Enhancing the Efficacy of Viral Vector Blood-Stage Malaria Vaccines

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Abstract

Replication-deficient adenovirus (Ad) and modified vaccinia virus Ankara (MVA) vectors expressing single *Plasmodium falciparum* antigens can induce potent T cell and antibody responses and have entered clinical testing using a heterologous prime-boost immunisation approach (Ad_MVA). This thesis describes a number of pre-clinical approaches aimed at enhancing the efficacy of these viral vectored vaccines targeting the blood-stage of malaria. First, the development of a highly efficacious malaria vaccine is likely to require a multi-antigen and/or multi-stage subunit vaccine. The utility of an Ad_MVA immunisation regime combining vaccines expressing the 42kDa C-terminus of the blood-stage antigen merozoite surface protein 1 ($MSP1_{42}$) and the pre-erythrocytic antigen circumsporozoite protein (CSP) in the *P. yoelii* mouse model was investigated. It was found that vaccine co-administration leads to maintained antibody responses and efficacy against blood-stage infection, but reduced secondary $CD8^+$ T cell responses and efficacy against liver-stage infection. $CD8^+$ T cell interference can be minimised by co-administering the MVA vaccines at separate sites, resulting in enhanced liver-stage efficacy. The mechanisms of $CD8^+$ T cell interference were explored. Second, Ad_MVA regimes expressing blood-stage antigens that can protect against *P. chabaudi* and *P. yoelii* blood-stage infection were tested against *P. berghei*, but did not confer protection. Similarly, IgG from rabbits immunised against *P. falciparum* MSP1 (PfMSP1) could not protect mice from a chimeric *P. berghei* parasite expressing PfMSP1. Third, two molecular adjuvants, the C4bp α-chain oligomerisation domain (IMX108/313) and the Fc fragment of murine IgG2a, were tested for their ability to enhance immunogenicity of recombinant adenoviruses when fused at the C-terminus of a blood-stage antigen. IMX108/313 was found to adjuvant T cell responses of small (< 80kDa) antigens and this was associated with antigen oligomerisation. However, the Fc fragment did not adjuvant responses. Finally, it was found that using a strong early promoter to drive antigen expression enhances the immunogenicity of single administration MVA vaccines, but that this did not enhance post-boost immunogenicity in an Ad_MVA regime.

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Abbreviations

αα	Amino acid
Ab	Antibody
ACK	Ammonium chloride potassium
Ad	adenovirus
Ad_MVA	Heterologous prime-boost immunisation with Ad and MVA (both expressing the same antigen) given eight weeks apart
AdHu5	Human adenovirus serotype 5
AdCh63	Simian adenovirus Ch63
ADCI	Antibody dependent cellular inhibition
AMA1	Apical membrane antigen 1
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
C4bp	C4b binding protein
C6	Simian adenovirus C6
C7	Simian adenovirus C7
C9	Simian adenovirus C9
CEF	Chicken embryo fibroblast
CIP	Calf Intestinal Phosphatase
CPE	Cytopathic effect
CRP	C-reactive protein
CSP	Circumsporozoite protein
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ETSR	3D7 allelic variant of *P. falciparum* $MSP1_{19}$
FCS	Foetal calf serum
FcγR	Fc γ receptor
FP9	Fowlpox strain FP9
g	Gravitational acceleration (9.80665 m/s^2)
GFP	Green fluorescent protein
GIA	Growth inhibition assay
GPI	Glycophosphatidylinositol
GSK	GlaxoSmithKline
GST	Glutathione S-transferase
h	Hour
HBe	Hepatitis B e antigen
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
i.d.	Intradermal
IFN-γ	Interferon-γ
IL-2	Interleukin 2
i.m.	Intramuscular
i.p.	Intraperitoneal

IQR	Interquartile range
i.v.	Intravenous
IFAT	Immunofluorescence antibody test
ifu	Infectious units
IgG	Immunoglobulin G
IMX108	Murine C4bp α-chain oligomerisation domain
IMX313	Hybrid C4bp α-chain oligomerisation domain
IQR	Inter-quartile range
LB	Lysogeny broth
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
ME	Multiple epitope string
MEM	Minimum essential medium
MHC	Major histocompatibility complex
min	Minutes
MSP1	Merozoite surface protein 1
MSP9	Merozoite surface protein 9
$MSP1_{19}$	19kDa fragment of MSP1
$MSP1_{33}$	33kDa fragment of MSP1
$MSP1_{42}$	42kDa fragment of MSP1
MVA	modified vaccinia virus Ankara
NYVAC	New York vaccinia virus
OUVS	Oxford University Veterinary Services
Pb	*Plasmodium berghei*
Pb ANKA	*P. berghei* ANKA strain

Pb ΔPM4	Attenuated *P. berghei* ANKA parasite lacking plasmepsin 4
Pb ΔSMAC	*P. berghei* ANKA lacking Schizont Membrane Associated Cytoadherence protein
PBMC	peripheral blood mononuclear cells
Pb NK65	*P. berghei* NK65 strain
Pb-PfM19	*P. berghei* ANKA parasite expressing $PfMSP1_{19}$
PBS	Phosphate buffered saline
PBS/T	0.05% Tween 20 in phosphate buffered saline
Pb XAT	Attenuated strain of *P. berghei* ANKA
Pc	*P. chabaudi*
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PfM115	*Plasmodium falciparum* MSP1 codon optimized vaccine antigen
PfM128	*Plasmodium falciparum* MSP1 codon optimized vaccine antigen including $PfMSP1_{19}$ 3D7
Pfs25	*P. falciparum* ookine surface protein 25
pfu	Plaque forming units
pRBC	Parasitised red blood cell
Py	*Plasmodium yoelii*
QKNG	FVO allelic variant of $PfMSP1_{19}$
RBC	Red blood cell
rpm	Revolutions per minute
RT	Room temperature

RTS,S	*P. falciparum* CSP vaccine made by GSK (**R**epeat NANP B cell epitope and **T** cell epitope of CSP fused to hepatitis B **s**urface antigen (RTS), co-administered with HB**s**Ag polypeptide (S))
s	Seconds
SAP	Serum amyloid P component
SFC	Spot forming cells
TAE	Tris-acetate-EDTA
TCR	T cell receptor
Th	T helper
TNFα	Tumour necrosis factor α
tPA	Tissue plasminogen activator
TRAP	Thrombospondin-related adhesion protein (also known as thrombospondin-related anonymous protein)
T-reg	Regulatory T cell
vp	Viral particles
VV	Vaccinia virus
WHO	World Health Organisation

Chapter 1

Introduction

1 Introduction

1.1 Malaria

Over 200 million cases of malaria occur each year resulting in almost 1 million deaths. Pregnant women and children under five are most at risk, and in Africa the disease accounts for 20% of all childhood deaths [1]. Malaria is caused by protozoan parasites of the *Plasmodium* genus. Four different species commonly infect humans – *P. falciparum, P. vivax, P. ovale* and *P. malariae.* Recently, *P. knowlesi* that primarily infects monkeys, has also been reported to cause human disease in South East Asia [2, 3].The most pathogenic strain is *P. falciparum*. In 2007 it was estimated that 1.38 billion people were at risk of *P. falciparum* malaria. Prevalence is by far the highest in Africa, where 0.35 billion people are considered to live in areas of high endemicity [4] (Figure 1.1). In addition to morbidity and mortality, malaria has a huge economic impact. Between 1965-1990 *P. falciparum* endemic countries had annual economic growth rates 1.3% lower than other countries [5].

1.2 *Plasmodium falciparum* life cycle

The life-cycle of *P. falciparum* can be divided into the three main stages outlined below and summarised in Figure 1.2.

Pre-erythrocytic stage: This can be divided into two sub-stages – the sporozoite stage and the liver-stage. During the sporozoite stage a female *Anopheles* mosquito bites a human host and injects haploid sporozoites. These migrate through the skin, and into the bloodstream. Within a few minutes sporozoites migrate to the liver, traverse Kuppfer cells and invade hepatocytes where asexual mitotic replication

occurs giving rise to trophozoites and then mature exo-erythrocytic schizonts containing 20-40,000 merozoites. These form over a period of 6-7 days. It is classically believed that the hepatocyte then ruptures, releasing merozoites into the blood. However, in the *P. yoelii* mouse model merozoites have been visualised leaving the liver in 'merosomes', membranous sacks containing hundreds of merozoites. These move through the circulation and finally rupture within the lung, causing free merozoites to be first released into the blood at a site far removed from the liver [6]. This has yet to be investigated for *P. falciparum*.

Blood/erythrocytic stage: Merozoites in the bloodstream invade red blood cells (RBCs) and divide mitotically from early trophozoite ring stages to late mature trophozoites and schizonts. The erythrocyte then ruptures releasing approximately 16 new merozoites into the bloodstream that infect new erythrocytes [7]. In *P. falciparum* each cycle of invasion, replication and erythrocyte rupture lasts 42 – 48 hours.

Sexual stage: The sexual stage takes place within the mosquito. During the blood-stage a subset of merozoites develop into male and female gametocytes in response to an unknown stimulus. Gametocytes are taken up by a mosquito when it feeds on an infected human and sexual reproduction then occurs in the mosquito midgut. A zygote is formed which develops into an ookinete and crosses the gut wall to form a sporozoite filled oocyst. Finally the oocyst bursts and sporozoites migrate to the mosquito's salivary glands. When this mosquito bites another human the cycle begins again.

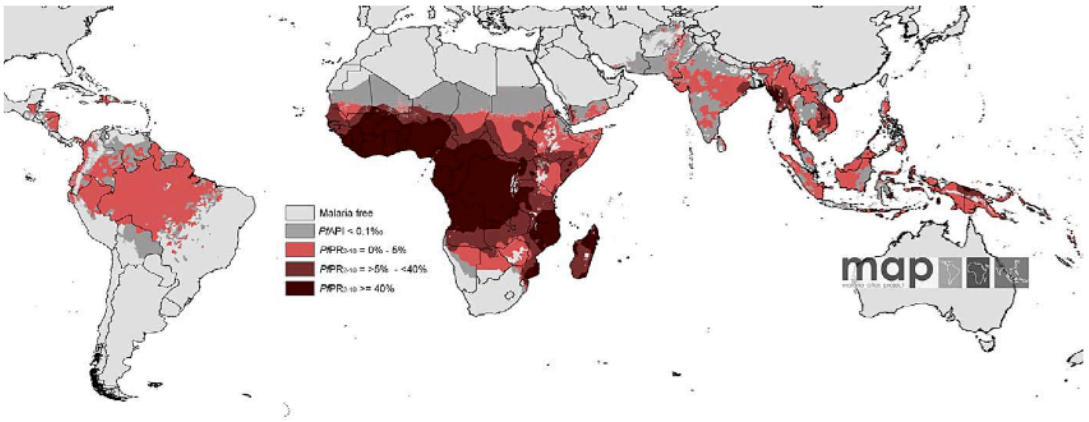


Figure 1.1: The spatial distribution of *P. falciparum* in 2007

The map indicates *P. falciparum* parasite rate, age standardised for the 2 – 10 year age group (PfPR2-10). The parasite rate is the proportion of the population found to carry asexual blood-stage parasites [8]. The map is divided into endemicity classes with low risk PfPR2-10 ≤ 5 % (light red), intermediate risk PfPR2-10 > 5 % to < 40% (medium red) and PfPR2-10 ≥ 40 % (dark red). Dark grey indicates areas of unstable risk where *P. falciparum* annual parasite incidence (PfAPI) is < 0.1 per 1000 people, and light grey indicates no risk. This figure is taken from Hay at al. 2009 [4].

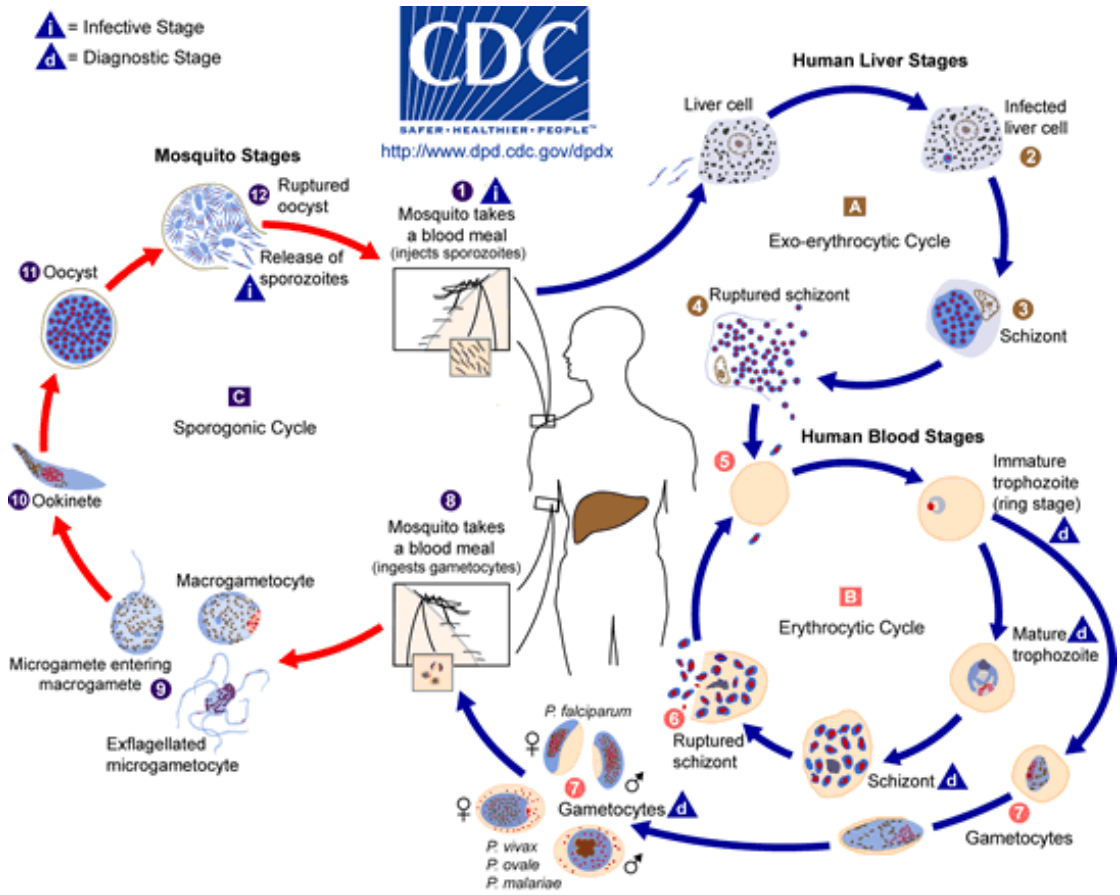


Figure 1.2: The malaria parasite life cycle

The *Plasmodium* life-cycle involves a human and mosquito host. Stages 1 – 7 occur within the human host and include the pre-erythrocytic- and blood-stages. Stages 8 – 12 occur within the mosquito and include the sexual-stage. This image was taken from the Centers for Disease Control and Prevention (CDC) website (http://www.cdc.gov/malaria/about/biology/).

1.3 Malaria pathogenesis

The pre-erythrocytic stage of *P. falciparum* infection is asymptomatic and lasts approximately seven days. Clinical symptoms develop during the erythrocytic stage and include bouts of nausea, fever and headaches, which coincide with the rupture of erythrocytic schizonts. Infection can lead to a wide spectrum of disease, from asymptomatic to severe disease pathology, including severe malarial anaemia, cerebral malaria, and death. There can be multiple causes of severe disease, but metabolic acidosis (usually lactic acidosis) is probably the most important determinant of survival and leads directly to respiratory distress [9, 10]. Lactic acidosis has multiple causes during severe malaria including increased lactic acid production by parasites due to direct stimulation by cytokines and reduced oxygen delivery to the tissues due to anaemia, vascular blockage and sequestration [9, 10].

1.4 The rationale for a vaccine against malaria

1.4.1 Do we need a vaccine?

In 2005 the World Health Assembly and the Roll Back Malaria partnership set a goal to reduce malaria cases and deaths, as recorded in 2000, by more than 50% by the end of 2010 and 75% by 2015. This target has resulted in some progress. Known malaria cases had risen from 233 million to 244 million between 2000 and 2005, but by 2009 had fallen to 225 million. Death rates have also fallen from 985,000 in 2000 to an estimated 781,000 in 2009 [1]. Nevertheless, the burden remains huge and reductions are a long way short of the target set for the end of 2010. The improvements have largely been achieved through increased coverage of insecticide-treated mosquito nets (ITNs). By the end of 2010 it was estimated that approximately 289 million ITNs had

been distributed in sub-Saharan Africa, which is enough to cover 76% of those at risk in the region. Despite this, it is estimated that only 42% of families owned at least one ITN in 2010 and that only 35% of children sleep under an ITN. Furthermore, the life-span of a long-lasting ITN is only 3 years, and therefore many are already in need of replacement. Whilst the increase in ITN usage is encouraging, continuing this trend and then maintaining coverage will be a massive challenge [1]. A second method contributing to the decrease in malaria incidence, is indoor residual spraying (IRS). In 2009, 75 million people (10% of the population at risk) were using this method as compared to only 13 million in 2005. However, both IRS and ITNs are reliant on a single class of insecticides, the pyrethroids, whose widespread use increases the risk of resistance developing in the mosquito population. In addition to the increased use of these control measures, the use of artemisinin-based combination therapy (ACTs) to treat malaria has also increased, from 11.2 million treatment courses in 2005 to 158 million in 2009. However, access to treatment continues to be a problem and the continued use of oral artemisinin-based monotherapies is increasing the incidence of resistance to artemisinins and threatening the efficacy of ACTs [1]. Other problems associated with treatment include non-compliance, cost, and unpleasant side effects. It is estimated that in 2010 adequate malaria control, using the currently available control strategies, would have required US$6 billion, but only US$1.8 billion was received [1].

In 2006 a target was set to produce by 2015 a partially effective vaccine with an efficacy of 50% for at least 1 year [11]. This vaccine would primarily be given to young children as part of the expanded program on immunisation (EPI) schedule [12]. It has been suggested that this goal is likely to be met by the current leading candidate

malaria vaccine, RTS,S [13]. However, in a recent trial in east Africa the estimated efficacy of RTS,S in infants at 12 months was only 39% [14]. The increased use of the partially effective control measures described above will also change the epidemiology of *P. falciparum* infection. Due to reduced acquirement of natural immunity against the disease-causing blood-stage through reduced exposure, peak incidence of malaria is likely to shift to older children and adults [12]. In order to justify deployment, a second generation vaccine will be required to provide greater than 80% efficacy for at least four years [11] and will need to be delivered by mass vaccination campaigns targeting subjects of all ages at risk of infection [12]. The target for such a second generation vaccine has been set for 2025 [11] and it is likely that this vaccine will target multiple life-cycle stages and will need to induce potent T cell and antibody responses. This vaccine, if developed, would have a huge impact on the lives of over 200 million people who are infected with *P. falciparum* each year.

1.4.2 Malaria vaccine strategies

Throughout the malaria life-cycle different antigens are expressed and different immune responses are required for protection. Therefore, subunit malaria vaccine strategies usually target antigen(s) from one of the three life-cycle stages. The majority of research to date has focused on vaccines targeting the pre-erythrocytic (sporozoite/liver) stage, and/or the blood-stage.

Pre-erythrocytic vaccines aim to completely prevent blood-stage infection (sterilising immunity), therefore preventing clinical disease and transmission. However, even a partially effective liver-stage intervention may result in reduced incidence of severe malaria. This has been observed in the field during trials of the

leading pre-erythrocytic vaccine candidate, RTS,S [15] and also following the use of ITNs [16]. The underlying mechanism is not known but it may be that a reduced liver burden results in blood-stage infection for a longer period of time before clinical threshold is reached, therefore allowing greater naturally acquired immunity to develop [17]. Alternatively the prevalence of parasites with differing virulence may be altered by effective immunisation [18]. A third possibility is that a partially effective liver-stage intervention reduces malaria transmission, and consequently the infection rate, thereby resulting in fewer cases of severe malaria.

Conversely, asexual **blood-stage vaccines** aim to reduce morbidity and mortality by decreasing parasite load. Such a vaccine may prevent merozoite invasion of red blood cells, prevent infected red cell adherence to vasculature, or act by direct killing of merozoites and/or infected cells. A highly effective blood-stage vaccine that could control parasitaemia at low levels, would also likely reduce transmission, although limited data are available to suggest the course of gametocytaemia in the face of significant immune pressure against the asexual stage.

It is increasingly recognised that a **multi-stage/multi-antigen** formulation that targets both stages is likely to be necessary to achieve the ambitious goals that have been set. The rationale for the development of vaccines targeting each of these stages and for a multi-stage vaccine is given in Figure 1.3.

Other subunit vaccine approaches include '**transmission blocking vaccines**' (TBVs) and '**anti-toxin**' vaccines. TBVs are an altruistic or "community" approach that would provide no direct / immediate benefit to the infected host, but rather would aim

to reduce transmission. They function by inducing antibodies to target the antigens expressed during the sexual and mosquito mid-gut stages of the parasite life-cycle, and therefore interfere with zygote and/or oocyst development when such antibodies are taken up in the mosquito's blood-meal. This is a rapidly increasing / recently revitalised area of research and could also be included in a multi-stage vaccine strategy [19]. Anti-toxin vaccines target plasmodial Glycophosphatidylinositol (GPI) anchors from merozoite surface proteins. These anchors can induce an immunopathological pro-inflammatory cytokine response [20], and neutralising antibodies against GPI can reduce severe malaria in the *P. berghei* mouse model [21]. Antibodies against the GPI anchor have also been associated with lack of disease in humans [22].

An alternative to subunit vaccine approaches is the use of attenuated whole parasites. Attenuated pathogens have been successfully deployed as efficacious vaccines against a variety of infectious diseases such as smallpox, polio, measles and yellow fever. *P. falciparum* sporozoites which have been radiation-attenuated or genetically-attenuated are being developed as possible whole-parasite vaccine strategies [23, 24].

This thesis focuses primarily on blood-stage vaccines but also the development of a multi-stage product, including a combination of pre-erythrocytic and blood-stage candidate vaccines.

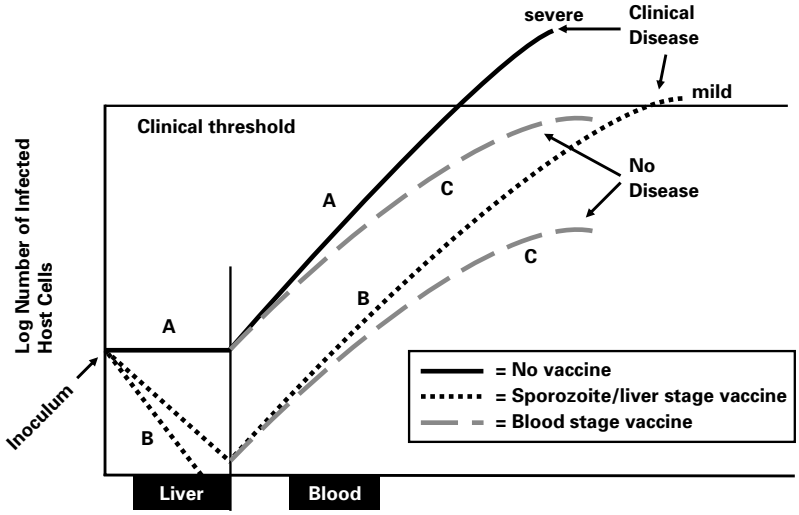


Figure 1.3: Graphical representation of parasite infection in an unvaccinated or vaccinated host

In an unvaccinated host (A/solid line) hepatocytes are infected according to the size of the sporozoite inoculum and rupture after > 5 days, releasing merozoites into the blood. These multiply logarithmically until there are enough parasites to cause clinical symptoms. An effective pre-erythrocytic stage vaccine (B/dotted line) will reduce the liver-stage burden. If this is 100% effective, there will be no blood-stage infection and therefore no clinical symptoms or transmission of infection. However, if the vaccine is < 100% effective, some parasites will emerge into the blood. Nevertheless, the overall clinical severity of the infection may be reduced (Section 1.4.2). An effective blood-stage vaccine (C, grey dashed lines) will limit the parasite replication rate in the blood and thus reduce peak parasitaemia, ideally to below the clinical threshold, therefore preventing disease although not infection. A multi-stage vaccine would aim not only to reduce liver-parasite burden, but also to slow the growth rate of parasites in the blood so that peak parasitaemia is well below the clinical threshold. This figure and text are adapted from Richie 2006 [17] but were originally published in [25]. Copyright © 2002 Karger.

1.4.3 Is a malaria vaccine feasible?

There is evidence to support the feasibility of protecting humans against both the liver- and blood-stages of malaria by vaccination. First, the development of a liver-stage vaccine is supported by studies carried out with irradiated sporozoites. It is possible to provide complete protection against experimental sporozoite challenge in rodents [26], *Grammomys surdaster* (the natural *P. berghei* rodent host) [27], non-human primates [28] and humans [29], by allowing X-irradiated infected mosquitoes to feed on the host prior to challenge. It is thought that the sporozoites travel to the liver and undergo partial development, but due to irradiation are unable to become merozoites. Nevertheless, they are capable of inducing immunity against challenge with infectious sporozoites. Immunity is thought to target the pre-erythrocytic stages as immunised hosts remain susceptible to blood-stage challenge [30], however the exact antigenic target(s) remain unknown [17].

Second, the rationale for a vaccine against the blood-stage of malaria comes from studies of naturally acquired immunity (NAI). Adults in malaria endemic regions develop NAI through repeated infections with the parasite. Although they remain susceptible to infection, they are capable of limiting the density of parasitaemia and the severity of clinical illness. This is the reason that children under the age of five years are most at risk of death due to infection [31]. Passive transfer of immunoglobulin (Ig) from clinically immune African adults can reduce parasite density and clinical symptoms in infected children [32] highlighting the important protective contribution of antibodies.

1.5 Mouse models of malaria

P. falciparum does not infect animals and therefore pre-clinical studies to test immunogenicity and efficacy of candidate vaccines/antigens must use other species of *Plasmodium*. However, no single murine model can replicate all the features of human *P. falciparum* malaria in terms of immunity or pathology. A large number of rodent malarias have been isolated in Africa, but the majority have been little studied and do not infect murines. Four species that were isolated in Central Africa during the late 1940s and 1950s do infect murine rodents and are used today in laboratory studies. These are *P. berghei, P. yoelii, P. chabaudi and P. vinckei.* The four species can be distinguished by small differences in morphological and developmental characteristics summarised in Table 1.1. *P. vinckei* is rarely used in vaccine studies, but the other three species are commonly used and are described in more detail below.

1.5.1 Plasmodium berghei

P. berghei was the first rodent malaria identified and isolated. In 1946, Vincke first described this parasite in blood films from the stomach contents of *Anopheles dureni*, and in 1948 in Kitanga, Congo, it was isolated from its natural mammalian host *Grammomys surdaster* [33]. It is now known to have at least nine natural hosts and can also infect a variety of laboratory hosts including rats, mice and hamsters. A number of well-characterised clones of *P. berghei* are available for laboratory studies. The most frequently used are ANKA, NK65, K173, SP11 and LUKA.

The life-cycle of *P. berghei* in the murine host involves infection of the liver for 48 – 52 hours. Blood-stage parasitaemia usually includes a lag phase at around 10% parasitised RBC (pRBC) for several days, after which time parasitaemia rises to

>50%. In some mouse strains including C57BL/6, TO and CBA, mice can die of a spectrum of causes, believed to model human cerebral malaria, despite only low levels of parasitaemia [34].

It is relatively "easy" to protect against the pre-erythrocytic-stage of *P. berghei* infection by vaccination. A large number of studies have been carried out using irradiated sporozoites [30], genetically attenuated sporozoites [35], peptides [19], or viral vectored vaccines [36]. Blood-stage *P. berghei*, however, has proved to be much more of a challenge, although until recently relatively few publications have acknowledged this. Despite being the earliest rodent malaria identified, only four publications report successful protection against blood-stage *P. berghei* by sub-unit vaccination [37-40]. A further two publications, both from 2010, report a failure to protect against *P. berghei* blood-stage by sub-unit vaccination [41, 42].

1.5.2 Plasmodium yoelii

The most widely used isolate, *P. yoelii yoelii* 17X was first collected in the Central African Republic. From this isolate the lethal clone *P. yoelii* YM (also known as 17XL) was generated (http://www.biology.ed.ac.uk/polopoly/documents/iiir/P_yoelii _isolates_clones.pdf). *P. yoelii* infects the murine liver for 43-48 hours. Unlike *P. falciparum* infection in humans, without intervention blood-stage *P. yoelii* YM/ 17XL parasitaemia develops rapidly to > 80% infected red blood cells (pRBC) resulting in death of the murine host.

P. yoelii is frequently used in studies of both liver- and blood-stage infection. Pre-erythrocytic protection can be induced using a variety of vaccination methods, but has primarily focussed on subunit vaccination against the circumsporozoite protein (PyCSP) (Section 1.6.1). Blood-stage immunity is primarily antibody mediated [43-45] and has been achieved using protein [45-47] and viral vectored vaccines [48]. The majority of research focuses on merozoite surface protein 1 (PyMSP1) (Section 1.6.2).

1.5.3 Plasmodium chabaudi

Plasmodium chabaudi is primarily used to study efficacy of blood-stage malaria vaccines as production of *P. chabaudi* sporozoites can be problematic. Both non-lethal (*P. chabaudi chabaudi* AS) and lethal (*P. chabaudi adami*) exist. Apical membrane antigen 1 (AMA1) and MSP1 protein vaccines can protect against blood-stage infection in this model [49, 50]. Similarly, viral vectored vaccines expressing PcAMA1 can reduce peak blood-stage parasitaemia [51]. In this model protection is primarily mediated by antigen-specific $CD4^+$ T cell responses independently of antibody [52-54].

1.5.4 Transgenic rodent malarias

P. falciparum cannot infect animal models and therefore chimeric parasite lines combining rodent and human malaria strains have been developed for the assessment of human malaria vaccines *in vivo* [55]. It is hoped that these chimeric parasites will increase understanding of vaccine-induced immune responses *in vivo* and aid the optimisation and selection of antigens / vaccine delivery vehicles prior to clinical testing [56]. Within these parasites DNA encoding a single antigenic region is replaced with DNA from an alternative parasite species by direct allelic replacement

[57] or insertion into knock-out parasite strains [58]. The majority of chimeric parasites developed have had a *P. berghei* or *P. falciparum* backbone and include *P. berghei* expressing *P. falciparum* $MSP1_{19}$ (Pb-PfM19) [57] or *P. falciparum* CSP [58, 59].

	P. berghei	*P. yoelii*	*P. chabaudi*	*P. vinckei*
Merozoites per schizont	12-18	12-18	6-8	6-12
Reticulocyte preference	Yes	Yes	No	No
Synchronous blood infection	No	No	Yes	Yes
Optimum temperature mosquito transmission	19-21	23-26	24-26	24-26
Oocyst size (µm)	<45	60-75	50	45-54
Sporozoites in glands (days post infection)	13-14	9-11	11-13	10-13
Mean diameter pre-erythrocytic schizonts (µm)	27	35-50	38-45	35
Duration of pre-erythrocytic cycle (h)	48-52	43-48	50-58	60-72
Duration of asexual blood stage cycle (h)	22-24	18	24	24
Developmental time gametocytes (h)	26-30	27	36?	27
Developmental time ookinete (h)	18-24	18-24	18-24?	18-24?
Microgamete size (µm)	15	16	-	-
Sporozoite size (µm)	11-12	14-16	10-15	11-21
Ookinete size (µm)	10-12	11	-	8-10

Table 1.1: Summary of the characteristics of rodent malarias parasites

This table was taken from the Leiden Malaria Research Group website (http://www.lumc.nl/con/1040/81028091348221/810281121192556/811070740182556/811070746282556/) and summarises data published elsewhere [60-63].

1.6 Antigenic targets for malaria vaccine development

The complex nature of the *Plasmodium* life cycle requires specialised protein expression for survival in both the invertebrate and vertebrate hosts, intracellular and extracellular survival, and invasion of multiple cell types. The genome consists of 5276 genes and over 2000 proteins have been identified [64]. A large number of antigens have already been identified as potential vaccine targets and many more will undoubtedly be identified in the future. However, the majority of vaccine research to date has focussed on a small number of antigens. Pre-erythrocytic vaccines have focussed primarily on circumsporozoite protein (CSP), thrombospondin related adhesion protein (TRAP), liver stage antigen 1 (LSA1) and liver stage antigen 3 (LSA3). A greater number of asexual blood-stage antigens have been identified, and in recent years a number of candidate gametocyte/transmission blocking antigens have also been identified including Pfs25, Pfs28, Pfs45/48, PfHAP2 and Pfs230. A selection of the leading candidate antigens and the stage at which they are expressed is shown in Figure 1.4.

In this thesis CSP, MSP1, AMA1 and MSP9 were used and are described in more detail below.

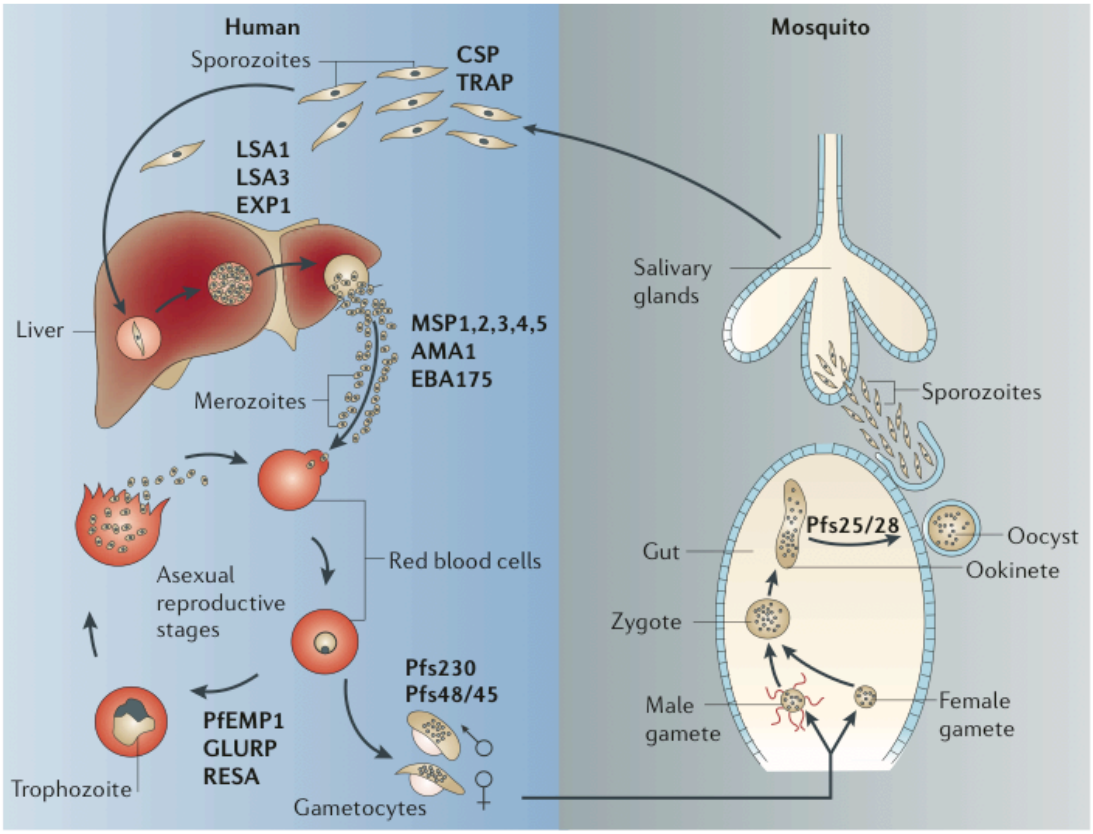


Figure 1.4: Candidate malaria vaccine antigens

A selection of candidate malaria antigens and the developmental stages at which they are expressed is shown. These include circumsporozoite protein (CSP), thromobospondin-related adhesion protein (TRAP), liver-stage antigen 1 (LSA1), LSA3, exported protein 1 (EXP1), merozoite surface protein 1 (MSP1), MSP2 – MSP5, apical membrane antigen 1 (AMA1), *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), ring infected erythrocyte surface antigen (RESA) [65], erythrocyte-binding antigen 175 (EBA175), glutamate rich protein (GLURP) and *P. falciparum* surface antigens (Pfs230, Pfs48/45 and Pfs25/28). This figure is taken from Winzeler, 2006 [66]. Copyright © 2006 Nature Publishing Group.

1.6.1 Circumsporozoite protein (CSP)

P. falciparum CSP is a 58kDa protein expressed abundantly on the surface of sporozoites [67] and also in exo-erythrocytic forms in the liver, and has therefore long been considered a good candidate vaccine antigen [68, 69]. It consists of a central repeat region with a non-repetitive region on either side. The central repeat region consists of 37-49 copies of asparagine-alanine-asparagine-proline (NANP) and a few asparagine-valine-aspartic acid-proline (NVDP) repeats. The function of the central repeat region is unknown, but it is highly conserved amongst *P. falciparum* strains, with mutations only altering the number of repeats. This central region contains immunodominant B cell epitopes, and antibodies against the central repeat region of this protein inhibit sporozoite invasion of hepatocytes [17]. The N-terminus is conserved amongst strains of *P. falciparum* and contains a 5 αα motif involved in sporozoite invasion of mosquito salivary glands [70] and binding to hepatocytes prior to invasion [71]. This motif is shared by all mammalian sporozoites [72]. The C-terminus is highly polymorphic and is involved in invasion of mosquito salivary glands, sporozoite motility and invasion of hepatocytes [73]. The leading candidate pre-erythrocytic vaccine, RTS,S, targets PfCSP and has shown significant efficacy against both infection and severe malaria in field studies [74].

The *P. yoelii* homologue of CSP (PyCSP) has been extensively studied. Protection against infection can be induced by a variety of vaccines expressing / encoding PyCSP [75-79] and is thought to be primarily due to $CD8^+$ T cells targeting the immunodominant epitope, $PyCSP_{280\text{-}288}$ [80, 81]; although a variety of other $CD4^+$ [82] and $CD8^+$ [83] T cell epitopes and antibody responses against the central repeat

region [76] may also contribute. Similarly, protection against *P. berghei* CSP is primarily mediated by $CD8^+$ T cells [36, 84].

1.6.2 Merozoite surface protein 1 (MSP1)

MSP1 is a leading blood-stage vaccine candidate and one of the most studied malaria parasite antigens. It is classically considered a blood-stage antigen, however it is also expressed during the late liver-stage of infection [85-87]. A 195 kDa *P. falciparum* MSP1 (PfMSP1) precursor is attached to the surface by a glycosylphosphatidylinositol (GPI) anchor and forms a protein complex with merozoite surface proteins 6 and 7 [88, 89]. During maturation proteolytic processing occurs resulting in fragments of 83, 28-30, 38-45 and 42 kDa. These remain non-covalently associated with MSP6 and MSP7 [90]. Prior to erythrocyte invasion further proteolytic processing of the 42 kDa fragment ($MSP1_{42}$) occurs into a 33 kDa ($MSP1_{33}$) and 10 kDa fragment ($MSP1_{19}$), (the 10 kDa fragment remains associated with the GPI anchor, resulting in a 19kDa fragment by electrophoresis). Membrane bound $MSP1_{19}$ remains on the merozoite surface during invasion of a red blood cell [91].

$MSP1_{19}$ has a flat disc-like structure with two epidermal growth factor (EGF)-like domains [92]. The structure is dependent upon six disulphide bonds and these provide some resistance to antigen processing [93, 94]. These structural constraints and the essential function of $MSP1_{19}$ result in a highly conserved structure [95], which is an attractive candidate malaria vaccine antigen.

Immunisation with $MSP1_{19}$ or $MSP1_{42}$ can protect both mice and macaques against blood-stage challenge [96-98]. High titre antibody responses against $MSP1_{19}$, but not $MSP1_{33}$, are reported to be essential for this protective immunity [99, 100]. The structure of the two EGF-like domains is necessary for the induction of protective immunity, as evidenced by immunisation with either domain alone, or following removal of the disulphide bonds by protein reduction, both of which abolishes protection in the *P. yoelii* model [101]. $CD4^+$ T helper (Th) epitopes within $MSP1_{33}$ can also provide help for $MSP1_{19}$-specific antibody responses [102]. The N-terminal $MSP1_{83}$ fragment can also induce partial immunity against *P. yoelii* [103], but $MSP1_{33}$ alone cannot [99, 102]. Furthermore, $CD8^+$ T cells targeting *P. yoelii* $MSP1_{33}$ can provide partial late liver-stage protection [86, 102].

Human adenovirus serotype 5 (AdHu5) and modified vaccinia virus Ankara (MVA) vectors expressing *P. yoelii* $MSP1_{42}$ ($PyMSP1_{42}$) given in a prime-boost regime, can provide partial liver-stage efficacy due to $CD8^+$ T cells targeting $PyMSP1_{33}$ [102], and protective blood-stage efficacy due to antibody responses against $PyMSP1_{19}$ [48, 102]. Phase I/IIa clinical trials of an optimized PfMSP1 vaccine, termed PfM128 [104], expressed by chimpanzee adenovirus serotype 63 (AdCh63) and MVA viral vectors has also been carried out at the Jenner Institute and showed a significant delay in diagnosis of patent parasitaemia [105].

1.6.3 Merozoite surface protein 9 (MSP9)

MSP9 (also known as acidic base repeat antigen, ABRA) was first identified in 1986 [106], but surprisingly has not been considered as a candidate vaccine antigen until

relatively recently. To date, homologues have been identified in seven *Plasmodium spp.* [38, 106, 107]. MSP9 is an 86.6 kDa protein synthesised by mature trophozoites and young schizonts and is located on the surface of merozoites and in the parasitophorous vacuole of an infected erythrocyte [106, 108, 109]. PfMSP9 has a cysteine-rich N-terminal region capable of binding to human erythrocytes [110, 111] and it has also been reported to bind to $MSP1_{42}$ [110, 112, 113]. Furthermore, it contains a chymotrypsin-like protease domain [114], and it has therefore been suggested that it may be involved in altering the erythrocyte membrane during invasion or proteolytic processing of other merozoite surface proteins [108, 110, 114]. The N-terminal region of both *P. falciparum* and *P. vivax* MSP9 are recognised by sera from naturally exposed individuals and antibodies against both the N- and C-termini of PfMSP9 can reduce parasite growth *in vitro* [115, 116]. Furthermore, T and B cell epitopes have been identified in mice [115, 117, 118]. A recent study induced protection against blood-stage *P. berghei* using recombinant PbMSP9 formulated in alum [38]. Protection was associated with high titre antibodies against PbMSP9.

1.6.4 Apical membrane antigen 1 (AMA1)

AMA1 (also previously known as Pf83 or Pk66) is expressed on the surface of sporozoites [119] and during the asexual blood-stages of the life-cycle in micronemes. After schizont rupture, the processed protein spreads circumferentially around the merozoite surface [120], where it plays an important role in erythrocyte invasion [121]. Vaccination with recombinant AMA1 protein can protect against blood-stage challenge in animal models [45, 122] and viral vectors expressing *P. chabaudi* AMA1 have similarly been found to protect against blood-stage challenge at the Jenner Institute [45]. AMA1-specific antibodies alone are sufficient for

protection in animal models [45], and in naturally exposed individuals high levels of antibodies to AMA1 are observed [123] which can inhibit parasite growth *in vitro* [124]. A role for $CD4^+$ T cells has also been identified in the *P. chabaudi* model, whereby $CD4^+$ T cell depletion reduces the protective immunity generated by PcAMA1 vaccines [53]. Furthermore, passive transfer of AMA1-specific $CD4^+$ T cell lines conferred some protection against *P. yoelii* and *P. chabaudi* in nude mice [53, 125].

1.7 Immunity to malaria and immune assays

1.7.1 Pre-erythrocytic immunity

Antibodies against sporozoites can prevent parasite invasion of the liver, but are not thought to be effective against intra-hepatic parasites. Antibodies targeting CSP are able to protect against *P. yoelii* sporozoite challenge [76]. Efficacy of RTS,S may also be partially antibody-mediated. A recent study in East Africa has suggested a non-linear relationship between anti-CSP antibody titres and protection conferred by RTS,S [14]. However, in previous studies a direct correlation with prevention of clinical disease was not observed [126, 127]. It has been suggested that anti-CSP antibodies may be able to directly neutralise sporozoites by inhibiting gliding motility and cell traversal. They may also act to opsonise sporozoites by activating the complement cascade, or may function through Fc γ receptor (FcγR)-mediated mechanisms to activate lysis by natural killer (NK) or NKT cells or engulfment by phagocytes [74]. CSP-specific antibodies induced by RTS,S are capable of inducing clearance of live sporozoites by phagocytes *in vitro*, and endocytic activity is significantly higher in protected individuals [128].

