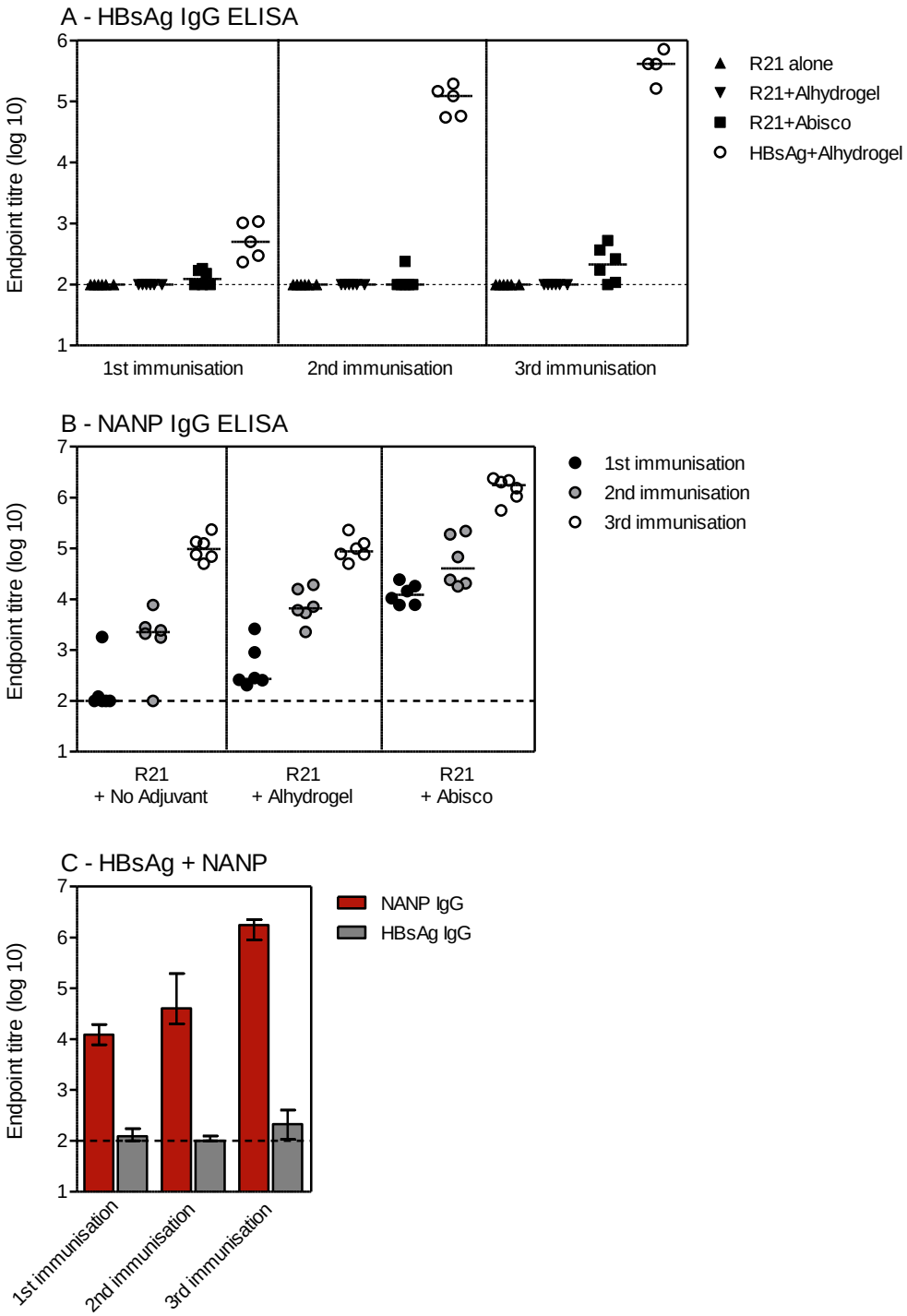


Figure 5.4 HBsAg induced antibody response after vaccination with R21

BALB/c mice were immunised i.m. with either 0.5µg R21 alone, 0.5µg R21 formulated with Alhydrogel or Abisco-100, or 0.5µg HBsAg + Alhydrogel. Three immunisations were given three weeks apart and blood was taken for immunology three weeks after each vaccination. HBsAg-specific IgG **(A)** and NANP-specific IgG **(B)** was assayed by ELISA after each immunisation. Relative NANP and HBsAg ELISA tires are compared **(C)**. Group median responses with interquartile range are shown. The dotted line indicates the limit of detection.

5.2.2 Comparative immunogenicity of the R21 particle vaccine and a highly immunogenic non-particulate CS recombinant protein with a range of adjuvants

The R21 lipid-protein particle has been produced because it has long been thought that particles have the ability to induce a greater immune response than recombinant proteins, either as particulate antigens or as simple antigens formulated with adjuvants that create particulate structures [431, 476-478]. It is therefore important to assess if this indeed holds true for R21. Is the CS antigen more immunogenic in the R21 particle or as a non-particulate recombinant protein? There has been a significant effort to produce an immunogenic non-particulate CS recombinant protein vaccine and four vaccines were recently generated and assessed head to head in a study to determine which elicited the highest immune responses and superior efficacy [479]. The most immunogenic non-particulate CS protein evaluated was produced in *E.coli* by Gennova (Pune, India) and this was obtained from PATH Malaria Vaccine Initiative (MVI, Washington DC) for assessment against R21. This assessment was performed with three different adjuvants Matrix M (similar to Abisco-100), MF59 (similar to AddaVax) and a bio-similar of the GSK adjuvant AS01 called LMQ.

5.2.2.1 Antibodies levels induced by R21 are greater than those induced by non-particulate CS protein

Groups of BALB/c mice were immunised twice, 8 weeks apart with 0.5µg or either R21 or CS formulated with three different adjuvants (Matrix M, MF59 or LMQ). Three weeks after the first immunisation, when comparing the groups that received the same adjuvant, the responses were between 2.6 and 4.7 fold higher in all groups receiving R21 compared to the groups receiving CS, and this was significantly higher

for the Matrix M and LMQ (*p<0.05 compared by Mann-Whitney test) (Figure 5.5-A). The same trend was seen after boost at week 11, with the responses being between 4.5 and 22 fold greater in the R21 groups (Figure 5.5-B). NANP-specific antibodies were also measured at week 20, three months after the boost to assess the durability of the response and the antibodies were very well maintained in all groups. There was only a small reduction in the IgG titres in all of the groups, between a 3.9 and 6.3 fold reduction and this was not significantly lower than the three week post boost time point (week 11) (Figure 5.5-D-F). Again the responses were higher in the R21 groups compared to the CS groups, between 3.3 and 19.8 fold, and this was significantly higher for the Matrix M and MF59 groups but not the LMQ group. There was also no significant difference between the responses induced by the three adjuvants for either R21 or CS, at any time point (compared by Mann-Whitney test, data not shown). Therefore it may be acceptable to pool together the data for the R21 groups or the CS groups at each time point and when compared by Mann-Whitney test, the responses to the particle vaccine, R21 are significantly higher at all time points than the responses to the non-particulate CS protein (Figure 5.5-C).

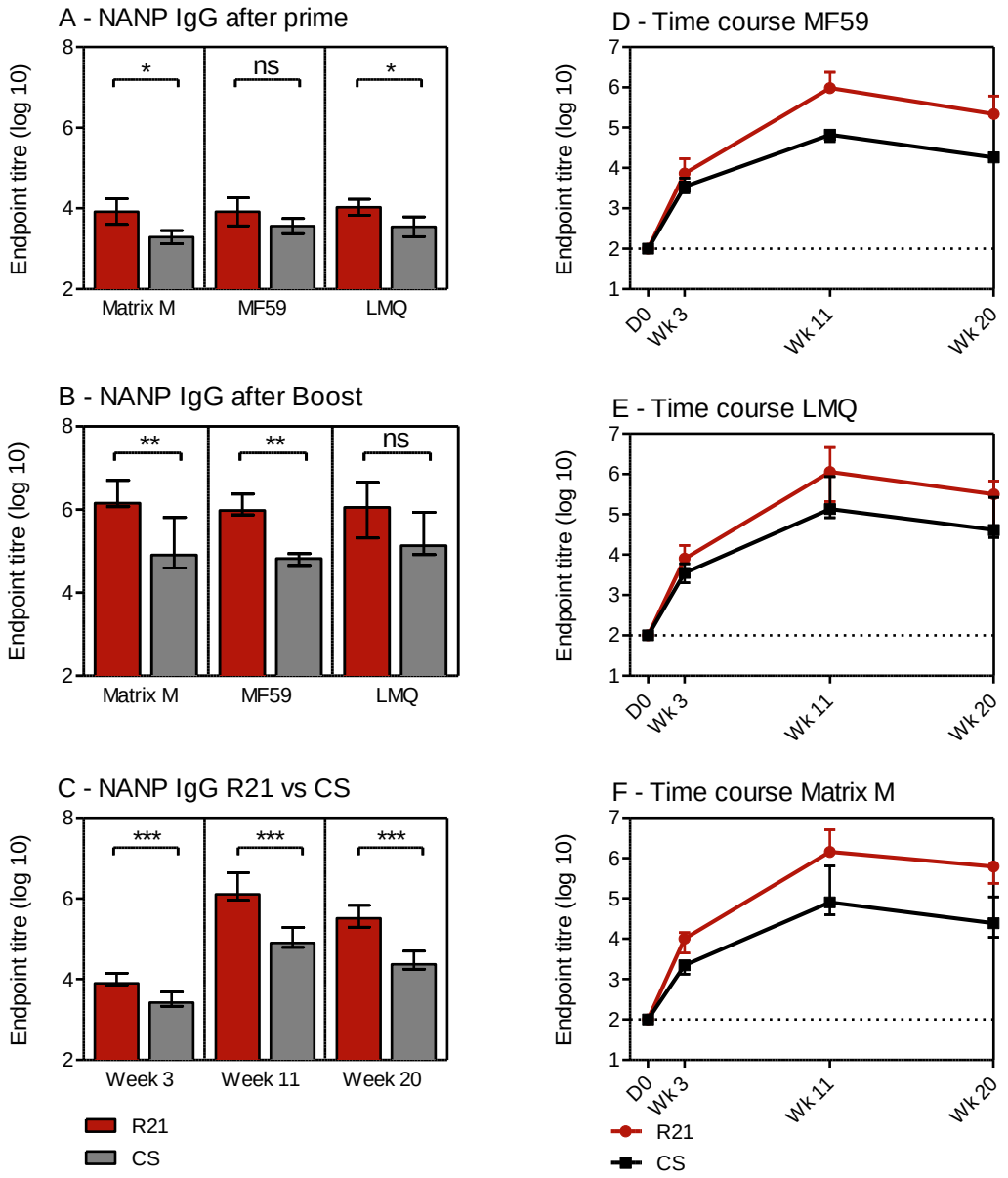


Figure 5.5 Comparative assessment of the humoral immunogenicity of R21 and recombinant non-particulate CS protein

BALB/c mice were immunised i.m. with 0.5µg R21 or CS formulated with adjuvant (MF59, Matrix M or LMQ). Two immunisations were given eight weeks apart and blood was taken 3 weeks after each vaccination and also 3 months after the final vaccination. NANP-specific IgG was assayed by ELISA after the prime **(A)** and boost **(B)**. ELISA data were combined for all R21 immunised mice and all CS immunised mice and compared at each time point in **(C)**. Time course of NANP-specific IgG titres are displayed for each of the three adjuvants, **(D)** MF59, **(E)** LMQ and **(F)** Matrix M. Group medians with interquartile range are shown and R21 groups compared to CS groups by Mann-Whitney test *p<0.05, **p<0.01, ***p<0.001.

5.2.2.2 T cell responses induced by R21 are greater than those induced by non-particulate CS protein

CS-specific T cell responses were also measured in the blood by ICS, two weeks after the boost vaccination. Isolated PBMC's were re-stimulated with a pool of CS peptides and frequencies of cytokine secreting CD4+ T cells were measured. Mice immunised with CS, only developed very low levels of CS-specific CD4+ T cells secreting IFNγ, TNF and IL2, with the highest number of responders seen after CS + Matrix M (Figure 5.6). Mice immunised with R21 particle however developed much higher frequencies of T cells, with the R21 + Matrix M or LMQ inducing the highest frequencies for all T cell subsets with very similar cytokine profiles.

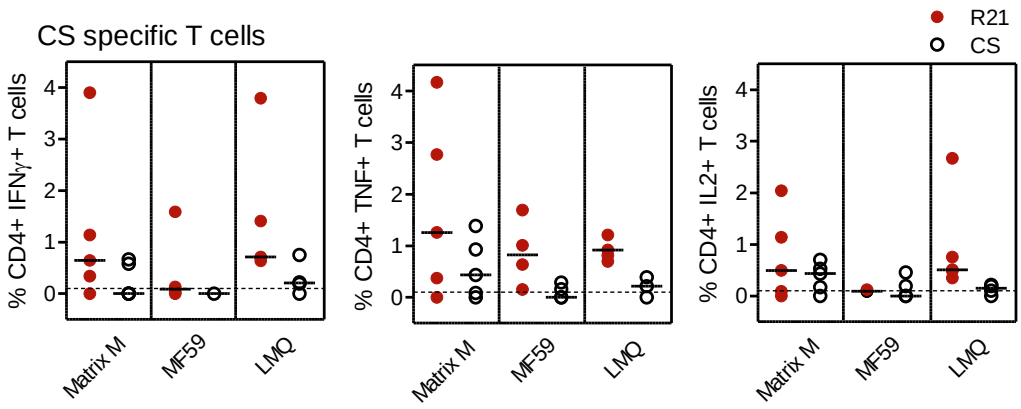


Figure 5.6 Comparative assessment of the cellular immunogenicity of R21 and recombinant non-particulate CS immunisation

BALB/c mice were immunised i.m. with 0.5µg R21 or CS formulated with adjuvant (MF59, Matrix M or LMQ). Two immunisations were given eight weeks apart and blood was taken 2 weeks after the final vaccination to assess CD4+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with a pool of overlapping CS peptides and three different cytokines were assessed (IFNγ, TNF and IL2). Results are presented as the percentage of CD4+ T cells expressing the cytokine. Median response is shown and the dotted line indicates background response.

5.2.3 Immunogenicity of ChAd63 – MVA ME.TRAP prime-boost regimen administered with a range of adjuvants alone

5.2.3.1 Cellular immune responses induced by the ChAd63 – MVA ME.TRAP prime-boost regimen are unaffected by the addition of certain adjuvants

To assess the effect of adjuvants on viral vectored vaccines, groups of BALB/c mice were immunised with the ChAd63 – MVA ME.TRAP eight week, prime-boost regimen, formulated with a range of adjuvants as indicated in figure. Cellular immune responses to the viral vectors were assayed after each immunisation using the BALB/c immunodominant H-2Kd CD8+ epitope Pb9 (SYIPSAEKI) present in the (multiple epitope) ME string of the viral vector insert. Two weeks after prime, IFNγ+ CD8+ T cells were detected in all groups by blood ICS and flow cytometry (Figure 5.7). The groups that received ChAd63 ME.TRAP in combination with Abisco-100 or AddaVax had slightly higher frequencies of IFNγ+ CD8+ T cells than ChAd63 ME.TRAP alone, but this was not significant (compared by Kruskal-Wallis with Dunn's multiple comparison test). The same trend was also seen in the levels of IL2+ CD8+ T cells and TNF+ CD8+ T cells detected but were also not statistically different in any group compared to the ChAd63 ME.TRAP alone group. After a boost immunisation with MVA ME.TRAP administered alone or with the same adjuvant as at prime, the cellular response was assayed in the spleen in an *ex-vivo* IFNγ ELISpot using the Pb9 epitope. The responses were not significantly different in the groups receiving ChAd63 - MVA ME.TRAP alone compared to the groups receiving the viral vector vaccines in combination with Abisco-100 or AddaVax, but were significantly lower in the groups receiving Carbopol or Carbopol with another adjuvant ($p<0.05$, compared by Kruskal-Wallis with Dunn's multiple comparison test) (Figure 5.7-D).

This indicates that although Carbopol does not appear to interfere with the induction of a cellular response by the ChAd63 ME.TRAP vaccine, detected in the blood at prime (Figure 5.7-A-C), it does interfere with the boosting of the response by the MVA ME.TRAP vaccine (Figure 5.7-D). Therefore since addition of either Abisco-100 or AddaVax does not interfere with the generation of antigen-specific T cells these two adjuvants may be suitable for use with R21 when the particle and viral vector regimens are combined.

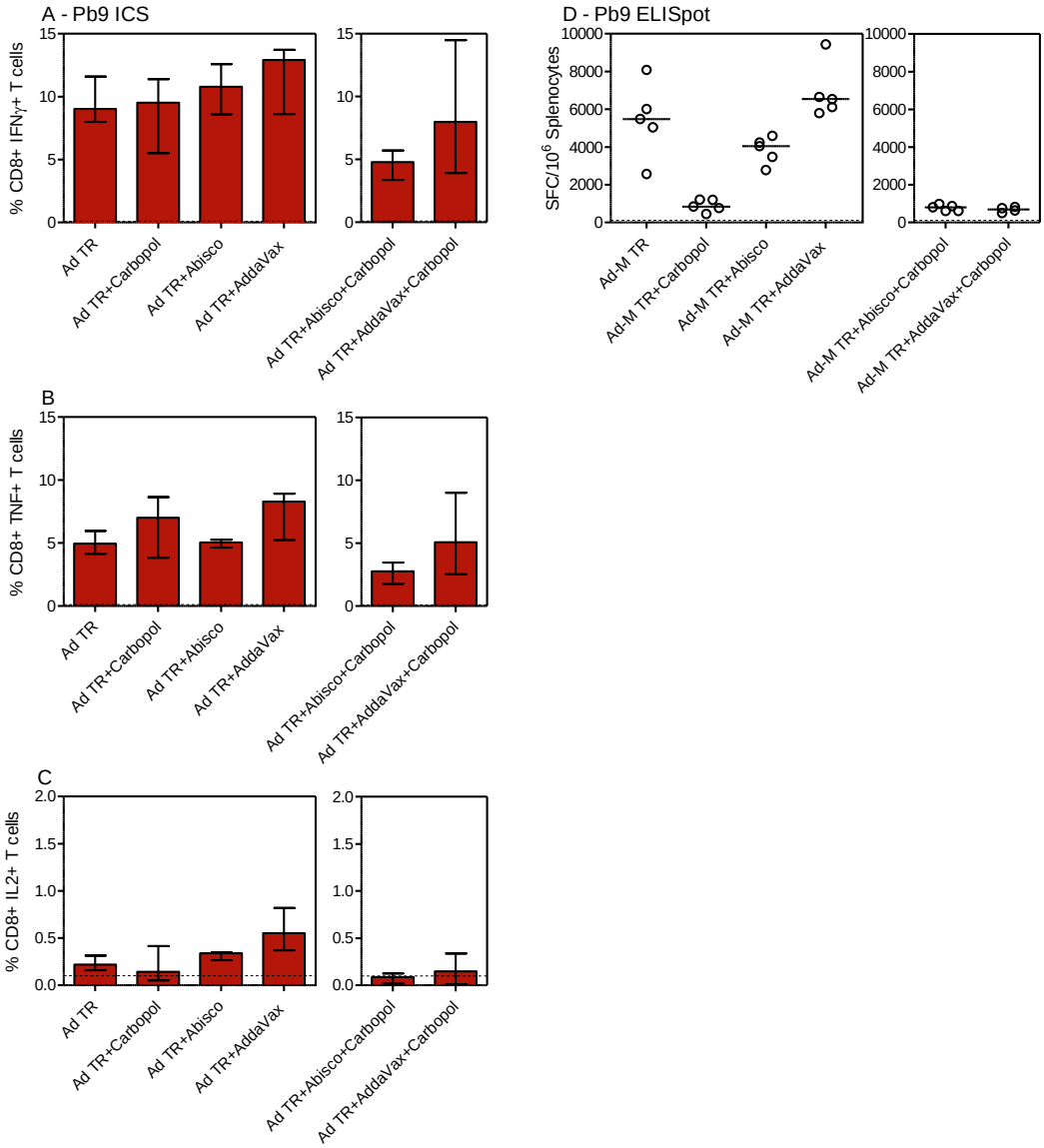


Figure 5.7 Cellular immune responses after vaccination with ChAd63 - MVA ME.TRAP alone or combined with adjuvant.

BALB/c mice were immunised i.m. with ChAd63 – MVA ME.TRAP 8 week prime-boost regimen, either alone (Ad-M TR) or combined with adjuvant. Blood was taken two weeks after the prime vaccination to assess CD8+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with the immunodominant H-2K^d CD8+ epitope Pb9 (SYIPSAEKI) present in the (multiple epitope) ME string of the viral vector insert and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD8+ T cells expressing IFNγ **(A)** TNF **(B)** or IL2 **(C)**. Spleens were taken three weeks after the final vaccination and Pb9-specific IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot **(D).** Group medians with interquartile range are shown and compared by Kruskal-Wallis with Dunn's multiple comparison test. The dotted line indicates background response.

5.2.3.2 Humoral immune responses induced by the ChAd63 – MVA ME.TRAP prime-boost regimen can be enhanced by adjuvants

To determine the effect of adjuvant on the induction of antibodies by the viral vectored vaccines, TRAP-specific IgG titres were also measured in the experiment described in the previous section (5.2.3.1). The addition of any of the tested adjuvants to the viral vectors did not interfere with the generation of TRAP-specific IgG responses at prime or at boost (Figure 5.8). Unexpectedly, three weeks after prime, the responses were slightly higher in all groups that received adjuvant compared to the group that received ChAd63 ME.TRAP alone, and this was significantly higher in the group that received AddaVax (*$p<0.05$, compared by Kruskal-Wallis with Dunn's multiple comparison test) (Figure 5.8-A). These responses in all groups continued to rise after prime as seen in samples assayed at week 8 (Figure 5.8-B), suggesting the response peaks later than antibodies induced by particle in adjuvant. These responses were then boosted by the MVA ME.TRAP immunisation administered alone or with the same adjuvant as at prime. Three weeks after boost the responses in the viral vector plus Abisco-100 or AddaVax groups were approximately 10 fold higher than the viral vector only group and this was significant for the Abisco-100 group (*$p<0.05$, compared by Kruskal-Wallis with Dunn's multiple comparison test) (Figure 5.8-C). The groups receiving Carbopol or Carbopol with another adjuvant however were not significantly different to the viral vector vaccines alone group. This indicates, all of these adjuvants tested are compatible with induction of antibodies by the viral vectors and two of the adjuvants, Abisco-100 and AddaVax, are able to enhance antibody responses approximately 10 fold. This may be beneficial to the efficacy of the viral vector regimen as TRAP antibodies may be able to prevent invasion of hepatocytes by sporozoites [207, 364].