The importance of cellular immune responses against the liver-stage *Plasmodium* infection has been clearly established [78, 129], with infected hepatocytes the primary target. This immunity is undoubtedly complex and may involve a variety of mechanisms in a diverse human population. A detailed study in the *P. yoelii* mouse model identified six distinct T cell dependent immune mechanisms that could confer protection against sporozoite challenge [130]. This was dependent on the mouse strain and vaccine delivery system and involved a combination of factors including $CD8^+$ cells, $CD4^+$ cells, NK cells, IFN-γ, IL-12 and nitric oxide (NO). Key conclusions from these data were that vaccines should aim to induce $CD8^+$ T cell responses and IFN-γ [130]. A comparative study of *P. yoelii* and *P. berghei* recently identified that IFN-γ and TNFα are crucial to non-cytolytic pathways that underlie $CD8^+$ T cell-mediated immunity to liver-stage infection with both of these parasites [84].

1.7.2 Blood-stage immunity

Immunity against blood-stage malaria is thought to be primarily antibody-mediated and may be associated with antibodies targeting either the surface of the infected erythrocyte or blood-stage merozoites [131, 132]. The first evidence for this came from passive transfer studies in which immunoglobulin (Ig) from naturally immune adults were used to treat children with severe malaria [32, 133]. A large number of epidemiological studies have subsequently associated antibodies against blood-stage antigens with protection against infection [131, 134-136]. The development of natural immunity with age is thought to be associated with induction of broad multiple strain-specific antibodies through repeated exposure, rather than cross-reactive antibodies. Furthermore, it has increasingly been recognised that antibodies against a single

antigen may be protective, neutral, or even interfere with protective antibodies ('blocking') [137] and that identifying antibody fine-specificity is important in identifying correlates of protection [138-140].

Further evidence for the crucial role of antibodies in blood-stage immunity comes from rodent malarias. Antibodies are central to protection against *P. yoelii*. Passive transfer of $MSP1_{19}$- or AMA1-specific antibodies can confer protection against lethal *P. yoelii* infection in BALB/c mice [43-45]. PyMSP1 and PyAMA1 protein-in-adjuvant formulations can also protect against lethal blood-stage challenge [45, 47] and similar to NAI in humans, antibody-mediated immunity is strain and species specific [141, 142]. Clearance of chronic *P. chabaudi* infection is also primarily dependent upon antibodies [143].

Antibody-mediated protection may occur through a variety of effector mechanisms [144]. Neutralising antibodies specific for merozoite antigens can inhibit erythrocyte invasion by merozoites, whilst antibodies specific for antigens on the surface of infected erythrocytes may prevent vascular adhesion or schizont rupture. Alternatively antibodies may act in coordination with cells, such as monocytes, to inhibit parasite replication (antibody-dependent cellular inhibition, ADCI) [145]. A large body of work is being carried out to develop functional antibody assays. These include the *in vitro* ADCI assay [146] and growth inhibition assay (GIA) and the antibody-dependent respiratory burst assay using opsonised merozoites mixed with neutrophils (ADRB) [147].

Studies using *P. chabaudi* have highlighted the importance of $CD4^+$ T cells in blood-stage protection against this parasite. Studies with knock-out mice have shown that control of peak infection levels with *P. chabaudi chabaudi* AS is mediated by $CD4^+$ Th1 cells, IL-12 and IFN-γ, in the absence of antibodies [148-152]. Clearance of the chronic phase requires $CD4^+$ Th1 or Th2 cells as well as B cells producing antibody [152-154]. Furthermore $CD4^+$ T cells against $MSP1_{33}$ and AMA1 raised by immunisation can provide help for B cell responses and mediate protection in the absence of antibody [52-54]. In *P. yoelii* the role of $CD4^+$ T cells is less well defined, but $CD4^+$ T cells targeting $MSP1_{33}$ are known to be crucial in providing help for $MSP1_{19}$ antibodies responses that protect against *P. yoelii.*

$CD8^+$ T cells against blood-stage antigens may be associated with increased cerebral malaria during *P. berghei* infection [155, 156]. However, they have also been associated with protection against blood-stage *P. yoelii* in adoptive transfer experiments [157]. Studies at the Jenner Institute in which individuals were immunised against PfMSP1 and developed strong $CD8^+$ T cell responses to this antigen, showed no evidence of reduced parasite multiplication rates. Importantly they also showed no evidence of immunopathology as compared to non-immunised individuals upon development of patent *P. falciparum* infection [105].

1.8 Viral vectored vaccines

Immunity to a variety of infectious diseases, including HIV, TB and malaria, will likely require potent T cell, as well as antibody, responses. Recombinant protein vaccines usually induce antibodies, but they are often poor at inducing cell-mediated

immunity. Furthermore, they often require co-administration of adjuvants, which can have unpleasant side-effects. Unlike protein-in-adjuvant formulations, a variety of vectored vaccines are being developed, many of which are capable of inducing both T cell and antibody responses. These include DNA vaccines, recombinant bacterial vaccines such as recombinant BCG and recombinant salmonella, as well as a growing number of attenuated viral vectored vaccines [158]. The work described in this thesis focuses on viral vectored vaccines, many of which have been found to be capable of inducing both strong T cell and antibody responses.

Following more than one immunisation with the same viral vector, there is potential for the development of anti-vector immunity. In order to overcome this problem a heterologous prime-boost approach was developed. This involves priming using one viral vector and then boosting using a second viral vector, both recombinant for the same target antigen. This approach maximises the host response to the vaccine insert. A large variety of prime-boost vaccination strategies utilising antigens from a variety of infectious diseases have been tested in both animals and humans. These studies have consistently found that DNA vectors, Fowlpox virus (FPV) and influenza virus are good at priming T cell responses, whilst poxviruses such as MVA and New York attenuated vaccinia virus (NYVAC), are good at boosting immune responses. Recombinant adenoviruses (Ad) have been identified as extremely good vectors for both priming and boosting antibody, as well as T cell, responses. These data have been summarised by Draper and Heeney, 2010 [158] and have led to growing recognition that the most efficient regimes, currently known, for inducing potent T cell and antibody responses are Ad_MVA, heterologous Ad_Ad, or DNA_Ad regimes (prime_boost). It has been suggested that Ad may be good at priming due to

prolonged high-level expression of antigen following immunisation, whereas MVA may be a poor priming agent, but good at boosting, due to a short burst of antigen expression from this virus [158, 159].

This thesis focuses on Ad_MVA prime-boost immunisation regimes and these viral vectors are described in more detail below.

1.8.1 Adenovirus

Adenoviruses form the family *Adenoviridae*. They are 60-90nm, non-enveloped, icosahedral particles with a protein capsid (240 hexons and 12 pentons) containing a 34-43 kbp double-stranded DNA genome [160]. Over fifty human adenovirus (AdHu) serotypes have been identified based on cross-neutralising antibody responses to hexon protein and the terminal knob of the penton fibre. These have been divided into 6 subgroups (A – F). AdHus cause mostly mild and self-limiting disease in immunocompetent individuals, but in immunodeficient adults or children disease can be life-threatening [161, 162].

Different subgroups of Ad have distinct tissue tropism and clinical outcomes, primarily due to use of differing primary receptors on the cell surface. Viruses from all subgroups (except group B) can bind the Coxsackievirus and adenovirus receptor (CAR), which is expressed on many cell types, although only at low levels on dendritic cells (DCs) [163]. Following binding to CAR, the virion is internalised due to interactions between integrins on the cell and Arg-Gly-Asp (RGD) motifs in the penton base [164]. Some subgroup B viruses do not bind CAR; for example, AdHu3

attaches to the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on APCs [165], whereas AdHu35 binds CD46 [166].

The adenovirus genome is well characterised and easy to manipulate [167]. For safe use as a vaccine vector, the adenovirus is rendered replication-deficient by deletion of the E1 gene. This can be supplied *in trans* for growth of replication deficient adenoviruses in tissue culture [168]. Most adenoviral vectors have been further modified by deletion of the E3 gene to provide additional transgene capacity.

The greatest problem with adenovirus-based vaccines is the presence of pre-existing immunity in the human population to prevalent adenoviruses such as AdHu5 and AdHu35. 35-80% of human adults are seropositive for AdHu5-neutralising antibodies and 5-15% for AdHu35 [169]. To overcome this problem a variety of simian adenoviruses, for which pre-existing immunity is not prevalent in humans, are being developed as vaccine vectors. In Kenya it was found that 23% of children had high titre neutralising antibodies to AdHu5, whilst only 4% had high titre antibodies to AdCh63 [170]. Simian adenoviruses, C6, C7, C9 and AdCh63 expressing PfMSP1 have been found to be immunogenic in mice and rabbits in both Ad_MVA and heterologous Ad_Ad immunisation regimes [104] and vectors expressing AdCh63 have been taken forward for human clinical trial [105, 171, 172].

The safety of adenoviral vectors as vaccines was questioned following a HIV trial in which 3000 HIV sero-negative volunteers were immunised with AdHu5 expressing the HIV-1 antigens gag, pol and nef (The Step Study). The trial was halted due to futility at the first interim analysis. However analysis also revealed a non-significant

trend for an increased HIV-1 infection rate in immunised volunteers who were AdHu5 sero-positive prior to immunisation [173]. Encouragingly, a detailed follow-up analysis of the data found no link between the vaccination and increased infection with HIV-1 [174], although such data have put in significant doubt the short-/medium-term use of this promising vector.

1.8.2 Modified Vaccinia virus Ankara (MVA)

MVA is a member of the *Poxviridae* family. Poxviruses are large, double-stranded DNA viruses, contained within an envelope and encoding more than 100 proteins. Wild-type viruses replicate in the cytoplasm using their own transcriptional machinery and are therefore non-oncogenic and non-integrating.

Wild-type Vaccinia virus (VV) was used as a vaccine extensively during the smallpox eradication campaign. However, safety concerns arose due to infection of immunocompromised individuals, and for this reason a variety of attenuated Vaccinia virus strains were developed. Amongst these was MVA, which was developed by more than 530 passages of wild-type chorioallantois vaccinia virus Ankara (CVA) strain in chick embryo fibroblasts (CEFs). This resulted in loss of over 15% of the genome and an inability to replicate in most mammalian cells [175-180]. Towards the end of the smallpox eradication campaign MVA was delivered to over 120,000 people, without serious side-effects, despite many individuals being immunocompromised and at risk from the previously used replicating Vaccinia virus vaccine [176, 181].

Importantly, MVA can efficiently express foreign genes up to 25 kbp [182, 183], with equivalent, if not greater, immunogenicity to wild-type VV recombinants [179, 184]. These properties make MVA a safe and immunogenic viral vector for use as a vaccine delivery platform.

Production of viral RNA from MVA is driven by viral promoters that fall into three different classes – early, intermediate and late. Early promoters act immediately after infection, as the enzymes required for RNA synthesis, capping and polyadenylation are brought into the cell within the infectious virion. However intermediate and late genes cannot be synthesised until after DNA replication as they require enzymes synthesised *de novo*. Classically foreign antigens are inserted into MVA after the P7.5 promoter which has the ability to regulate both early and late transcription from two different start sites within the same coding sequence [185]. However, other promoters such as modified H5 (mH5), short synthetic promoter (SSP) [186] or the late promoter, I1L, have also been described and could be used to increase the immunogenicity of MVA vaccines [187-189]. A variety of other promoters within the MVA backbone are currently under investigation at the Jenner Institute (T. Orubu, unpublished).

1.9 The current status of malaria vaccine development

1.9.1 Pre-erythrocytic vaccines

Complete protection of humans against *P. falciparum* sporozoite challenge was first demonstrated in 1973 [29]. Protection is long-lasting and efficacious against heterologous *P. falciparum* strains. However, it requires the bites of more than 1000 *P. falciparum* sporozoite-infected irradiated mosquitoes spread over 5 - 19 biting

sessions [23]. This is estimated to deliver between 100,000 and 300,000 irradiated sporozoites (Irr-spz) per individual [190]. Sanaria (Seattle, USA) are attempting to develop this model into a viable malaria vaccine, however a huge number of logistical challenges need to be overcome, including producing enough irradiated sporozoites to clinical GMP standard, storage of the vaccine and immunisation route. Sanaria have now developed technology to cryopreserve sporozoites and with a team of only six individuals can dissect more than 500 mosquitoes per hour. However, they have found in *P. yoelii* that cryopreservation leads to a requirement for 2 – 4 times as many Irr-spz for protection. Furthermore, immunisation by the intradermal (i.d.) route rather than intravenous (i.v) route also requires 2 – 4 times more Irr-spz. Therefore it may require 4 – 16 times more Irr-spz to protect people against *P. falciparum* if they are immunised with cryopreserved Irr-spz by the i.d. route [190]. This potentially amounts to immunisation with up to 4.8 million Irr-spz per individual! Clinical trials are currently underway to assess the effect(s) of dose, route and cryopreservation on the efficacy of Irr-spz vaccines, but it is likely that the high doses required and storage requirements will make this immunisation regime unfeasible for deployment or mass scale use.

An alternative whole parasite approach is the development of genetically-attenuated sporozoites, which cannot complete liver-stage development. However, in rodent models this approach has been limited by breakthrough blood-stage infections [24, 191]. More recently, double knock-outs have been found to prevent this problem in *P. yoelii* [192] and clinical trials are currently being carried out.

Two candidate vaccine approaches, targeting single pre-erythrocytic antigens rather than whole parasites, have shown significant efficacy against *P. falciparum* in clinical trials. The first is RTS,S, which is the leading candidate malaria vaccine currently in development. It has taken 30 years to develop from first conception to phase III trials, which are currently underway across Africa. RTS,S is based upon the CSP protein of *P. falciparum* strain NF54, clone 3D7 and consists of 19 copies of the central NANP repeats (R) and the entire C-terminal region of the protein ($\alpha\alpha$ 207-395) containing T cell epitopes (T). This is fused at the N-terminus to hepatitis B surface antigen (HBsAg) (S), which can form viral particles in the absence of DNA (altogether this construct is RTS). In order to stabilise the protein it is co-expressed in yeast with wild-type HBsAg (S), such that the final product is 25% RTS and 75% S. Seven separate clinical trials in malaria-naïve volunteers tested a variety of adjuvants, doses, and intervals between immunisations. In these studies protection ranged from 0 – 85.7%. A large number of field trials have also been carried out with variable results. In reported studies, efficacy against infection ranged from 11% - 71%; efficacy against clinical episodes has ranged from 25.6% - 59%; and efficacy against severe malaria has been 38.3 – 57.7% [74]. The longevity of protection has not been well-established, although is thought to be in the range of 18-24 months. In Mozambican babies, immunisation with RTS,S conferred protection (65.9%) against *P. falciparum* infection for 3 months, but protection had waned by 6 months [193]. However, Tanzanian infants were protected from infection for the whole 6 month trial period (65.2%) [194]. Phase III trials of RTS,S are currently underway across Africa and it is highly likely that this vaccine will be licensed for use within the next few years. However, given the increased awareness and implementation of existing control

strategies, such as ITNs, it is unlikely that this partially effective control measure will be highly cost-effective [12].

The second approach that has shown significant efficacy is viral vectored vaccines expressing ME-TRAP (a multiple epitope string (ME) from six pre-erythrocytic antigens fused to TRAP). ME-TRAP expressed by DNA and MVA vectors in a heterologous prime-boost regime induced partial protection against *P. falciparum* 3D7 strain sporozoite challenge, but not sterile protection in malaria naïve volunteers [195]. However, sterile protection was achieved in 2/5 volunteers by replacing DNA with the more immunogenic vector, Fowlpox strain FP9 (FP9) [196]. Disappointingly, however, immunogenicity of these constructs in the field was significantly lower [197-199]. Recently, more immunogenic viral vectors have been developed at the Jenner Institute. A phase I/IIa clinical trial of AdCh63 and MVA expressing ME-TRAP showed significant sterile efficacy in malaria naïve adults with 21% sterilely protected (3/14) and a further 36% (5/14) partially protected [171, 172]. Phase Ib safety and immunogenicity trials of this immunisation regime are now underway in Kenya and The Gambia.

1.9.2 Blood-stage vaccines

Over 30 Phase I/II trials of candidate blood-stage malaria vaccines have been carried out in recent years [200]. These have primarily been classical protein-in-adjuvant formulations of *P. falciparum* MSP1 or AMA1 either alone or in combination with other vaccines. However, the majority have not assessed efficacy.

One interesting blood-stage malaria vaccine candidate is 'Combination B'. This contains the blood-stage antigens RESA, MSP1 and MSP2 formulated in the adjuvant Montanide ISA 720. This vaccine reduced parasite density by 62% in children in Papua New Guinea. However, disappointingly the efficacy was specific to the homologous MSP2 allelic strain and there was no overall benefit in relation to clinical outcome [201].

AdCh63 and MVA viral vectors expressing an optimised *P. falciparum* MSP1 immunogen (termed PfM128) [104], were recently trialled in a small phase I/IIa study at the Jenner Institute. These vaccines were found to be safe and highly immunogenic and in the three individuals challenged with *P. falciparum* sporozoites a significant delay in onset of patent parasitaemia was observed ($P = 0.032$) [105]. Although the study was small, this is an extremely promising start for the alternative vectored delivery of blood-stage candidate antigens.

1.9.3 Multi-stage vaccines

It is widely acknowledged that a multi-stage and/or multi-antigen formulation is likely to be necessary to provide high-level efficacy. Any such vaccine will only warrant deployment if it provides significantly greater efficacy than RTS,S, as well as pre-existing pre-erythrocytic control measures such as ITNs, IRS and ACT. It is suggested that a second generation vaccine will need to provide greater than 80% efficacy for at least four years [11] in order to justify deployment.

One possible strategy that has been investigated pre-clinically is the combination of RTS,S with other recombinant protein-in-adjuvant vaccine candidates. Experiments

have been carried out in rhesus macaques to assess the merits of RTS,S/AS02$_A$ (a squalene-in-water emulsion containing monophosphoryl lipid A and the saponin, QS21) administration in combination with protein vaccines for the blood-stage antigens MSP1 and AMA1 [202]. This study found that AMA1-specific antibody responses are reduced when AMA1 is co-administered with either RTS,S or MSP1$_{42}$ either as a mixture, or at separate sites. AMA1-specific IFN-γ and IL-5 production was also reduced when AMA1 was co-administered with MSP1$_{42}$. This work highlighted the issue of antigenic competition in multi-component malaria vaccine formulations, and demonstrated that immune interference may be complex and antigen dependent. However, a wide variety of protein vaccines are routinely given together during childhood as part of the EPI schedule [203]. RTS,S has twice been trialled as part of the EPI schedule (in two different adjuvants, AS01$_E$ and AS02$_D$) along with diphtheria, tetanus, and whole-cell pertussis conjugate vaccine, hepatitis B (HepB) vaccine, Haemophilus influenzae type B (Hib) vaccine, oral polio vaccine (OPV), measles vaccine, and yellow fever vaccine. In both trials antibodies towards some antigens tended towards being lower when RTS,S was co-administered, but EPI responses all passed the pre-defined non-inferiority criteria and RTS,S thus does not significantly interfere with EPI [13, 194]. The impact of EPI on RTS,S immunogenicity was not directly assessed, but anti-PfCSP antibody titres were lower than in previous trials in which EPI and RTS,S had been staggered [15, 193], suggesting that co-administration with EPI vaccines may interfere with RTS,S immunogenicity [194].

Another body of work has been carried out investigating mixtures of DNA and poxvirus vaccines encoding four to nine malaria antigens in mice and macaques [204-209]. Similarly, this work established that mixtures of such vectored vaccines can lead to complex and antigen-specific interference for both antibody and T cell responses.

Despite these findings the majority of phase I/IIa clinical trials investigating multi-antigen and/or multi-stage malaria vaccines have not investigated the immunogenicity of each antigenic component alone, nor assessed individual contributions to efficacy. These include protein vaccines such as GMZ2 [210], PfCP2.9 [211] and combination B [212], as well as multi-antigen strings expressed in DNA plasmids or viral vectors such as NYVAC-*Pf*7 [213], and L3SEPTL polyprotein [214]. In another study, a mixture of five DNA plasmids exhibiting non-interference in pre-clinical models was identified and taken forward for clinical trial, but potential immune interference was not directly assessed in humans and the vaccine was not efficacious in a phase IIa sporozoite challenge trial [208]. Similarly, studies in the field of HIV-1 vaccines have used mixtures of 3-4 DNA and/or poxvirus or AdHu5 vaccines, but any potential effect(s) of immune interference between the individual components and their encoded antigens have not been reported [215, 216].

Two studies have been carried out in which individual components in a multi-antigen subunit/vectored vaccine have been directly assessed in humans when administered alone and in combination. The first was a phase Ia trial of the PEV3A vaccine containing virosome-formulated synthetic peptide antigens from *P. falciparum* AMA1 and CSP. Antibody responses were found to be unaffected by co-

administration of the antigens, but T cell responses were not assessed [217]. In a second study PEV3A was co-administered with a viral vectored vaccine regime, consisting of two vaccinations with FP9 and a third vaccination with MVA expressing ME-TRAP (FFM ME-TRAP) [218]. In this study total IgG responses against both AMA1 and CSP were not affected by the co-administration of the ME-TRAP vaccines, and in fact AMA1-specific IgG responses were increased after the third vaccination. However, CSP-specific IgG was less avid in the combination vaccine group and higher mean IFA titres were induced by immunization with PEV3A alone compared to the combination vaccine. Furthermore, the geometric mean IFN-γ ELISPOT response to ME-TRAP was 50 spot forming units (SFU)/10^6 peripheral blood mononuclear cells (PBMC) compared to a mean of approximately 450 SFU/10^6 PBMC in previous trials [195]. This suggests that T cell responses may have been affected by co-administration of these two vaccine candidates, although a control group to directly assess FFM ME-TRAP responses alone was not included in this study. Taken all together, these data highlight the importance of investigating the individual antigenic components within a multi-antigen vaccine and their impact on vaccine immunogenicity and efficacy in both pre-clinical and clinical studies.

1.10 Molecular adjuvants

The development of highly efficacious vaccines against difficult disease targets such as malaria, tuberculosis, HIV-1 and pandemic influenza, will likely require the induction of strong cellular immunity as well as antibody responses. Whilst classical protein-in-adjuvant vaccines primarily induce antibodies, viral vectored vaccines are being developed as a means of inducing both T cell and antibody responses [158]. The development of adjuvants to enhance the T cell responses induced by viral

vectored vaccines may be crucial to the ultimate development of highly effective vaccines against such difficult infectious diseases and also cancers.

1.10.1 The oligomerisation domain of the α-chain of C4b-binding protein (C4bp)

The oligomerisation domain of complement C4b-binding protein (C4bp) α-chain has been identified as an adjuvant of antibody responses in both protein and viral vectored vaccination regimes [48, 219]. Full-length native C4bp is an inhibitor of the classical and lectin pathways of complement activation. It consists of six to seven α-chains with or without a β-chain, linked together by an oligomerisation domain at the C-terminus of the α-chains [220]. This oligomerisation domain is 57 amino acid residues in humans and 54 amino acid residues in mice. It contains two cysteine residues and an amphipathic α-helix region, which are both necessary and sufficient for oligomerisation [221]. Previous studies have shown that fusion of this domain to other proteins causes them to oligomerise [219, 222-224]. When the murine C4bp oligomerisation domain (henceforth known as IMX108 [219]) was fused to the 19 kDa C-terminus of *P. yoelii* $MSP1_{19}$ recombinant protein it increased antigen-specific antibody responses following vaccination and enhanced protection against challenge with pRBC in mice [219]. Furthermore, if IMX108 is fused to $PyMSP1_{42}$ in AdHu5 and MVA viral vectored vaccines given in a prime-boost regime eight weeks apart, then clearly enhanced antibody responses are induced [48].

Due to induction of auto-antibodies against murine C4bp in mice following immunization with the fusion protein $PyMSP1_{19}$+IMX108, C4bp α-chain oligomerisation domains from a variety of other mammalian and avian species have

been tested [219]. All formed soluble heptameric proteins, and induced higher antigen-specific antibody titres in mice than $PyMSP1_{19}$ protein alone or in Freund's adjuvant. The most immunogenic form, termed IMX313, is a hybrid derived from two chicken C4bp α-chain oligomerisation domains. It was designed to minimize similarity to other mammalian C4bp α-chain domains and has less than 20% amino acid identity to human C4bp. The IMX313 sequence therefore minimizes the potential for auto-antibody induction in humans, and is a candidate "molecular adjuvant" for enhancement of vaccine-induced immune responses in humans.

1.10.2 IgG Fc fragment

Although the mechanism by which viral vectored vaccines prime immune responses is poorly understood, the role of professional antigen presenting cells (APC), such as DCs, is likely to be crucial. Therefore targeting antigens for uptake by DCs is one possible strategy for enhancing the potency of viral vectored vaccines.

DCs express Fc γ receptors (FcγR), which bind to the Fc portion of IgG and cause DC to take up immune complexes (IC) by endocytosis. Antigen internalised by DC may then be efficiently presented on MHC class II to stimulate $CD4^+$ T cell responses, or cross-presented on MHC class I and result in $CD8^+$ immune responses [225, 226]. In addition, binding of antibodies to the FcγR causes activation of the DC and results in up-regulation of both surface molecules and cytokines involved in antigen presentation [227].

A DNA vaccine construct consisting of Hepatitis B e (HBe) antigen fused to the human IgG1 Fc fragment has previously be shown to be highly immunogenic in

C57BL/6 mice as compared to the same vaccine without the Fc fragment. This vaccine enhanced $CD4^+$, $CD8^+$ and IgG antigen-specific responses and was dependent upon binding to an unidentified FcγR, as the enhanced responses were lost in FcγR knock-out mice, which lacked FcγRI, FcγRII and FcγRIII genes [228]. A number of studies have also found that protein-Fc fusion constructs are immunogenic [229-231], in one study investigating the HIV-1 antigen, gp120, finding significantly higher antibody responses if the protein included the human IgG1 Fc fragment. This was dependent on binding to FcγR, as 2 αα residue alterations that reduced FcγR binding 10-100 fold, led to reduced antigen titres [232]. The feasibility of this method for enhancing the immunogenicity of viral vectored vaccines has not previously been investigated.

1.11 Thesis Aims

- To assess the immunogenicity and efficacy of a multi-antigen/multi-stage viral vector formulation in the *P. yoelii* mouse model, and to investigate mechanisms of any observed antigenic interference with regard to T and/or B cell responses.

- To assess the feasibility of protecting against *P. berghei* and transgenic *P. berghei* blood-stage parasites using viral vectored vaccines.

- To investigate whether immunogenicity of blood-stage antigens can be enhanced using small molecular adjuvants.

- To assess the impact of promoter strength on immunogenicity of MVA vaccines.

1.12 Thesis overview

Chapter 2 describes materials and methods.

Chapter 3 investigates the immunogenicity and efficacy of AdHu5 and MVA viral vectored vaccines expressing *P. yoelii* CSP and $MSP1_{42}$. It then investigates a multi-stage vaccination strategy using a mixture of these two antigens in BALB/c mice.

Chapter 4 investigates the mechanisms of $CD8^+$ T cell interference observed post-boost when $PyMSP1_{42}$ and PyCSP vaccines are given as a mixture.

Chapter 5 investigates blood-stage protection against *P. berghei* and transgenic *P. berghei* expressing *P. falciparum* $MSP1_{19}$ by viral vectored vaccines.

Chapter 6 describes attempts to enhance blood-stage viral vectored vaccine immunogenicity using small molecular adjuvants in adenoviral vaccines or stronger promoters to drive antigen expression in MVA.

Chapter 7 summarises the results of this thesis, considers their contribution to the malaria vaccine field and discusses future directions.

Chapter 2

Materials and Methods

2 Materials and Methods

Details of all materials and methods used in this thesis are given here. For some assays detailed Jenner Institute protocols (J#) are provided in the Appendix.

2.1 Materials

2.1.1 Reagents

All commercially available reagents used in this study are described in Table 2.1 below.

Table 2.1: Reagents

Material	**Supplier**	**Catalogue Number**
293A cell line	Invitrogen	R705-07
Adenopure® Kit	PureSyn	70100
Agarose	Sigma	A9539
Alexa Fluor® 488 conjugated goat anti-mouse IgG (H + L)	Invitrogen	A-11001
Alkaline phosphatase conjugated goat anti-mouse total IgG	Sigma	A3562
Alkaline phosphatase conjugated goat anti-mouse total IgM	Sigma	A9688
Alkaline phosphatase conjugated goat anti-rabbit total IgG	Sigma	A8025
Ammonium chloride (NH_4Cl)	Sigma	A-4514
Ampicillin	Sigma	A5354

Antisedan™	Pfizer Animal Health	Oxford University veterinary services (OUVS)
AP conjugate substrate kit (ELISpot development)	BIO-RAD	170-6432
APC anti-mouse IFN-γ (clone XMG1.2)	eBioscience	17-7311-82
β-mercaptoethanol	Sigma	M3148
SIMGA*FAST*™ BCIP®/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium)	Sigma	B5655
Benzonase	Novagen	70664-3
Biotinylated rat anti-mouse IgG1	BD biosciences	553441
Biotinylated rat anti-mouse IgG2a	BD biosciences	553388
BSA (for restriction enzyme digests)	NEB	B9001S
BSA fraction V	Sigma	85040C
BugBuster® GST•Bind™ purification kit	Novagen	70794
Carbonate-Bicarbonate buffer	Sigma	C-3041
CD11c MicroBeads	Miltenyi Biotech Ltd	130-052-001
Caesium chloride (CsCl)	Sigma	203025
CFSE	Invitrogen	C34554
Chloramphenicol (γ-irradiated phial)	Sigma	C7795
CIP phosphatase (Calf intestinal phosphatase)	NEB	M0290
Collagenase/Dispase®	Roche	10 269 638 001
Colorplus Prestained Protein ladder, Broad range (10-230kDa)	NEB	P7711S

Cytofix/Cytoperm™ fixation permeabilisation kit with BD GolgiPlug (includes PermWash)	BD Biosciences	555028
DH5α competent *E. coli*, one-shot	NEB	C2987
DH5α competent *E. coli*, sub-cloning efficiency	Invitrogen	18265-017
Diethanolamine	Sigma	D8885
Diethanolamine Buffer (5x)	Fisher Scientific UK Ltd	34064
DMSO hybri-max®	Sigma	D2650
Domitor™	Pfizer Animal Health	OUVS
Donkey anti-mouse AP	Jackson Labs	715-055-150
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose	Sigma	D6546
Dulbecco's phosphate buffered saline	Sigma	D8537
EDTA 0.5M pH 8	Sigma	E7889
eFluor® 450 (Pacific Blue® replacement) anti-mouse CD4 (clone RM4-5)	eBioscience	48-0042
ExtrAvidin	Sigma	E2636
ExtrAvidin alkaline phosphatase conjugate	Sigma	E2636
Fc-block (anti mouse CD16/32)	eBioscience	16-0161-86
Ficoll®	Sigma	F2637
FITC anti-mouse TNFα (clone MP6-XP22)	eBioscience	11-7321-82
Foetal Calf Serum (FCS)	Sigma	F-2442
Formalin solution, neutral buffered, 10% (4% formaldehyde)	Sigma	HT5011
Gateway® LR Clonase™ II Enzyme Mix	Invitrogen	11791

Giemsa	Sigma	GS1L
Glycine	Sigma	G8898
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma	A3562
Goat anti-rabbit whole IgG alkaline phosphatase conjugate	Sigma	A8025
GolgiPlug	BD biosciences	555029
HCl	Sigma	S-7653
HEPES	Sigma	H3375
Immunopure IgG Binding Buffer	Pierce	21011
Immunopure IgG elution buffer	Pierce	21009
Immunopure Plus immobilized Protein G gel	Pierce	22852
IPTG (Isopropyl β-D-1 thiogalactopyranoside)	Sigma	I5502
Isofluorane	Pfizer Animal Health	OUVS
Isopropanol (propan-2-ol)	Fisher Scientific	P/7490/21
Kanamycin	Sigma	K0254
Ketastat™	Fort Dodge Animal Health	OUVS
$KHCO_3$	Sigma	P-9144
L-glutamine	Sigma	G-7513
LB agar tablet	Sigma	L7025
LB broth tablet	Sigma	L7275
Lipofectamine™ 2000	Invitrogen	11668
Magnesium chloride ($MgCl_2$)	Sigma	630-69
Methanol	Sigma	34860
MinElute gel extraction kits	QIAgen	28606

Minimal Essential Media (α-modification)	Sigma	M4526
Mouse IFN-γ ELISPOT kit	Mabtech	3321-2A
Na_2EDTA	Sigma	ED2SS
Nitrocellulose membrane	Bio-Rad	162-0117
OptiMEM	Invitrogen	11058-21
Orange G	Sigma	03756
p-nitrophenylphosphate substrate (pNPP)	Sigma	N-2765
Pacific Blue® anti-mouse CD4 (RM4-5)	eBioscience	57-0042-82
PBS (ready made, without Ca^{2+} and Mg^{2+})	Sigma	D8537
PBS 0.01M tablets	Sigma	P4417
PBS powder (Dulbecco's PBS for 10L)	Invitrogen	21600-069
PBS/Tween 0.01M powder	Sigma	P3563
pcDNA™4/TO	Invitrogen	V102020
pCR®-Blunt II-TOPO® PCR cloning kit	Invitrogen	K280020
PE anti-mouse CD11c (Clone N418)	eBioscience	12-0114-81
PE anti-mouse IL-2 (Clone JES6-5H4)	eBioscience	12-7021-82
Pen/Strep	Sigma	P-0781
pENTR™4	Invitrogen	11818-010
PerCP-Cy5.5 anti-mouse CD8 (clone 53-6.7)	BD Biosciences	551162
pGEM®-T Easy	Promega	A1360
pGEX-2T	GE Healthcare	28-9546-53
Precast minigels	Pierce	Various
Proteinase K	Invitrogen	25530
QIAgen plasmid midi kit	QIAgen	12143
QIAprep spin miniprep kit	QIAgen	27106
QIAquick® gel extraction kit	QIAgen	28706

Restriction buffers	NEB	Various
Restriction enzymes	NEB	Various
Rosetta™ 2 competent cells	EMD4Biosciences (Novagen)	71402
RPMI-1640	Sigma	R0883
S. O. C. medium	Invitrogen	15544-034
SDS	Gibco	15553-035
Smart ladder	Eurogentec	MW1700
Sodium azide (NaN_3)	Sigma	S8032
Sodium Bicarbonate ($NaHCO_3$)	Sigma	
Sodium Carbonate (NA_2CO_3)	Sigma	S-2127
SYBR safe (10000x in DMSO)	Invitrogen	S33102
T-REx™-293A cell line	Invitrogen	R71007
T4 DNA ligase and buffer (5x)	Invitrogen	15224-041
T4 DNA ligase and reaction buffer (10x)	NEB	M0202
Terrific Broth, modified	Sigma	T0918
Tetracycline (γ-irradiated phial)	Sigma	T8032
Tris base (Trizma® Base)	Sigma	T1503
Tris-acetate-EDTA (50x solution)	Fisher Scientific	BP1332-1
Tris-Hcl (Trizma® hydrochloride buffer solution)	Sigma	T2694
Trypan Blue	Sigma	T8154
TrypLE™ Express	Invitrogen	12605010
Tween® 20 (500mL, BioXtra, viscous liquid)	Sigma	P7949
ViraPower™ Adenoviral Gateway® Expression Kit	Invitrogen	K4930-00
Water (Double processed, tissue culture)	Sigma	W3500

Water (Sterile, RNAse and DNAse free)	Sigma	W4502

2.1.2 Equipment

All specialised equipment used in this study are described in Table 2.2 below.

Table 2.2: Equipment

Material	**Supplier**	**Catalogue No.**
CASY Counter	Schärfe Systems	N/A
Cell Strainer, 70μm	BD Falcon	352350
CyAN-ADP flow cytometer	Dako	N/A
MaxiSorp Nunc-Immuno 96 MicroWell™ Solid Plates	Thermo Scientific	460984
LS columns	Miltenyi Biotec Ltd	130-042-401
LSR II flow Cytometer	BD Biosciences	N/A
MAIP ELISPOT Plates	Millipore	MAIPS4510
Microvette serum collection tubes	Sarstedt	16.444
MidiMACS™ Separator and MultiStand	Miltenyi Biotec Ltd	130-042-301 & 130-042-303
MoFlo™ XDP	Beckman Coulter	N/A
Needles, 26G	BD Microlance	304300
Syringes, 0.5mL with 29G needle	BD microfine	324892
Syringes, 1mL, without needle (i.m. immunisation)	BD Plastipak	300013
14 mL Ultraclear tubes	Beckman Coulter	344060
Dialysis Cassette 3-12 mL and 0.5 – 3mL	Thermo Fisher	66453 &66455

2.1.3 Solutions and Buffers

Ammonium Chloride Potassium **(ACK) Lysis Buffer**: 8.29g ammonium chloride (NH_4Cl) (0.15M), 1g potassium bicarbonate ($KHCO_3$) (1mM), 37.2mg disodium ethylenediaminetetraacetic acid (Na_2EDTA) in 800mL distilled water (dH_2O). pH was adjusted to 7.2-7.4 with hydrochloric acid (HCl) before making a final solution up to 1L with dH_2O.

Antibiotic stock solutions:

1. **Ampicillin** (1000x): 100mg/mL in water. Ready to use solution stored at -20°C.
2. **Kanamycin** (2000x): 50mg/mL in 0.9% sodium chloride. Ready to use solution stored at 2-8°C.
3. **Chloramphenicol** (1000x): Dilute to 15mg/mL in methanol (MeOH). Store at 2–8°C.
4. **Tetracycline** (1000x): Dilute to 10mg/mL in ethanol (EtOH). Store at -20C.

Blood-Stage Culture Medium: 96ml RPMI-1640 plus 11mL foetal calf serum (FCS), 4.2mL 5% sodium bicarbonate ($NaHCO_3$) solution, and 5.5mg neomycin.

Complete Medium: Minimal Essential Media (MEM) (α-modification) was supplemented with 5mL L-glutamine (2mM), 5mL pen/strep (100U penicillin, 100μg streptamycin), 500μL β-mercaptoethanol (50μm) and 50mL batch-tested heat inactivated (56°C, 30 min) FCS (10%).

Coomassie Brilliant Blue Stain: 1.2g Brilliant Blue, 300mL MeOH, 60mL acetic acid: made up to 500mL in dH_2O.

Diethanolamine Buffer: 1 M diethanolamine buffer, pH 9.8, 0.5 mM magnesium chloride ($MgCl_2$): 97mL diethanolamine, 0.2g sodium azide (NaN_3) and 100mg magnesium chloride hexahydrate ($MgCl_2.6H_2O$) were added to 800 mL of dH_2O, pH was adjusted to 9.8 with HCl, and the solution was made up to 1L with dH_2O. Alternatively a 5x stock was purchased and diluted with dH_2O prior to use.

IgG purification neutralisation buffer: 1M Tris buffer, pH 9.0

IgG purification storage solution: 0.02% NaN_3 in dH_2O.

Laemmli non-reducing buffer: 125mM Tris-HCl pH6.3, 20% v/v glycerol, 4% w/v sodium dodecyl sulphate (SDS), 20mg/mL Brilliant Blue.

Laemmli reducing buffer: 125mM Tris-HCl pH6.3, 20% v/v glycerol, 4% w/v SDS, 20mg/mL Brilliant Blue, 1% v/v β-mercaptoethanol.

Lysogeny broth **(LB) agar:** 10 tablets in 500mL dH_2O (autoclaved the same day to avoid bacterial growth).

LB broth: 10 tablets in 500mL dH_2O (autoclaved the same day to avoid bacterial growth).

Loading dye: 0.4% orange G, 15% Ficoll®, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0): made up with autoclaved H_20.

Magnetic cell sorting (**MACS) buffer**: 100mL 10x PBS pH 7.4, 4 mL of 0.5 M EDTA pH 8.0 in 800mL dH_2O. 5g bovine serum albumin (BSA) Fraction V dissolved by gravity. The solution was sterilised through a 0.2µm Surfactant-Free Cellulose Acetate (SFCA) filter unit.

Phosphate buffered saline (**PBS**) 0.01M. Tablets dissolved in dH_2O (NaCl 0.138 M, KCl 0.0027 M, pH 7.4).

PBS/1% Formalin: 45mL ready made Dulbecco's PBS with 15mL formalin solution.

PBS/Tween (**PBS/T**) 0.01M. Sachets dissolved in dH_2O. (NaCl 0.138 M, KCl 0.0027 M, pH 7.4 Tween 0.05%). For washing plates, PBS powder was dissolved in 10L dH_2O with 5mL (0.05%) Tween-20.

Red Blood Cell Counting Solution: 10mL formalin, and 32g trisodium citrate ($Na_3C_6H_5O_7$) made up to 1L in dH_2O.

Tris-acetate-EDTA (**TAE**) **buffer**: Made up from 50x concentrate with dH_2O.

Transfer Buffer: 6g Tris base, 30g glycine and 200mL MeOH, made up to 1L in ultrapure dH_2O.

Tris-HEPES-SDS Running Buffer (1x): 100mM Tris, 100mM HEPES, 1% (~3mM) SDS, pH 8.0. (12.1g Tris Base (MW = 121), 23.8g HEPES (free acid MW = 238), 1g SDS (MW = 288) diluted to 1L in ultrapure dH_2O.

Western blot blocking buffer: 1.5g BSA in 50mL PBS.

Western blot washing buffer: 1mL Tween® 20 in 1L PBS.

6x SDS-PAGE Reducing Sample Buffer: 7mL 0.5M Tris pH 6.8 containing 0.4% SDS, 3mL glycerol, 1g SDS, 0.93g dithiothreitol (DTT), and 1.2mg bromophenol blue, made up to 10mL in water.

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

Transformation Storage Buffer (TSB): LB medium pH 6.1, 10% PEG, 5% DMSO, 10mM $MgCl_2$, 10mM $MgSO_4$.

Destain: 50mL methanol, 50mL acetic acid: made up 500mL in dH_2O.

2.2 Molecular Biology and cloning

2.2.1 Vaccine Antigens

For optimal expression in mammalian cells, a number of modifications were made to the antigens. Firstly, codon usage in *Plasmodium spps* differs substantially from that of mammals and optimising codon usage of DNA vaccines encoding *Plasmodium*

antigens for expression in mammalian cells can significantly enhance protein expression and immunogenicity [233]. Some antigens were codon optimised for expression in either mouse or human cells and were synthesised by Geneart AG (Regensburg, Germany). Some antigens were preceded by the human tissue plasminogen activator (tPA) leader sequence [234] (GenBank Accession No. K03021), which is known to enhance efficacy of MSP1 DNA-based vaccines [235, 236]. This was preceded by a Kozak sequence 5'-CGC GCC GCC ACC-3', which aids translation in eukaryotic cells [237]. Finally, some antigens included an in-frame PK-tag (αα sequence IPNPLLGLD) at the C-terminus of the antigen. This is recognised by anti-PK monoclonal antibody (also known as anti-V5), which enables detection of antigen expression in different vaccine delivery systems [238, 239]. Details of which antigens are codon optimised, include the Kozak sequence, tPA leader sequence and PK-tag are given in Table 2.3, page 83. Details of each individual antigen used are given below.

***P. yoelii* MSP1$_{42}$ (PyMSP1$_{42}$)**

P. yoelii YM MSP1 αα 1394-1757. This does not include the C-terminal GPI anchor signal sequence (αα 1758 – 1773), which may decrease immunogenicity [75, 240, 241]. AdHu5 and MVA vectors expressing this antigen were made by Dr. Simon Draper [48].

***P. yoelii* MSP1$_{42}$ IMX108 (PyMSP1$_{42}$-IMX108)**

P. yoelii YM MSP1 αα 1394-1754 fused at the C-terminus to the core oligomerisation domain of the α-chain of murine C4bp (αα 416-469), termed IMX108 [219]. Three C-terminal αα and the PK-tag were removed from PyMSP1$_{42}$

as compared to the construct without IMX108 described above. A shuttle vector encoding this antigen was originally made by J-B Marchand and F. Hill, Imaxio, France, and AdHu5 and MVA vectors expressing this antigen were made by Dr. Simon Draper [48].

***P. yoelii* CSP (PyCSP)**

P. yoelii YM CSP αα 1-356. This does not include the C-terminal GPI anchor signal sequence (αα 357-367), which has previously been shown to reduce immunogenicity [75, 240, 241]. AdHu5 and MVA vectors expressing this antigen were made by Dr. Sumi Biswas [102].

***P. berghei* $MSP1_{42}$ ($PbMSP1_{42}$)**

P. berghei ANKA MSP1 αα 1180-1541. This does not include the GPI anchor signal motif. AdHu5 and MVA vaccines expressing this antigen were made by Dr. Sumi Biswas (Dr. Anna Goodman, DPhil thesis).

***P. berghei* MSP9 (PbMSP9)**

P. berghei NK65 MSP9 αα 23–392. Five serine→alanine substitutions were made to remove potential N-glycosylation sites and an active serine protease site. This does not include the signal sequence (αα 1 – 22). AdHu5 and MVA vaccines expressing this antigen were made by Dr. Anna Goodman (DPhil thesis).

***P. berghei* AMA (PbAMA1)**

P. berghei ANKA strain AMA1 αα 21-479. GenBank Accession No. X07802. Six serine→alanine and one asparagine→glutamine substitutions were made to remove

potential N-glycosylation sites. AdHu5 and MVA vaccines expressing this antigen were made by Dr. Anna Goodman (DPhil thesis).

P. falciparum AMA1 3D7 (PfAMA1)

P. falciparum 3D7 AMA1 (GenBank Accession No. U65407) αα 25-546. This is the ectodomain only and does not include the N-terminal signal sequence, pro-sequence, the C-terminal transmembrane domain or cytoplasmic tail of PfAMA1. Five asparagine→glutamine αα substitutions were made to prevent N-glycosylation. AdHu5 and MVA viral vector vaccines expressing this antigen were made by Dr. Sumi Biswas [242]. This version of PfAMA1 was used unless stated otherwise.

P. falciparum AMA1 IMX313 (PfAMA1-IMX313)

P. falciparum 3D7 AMA1 αα 25-546 fused at the C-terminus to the optimised hybrid form of the C4bp α-chain oligomerisation domain, IMX313 [219]. This construct was made by F. Hill, Imaxio, France. AdHu5 and MVA vectors were made by Matthew Dicks and Simon Draper.

P. falciparum AMA1 FVO (PfAMA1 FVO)

P. falciparum FVO AMA1 αα 25-543. This antigen was provided by Prof. Richard Pleass (Liverpool School of Tropical Medicine). It includes the ectodomain only and does not include the N-terminal signal sequence, pro-sequence, the C-terminal transmembrane domain or cytoplasmic tail of PfAMA1. This is the native FVO sequence with no amino acid changes to prevent N-glycosylation. Construction of AdCh63 expressing this antigen is described in Section 2.2.8.

***P. falciparum* AMA1 murine IgG2a Fc (PfAMA1-Fc)**

P. falciparum FVO AMA1 αα 25-543 fused to the Fc portion of murine IgG2a (mIgG2aFc), NCBI Gene ID No. LOC100047788. Construction of AdCh63 expressing this antigen is described in Section 2.2.8.

PfM115

An 115kDa optimised *P. falciparum* MSP1 antigen. From N to C terminus it includes the conserved blocks: block 1 (αα 1-55), block 3 (αα 162-331) and block 5 (αα 369-399), block 12 from 3D7 strain (αα 1079-1170), Wellcome strain block 16 ($MSP1_{33}$), linked by a glycine-proline linker to 3D7 stain blocks 16 and 17 ($MSP1_{42}$). Unlike PfM128 it does not include Wellcome strain block 17 ($MSP1_{19}$). Three αα acid substitutions were also made in 3D7 block 17 to prevent potential N-glycosylation, avoid induction of blocking antibodies [137] and to aid antigen processing [93]. This antigen was designed and cloned into various adenoviral vectors and MVA by Dr. Simon Draper [243].

PfM128

A 128kDa optimised *P. falciparum* MSP1 antigen. From N to C terminus it includes the conserved blocks: block 1 (αα 1-55), block 3 (αα 162-331) and block 5 (αα 369-399), as well as block 12 from 3D7 strain (αα 1079-1170) and both the Wellcome and 3D7 alleles of blocks 16 and 17 ($MSP1_{42}$) (excluding the GPI anchor sequence), linked by a glycine-proline linker. Three αα acid substitutions were also made in block 17 to prevent potential N-glycosylation, avoid induction of blocking antibodies [137] and to aid antigen processing [93]. This antigen was designed and cloned into various adenoviral vectors and MVA by Dr. Sumi Biswas [243].

PfM128-IMX313

This antigen is identical to that described above except that a hybrid version of the α-chain oligomerisation domain of C4bp (termed IMX313) is fused at the C-terminus [219]. This construct was made by F. Hill, Imaxio, France and cloned into AdHu5 and MVA vectors by Matthew Dicks and Simon Draper.

Table 2.3: Summary of antigens used in this thesis

Antigen	**Codon optimised**	**Kozak Sequence**	**tPA**	**PK tag**
$PyMSP1_{42}$	No	Yes	Yes	Yes
$PyMSP1_{42}$-IMX108	No	Yes	Yes	No
PyCSP	No	Yes	Yes	Yes
$PbMSP1_{42}$	No	Yes	Yes	No
PbMSP9	Yes (mouse)	No	Yes	No
PbAMA1	Yes (mouse)	No	Yes	No
PfAMA1 3D7	Yes (human)	Yes	Yes	No
PfAMA1-IMX313	Yes (human)	Yes	Yes	No
PfAMA1 FVO	No	Yes	Yes	No
PfAMA1-Fc	No	Yes	Yes	No
PfM115	Yes (human)	No	No	No
PfM128	Yes (human)	No	No	No
PfM128-IMX313	Yes (human)	No	No	No

2.2.2 Agarose gel electrophoresis

DNA reaction products or PCR products were separated by 1% agarose gel electrophoresis (100V, 50min) in 1 x TAE buffer, stained using SYBR Safe, and visualised on a transilluminator. Size estimates were determined by comparison to Smart ladder DNA marker.

2.2.3 Restriction endonuclease digests

Digests were incubated at 37°C for 2h. 20μL reactions were performed as follows:

xμL	Total NEB Restriction Enzyme(s)
2μL	(10x) NEB restriction buffer
(2μL	(10x) bovine serum albumin (BSA) if required)
xμL	Plasmid DNA (0.5-1μg)
xμL	sterile distilled H_2O
Σ =	20 μL

Subsequent addition of calf intestinal phosphatase (CIP) (1 h, 37°C) minimised vector re-ligation where digestion would be expected to generate complimentary "sticky ends".

2.2.4 Ligation reaction

Digested DNA fragments were excised from agarose gels and purified using QIAgen MinElute gel extraction kits according to the manufacturer's instructions. Ligation was then performed overnight at 16°C. The reaction included ligation buffer, T4 DNA ligase, and approximately 1:3 cut plasmid DNA to insert DNA, made up to a volume of 20μL with dH_2O as described in Jenner Protocol 098 (J098) in the

Appendix. Controls were run in parallel and included using uncut plasmid DNA, no insert DNA or no ligase.

2.2.5 Preparation of agar plates

LB agar was melted in the microwave and allowed to cool in the water bath to 50°C. Antibiotic was added and gently mixed to a standard final concentration of 100μg/mL ampicillin or 25μg/mL kanamycin. Plates were poured and allowed to cool at room temperature (RT) and then stored at 4°C.

2.2.6 Bacterial Transformation

50μL maximum efficiency or sub-cloning efficiency DH5α *E. coli* were thawed on ice and incubated on ice for 30 min with 0.5-10μL ligation reaction, before heat-shock at 42°C in a water bath for 30 s and a further 2-5 min on ice. Bacteria were plated onto LB agar plate containing selection antibiotic. For ampicillin resistance selection, bacteria were plated out immediately. For kanamycin resistant selection, bacteria were incubated in 950μL S. O. C. medium at 37°C, 225 rpm for 1 h before plating 20 - 200μL. Plates were incubated at 37°C overnight for bacterial growth and then stored at 4°C.

2.2.7 Plasmid DNA preparation

Single colonies were selected from agar plates and cultured overnight in 2-3mL LB medium containing selection antibiotic(s). Plasmid constructs were then purified using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions. For greater quantities of DNA, constructs were amplified by further overnight culture in 150mL LB broth containing antibiotic(s) and purified using a QIAGEN Plasmid Midi Kit. DNA concentration was measured using a Nanodrop spectrophotometer and

plasmid identity was confirmed by analytical restriction digest. For long term storage, bacterial stocks were created - 1mL of bacterial culture containing the plasmid of interest was mixed 1:1 with 50% glycerol in complete media and stored at -80°C.

2.2.8 Restriction cloning into Adenovirus entry vector

The ViraPower™ Adenoviral Expression System was used to construct recombinant AdHu5 vaccines. This uses Gateway® technology to develop recombinant adenovirus containing the gene of interest under the control of a promoter of choice. AdCh63 vaccines were made using a novel gateway system developed by Dr. Matt Cottingham at the Jenner Institute, that was based on the AdHu5 Gateway® system. Both systems involve cloning of antigens into an entry vector and then into an adenoviral destination vector by site-specific recombination.

All adenoviral vectors used in this project used the 1.9kbp CMV promoter to drive antigen expression. This includes the regulatory element, enhancer and intron A. This promoter, along with a polylinker and BGH poly(A) transcription termination sequence were cloned into the entry vector pENTR™4 by Dr. Saranya Sridhar, University of Oxford [244]. This vector is termed pENTR™4.CMV.BGH. A second entry vector, pENTR™4.CMV.BGH.TO, was made by Dr. Sandy Douglas at the Jenner Institute. This includes two tetracycline operator sequences (TO) from pcDNA™4/TO that were inserted into the pENTR™4.CMV.BGH entry vector. The TO sequences allow repression of gene expression *in vitro*, which is necessary for antigens whose expression is detrimental to cell culture. Final AdHu5 constructs containing TO are cultured in T-REx™ 293 cells, (rather than 293 cells (Section 2.3.1)), which stably express the tetracycline repressor (TR) protein that binds to TO

sites and blocks transcription initiation. If tetracycline is added to viral culture medium it binds to TR protein and alters its conformation, causing it to be released from TO sites and allowing antigen expression to be driven by the CMV promoter. In the absence of the TR protein *in vivo* (i.e. following immunisation) constitutive antigen expression occurs.

Antigens were amplified from genomic DNA by PCR, synthesised by GeneArt (Germany), or cut from existing plasmids and cloned into either pENTR™4.CMV.BGH or pENTR™4.CMV.BGH.TO entry vectors. The constructs were checked by analytical digest or were sequenced by Eurofins MWG Operon (Germany).

PyCSP [102], PbMSP1$_{42}$ (A. Goodman, DPhil thesis) and PfAMA1 (3D7) [242] were cloned into pENTR™4.CMV.BGH by Dr. Sumi Biswas. PbAMA1 and PbMSP9 were cloned into pENTR™4.CMV.BGH.TO by Dr. Anna Goodman. PyMSP1$_{42}$ [48] and PfM128 [104] were cloned into pENTR™4.CMV.BGH by Dr. Simon Draper. All IMX108 and IMX313 constructs were cloned by F. Hill, Imaxio, France [48].

PfAMA1 (FVO native sequence) and PfAMA1-Fc were cloned into pENTR™4.CMV.BGH.TO as described in Figure 2.1. Briefly, plasmids containing PfAMA1 FVO and PfAMA1-Fc were kindly provided by Prof. Richard Pleass, (Liverpool School of Tropical Medicine) and antigens were amplified from these by PCR (J039) so that each amplified construct consisted of *SalI*-Antigen-*NotI*-*SphI*-*BamHI*. The following oligonucleotide primers were used (5' → 3'):

Forward: GTC GAC CAG AAC TAC TGG GAG CAT CC

PfAMA1 reverse: GGA TCC GCA TGC GCG GCC GCT CAG TAC GTA GGC TTA TGC TCT GGA ATA

PfAMA1-Fc reverse: GGA TCC GCA TGC GCG GCC GCT CAT TTA CCC AGA GAC CGG GA

Inserts were then ligated into the pCR®-Blunt II-TOPO® vector. The construct was then digested with *SalI* and *SphI* and inserted into pGEM®-T easy_tPA vector made by Dr. Simon Draper. tPA_PfAMA1±Fc was then inserted into the pENTR™4.CMV.BGH.TO entry vector by *BamHI* and *NotI* digestion and religation as shown.

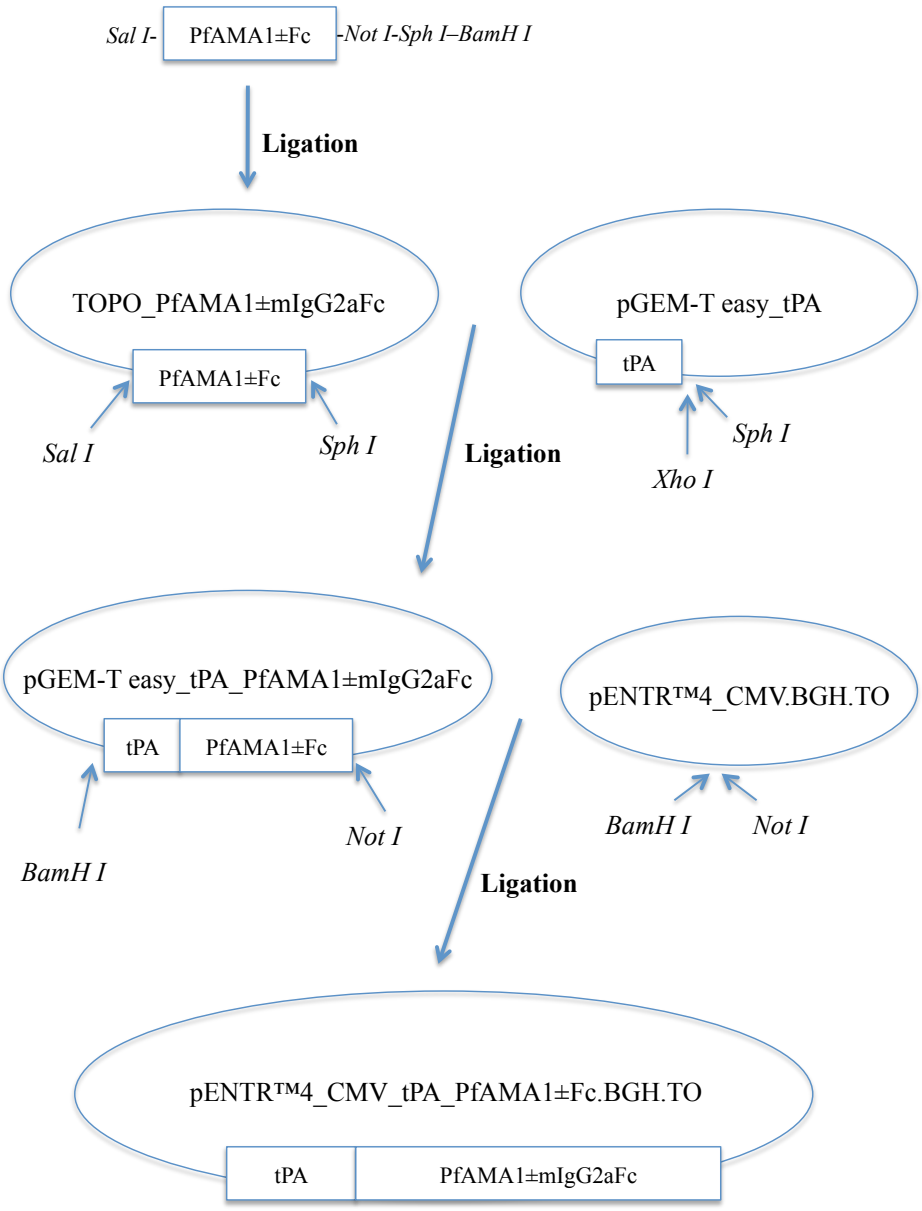


Figure 2.1: PfAMA1±Fc cloning scheme

2.2.9 Recombination into adenovirus destination vectors

For construction of AdHu5 vaccines, antigens with promoter and polyA tail were inserted into the 34.9kb E1- and E3-deleted adenovirus destination vector, pAd/PL-DEST at the E1 deletion site, between bases 519 and 2202, using site-specific recombination (J160). Specifically, a recombination reaction between attachment L (attL) sites within the entry clone and attachment R (attR) sites within the destination vector was performed using the LR clonase II enzyme mix. The reaction was terminated with proteinase K and transformed into bacteria. DNA was prepared and the correct construct was confirmed by analytical restriction digest. AdCh63 vectors were made in a similar way using the AdCh63 destination vector, pAdCh63-DEST developed by Dr. Matt Cottingham.

2.2.10 Restriction cloning into MVA

Antigens were inserted into the MVA-GFP or MVA-GFP2 shuttle vectors which drive antigen expression from the P7.5 promoter. PyMSP1$_{42}$ and PyMSP1$_{42}$-IMX108 were cloned by Dr. Simon Draper [48]. PyCSP [102], PbMSP1$_{42}$ (A. Goodman, DPhil thesis), PfAMA1 (3D7) [242] and PfM128 [104] were cloned by Dr. Sumi Biswas. PfM115 was cloned by Dr. Simon Draper [104]. PbAMA1 and PbMSP9 were cloned by Dr. Anna Goodman (DPhil thesis).

MVA-GFP3-SSP and MVA-GFP2-mH5 vectors were made by Dr. Matt Cottingham. PyMSP1$_{42}$ was inserted into these constructs as shown in Figure 2.2. Short synthetic promoter (SSP) is 40bp long and has both strong early and late activity. It was rationally designed for maximal activity based on studies that mutated P7.5 [185, 245]. The modified H5 promoter (mH5) is derived from the promoter for the H5

(previously known as H6) gene of Vaccinia virus WR strain [246]. It contains two base-pair alterations compared to the native sequence. It was designed by Moss *et al.* [187] and contains an A→T substitution (position -38) to remove an upstream ATG codon which might initiate translation and a T→A substitution (position -102) downstream of the transcription initiation site to enhance promoter strength [247].

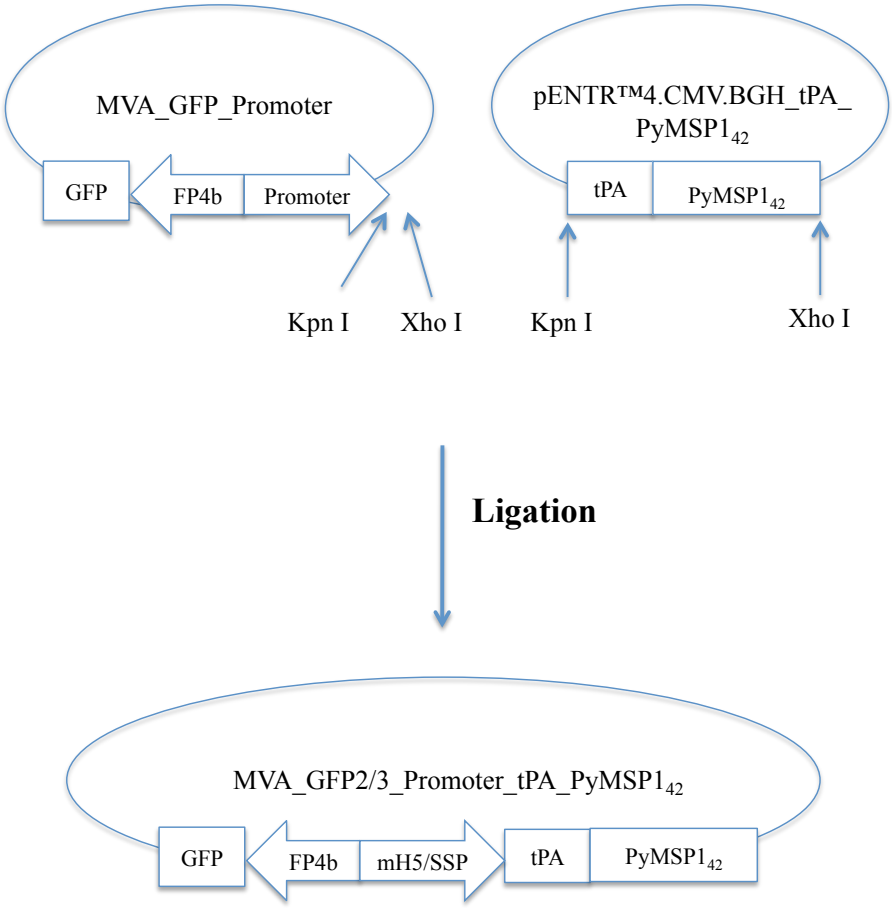


Figure 2.2: MVA-PyMSP1$_{42}$ promoters cloning scheme

2.2.11 Protein

PfAMA1 3D7 protein was a kind gift from Dr. Chetan Chitnis, ICGEB, India. PfAMA1 FVO protein was kindly given by Dr. Mike Blackman, NIMR, London.

$MSP1_{19}$ proteins were generated as recombinant *Plasmodium* glutathione S-transferase (GST)-fusion proteins expressed from pGEX-2T expression vectors in Rosetta competent cells. $PfMSP1_{19}$ 3D7 and $PyMSP1_{19}$ were amplified from genomic DNA by Dr. Simon Draper using Expand High Fidelity PCR (J039). $PfMSP1_{19}$ FVO was kindly provided by Dr. Fergal Hill (Imaxio, France) and $PbMSP1_{19}$ was amplified by Dr. Sumi Biswas from *P. berghei* genomic DNA kindly provided by Prof. Brendan Crabb (WEHI, Australia). The amplified DNA was cloned into a pGEM®-T Easy vector system and then into the GST expression vector, pGEX-2T, in frame with the upstream GST coding sequence [48, 104]. Plasmid constructs were verified by restriction enzyme digest.

Protein was expressed in Rosetta *E. coli* cells. These cells possess plasmids that supply six rare *E. coli* tRNAs, whose low abundance can limit translation of the A:T rich *Plasmodium* genomes from standard *E. coli*. Chemically competent Rosetta cells were bulked up by overnight growth in LB medium with chloramphenicol and stored in 200μL aliquots at -80°C before use.

pGEX-2T GST expression vectors were transformed into chemically competent Rosetta cells (as 2.2.6 but without heat shock at 42°C) and selected for chloramphenicol (tRNA plasmid) and ampicillin (pGEX-2T) resistance. Picked

colonies were grown in a 10mL started culture of Terrific Broth containing the same antibiotics before inoculation of 100mL cultures that were grown overnight at 37°C in the shaking incubator. Each culture was then made up to 500mL with fresh medium and incubated for a further 1 h as before. Protein expression was induced by addition of IPTG at a final concentration of 60μg/mL for a further 2 h. Cells were then harvested by centrifugation (6000 *g*, 15 min) and pellets stored at -20°C overnight. Bacteria were then lysed using BugBuster® and Benzonase according to the manufacturer's instructions. The suspension was centrifuged (15000 *g*, 20 min) and then lysate was ultracentrifuged (45000 *g*, 1h, 4°C). Extract was then loaded onto GST-affinity chromatography columns (GST•Bind purification kit). Proteins were eluted using 10mM reduced glutathione and collected in 1mL fractions. Protein concentration was measured using the nanodrop and fractions containing the majority of recombinant protein were stored in smaller aliquots at -20°C or at 4°C if in use. Proteins were checked by SDS-PAGE and Coomassie Brilliant Blue staining.

2.2.12 SDS-PAGE

Protein samples were diluted in 6x reducing loading dye and heated for 5 min at 95°C before loading onto 12% Tris-HCl precast gels and running at 200V for 45 min. Proteins were visualised by staining with Coomassie Brilliant Blue for 1 h at RT and destaining overnight in destain solution. Gels were transferred to 20% ethanol, 10% glycerol solution for 1 h and then dried between cellophane sheets soaked in 10% glycerol.

2.2.13 Western Blotting

Cell lysate was prepared by infection of 293 cells with AdHu5 vaccines of interest at a multiplicity of infection (MOI) of five. 30 h post infection the media was removed, cells were washed once with PBS, scraped, centrifuged (4000 rpm, 4 min) and re-suspended in non-reducing Laemmli buffer. Cells were then heated at 95°C for 5 min and sonicated for 1 min before storage at -20°C until use. For western blotting, samples were separated by SDS-PAGE on an 8-16% polyacrylamide pre-cast gel in Tris-HEPES-SDS running buffer. Proteins were blotted onto a nitrocellulose membrane by electrophoretic transfer. The membrane was then washed twice in PBS, blocked for 1 h with 3% BSA/PBS, washed again, incubated for 1 h in antigen-specific primary antibody diluted 1:1000 in 3% BSA/PBS, washed, incubated for 1 h with donkey anti-mouse-alkaline phosphatase diluted 1:3000, washed, and finally developed with SIGMA*FAST*™ BCIP®/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution. Colorplus prestained protein ladder, broad range (10-230 kDa), was included to determine the size of the proteins. Control proteins and non-recombinant AdHu5 infected lysate were included on each gel to confirm that primary antibody was antigen-specific. Antibody for detecting PfM128 antigen was polyclonal sera from mice immunised three times with recombinant $PfMSP1_{19}$-GST protein in PBS and for detecting PfAMA1 antigen polyclonal sera was used from mice immunised with viral vectored vaccines (AdCh63_MVA) encoding PfAMA1 [242].

2.2.14 Peptides

Peptides were synthesised by Protein Peptide Research or Invitrogen, Paisley, UK and dissolved in DMSO at a concentration of 20-50mg/mL and stored at -20°C. Working

stocks were diluted to 1mg/mL in PBS and stored at -20°C. Details of individual peptides used are given in Table 2.4 (Page 97) and all individual peptides used were >80% pure.

The $PyMSP1_{33}$ peptide pool was stored at 40μg/mL in complete media and consisted of 52 15mers, overlapping by 10αα, which corresponded to PyMSP1 αα 1394-1663. These were crude peptides, > 50% purity, except peptides containing the sequences of predicted epitopes (Peptides 6, 12, 16, 24, 27-29, 31, 32, 36, 40-42, 51 and 52) which were replaced with pure peptide [244].

The *P. yoelii / P. berghei* mixed peptide pool contained PbCSP αα1-60 (9 15mers, overlapping by 10αα) and PbCSP αα 261-340 (14 15mers, overlapping by 10αα). The relevant peptides were replaced with the available PyCSP peptides, $PyCSP_{280-288}$ and $PyCSP_{59-79}$.

The *P. falciparum* AMA1 FVO peptide pool consisted of 111 15mers overlapping by 10αα that spanned the entire length of PfAMA1 FVO native sequence. Peptides were of crude, >50% purity.

For PyCSP ELISAs a branched peptide was used, MAP4-Py1T-Py3 [248]. This is a multi-antigen peptide with 4 branches (MAP4). Each branch contains three copies of the PyCSP central repetitive sequence (QGPGAP) (Py3), known to be a B cell epitope, fused to a single copy of $PyCSP_{59-71}$ (Py1T).

Species	Antigen	Residues (αα)	Sequence	CD4 / CD8	MHC class I restriction	Ref.
P. yoelii	CSP	58 – 67	IYNRNIVNRL	CD8	K^d	[83]
	CSP	280 – 288	SYVPSAEQI	CD8	K^d	[249]
	CSP	59 - 79	YNRNIVNRLLGDALNGKPEEK	CD4	N/A	[250]
	$MSP1_{33}$	1421 - 1429	TYKSIKKHML	CD8	K^d *	[244]
	$MSP1_{33}$	1453 - 1462	DFLEVLSHEL	CD8	K^d *	[244]
	$MSP1_{33}$	1473 - 1481	YVIRNPYQL	CD8	D^b*	[244]
	$MSP1_{33}$	1513 - 1521	KFFNKMVEL	CD8	K^d *	[244]
	$MSP1_{33}$	1550 - 1558	KYIPILEDL	CD8	K^d *	[244]
	$MSP1_{33}$	1571 - 1580	EYSEELQNRL	CD8	K^d *	[244]
	$MSP1_{33}$	1599 - 1607	KYIQIDEKL	CD8	K^d *	[244]
P. falciparum	MSP1 (Well)	1354-1368	NKEKRD**KFLSSYNYI**	CD8	K^d *	[243]
	MSP1 (Well)	1359-1373	D**KFLSSYNYI**KDSID	CD8	K^d *	[243]
	AMA1 (Common)	151-165	VFGKGIIIENSKTTF	CD4	N/A	Un
	AMA1 (3D7)	206-220	**KYVKNLDEL**TLCSRH	CD8	K^d *	Un
	AMA1 (Common)	471-485	NKKIIAPRIFISDDK	CD4	N/A	Un
MVA	E3	140-148	VGPSNSPTF	CD8	D^d	[251]
	F2(G)	26-34	SPGAAGYDL	CD8	L^d	[251]

Table 2.4: Peptides

All individual peptides used in this thesis are listed. Throughout the thesis peptides are named according the species, antigen and αα sequence e.g. $PyCSP_{280-288}$. * indicates predicted MHC class I restriction according to the SYFPEITHI website developed at the University of Tübingen (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm). Predicted minimal $CD8^+$ T cell epitopes are shown in bold font [104]. 'Un' indicates unpublished epitopes identified at the Jenner Institute.

2.3 Virology

Transfection, purification and quantification of viruses was kindly carried out by either M. Dicks or the Jenner Institute Vector Core Facility. A brief description of the methods are given here and detailed Jenner Protocols (J#) are included in the Appendix.

2.3.1 Adenovirus transfection

Adenovirus transfection was carried out as described in J004. Briefly, recombinant destination adenovirus plasmid (pAd) was linearised by 2 h digestion at 37°C with *PacI* (for AdHu5) or *PmeI* (for AdCh63). Digestion was confirmed by running 10μL on an agarose gel. The remaining 85μL of restriction enzyme mix was then added to 215μL OptiMEM. This was added to 300μL 1:10 lipofectamine in OptiMEM, mixed, and incubated for > 20 min at RT. The mixture was added drop-wise onto pre-prepared 293 cell monolayers, or T-Rex™ 293 cell monolayers (for constructs including TO). Cells were then incubated for 4 h at 37°C, 5% CO_2 and then OptiMEM media replaced with 10% DMEM without antibiotics. Cells were incubated for a further 5-9 days and monitored daily for cytopathic effect (CPE). Cells were harvested when CPE was evident.

2.3.2 Adenovirus purification

Recombinant adenovirus was purified by caesium chloride (CsCl) density centrifugation (J005). Briefly, 293 or T-Rex™ 293 cells were infected with adenovirus and harvested on day 4-5 when CPE was evident. Cell pellets were re-suspended in lysis buffer, freeze-thawed, and incubated for 1 h at room temperature with 250 units/mL benzonase. The pellet was freeze-thawed twice more and then

centrifuged (900 *g*, 5 min). Three CsCl solutions were then prepared at 1.25g/mL, 1.35g/mL and 1.6g/mL CsCl in 10mM Tris, pH 7.8. Gradients were prepared by underlaying 5mL 1.25 g/mL CsCl with 5mL 1.35 g/mL CsCl in 14mL Beckman Ultraclear tubes. Virus supernatant was then added and centrifuged at 111,000 *g* for 2 h at 4°C. Lower band virus was then collected and diluted in 1.35g/mL CsCl underlayed with 1.6g/mL CsCl. This was then centrifuged at 111,000 *g* for 16-18 h at 4°C. The lower virus band was then dialysed against cold storage buffer using a dialysis cassette.

Some recombinant AdHu5 vaccines (made prior to 2008) were purified using the Puresyn Adenopure® Kit (J010). Briefly, an infected cell suspension was prepared by infection of 293 cells with the adenovirus of interest. When complete CPE was evident on day 4 – 5 post infection, cells were harvested and freeze-thawed three times. Cell debris was then pelleted by centrifugation (1500 *g*, 5 min) and the supernatant filtered using a Nalgene filtration unit. Benzonase was added to the supernatant for 30 min at 37°C and then dilution buffer added. Columns were then prepared and adenovirus was purified using Adenopump® column purification. Virus was eluted in elution buffer and filtered through a 0.22μm syringe filter.

The number of viral particles (vp) was determined by UV spectrophotometry (J106) and the infectious units (ifu) was determined by immunostaining (J006). Viruses were stored in aliquots at -80°C until use.

Adenoviruses purified by both CsCl density centrifugation and Adenopure were compared for *in vivo* immunogenicity using a variety of different antigenic inserts

including PyCSP and PyMSP1$_{42}$. Responses were not significantly different following purification by the two different methods (data not shown).

2.3.3 MVA transfection and purification

6-well plates of 0.5 x 10^6 chick embryo fibroblasts (CEFs) per well were prepared and incubated overnight at 37°C, 5% CO_2, to allow cells to become 60-80% confluent as described in J013. Cells were then infected with 0.5 x 10^6 plaque forming units (pfu) MVA-RFP (J014). Meanwhile, 5 µg recombinant MVA shuttle vector was linearised by *AatII* restriction digest to increase the efficiency of recombination (J017). CEF cell media was then replaced with transfection reagent containing the linearised MVA-GFP shuttle vector (J014, which has been recently replaced by J176) and cells were incubated for a further 2 days. During this time i*n vitro* recombination occurred, whereby Antigen-GFP replaced RFP at the TK locus in the MVA genome. CEFs containing recombinants expressing GFP were then separated from those expressing RFP by fluorescence using a MoFlo™ XDP cell sorter (J015, or J131 for more recent vaccines). The cell lysate obtained was used to infect fresh CEFs (J016). Pure recombinant MVA-GFP2-tPA-antigen was then isolated from any remaining MVA-RFP by repeated rounds of plaque picking (J018). Finally virus was bulked up and aliquoted (J019). The presence of the correct recombinant MVA and absence of parental MVA-RFP was confirmed by identity and purity PCR (J079). Virus titre was determined by counting GFP-expressing plaques in CEFs (J082).

2.4 Animals and Immunisations

2.4.1 Mice

Procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Oxford Animal Care and Ethical Review Committee for use under Project Licence (PPL 30/2414) and Personal Licence (PIL 30/7749). All mice were housed in specific pathogen-free (SPF) conditions at the Wellcome Trust Centre for Human Genetics.

5-8 week old female BALB/c (H-2^d), C57BL/6 (H-2^b) and CD-1 (outbred) mice were obtained from Harlan (U.K.) or Charles River Laboratories (U.K.).

Male and female Fc receptor common γ-chain (Fc$\gamma R^{-/-}$) on a BALB/c background and FcγRIIb$^{-/-}$ (CD32b) on a C57BL/6 background were originally provided by Prof. Richard Pleass (Liverpool School of Tropical Medicine) and kindly bred by Dr. Simon Draper at the Wellcome Trust Centre for Human Genetics.

2.4.2 Rabbits

New Zealand white rabbits were immunised and sera collected by Agrobio (France), Biogenes (Germany), or Spring Valley (USA).

PfAMA1±IMX313 experiments were funded by the PATH Malaria Vaccine Initiative (MVI) and were carried out by Spring Valley Laboratories USA.

2.4.3 Immunisation Route

Intradermal (i.d.) vaccinations were given into the ear using a 29G needle in a volume of 50μL (25μL/ear).

Intramuscular (i.m.) vaccinations were given into the quadriceps in 100μL (50μL/leg) using a 26G needle.

Intravenous (i.v.) injections were given in a volume of 100-200μL into the lateral tail vein using a 28G needle. To encourage vessel dilation mice were warmed for > 10 min at 37-38°C in a heatbox prior to injection.

Intraperitoneal (i.p.) injections up to 500μL were given using a 28G needle.

2.4.4 Anaesthetic

For i.d. and i.m. immunisations, mice were anaesthetised by inhalation of a mixture of isofluorane (2-3.5%) and oxygen (1-2L/min) in air. Alternatively, mice were injected i.p. with 75mg/kg Ketaset™ (ketamine hydrochloride) plus 1mg/kg Domitor™ (medetomidine hydrochloride) diluted in endotoxin-free water and were recovered from anaesthesia with 100μL i.p. of 1:10 (C57BL/6) or 1:100 (BALB/c) solution of Antisedan™ (atipamezole hydrochloride) in endotoxin-free water.

2.4.5 Vaccines

All vaccines were formulated in endotoxin-free PBS unless stated otherwise.

2.5 Collection and preparation of cells/sera

2.5.1 Isolation of splenocytes

Mice were culled by cervical dislocation and spleens were sterilely dissected into PBS. Spleens were mechanically homogenised using the base of a 5mL syringe and passed through a 70µm cell strainer and centrifuged (1250 rpm, 5 min). Supernatant was discarded and erythrocytes were removed by re-suspension of cells in ACK lysis buffer for 5 min, before the reaction was stopped by addition of > 25mL PBS. Cells were then centrifuged (1250 rpm, 5 min) and re-suspended in 3mL complete MEM. RBC 'ghosts' were removed using a pipette or by passing the cells through a cell strainer.

2.5.2 Isolation of PBMC

Mice were warmed at 37-38°C for >10 min to promote vessel dilation and tail veins were nicked using a sterile scalpel. 7-10 drops of blood were collected from the tail vein into 200µL 10mM EDTA in PBS and immediately mixed to prevent clotting. Cells were centrifuged (4000 rpm, 4 min) and supernatant removed. Cells were then incubated with ~1mL ACK lysis buffer for 5 min, centrifuged again and supernatant carefully removed. ACK lysis was repeated and cells were re-suspended in 350µL complete MEM.

2.5.3 Isolation of liver mononuclear cells

Mice were culled by cervical dislocation and livers were perfused with PBS by injection into the portal vein. Livers were dissected out, the gall bladder removed, and were mechanically homogenised using the base of a 5mL syringe. Cells were incubated for 1 h in complete MEM containing 0.7mg/mL collagenase and

0.03mg/mL DNase, then passed through a 70μm cell strainer. Cells were washed in PBS and under-laid with FICOLL before spinning at 2000 rpm without the brake for 45 min at 21°C to separate cells. Cells at the interphase were carefully removed, washed twice with PBS and re-suspended in complete MEM.

2.5.4 Isolation of $CD11c^+$ cells

Mice were culled by cervical dislocation and spleens were sterilely dissected into PBS. Spleens were then finely chopped using a sterile scalpel and re-suspended in complete MEM. Samples were rotated at RT for 30 min in the presence of 0.3mg/mL collagenase-dispase and 0.02mg/mL DNase I, before addition of 0.1M EDTA for a further 5 min to inactivate collagenase and disrupt DC-T cell interactions. Spleens were then mechanically homogenised and erythrocytes lysed as described in Section 2.5.1. After the final wash cells were re-suspended in cold MACS buffer at a concentration of 2.5 x 10^8 cells/mL. Cells were then incubated for 10 min at 4°C with 1:100 naïve BALB/c serum and 1:100 Fc-block before addition of 100μL CD11c MicroBeads per 10^8 cells and incubation for a further 15 min at 4°C. Cells were washed and re-suspended at 2 x 10^8 cells/mL in MACS buffer. $CD11c^+$ cells were then isolated by positive selection by passing splenocytes through a LS MACS column according to the manufacturer's instructions. $CD11c^+$ cells were then washed thoroughly in MACS buffer and resuspended in complete MEM. Purity of $CD11c^+$ cells was confirmed by flow cytometry following anti-mouse CD11c-PE staining.

2.5.5 Cell counting

Splenocytes were counted using a CASY counter. This was set to count cells between 5.5μM and 10.8μM in diameter. Samples were diluted 1:2000 (5μL in 10mL CASY) for counting and re-suspended at appropriate concentrations in complete medium.

PBMC were similarly counted with a CASY counter and set to count cells between 6.45μM and 10.8μM in diameter.

RBC were counted for blood-stage challenge. 5μL of blood from an infected donor mouse was diluted in 1mL RBC counting solution. Cells were counted on the Improved Neubauer Haemocytometer.

2.5.6 Collection of mouse sera

Mice were warmed at 37-38°C for >10 min to promote vessel dilation and tails nicked with a sterile scalpel before collection of < 200μL blood into microvette tubes. Alternatively, mice were culled by cervical dislocation and blood collected into eppendorf tubes by cardiac puncture. Samples were stored at 4˚C overnight to allow clotting, spun at 13,000 rpm for 4 min and then serum was transferred to clean eppendorfs and stored at -20˚C until use.

2.6 Cellular assays

2.6.1 Intracellular cytokine staining

Cellular immune responses in blood, spleen and liver were measured by intracellular cytokine staining (ICS). Isolated cells were plated at 100μL/well in 96-well U-bottom plates. They when then re-stimulated at 37˚C for 5 h in the presence of 1:1000 Brefeldin A (GolgiPlug) and peptides at a final concentration of 1μg/mL per peptide. Plates were then stored at 4˚C overnight.

Cells were transferred to V-bottom plates and centrifuged. Supernatant was then discarded, cells re-suspended in 1:100 anti-mouse CD16/CD32 (Fc-block) in PBS, and incubated for 15 min at 4°C. Cells were centrifuged again, and surface stained by incubation for 30 min with anti-mouse CD8a-PerCP-Cy5.5 (clone 53-6.7) and CD4-Pacific Blue (clone RM4-5) both diluted 1:100 in PBS. Cells were washed once in PBS and permeabilised by incubation in Cytofix/Cytoperm solution for 10-20 min. Cells were then washed twice in PermWash and stained intracellularly for 30 min with anti-mouse IFN-γ-APC clone XMG1.2 (1:200), IL-2-PE clone JES6-5H4 (1:100) and TNFα-FITC clone MP6-XP22 (1:100) diluted in PermWash. Finally, cells were washed twice in PermWash, once in PBS and then re-suspend in PBS/1% formalin solution. All wash steps were performed by centrifugation at 1250rpm for 5 min and all incubations were at 4°C.

Flow cytometry was carried out using a CyAn ADP Flow Cytometer or LSRII BD. Data were analysed using Flowjo version 8.7 (Tree Star, Inc.; USA). An example of the gating strategy used for all analysis is shown in Figure 2.3. Results presented show the stimulated response with background responses from un-stimulated control wells (typically < 0.05%) subtracted unless unstimulated responses are also shown. For some experiments, analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov/spice> [252]. Comparison of distributions was performed using a Student's T test and a partial permutation test as described [252].

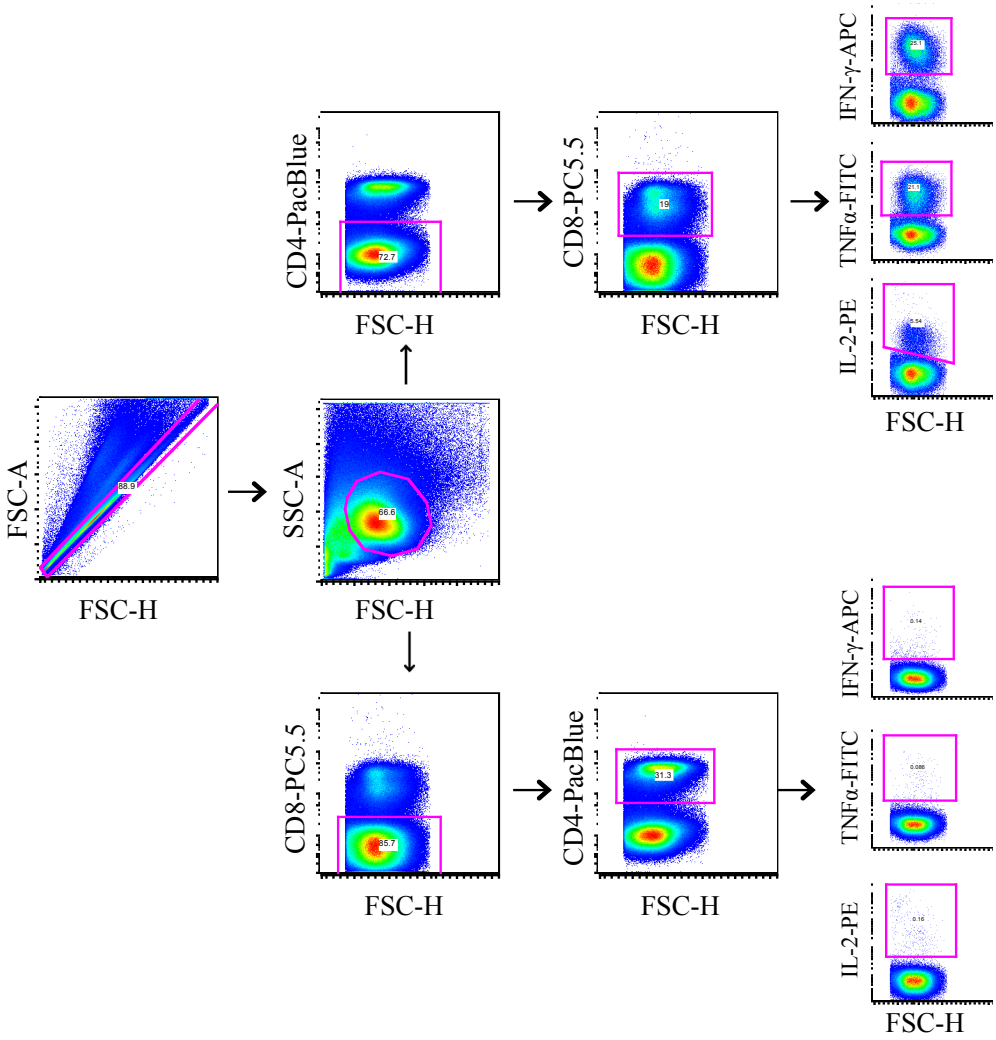


Figure 2.3: Example ICS gating strategy

All subsets were gated against 'forward scatter-height' (FSC-H), which measures the approximate size of cells. Analyses were only carried out on single cells. These 'singlets' were isolated by gating on FSC-H vs FSC-Area (FSC-A) before subsequent subset analysis to identify lymphocytes, $CD4^+$ and $CD8^+$ cells and expression of IFN-γ, TNFα and IL-2 from these subsets. Side scatter-area (SSC-A); PerCP-Cy5.5 conjugated anti-mouse CD8 (CD8-PC5.5); Pacific Blue conjuaged anti-mouse CD4 (CD4-PacBlue); APC conjugated anti-mouse IFNγ (IFNγ-APC); FITC conjugated anti-mouse TNFα (TNFα-FITC); PE conjugated anti-mouse IL-2 (IL-2-PE).