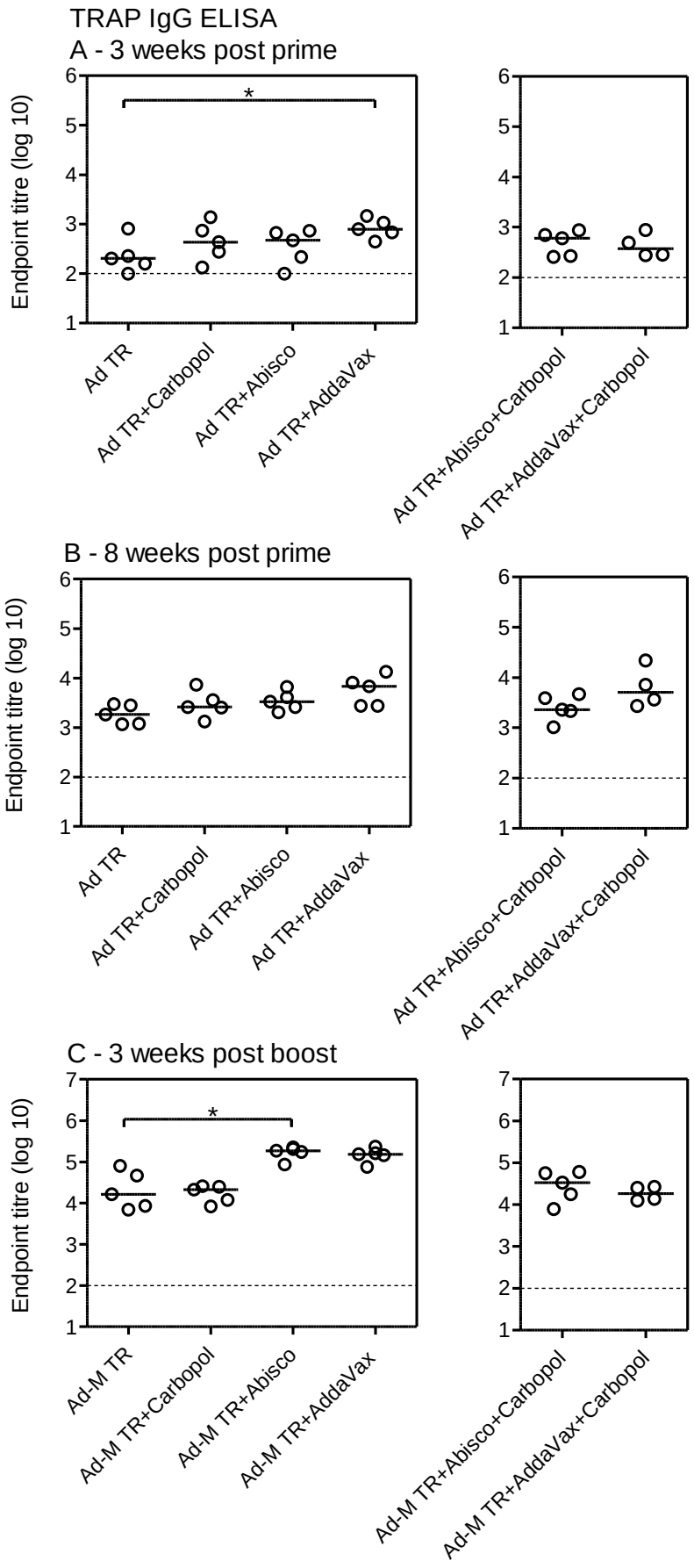
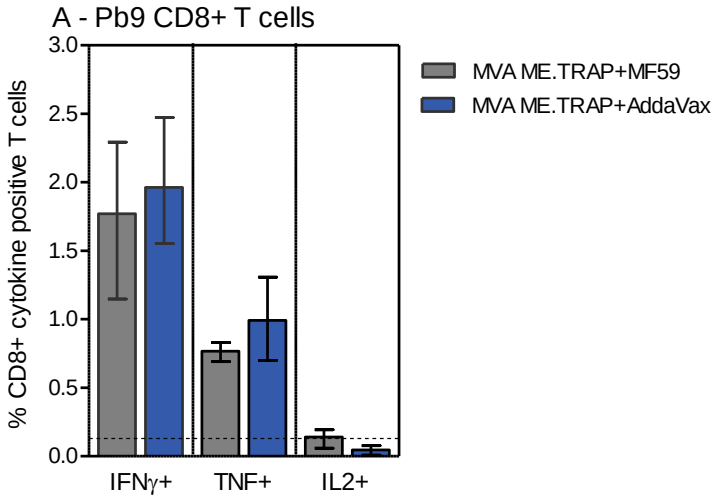

TRAP IgG ELISA
A - 3 weeks post prime
Endpoint titre (log 10)
*
Ad TR
Ad TR+Carbopol
Ad TR+Abisco
Ad TR+AddaVax
Ad TR+Abisco+Carbopol
Ad TR+AddaVax+Carbopol
B - 8 weeks post prime
C - 3 weeks post boost
Ad-M TR
Ad-M TR+Carbopol
Ad-M TR+Abisco
Ad-M TR+AddaVax
Ad-M TR+Abisco+Carbopol
Ad-M TR+AddaVax+Carbopol

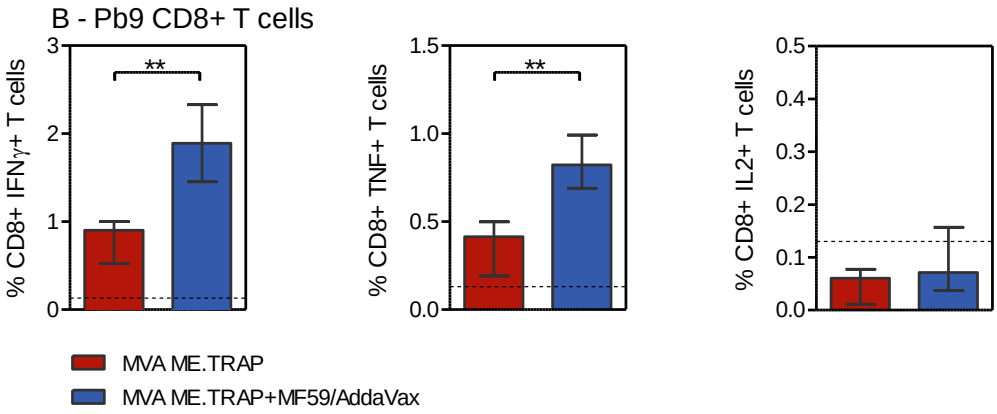
Figure 5.8 TRAP-specific IgG after vaccination with ChAd63 – MVA ME.TRAP alone or combined with adjuvant.

BALB/c mice were immunised i.m. with ChAd63 – MVA ME.TRAP 8 week prime-boost regimen, either alone (Ad-M TR) or combined with adjuvant. The viral vectors were formulated with either one adjuvant (left panel) or two adjuvants (right panel). Blood was taken and assayed by TRAP ELISA three weeks after the prime **(A)** 8 weeks after the prime **(B)** and three weeks after the boost **(C)**. Group medians are shown and compared by Kruskal-Wallis with Dunn's multiple comparison test *$p<0.05$. The dotted line indicates the limit of detection.

5.2.3.3 Cellular immune responses in the blood induced by MVA ME.TRAP vaccination can be enhanced by adjuvants

In the previous section the effect of adjuvants on the immunogenicity of ChAd63 ME.TRAP alone or ChAd63 – MVA ME.TRAP prime-boost regimen was assessed. But the effect of adjuvant on the MVA ME.TRAP vaccine alone was not evaluated. Here a small study is carried out to assess squalene based oil-in-water emulsions in combination with MVA ME.TRAP. Groups of BALB/c mice were immunised with a single vaccination of MVA ME.TRAP alone or in combination with adjuvant (MF59 or AddaVax) T cell responses were assessed to the Pb9 epitope in the blood by ICS one week after immunisation and by *ex-vivo* IFNγ ELISpot in the spleen three weeks after immunisation. At one week there is no difference in the frequencies of CD8+ T cells induced by the two squalene based oil-in-water adjuvants (Figure 5.9-A), but comparing MVA alone to MVA + MF59/AddaVax the responses are significantly higher for CD8+ IFNγ+ and CD8+ TNF+ T cells in the adjuvant group (**$p<0.01$, compared by Kruskal-Wallis with Dunn's multiple comparison test) (Figure 5.9-B). Interestingly, three weeks after immunisation this difference between MVA alone and MVA plus adjuvant groups is not seen in the spleen (Figure 5.9-C). This may indicate the phenotype of the T cells being detected in the blood is different from the phenotype of the T cells in the spleen.


A - Pb9 CD8+ T cells
% CD8+ cytokine positive T cells
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IFNγ+
TNF+
IL2+
MVA ME.TRAP+MF59
MVA ME.TRAP+AddaVax


B - Pb9 CD8+ T cells
% CD8+ IFNγ+ T cells
% CD8+ TNF+ T cells
% CD8+ IL2+ T cells
**
**
MVA ME.TRAP
MVA ME.TRAP+MF59/AddaVax

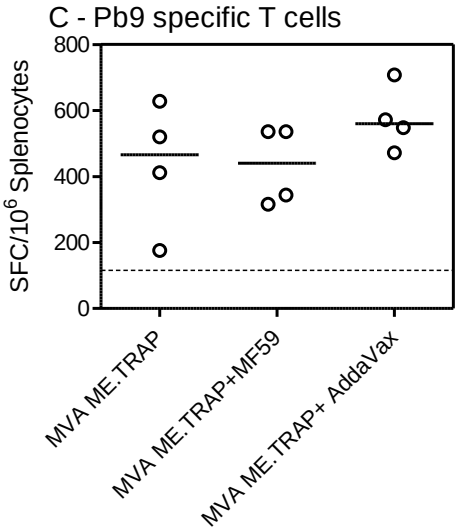

C - Pb9 specific T cells
SFC/10^6 Splenocytes
800
600
400
200
0
MVA ME.TRAP
MVA ME.TRAP+MF59
MVA ME.TRAP+ AddaVax

Figure 5.9 Cellular immune responses induced by MVA ME.TRAP vaccination alone or combined with adjuvant.

BALB/c mice were immunised i.m. with MVA ME.TRAP, either alone or combined with adjuvant (MF59 or AddaVax). Blood was taken one week after the prime vaccination to assess CD8+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with the Pb9 epitope and three different cytokines were assessed (IFNγ, TNF and IL2). Results are presented as the percentage of CD8+ T cells expressing cytokine. **(A)** MVA ME.TRAP with AddaVax compared to MVA ME.TRAP with MF59. **(B)** MVA ME.TRAP alone compared to MVA ME.TRAP with adjuvant (AddaVax/MF59). Spleens were taken three weeks after vaccination and antigen-specific, IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot using the Pb9 epitope **(C)**. Group medians with interquartile range are shown and compared by Kruskal-Wallis with Dunn's multiple comparison test **p<0.01. The dotted line indicates the limit of detection.

5.2.4 R21 + Adjuvant can be mixed and co-administered with the ChAd63 – MVA ME.TRAP prime-boost regimen.

During the assessment of the effect of adjuvant on the induction of immune responses by either the R21 (5.2.1) or the viral vector vaccines (5.2.3) two types of adjuvants have consistently enhanced immune responses to the greatest extent. These are the saponin based ISCOM adjuvants (Abisco-100 and Matrix M) and the squalene based oil-in-water emulsions (MF59 and AddaVax). They were both able to enhance the humoral and the cellular immunogenicity of R21, they did not interfere with the induction of the cellular immunogenicity by the viral vectors, and unexpectedly, they also enhanced the viral vector induced humoral immune responses. They were both therefore taken forward for assessment in combination with R21 and the ChAd63 – MVA ME.TRAP eight week prime-boost regimen. The aim of this study was to assess if the two vaccination strategies can be combined together without interference or reduction in immunogenicity. Groups of BALB/c mice were immunised with either two shots of R21 + adjuvant (MF59 or Abisco-100) eight weeks apart or the ChAd63 – MVA ME.TRAP prime-boost regimen, administered alone or combined together, as detailed in the table below.

Table 5.2 Co-administration study design

Vaccination regimens used to assess immunogenicity after co-administration of R21 + adjuvant and viral vector vaccines.

Gp	n	Particle + Adjuvant	Viral vectors	Interval
1	6	0.5µg R21 + Abisco	-	8 weeks
2	6	0.5µg R21 + MF59	-	8 weeks
3	6	-	ChAd63 - MVA ME.TRAP	8 weeks
4	6	0.5µg R21 + Abisco	ChAd63 - MVA ME.TRAP.	8 weeks
5	6	0.5µg R21 + MF59	ChAd63 - MVA ME.TRAP	8 weeks

5.2.4.1 Humoral and cellular responses induced by the R21 + adjuvant vaccine are unaffected by the addition of the viral vector regimen

Addition of ChAd63 – MVA ME.TRAP to the R21 + adjuvant vaccination did not interfere with the induction of the NANP-specific IgG (Figure 5.10-A+B CS-specific immune responses after vaccination with R21 + adjuvant alone or combined with viral vectors.) or the CS-specific T cell responses (Figure 5.10-C). All mice receiving R21 + adjuvant, either alone or combined with ChAd63 ME.TRAP developed high titres of NANP-specific IgG after the prime. These responses were boosted by the second immunisation and although the median antibody responses in the combination groups were slightly lower than those in the R21 in adjuvant groups, the difference was not statistically significant (compared by Mann-Whitney test). Interestingly, low titres of NANP-specific antibody responses were also detected in the group which received the ChAd63 – MVA ME.TRAP vaccine without R21 and this is presumably because 4 NANP repeats are included in the ME string of the ME.TRAP insert (data not shown). The same group also generated low levels of CS-specific T cell responses; again presumably due to the presence of the CS T cell epitopes in the ME string (Figure 5.10-C). The groups which received the viral vectors in combination with R21 + adjuvant generated the highest levels of CS-specific T cell responses, higher than R21 + adjuvant administered alone. This indicates that although there is no interference or enhancement of R21 antibody induction, R21 T cell induction may be enhanced by the ChAd63 – MVA ME.TRAP regimen, although the increase is only small and may simply be additive.

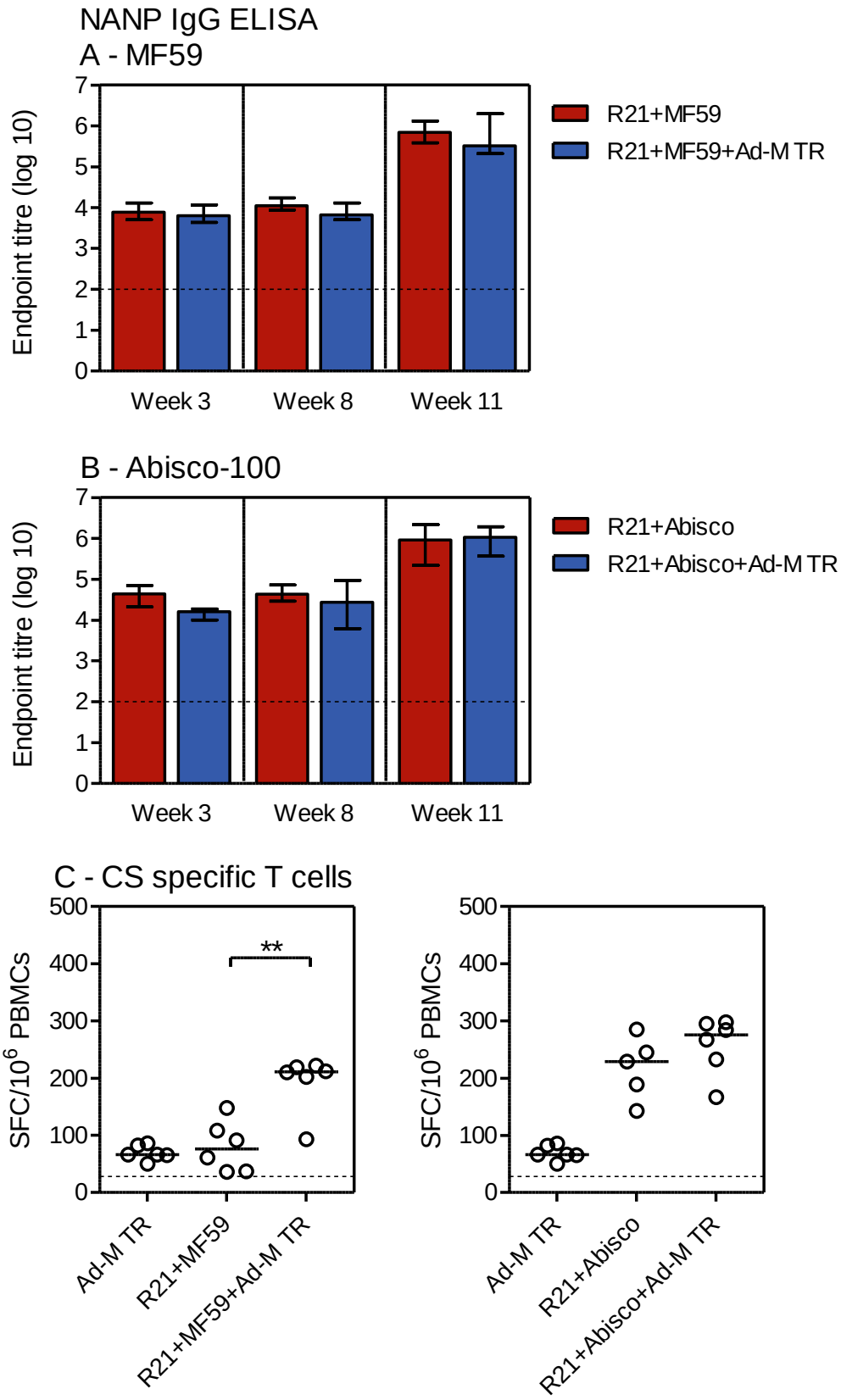

NANP IgG ELISA
A - MF59
Endpoint titre (log 10)
0
1
2
3
4
5
6
7
Week 3
Week 8
Week 11
R21+MF59
R21+MF59+Ad-M TR
B - Abisco-100
Endpoint titre (log 10)
0
1
2
3
4
5
6
7
Week 3
Week 8
Week 11
R21+Abisco
R21+Abisco+Ad-M TR
C - CS specific T cells
SFC/10^6 PBMCs
0
100
200
300
400
500
**
Ad-M TR
R21+MF59
R21+MF59+Ad-M TR
SFC/10^6 PBMCs
0
100
200
300
400
500
Ad-M TR
R21+Abisco
R21+Abisco+Ad-M TR

Figure 5.10 CS-specific immune responses after vaccination with R21 + adjuvant alone or combined with viral vectors.

BALB/c mice were immunised i.m. with ChAd63 - MVA ME.TRAP or 0.5µg R21 + adjuvant (Abisco-100 or MF59) or the particle and viral vectors combined together. Blood was taken three weeks after each vaccination and NANP IgG was assayed by ELISA. Responses after vaccination with MF59 are shown in **(A)** and responses after vaccination with Abisco-100 are shown in **(B)**. The dotted line indicates the limit of detection. Spleens were taken three weeks after final vaccination and antigen-specific, IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot using the a pool of overlapping CS peptides **(C)**. Responses after vaccination with MF59 (left panel) and Abisco-100 (right panel) and the dotted line indicates background response. Group medians with interquartile range are shown and compared by Mann-Whitney test **p<0.01.

5.2.4.2 Humoral and cellular responses induced by ChAd63 ME.TRAP or ChAd63 – MVA ME.TRAP are unaffected by the addition of the R21 + adjuvant vaccine

The R21 + adjuvant vaccine does not interfere with the generation of TRAP-specific T cell responses. Two weeks after the ChAd63 ME.TRAP prime, IFNγ+ CD8+ T cells were detected in the blood by ICS in all the groups that received ChAd63 ME.TRAP and as expected (Figure 5.11-A), were undetectable when only R21 + adjuvant was given (data not shown). Interestingly, the groups which received ChAd63 ME.TRAP in combination with R21 + adjuvant had higher frequencies of IFNγ+ CD8+ T cells and this was significant when compared by Mann-Whitney test, $p < 0.05$ for R21 + MF59 and $p < 0.01$ for R21 + Abisco. The same trend was also seen in the levels of TNF+ CD8+ or IL2+ CD8+ T cells, though only significant when comparing CD8+ TNF+ T cells induced by viral vectors with and without R21 + MF59 ($p < 0.05$). These slightly enhanced responses although not always significant are consistent with the trend seen in section 5.2.3.1 and 5.2.3.3, when adjuvant is combined with the viral vectors in the absence of R21. The responses were all boosted by the MVA ME.TRAP immunisation, but the enhanced response seen in the combination vaccine groups after prime was not seen in the blood one week after boost (Figure 5.11). Consistent with this ICS data one week after boost there was no significant difference in levels of the Pb9-specific T cell response detected in any group when measured in the spleen three weeks after boost (Figure 5.11-B). This again is consistent with the data seen in section 5.2.3.1 when adjuvants are assessed in combination with viral vectors in the absence of R21 and indicates that the particle vaccine has no effect on the induction of the cellular response induced by the viral vector vaccines. Instead, any enhancement of the response seen after prime is likely due to the adjuvant.

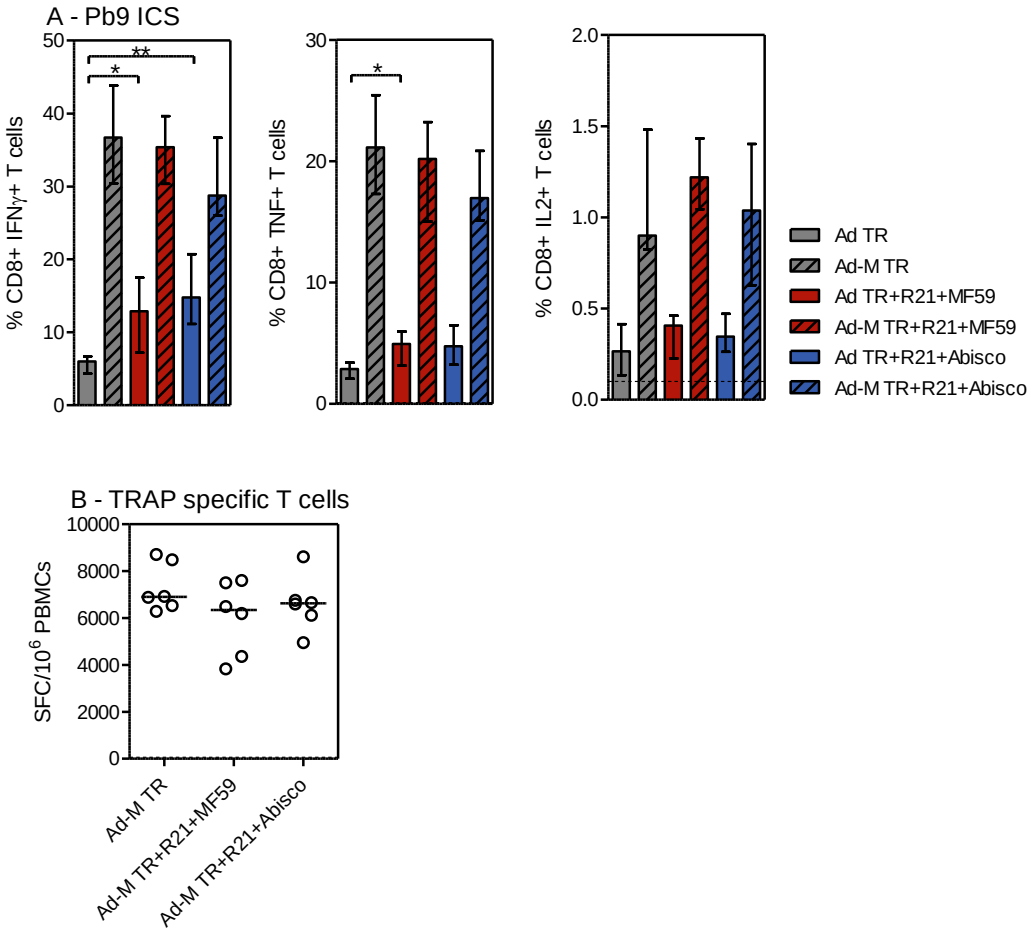


Figure 5.11 Viral vector induced cellular immune responses after vaccination alone or combined with R21 + adjuvant.

BALB/c mice were immunised i.m. with ChAd63 - MVA ME.TRAP 8 week prime-boost regimen alone or combined with 0.5µg R21 + adjuvant (Abisco-100 or MF59). Blood was taken two weeks after the prime and one week after boost vaccination for immunology. Frequencies of CD8+ cytokine secreting T cell were assayed by ICS. Cells were stimulated for six hours with the Pb9 epitope and three different cytokines were assessed (IFNγ, TNF and IL2). Results are displayed as the percentage of CD8+ T cells expressing IFNγ **(A)** TNF **(B)** or IL2 **(C)**. Spleens were taken three weeks after the final vaccination and Pb9-specific, IFNγ secreting T cells assayed in an *ex-vivo* IFNγ ELISpot **(D)** Group medians with interquartile range are shown and the dotted line indicates background response. Groups compared by Mann-Whitney test *p<0.05, **p<0.01.