2.6.2 Mouse *ex vivo* IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay

All ELISpot reagents were supplied in a mouse IFN-γ ELISpot kit from Mabtech. ELISpot plates were coated with 50μL/well of 5μg/mL mouse anti-IFN-γ mAb AN18 in carbonate-bicarbonate buffer and stored at 4°C overnight. Plates were then washed with PBS and blocked with complete MEM for at least 1 h at 37°C. Splenocytes (prepared as described in 2.5.1) were diluted to an optimal starting concentration in complete MEM and diluted 2-fold down the plate. Peptides were added at a final concentration of 1μg/mL and plates were incubated for 18 – 20 h at 37°C. Plates were then washed and responses detected by addition of 50ng/well biotin-conjugated rat anti-mouse IFN-γ mAb R46A2 in PBS for 2 h at RT. Plates were then washed again, before addition of 50ng/well Streptavidin Alkaline Phosphatase polymer in PBS for 1 h, washing, and development with Biorad AP conjugate development buffer. The reaction was stopped with tap water and when plates were dry spots were counted using an ELISPOT counter. Cells were plated in duplicate and the mean response in spot forming units (SFU) per 10^6 PBMC was calculated. Responses from un-stimulated control wells were subtracted.

2.6.3 *In vivo* cytotoxic T cell assay

Naive BALB/c splenocytes were prepared by mechanical disruption (as described in 2.5.1) as targets for an *in vivo* cytotoxic T cell killing assay (Figure 2.4). Cells were washed and re-suspend at 5 x 10^7 cells/mL in warm MEM (without additions, 37˚C), then split into two populations. One population of cells was labelled with a high concentration of CFSE (5μM) and the other with a low concentration of CFSE (0.5μM). After addition of CFSE cells were immediately incubated for 10 min at

37˚C, with regular mixing by inversion. The reaction was stopped by addition of cold complete MEM. Cells were then washed twice in > 20mL complete MEM and re-suspended at 1 x 10^7 cells/mL. Cells labelled with a high concentration of CFSE were incubated at 37˚C for 1 h with 200ng/mL peptide. Cells were then washed twice in PBS. For i.v. injection, a 1:1 ratio of high and low CFSE labelled cells were combined such that each mouse received a total of 2 x 10^7 cells in 200µL of PBS. These were then injected into BALB/c mice that had been previously primed with AdHu5 vaccines as indicated. Immunised mice were culled 6 h later and a single-cell suspension of splenocytes prepared as described in 2.5.1. CFSE intensity was analysed by flow cytometry. Each population was detected by its differential CFSE intensity. Specific killing was calculated using the following formulas:

- **Ratio** = $\frac{\text{percentage CFSE low}}{\text{percentage CFSE high}}$

- **Percentage specific killing** = $\left(1 - \left(\frac{\text{mean ratio control-primed}}{\text{ratio antigen-primed}}\right)\right) \times 100$

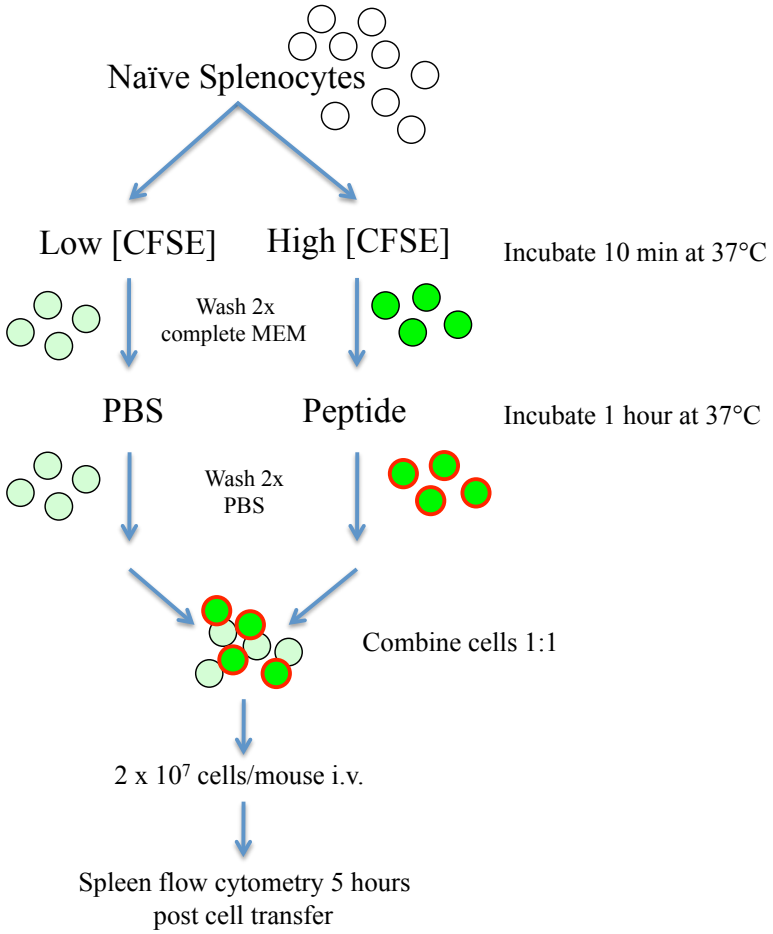


Figure 2.4 Schematic of the *in vivo* CTL killing assay

2.6.4 Peptide-pulsing and adoptive transfer of CD11c$^+$ cells

CD11c$^+$ cells were isolated as described in 2.5.4 and pulsed with 10μg/mL peptide for 16 h at 37°C. Cells were either pulsed with the 7 dominant PyMSP1 peptides, the 2 dominant PyCSP peptides, or a mixture of all 9 peptides. The concentration of DMSO was controlled across all wells at 0.24%. After pulsing, cells were washed thoroughly in PBS. Mice were injected i.d. in the ear with 2 x 10^5 CD11c$^+$ cells in a volume of 100μL (50μL/ear). In mice that received cells that had been pulsed separately, cells pulsed with each antigen were combined in a 1:1 ratio immediately before injection (giving 2 x 10^5 total cells).

2.7 Humoral assays

2.7.1 Indirect ELISA

All proteins/peptides were used at a concentration of 2μg/mL in PBS and were adsorbed to 96-well Nunc-Immuno Maxisorp plates by overnight incubation at RT wrapped in foil. Plates were washed 6 times in PBS 0.05% Tween 20 (PBS/T) and blocked for 1 h in PBS/T containing 10% skimmed milk powder. Pre-boost samples sera were diluted 1:100 and post-boost 1:1000 before serial dilution 3-fold down the plate and incubation for 2 h at RT. Plates were then washed in PBS/T and bound antibodies detected by incubation for 1 h with alkaline phosphatase conjugated goat anti-mouse total IgG or goat anti-rabbit IgG as appropriate. Alternatively bound mouse antibody isotypes were detected using biotin-conjugated rat anti-mouse IgG1, IgG2a at a concentration of 1μg/mL in PBS/T for 1 h, before washing and adding ExtrAvidin conjugated to alkaline phosphatase and incubation for 30 min. Plates were then washed and developed using 1mg/mL *p*-nitrophenylphosphate substrate (pNPP)

in diethanolamine buffer. Optical density was read at 405nm. Endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value 3 standard deviations greater than the OD_{405} for naïve mouse/rabbit serum. All samples were tested in duplicate and a high titre reference sample was included in all experiments and developed to the same endpoint each time.

Rabbit IgG ELISAs for PfAMA1±IMX313 were carried out using a standardized protocol [253] at the GIA reference center (LMVR, NIAID, NIH) with 3D7 PfAMA1 antigen provided by the Malaria Vaccine Development Branch, NIH, USA. A standard curve was generated using reference sera run in duplicate on every plate and one ELISA unit is the reciprocal dilution required to give an O.D. of 1 in the standardized assay [254].

2.7.2 Immunofluorescence antibody test (IFAT)

Sporozoite *P. yoelii* YM sporozoites were isolated from the salivary glands of infected mosquitoes and were dissected in PBS containing azide, which kills the sporozoites, before counting. Samples were diluted to 3 $x10^5$ sporozoites/mL and 10μL pipetted in each well on an 8-well microscope slide, such that each well contained approximately 3000 sporozoites. Slides were then air-dried, wrapped in foil and stored in a sealed bag with desiccant at -20ºC until use. For the IFAT, slides were thawed at RT in the sealed bag with dessicant prior to use. 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 h using 1% BSA in PBS/T. Following washing, serum samples were added at a dilution of 1:100 in PBS for 1 h. Slides were washed again and Alexa Fluor® 488 conjugated goat anti-mouse IgG

secondary antibody added at a dilution of 1:200 (in 1% BSA, PBS/T). Slides were incubated for 30 min, washed, and mounted with Mowiol® and a coverslip. Slides were dried at RT overnight in the dark.

Sporozoite IFATs were kindly carried out by Katharine Collins at the Jenner Institute.

Blood-stage *P. berghei* ANKA schizonts were prepared by overnight incubation of blood from an infected mouse (with > 5% pRBC) in blood-stage culture medium in a shaking incubator. The presence of schizonts was confirmed by Giemsa thin blood smear. Culture medium was gently spun, the pellet washed twice in PBS, and slides made. Slides were fixed for 30 min in 4% paraformaldehyde, washed twice in PBS, and incubated for 10 min in 0.1% Triton X100 to permeabilise infected erythrocytes. Slides were then washed again in PBS, incubated for 5 min in 0.01% sodium borohydride and given a final wash in PBS before blocking for 1 h in 1% BSA, 10% goat serum in PBS.

Serum samples from immunised mice were diluted 1:100 in PBS containing 1% BSA and 0.5% Tween 20 and were incubated on washed slides for 30 min. Slides were washed again, air dried, and secondary antibody (Alexa Fluor® 488 conjugated goat anti-mouse IgG) diluted 1:800 in PBS (1% BSA, 0.5% Tween 20) added for a further 30 min. Finally, slides were washed again and mounted in DAPI. Slides were set overnight before examination.

Blood-stage IFATs were kindly carried out by Dr. Andrew Williams at the Jenner Institute.

2.7.3 Rabbit IgG purification

Polyclonal rabbit IgG was purified from serum samples by Protein G affinity chromatography. Polypropylene IgG purification columns were packed with 2mL (settled) protein G gel according to the manufacturer's instructions and all buffers were warmed to RT before use. 10mL Immunopure IgG binding buffer was run through the column and discarded. 3mL of rabbit serum was diluted 1:1 in binding buffer was then run through the column, followed by a further 20mL of binding buffer. IgG was then eluted from the column by using 10mL Immunopure IgG elution buffer and collecting the sample in 300μL Tris pH9 (neutralisation buffer). Columns were washed with elution buffer and stored at 4ºC filled with storage buffer for re-use.

IgG samples required buffer exchanging into RPMI. This was done by transferring samples into an Amicon centrifugal filter device and adding RPMI-1640 to a total of 15mL. Samples were then centrifuged at 3095 *g* for 45 min, until ~250μL remained above the filter. The flow-through was discarded and the filter filled to 15mL again, this time with incomplete medium, before repeating centrifugation. Finally, the remaining sample was drawn from the filter using a 2mL syringe and 19G needle. The sides of the filter were then rinsed five times with ~300μL incomplete medium and the filter centrifuged for a brief period to draw the liquid to the bottom. The additional 300μL was then drawn up in the same needle and syringe as the rest of the sample. The sample was then pushed through a small diameter Millex-Gv filter into a sterile 1.5mL eppendorf.

IgG was quantified using a spectrophotometer at 280nm. The extinction coefficient for rabbit IgG is 1.44 (i.e. 1mg/mL of rabbit IgG has an OD of 1.44 at 280nm). The concentration of antibody was therefore calculated as follows:

Concentration of rabbit IgG (mg/mL) = (OD x dilution factor)/1.44

2.7.4 *In vitro* Growth Inhibition Assay (GIA)

Polyclonal rabbit IgG was purified (as described in 2.7.3). A standardized *in vitro* GIA was carried out at the PATH Malaria Vaccine Initiative GIA reference center (LMVR, NIAID, NIH) as previously described [254]. Briefly, synchronized *P. falciparum* parasites (late trophozoites and schizonts), culture medium and purified IgG were incubated for ~40 h in 96-well tissue culture plates. Parasite lactate dehydrogenase (pLDH) was then used to calculate relative parasitaemia, and the results were determined by the OD_{650}. Percent inhibition due to immune IgG was calculated as follows:

$$\% \text{ GIA} = 100 - \left[\frac{(A_{650} \text{ of pRBC with test IgG} - A_{650} \text{ of normal RBC})}{(A_{650} \text{ of pRBC without any IgG} - A_{650} \text{ of normal RBC})} \right] \times 100$$

Rabbit IgG from individual animals were tested at a final concentration of 5mg/mL and 2.5mg/mL against 3D7 strain *P. falciparum* parasites.

2.8 Parasitology

2.8.1 Parasite strains

***P. yoelii* YM** lethal parasites were kindly provided by Dr G. A. Butcher, Imperial College, London, UK [48, 102].

***P. berghei* ANKA** strain 234 (wild-type *P. berghei*) [244, 255].

***P. berghei* NK65** (MR4 Number: MRA-268) was obtained through the Malaria Research and Reference Reagent Resource Centre (MR4) and was deposited by Prof. V. Nussenzweig, New York University, USA.

Pb-PfM19 and Pb-PbM19. These parasite lines were kindly provided by Dr B. S. Crabb and Dr T. F. de Koning-Ward, Walter and Elisa Hall Institute (WEHI, Australia). *P. berghei* ANKA chimeric parasite lines were generated using an allelic replacement approach [57]. Pb-PfM19 expresses *P. falciparum* strain D10 MSP-1_{19} (MAD20 allele) in place of wild-type *P. berghei* MSP-1_{19}, and Pb-PbM19 was generated as a transfection control, replacing wild-type *P. berghei* MSP-1_{19} with the identical wild-type sequence. Identity PCR to confirm parasite identity was carried out by Dr. Anna Goodman.

2.8.2 Quantification of blood-stage parasitaemia

Blood-stage parasitaemia was quantified by Giemsa-stained thin blood smear. Blood smears were made by snipping <1mm from the mouse tail tip and spreading one drop of blood on a glass slide. Slides were air-dried and fixed in methanol for > 1 min and

then stained in 5% Giemsa (diluted in dH_2O) for at least 30 min. Slides were then dried and examined on a light microscope at 100x under oil immersion. Each field of view contained approximately 500 RBC and a mouse was deemed uninfected upon the absence of patent parasitaemia in 50 fields of view. The percentage of pRBCs was calculated, according to Table 2.5.

Table 2.5: Number of fields of view counted to calculate pRBC

% Parasitaemia (P)	No. RBCs Counted	No. Fields of View @ 100x magnification	No. Parasites (p)
P ≥ 10%	500	1	**p** ≥ 50
10 > **P** ≥ 1%	2500	5	250 > **p** ≥ 25
1% > **P** > 0.1%	5000	10	50 > **p** > 5
P ≤ 0.1%	25000	50	**p** ≤ 25

2.8.3 Cryopreservation of blood-stage parasites

Donor mice were infected either from naïve infected mice or from an aliquot of parasite-infected blood which was thawed and immediately injected i.p.. Blood-stage parasitaemia was monitored until ~10-20%. Donor mice were culled by cervical dislocation and blood was rapidly collected by cardiac puncture into 10x heparin (final heparin concentration 30U/mL blood). Blood was mixed with an equal volume of ice-cold 20% DMSO in blood-stage culture medium, divided into 0.1mL aliquots and snap frozen in liquid nitrogen. Aliquots were stored at -196°C in liquid nitrogen.

2.8.4 pRBC challenge

Three days prior to *P. yoelii* challenge and 5-6 days prior to *P. berghei* challenge, an aliquot of infected blood was thawed and 50-100μL immediately injected i.p. into a donor mouse. On the day of challenge, the RBC/mL of blood was counted (Section 2.5.5) and the percentage parasitaemia calculated (Section 2.8.2) in order to quantify the number of infected RBC (pRBC) per μL of blood (pRBC/μL). Immediately prior to challenge, the donor mouse was bled by nicking the tail and blood was diluted to 10^5 pRBC/mL for *P. yoelii* and 10^3 pRBC/mL for *P. berghei* in PBS such that 100μL for i.v. injection contained 10^4 pRBC and 10^2 pRBC respectively. Blood-stage parasitaemia was monitored once daily from day 3 post-challenge by Giemsa-stained thin blood smear (Section 2.8.2) and at the peak of infection health status was monitored twice daily. Mice were culled if they reached the humane end-point of >70% parasitaemia. Mice that were protected did not reach these levels of parasitaemia and were monitored for at least 30 days post-challenge.

2.8.5 Sporozoite challenge

Laboratory-reared female *Anopheles stephensi* mosquitoes (J144) were infected by blood feed on a *P. yoelii* YM infected TO mouse (15-25% parasitaemia), then maintained at 23.5 - 25°C for 15 days (J146). This work was carried out by Mr Mark Tunnicliff at the Peter Medawar Building, University of Oxford. Mosquito salivary glands were dissected on day 15 and collected in a glass homogeniser containing RPMI-1640. Following homogenisation, sporozoites were counted using a haemocytometer and diluted to the desired concentration in RPMI-1640 on ice. Mice were infected with 50, 250 or 1000 spz by i.v. injection in a volume of 100μL. From

day 4 or 5 post-challenge, mice were monitored daily by quantification of blood-stage parasitaemia (Section 2.8.2).

2.9 Statistics

Data were analysed using GraphPad Prism v5.01. The normality of the data was determined using the Kolmogorov-Smirnov one-sample test. All data were compared using non-parametric statistics, due small group size of ≤ 8 mice/group. Two groups were compared using a Mann-Whitney U test and multiple groups were analysed by Kruskall-Wallis test with post-hoc Dunn's multiple comparison test. Paired data, such as antibody responses in a single mouse over time, were assessed by Friedman test and non-parametric spearman's rank correlations were performed.

In all cases $P \leq 0.05$ was considered significant (*$P \leq 0.05$, ** $P \leq 0.01$ and ***$P \leq 0.001$).

Chapter 3

A combination vaccine strategy targeting both liver- and blood-stage malaria

3 A combination vaccine strategy targeting both liver- and blood-stage malaria

3.1 Introduction

It is widely acknowledged that a multi-stage and/or multi-antigen formulation malaria vaccine will likely be necessary to provide high-level efficacy against *P. falciparum*. The feasibility of a combination vaccine strategy targeting both the liver- and blood-stages of malaria has not previously been assessed with the clinically relevant Ad_MVA prime-boost vectored vaccine platform. The work described in this chapter sought to evaluate in mice the co-administration of vectors expressing the *P. yoelii* antigens CSP (PyCSP) and $MSP1_{42}$ ($PyMSP1_{42}$) in an Ad_MVA prime-boost regime.

Viral vectored vaccines expressing PyCSP are reported to be protective against liver-stage infection [75, 77-79, 256, 257]. In BALB/c mice protection is thought to be largely dependent upon $CD8^+$ T cell responses against the dominant $CD8^+$ T cell epitope, $PyCSP_{280-288}$ [249, 258]. A sub-dominant $CD8^+$ T cell epitope ($PyCSP_{58-67}$) has also been identified [83]. A $CD4^+$ T cell epitope [250] (sometimes referred to as Py1, but known as $PyCSP_{59-79}$ throughout this study) provides help for antibody induction against peptides from the repetitive central region $(QGPGAP)_n$. Passive transfer of PyCSP-specific monoclonal antibodies can protect against *P. yoelii* sporozoite challenge [259], and protection by polyclonal anti-PyCSP antibodies induced by vaccination has also been demonstrated [76]. However, in the majority of studies protection is $CD8^+$ T cell, rather than antibody, mediated. A schematic of the PyCSP epitopes in BALB/c mice is shown in Figure 3.1. AdHu5 and MVA vectors

expressing PyCSP have been generated at the Jenner Institute [102], but have not previously been extensively tested for both immunogenicity and protection.

Extensive studies using AdHu5 and MVA vectors expressing $PyMSP1_{42}$ vaccines have been carried out previously at the Jenner Institute by Draper *et al.*. Priming with 5×10^{10} vp AdHu5 and boosting with 5×10^{7} pfu MVA both expressing $PyMSP1_{42}$ protects 76% of BALB/c mice against challenge with 10^{4} *P. yoelii* infected RBC (pRBC) [48]. These mice all developed patent blood-stage parasitaemia, but infection was controlled by vaccine-induced $PyMSP1_{19}$ antibodies. The same immunisation regime protected 100% of BALB/c mice challenged with 50 *P. yoelii* sporozoites [102]. The parasite virulence between the two challenge models is considered comparable [102], and this enhanced survival is attributed to partial protection against the liver-stage infection due to $PyMSP1_{33}$-specfic $CD8^{+}$ T cell responses. Protection is further improved if the vaccine constructs included the molecular adjuvant IMX108. This is the α chain oligomerisation domain of murine C4bp and is described in more detail in Chapter 5. Immunisation with the same regime encoding $PyMSP1_{42}$-IMX108 protected 100% of BALB/c mice against pRBC challenge, with an absence of patent parasitaemia in 59% [48]. This protection was associated with significantly higher antigen-specific total IgG titres and a shift towards T_H1 associated antigen-specific IgGs. The responses induced against $PyMSP1_{42}$ in BALB/c mice are summarised in Figure 3.1.

The work presented in this chapter investigates the immunogenicity and efficacy of both single-shot AdHu5-PyCSP and Ad_MVA PyCSP prime-boost immunisation regimes. Dosing studies related to priming and boosting with the Ad_MVA

PyMSP1$_{42}$ immunisation regime (with or without IMX108) are also performed. Finally, it is investigated whether mixing the PyCSP and PyMSP1$_{42}$-IMX108 vaccines in an Ad_MVA regime is a feasible method of developing an immunogenic and efficacious multi-stage malaria vaccine.

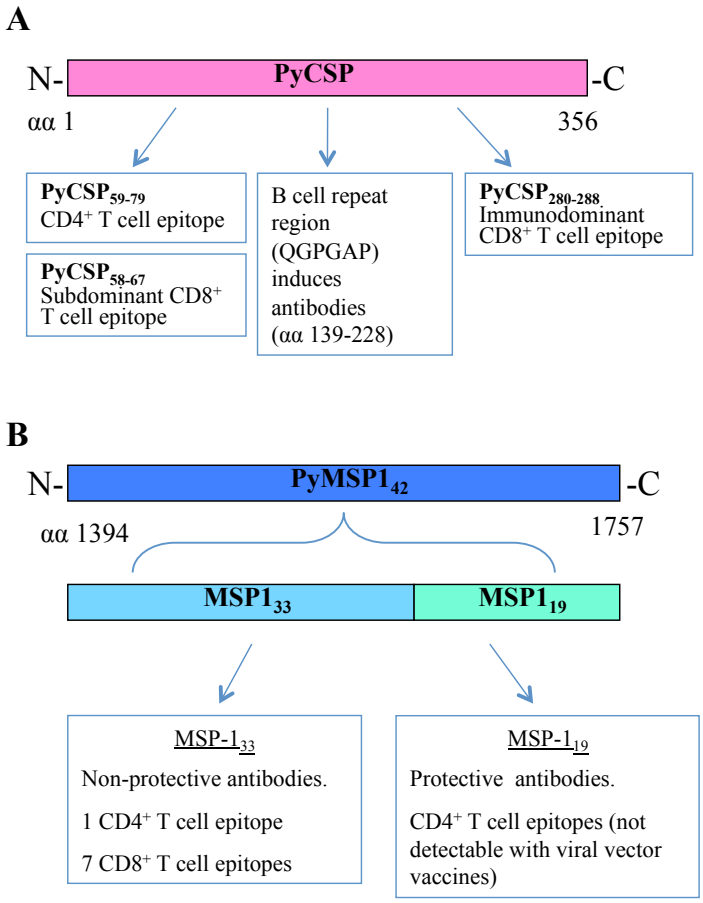


Figure 3.1: Schematic representation of PyCSP (A) and PyMSP1$_{42}$ (B) epitopes in BALB/c mice

Both diagrams show the length of the antigen in amino acids (αα). For each PyCSP epitope the subscript indicates its αα position within the protein [76, 83, 249, 250]. PyMSP1$_{42}$ is cleaved into two fragments, PyMSP1$_{33}$ and PyMSP1$_{19}$ by proteolytic processing during erythrocyte invasion. The epitopes within each of these fragments are summarised [99, 102, 244, 260].

3.2 Results

3.2.1 Immunogenicity and efficacy of PyCSP vaccines

3.2.1.1 Humoral immune responses to AdHu5 and MVA PyCSP vaccines

The induction of PyCSP-specific antibodies was investigated by collection of sera 3 and 8 weeks after priming with AdHu5-PyCSP and 2 weeks after boosting with MVA-PyCSP. Total IgG responses were assayed by ELISA against the PyCSP-specific branched peptide MAP4-Py1T-Py3, which contains the central repeat region (Py3 indicates three copies of the QGPGAP repeat) (Figure 3.2A). Three weeks after the prime, none of the mice primed with 10^7 vp AdHu5-PyCSP had developed a detectable CSP-specific response, whilst following the three higher doses all mice had seroconverted. Responses were significantly lower in mice immunised with 10^7 vp AdHu5-PyCSP compared to 10^{10} vp. By week 8 only one mouse immunised with the lowest dose had developed detectable IgG responses. After the MVA boost, CSP-specific total IgG increased in all four groups and responses were not significantly different between the groups. However, in a separate experiment responses were assayed only at day 68 (12 days post MVA-PyCSP boost) and were significantly lower if mice had been primed with a dose of 10^7 vp (Figure 3.2B). To confirm that the antibodies induced were conformation-specific and capable of binding *P. yoelii* sporozoites, an immunofluorescence antibody test (IFAT) was carried out by Katharine Collins at the Jenner Institute. Sera from mice immunised with 1 x 10^{10} vp AdHu5 PyCSP and boosted eight weeks later with 1 x 10^7 pfu MVA PyCSP were tested and found to be capable of binding *P. yoelii* sporozoites at a dilution of 1:100 (Figure 3.3).

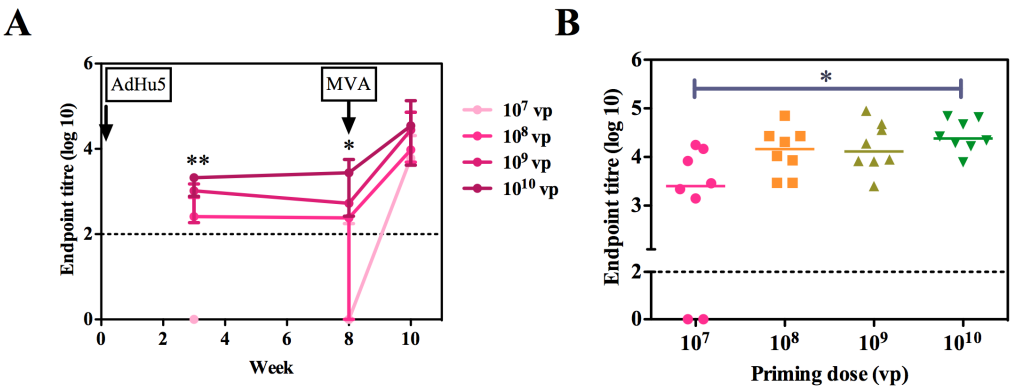


Figure 3.2: Antibody responses following immunisation with Ad_MVA PyCSP

BALB/c mice were immunised i.d. at week 0 with 10^7, 10^8, 10^9 or 10^{10} vp AdHu5-PyCSP and boosted 8 weeks later with 10^7 pfu MVA-PyCSP. Sera were collected at weeks 3, 8 and 10 and total IgG against PyCSP was assayed by ELISA at all time-points (A). n = 4 mice/group. Median and range are shown. ** $P < 0.01$, 10^7 vp AdHu5-PyCSP differs from 10^{10} vp AdHu5-PyCSP at week 3 and * $P < 0.05$, 10^7 vp AdHu5-PyCSP differs from 10^{10} vp AdHu5-PyCSP at week 8 by Dunn's multiple comparison test.

In a separate experiment using the same immunisation regime, responses were measured 12 days post-boost (B). n = 8 mice/group and bars represent median titre. * $P < 0.05$, 10^7 vp AdHu5-PyCSP differs from 10^{10} vp AdHu5-PyCSP by Dunn's multiple comparison test.

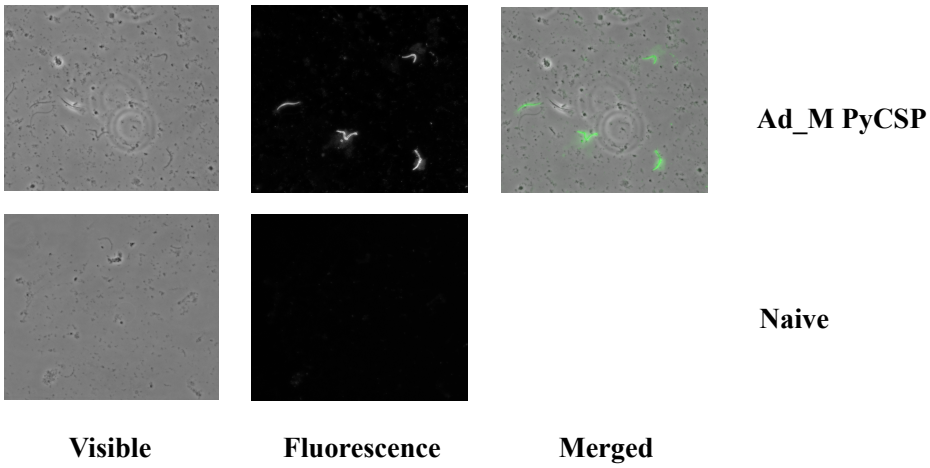


Figure 3.3: Visible and fluorescence images of *P. yoelii* sporozoites immunostained with PyCSP antibodies raised in mice.

BALB/c mice were immunised i.d. with 1 x 10^{10} vp AdHu5-PyCSP and boosted with 1 x 10^{7} pfu MVA-PyCSP. Sera were collected two weeks later and were used at a dilution of 1:100 to stain *P. yoelii* sporozoites. Sera from naïve (non-immunised) mice were also tested. Bound antibodies were detected using goat anti-mouse IgG conjugated to Alexa Fluor™ 488.

3.2.1.2 Cellular immune responses to AdHu5 and MVA PyCSP

It has been shown that that IFN-γ and TNFα are important components of the non-cytolytic pathways underlying $CD8^+$ T cell-mediated efficacy against intracellular liver-stage malaria infection in mice [84, 261], and also that $CD4^+$ T cells simultaneously producing IFN-γ, TNFα and IL-2 are associated with protection against *Leishmania major* in a murine challenge model [262]. Therefore, production of IFN-γ, TNFα and IL-2 from T cells following vaccination was measured and the multi-functionality of the cells was assessed. The so-called "quality" of the antigen-specific cells was analysed in terms of production of all three cytokines.

Both single-shot AdHu5 and AdHu35 vectors expressing PyCSP have been previously shown to be immunogenic and highly efficacious against sporozoite challenge [75, 77, 78]. Therefore immunogenicity following a single immunisation with the AdHu5-PyCSP vaccine was investigated. BALB/c mice were immunised i.d. with 10^9 or 10^{10} vp AdHu5-PyCSP. Two weeks later the mice were culled and cellular responses in both the spleen and liver were analysed by ICS following stimulation with three different peptides, $PyCSP_{280\text{-}288}$, $PyCSP_{58\text{-}67}$ and $PyCSP_{59\text{-}79}$. In a separate experiment, twelve days after immunisation with four different doses of AdHu5-PyCSP cellular immune responses in the blood were assayed. In this case cells were only stimulated with the dominant $CD8^+$ T cell epitope ($PyCSP_{280\text{-}288}$) and the sub-dominant $CD8^+$ T cell epitope ($PyCSP_{58\text{-}67}$). Following both experiments, expression of IFN-γ, TNFα and IL-2 was analysed from $CD8^+$ T cells (Figure 3.4). These data confirm that the AdHu5-PyCSP vaccine is also immunogenic for T cell responses. As expected, the strongest $CD8^+$ T cell responses were observed following stimulation with the published dominant $CD8^+$ T cell epitope, $PyCSP_{280\text{-}288}$, with

relatively weaker responses to the sub-dominant epitope, $PyCSP_{58-67}$. Almost no $CD8^+$ T cell response was observed in the spleen or the liver following stimulation with the longer peptide, $PyCSP_{59-79}$, which is reported to contain a $CD4^+$ T cell epitope [250].

In all three organs tested, the responding cells produced the greatest amount of IFN-γ, with less TNFα and very little IL-2. The proportion of cells producing all three cytokines simultaneously (3+) was relatively small but comparable between spleen and liver. However, in the blood almost no 3+ cells were detected due to lack of an IL-2 response. In all three organs, cells producing two cytokines (2+) predominantly produced IFN-γ and TNFα but not IL-2, whilst single cytokine producing cells (1+) primarily produced IFN-γ only. In the spleen and liver the dose of vaccine used did not alter the "quality" of the response in terms of the proportion of cells producing each combination of cytokines. However, in the blood the "quality" of the response at the lowest dose was more difficult to assess accurately due to the extremely low magnitude of the response induced.

In the first experiment (in which responses were measured in the spleen and liver), responses in the spleen to $PyCSP_{280-288}$ were significantly higher (in terms of production of both IFN-γ and TNFα) when 10^{10} vp of AdHu5 vaccine was administered as compared to 10^9 vp. However, this was not reflected in the liver, where both doses performed comparably. Despite this difference, responses in the spleen and liver correlated ($P < 0.0001$ and $r = 0.7736$ by Spearman's correlation for IFN-γ, $n = 23$), although the magnitudes of responses in the liver were 10-20 fold higher than in the spleen for all three cytokines.

Production of IFN-γ, TNFα and IL-2 from $CD4^+$ cells was also assessed in the spleen and the liver. However, in both organs, $CD4^+$ T cell responses following stimulation with the published $CD4^+$ T cell epitope, $PyCSP_{59-79}$, were no higher than background (Unstim) or controls stimulations with the $CD8^+$ T cell epitopes, $PyCSP_{280-288}$ and $PyCSP_{58-67}$ (Figure 3.5). In the spleen, IFN-γ and TNFα background responses and stimulated responses were extremely low at <0.05% of total $CD4^+$ cells, background IL-2 was high with ~0.15% of $CD4^+$ cells producing IL-2. In the liver background responses for all three cytokines were high at ~0.5% of all $CD4^+$ cells. In these experiments ICS analysis was not performed on naïve mice, therefore it could not be determined whether this background response is induced by vaccination or is present pre-vaccination.

$CD8^+$ T cell responses were next assessed after boosting with MVA-PyCSP. Mice were primed with four different doses of AdHu5-PyCSP as previously, and eight weeks later all the mice were boosted with 1 x 10^7 pfu MVA-PyCSP. Responses in the blood were measured twelve days later by ICS (Figure 3.6). These data show that the priming dose can influence post-boost immunogenicity. Mice that had been primed with 10^9 or 10^{10} vp AdHu5-PyCSP produced significantly more IFN-γ than mice primed with 10^7 vp AdHu5-PyCSP, although one mouse at the top dose did not respond to boosting. As after the AdHu5-PyCSP prime, IL-2 was not detected in peptide stimulated PBMCs post-boost. The "quality" of the multi-functional response was not altered by boosting in mice primed with 10^8, 10^9, or 10^{10} vp AdHu5-PyCSP. Also in this case, the multifunctional "quality" of the response in mice primed with 10^7 vp AdHu5-PyCSP shifted and was no longer significantly different to the other doses post-boost. Post-boost $CD4^+$ T cell responses were not tested here, but have

previously been tested in the spleen and were found to be < 0.1% (Dr. Sumi Biswas, DPhil thesis).

Overall, these data demonstrate that both AdHu5-PyCSP and MVA-PyCSP are immunogenic. Following AdHu5 priming, $CD8^+$ T cell and antibody responses are induced and both of these were significantly boosted by MVA-PyCSP. A high dose of either 10^9 or 10^{10} vp AdHu5-PyCSP gave the strongest responses post-prime and these were maintained post-boost.

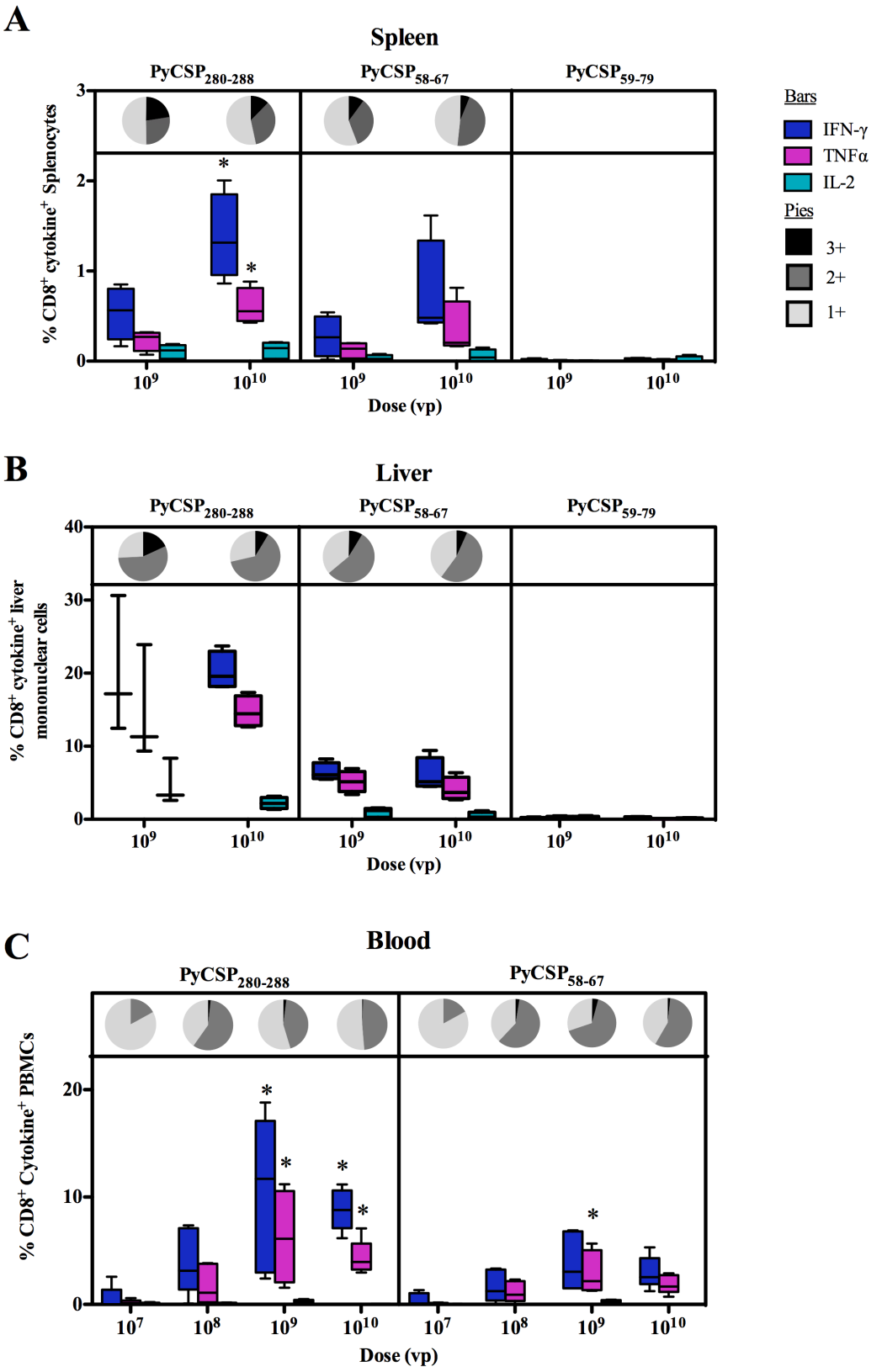

A
Spleen
PyCSP280-288
PyCSP58-67
PyCSP59-79
% CD8+ cytokine+ Splenocytes
Dose (vp)
Bars
IFN-γ
TNFα
IL-2
Pies
3+
2+
1+
B
Liver
% CD8+ cytokine+ liver mononuclear cells
C
Blood
% CD8+ Cytokine+ PBMCs

Figure 3.4: CD8$^+$ T cell responses following immunisation with AdHu5-PyCSP

Production of IFN-γ, TNFα and IL-2 from CD8$^+$ T cells in the spleen (A), liver (B) or blood (C), two weeks (A and B) or three weeks (C) after i.d. immunisation of BALB/c mice with the indicated doses of AdHu5-PyCSP. Box and whisker plots show median, interquartile range (IQR) and range. The peptide used to stimulate the cells is indicated above the bars and un-stimulated responses are subtracted. n = 4/group in the spleen, n = 3-4/group in the liver and n = 5/group in the blood. The pies indicate the proportion of cells producing all three cytokines (3+: IFN-γ$^+$TNFα$^+$IL-2$^+$), two cytokines (2+: predominantly IFN-γ$^+$TNFα$^+$IL-2$^-$) or a single cytokine (1+: predominantly IFN-γ$^+$TNFα$^-$IL-2$^-$). Pies are not shown if responses were not above background. * $P < 0.05$ compared to production of the same cytokine following immunisation with the lowest dose of AdHu5-PyCSP and stimulation with the same peptide.

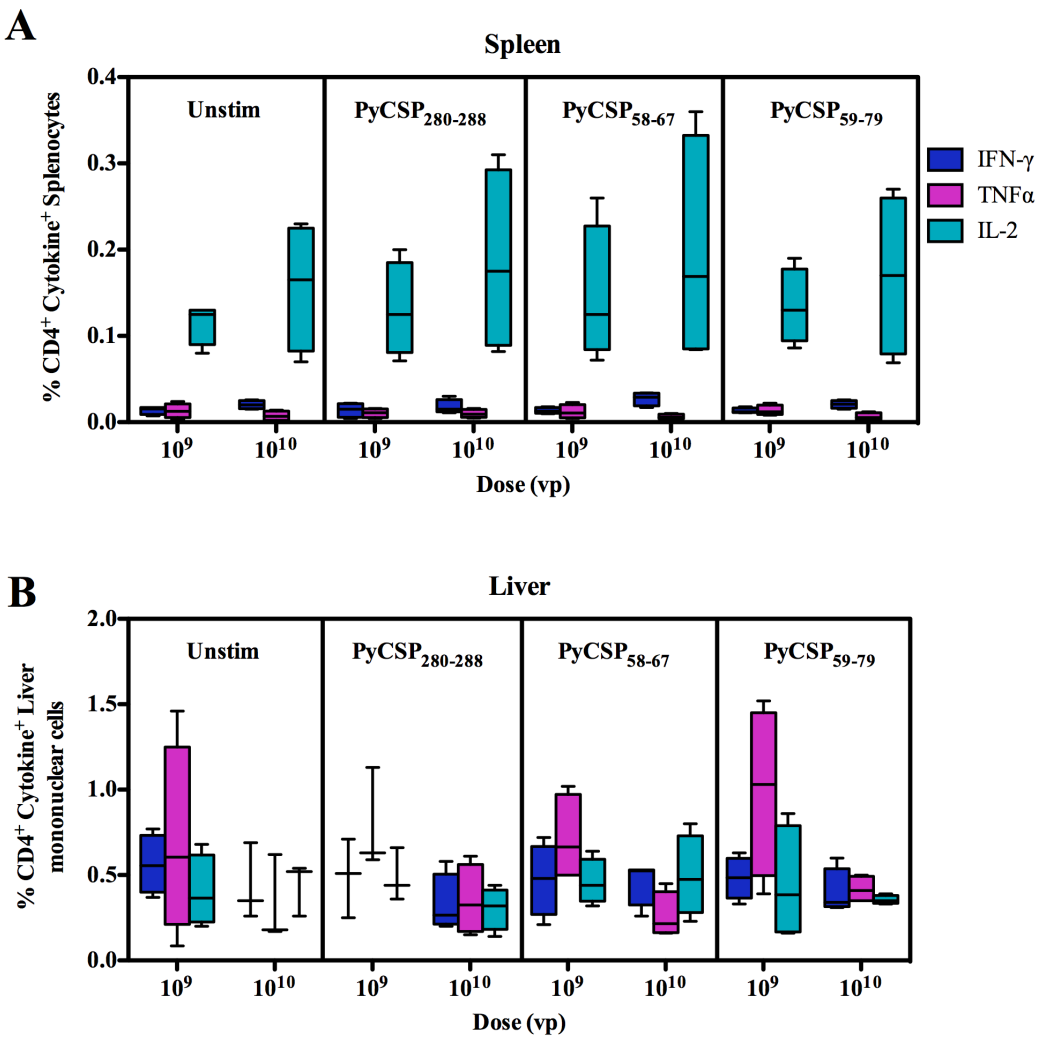


Figure 3.5: CD4$^+$ T cell responses following immunisation with AdHu5-PyCSP

Production of IFN-γ, TNFα and IL-2 from CD4$^+$ T cells in the spleen (A) and liver (B) two weeks after i.d. immunisation of BALB/c mice with 10^9 or 10^{10} vp AdHu5-PyCSP. Box and whisker plots show median, IQR and range. Cells were either unstimulated or were stimulated with the indicated peptides. (Unstimulated responses are not subtracted). n = 4/group in the spleen, n = 3-4/group in the liver.