Furthermore, the R21 + adjuvant vaccine does not interfere with the generation of TRAP-specific IgG responses (Figure 5.12). Interestingly after prime, the groups which received the combination vaccine had higher median endpoint titres than the group which only received ChAd63 ME.TRAP and this was significant for the viral vector group with or without R21 + MF59 (compared by Mann-Whitney test). The primed responses were boosted by the MVA ME.TRAP immunisation and the responses in the combination groups were approximately three fold higher than the viral vector regimen only group. This suggests that the addition of the R21 + adjuvant vaccine does not interfere with the generation of TRAP-specific IgG, but instead enhances it. This is again consistent with the data seen in the previous experiment (5.2.3.2) where TRAP-specific IgG responses where enhanced 10 fold by the addition of adjuvant (Abisco-100 or AddaVax) to the ChAd63 – MVA ME.TRAP prime-boost regimen (Figure 5.8). This again suggests that the adjuvant not the particle vaccine is able to enhance the immune responses induced by the viral vectors.

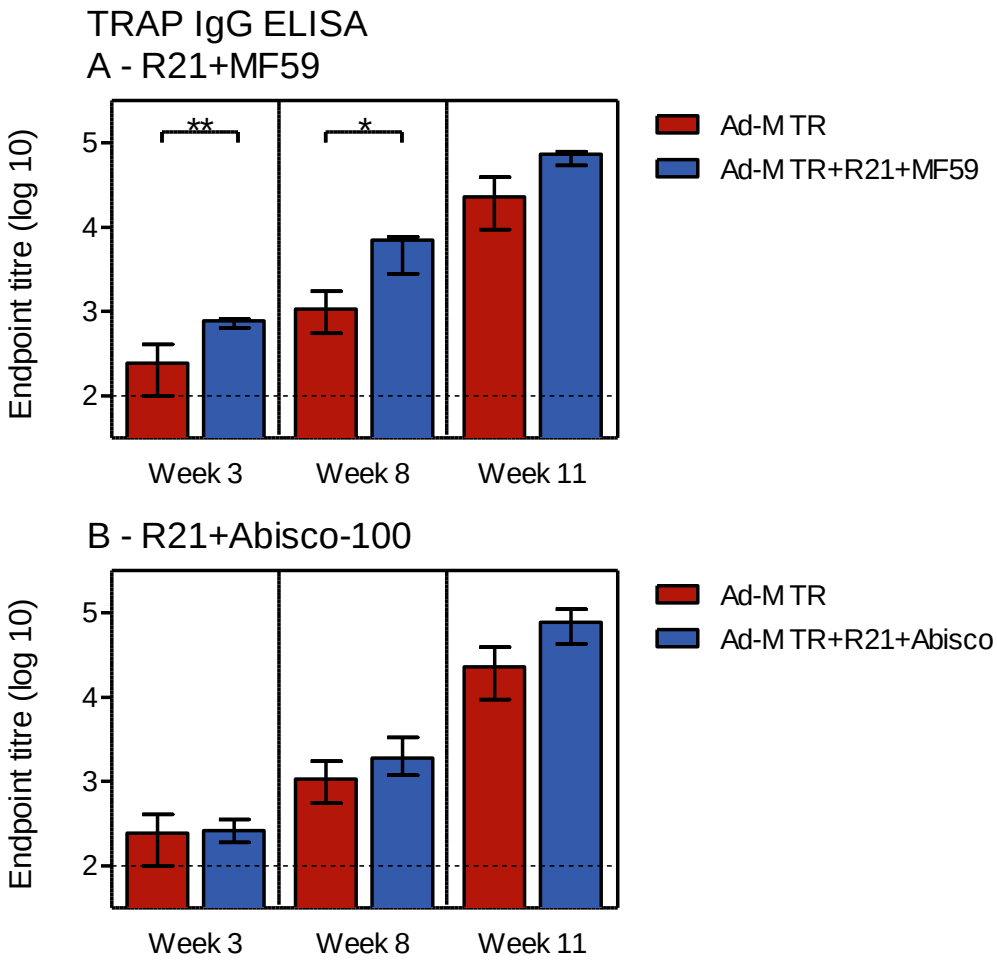


Figure 5.12 Viral vector induced humoral immune responses after vaccination alone or combined with R21 + adjuvant.

BALB/c mice were immunised i.m. with either ChAd63 ME.TRAP - MVA ME.TRAP alone or combined with 0.5µg R21 + adjuvant (Abisco-100 or MF59). Blood was taken three weeks after each vaccination for immunology and TRAP-specific IgG was assayed by ELISA. Reponses to vaccination with R21 + MF59 are shown in **(A)** and responses to vaccination with R21 + Abisco-100 are shown in **(B)**.Group median responses with interquartile range are shown and groups compared by Mann-Whitney test, *p<0.05, **p<0.01. The dotted line indicates the limit of detection.

5.3 Discussion

The main aim of this thesis was to develop an immunogenic CS-based particle vaccine that could induce strong immune responses to the *P. falciparum* CS protein for use in a multi-component vaccination strategy in humans. In this chapter R21 is shown to induce very high levels of anti-CS antibodies to the NANP repeat B cell epitope and good levels of T cells in BALB/c mice. R21 is highly immunogenic when administered without adjuvant; however antibodies and T cells can be further enhanced with a range of safe well tolerated adjuvants. Superior antibody titres are achieved with saponin-based ISCOM adjuvants (Abisco-100 and Matrix M) and squalene-based o/w emulsions (MF59 and AddaVax). T cell induction is also enhanced with both types of adjuvants, but levels were much higher after administration of R21 with Abisco-100 or Matrix M. This chapter however only assesses the magnitude of immune responses generated after vaccination but in order to fully evaluate these adjuvants it is important to assess the quality or type of immune response induced. Adjuvants have the ability to modulate the type and function of the response, so even though the magnitude may be similar the isotype may be skewed towards either Th1 or Th2 isotype and function of antibodies of T cells could be different. Therefore assessing qualities to determine phenotype, avidity/affinity of immune components and induction of memory responses would provide important information. On assessing the optimal regimen to use for the induction of immune responses it was found that two immunisation of R21 + Abisco-100 given 8 weeks apart induced strong responses that were not improved by additional immunisations or by altering the vaccination intervals. This is desirable as it will allow the R21 + adjuvant vaccine to be easily combined with the existing effective ChAd63 – MVA ME.TRAP 8 week prime-boost regimen in a multi-component vaccine strategy.

In addition to simply producing a CS-based particle vaccine we aimed to improve the design of the particle in order to induce stronger CS-specific immune responses compared to those achieved by the leading malaria vaccine candidate RTS,S. To do this R21 was generated from a single CS-HBsAg fusion protein in contrast to RTS,S which is formed from two proteins, the CS-HBsAg fusion protein and unfused HBsAg. R21 therefore results in a particle composed of 100% CS fusion protein where as RTS,S is only 20% fusion protein and 80% HBsAg. To assess if the R21 particle can induce stronger CS-specific immune responses it would need to be compared directly to RTS,S, which is unfortunately unavailable to us at this time. Therefore a comparison was made between immune responses after immunisation with R21 or HBsAg to assess the degree of reduction in HBsAg-specific antibodies induced by the fusion protein. It was found that after R21 immunisation, the majority of the antibody response was induced towards the malaria antigen. Only low levels of HBsAg-specific antibodies were induced when R21 was administered in the most potent adjuvant in comparison to immunisation with the same dose of HBsAg + Alhydrogel. This indicates that the majority of the surface or the particle is covered by CS, and R21 will potentially induce higher CS immune responses than RTS,S. The enhanced level of CS antigen on the surface of R21 may result in greater CS humoral responses not only because of the greater amount of malaria antigen available but also because it may mimic the high level of epitope density present of the surface of many pathogens [444, 480-482]. This repetitive display of the NANP B cell antigen may enhance the recognition of antigen by B cell receptors (BCRs), improve crosslinking of BCRs and therefore enhance B cell activation and antibody production [480, 483-487]. However the epitope coverage of the surface of the particle is yet to be demonstrated, but methods such as immunogold labelling with antibodies to CS and HBsAg with transmission electron microscopy could be used to evaluate this. In addition to the repetitive display of epitope on the surface of the particle the size of the particle may also contribute to the superior immunogenicity of

R21. Particles due to their size enhance uptake by and activation of DCs and therefore generation of T helper cells or cytotoxic T cell responses [478, 488]. In agreement with this, administration of the CS antigen in particulate form as R21 was shown to be more immunogenic than non-particulate CS recombinant protein with improved induction of both humoral and cellular responses. These two vaccines were given in three different types of adjuvants (emulsion, liposomes and ISCOMs) which are also known to enhance immunogenicity due to delivery of antigen as various particle structures, and accordingly the non-particulate CS was able to induce very strong humoral immune responses [478]. However R21 immunogenicity was still superior, suggesting administration of CS with adjuvant is not sufficient to induce the exceptionally high levels of responses seen with R21, and the particulate nature of the R21 itself also contributes to immunogenicity. This study also showed that when R21 is administered in a bio-similar of the GSK AS01 adjuvant (LMQ), which has been the most immunogenic and efficacious adjuvant used with RTS,S [300], immunogenicity is not enhanced above that achieved by Matrix M. This ability to induce comparable levels of antibodies and T cells without the need for access to the GSK proprietary adjuvant AS01 is potentially beneficial for the future development of this and many other vaccine candidates.

Finally, the ability to mix and administer the particle in adjuvant and viral vector vaccines together without immunological interference was assessed. Immune interference is common although it has not always been assessed in multi-antigen or multi-component vaccine strategies. It has been clearly seen in studies combining RTS,S with protein in adjuvant vaccines [489], and in some studies combining viral vectors with different antigenic inserts [490-494] but not in others [495]. Immune interference therefore appears to be complex and antigen dependant, it is likely due to competition for antigen presentation on APCs although mechanisms are unclear and it and can sometimes be overcome by immunisation at separate sites [490].

Presumably, combining multiple antigens using different vaccine technologies would be more successful because they activate the immune system via difference mechanisms, but this is not always the case. However, in this study no interference was observed with the induction of immune responses by either R21 + adjuvant or the ChAd63 – MVA ME.TRAP viral vector regimen when they were mixed and administered at the same site. This is similar to the results seen in a recent study where immunogenicity was unaffected when FP9 and MVA viral vectors expressing *P. berghei* CS were combined with *P. berghei* CS-based hepatitis B core particle vaccine [464]. In addition to assessing immune interference, combining adjuvants with viral vectors was found to enhance the antibodies induced to the insert expressed by the viral vectors and this result has since been repeated in another study in our group using viral vectors vaccines for Rift Valley fever [496]. Enhanced induction of TRAP-specific antibodies may be beneficial as it is still unclear if antibodies to TRAP can contribute to protection [364].

In summary, in this chapter R21 has been shown to induce high levels of CS-specific antibodies and good levels of T cells and no antigenic interference was seen when this vaccine was combined with the viral vector vaccines. Both the saponin-based ISCOM adjuvants and the squalene-based o/w emulsions were able to enhance the immunogenicity of R21 effectively and also enhanced the induction of antibodies to the viral vector insert. This study however does not assess whether these immune responses measured are functional or protective and this will therefore be assessed in the following chapter.

6 Protective efficacy of CS-based particles and TRAP based viral vector vaccines in BALB/c mice using a transgenic parasite model

6.1 Introduction

R21 and ChAd63 – MVA ME.TRAP have been shown in the previous chapter to be immunogenic in BALB/c mice administered either alone or in several adjuvants (Chapter 5). The most potent adjuvants were then selected and used with R21 to assess immunological interference when the two vaccination regimens were combined and no interference was observed (Chapter 5). In these studies the CS or TRAP specific antibodies and T cells were measured after immunisation but these measures of immunogenicity do not necessarily indicate a functional protective immune response. Also, while CS and TRAP specific antibodies and or T cells have been shown to inhibit liver stage parasites and prevent or reduce blood-stage infection in a number of studies (reviewed in Chapter 1), other components may contribute to protection that have not been measured here. Therefore it is also useful to assess the functionality or protective capacity of the entire vaccine induced immune response. Following demonstration of protective efficacy mechanistic experiments can then also be performed to help determine the immune components contributing to protection.

A number of *in-vitro* functional assays exist to assess the efficacy of candidate malaria vaccines. However, the development and use of these assays is most advanced for assessing blood-stage and transmission blocking vaccine efficacy. Although they have been used for pre-erythrocytic vaccines [197, 200, 229], they have not been as well standardised between laboratories and have not always

yielded consistent results [428, 429, 497]. The main problems that have limited the development and wide spread use of these assays for the pre-erythrocytic stage is the difficulty of culturing human hepatocytes and the low level of infectivity of the cultured cells by *P. falciparum* [428]. These assays are currently being optimised by students at the Jenner Institute, with the aim of developing robust reproducible assays that can aid evaluation of candidate malaria vaccines in the future.

Currently experimental malaria vaccines are routinely assessed in murine malaria sporozoite challenge studies. However it has been difficult to assess the protective efficacy of *P. falciparum* based vaccines pre-clinically because *P. falciparum* does not infect mice, which are the most versatile and commonly used animal model available. *P. falciparum* does infect some non-human primates, but this model is expensive and not easily accessible for routine studies. Some other species of malaria infect mice and *P. berghei, P. yoelii* and *P. chabaudi* have commonly been used to study malaria infections in murine models, but these malaria species are not natural pathogens of the laboratory mouse [166]. This can complicate interpretation of results as it means no evolutionary adaptation of the parasite has occurred alongside the murine immune system, which is in stark contrast to malaria species that infect humans. Hence proteins that are identified as important targets in human malaria may not exist or may have different functions in the species that infect mice. These three malaria species also differ significantly in virulence and in the detail of the mechanisms of a protective immune response required to control infection. Therefore, the relevance of each of these murine models for assessing vaccines against human malaria is questioned [167]. Nonetheless despite the differences, there are also a number of similarities between these models and human malaria and many of the recent advances in malaria vaccine development have come from studies in animal models which have translated to some degree to human infection models [182].

An alternative model that is being increasingly developed is the use of transgenic (Tg) *P. berghei* parasites that express one or more *P. falciparum* genes [171, 176-178]. One method for generating Tg parasite is the replacement of the *P. berghei* gene with the *P. falciparum* version under the control of the relevant *P. berghei* promoter. However, this strategy can result in reduced parasite fitness, where the parasite has altered development or infectivity due to the loss of a required gene [498]. Alternatively the addition strategy can be used where the *P. falciparum* gene can be inserted at a different dispensable point in the genome. This is less likely to affect fitness as the parasite retains its original version of the gene. The 230p locus in *P. berghei* is considered silent and replacement of this gene has no impact on parasite behaviour or survival; therefore the *P. falciparum* antigen can be expressed under the control of the *P. berghei* UIS4 promoter by insertion at the 230p locus [169, 362]. The CS Tg parasite used in this study was developed by a DPhil student, Ahmed Salman, at Leiden University Medical Centre. This parasite used the addition strategy with the *P. falciparum* CS under the control of the *P. berghei* UIS4 promoter which is expressed at both the sporozoite and liver stage similar to the native expression profile of CS [321, 499]. This parasite has similar levels if infectivity and virulence to the wild type *P. berghei* and can therefore be used to assess the protective capacity of the *P. falciparum* CS or *P. berghei* TRAP immune responses in murine models.

The pre-erythrocytic stage of infection is seen as an attractive bottleneck during the malaria parasite life cycle for targeting and eliminating the parasite, because in comparison to the erythrocytic stage, only very few sporozoites are inoculated by the mosquito and this results in similarly low numbers of infected hepatocytes [13, 500]. However, due the parasites sophisticated immune evasion mechanisms there is significant potential for at least one sporozoite to avoid elimination by the immune system, and this one parasite will result in a blood stage infection which could rapidly

multiply [501]. For this reason a sporozoite stage vaccine is considered ‘leaky’ and even the highest levels of vaccine-induced immune responses to a single antigen may not be able to confer complete sterile protection. This is demonstrated by the RTS,S results, where RTS,S induces extremely high levels of CS antibody yet prevention of blood stage infection is relatively low. It has been estimated that RTS,S is actually very effective and can reduce the liver to blood stage parasite inoculum by 96.1% [469] and in some cases the surviving parasites originate from only one surviving sporozoite. Similar analysis has been done on PCR data from sporozoite challenged volunteers vaccinated with T cell inducing viral vectors expressing ME.TRAP, and a similar reduction in liver to blood stage inoculum is estimated >90% [383, 387]. It is therefore hypothesised that if the antibodies induced by the RTS,S and TRAP based viral vectors can eliminate >95% of the sporozoites before they enter the liver cells, then the T cells targeting the infected hepatocytes will have a better chance at clearing the remaining parasites and preventing blood stage infection [450]. Evidence that this increase in efficacy can be achieved and is more than additive was generated in a *P. berghei* model, where mice were immunised with either an antibody inducing CS-based particle vaccine or a CS-based T cell inducing viral vector regimen [464]. No immunological interference was detected and efficacy of the individual vaccines was 12% and 37% alone for the particle and the viral vector based vaccines respectively, and when they were combined together efficacy increased to 90%. This vaccine strategy uses the same antigen in two vaccines that stimulate different arms of the immune response; however targeting multiple antigens during the pre-erythrocytic stage may be more effective than a single antigen, particularly in human infection involving fully adapted parasites. This is supported most strongly by the success of the whole sporozoite based vaccine approaches. In these studies immunisation with either radiation attenuated or genetically attenuated parasites, or sporozoites administered under drug cover have all demonstrated very high levels of efficacy in mouse studies and this efficacy has

translated into human trials [114, 116]. The mechanism of protective efficacy in these models is not well defined, though a number of studies have shown it may be mediated by immune responses targeting multiple pre-erythrocytic stage antigens dominated largely by CD8+ T cells [264, 312, 314]. Despite the promising results obtained by these vaccination approaches though, the lack of efficacy elicited by needle and syringe administration and the logistical challenges of deployment of whole parasite vaccines mean this approach is unlikely to be successful in the near future. Therefore eliciting high levels of protection in humans by targeting multiple antigens from the pre-erythrocytic stage with subunit vaccination is an attractive strategy.

As mentioned in the previous chapter concurrent induction of both high titres of CS antibodies and high frequencies of TRAP specific T cells is a promising approach for improving pre-erythrocytic vaccine efficacy. The antibodies could target sporozoites and thereby reduce the invasion of liver cells and the CD4+ and CD8+ T cells could target and clear the intra-hepatic parasites that successfully evade the antibodies. Therefore, the main aims of this study were firstly, to determine if the immune response induced by vaccination with R21 is protective in BALB/c mice in a transgenic parasite model and whether this efficacy is affected by adjuvant. The second aim is to determine if the efficacy of R21 can be enhanced by combination with TRAP based viral vector vaccines. Finally this study will also assess the efficacy of the leading malaria vaccine, RTS,S/AS01B alone or combined with viral vectors in the transgenic parasite model. All animal handling and immunisations were performed by Rebecca Hillson a research technician at the Jenner Institute. Preparation of all vaccines, dissection of mosquitos, preparation of sporozoites, counting of blood smears as well as processing of all samples, all immunological assays and all data analysis was performed by Katharine Collins.

6.2 Results

6.2.1 R21 efficacy is affected by adjuvant

The R21 particle vaccine produced in Chapter 4 has been shown to be most immunogenic when administered with two types of adjuvants, the saponin-based ISCOMs (Matrix M and Abisco-100) and the squalene-based o/w emulsions (MF59 and AddaVax). These adjuvants were therefore used to assess the ability of R21 vaccination to protect against sporozoite challenge. Since R21 contains epitopes from the *P. falciparum* CS protein and *P. falciparum* does not infect mice, a model using a transgenic *P. berghei* parasite which expresses the *P. falciparum* CS antigen (in addition to the *P. berghei* copy of CS) was used (Tg Pb-PfCS). This transgenic parasite was produced by Ahmed Salman, a student at the Jenner Institute, in collaboration with Dr Chris Janse and Dr Shahid Khan of Leiden University Medical Centre.

A standard challenge dose of 1000 sporozoites per mouse injected i.v. was used for all challenge experiments, and consistent with the R21 immunogenicity studies they were performed in BALB/c mice. R21 + adjuvant was given twice, eight weeks apart and mice were challenged three weeks after the boost, unless otherwise indicated. Immunogenicity was measured after prime and prior to challenge, and mice were monitored from day five after challenge by thin-film blood smears for the development of blood stage parasitaemia. Mice were considered sterilely protected if they were slide negative at day 14 after challenge and partial vaccine efficacy was assessed as a significant delay in the development of 1% parasitaemia in the blood compared to control mice determined by comparison of Kaplan-Meier survival curves by Log-rank (Mantel-Cox) Test (Figure 6.1).

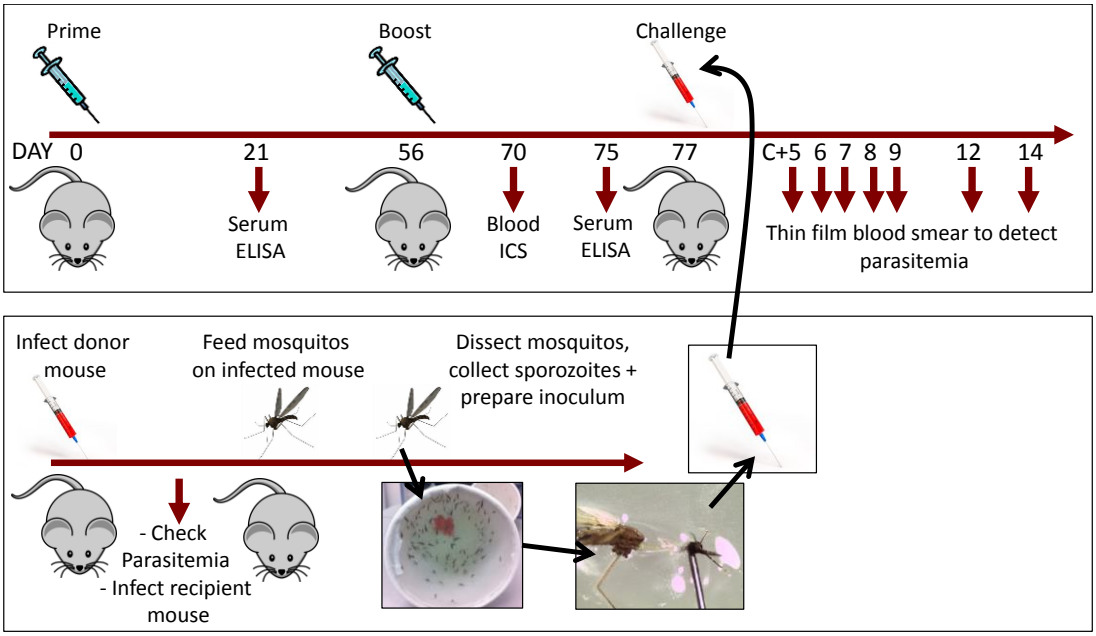


Figure 6.1 Schematic representation of the sporozoite challenge studies in mice.

The top panel shows the vaccination and challenge schedule, samples were taken for immunology at after prime and prior to challenge and thin-film blood smears were taken from day 5 after challenge to assess development of parasitaemia in the blood. The bottom panel show the process for generating the sporozoites for the challenge inoculum.

6.2.1.1 Two Saponin based ISCOM adjuvants elicit equivalent sterile efficacy when administered with R21

In the previous chapters two very similar saponin-based ISCOM adjuvants Abisco-100 and the clinical formulation Matrix M have been used to assess the immunogenicity of R21 and have been shown to enhance both the cellular and humoral immune responses to the same extent. Here, in the transgenic challenge model they both also elicited significant levels of protective efficacy when comparing R21 + adjuvant vaccinated mice to control mice by Log-rank (Mantel-Cox) Test. R21 + Abisco-100 sterilely protected 100% of the challenged mice (8/8) ($p < 0.0001$) (Figure 6.2-B) and R21 + Matrix M sterilely protected 87.5% (7/8) ($p = 0.0002$) and this was confirmed in a second independent challenge (7/8) ($p < 0.0001$) (Figure 6.2-C+D). No protection was conferred by immunisation with the adjuvants alone in the same regimen when compared to unvaccinated mice ($p = 0.977$) (Figure 6.2-A) and there was no difference between the level of efficacy elicited by the two R21 + adjuvant groups (Matrix M or Abisco-100) ($p = 0.309$) (Figure 6.2-E). There was also no difference in the level of NANP specific IgG measured in the two groups after either prime or boost, suggesting that if a threshold of anti-NANP IgG is required to achieve sterile efficacy, then immunisation of R21 with these adjuvants may have exceed this level similarly. These data also indicate as expected that the two very similar adjuvants Abisco-100 and Matrix M are able to enhance not only the magnitude but also the protective capacity of the immune responses induced by R21 to a similar extent.