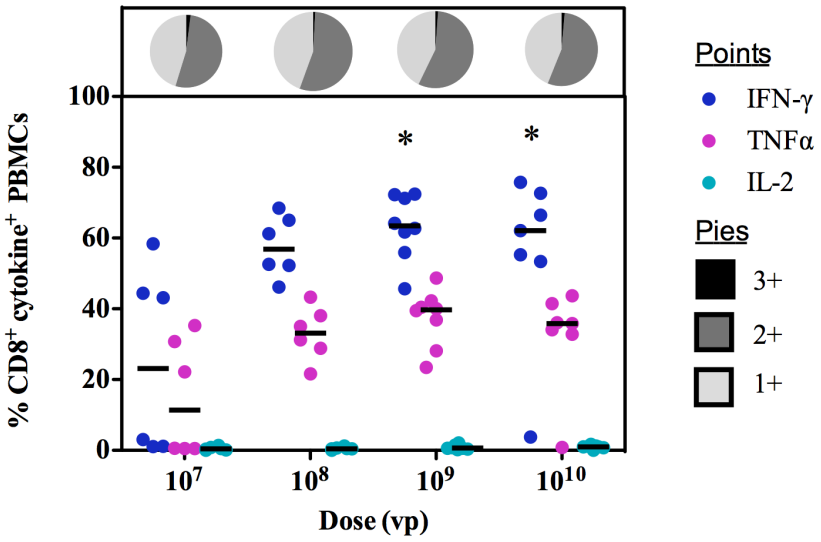


Figure 3.6: $CD8^+$ T cell responses in peripheral blood PBMCs following immunisation with Ad_MVA PyCSP

BALB/c mice were immunised i.d. with 10^7, 10^8, 10^9 or 10^{10} vp AdHu5-PyCSP and eight weeks later all mice were boosted i.d. with 1 x 10^7 pfu MVA-PyCSP. Twelve days post-boost mice were bled and cells stimulated with $PyCSP_{280-288}$ before ICS. Production of IFN-γ, TNFα and IL-2 from $CD8^+$ T cells is shown. Points represent individual mice and lines show median responses for n = 6-8 mice/group. The pies indicate the proportion of cells producing all three cytokines (3+: IFN-γ^+TNFα^+IL-2^+), two cytokines (2+: predominantly IFN-γ^+TNFα^+IL-2^-) or a single cytokine (1+: predominantly IFN-γ^+TNFα^-IL-2^-). * $P < 0.05$ for the same cytokine compared to priming with 10^7 vp AdHu5-PyCSP by Dunn's post-hoc multiple comparison text.

3.2.2 Efficacy of PyCSP vaccines against *P. yoelii* sporozoite challenge

Protective efficacy of a malaria challenge can be assessed according to several criteria. The primary outcome following challenge with a lethal strain of malaria is survival, regardless of whether mice are immunised with a blood or liver-stage vaccine. Following immunisation with a vaccine that targets only the liver-stage of infection, any parasites that emerge in the blood will inevitably result in death of the mouse. Therefore following a liver-stage vaccine regime, survival indicates sterile protection. If mice are not sterilely protected, efficacy of a liver-stage vaccine may also be assessed by the reduction of liver parasite burden. This may be quantified in terms of the number of parasites that emerge in the blood on day five post-challenge, or alternatively by real-time RT-PCR to quantify parasites in the liver [102, 263].

A number of studies have shown that a single vaccination with an adenoviral vaccine expressing PyCSP is sufficient to provide protection against a standard *P. yoelii* sporozoite challenge [75, 77, 78]. However, despite the development of both antibody and $CD8^+$ T cell responses to single-shot AdHu5-PyCSP, in this study little efficacy was observed when mice were challenged with 50 sporozoites two weeks after immunisation. Overall only two mice that had been immunised with 10^9 vp AdHu5-PyCSP, and one mouse immunised with 10^{10} vp AdHu5-PyCSP survived (ie. were sterilely protected) (Table 3.1). However, a significant delay to onset of patent blood-stage parasitaemia was observed compared to naïve mice in the 10^8 and 10^9 vp AdHu5-PyCSP groups (Figure 3.7). This was assessed by analysing parasitaemia on day 5 post-challenge and indicates a small but significant reduction in liver-parasite burden.

In order to assess the efficacy of an Ad_MVA PyCSP prime-boost immunisation regime, mice were challenged with 50 sporozoites two weeks after the MVA boost (Table 3.2). Unfortunately due to non-viability of the parasites on three occasions (and therefore not all the naïve mice becoming infected), the true level of efficacy could not be identified. On the third occasion none of the naïve mice were infected (data not shown). This was likely due to poor sporozoite viability caused by temperature fluctuations in the insectary during the mosquito stage of the *P. yoelii* life-cycle. Nevertheless, for all four immunisation regimes tested, survival was greater than that observed in naïve mice, suggesting that the vaccine did have some effect on the liver parasite burden. In agreement with the $CD8^+$ T cell responses (Figure 3.6), survival was comparable between the top three doses, but lower in mice primed with 10^7 vp AdHu5-PyCSP.

Dose AdHu5-PyCSP (vp)	**10^7**	**10^8**	**10^9**	**10^{10}**	**Naïve**
No. survived / No. challenged	0/5	0/5	2/5	1/5	0/5
% sterilely protected	0%	0%	40%	20%	0%

Table 3.1: Protective efficacy of AdHu5-PyCSP immunisation against *P. yoelii* sporozoite challenge

BALB/c mice were immunised with AdHu5-PyCSP at the indicated doses. Two weeks later they were challenged with 50 sporozoites i.v. and monitored for survival.

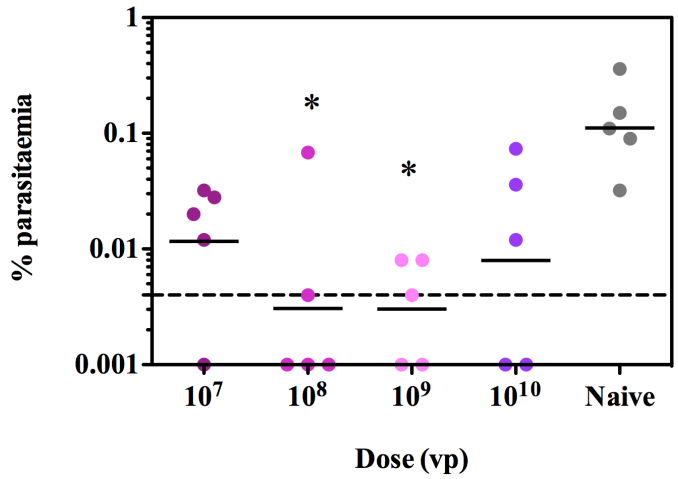


Figure 3.7: Parasitaemia five days post sporozoite challenge

Female BALB/c mice were immunised i.d. with 10^7 – 10^{10} vp AdHu5-PyCSP. Two weeks later mice were challenged i.v. with 50 *P. yoelii* sporozoites. Parasitaemia five days after challenge is shown. Points represent individual mice and bars indicate the geometric mean parasitaemia. * $P < 0.05$, parasitaemia on day 5 post challenge in mice immunised with 10^8 or 10^9 vp AdHu5-PyCSP compared to Naïve mice by Dunn's post-hoc multiple comparison test.

Priming Dose (vp)	10^7	10^8	10^9	10^{10}	Naïve
No. survived / no. challenged	8/15	14/16	13/16	13/16	7/16
% sterilely protected	53%	87.5%	81.3%	81.3%	43.8%

Table 3.2: Protective efficacy of Ad_MVA-PyCSP immunisation against *P. yoelii* sporozoite challenge

BALB/c mice were immunised with 10^7 – 10^{10} vp AdHu5-PyCSP as indicated and boosted 8 weeks later with 10^7 pfu MVA-PyCSP. Two weeks after MVA immunisation mice were challenged with 50 sporozoites. Due to non-viability of parasites, not all the naïve mice were infected.

3.2.3 Immunogenicity and efficacy of Ad_MVA PyMSP1$_{42}$-IMX108

The vaccine doses used in previous mouse studies of Ad_MVA PyMSP1$_{42}$ immunisation were relatively high, and a combination vaccine strategy containing a mixture of vaccines targeting PyMSP1$_{42}$ and PyCSP will not be able to use each of these vaccines at such a high dose. For this reason the protective efficacy of lower doses of AdHu5 and MVA expressing PyMSP1$_{42}$ is investigated here, both with and without C-terminal fusion of the expressed antigen to IMX108.

BALB/c mice were primed with either 10^9 or 10^{10} vp AdHu5-PyMSP1$_{42}$ ± IMX108 and boosted eight weeks later with 10^6 or 10^7 pfu MVA-PyMSP1$_{42}$ ± IMX108. Two weeks post-boost antibody responses were assayed in the serum by ELISA. Only the boosting dose consistently had a significant effect on total IgG titres, with the higher dose (1 x 10^7 pfu MVA) inducing greater amounts of IgG (Figure 3.8A). For IgG2a the effect of the boosting dose is highly significant (P = 0.008) by factorial ANOVA, whilst effect of priming dose and IMX108 are not (Figure 3.8B). Responses tended towards being higher if the vaccine included IMX108, but only if a high dose prime was given. This is in agreement with previously reported data for high dose vaccination which found that antibody responses were consistently increased if the construct included IMX108, however in that study lower vaccine doses were not tested [48].

Two weeks post-boost mice were challenged with 10^4 *P. yoelii* (pRBC). Table 3.3 (Experiment 1) summarises survival and parasitaemia in the different groups. None of these groups were significantly different due to the small numbers of mice used.

However with a high dose prime, the presence of IMX108 consistently led to 1/6 extra mice surviving. Peak parasitaemia also tended to be lower if the construct included IMX108 as compared to the same dose without adjuvant (Figure 3.8C). However, all mice except one developed patent parasitaemia, in contrast to previous studies with the even higher doses of 5 x 10^{10} vp AdHu5 and 5 x 10^{7} pfu MVA expressing PyMSP1$_{42}$-IMX108 [48]. Overall, peak parasitaemia negatively correlates with both total IgG and IgG2a antibody titre, demonstrating that higher antibody titres result in lower parasitaemia (Figure 3.8, C and D). IgG1 titres were not measured in this study. These results are consistent with previous studies which have shown that protection is dependent upon PyMSP1$_{19}$-specific antibodies.

These data demonstrate that the highest level of protection can be achieved with a boosting dose of 1 x 10^{7} pfu MVA, due to induction of a greater amount of antigen-specific IgG. Although results were not significant, protection also tended towards being greater if the construct included IMX108. Therefore, it was decided that in all future studies in which PyMSP1$_{42}$ vaccines would be combined with PyCSP vaccines, the blood-stage construct should be PyMSP1$_{42}$-IMX108 and the boosting dose should be the maximum feasible dose of 1 x 10^{7} pfu MVA to attempt to maximise antibody responses. Given that following a 1 x 10^{7} pfu MVA boost, survival also tended to be higher if mice had received a high dose prime, it was decided to investigate further the effect of priming dose.

BALB/c mice were primed with 5 x 10^{10} vp, 1 x10^{10} vp or 1 x10^{9} vp AdHu5-PyMSP1$_{42}$-IMX108 and all boosted eight weeks later with 1 x 10^{7} pfu MVA-PyMSP1$_{42}$-IMX108. Two weeks later mice were challenged with 10^{4} pRBC i.v. and

parasitaemia monitored. Results are shown in Table 3.3 (Experiment 2) and Figure 3.9. Surprisingly, 1/5 mice primed with 1 x10^{10} vp died. However, prior to death parasitaemia was only 32%. Given that the humane end point is > 80% parasitaemia, this suggests that this mouse may not have been immunologically fit. All other mice survived, with no patent parasitaemia observed in 4/5 mice primed with 5 x 10^{10} vp or 1 x10^{10} vp. Following the lowest dose of 1 x 10^{9} vp, 3/5 mice developed patent parasitaemia. Although these differences did not reach significance given the small number of mice used, they do suggest that a higher priming dose gives better protection. Antibodies were not assessed in these mice prior to challenge and therefore the impact of the higher dose on antibody titre is not known. However, given the previously published data and the negative correlation between anti-PyMSP1_{19} antibody titres and peak parasitaemia observed previously, it is likely that the higher AdHu5 dose used in this experiment resulted in higher PyMSP1_{19}-specific antibody titres.

Adjuvant	AdHu5 (vp)	MVA (pfu)	No. survived/ No. challenged (% protection)	No. sterile / No. challenged (% sterile)	Geometric mean (range) % parasitaemia of protected mice
Experiment 1					
IMX108	1×10^{10}	1×10^{7}	5/5 (100%)	0/5 (0%)	0.3773 (0.025 – 12.67)
		1×10^{6}	4/6 (66%)	0/6 (0%)	11.29 (1.44 – 34.29
	1×10^{9}	1×10^{7}	4/6 (66%)	1/6 (16.6%)	0.2882 (0 – 7.9)
		1×10^{6}	4/6 (66%)	0/6 (0%)	16.00 (1.5 – 37.07)
Nil	1×10^{10}	1×10^{7}	5/6 (83%)	0/6 (0%)	2.341 (0.37 – 10.6)
		1×10^{6}	3/6 (50%)	0/6 (0%)	34.47 (24.67 – 46.96)
	1×10^{9}	1×10^{7}	4/6 (66%)	0/6 (0%)	0.6826 (0.01 – 38.32)
		1×10^{6}	3/6 (50%)	0/6 (0%)	14.27 (7.8 – 26.6)
Experiment 2					
IMX108	5×10^{10}	1×10^{7}	5/5 (100%)	4/5 (80%)	0.0000175 (0 – 1.64)
	1×10^{10}	1×10^{7}	4/5 (80%)	4/5 (80%)	0
	1×10^{9}	1×10^{7}	5/5 (100%)	2/5 (40%)	0.0019 (0 – 2.32)

Table 3.3: Impact of both dose and IMX108 on survival following pRBC challenge

BALB/c mice were vaccinated i.d. with 10^{10} or 10^{9} vp AdHu5-PyMSP1$_{42}$ and boosted eight weeks later with 10^{7} or 10^{6} pfu MVA-PyMSP1$_{42}$ either with or without IMX108 fused to the antigen C-terminus as indicated. Two weeks post-boost mice were challenged with 10^{4} *P. yoelii* pRBCs. The numbers protected and numbers that did not develop patent blood-stage parasitaemia (sterilely protected) are shown, as well as the geometric mean and range of blood-stage parasitaemias for mice that survived the challenge. In all experiments naïve mice were included and all succumbed to infection (data not shown).

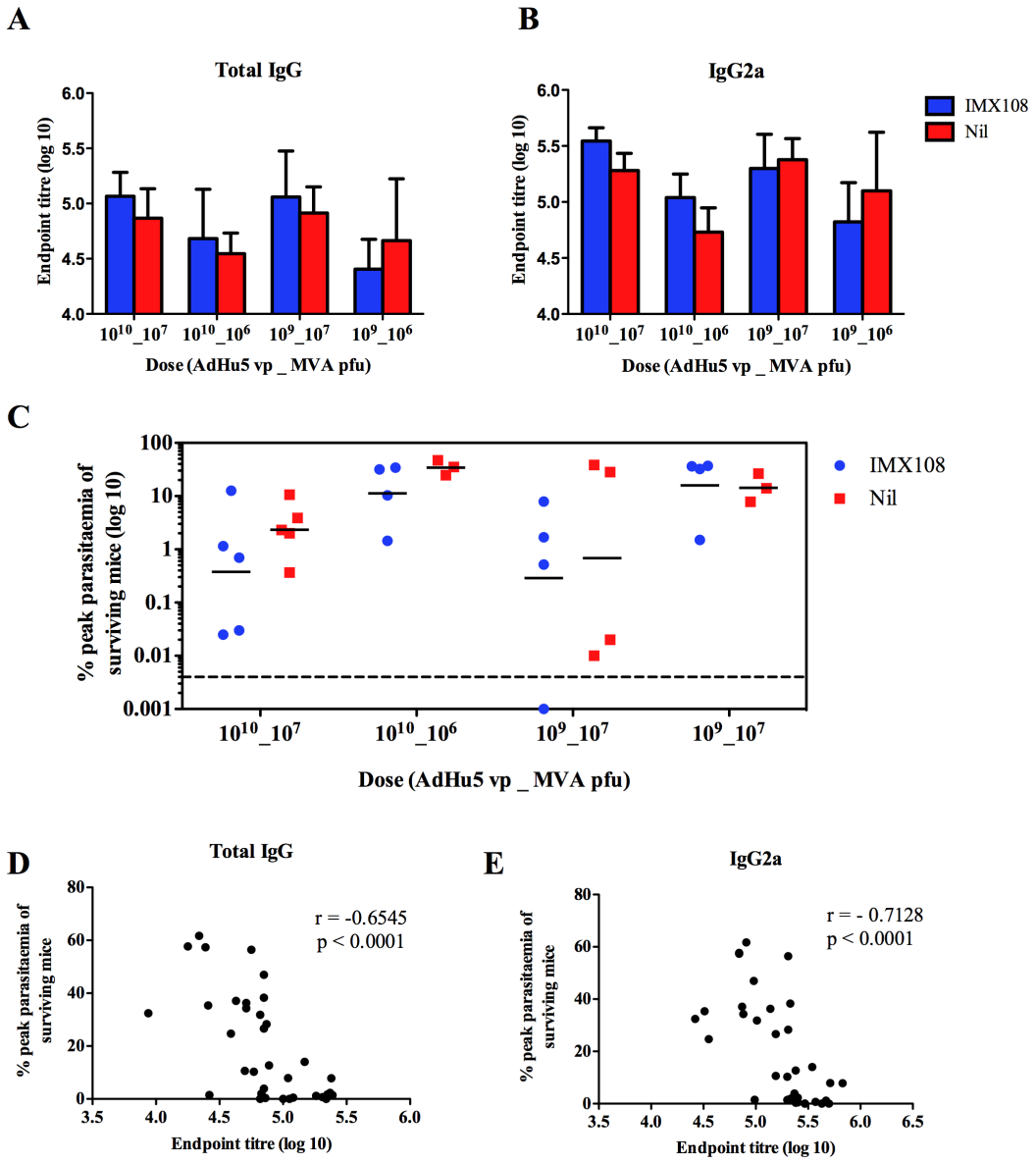

A
Total IgG
Endpoint titre (log 10)
6.0
5.5
5.0
4.5
4.0
10^10_10^7
10^10_10^6
10^9_10^7
10^9_10^6
Dose (AdHu5 vp _ MVA pfu)
B
IgG2a
IMX108
Nil
C
% peak parasitaemia of surviving mice (log 10)
100
10
1
0.1
0.01
0.001
D
Total IgG
r = -0.6545
p < 0.0001
% peak parasitaemia of surviving mice
Endpoint titre (log 10)
E
IgG2a
r = - 0.7128
p < 0.0001

Figure 3.8: Impact of dose and IMX108 on the immunogenicity and efficacy of Ad_MVA PyMSP1$_{42}$

BALB/c mice were vaccinated i.d. with 10^{10} or 10^{9} vp AdHu5-PyMSP1$_{42}$ and boosted eight weeks later with 10^{7} or 10^{6} pfu MVA-PyMSP1$_{42}$ either with or without IMX108 fused to the antigen C-terminus. PyMSP1$_{19}$-specific total IgG (A) and IgG2a (B) endpoint titres in the serum were measured by ELISA prior to challenge. Mice were challenged two weeks post-boost with 10^{4} *P. yoelii* pRBCs (Table 3.3 indicates survival in each group). Peak blood-stage % parasitaemias for mice that survived challenge are shown (C) with lines indicating the geometric mean peak parasitaemia and the dashed line indicating the lower limit of detection. Both total IgG titre (D) and IgG2a titre (E) correlate with peak parasitaemia in mice that survived challenge.

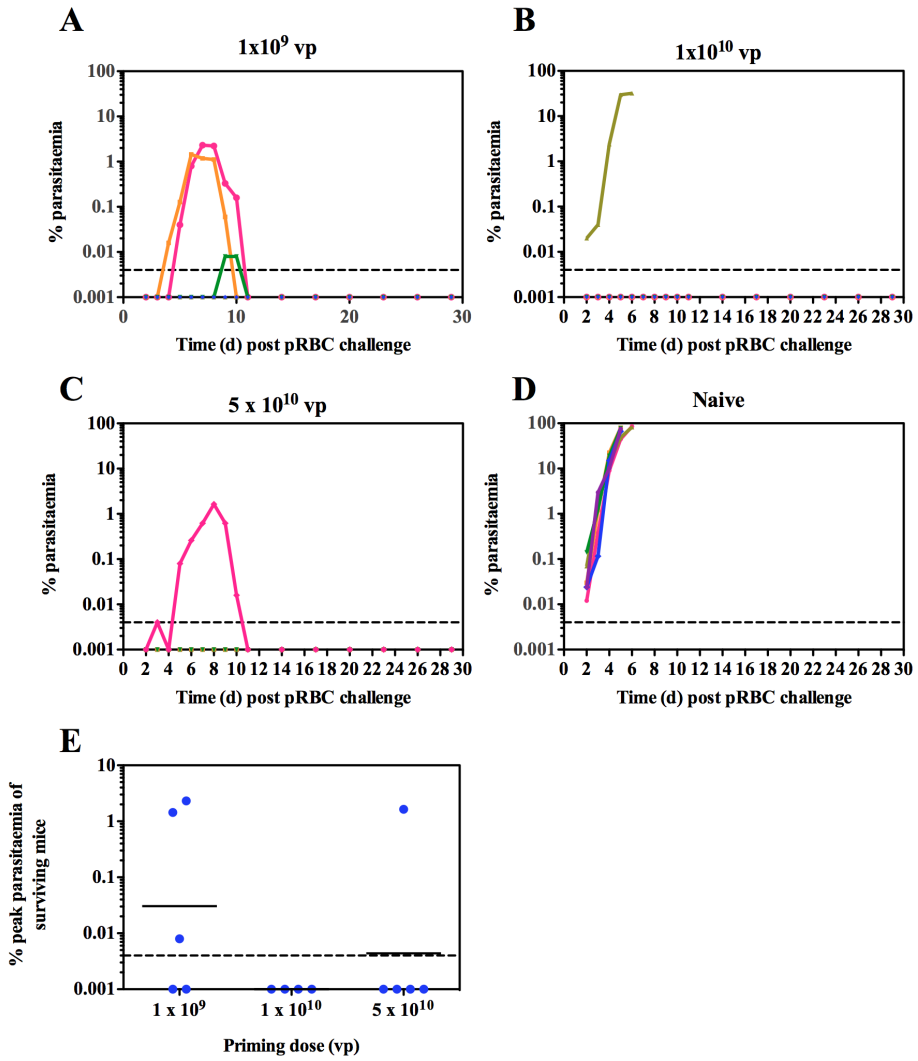


Figure 3.9: Impact of priming dose on protective efficacy of Ad_MVA PyMSP1$_{42}$-IMX108 against *P. yoelii* pRBC challenge

BALB/c mice were primed i.d. with 1 x10^9 vp (A), 1 x10^{10} vp (B) or 5 x10^{10} vp (C) AdHu5-PyMSP1$_{42}$-IMX108 and all boosted eight weeks later with 1 x 10^7 pfu MVA-PyMSP1$_{42}$-IMX108. Two weeks post-boost mice were challenged with 10^4 *P. yoelii* pRBC i.v. and parasitaemia monitored. A group of naïve mice was also challenged (D). Peak blood-stage % parasitaemias for mice that survived challenge are shown (E). n = 5 mice/group immunised and 6 naïve mice. Lines indicate the geometric mean peak parasitaemia and the dashed line indicates the limit of detection (0.004%).

3.2.4 Immunogenicity of a mixture of PyMSP1 and PyCSP vaccines in an Ad_MVA prime-boost regime

Based on the assessment of both Ad_MVA PyMSP1$_{42}$ and Ad_MVA PyCSP vaccination regimes, doses of each vaccine were established to use in an Ad_MVA regime with a mixture of both antigens. Each antigen was kept at a 1:1 ratio in the first instance. Given the previously established immunogenicity and protection data it was decided to use the maximum dose feasible – priming with 1 x 10^{10} vp of each AdHu5, and boosting with 1 x 10^{7} pfu of each MVA. The vaccines were delivered as a mixture and in order to control for viral load AdHu5 and MVA vaccines with no antigenic insert were included in mice receiving vaccines targeting only a single antigen. The vaccination regime is summarised in Table 3.4.

Antigens	AdHu5 Prime (Mixed)	MVA Boost (Mixed)
MSP1 + CSP	$1x10^{10}$ vp AdHu5-PyMSP1$_{42}$-IMX108 $1x10^{10}$ vp AdHu5-PyCSP	$1x10^{7}$ pfu MVA-PyMSP1$_{42}$-IMX108 $1x10^{7}$ pfu MVA-PyCSP
MSP1 + Control	$1x10^{10}$ vp AdHu5-PyMSP1$_{42}$-IMX108 $1x10^{10}$ vp AdHu5-Control	$1x10^{7}$ pfu MVA-PyMSP1$_{42}$-IMX108 $1x10^{7}$ pfu MVA-Control
CSP + Control	$1x10^{10}$ vp AdHu5-Control $1x10^{10}$ vp AdHu5-PyCSP	$1x10^{7}$ pfu MVA-Control $1x10^{7}$ pfu MVA-PyCSP

Table 3.4: Summary of immunisation regimes used to investigate a PyCSP and PyMSP1$_{42}$ combination vaccine strategy

In order to investigate the feasibility of an Ad_MVA vaccination regime targeting both PyMSP1 and PyCSP the above immunisation regimes were used. The viruses used were mixed, and then mice were vaccinated i.d. in the ear in a total volume of 50µL, with 25µL given into each ear. There was an eight-week interval between prime and boost. Where only a single malaria antigen was given control vaccines were included to control for viral load. Throughout this study the vaccine regimes are named according to the antigens included as shown here.

3.2.4.1 Humoral immune responses to a mixture of Ad_MVA PyMSP1 and PyCSP vaccines.

BALB/c mice were immunised against the $PyMSP1_{42}$ and/or PyCSP antigens either individually or as a mixture, utilizing the previously described Ad_MVA prime-boost immunisation regime (Table 3.4). The kinetics of the total IgG antibody responses over time against the $PyMSP1_{19}$ and PyCSP antigens were measured by ELISA (Figure 3.10A and B). Antibody responses to each antigen were specific, with no cross-reactivity observed. Responses to both antigens were also detectable two weeks after the AdHu5 prime, and were not significantly affected following co-administration of the two AdHu5 vaccines as a mixture. Following the MVA immunisation at day 56, responses to both antigens were significantly boosted ($P <$ 0.0001 by paired t-test for both antigens) and no significant differences in $PyMSP1_{19}$- or PyCSP-specific IgG titres were observed, either at week 10 or 18, between mice that had received vaccines targeting a single antigen and those that had received the vaccine mixture targeting both antigens. Furthermore, administration of the vaccine mixture did not affect the titre of $PyMSP1_{19}$-specific or PyCSP-specific IgG1 or IgG2a two weeks after the MVA boost (Figure 3.10, C and D).

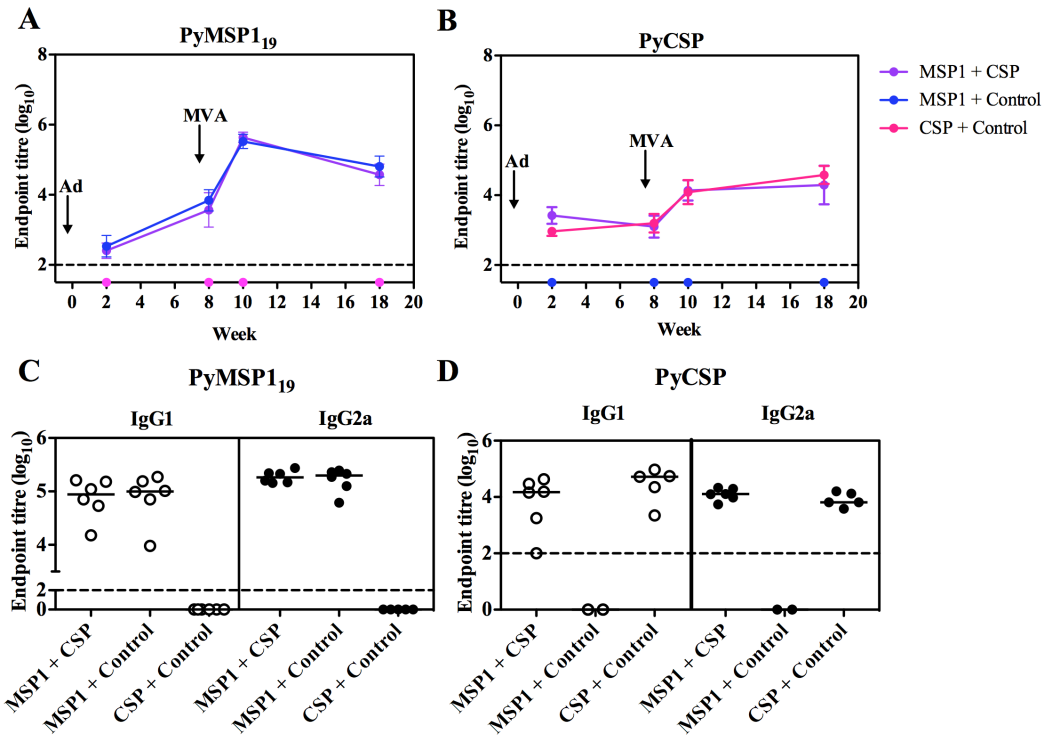


Figure 3.10: Antigen-specific IgG is maintained in a multi-stage vaccine regime

BALB/c mice were immunised i.d. with the AdHu5_MVA vaccine regimes described in Table 3.4. Serum was collected two and eight weeks post-prime and two and ten weeks post-boost (weeks 10 and 18). Total IgG responses at all time-points were measured against PyMSP1$_{19}$-GST protein (A) and PyCSP-specific MAP4-Py1T-Py3 branched peptide (B) by ELISA. Data are means ± standard deviation for six mice per group. These data are representative of two independent experiments. IgG1 and IgG2a responses were measured two weeks post-boost against PyMSP1$_{19}$ (C) and PyCSP (D). Bars indicate the median response. The dashed line indicates the limit of detection.

3.2.4.2 Cellular immune responses to a mixture of Ad_MVA PyMSP1 and PyCSP vaccines.

BALB/c mice were immunised as before (Table 3.4) and PBMCs were isolated from the blood two and eight weeks after the Ad prime, and two and nine weeks after the MVA boost, in order to assess the affect of mixing the Ad and MVA vaccines on transgene-specific $CD8^+$ T cell responses. Production of IFN-γ from $CD8^+$ cells was assessed by ICS following re-stimulation with a pool of overlapping peptides against $PyMSP1_{33}$ (Figure 3.11A) or $PyCSP_{280\text{-}288}$ peptide (Figure 3.11B). Following AdHu5 priming there was comparable production of antigen-specific IFN-γ from $CD8^+$ T cells in mice immunised with either a single antigen or both antigens. However two weeks after the MVA boost, the two-antigen vaccine regime resulted in significantly lower antigen-specific $CD8^+$ IFN-γ^+ responses to both PyMSP1 and PyCSP in comparison to the single antigen regimes. $CD8^+$ IFN-γ^+ responses were also measured in the spleen two weeks post-MVA (Figure 3.11C). These data confirmed the responses observed in the blood, with significantly lower responses to the $PyMSP1_{33}$ peptide pool and $PyCSP_{280\text{-}288}$ detected in mice receiving the two-antigen regime in comparison to a single antigen given alone. Additionally (due to the availability of cells), responses were also measured against the sub-dominant PyCSP epitope, $PyCSP_{58\text{-}67}$ [83]. These data confirmed that responses to both reported class I $H\text{-}2^d$ epitopes within PyCSP are lower in mice receiving the vaccine mixture in comparison to when Ad_MVA PyCSP is given alone. These data also indicated that the total magnitude of the $CD8^+$ IFN-γ^+ T cell response in the two-antigen regime is no greater than when the Ad_MVA PyCSP vaccines are given alone.

In addition to IFN-γ, production of TNFα and IL-2 was also measured and the multi-functionality of the $CD8^+$ T cells was assessed. The so-called "quality" of the antigen-specific cells was analysed in terms of production of all three cytokines in blood, spleen and liver two weeks after the MVA boost immunisation (Figure 3.12). Responses in the blood and spleen were measured against both the $PyMSP1_{33}$ peptide pool (Figure 3.12A) and $PyCSP_{280\text{-}288}$ peptide (Figure 3.12B). As seen previously for IFN-γ, total production of antigen-specific TNFα and IL-2 was significantly reduced in mice immunised against both antigens in comparison to a single antigen. However, within each compartment the proportion of cells (Figure 3.12, A and B pie charts) that were producing all three cytokines (IFN-γ+TNFα+IL-2), two cytokines (predominantly (IFN-γ+TNFα) or a single cytokine (predominantly IFN-γ), was largely unaffected by immunising against both antigens. Due to the relatively small number of cells isolated, liver mononuclear cells could not be stimulated with each antigen separately. Instead, they were stimulated with a single peptide pool containing $PyMSP1_{33}$ peptide pool, $PyCSP_{280\text{-}288}$ and $PyCSP_{58\text{-}67}$ (Figure 3.12C). Whilst conclusions about the contribution of each individual antigen cannot be made from these data, it was clear that the total cytokine production from liver mononuclear cells of mice that had received the MSP1 + CSP vaccine regime was no higher than in mice that had received the PyCSP vaccines alone – in agreement with data from the spleen and blood. As in previous experiments with Ad_MVA PyCSP (Section 3.2.1.2), $CD8^+$ T cells expressing IL-2 following re-stimulation were undetectable in the blood, but observed in both the spleen and liver.

$CD4^+$ T cell responses were also assessed in the spleen two weeks post-boost (Figure 3.13). Comparable production of IFN-γ from cells stimulated with $PyMSP1_{33}$ peptide

pool was observed in mice immunised with either MSP1 + CSP or MSP1 + Control. However, in mice immunised with MSP1 + CSP, as compared to CSP + Control, IFN-γ production was significantly lower following $PyCSP_{59\text{-}79}$ stimulation. Surprisingly, a $CD4^+$ T cell response was also detected following stimulation with the sub-dominant $CD8^+$ T cell epitope, $PyCSP_{58\text{-}67}$. This was comparable in magnitude to the response induced against the longer $CD4^+$ epitope, $PyCSP_{59\text{-}79}$, and was not significantly reduced by immunisation with a mixture of the two antigens.

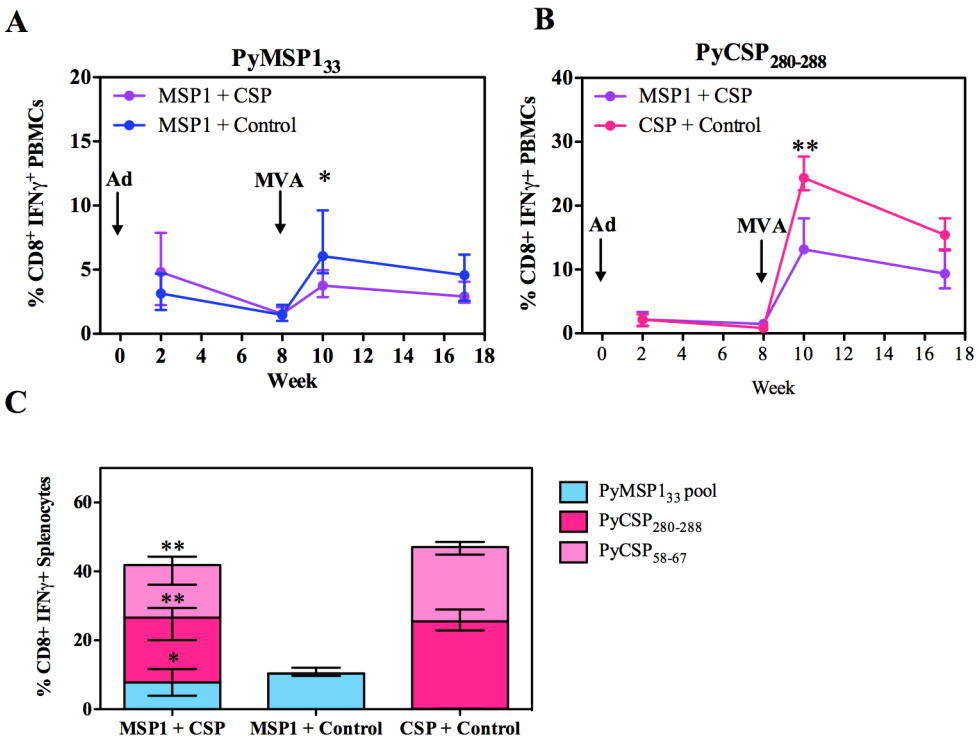


Figure 3.11: Antigen-specific CD8$^+$ T cell responses are lower post-boost following a two-antigen vaccine regime than a single antigen regime

BALB/c mice were immunised i.d. with the regimes described in Table 3.4. The percentages of CD8$^+$ PBMCs producing IFN-γ were measured in the blood by ICS at the indicated time-points. PBMCs were re-stimulated with the PyMSP1$_{33}$ peptide pool (A) or PyCSP$_{280-288}$ (B). Data are median ± interquartile range for 6-12 mice per group. Two weeks post boost CD8$^+$ IFN-γ$^+$ responses were measured in the spleen against the indicated peptides (C). Bars represent median responses ± range for six mice per group. * $P < 0.05$, ** $P < 0.01$ by Mann Whitney test, excluding negative controls.

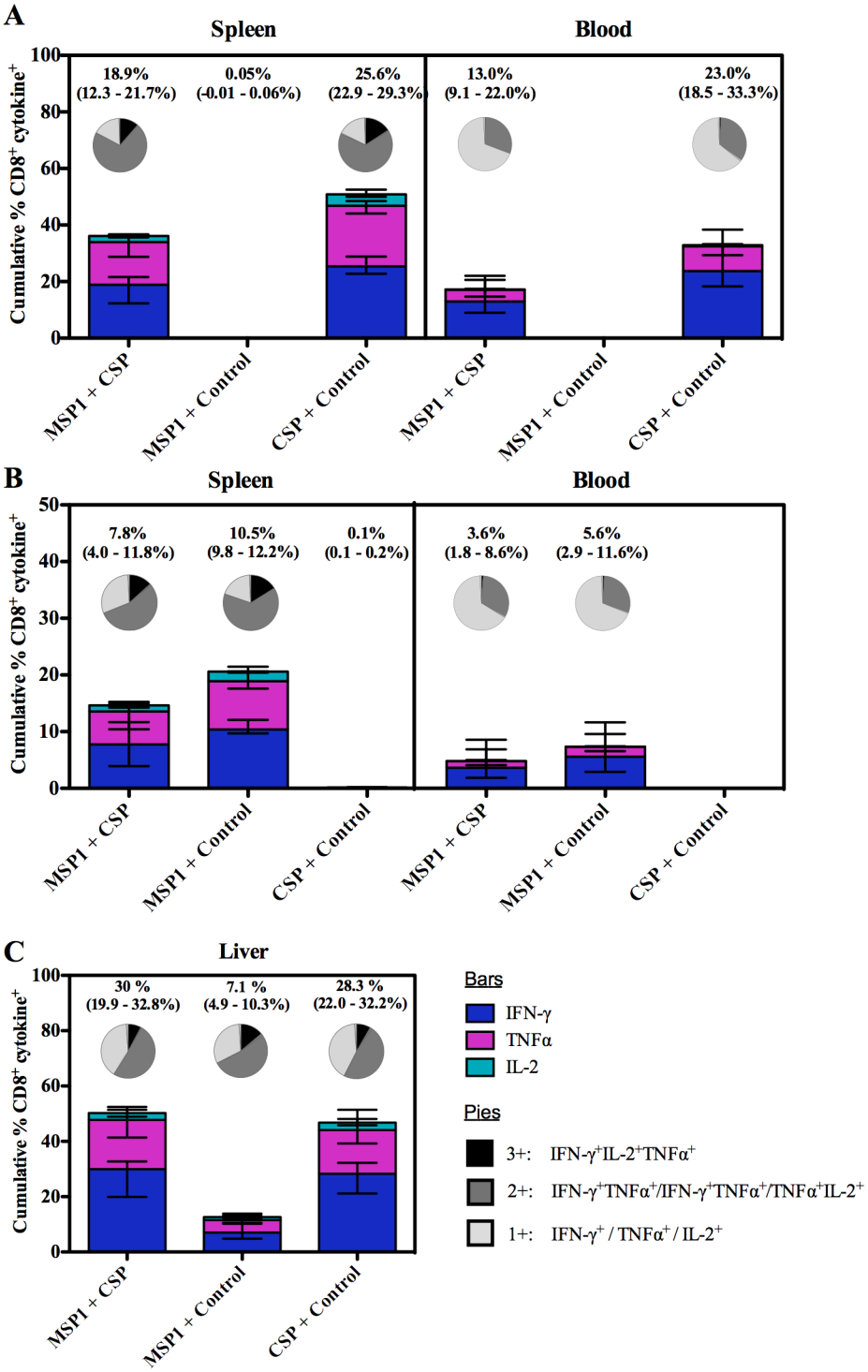

A
Spleen
Blood
Cumulative % CD8+ cytokine+
18.9% (12.3 - 21.7%)
0.05% (-0.01 - 0.06%)
25.6% (22.9 - 29.3%)
13.0% (9.1 - 22.0%)
23.0% (18.5 - 33.3%)
MSP1 + CSP
MSP1 + Control
CSP + Control
B
Spleen
Blood
7.8% (4.0 - 11.8%)
10.5% (9.8 - 12.2%)
0.1% (0.1 - 0.2%)
3.6% (1.8 - 8.6%)
5.6% (2.9 - 11.6%)
C
Liver
30 % (19.9 - 32.8%)
7.1 % (4.9 - 10.3%)
28.3 % (22.0 - 32.2%)
Bars
IFN-γ
TNFα
IL-2
Pies
3+: IFN-γ+IL-2+TNFα+
2+: IFN-γ+TNFα+/IFN-γ+TNFα+/TNFα+IL-2+
1+: IFN-γ+ / TNFα+ / IL-2+

Figure 3.12: The quantity, but not the quality, of $CD8^+$ T cells is affected following immunisation against two antigens

In addition to IFN-γ, TNFα and IL-2 were measured by ICS in both the blood and the spleen following re-stimulation with $PyCSP_{280\text{-}288}$ (A) or the $PyMSP1_{33}$ peptide pool (B), or in the liver following re-stimulation with a mixed peptide pool containing the $PyMSP1_{33}$ peptide pool, $PyCSP_{280\text{-}288}$ and $PyCSP_{58\text{-}67}$ (C). Bars indicate the median percentage of cells producing each of the three cytokines. The pies indicate the proportion of cells producing all three cytokines (black), two cytokines (dark grey) or a single cytokine (light grey) and the numbers above the pies indicate the total percentage of responding cells (± range) producing any cytokine in response to stimulation with the indicated peptides.

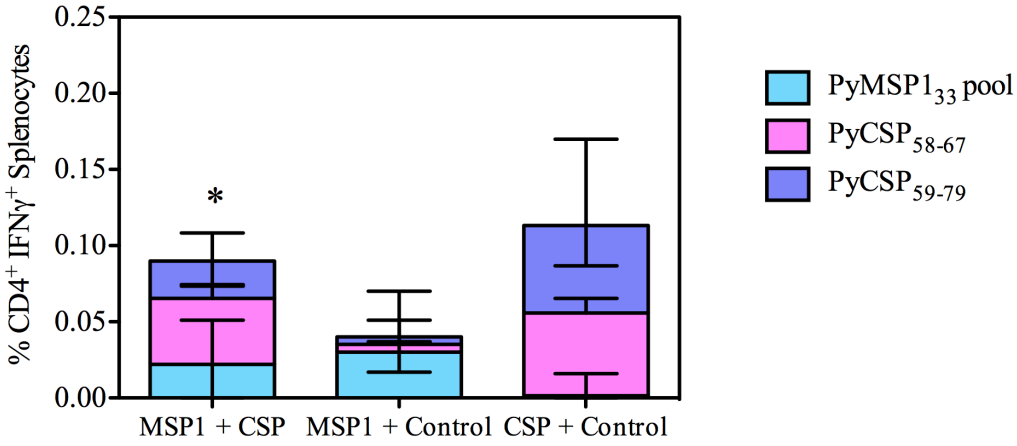


Figure 3.13: Antigen-specific CD4$^+$ T cell responses following a two-antigen vaccine

BALB/c mice were immunised i.d. with the regimes described in Table 3.4. The percentages of CD4$^+$ splenocytes producing IFN-γ were measured by ICS following stimulation with the indicated peptides. Bars represent median responses ± range for six mice per group. * $P < 0.05$ for MSP1 + CSP compared to CSP + Control following PyCSP$_{59\text{-}79}$ stimulation by Mann Whitney test.

3.2.4.3 Protection against P. yoelii sporozoite challenge

$CD8^+$ T cell responses, induced by viral vectored vaccines encoding either $PyMSP1_{42}$ or PyCSP, have been previously reported to provide partial or sterilising liver-stage protection against *P. yoelii* sporozoite challenge [77-79, 102]. In order to assess whether the reduced $CD8^+$ T cell responses post-boost in the MSP1 + CSP vaccine regime had an impact on efficacy against sporozoite challenge, mice were challenged intravenously 2 or 10 weeks after the MVA boost with either 1000 or 50 sporozoites respectively (Table 3.5). 50 sporozoites are routinely used in the *P. yoelii* challenge model [48, 158, 264]. However, it was already known that the Ad_MVA $PyMSP1_{42}$ vaccine regime could protect against a high dose challenge of 250 sporozoites [265] two weeks after the MVA boost immunisation [102]. Therefore in order to maximise any protective differences between immunisation groups, an even higher challenge dose of 1000 sporozoites was used at the two weeks post boost challenge time-point (Table 3.5 and Figure 3.14). In both experiments fewer mice were sterilely protected following immunisation with the MSP1 + CSP regime in comparison to the CSP alone regime. Following challenge two weeks post boost, only 1/6 mice was sterilely protected in the MSP1 + CSP group, compared to 4/6 in the CSP alone group, and a similar trend was observed at the late 10 week challenge time-point. Sterilising liver-stage efficacy had therefore been reduced following co-administration of the MSP1 + CSP vaccines, indicating that a mixed population of $CD8^+$ T cells against $PyMSP1_{33}$ and PyCSP is less protective in this model than a similar quantity of cells targeting PyCSP alone. However, despite this reduced efficacy at the liver-stage, and in agreement with the $PyMSP1_{19}$ antibody data (Figure 3.10A), overall survival in the MSP1 + CSP vaccinated mice remained high following the onset of blood-stage parasitaemia, with 5/6 (83%) surviving in each challenge experiment in comparison

to 100% survival following immunisation with the MSP1 vaccine alone (Figure 3.14A). For mice challenged ten weeks post-boost, sera were collected and, as before, peak parasitaemia correlated significantly with pre-challenge total PyMSP1$_{19}$-specific IgG ELISA titre (Figure 3.14B). These data suggest that, following the onset of blood-stage parasitaemia, survival in this model remains dependent on PyMSP1$_{19}$ antibody titres. These data also confirmed that the Ad-M PyMSP1$_{42}$ vaccine regime by itself maintains 100% survival following both a high dose challenge with 1000 sporozoites at the day 70 time-point, and also following a challenge ten weeks post-boost with 50 sporozoites.

Immunisation Regime	No. survived / No. challenged (% survival)	No. sterilely protected / No. challenged (% sterile)	Geometric mean (range) peak parasitaemia of surviving mice
A) 1000 sporozoites, 2 weeks post boost			
MSP1 + CSP	5/6 (83%)	1/6 (16%)	4.62% (0 – 28%)
MSP1 + Control	6/6 (100%)	1/6 (16%)	1.80% (0 – 7.9%)
CSP + Control	4/6 (66%)	4/6 (66%)	N/A
Naïve unimmunised	0/6 (0%)	0/6 (0%)	N/A
B) 50 sporozoites, 10 weeks post boost			
MSP1 + CSP	5/6 (83%)	2/6 (33%)	4.53% (0 – 11.8%)
MSP1 + Control	6/6 (100%)	1/6 (16%)	0.20% (0 – 17.6%)
CSP + Control	3/6 (50%)	3/6 (50%)	N/A
Naïve unimmunised	0/6 (0%)	0/6 (0%)	N/A

Table 3.5: Efficacy of the vaccine mixture against *P. yoelii* sporozoite challenge

BALB/c mice were immunised i.d. with the regimes described in Table 3.4 and challenged i.v. with sporozoites at the indicated time-points. Survival, sterile protection and geometric mean peak parasitaemia are summarised.

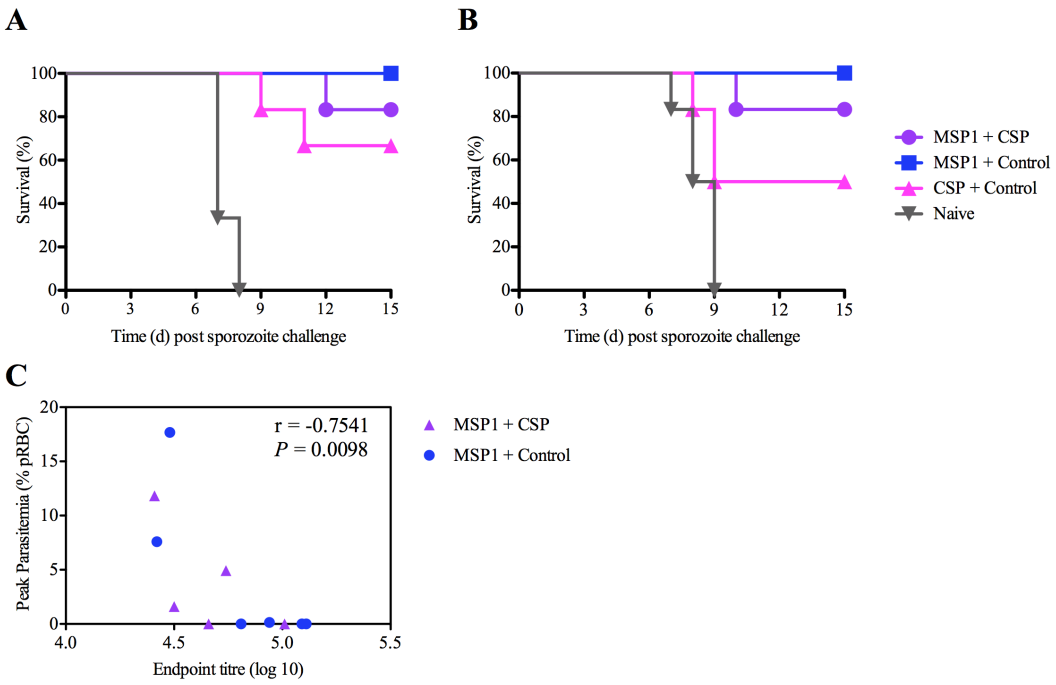


Figure 3.14: Survival and peak parasitaemia following sporozoite challenge

BALB/c mice were challenged with sporozoites as described in Table 3.5. Kaplan-Meier curves show survival following sporozoite challenge two weeks post-boost (A) and ten weeks post-boost (B). Following challenge ten weeks post-boost, PyMSP1$_{19}$-specific total IgG titres correlate with peak parasitaemia by Spearman's Rank correlation analysis (C).

3.3 Discussion

In the first part of this chapter the immunogenicity and efficacy of both AdHu5-PyCSP and Ad_MVA PyCSP immunisation was investigated. Encouragingly, it was found that the vaccines were immunogenic, with AdHu5-PyCSP capable of priming both antibody and $CD8^+$ T cell responses that are significantly boosted by MVA-PyCSP. $CD4^+$ T cell responses were not detected above background after priming and were not tested post-boost in these experiments. However, a previous experiment carried out at the Jenner Institute found that they were less than 0.1% of the total $CD4^+$ T cell population and this was confirmed in later experiments in which PyCSP vaccines were mixed with $PyMSP1_{42}$ vaccines (Figure 3.13).

Disappointingly, and in contrast to published studies [75, 77, 78], our AdHu5-PyCSP vaccine given alone did not provide significant protection against *P. yoelii* sporozoite challenge. It may be that our vaccine is not as immunogenic as others, however given the variability between laboratories and the lack of standardised assays, it is impossible to establish this. Variations in mice and even in animal facilities are also known to influence immunogenicity and protection [266]. In these initial studies, the level of protective efficacy of Ad_MVA PyCSP was not established. Three independent experiments were carried out, but on all three occasions patent infection failed to develop in every naïve mouse due to poor sporozoite viability. However, the combination vaccine studies in which a group of mice were immunised with CSP + Control prior to sporozoite challenge confirmed that the Ad_MVA PyCSP vaccines are capable of providing protection, with 66% of mice surviving a high dose challenge of 1000 sporozoites two weeks post-boost.

During this study the mechanism of protection by PyCSP vaccines was not investigated, however, in agreement with the majority of published studies, it is likely to be largely dependent on $CD8^+$ T cell responses to the dominant $CD8^+$ T cell epitope [80, 81, 249, 267]. This is supported by the combination vaccine data in which reduced liver-stage protection was observed when $CD8^+$ T cell responses were reduced.

The second part of this study investigated the impact of IMX108, as well as various doses of both AdHu5 prime and MVA boost, on blood-stage protection induced by Ad_MVA $PyMSP1_{42}$. It was found that the boosting dose of MVA had the greatest impact on antibody responses and therefore on protection. However, the inclusion of IMX108 also led to a small increase in both antigen-specific IgG titres and protection against pRBC challenge. For this reason the $PyMSP1_{42}$-IMX108 construct was used throughout the combination vaccine studies. Encouragingly during these combination studies it was found that the Ad_MVA $PyMSP1_{42}$ immunisation regime was capable of protecting 100% of mice against an extremely high dose challenge with 1000 *P. yoelii* sporozoites (Table 3.5).

In the final part of this chapter, a multi-stage and multi-antigen vaccine regime (using a co-administered clinically-relevant viral vectored vaccine delivery platform) was investigated in a preliminary series of immunogenicity and protection experiments. In the *P. yoelii* mouse model it was shown that co-administration of a mixture of viral vectored vaccines expressing two different malaria antigens in an AdHu5-MVA prime-boost regime results in potent antibody responses against both antigens.

However, $CD8^+$ T cell responses were significantly reduced after the MVA boost immunisation if the two vaccines were given as a mixture and this resulted in reduced liver-stage protection.

An important finding from these experiments is that antibody responses are maintained against both antigens following Ad_MVA vaccination in BALB/c mice. This was confirmed by sporozoite challenge in which no significant differences were observed in survival between mice immunised against $PyMSP1_{42}$ alone, as compared to mice immunised against both $PyMSP1_{42}$ and PyCSP. These results are in agreement with a study in which two separate AdHu5 vectors expressing *P. falciparum* ookinete surface protein (Pfs25) and *P. vivax* ookinete surface protein (Pvs25) were given as a vaccine mixture and antigen-specific IgG towards both antigens was maintained [268]. Similarly, combining the RTS,S (CSP vaccine) with $MSP1_{42}$ and AMA1 protein vaccines in rhesus macaques showed no interference between antibody responses against RTS,S and $MSP1_{42}$ [202]. However the authors did report that $MSP1_{42}$ immunisation interfered with both AMA1-specific antibodies and IFN-γ production, and it will be important in future studies to ascertain whether antigenic interference is an antigen-specific phenomenon, or related to specific vaccine delivery platforms. Future clinical studies using viral vectored vaccines encoding liver- and blood-stage malaria antigens should establish whether interference between antigen-specific antibody responses occurs following co-administration of viral vectored vaccines encoding *P. falciparum* antigens in humans [269].

This study also investigated the multi-functionality of the vaccine-induced $CD8^+$ T cells in the spleen, liver and the blood in terms of production of IFN-γ, TNFα and IL-2. It was found that after the MVA boost, strong $CD8^+$ T cell responses were detected that included 3+ cells (IFN-γ^+TNFα^+IL-2^+) in the spleen and liver. However, very few 3+ cells were detected in the PBMC, due to the lack of $CD8^+$ T cells producing IL-2. Phase I trials of AdCh63 MVA expressing PfM128 have observed a similar lack of antigen-specific IL-2 from $CD8^+$ T cells in human PBMC [105]. IL-2 is involved in expansion of $CD8^+$ T cell populations, but does not have a direct effector function. Given that IL-2 is primarily expressed by 'central memory' $CD8^+$ T cells in mice, which are predominantly found in secondary lymphoid organs, whilst 'effector memory' $CD8^+$ T cells rarely produce IL-2 [270, 271], this suggests that Ad_MVA vaccination regimes induce primarily effector memory $CD8^+$ T cells that circulate in the blood. This data is in agreement with Reyes *et al.*, who assessed the memory phenotype of peripheral PBMC following immunisation with Ad_MVA expressing *P. berghei* CSP and found that the greatest proportion of cells were effector memory ($CD62L^-CD127^+$), followed by effector cells ($CD62L^-CD127^-$) and the smallest proportion of cells were central memory ($CD62L^+CD127^+$) [272].

This study also found that following an Ad_MVA vaccination regime, in which the MVA vaccines were given as a mixture, reduced $CD8^+$ T cell responses to each individual antigen were observed. However, the magnitude of the total $CD8^+$ IFN-γ^+ T cell response was the same whether mice were immunised with the PyCSP vaccines alone, or with both the $PyMSP1_{42}$ and PyCSP vaccines (Figure 3.11C). Despite this, following sporozoite challenge, liver-stage protection was reduced in mice receiving the two-antigen regime, showing that in this case it was more beneficial to have a

high number of T cells against PyCSP than the same number of T cells targeting PyCSP and $PyMSP1_{42}$. Given the potential for antigenic interference, this highlights the importance of careful consideration of the antigens used in a multi-antigen vaccine. It also suggests that in the *P. yoelii* mouse model, $CD8^+$ T cells against PyCSP are more efficient at killing the pre-erythrocytic parasites than those against $PyMSP1_{42}$. This may be because CSP is expressed throughout the sporozoite and early pre-erythrocytic stages of the parasite life-cycle [19], whilst PyMSP1 is expressed only during the late-liver stage [86].

The reduced $CD8^+$ T cell response to each individual antigen observed in these studies may occur for a number of reasons. The effect could be systemic or local, and at the local level there could be a number of possible causes including competition for presentation of antigens on MHC class I molecules. Alternatively, several studies have found that $CD8^+$ T cell interference occurs when $CD8^+$ T cells compete for antigenic epitopes presented on the same APC surface [273]. The mechanism of $CD8^+$ T cell interference is explored in detail in Chapter 4.

Chapter 4

Mechanisms of $CD8^+$ T cell interference in a *P. yoelii* combination vaccine strategy.

4 Mechanisms of $CD8^+$ T cell interference in a *P. yoelii* combination vaccine strategy.

4.1 Introduction

As described in Chapter 3, it was found that priming by co-administration of AdHu5 vaccines and boosting by co-administration of MVA vaccines expressing PyCSP and $PyMSP1_{42}$ (as a mixture) leads to maintained antibody responses but reduced secondary $CD8^+$ T cell responses against both antigens after the MVA boost in BALB/c mice. This results in reduced vaccine-mediated protective efficacy against *P. yoelii* liver-stage infection but maintained efficacy against blood-stage infection. Here, studies were undertaken in an attempt to overcome the $CD8^+$ T cell interference and to identify the mechanism(s) of this interference.

4.2 Results

4.2.1 $CD8^+$ T cell interference is not completely overcome by lowering vaccine dose

One possible explanation for the observed $CD8^+$ T cell interference following the co-administration of the MVA $PyMSP1_{42}$ and PyCSP booster vaccines (but not AdHu5 priming vaccines), was that it was a systemic or spatial effect. Given the relatively high overall magnitude of the responses induced against each antigen individually, it may not be physiologically possible to expand both antigen-specific populations to the same extent when the vaccines are co-administered. T cell responses induced by vectored vaccines follow a dose response curve [274], and it was therefore hypothesised that lowering the dose of the vaccines might reduce the overall magnitude of the response induced and thus prevent the observed $CD8^+$ T cell

interference. BALB/c mice were thus immunised as described in Table 3.4, but primed with 10^8 vp of each AdHu5 vaccine (rather than 10^{10} vp) and boosted with 10^6 pfu of each MVA vaccine (rather than 10^7 pfu). Two weeks after the MVA boost, mice were culled and splenocytes were re-stimulated with the $PyMSP1_{33}$ peptide pool or $PyCSP_{280\text{-}288}$ before assaying responses by ICS (Figure 4.1). In this case, PyCSP-specific responses were significantly lower (P = 0.0087 by Mann Whitney test) than those observed previously with a median response of 13.7% of $CD8^+$ T cells producing IFN-γ after CSP only vaccination, in comparison to 25.4% observed in the previous experiment (Figure 3.11), and now significant $CD8^+$ T cell inference was not observed for this antigen. However, despite the reduced magnitude of the PyCSP $CD8^+$ T cell population, significant interference was still observed following vaccine co-administration for the relatively weaker responses against $PyMSP1_{33}$. TNFα and IL-2 responses were similarly reduced in the vaccine mixture as compared to MSP1 vaccination alone (data not shown). Given overall responses of greater total magnitude were previously observed in the spleen (Figure 3.11C), these data indicate that physiological space is not the only contributing factor to $CD8^+$ T cell interference in this model.

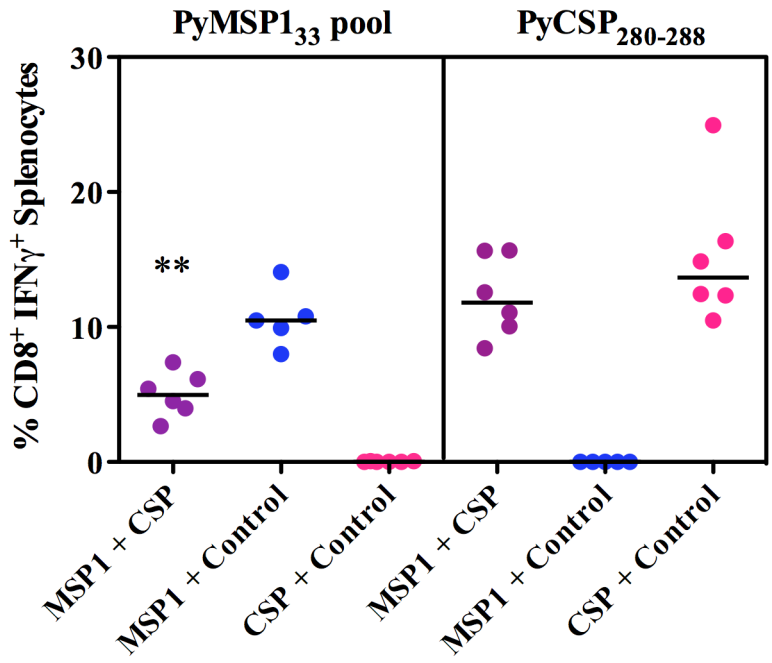


Figure 4.1: CD8$^+$ T cell interference is not completely overcome by lowering doses of vaccines

BALB/c mice were immunised i.d with the same regimes as described in Table 3.4, but using lower doses of vaccines (1 x 10^8 vp of each AdHu5 and 1 x 10^6 pfu of each MVA). Antigen-specific CD8$^+$ IFN-γ$^+$ responses were measured as before in the spleen two weeks post-boost by ICS. ** $P < 0.01$ by Mann Whitney test, excluding negative controls.

4.2.2 $CD8^+$ T cell interference can be minimised by immunisation at separate sites

A second possibility was that $CD8^+$ T cell interference was a local effect, occurring at the draining lymph node sites of T cell boosting, that could be overcome by immunizing with the vectored vaccines at physically separated sites. To investigate this, BALB/c mice were primed as usual with a mixture of 10^{10} vp of each AdHu5 vaccine and then boosted eight weeks later with 10^7 pfu of each MVA vaccine, except that the two different MVA vaccines were given into separate ears. Two weeks after the MVA boost, PBMCs were isolated and re-stimulated as before with the indicated peptides before analysis by ICS (Figure 4.2A). PyCSP-specific $CD8^+$ IFN-γ^+ T cell responses were slightly lower in mice immunised against both antigens as compared to against CSP alone, however the difference was small and no longer significant. Importantly, these PyCSP-specific responses were maintained at a similar magnitude to those seen previously when the vaccines had been given as a mixture, rather than at the lower magnitude observed following reduced dose immunisation. $PyMSP1_{33}$-specific $CD8^+$ IFN-γ^+ T cell responses were, however, still significantly lower in mice immunised against $PyMSP1_{42}$ + PyCSP compared to $PyMSP1_{42}$ alone. Similar results were observed for TNFα and IL-2, and the multi-functionality of the cells was unaffected by immunizing at separate sites, in agreement with that observed previously (data not shown). Furthermore, total IgG responses against both antigens remained unaffected by immunizing with antigens at separate sites, and were maintained at the same level as when the two MVAs had been given as a mixture (Figure 4.2 B and C).

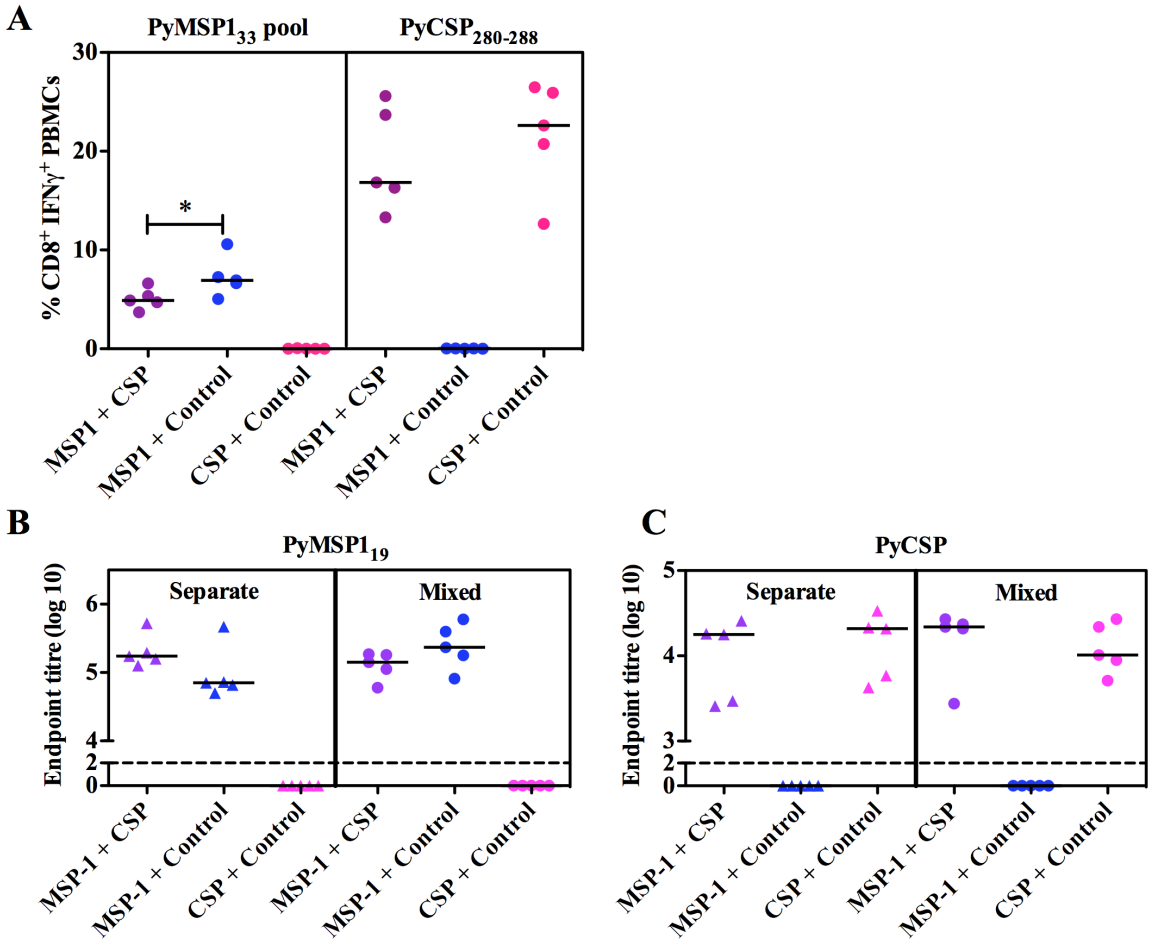


Figure 4.2: Immunogenicity following separate sites immunisation of MVA

BALB/c mice were primed i.d. with a mixture of AdHu5 vaccines as described in Table 3.4 but eight weeks later the two MVA vaccines were co-administered i.d. at separate sites – one given in each ear. Twelve days after immunisation with MVA, the production of antigen-specific IFN-γ from $CD8^+$ PBMCs was measured by ICS. Bars indicate median responses. * $P < 0.05$ by Mann Whitney test, excluding negative controls. These data are representative of two independent experiments. Total IgG against both $PyMSP1_{19}$ (B) and PyCSP (C) were also measured. The dashed line indicates the lower limit of detection and bars indicate median response.

4.2.3 Immunisation at separate sites improves efficacy against *P. yoelii* sporozoite challenge

Two weeks following immunisation with the MVA vaccines at separate sites, mice were challenged by i.v. injection of 1000 sporozoites. In this instance, on day five post-challenge, parasitaemia was undetectable in mice immunised against PyMSP1$_{42}$ + PyCSP, whereas mice immunised with either antigen alone had already begun to develop patent blood-stage infection (Figure 4.3A). Given PyMSP1$_{19}$ antibody responses remained comparable in the MSP1 + CSP and MSP1-only immunised groups, and given the observed reduced interference in CD8$^+$ T cell responses against both antigens, this reduction in patent parasitaemia at day 5 post-challenge indicates that when the two recombinant MVA vaccines are given at separate sites, mice immunised against both antigens had enhanced liver-stage protection compared to when they were immunised with either antigen alone. However, all mice in the co-administration and MSP1-only immunised groups went on to eventually develop a patent blood-stage parasitaemia but, like before, high-level survival was maintained in both groups of mice possessing antibodies against PyMSP1$_{19}$ (Figure 4.3B and Table 4.1). Ultimately, delayed onset blood-stage parasitaemia combined with subsequent survival in all mice, indicated a more beneficial effect of PyMSP1$_{42}$ and PyCSP co-immunisation following separate site administration of the MVA boosting vaccines in comparison to that previously seen when the vaccines were administered as a mixture.

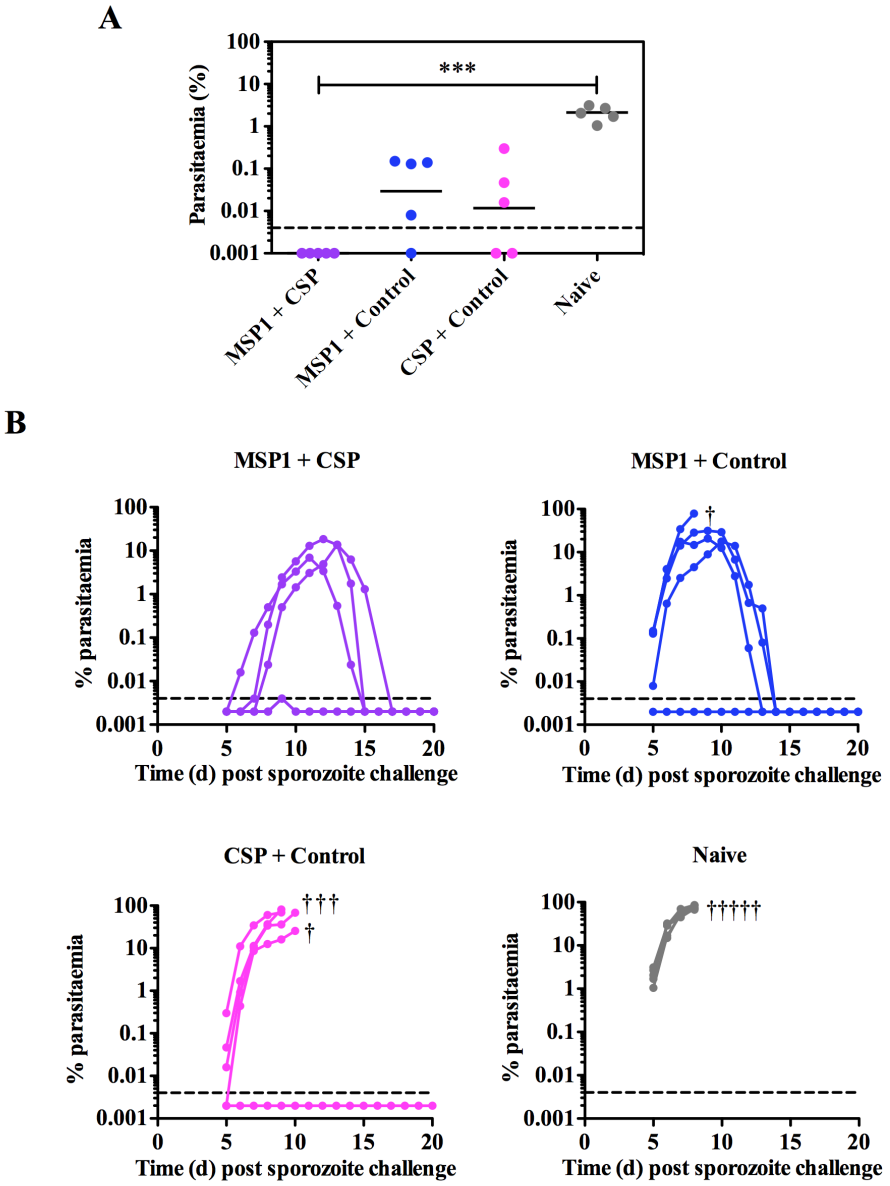


Figure 4.3: Efficacy against sporozoite challenge in mice immunised with MVA at separate sites

BALB/c mice that had been immunised with MVA at separate sites were challenged i.v. with 1000 sporozoites fourteen days after MVA. (A) Parasitaemia on day 5 post-challenge. Bars represent geometric mean parasitaemia. *** $P < 0.001$ by Dunnett's multiple comparison test, comparing all groups to MSP1+ CSP. (B) Parasitaemia over time for the different vaccine groups. Lines indicate percentage of infected RBC for individual mice of 5-6 per group. † indicates that mice were culled.

Immunisation Regime	No. survived / No. challenged (% survival)	No. sterilely protected / No. challenged (% sterile)	Geometric mean (range) peak parasitaemia of surviving mice
1000 sporozoites, 2 weeks post separate site boost			
MSP1 + CSP	5/5 (100%)	1/5 (20%)	1.63% (0 – 18.4%)
MSP1 + Control	4/5 (80%)	1/5 (20%)	22.6% (0 – 31.29%)
CSP + Control	1/5 (20%)	1/5 (20%)	N/A
Naïve unimmunised	0/6 (0%)	0/6 (0%)	N/A

Table 4.1: Efficacy of separate site immunisation against *P. yoelii* sporozoite challenge

BALB/c mice were immunised i.d. with the regimes described in Table 3.4, but with the MVA boost vaccines given at separate sites. Mice were challenged i.v. with 1000 sporozoites two weeks post-boost. Survival, sterility and geometric mean peak parasitaemia are summarised here.

4.2.4 $CD8^+$ T cell interference in C57BL/6 and outbred mice

Having identified that $CD8^+$ T cell interference could be overcome for PyCSP-specific responses and minimised for $PyMSP1_{33}$-specific responses by immunisation with the two recombinant MVAs at separate sites, we sought to identify the mechanism of interference. One possibility was that it was due to peptides competing for presentation on MHC class I molecules on the surface of APCs [275]. In BALB/c mice all seven dominant epitopes within the $PyMSP1_{33}$ peptide pool are predicted to be H-$2K^d$-restricted, as well as $PyCSP_{280\text{-}288}$ [249] and $PyCSP_{58\text{-}67}$ [83]. Therefore, we investigated whether competition occurred in C57BL/6 or outbred mice immunised with the regimes described in Table 3.4. In both strains of mice, T cell and antibody responses were assessed two weeks post-boost. In C57BL/6 mice, $PyMSP1_{33}$-specific production of IFN-γ from $CD8^+$ T cells was clearly reduced in mice immunised against both antigens, as compared to MSP1 only, although this result was not significant due to one low responder in the MSP1 only group (Figure 4.4A). There are no published PyCSP epitopes in C57BL/6 mice and peptides were not available against full-length PyCSP to test responses. Instead, we used a mixed pool of peptides containing overlapping *P. berghei* CSP (PbCSP) peptides with the relevant peptides replaced with the available PyCSP peptides - $PyCSP_{280\text{-}288}$, $PyCSP_{59\text{-}59}$ and $PyCSP_{58\text{-}67}$ (Py/PbCSP pool). There are 13/119 αα changes as compared to PyCSP. However, following stimulation with the Py/PbCSP pool, only 2/6 mice immunised with CSP + Control had $CD8^+$ IFN-γ^+ responses that were detectable above background and even these responses were < 0.8% of total $CD8^+$ T cells (Figure 4.4B). Given the lack of published PyCSP epitopes in C57BL/6 mice, it is likely that this result is real, however, it is possible that responses were missed due to the differences in the peptide pool compared to the vaccine sequence.

Antigen-specific $CD4^+$ T cell responses were not detectable for either antigen above background (data not shown). $PyMSP1_{19}$-specific total IgG responses two weeks post-boost were higher in mice immunised against both antigens, compared to mice immunised against MSP1 only, whilst PyCSP-specific total IgG responses were comparable between mice immunised against both antigens or only PyCSP, similar to responses observed in BALB/c mice.

In outbred CD-1 mice only 2/6 mice immunised against either $PyMSP1_{42}$ or PyCSP alone responded to restimulation of splenocytes with that antigen. However, 4/6 mice immunised against both antigens responded. Antigen-specific responses tended towards being lower in mice immunised against both antigens, as compared to a single antigen, however, with such small numbers of responding mice these results are not conclusive (Figure 4.5A). The same four mice responded to both antigens in the combination vaccine group and given that the seven known $CD8^+$ T cell epitopes in $PyMSP1_{33}$ and two $CD8^+$ T cell epitopes in PyCSP are K^d-restricted, it is possible that these mice are all $H\text{-}2^d$ restricted. Future studies should analyse outbred mice for MHC class I restriction and should also use a greater number of mice to increase power for statistical analysis in responding mice.

$CD4^+$ IFN-γ^+ T cell responses in outbred mice were barely detectable above background (Figure 4.5B). $PyMSP1_{33}$-specific responses were marginally higher in mice immunised against $PyMSP1_{42}$ only, and one mouse immunised against PyCSP had a small PyCSP-specific response. Unlike BALB/c or C57BL/6 mice, $PyMSP1_{19}$-specific IgG responses were lower in mice immunised against both antigens as

compared to MSP1 only and surprisingly, not all outbred mice developed a PyCSP-specific antibody response following immunisation with PyCSP vaccines. Only 3/6 mice immunised with either MSP1 + CSP or CSP + Control sero-converted, however, in the mice that responded antibody titres were comparable between groups (Figure 4.5 C and D).

Disappointingly, conclusive efficacy data from sporozoite challenges were not achieved following the combination vaccine strategy in C57BL/6 or CD-1 mice. C57BL/6 mice could not be challenged due to a failure to isolate enough viable sporozoites from infected mosquitoes. CD-1 mice were challenged with 50 sporozoites two weeks post-boost, however, some non-specific control of blood-stage parasitaemia was observed. In 5/6 naïve mice and 1/6 CSP + Control immunised mice parasitaemia failed to reach the humane end-point of >70% despite patent blood-stage infection (Figure 4.6). Nevertheless, some conclusions can be drawn. Data collection began on day 5 post-challenge and by this time parasitaemia was already as high as 22% in some CSP immunised or naïve mice, suggesting that liver-stage control was not achieved with this vaccine. Blood-stage protection appeared to be more effective with low parasitaemia observed in all mice immunised against MSP1. Patent parasitaemia was not observed in 1/6 MSP1 + CSP immunised and 3/6 MSP1 + Control immunised mice, and parasitaemia remained low in all MSP1 immunised mice when the experiment was ended on day 12 post-challenge (Figure 4.6). These data suggest that PyMSP1$_{19}$-specific antibodies were acting to control the blood-stage infection. However, given the failure of CSP immunised and naïve mice to succumb to infection, firm conclusions cannot be drawn from these data.

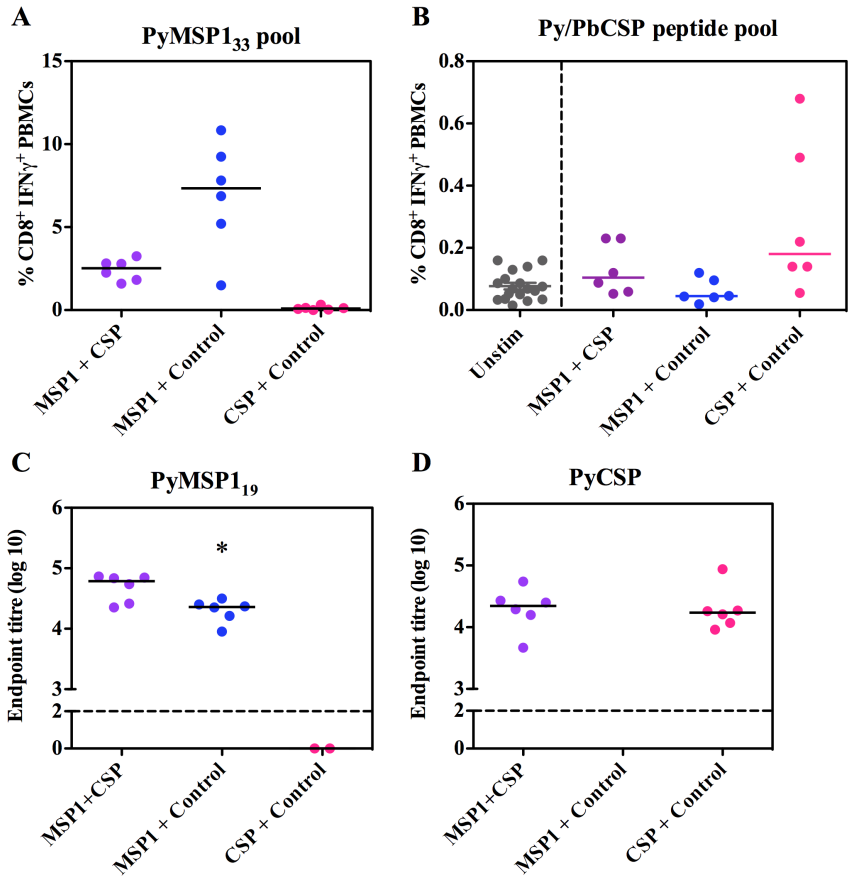


Figure 4.4: Cellular and humoral immunity following PyMSP1$_{42}$ and PyCSP immunisation in C57BL/6 mice

C57BL/6 mice were immunised with PyMSP1$_{42}$ or PyCSP vaccines or a combination of the two as described in Table 3.4. Two weeks post-boost mice were bled and CD8$^+$ IFN-γ$^+$ T cell responses assessed by ICS following stimulation with PyMSP1$_{33}$ peptide pool (A) or Py/PbCSP peptide pool (B). Following PyMSP1$_{33}$ stimulation, un-stimulated background responses (<0.2% as shown in B) were subtracted for each individual mouse. For Py/PbCSP stimulation, background responses were not subtracted and are shown. PyMSP1$_{19}$- (C) and PyCSP-specific (D) total IgG titres were measured in the sera two weeks post-boost. Data points indicate individual mice and bars are median responses for n = 6 mice/group. * $P < 0.05$ by Mann Whitney test, excluding negative controls.

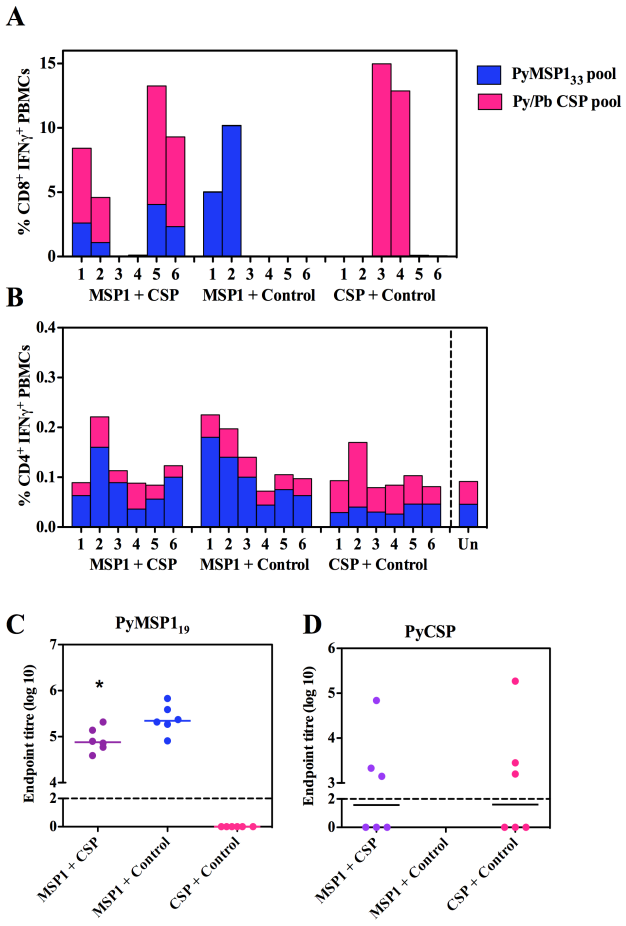


Figure 4.5: Cellular and humoral immune responses following PyMSP1$_{42}$ and PyCSP immunisation in CD-1 outbred mice

CD-1 mice were immunised i.d. with PyMSP1$_{42}$ or PyCSP vaccines or a combination of the two as described in Table 3.4. Two weeks post-boost mice were bled and $CD8^+$ (A) and $CD4^+$ (B) IFN-γ^+ T cell responses assessed by ICS following stimulation with PyMSP1$_{33}$ peptide pool or Py/PbCSP peptide pool. Bars indicating the response for each individual mouse of n = 6 mice/group are shown. For $CD8^+$ T cell responses, background un-stimulated responses are subtracted, whilst for $CD4^+$ data background responses were not subtracted and the mean unstimulated (un) is shown. PyMSP1$_{19}$- (C) and PyCSP-specific (D) total IgG titres were measured in the sera two weeks post-boost. Data points indicate individual mice and bars are median responses. * $P <$ 0.05 by Mann Whitney test, excluding negative controls.