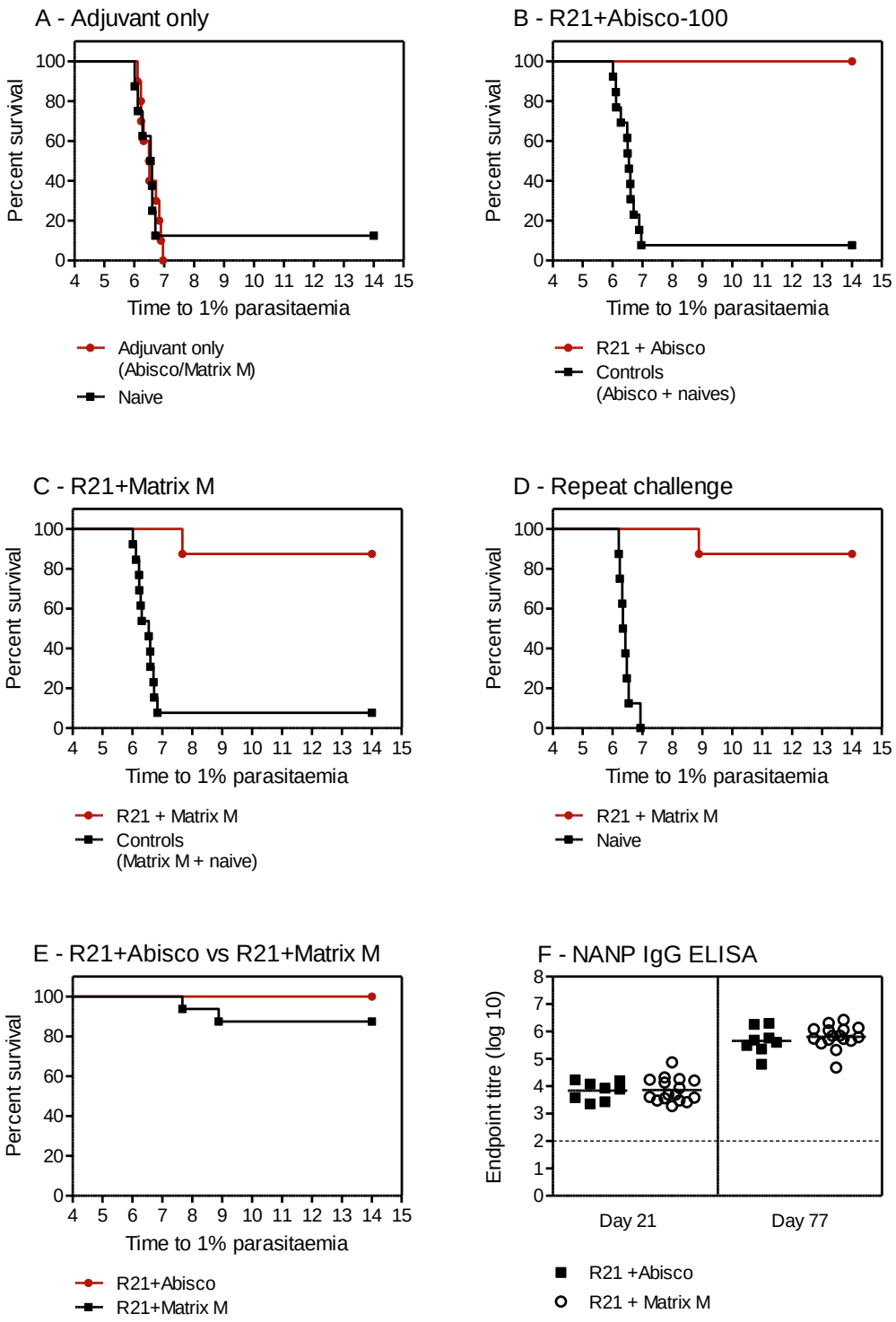
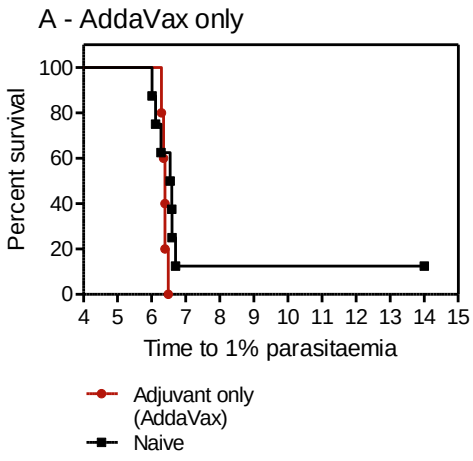

A - Adjuvant only
Percent survival
Time to 1% parasitaemia
Adjuvant only
(Abisco/Matrix M)
Naive
B - R21+Abisco-100
Percent survival
Time to 1% parasitaemia
R21 + Abisco
Controls
(Abisco + naives)
C - R21+Matrix M
Percent survival
Time to 1% parasitaemia
R21 + Matrix M
Controls
(Matrix M + naive)
D - Repeat challenge
Percent survival
Time to 1% parasitaemia
R21 + Matrix M
Naive
E - R21+Abisco vs R21+Matrix M
Percent survival
Time to 1% parasitaemia
R21+Abisco
R21+Matrix M
F - NANP IgG ELISA
Endpoint titre (log 10)
Day 21
Day 77
R21 +Abisco
R21 + Matrix M

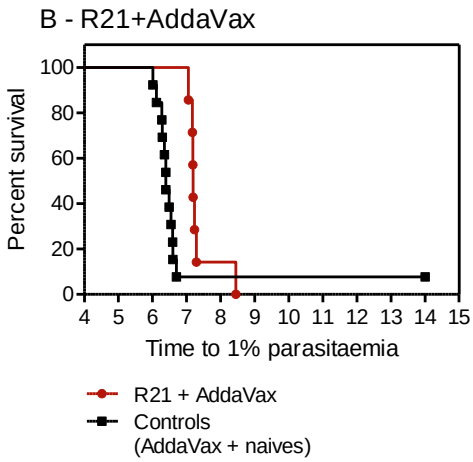
Figure 6.2 Protective efficacy elicited by saponin-based ISCOM adjuvants with R21 in a transgenic sporozoite model.

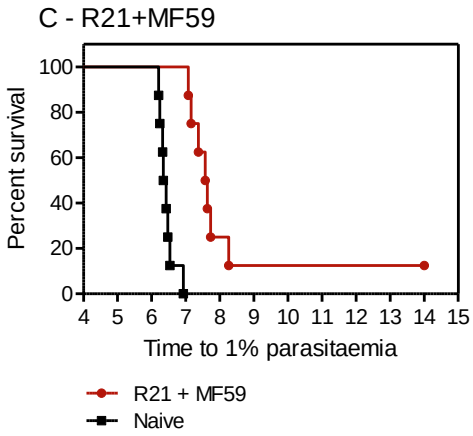
BALB/c mice were immunised i.m. with 0.5µg R21 + adjuvant (Abisco-100 or Matrix M), twice eight weeks apart (n=8/group). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice. Two groups of adjuvant control mice (n=5/group) were also challenged three weeks after receiving two shots of adjuvant (Abisco-100 or Matrix M) i.m. eight weeks apart. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** Adjuvant control = no significant difference, **(B)** R21 + Abisco-100 $p<0.0001$, **(C)** R21 + Matrix M $p=0.0002$, **(D)** R21 + Matrix M repeat $p<0.0001$, **(E)** R21 + Abisco vs R21 + Matrix M = no significant difference. Blood was taken three weeks after each vaccination (Day 21 and Day 77) for immunology and NANP specific IgG was assayed by ELISA **(F)**, group mean responses shown and the dotted line indicates the limit of detection. Groups compared by One-way ANOVA with Bonferroni's multiple comparison test.

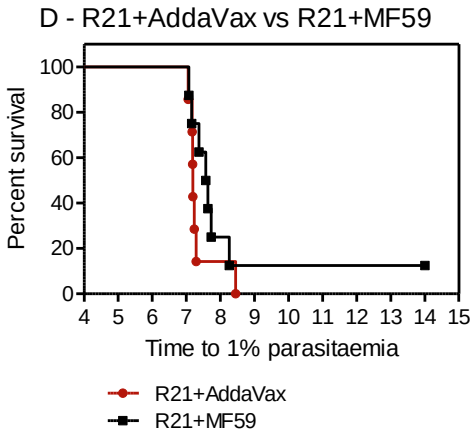
6.2.1.2 Two squalene oil-in-water emulsion adjuvants elicit equivalent partial efficacy when administered with R21

In previous chapter the two squalene o/w emulsion adjuvants MF59 and AddaVax have also been used to assess the immunogenicity of R21 and have been shown to enhance the humoral immune responses and to a lesser extent the cellular immune responses to a similar level. In the transgenic sporozoite challenge model they are also both able to elicit significant levels of protective efficacy, however, R21 + AddaVax did not sterilely protect any mice (0/7) but did significantly delay the development of blood stage parasitaemia compared to control mice ($p = 0.026$) (Figure 6.3-B) R21 + MF59 sterilely protected 12.5% of the vaccinated mice (1/8) and significantly delayed blood stage parasitaemia in the remaining 7 ($p < 0.0001$) (Figure 6.3-C). Immunisation with AddaVax alone did not confer any protective efficacy ($P = 0.141$) (Figure 6.3-A) and there was no difference between the levels of protection elicited by the two squalene o/w emulsion adjuvants administered with R21 ($p = 0.220$) (Figure 6.3-D). There was also no difference in the level of NANP-specific IgG detected either after prime or boost, and despite high CS antibody titres R21 + AddaVax/MF59 failed to elicit sterile protection in more than one mouse (Figure 6.3-E). This study indicates that as expected these two adjuvants are very similar and may act in the same way as they are able to enhance not only the magnitude but also the protective ability of the immune response induced by R21 in a similar fashion.


A - AddaVax only
Percent survival
100
80
60
40
20
0
4 5 6 7 8 9 10 11 12 13 14 15
Time to 1% parasitaemia
Adjuvant only
(AddaVax)
Naive


B - R21+AddaVax
Percent survival
100
80
60
40
20
0
4 5 6 7 8 9 10 11 12 13 14 15
Time to 1% parasitaemia
R21 + AddaVax
Controls
(AddaVax + naives)


C - R21+MF59
Percent survival
100
80
60
40
20
0
4 5 6 7 8 9 10 11 12 13 14 15
Time to 1% parasitaemia
R21 + MF59
Naive


D - R21+AddaVax vs R21+MF59
Percent survival
100
80
60
40
20
0
4 5 6 7 8 9 10 11 12 13 14 15
Time to 1% parasitaemia
R21+AddaVax
R21+MF59

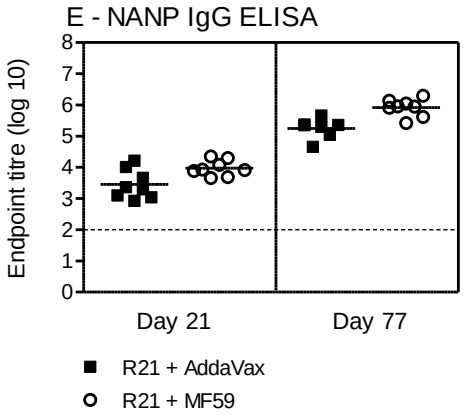

E - NANP IgG ELISA
Endpoint titre (log 10)
8
7
6
5
4
3
2
1
0
Day 21
Day 77
R21 + AddaVax
R21 + MF59

Figure 6.3 Protective efficacy elicited by squalene o/w emulsions with R21 in a transgenic parasite model.

BALB/c mice were immunised i.m. with 0.5µg R21 + adjuvant (AddaVax of MF59) twice, eight weeks apart (n=7/group and n=8/group, respectively). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice. Adjuvant control mice (n=5) were also challenged three weeks after receiving two shots of AddaVax i.m. eight weeks apart. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** Adjuvant control = no significant difference, **(B)** R21 + AddaVax p=0.026, **(C)** R21 + MF59 p=<0.0001, **(D)** R21 + AddaVax vs R21 + MF59 = no significant difference. Blood was taken three weeks after each vaccination (Day 21 and Day 77) for immunology and NANP specific IgG was assayed by ELISA **(E)**, group mean responses shown and the dotted line indicates the limit of detection. Groups compared by One-way ANOVA with Bonferroni's multiple comparison test.

6.2.1.3 R21 is most protective when administered with the saponin-based ISCOM adjuvants Abisco-100 or Matrix M

R21 elicits varying degrees of efficacy determined in the previous sections (0 and 6.2.1.2) by the type of adjuvant it is formulated with. Since the same types of adjuvants elicit the same level of efficacy the data can be combined from the independent experiments and analysed together. Therefore, R21 + Abisco/Matrix M sterilely protects 91.7% of the vaccinated mice (22/24) ($p < 0.0001$) whereas R21 + AddaVax/MF59 only sterilely protects 6.7% (1/15) but it does also delay the development of blood stage parasitaemia in the remaining mice resulting in significant protection ($p < 0.0001$). Although both types of adjuvants are statistically very effective, the efficacy elicited by R21 + Abisco/Matrix M is clearly superior as it sterilely protects a greater percentage of the vaccinated mice compared to R21 + AddaVax/MF59, and this is significantly greater if you compare the two groups by Log-rank (Mantel-Cox) Test ($p < 0.0001$) (Figure 6.4-A). Interestingly though, although protective efficacy is very different between the two types of adjuvants, there is no difference in the level of NANP specific IgG detected, either after prime or just prior to challenge (Figure 6.4-B). These data suggest therefore that efficacy of R21 is not mediated solely by the magnitude of the CS specific IgG response, either the type/quality of the IgG response may be important or other components of the immune system may contribute to the protective immune response.

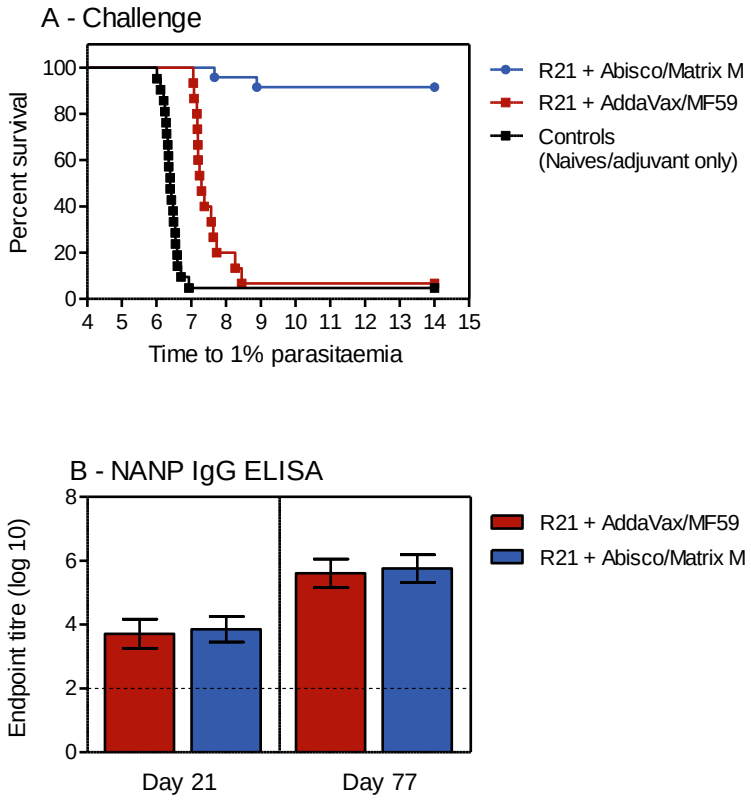


Figure 6.4 Comparative assessment of efficacy elicited by R21 administered in different adjuvants in a transgenic parasite model.

Combined analysis of data from independent experiments where BALB/c mice were immunised i.m. with 0.5µg R21 + adjuvant, twice, eight weeks apart (Abisco-100/Matrix M n=24) of (AddaVax/MF59 n=15). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with control mice (naïve + adjuvant only mice, n=21). Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graph and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** R21 + Abisco/Matrix M vs R21 + AddaVax/MF59 $p<0.0001$. Blood was taken three weeks after each vaccination (Day 21 and Day 77) for immunology and NANP specific IgG was assayed by ELISA **(B)**, group mean responses with SD shown and dotted line indicates the limit of detection. Groups compared by One-way ANOVA with Bonferroni's multiple comparison test.

6.2.1.4 CD4+ T cell levels at the time of infection may have a role in the protective efficacy elicited by R21 + adjuvant immunisation

As mentioned in the previous section, the level of protective efficacy differs between mice immunised with R21 formulated with either Abisco-100/Matrix M or AddaVax/MF59 (Figure 6.5-A). There was however no difference in the level of anti-NANP antibodies detected in these two groups, but interestingly the frequencies of CS specific CD4+ T cell detected in the blood just prior to challenge were higher in the group where most mice were sterilely protected (Figure 6.5-B). This difference was seen in the frequencies of all the cytokine secreting CD4+ populations measured (IFNγ+, TNF+ and IL2+) and was statistically significant for each $p = 0.0019$, $p = 0.0002$, $p = 0.0005$ respectively (Un-paired t test). The levels of CD8+ T cells were also measured and as expected for protein immunisation, they were very low (mean population frequencies between 0.02-0.18%) and were not different between the groups (Figure 6.5-C).

This finding, that frequencies of antigen specific CD4+ T cells are higher in the sterilely protected mice is simply an association and only indicates that CD4+ T cell may have a role in protective efficacy. To further assess the contribution of CD4+ T cells in sterile protection, T cell depletion studies were set up using monoclonal antibodies to deplete T cell subsets prior to challenge, from mice vaccinated with R21 + Matrix M. Unfortunately problems in the insectary meant that no sporozoites were available to complete the challenge study, a repeat is planned to validate this observation.

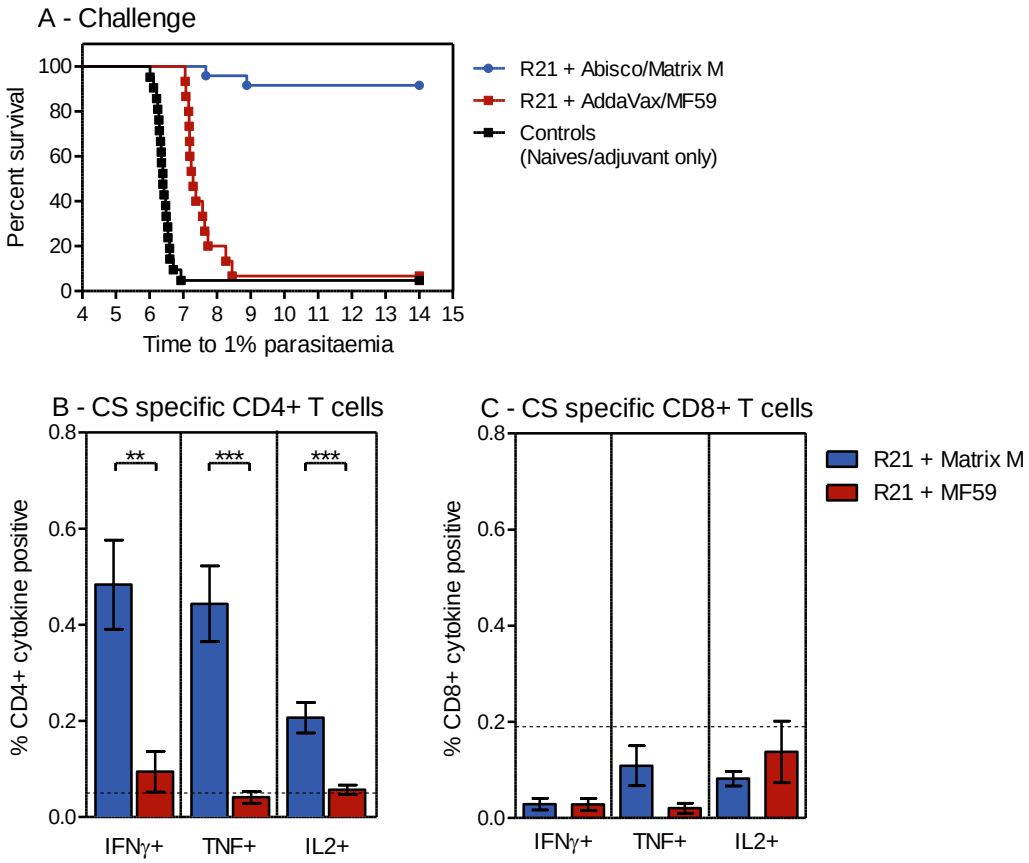


Figure 6.5 R21 + adjuvant induces CS specific T cells in the blood.

(A) Combined efficacy analysis of data from two independent experiments where BALB/c mice were immunised i.m. with 0.5µg R21 + adjuvant, twice, eight weeks apart (Abisco-100/Matrix M n=24) of (AddaVax/MF59 n=15). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with control mice (naïve and adjuvant only mice, n=21). Time to develop 1% parasitaemia was calculated using linear regression, the results presented in the Kaplan-Meier survival graph and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** R21 + Abisco/Matrix M vs R21 + AddaVax/MF59 $p<0.0001$. Blood was taken three weeks after the final vaccination to assess CD4+ and CD8+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with a pool of overlapping CS peptides and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD4+ **(B)** or CD8+ **(C)** T cells expressing the cytokine. Group means with SEM are shown and the groups compared by Unpaired t test **$p<0.01$, ***$P<0.001$ and dotted line indicates background response (data from one experiment n=8/group)

6.2.2 R21 + Matrix M efficacy is maintained 14 weeks after vaccination

The efficacy elicited by immunisation with R21 + Matrix M described previously was assessed at three weeks after final immunisation, so in order to determine how durable this protection is, mice were also challenged seven weeks and 14 weeks after immunisation. Efficacy was maintained very well at seven weeks post boost immunisation with 75% of mice sterilely protected (6/8) and this was not significantly different when compared to efficacy at three weeks post immunisation ($p = 0.4468$, by Log-rank (Mantel-Cox) Test). At 14 weeks post immunisation, sterile efficacy was reduced to 50% (2/4) and this was 37% lower than the efficacy at three weeks (Figure 6.6-A). This was not significantly lower but this is likely due to the small number in the group ($p = 0.0636$). NANP-specific IgG titres were also measured in these mice prior to challenge and there was no difference in the levels between the three groups (Figure 6.6-B) (compared by One-way ANOVA with Bonferroni's multiple comparison test).

Sterile efficacy 14 weeks after immunisation is 37% lower than efficacy three weeks after immunisation. This reduction in protective efficacy can however be boosted to 100% if mice are challenged once (three weeks post immunisation) within the 14 weeks (Figure 6.6-C). Therefore efficacy after vaccination and one sporozoite infection is very durable and 100% sterile efficacy is maintained for at least 14 weeks. Interestingly NANP-specific IgG titres measured 14 weeks after immunisation are not boosted by challenge above the level of mice that were unchallenged at this point (Figure 6.6-D). To confirm the contribution of vaccination to this sterile efficacy or if sterile efficacy is simply conferred by sporozoite infection, additional control groups would be required. A control group of mice would need to be included which would be challenged at the same time as the vaccinated group and then drug

cleared before blood stage infection and re-challenged at 14 weeks. It would also be necessary to ensure no residual chemotherapy remained in the control mice, and so including a further control group which received chemotherapy only would also be necessary.

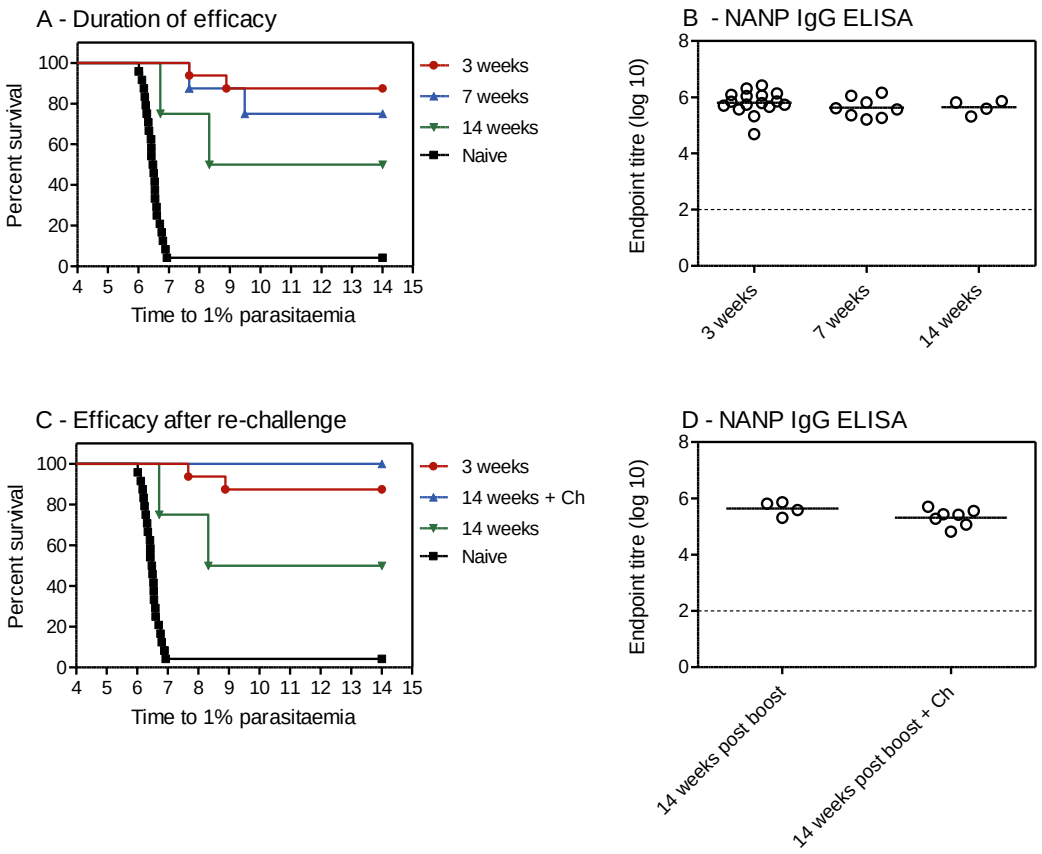


Figure 6.6 Duration of protective efficacy elicited by R21 + Matrix M immunisation in a transgenic sporozoite model.

BALB/c mice were immunised i.m. with 0.5µg R21 + Matrix M twice, eight weeks apart. Mice were challenged either three weeks (n=16), seven weeks (n=8) or 14 weeks (n=4) after vaccination, or 14 weeks after vaccination and challenge (n=7) by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice at each challenge. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** Duration of efficacy, **(C)** Efficacy after re-challenge. Blood was taken prior to challenge for immunology and NANP specific IgG was assayed by ELISA **(B+D)**. Group mean responses are shown and compared by One-way ANOVA with Bonferroni's multiple comparison test and the dotted line indicates limit of detection.

6.2.3 R21 induced greater protective efficacy than non-particulate recombinant CS protein

R21 is not only more immunogenic but also more efficacious than non-particulate CS recombinant protein. In the previous chapter mice were immunised with 0.5µg of either R21 or non-particulate CS in a range of adjuvants and all CS-specific immune responses measured were higher in mice immunised with R21 compared to CS. In this study mice were immunised with 0.5µg of R21 or non-particulate CS formulated with Matrix M, two shots were given, eight weeks apart, and mice were challenged with transgenic parasites (Tg Pb-PfCS) three weeks after the boost. R21 + Matrix M protected 87.5% of the vaccinated mice (14/16) and CS + Matrix M protected only 42.5% (3/7) and this was significantly lower by Log-rank (Mantel-Cox) Test ($p = 0.0145$) (Figure 6.7-A). The CS specific immune responses were also assessed prior to challenge and as seen previously the levels of both NANP-specific IgG and CS-specific and T cells were higher in the R21 + Matrix M groups (Figure 6.7-B+C). This was a significant difference for the antibodies at both time points measured, but not significant for the T cells. The CS antigen is therefore both more immunogenic and efficacious when administered as the R21 particle compared to recombinant protein and the induction of sterile efficacy is not solely due to the ISCOM adjuvants.

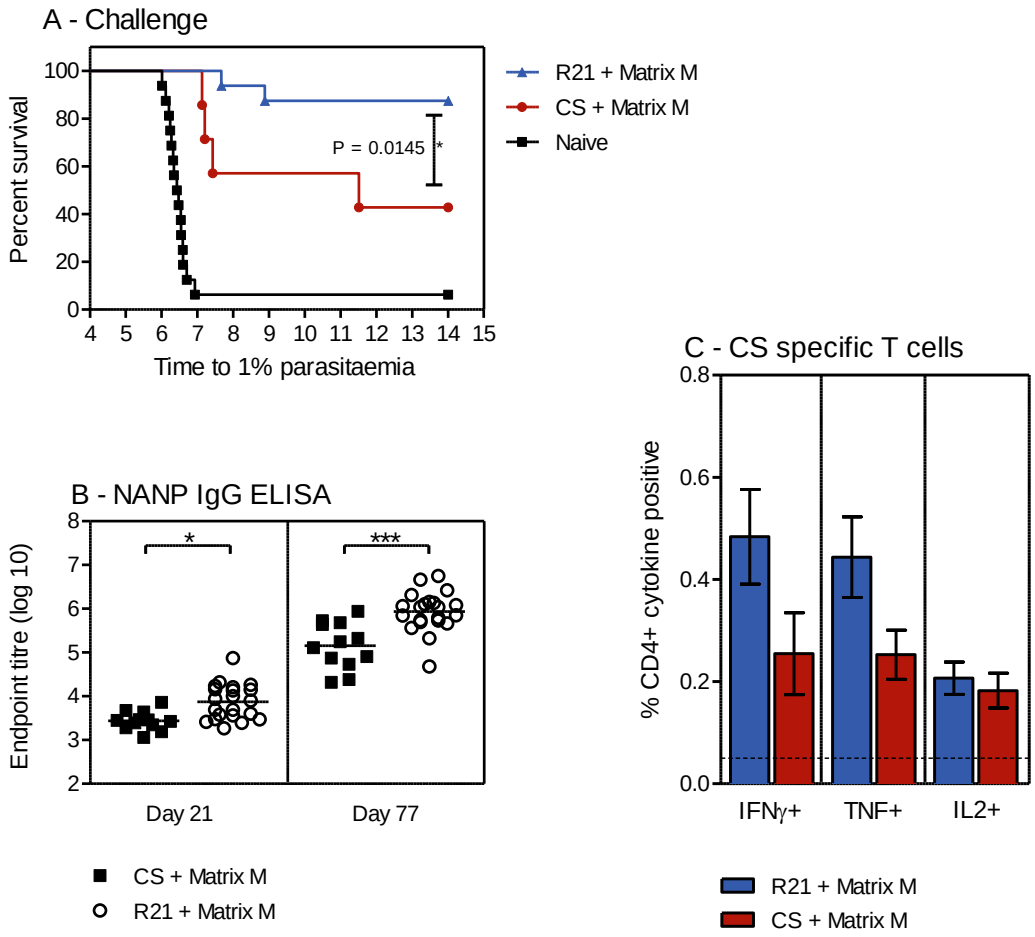


Figure 6.7 Comparative assessment of efficacy elicited by R21 + Matrix M and CS + Matrix M immunisation in a transgenic sporozoite model.

BALB/c mice immunised i.m. with two shots of 0.5µg R21 + Matrix M (n=16) or 0.5µg CS + Matrix M (n=7), eight weeks apart, were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (TG Pb-PfCS) along with naïve mice (n=16). Time to develop 1% parasitaemia was calculated using linear regression, the results presented in a Kaplan-Meier survival graph and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** R21 + Matrix M vs CS + Matrix M (p=0.0145) (efficacy data from two independent experiments). **(B)** NANP-specific IgG was assayed by ELISA three weeks after each vaccination (Day 21 and Day 77). Group mean responses are shown and compared by One-way ANOVA with Bonferroni's multiple comparison test, *p<0.5, ***p<0.001 (ELISA data from three independent experiments). **(C)** Three weeks after the final vaccination CD4+ and CD8+ cytokine secreting T cell frequencies were assessed by ICS in the blood. Cells were stimulated for six hours with a pool of overlapping CS peptides and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD4+ T cells expressing the cytokine. Group means with SEM are shown and compared by Unpaired t test (data from one experiment), dotted line indicates the background response.

6.2.4 NANP-specific IgG protects mice in a dose dependant manner

High levels of CS or NANP-specific antibodies have been shown in a number of studies to protect against malaria. Therefore, it is possible that efficacy elicited by R21 is mediated by the very high levels of NANP-specific IgG induced by vaccination. To determine if efficacy is mediated by antibodies, total IgG was purified from the serum of mice immunised with R21 + Matrix M (2 shots, 8 weeks apart). Three groups of naïve mice received different doses of this purified IgG (150µg, 750µg, 1.5mg) which was passively transferred by i.v. injection 5 hours prior to challenge with transgenic parasites (Tg Pb-PfCS). Efficacy of the passively transferred IgG was then assessed, by delay in time to development of 1% parasitaemia in the blood (Figure 6.8-A-D). A control group also received IgG purified from unvaccinated mice prior to challenge and this conferred no efficacy compared to naïve mice by Log-rank (Mantel-Cox) Test ($p = 0.187$). The IgG from the vaccinated mice however did confer protection and it did so in a dose dependant manner. Only two mice were sterilely protected, one in the 150µg dose group and 1 in the 1.5mg dose group, but all groups were partially protected. The delay in the time to 1% parasitaemia was significant in each group compared to the naïve mice and this increased with the dose of IgG transferred. Time to 1% parasitaemia in days in each group was: naïve = 6.51, 150µg = 6.83, 750µg = 7.64 and 1.5mg = 7.74, and therefore the degree of statistically significant delay also increased with dose: 150µg $p = 0.0264$, 750µg $p = 0.0003$, 1.5mg $p < 0.0001$, as determined by Log-rank (Mantel-Cox) Test compared to the naïve mice. These data suggest that vaccine induced IgG, presumably NANP-specific antibodies, are able to confer some degree of protection alone. NANP-specific IgG titres were measured 1 day after challenge (Figure 6.8-E) and all mice had good titres that increased in accordance with the IgG dose i.e. the titres in the 1.5mg group were approximately 10 fold higher than those

in the 150µg group. These NANP-IgG titres also correlated with the efficacy, measured as time to 1% parasitaemia (Spearman r = 0.5991, p = 0.0032) (Figure 6.8-F). It may therefore be the case that if the dose of IgG had been further increased, efficacy may also have increase and sterile efficacy may have eventually been reached.

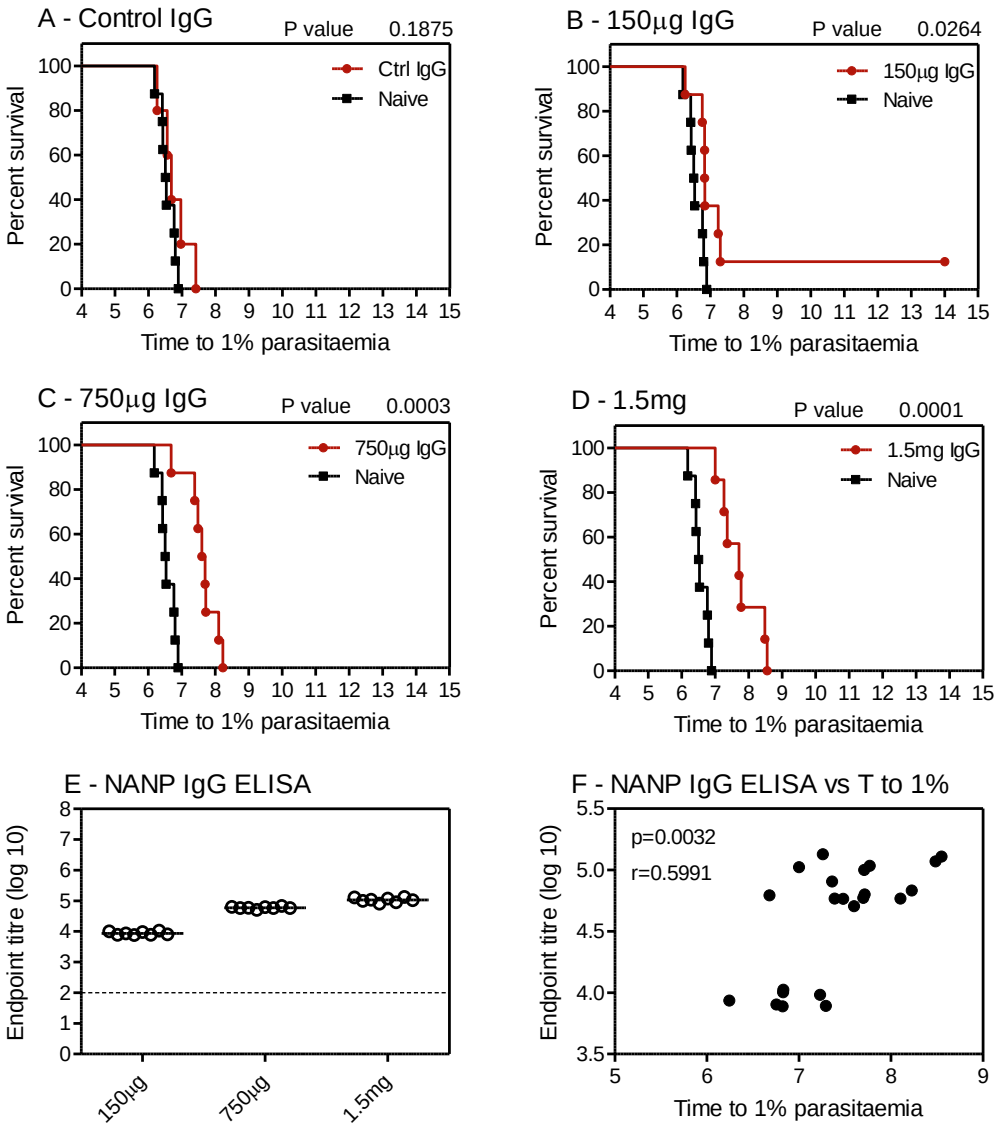


Figure 6.8 Efficacy elicited by passive transfer of IgG from mice immunised with R21 + Matrix M in a transgenic sporozoite model.

IgG was purified from BALB/c mice immunised i.m. with two shots of 0.5µg R21 + Matrix M eight weeks apart. The purified IgG was transferred by i.v. injection into naïve BALB/c mice and five hours later mice were challenged by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with naïve mice (n=8/group). Time to develop 1% parasitaemia was calculated using linear regression, the results presented in a Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(B)** 150µg IgG p=0.0264, **(C)** 750µg IgG p=0.0003 **(D)** 1.5mg IgG p<0.0001. **(A)** Control mice were also challenged 5 hours after receiving IgG purified from unvaccinated mice, no significant difference. **(E)** NANP specific IgG was assayed by ELISA prior to challenge, group mean responses are shown and dotted line indicates the limit of detection. **(F)** NANP IgG correlates with time to 1% parasitaemia, assessed by Spearman's rank correlation (Spearman r=0.5991 p=0.0032).

However, this idea that increasing the dose of IgG further would have resulted in sterile efficacy is not entirely supported by the data. In agreement with this notion, in previous experiments, R21 + Matrix M (and R21 + Abisco-100) immunisation has indeed induced higher titres of NANP-specific IgG than those detected in the mice after IgG transfer (Figure 6.9-B), and this has resulted in sterile efficacy. But in contrast, immunisation with R21 + MF59 (or R21 + AddaVax) also induces similarly high levels of NANP-specific IgG (Figure 6.9-B). So if it is simply the magnitude of NANP-specific IgG responsible for protection then the R21 + MF59 immunised mice would also have also been sterilely protected, but only 12.5% efficacy was achieved. Interestingly the level of efficacy elicited by the highest dose of transferred IgG is equivalent to the efficacy of the R21 + MF59 induced immune response (Figure 6.9-A) ($p = 0.624$), and a similarity between these two partially protective regimens is the absence of a T cell response.

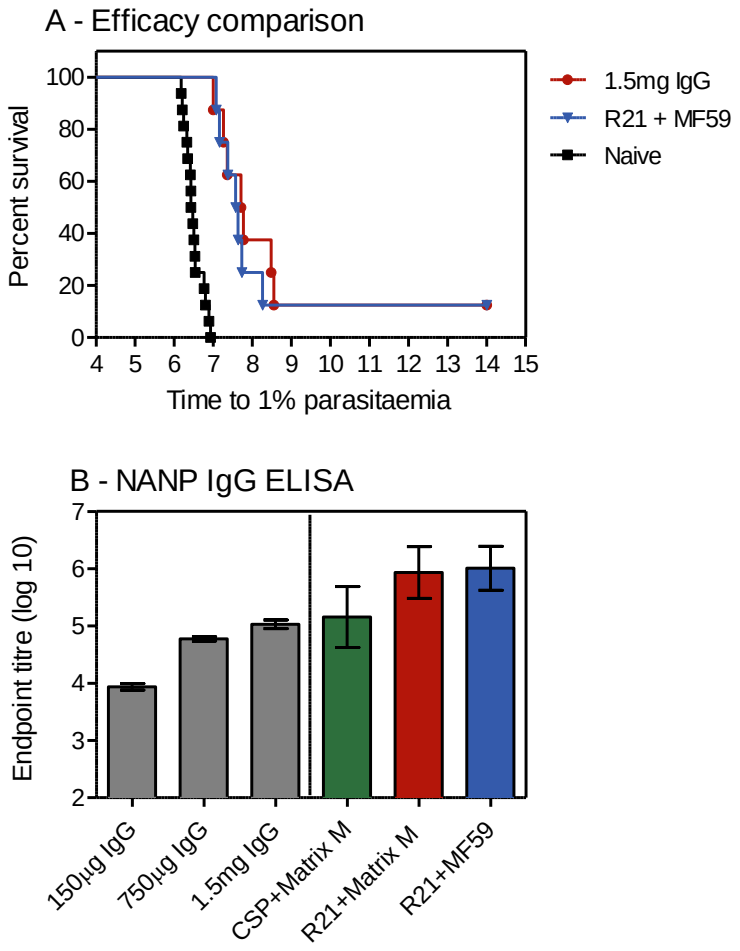


Figure 6.9 Comparison of IgG passive transfer groups with vaccinated mice

Comparison of data from different experiments where mice were either immunised with 0.5µg R21 + MF59, twice eight weeks apart (n=8), or received 1.5mg of IgG purified from BALB/c mice immunised i.m. with two shots of 0.5µg R21 + Matrix M eight weeks apart (n=8). Mice were challenged by i.v. injection with 1000 sporozoites (Tg Pb-PfCS) along with naïve mice (n=8). Time to develop 1% parasitaemia was calculated using linear regression. The results are presented in a Kaplan-Meier survival graph and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** R21 + MF59 vs 1.5mg IgG, no significant difference. **(B)** NANP-specific IgG assayed by ELISA prior to challenge for mice receiving different doses of IgG by passive transfer and mice immunised with different vaccines, group means with SD responses are shown.

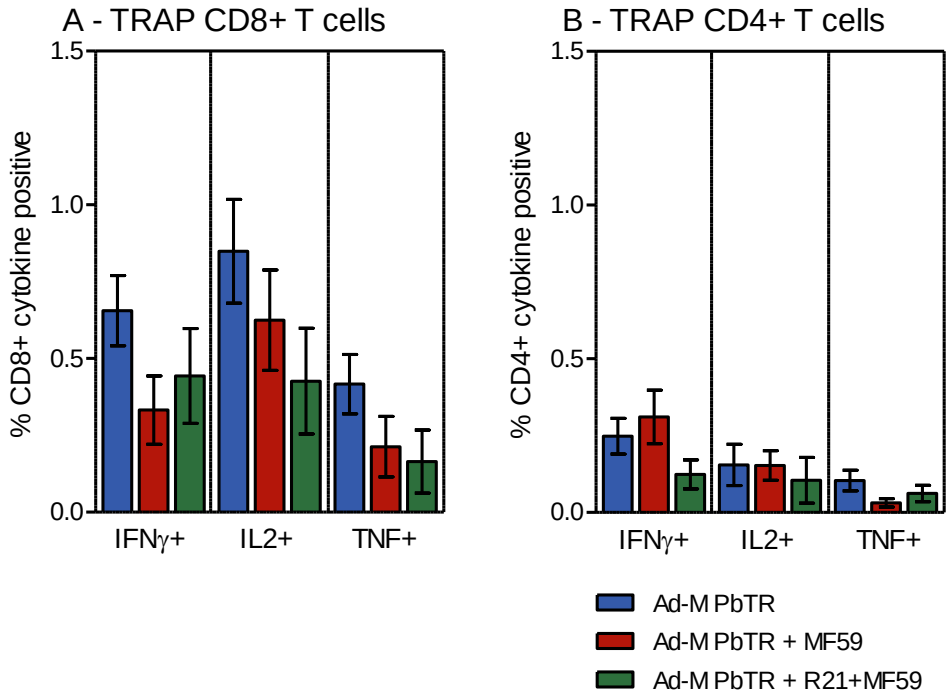
6.2.5 R21 + MF59 efficacy can be enhanced by co-administration with the ChAd63 – MVA PbTRAP prime-boost regimen

The aim of this study was to assess whether combining two different vaccination strategies that target different areas of the pre-erythrocytic stage of malaria results in an increase in protection. In the previous chapter immunological interference of R21 + adjuvant combined with viral vectored vaccines was assessed and no reductions in immune responses induced by either vaccine regimen were observed. These studies were carried out with the ChAd63 – MVA ME.TRAP heterologous prime boost regimen used in clinical trials, that was shown to be partially protective in humans in Chapter 3 [356, 357]. However this vaccine contains Pb9 in the ME string, which is the strong *P. berghei* H-2^{d} restricted epitope from CS that is able to confer sterile protection in BALB/c mice, therefore efficacy measured by these vaccines would reflect CS-specific responses rather than TRAP. So in order to assess the ability of TRAP-specific T cells induced by viral vectors to protect in the transgenic sporozoite challenge in the model used here, viral vectors expressing *P.berghei* TRAP without the ME string were used. Therefore the TRAP-specific immune responses induced by viral vectors can target the native *P. berghei* parasite and CS-specific immune responses induced by R21 can target the copy of *P. falciparum* CS that has been added to the transgenic *P. berghei* parasite.

In order to determine if combining the vaccine strategies results in increased efficacy the suboptimal R21 + adjuvant regimen, R21 + MF59, was selected for assessment with ChAd63 – MVA PbTRAP. MF59 was chosen for use with R21 because this formulation did not elicit 100% sterile efficacy so it would therefore be possible to detect an increase in efficacy in the combination regimen.