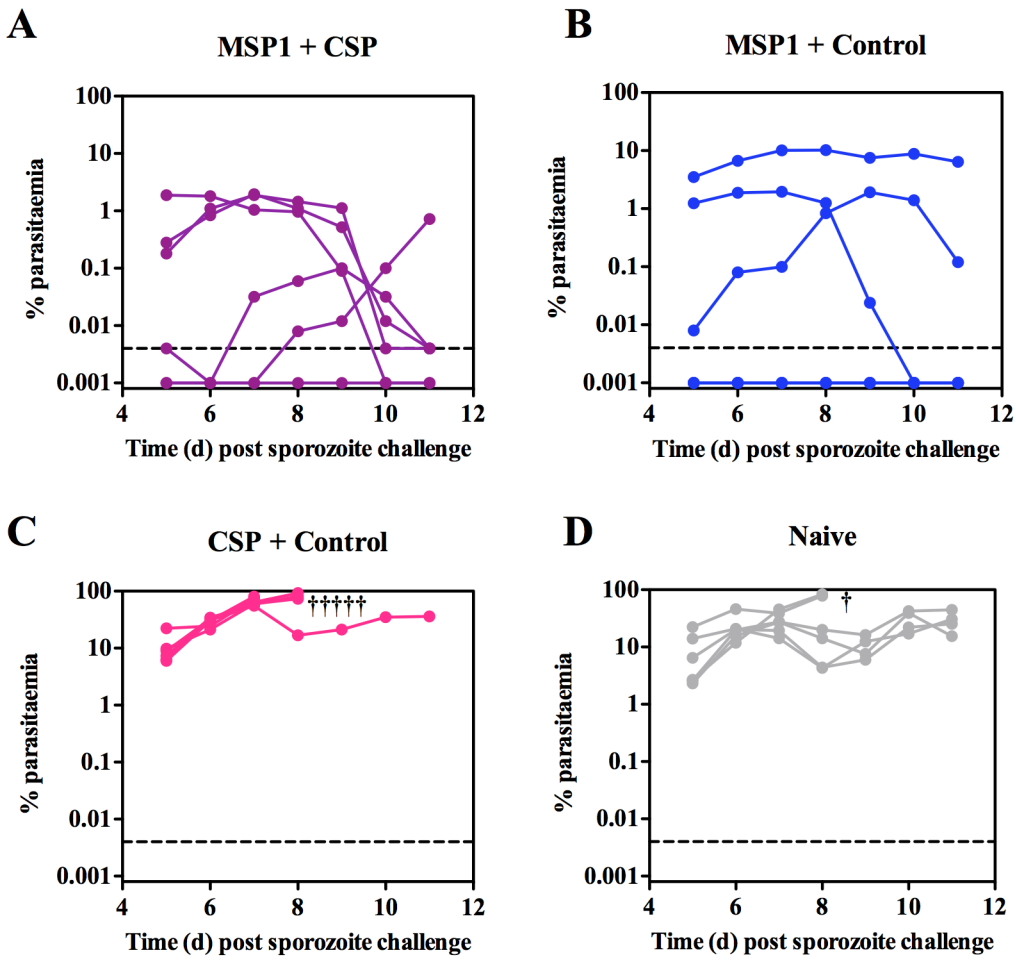


Figure 4.6: Protective efficacy of PyMSP1$_{42}$ and PyCSP vaccines against *P. yoelii* sporozoite challenge in CD1 outbred mice

CD-1 mice were immunised i.d. with PyMSP1$_{42}$ or PyCSP vaccines or a combination of the two as described in Table 3.4. Two weeks post-boost mice were challenged i.v. with 50 *P. yoelii* sporozoites. Parasitaemia was monitored from day 5 post challenge in mice immunised with MSP1 + CSP (A), MSP1 + Control (B), CSP + Control (C) or naïve mice (D). Parasitaemia in individual mice of n = 6 mice/group is shown. The dashed line indicates the lower limit of detection (0.004%). † indicates that mice were culled due to > 70% parasitaemia. The experiment was terminated on day 11 post-challenge.

4.2.5 $CD8^+$ T cell interference is not due to competition for presentation on MHC class I.

We further assessed the role of MHC class I restriction in $CD8^+$ T cell interference by measuring responses against epitopes with a different MHC class I restriction within the MVA vector backbone. Splenocytes from BALB/c mice, harvested two weeks after the MVA boosting immunisation, were re-stimulated with F2(G) (H-$2L^d$-restricted) and E3 (H-$2D^d$-restricted) peptides [251] (Figure 4.7). For both epitopes encoded within the MVA vector, IFN-γ production from *de novo* primed $CD8^+$ T cells was also significantly lower in mice that had been immunised with the two malaria antigen regime compared to when mice were immunised with MSP1 alone, despite all mice receiving the same viral load. $CD8^+$ T cell interference was therefore occurring with respect to MVA-specific responses as well as antigen-specific responses regardless of epitope MHC class I restriction.

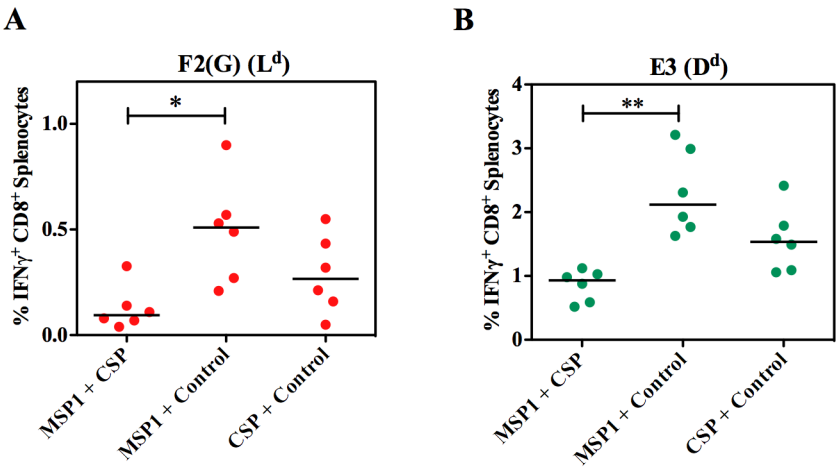


Figure 4.7: CD8$^+$ T cell interference is not due to competition for presentation on MHC class I molecules

BALB/c mice were immunised i.d. with the regimes described in Table 3.4. Two weeks after the boost, MVA-specific responses were measured in the spleen by ICS. CD8$^+$ IFN-γ$^+$ responses were measured following re-stimulation with the H-2L^d-restricted peptide F2(G) (A), and H-2D^d-restricted peptide E3 (B). * $P < 0.05$, ** $P < 0.001$ by Dunn's multiple comparison test.

4.2.6 $CD8^+$ T cell interference is not due to presentation of both antigens on the same APC

It was hypothesised that interference could also be due to some other aspect of presentation of both antigens on the same APC. It has previously been demonstrated that $CD8^+$ T cell interference can occur when $CD8^+$ T cells compete for access to APCs when both antigens are presented on the same APC surface [273]. Whilst the exact cause of the competition is not known, it has been suggested it may be due to limited space around the APC, perhaps limiting access to antigen (peptide:MHC complexes), co-stimulatory molecules and/or survival factors. In order to investigate this phenomenon here, BALB/c mice were immunised with a mixture containing 1 x 10^{10} vp each of AdHu5-PyMSP1$_{42}$ and AdHu5-PyCSP. Eight weeks later, rather than boosting with MVA, the responses were boosted with 2 x 10^5 peptide-pulsed $CD11c^+$ dendritic cells (DCs), as previously reported [276]. DCs were isolated by positive selection and were > 90% $CD11c^+$. The DCs were pulsed with PyMSP1$_{33}$ peptides and PyCSP peptides, either i) mixed together or ii) pulsed separately and then cells mixed immediately prior to i.v. injection. Two weeks later $CD8^+$ IFN-γ^+ responses were measured against the two antigens in the blood by ICS (Figure 4.8). In the majority of mice both PyMSP1$_{33}$ and PyCSP-specific responses were boosted by injection of peptide-pulsed $CD11c^+$ DC compared to non-pulsed DC, but the level of boosting did not reach statistical significant due to at least one non-responder in each group. Nevertheless this experiment demonstrated that responses were not different, irrespective of whether the DCs had been pulsed with peptides specific for both antigens either separately or as a mixture. These data thus suggest that T cell

interference is not occurring due to $CD8^+$ T cells competing when both antigens are presented on the same APC.

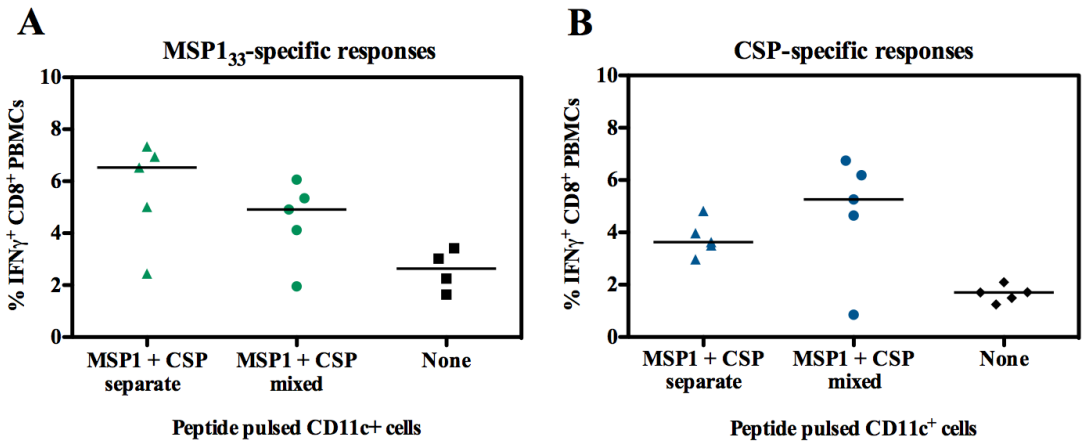


Figure 4.8: Enhanced immunogenicity when vaccines are given at separate sites is not due to presentation of antigens on separate APCs

BALB/c mice were primed i.d. with a mixture of AdHu5 vaccines as described in Table 3.4. Eight weeks later they were boosted i.d. with 2 x 10^5 CD11c$^+$ cells pulsed either i) with a mixture of the seven known PyMSP1$_{33}$ peptide epitopes and the two known PyCSP peptide epitopes, or ii) separately with the PyMSP1$_{33}$ peptides and PyCSP peptides before mixing the cells in a 1:1 ratio immediately prior to injection to give a total of 2 x 10^5 cells. A third group of mice were injected with 2 x 10^5 non-pulsed CD11c$^+$ cells (none). Two weeks after immunisation with peptide pulsed DCs, PBMCs were isolated and re-stimulated with peptides specific for PyMSP1$_{33}$ (A) and PyCSP (B) before ICS.

4.2.7 Cells presenting two antigens are better targets for killing at the time of MVA boost immunisation

It was hypothesised that secondary $CD8^+$ T cell responses may be lower when the two recombinant MVA vaccines expressing both antigens are given together at the same site due to dual infection of cells at the vaccination site. Infection of a single cell with more than one MVA has been demonstrated *in vitro* [277], and is likely to occur *in vivo* given the relatively high doses of MVA used. These cells may be better targets for killing by pre-existing transgene-specific cytotoxic $CD8^+$ T cells specific for both $PyMSP1_{33}$ and PyCSP, allowing less time for antigen expression, processing and/or cross-presentation to APCs, in comparison to when the two vaccines are given separately (Figure 4.9). In order to investigate this an *in vivo* CTL killing assay was carried out. In the first experiment, BALB/c mice were primed by i.d. immunisation with 2×10^{10} vp $AdPyMSP1_{42}$ + AdPyCSP, $AdPyMSP1_{42}$ + AdControl or AdControl only and then left to rest for eight weeks, as in all previous immunogenicity and efficacy studies. At the time the MVA-boost immunisation would normally be given, the mice were injected with CFSE-labelled, peptide-pulsed splenocytes prepared as described in Figure 2.4. Briefly, naïve splenocytes labelled with a high concentration of CFSE (CFSE high) were pulsed with either $PyMSP1_{33}$ peptide pool or a mixture of $PyMSP1_{33}$ peptide pool and $PyCSP_{280\text{-}288}$ peptides. To control for non-specific killing un-pulsed cells were labelled with a low concentration of CFSE (CFSE low). 2×10^7 cells containing an equal number of CFSE high and CFSE low cells were injected i.v. into the BALB/c mice that had been immunised with AdHu5 vaccines eight weeks earlier. Five hours after the cells were injected, the mice were culled and antigen-specific killing was calculated by analysing CFSE-fluorescence intensity (Figure

4.10A). Specifically, the ratio of the size of the CFSE low population (antigen non-specific killing) to the size of the CFSE high population (antigen-specific killing) following priming with the different immunisation regimes was calculated. These data showed that if cells were pulsed with $PyMSP1_{33}$ and PyCSP peptides mixed together (and therefore cells were presenting both antigens) then in mice primed against both antigens approximately 40% of the cells are killed within 5 hours. This was in comparison to approximately 20% killing seen when mice had been primed only against $PyMSP1_{42}$, or when cells pulsed with only $PyMSP1_{33}$ peptides were transferred into mice primed against either $PyMSP1_{42}$ alone or both antigens (Figure 4.10B). These data imply that priming mice against two malaria antigens could lead to a greater level of cellular killing following co-administration of two recombinant MVA booster vaccines as mixture, and this is associated with reduced boosting of $CD8^+$ T cell responses against both antigens.

In a second experiment, mice were primed with a mixture of $AdPyMSP1_{42}$ + AdPyCSP or AdControl and then injected with CFSE-labelled cells 8 weeks later as in the previous experiment. However, in this experiment the 'CFSE high' cells were either pulsed with $PyMSP1_{33}$ only, $PyCSP_{280\text{-}288}$ only, a mixture of $PyMSP1_{33}$ and $PyCSP_{280\text{-}288}$ peptides, or were pulsed separately with $PyMSP1_{33}$ and $PyCSP_{280\text{-}288}$ peptides and the cells mixed immediately before injection. Again, the percentage of injected cells that were killed was higher if the injected cells had been pulsed with peptides against both antigens as compared to a single antigen, however, the magnitude of killing was not different between mice injected with cells pulsed with peptides against both antigens as a mixture, or pulsed separately but injected simultaneously (Figure 4.11). Therefore, although enhanced killing is observed when

mice are previously primed against both antigens, the observations using this model assay failed to account for the improved $CD8^+$ T cell immunogenicity seen against the PyCSP antigen following co-administration of the two MVA vaccines at separate sites. It should be noted, however, that these experiments were small, with only 4 mice per group, therefore these experiments should be repeated with a larger number of mice per group before firm conclusions are drawn.

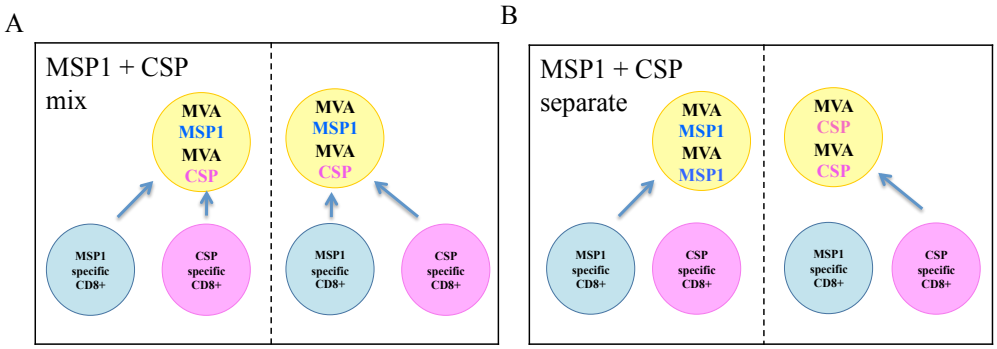


Figure 4.9: Diagram of potential killing of MVA-infected cells during boosting

In mice immunised with a mixture of MVA-PyMSP1$_{42}$ (MVA-MSP1) and MVA-PyCSP (MVA-CSP) (A) each MVA infected cell could be targeted by two CTL populations whereas in mice immunised with the same vaccines at separate sites (B) each MVA infected cell only being targeted by a single CTL population, reducing the chances of a killing event occurring. Blue and pink cells represent pre-existing PyMSP1 and PyCSP-specific CD8$^+$ T cells present at the time of the boost due to priming with a mixture of AdHu5-PyMSP1$_{42}$ and AdHu5-PyCSP. Yellow cells indicate those infected with MVA at the time of the boost. The dashed line separates immunisation sites and arrows indicate the potential for killing by antigen-specific CTLs.

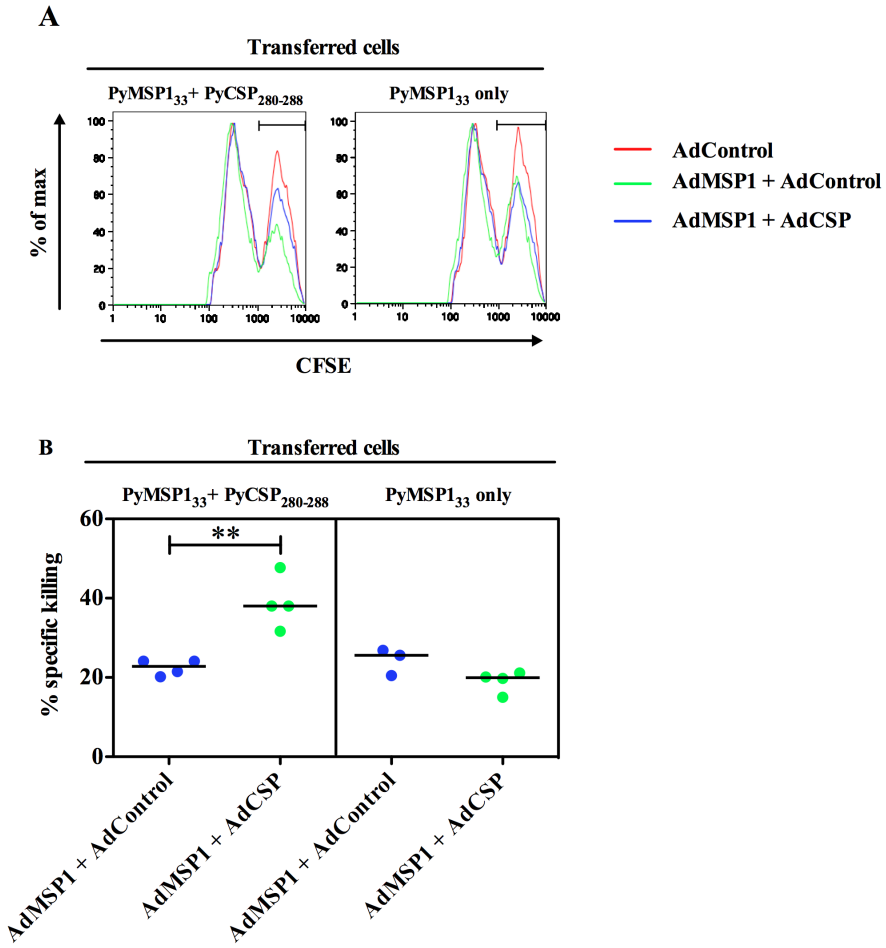


Figure 4.10: Antigen-specific killing occurs at the time of the MVA boost

BALB/c mice were immunised i.d. with AdControl, AdMSP1 + AdControl, or AdMSP1 + AdCSP as indicated. Eight weeks later they were injected with 2 x 10^7 cells containing an equal number of CFSE high and CFSE low cells, where CFSE high cells had been pulsed with either PyMSP1$_{33}$ and PyCSP$_{280-288}$ peptides, or PyMSP1$_{33}$ peptides only as shown (transferred cells). After 5 h mice were culled and splenocytes analysed by flow cytometry and antigen specific killing was calculated. Representative histograms of the intensity of CFSE staining showing the proportion of CFSE high to CFSE low cells recovered following priming with the different vaccines are shown (A) and antigen specific killing was calculated (B). ** $P = 0.003$ by Mann Whitney test. Bars indicate median response.

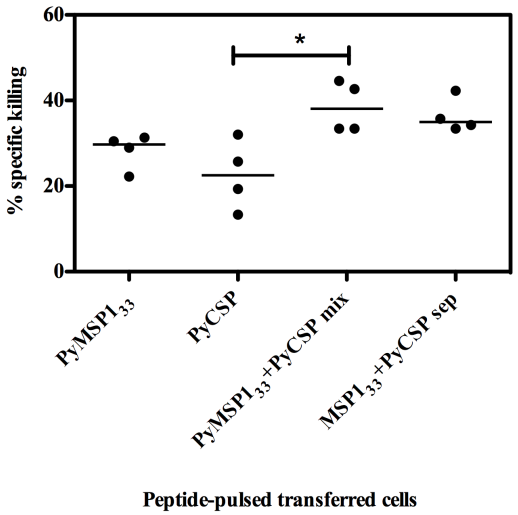


Figure 4.11: Antigen-specific killing is comparable between cells pulsed with a mixture of peptides, or with peptides pulsed separately

BALB/c mice were immunised i.d. with a mixture of AdHu5-PyMSP1$_{42}$ and AdHu5-PyCSP or with AdHu5-Control. Eight weeks later they were injected with 2 x 10^7 cells containing an equal number of CFSE high and CFSE low cells, where CFSE high cells had been pulsed with either PyMSP1$_{33}$ peptide pool only, PyCSP$_{280\text{-}288}$ peptide only, or PyMSP1$_{33}$ peptide and PyCSP$_{280\text{-}288}$ either mixed together (mix) or with half the cells pulsed with each (sep). After 5 h mice were culled and splenocytes analysed by flow cytometry and percentage antigen-specific killing in antigen-immunised mice was calculated. Points represent individual mice and the lines show median responses. * $P < 0.05$ by Dunn's multiple comparison test.

4.3 Discussion

It was shown in the *P. yoelii* mouse model that co-administration of a mixture of viral vectored vaccines expressing two different malaria antigens in an AdHu5-MVA prime-boost regime results in strong antibody responses against both antigens. However, $CD8^+$ T cell responses were reduced after the MVA boost immunisation, in three strains of mice, if the two vaccines were given as a mixture, but this effect was reduced if the two MVA boost vaccines were co-administered at separate sites. Importantly, maintaining high-level $CD8^+$ T cell and antibody responses against both the PyCSP and $PyMSP1_{42}$ antigens, by immunizing at separate sites, was associated with the highest levels of efficacy against a stringent high dose challenge with *P. yoelii* sporozoites.

$CD8^+$ T cell interference may occur for a number of reasons. The first hypothesis was that it could be a systemic or spatial effect, due to the large magnitude of the $CD8^+$ T cell responses induced in BALB/c mice against these two antigens. In order to address this question the doses of vaccines were lowered, giving 100-fold less AdHu5 and 10-fold less MVA. This reduced the overall magnitude of the antigen-specific immune responses, in agreement with other dosing studies in mice for similar viral vectored vaccines [274]. In this case PyCSP-specific $CD8^+$ T cell interference was no longer observed, although the $PyMSP1_{33}$-specific response was still reduced by more than 50% in the two-antigen regime, indicating that whilst the magnitude may have been contributing to competition, it is not the sole cause. Additionally, altering the ratio of the vaccines, such that mice were boosted with 10-fold more MVA-

PyMSP1$_{42}$ than MVA-PyCSP in the two-antigen regime, could not overcome the PyMSP1$_{33}$-specific CD8$^+$ T cell competition (data not shown).

It has been found that co-administering DNA vaccines at separate sites can overcome CD8$^+$ T cell interference [204]. In agreement with that, it was found that administration of the two recombinant MVA vectors at separate sites eliminated PyCSP-specific CD8$^+$ T cell interference. PyMSP1$_{33}$-specific competition did still occur, but in this case it was minimised, with 70% of the response maintained in the mice immunised against both antigens, whilst in previous experiments the response had been reduced by more than 50%. Furthermore, when these mice were challenged with *P. yoelii* sporozoites, those immunised at separate sites demonstrated enhanced liver-stage efficacy as determined by day 5 blood-stage parasitaemias (Figure 4.3).

A second hypothesis was that interference occurred due to competition for presentation of processed peptides on MHC class I molecules [275]. Given that all known PyMSP1$_{33}$ and PyCSP epitopes are H-2K^d-restricted in BALB/c mice, immunogenicity was investigated in two other mouse strains. In C57BL/6 mice the one known PyMSP1$_{33}$ epitope ($\alpha\alpha$1473 – 1481 YVIRNPYQL) is H2-D^b-restricted (Dr. S. Draper, unpublished observations), whilst in PyCSP no H-2^b-restricted epitopes are published. Despite the lack of detectable PyCSP-specific responses in these mice, PyMSP1$_{33}$-specific responses were nevertheless reduced in the combination vaccine, suggesting that interference is not dependent on a CD8$^+$ T cell response to both antigens. However, it is possible that PyCSP-specfic responses were missed due to the use of a Pb/PyCSP mixed peptide pool, rather than peptides spanning the entire length of PyCSP. In outbred mice, relatively few mice responded

to vaccination, making conclusions difficult, however, in responding mice responses tended to be lower if mice had been immunised against both antigens. Even in responding mice, PyCSP did not appear to provide any liver-stage protection. To the best of our knowledge, liver-stage efficacy due to PyCSP-specific $CD8^+$ T cells has never been demonstrated in outbred mice. Disappointingly, our vaccines did not result in sero-conversion of all PyCSP immunised mice. This is in contrast to the work of Wang *et al*. [76], who induced PyCSP-specific antibody-mediated protection against *P. yoelii* sporozoites in outbred mice with comparable antibody titres to those observed in inbred strains. It is possible this is due to a lack of $CD4^+$ T cell help induced by the vaccines tested here, but this will require further investigation.

As these data could not conclusively confirm whether $CD8^+$ T cell interference was due to competition for peptide presentation on the same MHC class I molecules, we also tested immunogenicity to epitopes within the MVA backbone that were known to have a different MHC class I restriction. These mice had not previously been exposed to MVA, and therefore MVA-specific T cell responses were primary, rather than secondary. Nevertheless, the responses were reduced when the vaccines were given as a mixture. These data clearly demonstrated that responses were reduced at the time of the boost in a combination vaccine, regardless of MHC class I restriction. Furthermore, as the MVA proteins are present within the virus prior to injection, these results suggest that interference is not due to a problem with translation of both antigens in co-infected cells.

A number of studies have found that $CD8^+$ T cell interference occurs when $CD8^+$ T cells compete for antigenic epitopes presented on the same APC surface [273]. The

cause is not known, but it may be due to limited space around the APC, perhaps limiting access to co-stimulatory molecules or survival factors. It has previously been shown that peptide-pulsed DCs can boost MVA primed immune responses [276], and so to investigate whether this was the case here, mice primed with the mixture of Ad-PyCSP and $PyMSP1_{42}$ were boosted with peptide-pulsed $CD11c^+$ DCs that had either been pulsed with peptides from both antigens together or separately. A modest increase was observed in the production of IFN-γ from $CD8^+$ T cells when Ad-primed responses were boosted with peptide-pulsed DCs, but there was no significant difference in the magnitude of the $CD8^+$ IFN-γ^+ response between those that had been pulsed with peptide separately or together. This is in contrast to the findings of Kedl *et al.* [273], who show that when mice are immunised with DC pulsed with two different peptides, in the presence of high-affinity T cells specific for one of the peptides, then responses to both peptides are impaired if they are presented on the same APC, but are impaired only to one peptide if they are presented on separate APCs. One caveat of these data is that antigen presentation may be different when two MVAs infect an APC, as compared to when APC are peptide-pulsed, as peptide-pulsing does not require antigen internalisation and processing for presentation on MHC class I.

The next hypothesis was that the killing of MVA infected cells at the time of the boost by cytotoxic $CD8^+$ T cells (CTLs), previously primed by the AdHu5 vaccines, may also be a crucial factor in determining the magnitude of the secondary $CD8^+$ T cell response. A published study of secondary influenza infection showed that $CD8^+$ T cells secreting perforin rapidly terminate antigen presentation in this model [65]. This killing may represent a negative-feedback loop to prevent excessive lymphocyte

activation and immunopathology [65]. Furthermore, studies with MVA and Vaccinia virus have shown that during secondary $CD8^+$ T cell responses the timing of antigen presentation is crucial, with responses only detected to antigens expressed early during the viral life cycle [278]. In that study a preference for immune responses to early antigens was not observed during primary $CD8^+$ T cell response and during secondary responses was dependent on the presence of primed T cells. However, a mechanism was not identified [278]. An *ex-vivo* CTL assay using CMV-specific T cell clones and CMV peptide-pulsed human PBMC showed that monocytes, B cells, T cells and DCs are rapidly killed by CMV-specific cytotoxic T cells, with 20% of monocytes killed within 1 hour [279]. The results presented here (Figure 4.10), using an *in vivo* killing assay, showed that following injection of peptide-pulsed splenocytes, cells pulsed with both $PyMSP1_{33}$ and PyCSP peptides together were almost twice as likely to be killed within 5 hours as those that had been pulsed with peptides corresponding to a single antigen. Taken together these data suggest that the more rapid clearance of MVA co-infected cells, that express both the $PyMSP1_{42}$ and PyCSP antigens, results in a reduced period of antigen expression and presentation, and consequently a reduced secondary $CD8^+$ T cell response. However, it was also observed that enhanced killing occurred when cells were pulsed with peptides separately and the cells mixed immediately before injection (Figure 4.11). This does not appear to be in agreement with the immunogenicity data, which suggested that enhanced immune responses were induced by immunising at separate sites (Figure 4.2). This could reflect differences in systemic compared to local CTL activity, or otherwise, alternative mechanisms may yet explain the reasons for improved immunogenicity following separate site immunisation with two recombinant MVAs.

The outcomes from this study imply that immunizing at separate sites may be necessary to prevent antigenic interference. However, this may be neither practical, nor financially viable in the field. A more feasible approach is the development of multivalent MVA vectors, which express a number of malaria antigens from a single virus. Establishing whether $CD8^+$ T cell interference occurs in humans, and the mechanism of interference, will be crucial in deciding which is the more effective approach to developing a multi-antigen or multi-stage malaria vaccine. Phase I/IIa clinical trials are currently underway at Oxford University to assess the efficacy of AdCh63-MVA vectored vaccines expressing *P. falciparum* MSP1, AMA1 and ME-TRAP alone and in combination. Based on the pre-clinical work presented here, vaccines are being co-administered at separate sites in the first instance.

In summary, this work has shown that Ad_MVA viral vectored vaccines expressing the antigens PyCSP and $PyMSP1_{42}$, can induce enhanced protection against *P. yoelii* sporozoite challenge compared to either antigen given alone, provided MVA vaccinations are given at separate sites. When MVA vaccines are given as a mixture reduced $CD8^+$ T cell responses are observed. This $CD8^+$ T cell interference is not due to peptide epitope competition for presentation on same MHC class I molecules, nor is it at the level of the APC. The data presented here suggest that enhanced killing of cells presenting both antigens at the time of the boost may occur, regardless of whether the vaccines are given at separate sites or as a mixture. The exact mechanism of $CD8^+$ T cell interference that occurs following administration of a MVA vaccine mixture thus remains to be fully elucidated. Overall, these data suggest that individual MVA vaccines administered at separate sites, rather than a multivalent MVA, is

likely to be necessary for the development of potent immune responses to multiple antigens by Ad_MVA immunisation in humans.

Classical protein-in-adjuvant vaccines do not induce strong cellular immunity and therefore T cell interference in multi-antigen vaccination regimes is not routinely assessed. However, multi-antigen viral vectored and DNA vaccines which induce stronger T cell responses are now being tested for a wide variety of diseases including HIV-1, tuberculosis, malaria and influenza [158]. Understanding T cell interference further will be critical in developing effective multi-component vaccines against these difficult infectious diseases.

Chapter 5

Vaccine efficacy against blood-stage *P. berghei*

5 Vaccine efficacy against blood-stage *P. berghei*.

5.1 Introduction

This chapter is divided into two sections. The first section investigates protection against blood-stage *P. berghei* using viral vectored vaccines. In the second section this work is extended to investigate protection against a transgenic *P. berghei* parasite expressing *P. falciparum* $MSP1_{19}$.

5.1.1 Vaccines against blood-stage *P. berghei*

Blood-stage *P. yoelii* infection can be successfully controlled by a variety of subunit vaccination methods, including protein-in-adjuvant and viral vectored vaccines [48, 97-99, 102, 280]. However, only four publications to date demonstrate protection against blood-stage *P. berghei* by subunit vaccination. These include a recombinant *Salmonella* vaccine encoding regions of blocks 3 and 4 of PbMSP1 [37], PbMSP9 N-terminus recombinant protein formulated in alum [38], an unconvincing publication on $PbMSP1_{19}$ recombinant protein in alum [39], and partial protection following immunisation with recombinant PbAMA1 in Freund's adjuvant [40]. More recently, several examples of failed protection by vaccines targeting PbMSP1 and PbAMA1 have been reported [41, 42]. In the study by Yoshida *et al*., blood-stage vaccine efficacy against *P. berghei* or *P. yoelii* were compared head-to-head using baculoviral based vaccines (BBV) targeting *P. berghei* or *P.yoelii* $MSP1_{19}$ and AMA1. This study found that BBV vaccines could induce complete or partial protection against *P. yoelii* but not against *P. berghei* [41]. Similarly, AdHu5 and MVA vectored vaccines expressing *P. berghei* $MSP1_{42}$ given in a prime-boost regime failed to protect BALB/c, C3H or C57BL/6 mice against challenge with *P. berghei* ANKA pRBC or

sporozoites (Dr. A. Goodman, DPhil thesis), despite the ability of AdHu5_MVA targeting PyMSP1 to protect against *P. yoelii* pRBC [48].

Immune responses induced by viral vectored vaccines can be boosted by protein-in-adjuvant immunisation. Protein boosting enhances antibody titres, with the highest titres observed following Ad_Protein or Ad_MVA_Protein immunisation regimes [281, 282]. Therefore, in order to induce greater titres of $PbMSP1_{19}$-specific antibodies, a further booster vaccination with $PbMSP1_{19}$ protein in Montanide ISA720 was given to mice previously immunised with AdHu5_MVA-$PbMSP1_{42}$. However this did not increase protection against *P. berghei* ANKA pRBC (Dr. A. Goodman, DPhil thesis). Similarly, five immunisations of mice with $PbMSP1_{19}$ protein in complete Freund's adjuvant and incomplete Freund's adjuvant failed to protect against challenge with *P. berghei* ANKA pRBC. This was despite the ability of all of these vaccination regimes to induce high-titre $PbMSP1_{19}$-specific antibodies detectable by ELISA and capable of binding merozoites in an immunofluorescence assay (IFAT) (Dr. A. Goodman, DPhil thesis).

Studies of infection and drug cure, or immunisation with formalin killed pRBC [283, 284], show that protection against *P. berghei* is possible, although it is generally harder to protect against than other rodent malarias and heterologous immunity is much lower [41, 285]. There is strong evidence of the role of antibodies in protection against blood-stage *P. berghei*. Nunes *et al*. used an infection and drug cure model in $AID^{-/-}$ $\mu s^{-/-}$ mice (whose B cells cannot undergo Ig class switching nor secrete antibody, but rather have surface IgM) to demonstrate that immunity to Pb NK65 is dependent upon secreted antibody [286]. Furthermore, passive transfer of

hyperimmune sera from mice that had been infected with *P. berghei* NK65 (and drug cured) into naïve C57BL/6 mice led to significantly reduced parasitaemia upon challenge. However, the infection was not completely cleared (indicating that antibody is not solely responsible for protection), nor were the isotypes of IgG required or antigenic targets identified [286].

There is some evidence for the role of Fc-mediated mechanisms in antibody-mediated protection against *P. berghei*. Humans and mice have both activatory (A) and inhibitory (I) FcγRs, and in both species the inhibitory receptor is FcγRIIb (CD32b). Different isotypes of IgG have varying affinities for FcγRs, giving rise to an A:I ratio for each subtype [287, 288]. In particular, mouse IgG1 preferentially binds to FcγRIIb and FcγRIII leading to inhibitory signalling, whilst IgG2a preferentially binds to FcγRIV leading to activatory signalling (Figure 5.1). Adoptive transfer studies with the non-lethal/radiation attenuated strain of *P. berghei* NK65, known as Pb XAT, have shown that protection against this parasite requires IgG2a but not IgG1 [289]. Although this does not directly implicate an immune mechanism mediated by FcγRs, studies using FcR common γ-chain (common to FcγRI, FcγRIII and FcγRIV) knockout ($Fc\gamma R^{-/-}$) mice have shown that FcγRs are essential in mediating blood-stage protection against Pb XAT [290]. Additionally, experiments with attenuated plasmepsin 4-deficient (ΔPM4) *P. berghei* have indicated that the spleen is essential for antibody-mediated clearance of this parasite, probably via Fc-mediated phagocytosis by splenic macrophages [291], although this mechanism remains to be fully established.

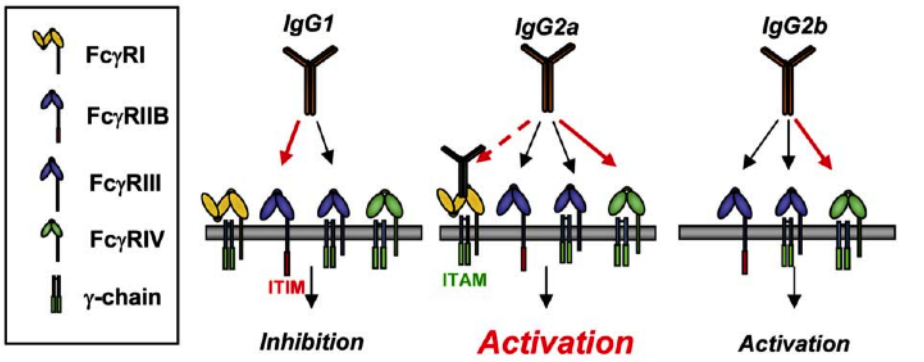
Further evidence for the importance of FcγR against *P. berghei* comes from studies with transgenic *P. berghei* expressing $PfMSP1_{19}$ (termed Pb-PfM19). Protection against Pb-PfM19 by passive transfer of a human mAb (termed JS1), which is specific for $PfMSP\text{-}1_{19}$ is observed in transgenic mice which express the human FcγRI (CD64), but not in wild-type mice [292]. This indicates that protection is dependent upon the interaction between this humanised IgG1 mAb and the human FcγR CD64. Interestingly, protection cannot be achieved in wild-type mice using the same human mAb JS1 Fab fragment fused to mouse IgG1 Fc [293], indicating that in the murine system, the mouse IgG1 isotype is not effective against *P. berghei.* Human mAb JS1 Fab fused to mouse IgG2a, IgG2b, IgG3, IgM and IgA Fc are now being made (Prof. Richard Pleass, personal communication). If *P. berghei* is a good model of Fc-mediated protection then it is hypothesised that IgG2a and possibly IgG2b Fc regions, when fused to JS1 Fab, will be protective due to preferential binding to the activatory receptor FcγRIV, rather than the inhibitory receptor FcγRIIb.

In this chapter protection against *P. berghei* following viral vectored vaccines expressing $PbMSP1_{42}$, PbAMA1 and PbMSP9 is assessed using both *P. berghei* ANKA and *P. berghei* NK65. Vaccine efficacy in mice lacking the inhibitory receptor FcγRIIb is then assessed.

5.1.2 Transgenic *P. berghei* expressing *P. falciparum* $MSP1_{19}$

P. falciparum does not infect rodents, therefore transgenic parasites have been developed to test the efficacy of vaccines targeting *P. falciparum* antigens in rodent models. A transgenic *P. berghei* ANKA parasite which contains *P. falciparum* $MSP1_{19}$ ($PfMSP1_{19}$), in place of $PbMSP1_{19}$, has been generated in order to test the

efficacy of vaccines targeting $PfMSP1_{19}$ [57]. Efficacy against this parasite, termed Pb-PfM19, has been assessed in BALB/c and C57BL/6 mice following immunisation with Ad_MVA encoding PfM115 (an 115kDa antigen identical to PfM128 but without the Wellcome strain 19kDa fragment), and Ad_MVA expressing PfM128 followed by $PfMSP1_{19}$ recombinant protein in Montanide ISA720. However, no protective efficacy was observed (Dr. S. Draper and Dr. A. Goodman, DPhil theses). Similarly, Yoshida *et al*. found that BBV vaccines targeting $PbMSP1_{19}$ could not protect against Pb-PfM19 [41]. As far as is known protection against Pb-PfM19 has not yet been demonstrated by active immunisation of mice with vaccines targeting PfMSP1 (Prof. Brendan Crabb, personal communication). There is, however, evidence of $PfMSP1_{19}$-specific antibody-mediated immunity to Pb-PfM19 from adoptive transfer studies in which human or rabbit anti-$PfMSP1_{19}$ IgG was used to passively immunise mice [56, 292, 294]. Protection has also been achieved by repeated infection and drug-cure with Pb-PfM19 followed by subsequent challenge with Pb-PfM19 pRBC. In this chapter protection against Pb-PfM19 following passive transfer of serum or IgG from rabbits immunised with viral vectored vaccines expressing PfM128 or PfM115 is investigated.



	IgG1 (A/I)	IgG2a (A/I)	IgG2b (A/I)
FcγRIII/FcγRIIB	0.1	1.6	0.5
FcγRIV/FcγRIIB	n.a.	70	7

Figure 5.1: Antibody isotype specific triggering of individual FcγRs in mice

Black arrows indicate low affinity interactions and red arrows show the preferential interactions of the indicated antibody isotypes with FcγRs. The dashed red arrow indicates that interaction may be blocked due to monomeric IgG2a continuously binding FcγRI. The table shows the A:I ratios of the individual FcγRs based on the affinities of each antibody isotype. This figure is taken from Nimmerjahn and Ravetch, 2006 [295]. Copyright © 2006 Elsevier Inc..

5.2 Results

5.2.1 Immunogenicity of viral vectored vaccines expressing PbMSP1$_{42}$, PbAMA1 and PbMSP9.

Following the failure of AdHu5_MVA PbMSP1$_{42}$ vaccination regimes to protect against blood-stage *P. berghei* ANKA challenge (Dr A. Goodman, DPhil thesis), AdHu5 and MVA vectors expressing PbAMA1 and PbMSP9 were developed at the Jenner Institute by Dr Anna Goodman. These antigens were chosen due to published examples demonstrating that active immunisation against PbAMA1 [40] and PbMSP9 [38] can protect against *P. berghei* pRBC challenge.

Reagents were not available to test the induction of antibodies against PbAMA1 or PbMSP9 by ELISA, or cellular responses by ICS or ELISPOT. Therefore, the induction of conformation-specific antibodies by vaccination was assessed by immunofluorescence antibody test (IFAT). Sera from AdHu5_MVA immunised BALB/c mice were assessed for binding to *P. berghei* ANKA mature schizonts (Figure 5.2). These experiments were performed by Dr. Andrew Williams. These data demonstrate that the vaccines are immunogenic and that antibodies capable of binding to schizonts are produced following AdHu5_MVA immunization with PbMSP1$_{42}$ (as observed previously), PbAMA1 and PbMSP9, but not following immunisation with a control antigen (ovalbumin, OVA).

The availability of PbMSP1$_{19}$-GST protein allowed assessment of the induction of PbMSP1$_{19}$-specific IgG following Ad_MVA PbMSP1$_{42}$ immunisation, or immunisation with a mixture of all three antigens (Figure 5.3). Immunisation with

AdHu5_MVA-PbMSP1$_{42}$ alone induced PbMSP1$_{19}$-specific IgG and a significant increase in IgG titres was observed following the MVA boost, as previously described (Dr. Anna Goodman, DPhil thesis). However, surprisingly, no PbMSP1$_{19}$-specific IgG was detectable following either prime or boost by immunisation with a mixture of AdHu5 vaccines expressing all three antigens – PbMSP1$_{42}$, PbAMA1 and PbMSP9. These data indicate that the addition of PbAMA1 and PbMSP9 vaccines interfered with the induction of immunity to PbMSP1$_{42}$. Unfortunately the impact on the vaccine mixture on MSP9- and AMA1-specific antibody responses cannot yet be assessed by ELISA. However, IFATs are currently underway to assess whether any parasite-binding antibodies are induced by vaccination against all three antigens simultaneously.

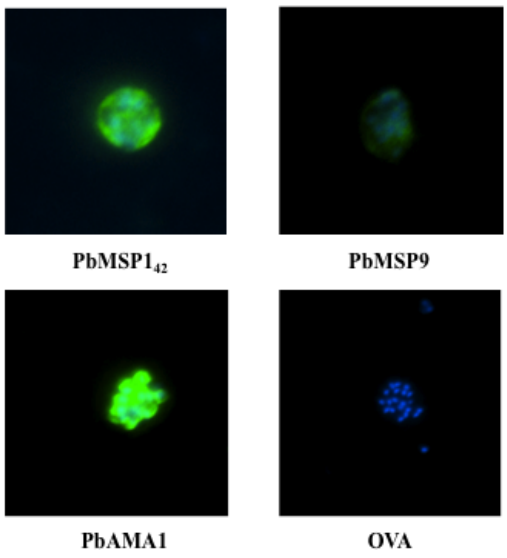


Figure 5.2: Fluorescence images of *P. berghei* ANKA schizonts immunostained with PbMSP1$_{42}$, PbMSP9, PbAMA1 and OVA antibodies raised in mice

P. berghei ANKA schizonts were fixed with paraformaldehyde and permeabilised with Triton X-100. Sera from mice immunised i.d. with 1 x 10^{10} vp AdHu5 and boosted with 1 x 10^{7} pfu MVA expressing the indicated antigens were tested at a dilution of 1:100. Bound antibodies were detected using goat anti-mouse IgG conjugated to Alexa Fluor™ 488 (green) and cell nuclei were visualised by DAPI staining (blue). Merged images are shown.