6.2.5.1 ChAd63 – MVA PbTRAP prime-boost regimen induces TRAP-specific T cells and antibodies and can be combined with R21 + MF59 without immunological interference

Induction of PbTRAP specific immune responses by ChAd63 – MVA PbTRAP was unaffected by the addition of MF59 alone or R21 + MF59. BALB/c mice were immunised with ChAd63 – MVA PbTRAP in an 8 week prime-boost regimen either alone, combined with MF59, or combined with 0.5µg R21 + MF59. PbTRAP specific T cell frequencies were measured in the blood by ICS 1 week after the boost and both CD4+ and CD8+ T cells were detected. Frequencies of CD8+ T cells were higher than CD4+ T cells, and there were no differences between the three groups in the frequencies of any T cell subset measured (IFNg+, TNF+ and IL2+) (compared by One-way ANOVA with Bonferroni's multiple comparison test) (Figure 6.10-A+B). PbTRAP-specific IgG was also measured 3 weeks after boost and there was no reduction or enhancement of the responses by the addition of MF59 of R21 + MF59 (Figure 6.10-C). Induction of NANP-specific IgG by R21 + MF59 was also unaffected by the addition of the ChAd63 – MVA PbTRAP. Immunised mice induced high levels of NANP-specific IgG and the responses were neither reduced nor enhanced by co-administration with viral vectors (compared by One-way ANOVA with Bonferroni's multiple comparison test) (Figure 6.10-D).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1.0
0.5
0.0
IFNγ+
IL2+
TNF+
B - TRAP CD4+ T cells
% CD4+ cytokine positive
1.5
1.0
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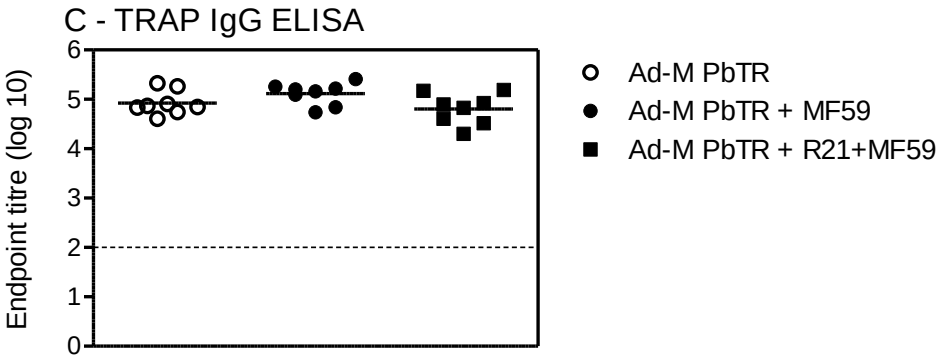
IFNγ+
IL2+
TNF+
Ad-M PbTR
Ad-M PbTR + MF59
Ad-M PbTR + R21+MF59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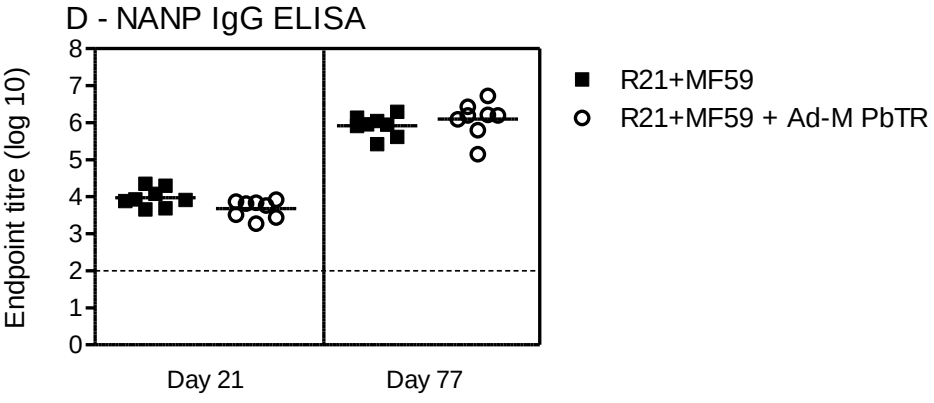
5
4
3
2
1
0
Ad-M PbTR
Ad-M PbTR + MF59
Ad-M PbTR + R21+MF59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7
6
5
4
3
2
1
0
Day 21
Day 77
R21+MF59
R21+MF59 + Ad-M PbTR

Figure 6.10 No interference with induction of T cells and IgG in combination regimens

BALB/c mice were immunised i.m. with ChAd63 – MVA PbTRAP 8 week prime-boost regimen, either alone (Ad-M PbTR) combined with MF59 (Ad-M PbTR+MF59) or combined with 0.5µg R21 + MF59 (Ad-M PbTR+R21+MF59). Blood was taken one week after the final vaccination to assess CD4+ and CD8+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with a pool of overlapping PbTRAP peptides and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD8+ **(A)** or CD4+ **(B)** T cells expressing the cytokine. Blood was also taken three weeks after the final vaccination for immunology and PbTRAP-specific IgG was assayed by ELISA **(C)**. BALB/c mice immunised i.m. with the 0.5µg R21 + MF59 either alone or combined with the ChAd63 – MVA PbTRAP and NANP-specific IgG measured after prime and boost **(B).** Group means with SEM are shown and the dotted line indicates limit of detection. Groups compared by One-way ANOVA with Bonferroni's multiple comparison test.

6.2.5.2 ChAd63 – MVA PbTRAP prime-boost regimen elicits partial efficacy

Immunisation with the ChAd63 – MVA PbTRAP prime-boost regimen induces TRAP-specific immune responses that are able to significantly delay the time to development of 1% parasitaemia in the blood in the transgenic challenge model. The viral vector regimen administered alone sterilely protected 37.5% of the challenged mice (3/8) and significantly delayed the time to 1% parasitaemia in the rest ($p = 0.0003$, by Log-rank (Mantel-Cox) Test) (Figure 6.11-A). The viral vector regimen combined with MF59 however, did not sterilely protect any mice (0/8) but again, there was a significant delay to 1% parasitaemia ($p = 0.0042$) (Figure 6.11-B). Addition of MF59 slightly reduced the efficacy of the ChAd63 – MVA PbTRAP prime-boost regimen because it did not sterilely protect any mice, but when these survival curves were compared together they were not significantly different ($p = 0.065$) (Figure 6.11-C). So overall the combined efficacy of ChAd63 – MVA PbTRAP +/- MF59 is 18.75% (3/16) with a median time to 1% parasitaemia of 7.04 days compared to 6.38 days for naïves and this is significant compared by Log-rank (Mantel-Cox) Test $p < 0.0001$ (Figure 6.11-D).

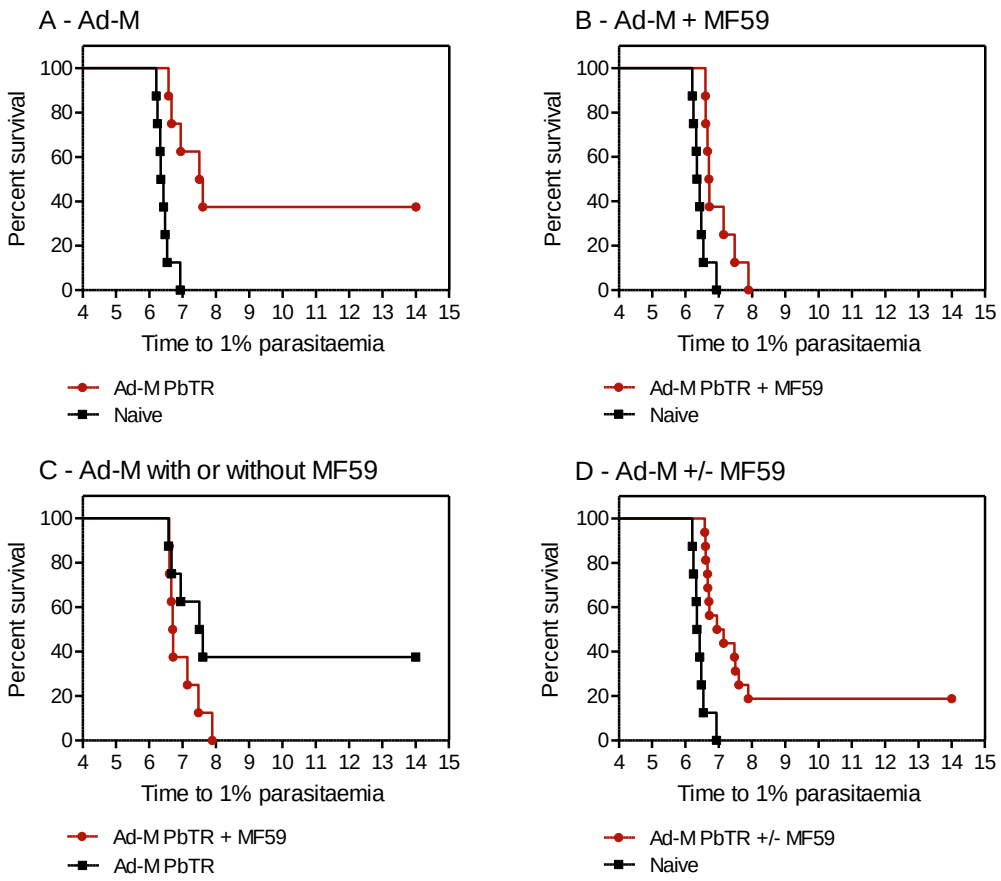


Figure 6.11 ChAd63 – MVA PbTRAP is partially protective in a transgenic parasite model

BALB/c mice were immunised i.m. with the ChAd63 – MVA PbTRAP 8 week prime boost regimen, either alone (Ad-M PbTR) or combined with MF59 (Ad-M PbTR+MF59). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** Ad-M PbTR p=0.0003, **(B)** Ad-M PbTR+MF59 p=0.0042, **(C)** Ad-M PbTR vs Ad-M PbTR+MF59 = no significant difference (p = 0.0655), **(D)** Ad-M PbTR+/-MF59 p<0.0001.

6.2.5.3 Efficacy is enhanced by the combination regimen

Both R21 + MF59 and the ChAd63 – MVA PbTRAP prime boost regimen elicit significant delay in the development of blood stage parasitaemia when administered alone (Figure 6.12-A+B). When the two vaccination strategies are mixed and co-administered together this significantly increases the efficacy above the level elicited by each vaccination strategy alone. The combination sterilely protects 62.5% of the challenged mice (5/8) and delays time to 1% parasitaemia in the rest, compared by Log-rank (Mantel-Cox) Test to R21 + MF59 which sterilely protects 12.5%, $p = 0.0112$ (Figure 6.12-C). The combination compared to ChAd63 – MVA PbTRAP +/- MF59 which sterilely protects 37.5% is also significantly more efficacious ($p = 0.013$) (Figure 6.12-D). Therefore, R21 + MF59 can be successfully combined with a viral vector regimen that targets a different area of the pre-erythrocytic stage of malaria without immunological interference and results in a statistically significant increase in efficacy.

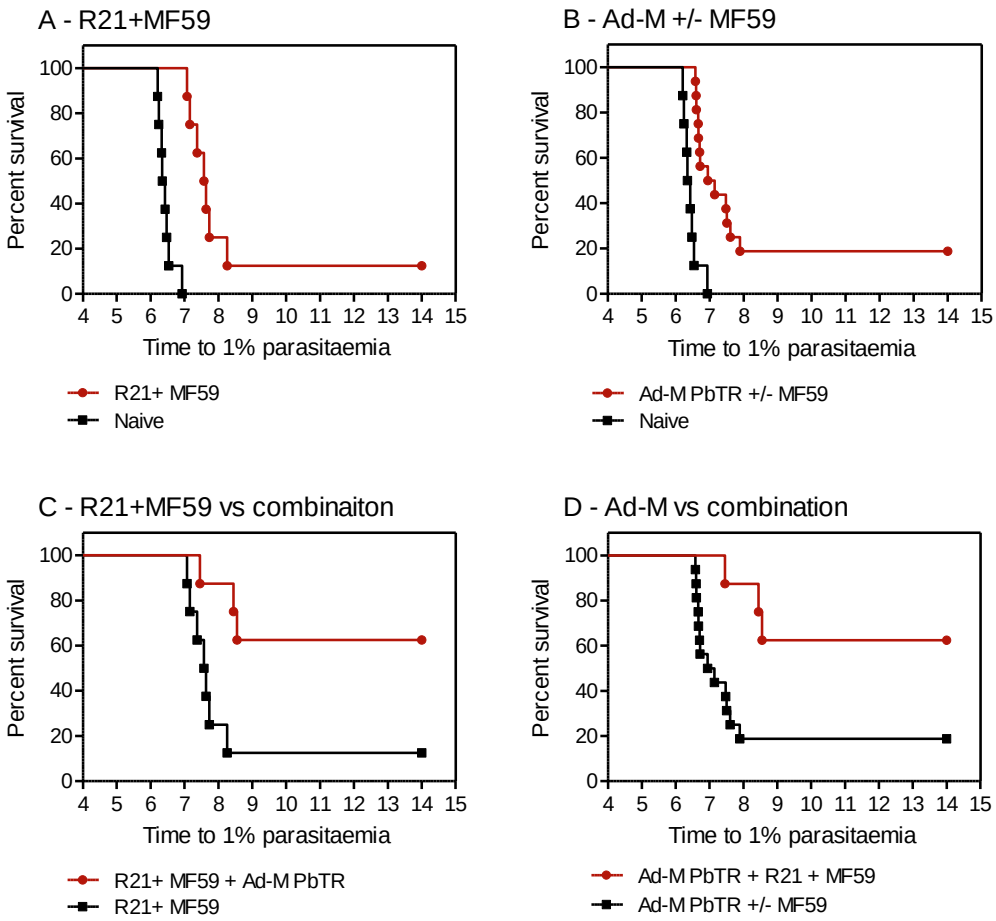


Figure 6.12 Efficacy is enhanced by combination regimen

BALB/c mice were immunised i.m. with the ChAd63 – MVA PbTRAP 8 week prime-boost regimen, both alone and combined with MF59 (n=16) (Ad-M PbTR+/-MF59). Or 0.5µg R21 + MF59 alone (n=8) or combined with ChAd63 – MVA PbTRAP (n=8). Mice were challenged three weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** R21+MF59 $p<0.0001$, **(B)** Ad-M PbTR+/-MF59 $p<0.0001$ **(C)** R21+MF59 vs R12+MF59 + Ad-M PbTR $p=0.0112$, **(D)** Ad-M PbTR + R21+MF59 vs Ad-M PbTR+/-MF59 $p=0.0134$.

6.2.6 RTS,S/AS01B efficacy can be enhanced by co-administration with ChAd63 – MVA ME.TRAP prime-boost regimen

The previous section indicated that combining vaccine strategies together, that target different parts of the pre-erythrocytic stage of infection, can result in enhanced efficacy in the transgenic sporozoite model used here. To further evaluate this finding, two of the leading candidate malaria vaccine regimens in development, ChAd63 – MVA ME.TRAP prime-boost (assessed in humans in Chapter 3) and RTS,S/AS01B [336] were assessed in combination in the transgenic sporozoite model. This study was to primarily assess if combining the two vaccine regimens resulted in any immunological interference and in addition mice were also challenged to assess any negative impact of the viral vectors on the efficacy of RTS,S/AS01B. However, this challenge model is not able to assess the efficacy of the TRAP induced immune response because the viral vector vaccines contain the Pb9 epitope, which is the immunodominant CS epitope that can confer protection in BALB/c mice [237]. So although efficacy of the viral vector regimen is evaluated in this study, it is assessing primarily the protection elicited by the CS epitope present in the vaccines and not TRAP. Importantly though, this model will tell us if there is a potential for immunological interference that results in a reduction in the efficacy elicited by RTS,S/AS01B, and this will help inform which regimens are used when combining these vaccines in Phase1/2a clinical trials in the near future.

6.2.6.1 RTS,S induced antibodies and T cells are unaffected by co-administration or mixing with ChAd63 – MVA ME.TRAP

To assess the effect of viral vectors on RTS,S/AS01B induced immune responses, BALB/c mice were immunised with RTS,S/AS01B alone or combined with the viral vector vaccines in a number of different regimens detailed in Table 6.1. The vaccination regimens chosen were to assess immunological interference when the two vaccines are mixed and administered together, which would be the most deployable regimen. So the key comparison is the RTS,S vaccinated mice (group 1. R-R-R) compared to the mixture group (group 8. AR-MR-MR MIX). In order the determine if any interference observed in this mixture group could be overcome by co-administration at separate sites, groups 3,5 and 6 were included and to determine if interference could be avoided by staggered immunisation group 7 was included.

Table 6.1 RTS,S AS01 with viral vectors co-administration study design

Vaccination regimens used in the co-administration study. **R** = 5µg RTS,S/AS01B, **A** = 1 x 10^8 ifu ChAd63 ME.TRAP, **M** = 1 x 10^6 pfu MVA ME.TRAP. In groups 3, 4, 5 and 6 when two vaccines are given at the same time, viral vectors are administered into one limb and RTS,S/AS01B is administered into the other. In group 8, viral vectors and RTS,S/AS01B are mixed prior to administration.

Gp \ Day	0	7	14	28	42	56
1.R-R-R			R	R	R	Transgenic sporozoite challenge (*P.berghei* + *P.falciparum* CS)
2.A-M			A		M	
3.AR-R-MR			A + R	R	M + R	
4.A-M-M			A	M	M	
5.AR-MR-MR			A + R	M + R	M + R	
6.AMR-AMR-AMR			A + M + R	A + M + R	A + M + R	
7.R-A-R-R-M	R	A	R	R	M	
8.AR-MR-MR MIX			A + R	M + R	M + R	
9.Naïve						

All group receiving RTS,S/AS01B developed high titres of NANP-specific IgG and these were measured two weeks after the final immunisation, one day before challenge (Figure 6.13-A). Interestingly, when comparing each combination group to the RTS,S alone group two groups developed significantly higher antibody titres (One-way ANOVA with Dunnetts's multiple comparison post-test comparing all groups to R-R-R). These two groups were the AMR-AMR-AMR co-administration group which received the viral vectors mixed and injected into one limb and the RTS,S/AS01B into the other limb ($p < 0.01$), and the AR-MR-MR mixture group, which received the viral vector and the RTS,S/AS01B mixed and injected into the same limb ($p < 0.001$). CS-specific T cells responses were also measured one week after the final vaccination and there was no significant difference between the RTS,S/AS01B alone group and the co-administration or mixture groups in frequencies of any CD4+ subsets measured (groups compared by One-way ANOVA with Dunnetts's multiple comparison post-test comparing all groups to R-R-R) (Figure 6.13-B). Therefore RTS,S/AS01B induced immune responses are not negatively affected by combination with the viral vectors in any regimen and interestingly the NANP-specific IgG induction is enhanced in two of the combination groups.

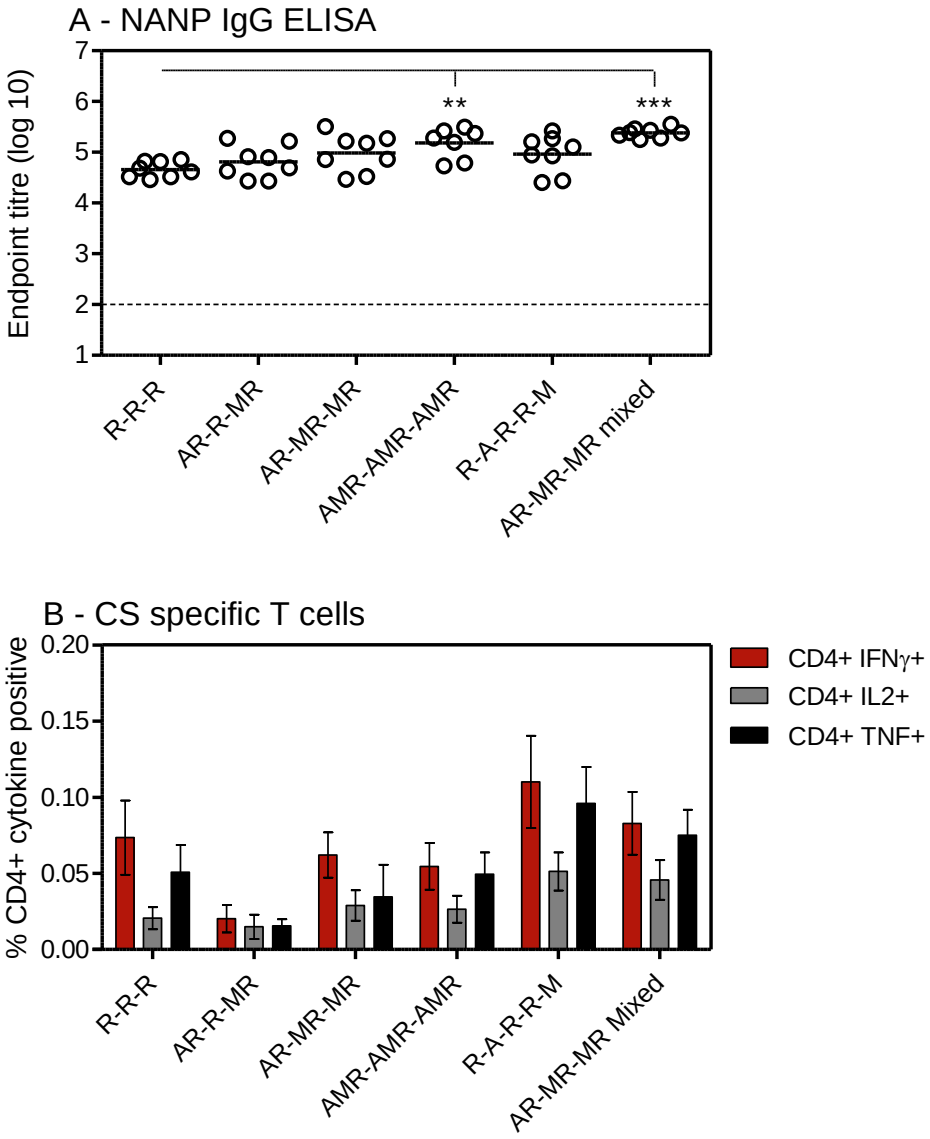


Figure 6.13 Assessment of interference with induction of CS-specific responses in combination regimens

BALB/c mice were immunised i.m. with RTS,S/AS01B alone or combined with the ChAd63 – MVA ME.TRAP, in various regimens detailed in Table 6.1. Blood was taken two weeks after the final vaccination and TRAP-specific IgG was assayed by ELISA **(A)** Blood was also taken one week after the final vaccination to assess CD4+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with a pool of CS peptides. Results are expressed as the percentage of CD4+ T cells secreting each cytokine **(B)**. Group means with SEM are shown and the dotted line indicates limit of detection. Groups compared by One-way ANOVA with Dunnetts's multiple comparison post-test comparing all groups to R-R-R **p < 0.01 and ***p < 0.001.

6.2.6.2 Viral vector induced T cells are unaffected by co-administration or mixing with RTS,S/AS01B

All mice receiving the viral vectors developed high frequencies of Pb9-specific CD8+ T cells. T cell responses were measured one week after the final vaccination by ICS and flow cytometry after stimulation for 6 hours with the dominant Pb9 epitope (Figure 6.14-A). There are differences between the groups in the frequencies of T cells detected, and the same trend is seen for each cytokine positive population. When analysing this data only certain groups should be compared. Firstly there is no difference between the T cell responses induced by vaccination with viral vectors alone in the standard prime-boost regimen (A-M) and the responses induced when this regimen is co-administered with RTS,S/AS01B (AR-R-MR). There is also no difference between the responses when the viral vector regimen includes an additional MVA vaccination (A-M-M) and the corresponding RTS,S/AS01B co-administration group (AR-MR-MR). There is however, a significantly greater response when viral vectors and particle vaccine in the AR-MR-MR regimen are mixed and administered together compared to co-administration at separate sites (groups compared by One-way ANOVA with Bonferroni's multiple comparison post-test comparing selected pairs of groups *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Staggered immunisation gives the highest T cell responses, but this is likely due to the increase in time interval between the ChAd63 and MVA vaccinations, which has been shown by studies in our group to improve responses. Finally, administering both viral vectors together in the co-administration regimen compared to single vector (AR-MR-MR vs AMR-AMR-AMR) does not affect the induction of CD8+ T cells to the Pb9 epitope. The mixed vector co-administration regimen is interestingly the only regimen though that appears to affect the induction of TRAP-specific IgG, with a reduction observed. With all other co-administration regimens there is no significant reduction or enhancement in the TRAP antibody response compared to the viral vector alone groups (Figure 6.14).

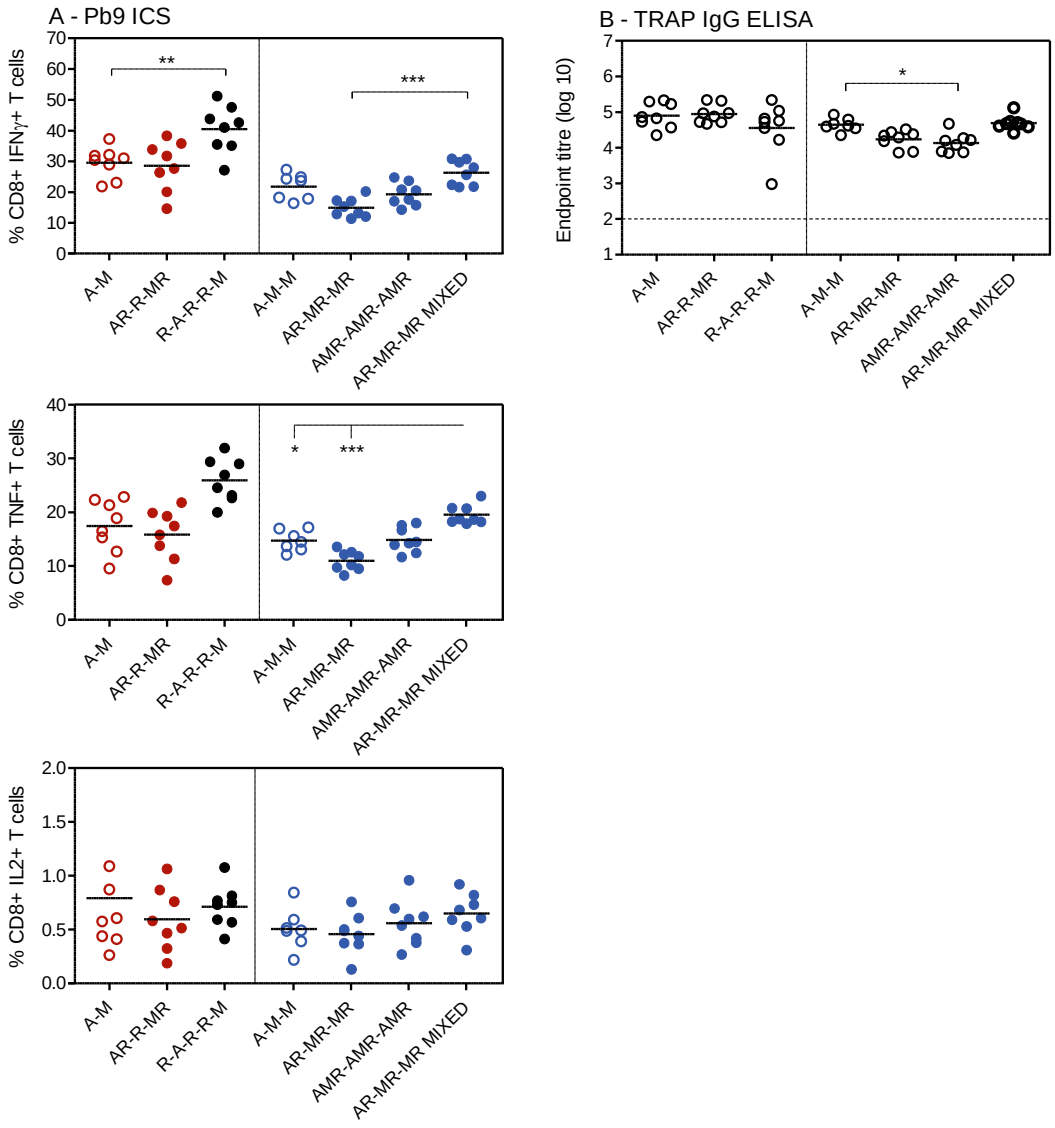
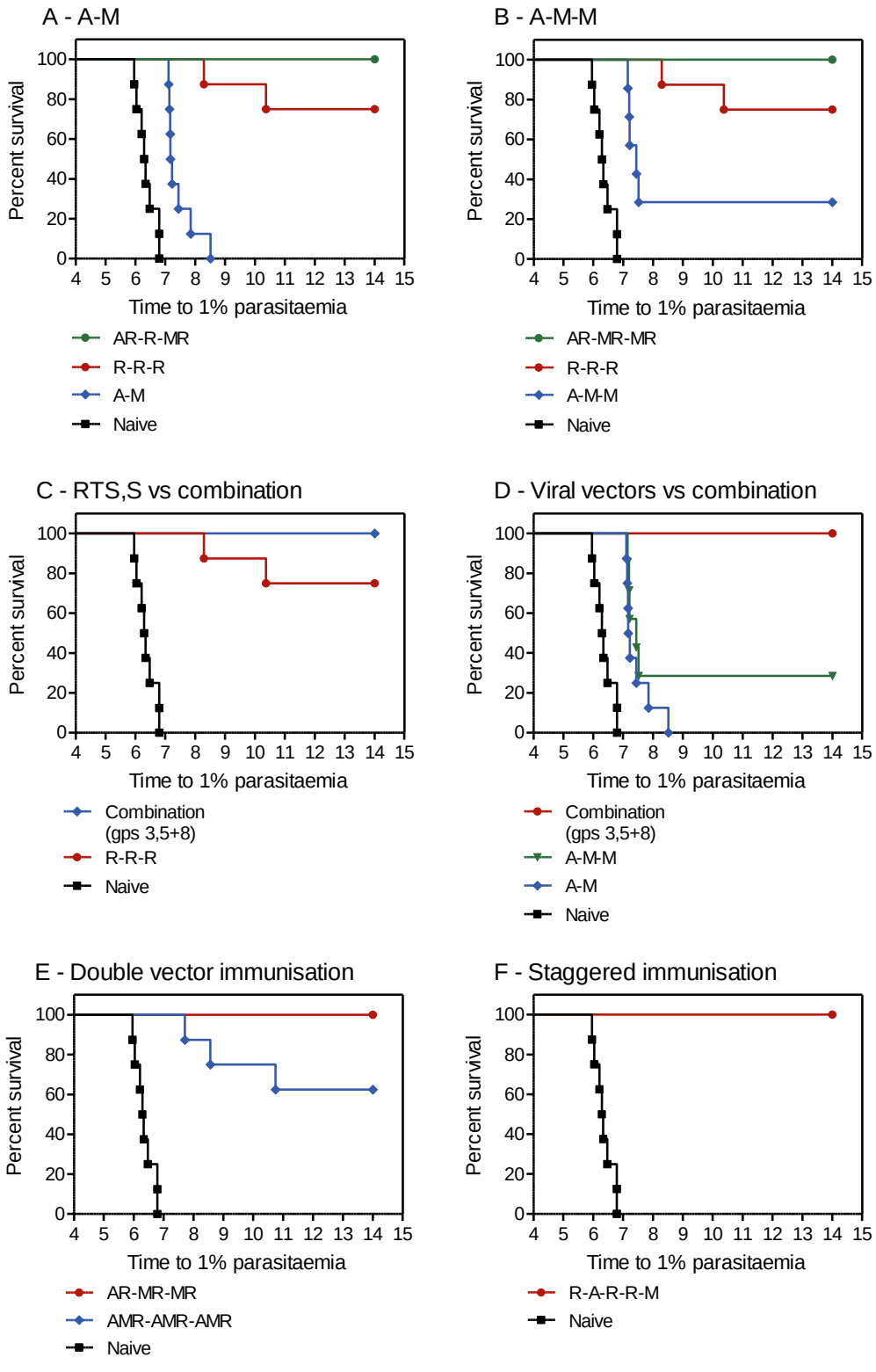


Figure 6.14 Assessment of interference with induction of responses to the viral vector insert in combination regimens

BALB/c mice were immunised i.m. with ChAd63 - MVA ME.TRAP prime-boost regimen, alone or combined with RTS,S/AS01B in various regimens detailed in Table 6.1. Blood was taken one week after the final vaccination to assess CD8+ cytokine secreting T cell frequencies by ICS. Cells were stimulated for six hours with the Pb9 peptide and three different cytokines were assessed (IFNγ, TNF and IL2). Results are expressed as the percentage of CD8+ T cells secreting each cytokine **(A)** Blood was also taken two weeks after the final vaccination and TRAP-specific IgG was assayed by ELISA **(B)**. Group means are shown and dotted line indicates limit of detection. Groups compared by One-way ANOVA with Bonferroni's multiple comparison post-test comparing selected pairs of groups *p < 0.05, **p < 0.01 and ***p < 0.001.

6.2.6.3 Efficacy is enhanced by combining RTS,S/AS01B and viral vectors in a number of immunisation regimens

All vaccinated groups result in a significant delay to 1% parasitaemia when compared to the naïve mice by Log-rank (Mantel-Cox) test (Table 6.2 and Figure 6.15). The viral vector prime boost regimens A-M and A-M-M were able to significantly delay development of blood stage parasitaemia and the A-M-M also sterilely protected 28.6% of challenged mice (2/7) ($p < 0.001$ for both comparisons). The RTS,S/AS01B regimen sterilely protected 75% of the mice (6/8) and co-administration of RTS,S/AS01B and viral vectors increased efficacy above single vaccine regimen alone (Table 6.2 and Figure 6.15). When combing the vaccine regimens there is no difference in efficacy between staggered immunisation and co-administration at separate sites (gp7 vs gp5). There is also no difference in efficacy between co-administration at separate sites and the vaccines mixed and administered at the same site (gp5 vs gp8). The efficacy is slightly reduced with double vector immunisation is used in the co-administration regimen compared to single vector immunisation, though this is not significant. Finally, as seen with the immunogenicity data in the previous section, there is no interference in the induction of a protective immune response when RTS,S/AS01B and viral vectors are mixed and co-administered together.



Comparison	Log-rank (Mantel-Cox) Test
R-R-R vs combination (gp3,5+8)	P = 0.0113
A-M vs combination (gp3,5+8)	P < 0.0001
A-M-M vs combination (gp3,5+8)	P < 0.0001

Figure 6.15 RTS,S/AS01B and ChAd63 – MVA PbTRAP is partially protective in a transgenic parasite model and efficacy is enhanced by combination.

BALB/c mice were immunised i.m. with ChAd63 – MVA ME.TRAP 8 week prime-boost regimen, alone or combined with RTS,S/AS01B in various regimens detailed in Table 6.1. Mice were challenged two weeks after the final vaccination by i.v. injection of 1000 sporozoites (Tg Pb-PfCS) along with eight naïve mice. Blood stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression. The results are presented in the Kaplan-Meier survival graphs and survival curves were compared by Log-rank (Mantel-Cox) Test. **(A)** A-M alone or combined with RTS,S/AS01B **(B)** A-M-M alone or combined with RTS,S/AS01B **(C)** RTS,S vs combination ($p = 0.0113$), **(D)** Viral vectors vs combination ($p < 0.0001$), **(E)** Double vector vs single vector immunisation, **(F)** staggered immunisation. Table shows the comparison of single vs combination regimens compared by Log-rank (Mantel-Cox) Test.

Table 6.2 Efficacy of each immunisation regimen compared to naïve mice

Mice were considered sterilely protected if they were slide negative at day 14 after challenge and partial vaccine efficacy was assessed as a significant delay in the development of 1% parasitaemia in the blood compared to control mice determined by comparison of Kaplan-Meier survival curves by Log-rank (Mantel-Cox) Test.

Group	no. protected/ no. challenged	% sterile efficacy	T to 1% (median)	P value (compared to naïve)
1.R-R-R	6/8	75%	14	< 0.0001
2.A-M	0/8	0%	7.19	< 0.0001
3.AR-R-MR	8/8	100%	14	< 0.0001
4.A-M-M	2/7	28.6%	7.44	< 0.0001
5.AR-MR-MR	8/8	100%	14	< 0.0001
6.AMR-AMR-AMR	5/8	62.5%	14	< 0.0001
7.R-A-R-R-M	8/8	100%	14	< 0.0001
8.AR-MR-MR MIX	8/8	100%	14	< 0.0001
9.Naïve	0/8	0%	6.31	

6.3 Discussion

Studies described in the previous chapter showed R21 + adjuvant to be highly immunogenic when used alone or when combined with the ChAd63 – MVA ME.TRAP regimen. The most potent adjuvants for enhancing both antibody and T cell induction by R21 were found to be the saponin-based ISCOM adjuvants. Equally effective at enhancing the induction of high antibody titres, but less so at enhancing T cell induction were the squalene-based o/w emulsions. The aim of this chapter was to evaluate the ability of the R21 induced immune response to protect against sporozoite challenge in a pre-clinical model. R21 administered with Abisco-100 or Matrix M was shown induce almost complete sterile protection (100% and 87.5, respectively). In contrast, R21 + AddaVax or MF59 induced a significant delay in the time to development of 1% parasitaemia, yet administration with AddaVax did not sterilely protect any mice and administration with MF59 only sterilely protected 1 out of 8 challenged mice. Although both types of adjuvants formulated with R21 elicit significant protection, when the survival curves were compared, immunisation with R21 + Abisco/MatrixM was significantly more protective than immunisation with R21 + AddaVax/MF59 ($p < 0.0001$). R21 administered with both types of adjuvants however induced similar levels of NANP-specific IgG detected after prime and prior to challenge and this suggest that the magnitude of the CS-specific antibody response does not mediate efficacy alone. Potentially, the type/quality of the IgG response may be important, or alternatively other components of the immune system such as T cells may contribute to the protective immune response. Further assessment of the IgG isotypes induced by these two different adjuvants and assessment of the avidity of the IgG, may identify important differences between the antibody responses [178, 502, 503]. Additionally, although the magnitude of the CS-specific IgG responses were very similar in these two groups, analysis of CS-specific T cells responses identified the more protective R21 + Abisco/Matrix M as being

more effective at inducing cellular responses than R21 + AddaVax/MF59. This reflects results seen in humans immunised with RTS,S where efficacy was significantly enhanced when RTS,S was administered in the AS01 adjuvant compared to the AS02 adjuvant [300]. These two adjuvant formulations are very similar and both contain MPL and QS21, but AS01 is a liposome formulation whereas AS02 is an emulsion. Studies have also shown that RTS,S/AS01B as well as being more protective, also induces greater cellular responses to CS [469]. Nevertheless, the results in this chapter simply identify an association with the frequencies of antigen specific CD4+ T cells and protective efficacy, which only indicates that CD4+ T cell may have a role in protection. To further assess the contribution of CD4+ T cells in sterile protection mechanistic studies would need to be performed. T cell depletion studies could be carried out in mice vaccinated with R21 + Matrix M, where monoclonal antibodies could be used to deplete T cell subsets prior to sporozoite challenge and the impact on efficacy assessed.

The protection elicited by immunisation with R21 + Matrix M was also found to be relatively durable; when assessed at 7 and 14 weeks after vaccination, sterile protection remained high at 75% and 50%, respectively. Moreover, it was found that the vaccine induced efficacy could be maintained at 100% 14 weeks post vaccination if mice received sporozoite challenge once during the 14 week interval (3 weeks post vaccination). The ability to enhance the durability of the vaccine induced protection with exposure to sporozoites post vaccination is encouraging, though studies have previously shown that live sporozoite inoculation under drug cover can protect mice. These studies however have required inoculation of very large doses of sporozoites [316, 504]. In a study in BALB/c mice, 20,000 sporozoites were required to achieve >90% protection and immunisation with a single dose of 4000 sporozoites did not protect any mice [317]. So in this study with R21, it is unlikely that exposure of BALB/c mice to 1000 sporozoites during challenge conferred protection on its

own. Nonetheless, the contribution of vaccination to the sterile efficacy needs to be confirmed with repeat experiments which should also use additional control groups. These could include unvaccinated mice which would be challenged at the same time as the vaccinated group and then drug cleared before blood stage infection develops, and then re-challenged at 14 weeks with the vaccinated mice. This would confirm if protection was simply elicited by sporozoite infection. Nevertheless, the ability to boost efficacy induced by R21 when it wanes, by exposure to sporozoites or natural infection, could be useful in the field. Interesting, there is no increase in the level of NANP-specific IgG in the mice that received an extra challenge, so it does not appear that exposure to sporozoite challenge after vaccination boosts the CS antibody response and it is possible that improved protection is mediated by immune responses generated towards other pre-erythrocytic stage antigens.

This study has also shown that CS antigen is more immunogenic and protective when administered in the R21 particle compared to non-particulate CS protein. This is irrespective of the use of potent adjuvants. CS + Matrix M sterilely protected 42.5% of mice, compared to 87.5% protected by R21 + Matrix M and this could be due to significantly lower levels of CS-specific antibodies and lower frequencies of CS-specific T cells detected prior to challenge. To evaluate the contribution of R21 induced antibodies to the protective mechanism, IgG transfer experiments were performed. Transfer of IgG purified from mice vaccinated with R21 + Matrix M was able to protect naïve mice in a dose dependant manner. However, transfer of the highest dose, was only able to confer partial protection. The level of NANP-specific IgG measured in the mice on the day after challenge increased in accordance with the dose transferred and also correlated strongly with protective efficacy (Spearman $r = 0.5991$, $p = 0.0032$). This suggests that it may be possible to achieve sterile efficacy by transferring a greater dose of IgG prior to challenge. However, having higher titres of NANP-specific IgG only resulted in sterile protection in mice

vaccinated with R21 + Matrix M and not for mice vaccinated with R21 + MF59, despite having the same level of IgG. Interestingly, R21 + MF59 immunised mice develop significantly lower frequencies (equivalent to background response) of CS-specific T cells than R21 + Matrix M immunised mice and CS-specific T cells are also absent from the mice which received the transferred IgG. If the survival curves are compared for the mice in these two groups that had no CS-specific T cells (R21 + MF59 and 1.5mg IgG transfer) they are not significantly different ($p = 0.624$), by Log-rank (Mantel-Cox) Test) (Figure 6.9-A). This could suggest that NANP-specific IgG is only able to confer partial efficacy in this model, and in order to reach sterile efficacy a T cell component is required. Alternatively, it could simply imply that the level of efficacy is the same, but the mechanism of protection is different. Consistent with the hypothesis that CD4+ T cells contribute to protection, NANP-specific IgG titres are almost identical in mice immunised with CS + Matrix M and mice receiving passive transfer of 1.5mg IgG (Figure 6.9-B). But again, the CD4+ T cell frequencies are higher in the CSP + Matrix M group. They are also higher than those in R21 + MF59 group and accordingly the level of sterile efficacy is higher in CS + Matrix M immunised mice than both groups that lack CD4+T cells. The relative levels of immune response for each group have been graded and detailed in Table 6.3, and a trend is evident where efficacy appears to increase with the magnitude of the CD4+ T cell response. Consistent with the R21 data, CD4+ T cells have been implicated in protection elicited by RTS,S/AS01 immunisation [300, 421, 470-474, 505, 506], and a mathematical model which was used to assess the contribution of immune components to protection against sporozoite challenge in naïve adults, showed that a certain level of efficacy is achieved by the vaccine induced CS-specific antibodies and this can be enhanced by vaccine induced CD4+ T cells [469]. However for R21 immunisation further mechanistic studies are needed to assess the contribution of antibodies and T cells to protection. Furthermore, since the types of immune responses induced by R21 immunisation with different adjuvants are clearly different,

evidenced so far by the differential induction of T cells, there may be other differences such as the isotype and function of the IgG induced, and therefore protection could be conferred by different mechanisms.

Table 6.3 Comparison of immunogenicity and efficacy of CS-based vaccination or passive transfer regimens tested in the transgenic sporozoite challenge model.

CS-specific antibodies are scored based on the relative magnitude of the NANP-specific IgG response measured by ELISA prior to challenge. CS-specific T cells are scored based on relative frequencies of CD4+ cytokine secreting T cells measured by ICS prior to challenge. Sterile efficacy is the percentage of mice that do not develop blood stage parasitaemia by day 14 after challenge and partial efficacy is the median time to 1% parasitaemia per group (T to 1%).

Vaccine	CS specific antibodies	CS specific CD4+ T cells	% sterile efficacy	T to 1%
R21 + Matrix M	+++	+++	87.5%	14
CSP + Matrix M	++	++	42.5%	11.51
R21 + MF59	+++		12.5%	7.6
1.5mg IgG Transfer	++		12.5%	7.73

Finally, this study showed that efficacy induced by immunisation with R21 can be enhanced by co-administration with TRAP based viral vector vaccines. No interference in the induction of immune responses to either vaccine was observed and efficacy to individual vaccines was 12.5% and 37.5% for the R21 and viral vectors, respectively, and increased to 62.5% in the combination regimen. A similar result was also seen when RTS,S/AS01B was combined with ChAd63 – MVA ME.TRAP. Although assessing efficacy was not a primary endpoint for this study, it did show that the viral vectors do not interfere the induction of immune responses or with the level of protection elicited by RTS,S/AS01B. These results are similar to a

previously published study which was introduced at the beginning of this chapter, where a Hepatitis B core particle vaccine was combined with a viral vector regimen. No interference in immunogenicity was seen and efficacy was synergistically enhanced in the combination group [464]. This supports the hypothesis that targeting both the sporozoites and the liver stage parasite with both cellular and humoral responses, utilizing two different antigens is able to overcome the ‘leaky’ effect of a sporozoite vaccine. These combination vaccine regimens should therefore be assessed in humans as a priority as an efficient method for improving vaccine efficacy with products that have already be proven to be safe and partially effective in clinical trials.

7 Concluding remarks

7.1 Overview

Malaria subunit vaccine development has focused predominately on the generation of vaccines that induce either humoral or cellular responses, and although some have demonstrated partial efficacy there is still no licensed vaccine available. This study demonstrates that the liver stage T cell inducing vaccine regimen ChAd63 – MVA ME.TRAP is immunogenic and partially protective in humans (21%). Though this level of efficacy is suboptimal, there is the only one subunit vaccine regimen able to elicit greater efficacy in humans, and that is the CS-based VLP vaccine RTS,S/AS01B. This vaccine predominantly induces CS-specific antibodies that target the sporozoite stage of infection and has been shown to elicit between 30-50% efficacy in a recent large phase 3 trial in Africa.

This thesis aimed to improve pre-erythrocytic subunit vaccine efficacy using two approaches. Firstly by generating a potentially more immunogenic CS-based particle vaccine and secondly by using this vaccine in a multi-component vaccination strategy with TRAP based viral vectors. In this study an improved version of RTS,S called R21, has been produced by expressing a single CS-HBsAg fusion protein in the yeast *Pichia pastoris.* R21 is potentially more immunogenic than RTS,S as it is composed of a much higher percentage of CS antigen. R21 was shown to be immunogenic when administered alone or in a range of adjuvants and elicited sterile efficacy when formulated with ISCOM adjuvants. Assessment of this multi-component regimen demonstrated that these two vaccine strategies that predominantly induce different arms of the immune response and elicit partial protection in humans can be combined in a murine malaria model without immunological interference. Furthermore, administering R21 together with TRAP based viral vectors resulted in a synergistic enhancement of protective efficacy.

7.2 Conclusions and future directions

7.2.1 ChAd63 – MVA ME.TRAP vaccination in humans

The initial aim of this thesis was to assess in humans the immunogenicity and efficacy of ChAd63 - MVA ME.TRAP, a T cell inducing viral vector vaccine regimen that targets the liver-stage of infection. Here this vaccine regimen was shown to be safe and immunogenic in naïve adults and was able to elicit 21% sterile efficacy after CHMI. These vaccines used in the heterologous prime-boost regimen induced very high levels of antigen-specific T cells and moderate levels of TRAP-specific antibodies. Although the immunogenicity of ChAd63 ME.TRAP alone was exceptional, both antibodies and T cells were boosted significantly by the MVA boost. Moreover, ChAd63 ME.TRAP was not protective when used alone, or when mixed and administered with MVA ME.TRAP. This suggests that the protective capacity of the immune response is altered by the MVA boost vaccine, either by increasing the magnitude of the response and overcoming a threshold required for protection or by altering the quality and functionality of the immune components. ChAd63 followed by MVA was found to be the most immunogenic and efficacious combination of TRAP based viral vectors assessed at the Jenner Institute to date [355, 357, 381, 384, 385, 387]. While this regimen did not induce complete sterile protection, the vaccine-induced responses appear very effective at eliminating the liver stage parasites, and it is estimated that the delay in parasitaemia corresponds to a 96% reduction in liver parasite burden [357, 383]. The ChAd63 – MVA prime-boost regimen has since been shown to induce high levels of T cells and antibodies to other *P. falciparum* malaria antigens including CS (unpublished), AMA1 and MSP1 [493, 507-509], as well as *P. vivax* antigens [510]. Furthermore, it was shown that once the effector response had contracted it was possible to re-boost the T cell response with a third vaccination of either ChAd63 or MVA. Both vectors were able

to re-boost the response back to the level reached after the original boost, suggesting anti-vector immunity does not impair the immunogenicity of these vectors. This indicates that it may be possible to re-boost efficacy if it wanes over time and furthermore there may be little or no interference if multiple vaccines are produced for different diseases using the same viral vectors. The same heterologous prime-boost regimen could therefore be used to target other diseases for which T cells may be required for protection such as influenza, HIV and tuberculosis, and similar regimens are being evaluated [511-514]. Efficacy elicited by ChAd63 - MVA ME.TRAP appears to be mediated predominantly by high affinity T cells that secrete IFNγ [357]. Moreover, analysis of the multi-functionality of the T cells by ICS identified CD8+ T cells that secrete only IFNγ (without IL2 of TNF) as being most strongly associated with protection. This vaccine regime is now being assessed in adults and children in Africa, and it will be interesting to see if this association is also seen in larger data sets and in pre-exposed individuals. Further work could be carried out to fully characterise the responses induced by ChAd63 – MVA ME.TRAP vaccination. For example, memory T cells were assessed in this study with the cultured ELISpot assay which had previously identified an association with TRAP-specific memory cells and protective efficacy [386, 415]. However this association was not seen with ChAd63 – MVA ME.TRAP. This could be due to the small sample size assessed or it may be that the assay used did not have the ability to identify a population of memory cells required for protection. The cultured ELISpot identifies all memory T cells and does not differentiate between CD4+, CD8+, effector memory or central memory subsets. It is thought that during the 10 days of culture both the effector and effector memory T cells die and hence this assay may preferentially assess central memory responses. It may be informative to look at and clarify the memory T cell subsets in more detail by flow cytometry and this may have a greater ability to detect specific populations involved in protection [105, 422, 515]. The prime-boost regimen also induced good levels of TRAP-specific antibodies, an

improvement on previous viral vector regimens [385], however these did not correlate with efficacy. This could imply that the TRAP-specific IgG does not have a role in protection or it could indicate that the assay was unable to identify IgG that is functional and protective. The assay used measures total IgG, therefore assessing the isotype [502] and the avidity of the IgG may reveal a subset of antibodies that associate with efficacy. Furthermore, the functional ability of both the T cell and antibody subsets identified in the immunological assays could be confirmed *in vitro.* Assays could be used to either assess the ability of antibodies to inhibit sporozoite invasion of hepatocytes, or the development of liver stage parasites, or they could assess the ability of T cells to kill infected hepatocytes [428, 429]. These assays are currently being developed and optimised in our laboratory, and would help provide important information about the mechanisms involved in the protective response induced by vaccination in humans. Furthermore, identifying the protective mechanisms could inform the design and optimisation of future vaccines so they can elicit the correct type of response.

Although ChAd63 – MVA ME.TRAP is very effective at inducing CD8+ T cells and induces good levels of antibodies to the viral vector transgene, the level of efficacy is suboptimal for a deployable cost-effective malaria vaccine. Nonetheless targeting the pre-erythrocytic stage of infection still appears the most attractive option for a vaccine that would prevent both infection and transmission. The most successful malaria vaccines evaluated to date have all targeted the pre-erythrocytic stage. The highest level of efficacy elicited by subunit vaccination has been with RTS,S/AS01B (30 – 50%) [348, 349] and the highest level of sterile efficacy achieved by any vaccine technology has been by whole sporozoite vaccination [116]. This was demonstrated most successfully with CPS immunisation where complete protection is induced in humans by <45 sporozoite infected mosquito bites [308, 309]. However, since the logistics and current issues associated with delivering and

deploying whole sporozoite vaccines may be impossible to overcome in the near future, it was hypothesised that this ChAd63 – MVA ME.TRAP viral vector regimen could be combined with a CS-based particle like RTS,S, to improve efficacy. The theory is that the CS-specific antibodies could target and eliminate the majority (>90%) of sporozoites, leaving only a few infected hepatocytes [469]. This very low number of infected hepatocytes could then be more easily targeted and eliminated by the cellular response induced by TRAP based viral vector vaccines. Therefore the second major aim of this thesis was to generate a CS-based particle similar to the partially protective vaccine RTS,S that could be used in a multi-component vaccine strategy with the viral vector vaccine regimens developed at the Jenner Institute.

7.2.2 Generation of a novel CS-based particle vaccine R21

Since RTS,S was developed almost 30 years ago the aim was also to improve the design of the particle in order to enhance the induction of a protective immune response. Hence, in this study a CS-based particle vaccine was designed and successfully produced and purified consisting of a single CS-HBsAg fusion protein. This was a novel finding as it had previously been found that CS-HBsAg particles would only form with the co-expression of a considerable excess of HBsAg [443]. This is potentially an improvement upon RTS,S as it means a greater amount of the particle will be composed of the CS antigen instead of the HBsAg and may result in a greater proportion of the immune response being generated towards the malaria antigen. In addition, the increase in density and repetitive display of CS antigen on the surface of the VLP may enhance B cell activation and antibody production [433, 444, 480, 483-487]. In order to confirm an enhancement in CS-specific immune responses and a reduction in HBsAg immunogenicity, R21 would need to be directly compared to RTS,S, which was unavailable at the time of this study, but is now under investigation. However, the accessibility of CS antigen and the relative inaccessibility of HBsAg on the surface of the particle was demonstrated by ELISA.

This was confirmed in immunogenicity experiments that assessed the induction of antibodies to HBsAg and CS. It was found that in comparison to the same dose of HBsAg, R21 was very poor at inducing HBsAg-specific IgG, therefore HBsAg is likely inaccessible to the BCRs. Further studies could be performed to confirm this, such as immunogold labelling with EM that would allow visualisation of intact particle and the recognition of antigen on the surface [452].

The work in this thesis assessed the particulate nature of the R21 particle using relatively simple methods; however the structure could be fully evaluated using techniques that have successfully characterised HBsAg particles [448, 453-455]. In particular, the structural composition and interaction of both the lipid and protein components could be determined [456]. This is of interest because studies have shown that the lipid content directly influences the immunogenicity of the HBsAg particles [442, 457, 458]. The method developed here for the generation and purification of immunogenic chimeric HBsAg particles is relatively simple and quick. It could therefore be used to rapidly produce multiple vaccine candidates displaying different antigens for pre-clinical evaluation. Furthermore, assessment of a newly developed system for affinity purifying recombinant proteins using a four amino acid C-terminal EPEA tag is ongoing at the Jenner Institute and this could simplify purification further [459]. This tag may also be suitable for clinical vaccine products which would enable rapid translation of pre-clinical vaccines in clinical trials with minimal product development time. The development of this process could therefore accelerate the pre-clinical and clinical evaluation of candidate vaccines for malaria and other diseases.

7.2.3 R21 immunogenicity

Following purification, the ability of the particle to induce a good immune response was assessed. R21 was found to be immunogenic in BALB/c mice at very low dose,

either used alone or in a range of adjuvants. The most potent adjuvants for the induction of antibodies and T cells were found to be the saponin-based ISCOMS. Equally effective at enhancing the induction of high antibody titres, but less so at enhancing T cell induction were the squalene-based o/w emulsions. Importantly when compared to a bio-similar of the GSK proprietary adjuvant AS01B, Matrix M was found to induce equivalent levels of antibodies and T cells. This could have important implications for the future development of this and many other vaccines, as Matrix M and other ISCOM adjuvants are more readily available for evaluation with experimental vaccines. Furthermore, as evidenced by RTS,S studies, the use of an appropriate adjuvant can greatly affect the induction of a protective immune response [300, 340]. Therefore lack of access to effective adjuvants can massively hamper discovery of protective vaccines. The majority of the immune response induced by R21 was to the CS antigen and not towards the HBsAg (which constitutes ~50% of the particle) suggesting that the surface of the particle is covered in CS. The CS antigen was also found to be more immunogenic when delivered in particulate form as R21, compared to non-particulate recombinant CS protein, irrespective of the adjuvant used to enhance the response. This finding supports the idea that particles are inherently more immunogenic than non-particulate recombinant proteins due to their size and/or the repetitive organisation and surface display of antigen [478, 480, 483-488], thus highlighting the importance of generating chimeric VLP vaccines for the induction of both humoral and cellular immune responses [444, 480-482]. R21 was found to induce almost complete sterile protection when administered with ISCOM adjuvants (Abisco/Matrix M) and in contrast, only induced a significant delay in the development of 1% parasitaemia when administered with squalene based o/w emulsions (AddaVax/MF59). Although both types of adjuvants formulated with R21 elicit significant protection, when the survival curves were compared, immunisation with R21 + Abisco/Matrix M was significantly more protective than immunisation with R21 + AddaVax/MF59 ($p <$

0.0001). It should be considered that the model used to assess protective efficacy employed a transgenic *P. berghei* parasite expressing the native *P. berghei* form of CS (*Pb*CS) in addition to the *P. falciparum* version (*Pf*CS) used in R21. Therefore the parasite is able to use both CS proteins to invade hepatocytes, whereas immune responses will only be induced towards the *P. falciparum* version [178, 498]. It may be possible that if the sporozoite is not neutralised completely and very rapidly by the immune responses to the *Pf*CS before it makes contact with a hepatocyte then the parasite may have the opportunity to utilize *Pb*CS for invasion. This model is therefore quite stringent, as protection would require a very efficient and effective response. This may also account for the incomplete sterile protection elicited by R21 + Abisco/Matrix M as the invasion of only one hepatocyte would result in the development of blood stage parasitaemia, albeit delayed. Interestingly, although there was a significant difference in the level of protective efficacy elicited by R21 + Abisco/Matrix M and R21 + AddaVax/MF59 there was no difference in the magnitude of NANP-specific IgG titres prior to sporozoite challenge. This therefore indicates that protective efficacy is not mediated solely by the magnitude of the CS-specific antibody response. One explanation for this could be the induction of functionally different antibody responses, and assessing avidity or isotype of the NANP-specific IgG might identify different types of antibodies. This would be expected as these different classes of adjuvants are known to skew the immune responses in slightly different ways and different antibody isotypes have different functions [502]. Further assays could be used to assess the functional activity of the IgG isotypes detected, such as assessing the complement activity of IgG1 [516, 517], and assessing inhibition of motility or invasion, or inhibition of liver stage parasite development [428, 518]. Furthermore, as seen in other studies high antibody titres that are also of high avidity may be more protective [178, 503]. An alternative explanation for the different levels of protective efficacy could be due to the different levels of CS-specific T cells detected prior to challenge. R21 + Abisco/Matrix M was more

effective at inducing cellular responses and also more protective. Similarly transfer of high doses of IgG did not sterilely protect mice. This could be because the amount of NANP-specific IgG transferred was not high enough, or because there were no CS-specific T cells present. These results are in agreement with other studies which have shown CS-specific T cells contribute to protective efficacy in transgenic parasite models [479, 503]. Furthermore, it reflect results seen with the assessment of RTS,S in humans, where induction of cellular immune responses and also efficacy were superior with the adjuvant AS01 compared to AS02 and CD4+ T cells have been associated with protection [300, 421, 470-474, 505, 506]. Moreover, a mathematical model was used to assess the contribution of immune components to protection against sporozoite challenge in naïve adults, and indicated that a level of efficacy is achieved by the vaccine induced CS-specific antibodies and this can be enhanced by vaccine induced CD4+ T cells [469]. However, the results presented here only identify a possible association between CS-specific T cell induction and protective efficacy. Murine mechanistic studies could be used to further assess the contribution and function of the different immune components with protection by depleting or transferring vaccine induced T cell subsets or IgG prior to challenge, though the mechanisms of protection identified this way may not translate into humans. Alternatively, primary human hepatocytes or human hepatocyte cell lines could be used to assess the functionality of T cells in hepatocyte killing assays as well as the functionality of IgG in inhibition of invasion and liver stage development assays.

7.2.4 Multi-component vaccination assessing immune interference and enhancement of efficacy

Following assessment of R21 in adjuvant, the final aim was to determine if R21 can be successfully combined with viral vector vaccines in a multi-component vaccination strategy. No interference was observed with the induction of immune responses by

either R21 + adjuvant or the ChAd63 – MVA ME.TRAP regimen when the vaccines were mixed and administered at the same site. Furthermore, the efficacy induced with R21 in the transgenic parasite model can be synergistically enhanced by co-administration with PbTRAP based viral vectors. Efficacy of the individual vaccine was 12.5% and 37.5% for the R21 and viral vectors respectively, and this increased to 62.5% in the combination regimen. A similar result was seen when the two leading clinical malaria vaccines RTS,S/AS01B and ChAd63 – MVA ME.TRAP were combined in BALB/c mice. This study was not designed to assess TRAP-mediated efficacy of the viral vectors, because the ME.TRAP vaccine contains the Pb9 immunodominant CS epitope that is able to confer protection on its own. However the study did demonstrate that these vaccines can be mixed and co-administered at the same site without any interference and that ChAd63 – MVA ME.TRAP does not interfere with the protective efficacy induced by RTS,S/AS01B. These results reflect those seen in a study where immunogenicity was unaffected when FP9 and MVA viral vectors expressing *P. berghei* CS were combined with *P. berghei* CS-based hepatitis B core particle vaccine, and efficacy was enhanced [464]. Similar studies could be performed using the newly generated transgenic *P. berghei* parasite that contains *P. falciparum* CS replacement instead of addition, and this could simplify the interpretation of results. In addition, both mechanistic studies and functional assays using human hepatocytes as described above could be used to assess the contribution to protection of the immune components induced by the individual vaccine regimens. Furthermore these studies could be repeated in outbred strains of mice which have more diverse MHC repertoires and the results may therefore be more reflective of those seen with vaccination in humans. However none of these models are currently able to effectively predict the efficacy of malaria vaccines in humans. Therefore, since these findings indicate that a multi-component malaria vaccine comprising R21 and viral vectors may be a feasible approach, R21 is currently being manufactured to GMP. This vaccine is being taken forward for

evaluation in Phase 1/2a clinical trials with the future aim of combining it with the ChAd63 - MVA ME.TRAP regimen in humans. It will however be important to determine if the immunogenicity and durability of efficacy elicited by R21 is greater than that achieved by RTS,S and these studies are now underway.

7.3 Final remarks

This study describes a multi-component vaccination strategy for the concurrent induction of humoral and cellular mediated immunity using viral vector vaccines combined with particle in adjuvant. This vaccination strategy could be employed to induce immunogenicity against multiple antigens and multiple stages of malaria and may also be applied to other diseases where T cells and antibodies might be required for protection. In addition the development of a simple purification strategy may allow the rapid production of candidate particle vaccines for evaluation in multi-component regimens. Taken together the results in this thesis support the hypothesis that targeting both the sporozoites and the liver stage parasites with both cellular and humoral responses, utilizing two different antigens is able to overcome the ‘leaky’ effect of a sporozoite vaccine. Therefore these combination vaccine regimens should be assessed in humans as a priority as an efficient method for improving vaccine efficacy with products that have already be proven to be safe and partially effective in clinical trials.

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Appendix

- CS total peptide pool used for murine *ex vivo* ELISpots and ICS assays

CS peptide pool	Sequence
1	DPNANPNANPNANPN
2	PNANPNANPNANPNA
3	NANPNANPNANPNAN
4	ANPNANPNANPNANP
5	NPNANPNANPNANPN
6	PNANPNANPNANPNA
7	NANPNANPNANPNKN
8	ANPNANPNKNNQGNG
9	NPNKNNQGNGQGHNM
10	NQGNGQGHNMPNDPN
11	QGHNMPNDPNRNVDE
12	PNDPNRNVDENANAN
13	RNVDENANANSAVKN
14	NANANSAVKNNNNEE
15	SAVKNNNNEEPSDKH
16	NNNEEPSDKHIKEYL
17	PSDKHIKEYLNKIQN
18	IKEYLNKIQNSLSTE
19	NKIQNSLSTEWSPCS
20	SLSTEWSPCSVTCGN
21	WSPCSVTCGNGIQVR
22	VTCGNGIQVRIKPGS
23	GIQVRIKPGSANKPK
24	IKPGSANKPKDELDY
25	ANKPKDELDYANDIE
26	DELDYANDIEKKICK
27	ANDIEKKICKMEKCS
28	KKICKMEKCSSVFNV

ME.TRAP Peptide pools for human *ex vivo* and cultured ELISpot assays

TRAP T9/96 pool 1 (pep 1-10)	**Sequence**
1	MNHLGNVKYLVIVFLIFFDL
2	VIVFLIFFDLFLVNGRDVQN
3	FLVNGRDVQNNIVDEIKYSE
4	NIVDEIKYSEEVCNDQVDLY
5	EVCNDQVDLYLLMDCSGSIR
6	LLMDCSGSIRRHNWVNHAVP
7	RHNWVNHAVPLAMKLIQQLN
8	LAMKLIQQLNLNDNAIHLYV
9	LNDNAIHLYVNVFSNNAKEI
10	LNDNAIHLYVNVFSNNAKEI
TRAP T9/96 pool 2 (pep 10-20)	
11	IRLHSDASKNKEKALIIIRS
12	KEKALIIIRSLLSTNLPYGR
13	LLSTNLPYGRTNLTDALLQV
14	TNLTDALLQVRKHLNDRINR
15	RKHLNDRINRENANQLVVIL
16	ENANQLVVILTDGIPDSIQD
17	TDGIPDSIQDSLKESRKLSD
18	SLKESRKLSDRGVKIAVFGI
19	RGVKIAVFGIGQGINVAFNR
20	GQGINVAFNRFLVGCHPSDG
TRAP T9/96 pool 3 (Pep 21-50)	
21	FLVGCHPSDGKCNLYADSAW
22	KCNLYADSAWENVKNVIGPF
23	ENVKNVIGPFMKAVCVEVEK
24	MKAVCVEVEKTASCGVWDEW

25	TASCGVWDEWSPCSVTCGKG
26	SPCSVTCGKGTRSRKREILH
27	TRSRKREILHEGCTSEIQEQ
28	EGCTSEIQEQCEEERCPPKW
29	CEEERCPPKWEPLDVPDEPE
30	EPLDVPDEPEDDQPRPRGDN
TRAP T9/96 pool 4 (Pep 21-50)	
31	DDQPRPRGDNSSVQKPEENI
32	SSVQKPEENIIDNNPQEPSP
33	IDNNPQEPSPNPEEGKDENP
34	NPEEGKDENPNGFDLDENPE
35	NGFDLDENPENPPNPDIPEQ
36	NPPNPDIPEQKPNIPEDSEK
37	*PPNPPNPPNPDIPEQKPNIP*
38	DIPEQKPNIPEDSEKEVPSD
39	EDSEKEVPSDVPKNPEDDRE
40	VPKNPEDDREENFDIPKKPE
TRAP T9/96 pool 5 (Pep 21-50)	
41	ENFDIPKKPENKHDNQNNLP
42	NKHDNQNNLPNDKSDRNIPY
43	NDKSDRNIPYSPLPPKVLDN
44	SPLPPKVLDNERKQSDPQSQ
45	ERKQSDPQSQDNNGNRHVPN
46	DNNGNRHVPNSEDRETRPHG
47	SEDRETRPHGRNNENRSYNR
48	RNNENRSYNRKYNDTPKHPE
49	KYNDTPKHPEREEHEKPDNN
50	REEHEKPDNNKKKGESDNKY
TRAP T9/96 pool 6 (Pep 51-57)	
51	KKKGESDNKYKIAGGIAGGL

52	KIAGGIAGGLALLACAGLAY
53	ALLACAGLAYKFVVPGAATP
54	KFVVPGAATPYAGEPAPFDE
55	YAGEPAPFDETLGEEDKDLD
56	TLGEEDKDLDEPEQFRLPEE
57	EPEQFRLPEENEWN
ME pool	
ls8	KPNDKSLY
cp26	KPKDELDY
ls6	KPIVQYDNF
tr42/43	ASKNKEKALII
tr39	GIAGGLALL
cp6	MNPNDPNRNV
st8	MINAYLDKL
ls50	ISKYEDEI
tr26	HLGNVKYLV
ls53	KSLYDEHI
tr29	LLMDCSGSI
NANP	NANPNANPNANPNANP
TRAP AM	DEWSPCSVTCGKGTRSRKRE
cp39	YLNKIQNSL
la72	MEKLKELEK
ex23	ATSVLAGL
CSP	DPNANPNVDPNANPNV
38H(BCG)	QVHFQPLPPAVVKL
FTTp	QFIKANSKFIGITE

- PbTRAP total peptide pool used for murine *ex vivo* ELISpot and ICS assays

PbTRAP	**Sequence**
1	MKLLGNSKYFFVVLLLCISV
2	FVVLLLCISVFLNGQEILDE
3	FLNGQEILDEIKYSEEVCNE
4	IKYSEEVCNEQIDLHILLDG
5	QIDLHILLDGSGSIGHSNWI

6	SGSIGHSNWISHVIPMLTTL
7	SHVIPMLTTLVDNLNISRDE
8	VDNLNISRDEINISMTLFST
9	INISMTLFSTYARELVRLKR
10	YARELVRLKRYGSTSKASLR
11	YGSTSKASLRFIIAQLQNNY
12	FIIAQLQNNYSPHGTTNLTS
13	SPHGTTNLTSALLNVDNLIQ
14	ALLNVDNLIQKKMNRPNAIQ
15	KKMNRPNAIQLVIILTDGIP
16	LVIILTDGIPNNLKKSTTVV
17	NNLKKSTTVVNQLKKKDVNV
18	NQLKKKDVNVAIIGVGAGVN
19	AIIGVGAGVNNMFNRILVGC
20	NMFNRILVGCGKLGPCPYYS
21	GKLGPCPYYSYGSWDQAQTM
22	YGSWDQAQTMIKPFLSKVCQ
23	IKPFLSKVCQEVEKVALCGK
24	EVEKVALCGKWEEWSECSTT
25	WEEWSECSTTCDNGTKIRKR
26	CDNGTKIRKRKVLHPNCAGE
27	KVLHPNCAGEMTAPCKVRDC
28	MTAPCKVRDCPPKPVAPPVI
29	PPKPVAPPVIPIKVPDVPVK
30	PIKVPDVPVKPVEPIEPAEP
31	PVEPIEPAEPAEPAEPAEPA
32	AEPAEPAEPAEPAEPAEPAE
33	EPAEPAEPAEPAEPAEPAEP
34	PAEPAEPAEPAEPAEPAEPA
35	AEPAEPAEPAEPAEPAEPAE
36	EPAEPAEPAEPAEPAKPAEP
37	PAEPAKPAEPAEPAEPAEPA
38	AEPAEPAEPAEPVNPDNPIL
39	EPVNPDNPILPIKPEEPSGG
40	PIKPEEPSGGAEPLNPEVEN
41	AEPLNPEVENPFIIPDEPIE

42	PFIIPDEPIEPIIAPGAVPD
43	PIIAPGAVPDKPIIPEESNE
44	KPIIPEESNELPNNLPESPS
45	LPNNLPESPSDSQVEYPRPN
46	DSQVEYPRPNDNGDNSNNTI
47	DNGDNSNNTINSNKNIPNKH
48	NSNKNIPNKHVPPTDDNPYK
49	VPPTDDNPYKGQEERIPKPH
50	GQEERIPKPHRSNDEYIYYN
51	RSNDEYIYYNNANNNDKLEP
52	NANNNDKLEPEIPSKDYEEN
53	EIPSKDYEENKSKKQSKSNN
54	KSKKQSKSNNGYKIAGGIIG
55	GYKIAGGIIGGLAIIGCIGV
56	GLAIIGCIGVGYNFIAGSSA
57	GYNFIAGSSAAAMAGEAAPF
58	AAMAGEAAPFEDVMADDEKG
59	EDVMADDEKGIVENEQFKLP
60	DEKGIVENEQFKLPEDNDWN