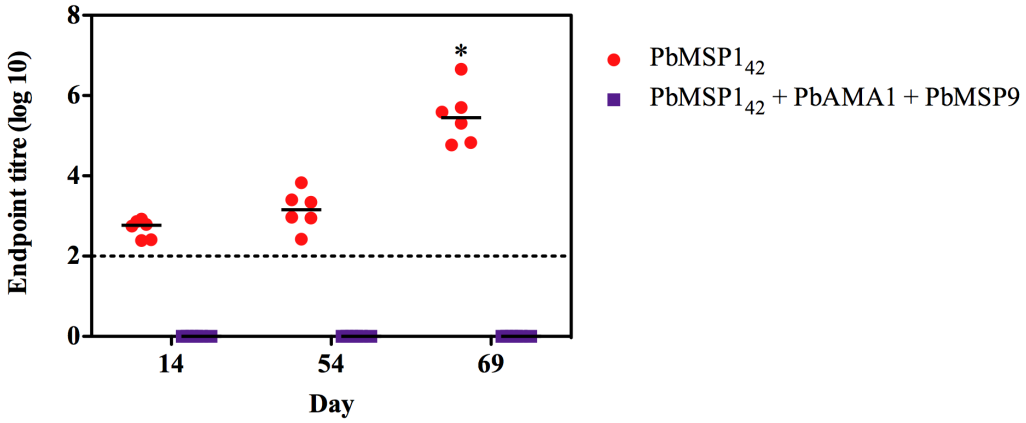


Figure 5.3: PbMSP1$_{19}$-specific total IgG following Ad_MVA PbMSP1$_{42}$ immunisation

Female BALB/c mice were immunised i.d. with 1 x 10^{10} vp AdHu5 and boosted eight weeks later i.d. with 1 x 10^{7} pfu MVA expressing PbMSP1$_{42}$ (red circles), or with 3 x 10^{10} vp AdHu5 and 3 x 10^{7} pfu MVA containing an 1:1:1 mixture of vaccines expressing PbMSP1$_{42}$, PbAMA1 and PbMSP9 (purple squares). Sera were collected on days 14 and 54 post-prime and 2 weeks post-boost (day 69) and assayed for total PbMSP1$_{19}$-specific IgG by ELISA. Points represent individual mice of 6 per group and bars indicate median titre. * P = 0.0313 for PbMSP1$_{42}$-specific IgG titre on day 69 compared to PbMSP1$_{42}$-specific IgG titre on day 54 (pre-boost) following immunisation against PbMSP1$_{42}$ alone by a repeated Wilcoxon matched-pairs signed rank test.

5.2.2 Efficacy of viral vectored vaccines against wild-type *P. berghei* ANKA and *P. berghei* NK65

Efficacy of the new vaccines targeting PbAMA1 and PbMSP9 were assessed in BALB/c mice by challenge with Pb ANKA pRBC, the *P. berghei* strain against which AdHu5-MVA-PbMSP1$_{42}$ had failed to induce protection. In this experiment mice were immunised with 1 x 10^{10} vp AdHu5 expressing either PbAMA1, PbMSP9 or no antigenic insert (Control) and eight weeks later were boosted with 1 x 10^{7} pfu MVA expressing the same antigen. Two weeks later mice were challenged with 5 x 10^{2} Pb ANKA pRBC and monitored daily. Similar to previous experiments with Ad_MVA PbMSP1$_{42}$, no significant efficacy was observed compared to the control immunised mice (Figure 5.4). By day 7 post-challenge 8/8 PbAMA1 immunised mice were culled, compared to 1/8 PbMSP9 immunised and 3/8 control immunised mice. This was not due to a difference in parasitaemia between groups, but rather reflected more severe subjective symptoms such as piloerection, hunching and lack of movement. However, all remaining mice had succumbed to infection by day 13 post-infection.

In all four published examples of protection against wild-type blood-stage *P. berghei* by sub-unit vaccination, the challenge strain was *P. berghei* NK65 (Pb NK65) rather than Pb ANKA [37-39]. The non-lethal / attenuated strain Pb XAT is also derived from *P.* berghei NK65 [296]. Pb NK65 infection results in a greater number of latent merozoites, i.e. merozoites are in the blood for longer before invading a new RBC [297]. Pb NK65 is also known i) to have a greater attraction to immature RBC (reticulocytes), ii) to be more likely to multiply infect the same cell, and iii) to produce more rings early in infection [298], leading to a less synchronous infection

than Pb ANKA. Given these published data, it was assessed whether efficacy could be achieved instead against Pb NK65. Mice were immunised with 1 x 10^{10} vp AdHu5 expressing either PbMSP1$_{42}$, PbAMA1 or PbMSP9, or a mixture of all three vaccines (a total of 3 x 10^{10} vp) and eight weeks later were boosted with 1 x 10^{7} pfu MVA expressing the same antigen or, again, a mixture of all 3 vaccines (3 x 10^{7} pfu total). Two weeks post boost mice were challenged with 5 x 10^{2} Pb NK65 pRBC (Figure 5.5). However, all mice were culled by day 7 because no differences observed between groups. Parasitaemia in Ad_MVA PbMSP9 immunised mice was slightly lower than Ad_MVA PbMSP1$_{42}$ immunised or naïve mice on day 5 ($P < 0.05$), but this effect had disappeared by day 6.

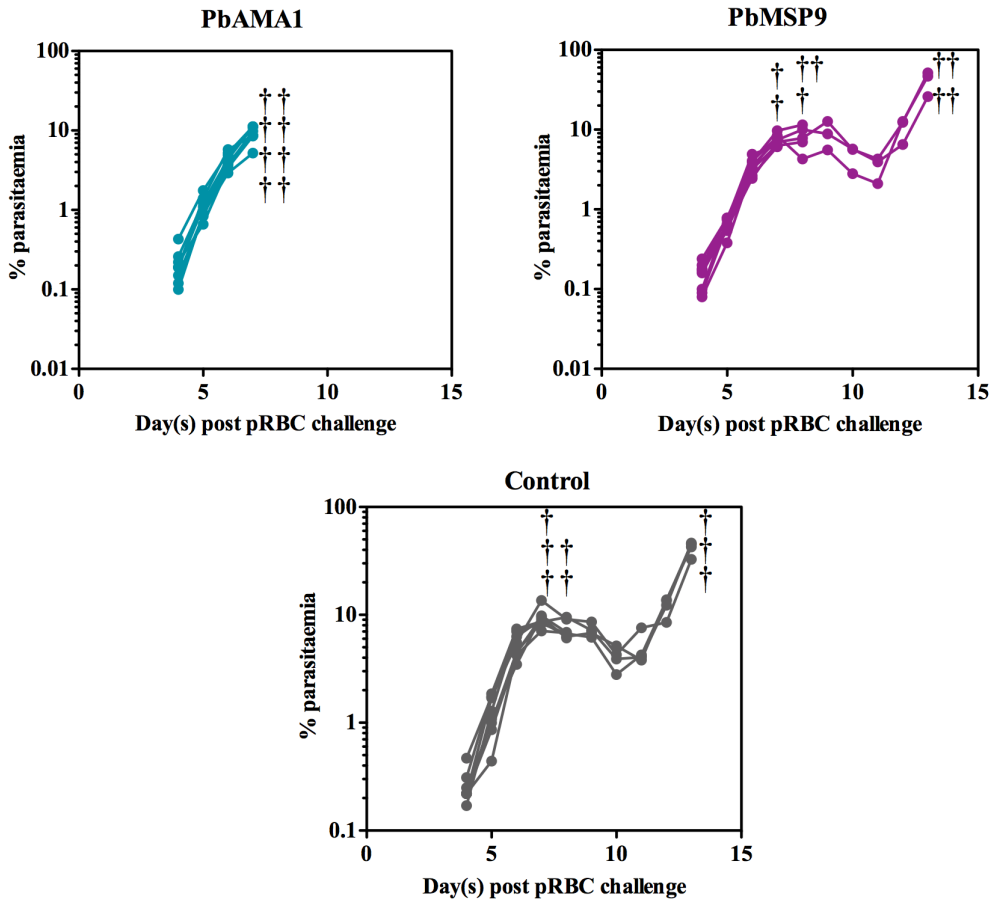


Figure 5.4: Efficacy study of Ad_MVA PbAMA1 and Ad_MVA PbMSP9 immunisation against Pb ANKA pRBC infection

BALB/c mice were immunised i.d. with 1 x 10^{10} vp AdHu5 and boosted thirteen weeks later with 1 x 10^{7} pfu MVA encoding either PbAMA1, PbMSP9 or no antigen (Control). Two weeks post-boost mice were challenged with 5 x 10^{2} Pb ANKA pRBC and monitored for parasitaemia. Lines represent individual mice of 8 mice/group. † indicates that mice were culled.

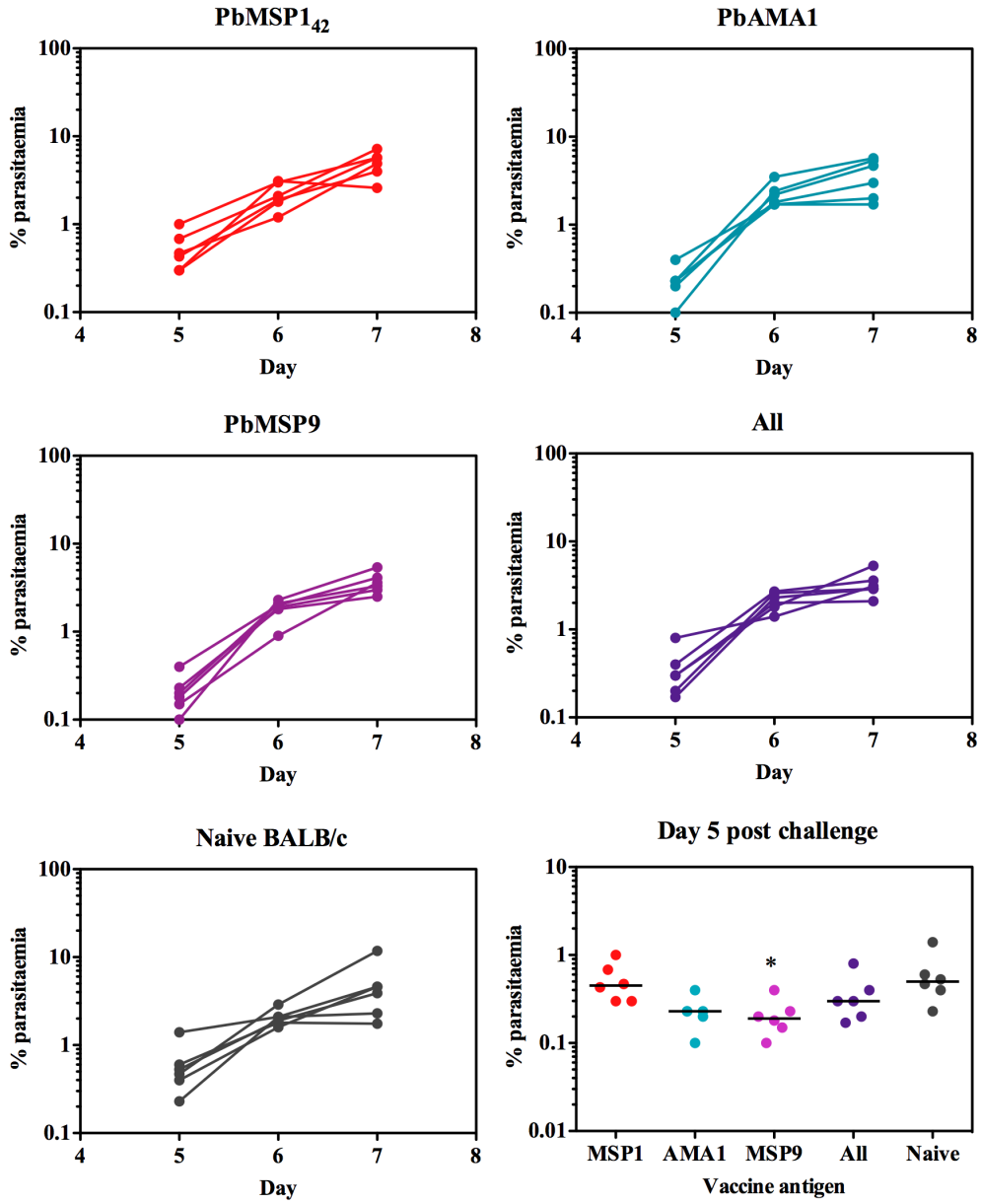


Figure 5.5: Efficacy study of Ad_MVA PbMSP1$_{42}$, PbAMA1, PbMSP9 immunisation against Pb NK65 pRBC infection

BALB/c mice were immunised i.d. with 1 x 10^{10} vp AdHu5 and boosted eight weeks later with 1 x 10^{7} pfu MVA encoding either PbMSP1$_{42}$, PbAMA1 or PbMSP9. A fourth group of mice were immunised with a mixture of all 3 antigens at both prime and boost (All). Two weeks post-boost mice were challenged with 5 x 10^{2} Pb NK65 pRBC and monitored for parasitaemia. A control group of non-immunised mice were

also challenged (Naïve). Lines represent individual mice of 6 mice/group. Day 5 parasitaemia is also shown with points representing individual mice and bars indicating median parasitaemia. * $P < 0.05$ for PbMSP9 parasitaemia on day 5 post challenge compared to PbMSP1$_{42}$ immunised and Naïve mice by Dunn's multiple comparison test.

5.2.3 The role of FcγRIIb (CD32b) in antibody-mediated protection against *P. berghei*

Viral vectored vaccines are known to induce both IgG1 and IgG2a [48]. Given the evidence for the role of Fc-mediated mechanisms in protection against blood-stage *P. berghei*, it is possible that the inhibitory effect of IgG1 binding to FcγRIIb may be preventing activatory signals induced by IgG2a. Here, using FcγRIIb knockout mice ($CD32b^{-/-}$) it is investigated whether removing the inhibitory signals induced through FcγRIIb allows protection against *P. berghei* (possibly mediated by antibody Fc effector functions such as ADCI) to be observed.

To investigate this, $CD32b^{-/-}$ mice on a C57BL/6 background were immunised i.d. with an AdHu5_MVA prime-boost regime expressing either $PbMSP1_{42}$ or no antigen (Control). In this preliminary experiment wild-type C57BL/6 immunised mice were not included, however it is already known that these viral vectored vaccines do not protect wild-type C57BL/6 mice against *P. berghei* (Section 5.1.1). $PbMSP1_{19}$-specific IgG responses were induced by immunisation (Figure 5.6). Unlike immunisation of BALB/c mice, responses were not detectable two weeks post-prime, and post-boost responses were lower than those observed in BALB/c mice (Figure 5.3). This is in contrast to a previous study using wild-type C57BL/6 mice in which $PbMSP1_{19}$-specific responses induced by Ad_MVA $PbMSP1_{42}$ immunisation were not significantly different between BALB/c and C57BL/6 mice two weeks post-boost (Dr A. Goodman, DPhil thesis). Although wild-type C57BL/6 mice were not included in this experiment and responses cannot be directly compared, the results suggest that Ad_MVA immunisation in $CD32b^{-/-}$ mice may induce lower responses than in

C57BL/6 wild-type mice. Future experiments should directly compare immunogenicity in CD32b$^{-/-}$ and wild-type C57BL/6 mice following both priming and boosting.

Two weeks post-boost, mice were challenged with *P. berghei* ANKA pRBC and monitored for parasitaemia (Figure 5.7). Disappointingly no significant differences in parasitaemia were observed in the PbMSP1$_{42}$ immunised compared to control immunised or naïve mice. The lack of the FcγRIIb did not appear to influence infection in naïve mice either, with no significant differences observed in parasitaemia between naïve CD32b$^{-/-}$ and naïve wild-type C57BL/6 mice. In some groups symptoms were more severe than others, with cerebral malaria (CM) developing in a subset of mice. This is commonly observed following infection of C57BL/6 mice with *P. berghei* [299]. In order to make decisions on which mice to cull, a scoring system was developed which involved studying appearance, movement, behaviour and weight loss. This was based on the work of Carroll *et al.* [300] and was implemented from day 6 post-infection. Based on this scoring system naïve wild-type C57BL/6 mice were culled earliest, followed by naïve CD32b$^{-/-}$ mice, then control and PbMSP1$_{42}$ immunised mice, many of which still had very few symptoms on day 9, at which point all remaining mice were sacrificed. However, due to breeding schedules and the exploratory nature of this experiment, mice were not age matched and were much smaller in the naïve groups, with an median peak weight of 16.5g for naïve wild-type C57BL/6 mice, 22.6g for naïve CD32b$^{-/-}$ mice, and 26.0g for immunised CD32b$^{-/-}$ mice (data not shown). It could be that symptoms of CM onset earlier in younger and smaller mice and future experiments should ensure that mice are both age, weight and gender matched to avoid these confounding factors.

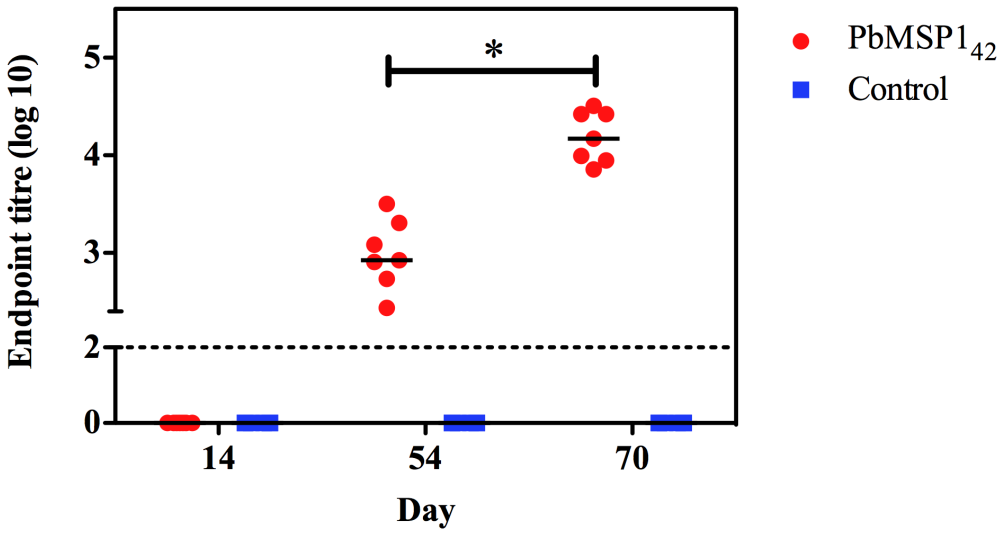


Figure 5.6: PbMSP1$_{19}$-specific total IgG responses following Ad_MVA PbMSP1$_{42}$ immunisation in CD32b$^{-/-}$ mice

Five male and two female 11 week old CD32b$^{-/-}$ mice were immunised i.d. with 1 x 10^{10} vp AdHu5-PbMSP1$_{42}$ and boosted 8 weeks later with 1 x 10^{7} pfu MVA-PbMSP1$_{42}$. At the same time, three male and two female age matched controls were immunised with AdHu5 and boosted with MVA expressing no antigenic insert (Control). On days 14, 54 (pre-boost) and 70 (2 weeks post-boost) sera were collected and PbMSP1$_{19}$-specific total IgG measured by ELISA. Dots represent individual mice and bars show median endpoint titre. * P = 0.0156 between day 54 (pre-boost) and day 70 (post-boost) in PbMSP1$_{42}$ immunised mice by Wilcoxon matched pairs signed rank test . The dashed line indicates the lower limit of detection.

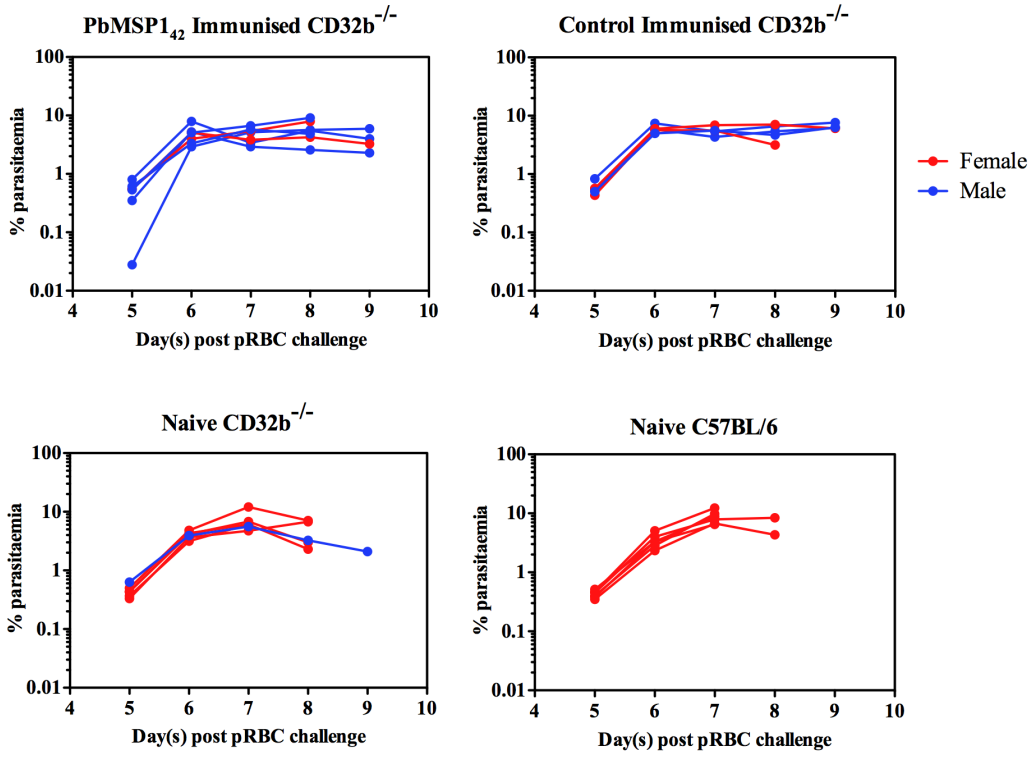


Figure 5.7: Efficacy of AdHu5_MVA PbMSP1$_{42}$ in CD32b$^{-/-}$ mice

Male (blue) and female (red) CD32b$^{-/-}$ mice were immunised i.d. with 1 x 10^{10} vp AdHu5 and boosted eight weeks later with 1 x 10^{7} pfu MVA encoding either no antigen (Control) or PbMSP1$_{42}$. Two weeks post-boost mice were challenged with 5 x 10^{2} *P. berghei* ANKA pRBC and monitored for parasitaemia. Control groups of non-immunised (Naïve) wild-type C57BL/6 and CD32b$^{-/-}$ mice were also challenged. Lines represent individual mice of 5-7 mice/group.

5.2.4 Protective efficacy of rabbit PfMSP1-specific IgG against Pb-PfM19

Protection against Pb-PfM19 by active immunisation has so far not been possible, but protection by adoptive transfer of rabbit $PfMSP1_{19}$-specific IgG has been reported twice to lead to sterile protection [56, 294]. In three separate studies carried out for the Jenner Institute, New Zealand white rabbits have been immunised with PfMSP1-specific vaccines. In the first study carried out at Agrobio (France) rabbits were primed with AdHu5 or the simian adenovirus C7 and boosted with MVA, C6 or AdHu5 viral vectored vaccines expressing the optimised PfMSP1 antigen, PfM115 [104]. In the second study performed by Biogenes (Germany), rabbits were primed i.d. with AdHu5 and boosted with MVA expressing the PfM128 antigen [104], which is identical to PfM115 but also includes $PfMSP1_{19}$ from the FVO *P. falciparum* strain. Finally, in the third study carried out at Agrobio (France), rabbits were immunised with AdHu5 or AdCh63 and boosted with MVA expressing PfM128 with all vaccines given i.m. In one group rabbits were immunised with a mixture of vectors expressing both PfM128 with PfAMA1 [242]. In all experiments vaccine doses were 5×10^{10} vp adenovirus and 1×10^{8} pfu MVA. Given the availability of rabbit sera from these studies, it was investigated whether these sera, when passively transferred, could protect against challenge with the Pb-PfM19 parasite.

All immunised rabbits developed an anti-$PfMSP1_{19}$-specific IgG response two weeks post-boost regardless of the vaccination regime used (Figure 5.8). Sera from PfM115 i.d. immunised rabbits had previously been assayed for $PfMSP1_{19}$ 3D7-specific and full length PfMSP1-specific total IgG by ELISA [104], and the results in this repeat experiment were comparable to that data, with no difference observed between those

groups. Overall, PfMSP1$_{19}$-specific IgG responses were comparable between all groups except AdHu5_MVA expressing PfM115 given i.m., in comparison to AdCh63_MVA immunisation expressing PfM128 and PfAMA1 in a vaccine mixture when tested against PfMSP1$_{19}$ 3D7 strain. Overall, the vaccine mixture induced the lowest PfMSP1$_{19}$ responses to both alleles, which suggests some antigenic interference.

A subset of sera from PfM115 and PfM128 immunised rabbits had previously been analysed for the correlation between 3D7 and FVO PfMSP1$_{19}$-specific IgG responses [104]. Here, a larger data set was analysed and a significant correlation was confirmed for both PfM115 and PfM128 (Figure 5.9). Overall, these antibody data demonstrate remarkable consistency between immunogenicity induced by viral vector vaccines used at different times and in different locations.

In order to assess whether sera from these rabbits could protect mice against challenge with Pb-PfM19 pRBC, rabbit sera were diluted 1:10 in PBS and 500μL injected i.p. into BALB/c mice on days -1, 0 and +1 with respect to Pb-PfM19 challenge on day 0 (i.e. a total of 150μL rabbit serum per mouse). The challenge was carried out by i.p. injection of 5 x 10^3 Pb-PfM19 pRBC. A range of protection was observed, with 6/33 sterilely protected and a further 9/33 having a delay to onset of patent-parasitaemia, whilst all naïve mice succumbed to infection. For ease of presentation only the parasitaemia data on day 5 post-challenge are presented (Figure 5.10A). Given these surprising results, and the first positive readout with regard to protection outcome using this mouse model at the Jenner Institute, a second experiment was undertaken to include all necessary and relevant controls. In this

second experiment sera from Ad_MVA PfAMA1 immunised or Control (no antigenic insert) immunised rabbits were tested in a similar manner and again a variety of parasitaemias were observed, indicating that the effect of rabbit sera on Pb-PfM19 is non-specific (Figure 5.10B).

Given that previously published studies had used purified IgG rather than whole sera, IgG were purified from a subset of rabbits and used in the assay. These rabbits were all from the third rabbit study carried out at Agrobio and had all been immunised i.m. with 5 x 10^{10} vp AdCh63 or AdHu5 expressing PfM128 and boosted with 1 x 10^{8} pfu MVA-PfM128. Some rabbits had also been immunised against PfAMA1 as indicated. Day 0 pre-immune IgG from the same rabbits and from control immunised rabbits were also purified. 0.5mg IgG was transferred on days -1, 0 and +1, and on day 0 mice were challenged with 5 x 10^{2} PbPfM19 pRBC by i.v. injection, however no differences in parasitaemia between groups was observed (Figure 5.11).

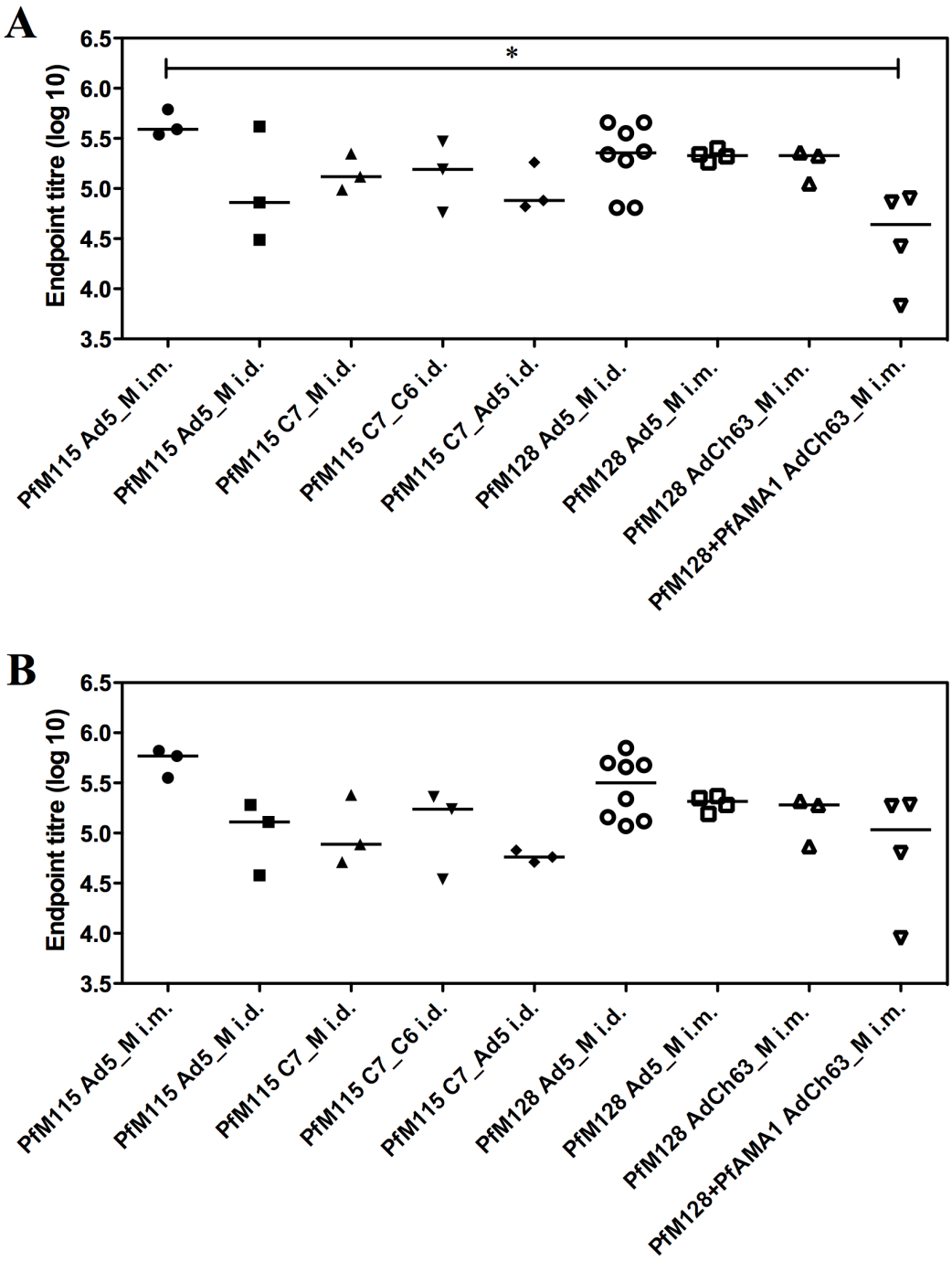

A
B
Endpoint titre (log 10)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
*
PfM115 Ad5_M i.m.
PfM115 Ad5_M i.d.
PfM115 C7_M i.d.
PfM115 C7_C6 i.d.
PfM115 C7_Ad5 i.d.
PfM128 Ad5_M i.d.
PfM128 Ad5_M i.m.
PfM128 AdCh63_M i.m.
PfM128+PfAMA1 AdCh63_M i.m.

Figure 5.8: PfMSP1$_{19}$-specific IgG following PfM115 or PfM128 immunisation of rabbits

New Zealand white rabbits were immunised with vaccines expressing PfM115 (containing PfMSP1$_{19}$ 3D7 allelic variant) or PfM128 (containing both the 3D7 and FVO allelic variants of PfMSP1$_{19}$) by the i.m. or i.d. route as indicated. Viral vectors were given in an eight week prime-boost regime (Prime_Boost) using human adenovirus serotype 5 (Ad5), simian adenovirus serotype 7 (C7), simian adenovirus serotype 6 (C6), chimpanzee adenovirus 63 (AdCh63) or MVA (M), AdCh63_M expressing PfM128 was also mixed with AdCh63_M expressing AMA1. Two weeks post-boost sera was collected and analysed for 3D7 (A) or FVO (B) PfMSP1$_{19}$-specific responses. Points indicate individual rabbits and bars are median titres. * P <0.05 3D7 MSP1$_{19}$-specific total IgG following immunisation with PfM115 Ad5_M i.m compared to PfM128+PfAMA1 AdCh63_M by Dunn's multiple comparison test.

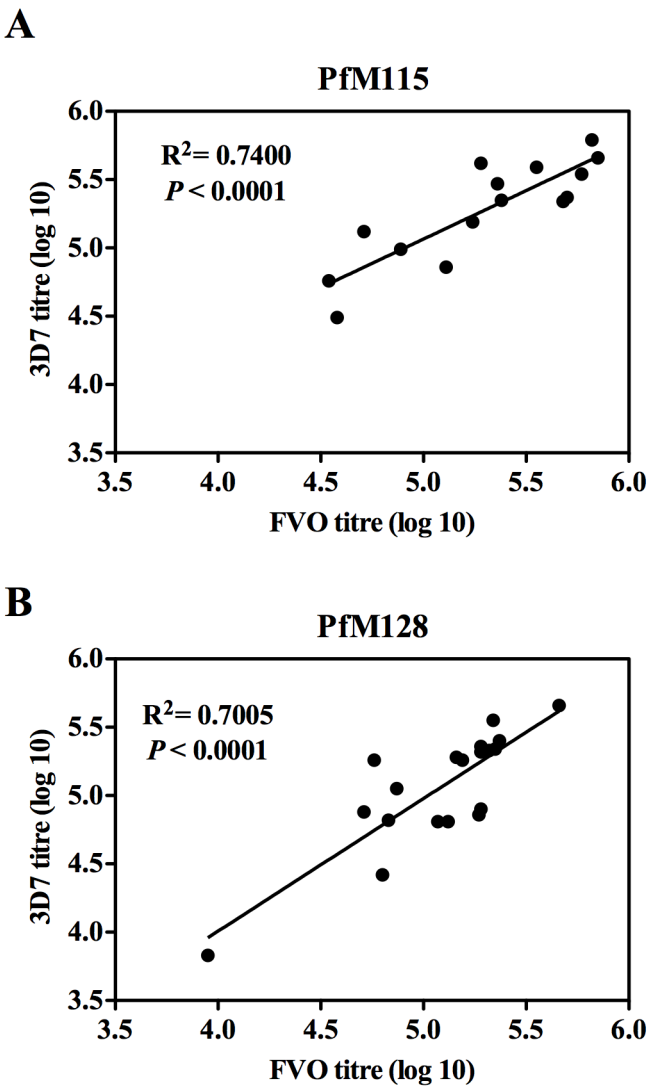


Figure 5.9: Correlation of total IgG responses to PfMSP1$_{19}$ allelic variants

The data shown in Figure 5.8 were analysed for correlation between allelic variants. Antibody titres from individual rabbits and linear regression lines are shown, with Pearson rank correlations (R^2) and *P* values.

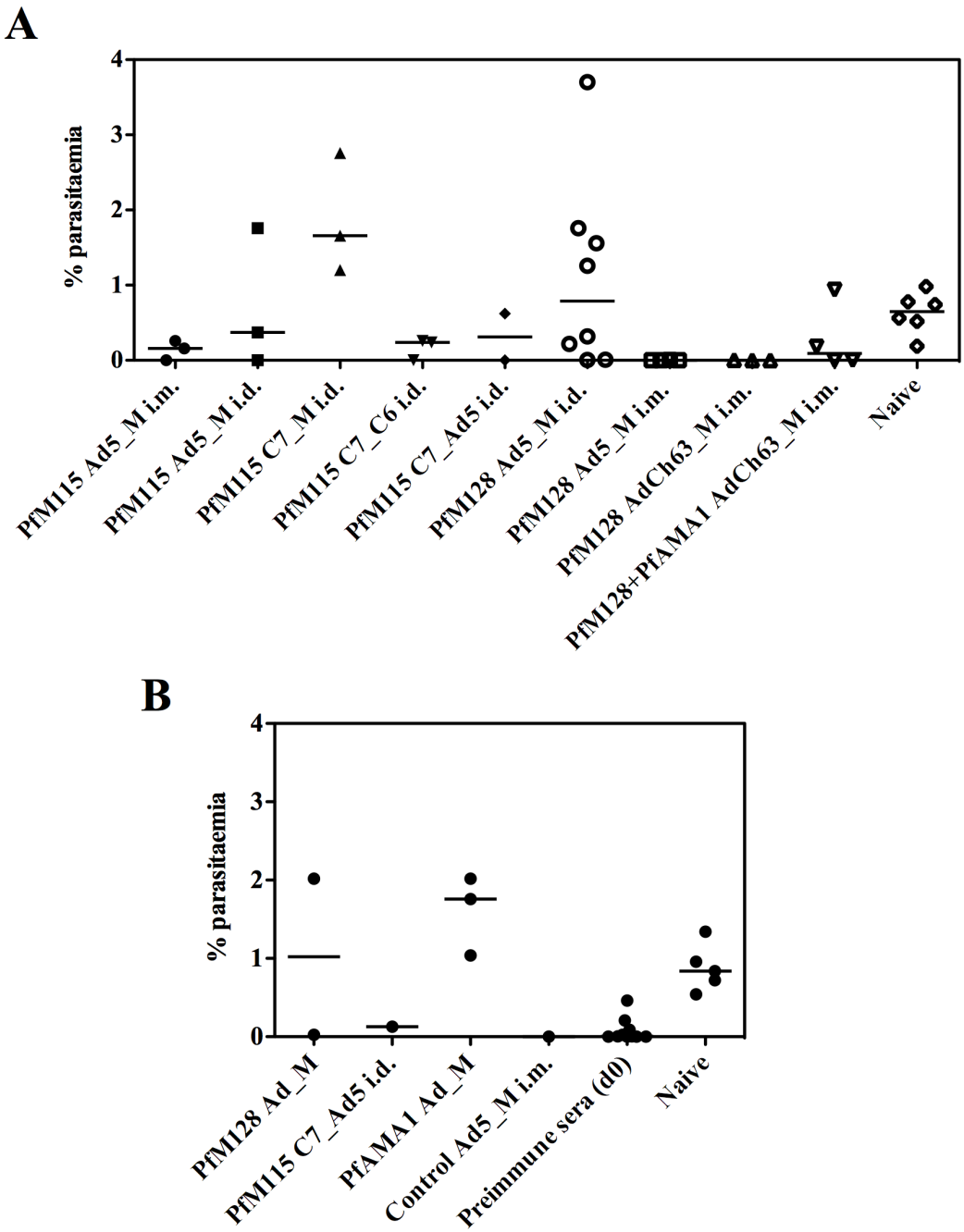

A
% parasitaemia
0
1
2
3
4
PfM115 Ad5_M i.m.
PfM115 Ad5_M i.d.
PfM115 C7_M i.d.
PfM115 C7_C6 i.d.
PfM115 C7_Ad5 i.d.
PfM128 Ad5_M i.d.
PfM128 Ad5_M i.m.
PfM128 AdCh63_M i.m.
PfM128+PfAMA1 AdCh63_M i.m.
Naive
B
% parasitaemia
0
1
2
3
4
PfM128 Ad_M
PfM115 C7_Ad5 i.d.
PfAMA1 Ad_M
Control Ad5_M i.m.
Preimmune sera (d0)
Naive

Figure 5.10: Day 5 Pb-PfM19 parasitaemia after passive transfer of rabbit sera

500μL/day of rabbit sera diluted 1:10 in PBS from a single rabbit was used to passively immunise each BALB/c mouse i.p. on three consecutive days. On the second day mice were challenged i.p. with 5 x 10^3 Pb-PfM19 pRBC and monitored for parasitaemia. In the first experiment (A) mice were all given sera taken on day 70 from PfMSP1 immunised rabbits as indicated, in the second experiment (B) mice were given sera taken on day 70 from rabbits immunised with the PfM128, PfM115, PfAMA1 or control. If not specified, 'Ad' indicates that some rabbits received AdHu5 and other AdCh63 vaccines by either the i.d. or i.m. route. 'Control' indicates that sera was transferred from rabbits immunised with AdHu5_M with no antigenic insert. Pre-immune sera were the day 0 samples from all rabbits that had resulted sterile protection in 'A' and a selection of samples that had resulted in < 0.008 % parasitaemia on day 5 in 'A'. Naïve mice did not receive any sera. Points indicate individual mice and bars indicate median parasitaemia.

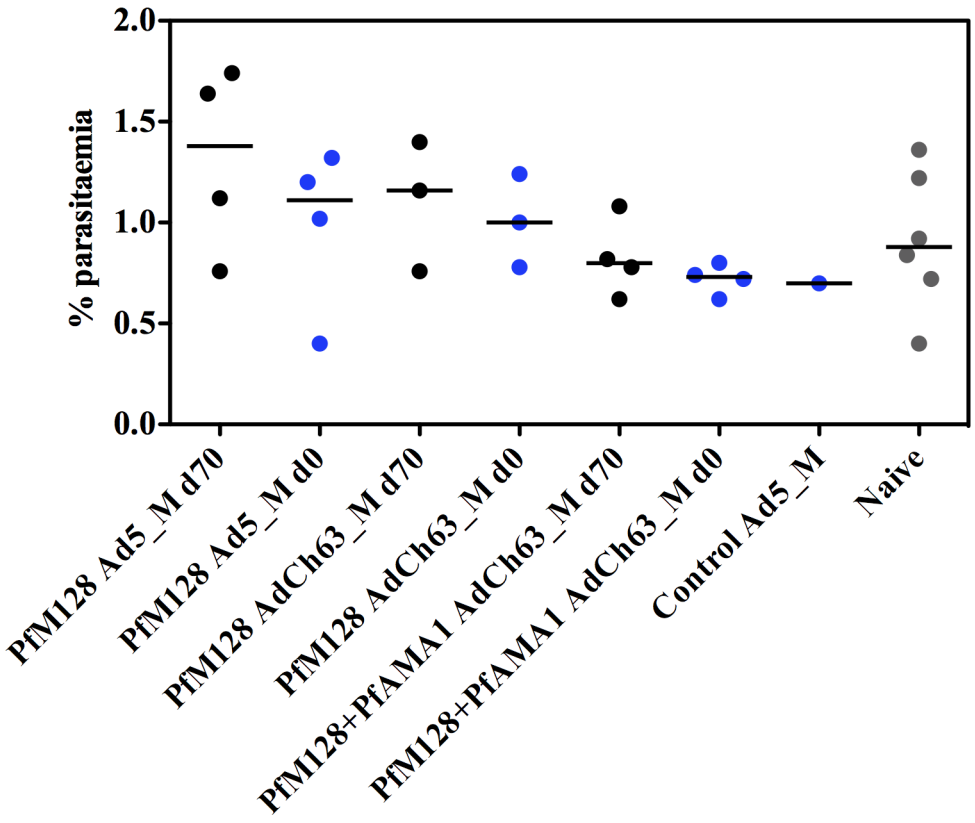


Figure 5.11: Day 5 Pb-PfM19 parasitaemia after passive transfer of purified rabbit polyclonal IgG

IgG was purified from sera of New Zealand white rabbits prior to immunisation (d0) and two weeks post-immunisation (d70) following immunisation with the indicated regimes (Prime_Boost). 0.5mg/day of purified IgG from a single rabbit was used to passively immunise each BALB/c mouse on three consecutive days. On the second day mice were challenged i.v. with 5 x 10^2 Pb-PfM19 pRBC. Naïve mice did not receive any IgG. Parasitaemia on day 5 post-challenge is shown. Points indicate individual mice and bars indicate median parasitaemia.

5.3 Discussion

The data presented here demonstrate that AdHu5 and MVA vaccines given in a prime-boost regime and expressing the blood-stage antigens PbMSP1$_{42}$, PbAMA1 and PbMSP9 cannot protect against wild-type *P. berghei*. Despite evidence that protection against *P. berghei* may be partly Fc-mediated, protection was not achieved in mice lacking the inhibitory FcγR, CD32b. Finally, anti-PfMSP1$_{19}$ IgG from rabbits immunised with viral vectored vaccines did not protect mice from Pb-PfM19 following passive transfer.

5.3.1 Immunogenicity of viral vectored vaccines expressing PbMSP1$_{42}$, PbAMA1 and PbMSP9 .

In the first part of this chapter protection against wild-type *P. berghei* blood-stage infection by immunisation with viral vectors expressing blood-stage antigens was assessed. Three antigens, PbMSP1$_{42}$, PbAMA1 and PbMSP9, were expressed in AdHu5 and MVA viral vectors and tested for both immunogenicity and efficacy. Vaccines targeting all three antigens have previously been reported to protect against *P. berghei* [37-40] and an identical AdHu5_MVA vaccination approach can protect against blood-stage challenge in the *P. yoelii* and *P. chabaudi* rodent malaria models [48, 51].

Following immunisation of BALB/c mice with AdHu5_MVA expressing these antigens, antibodies capable of binding to *P. berghei* ANKA schizonts were observed, indicating that all three vaccination regimes were immunogenic. With the exception of PbMSP1, antibody responses were not tested by ELISA due to lack of recombinant

protein. AdHu5_MVA PbMSP1$_{42}$ immunisation induced PbMSP1$_{19}$-specific IgG that was increased following MVA boosting, as previously observed (Dr. Anna Goodman, DPhil thesis). However, in one experiment mice were primed with a mixture of AdHu5 vaccines and boosted with a mixture of MVA vaccines expressing all three antigens. This regime resulted in a complete abrogation of PbMSP1$_{19}$-specific antibody responses normally observed following AdHu5_MVA-PbMSP1$_{42}$ immunisation. IFATs are currently underway to assess whether immunising against all three antigens simultaneously prevents induction of all *P. berghei*-specific antibodies, or whether antibodies to PbAMA1 or PbMSP9 are still induced (although it will not distinguish between the two).

The absence of a PbMSP1$_{19}$-specific antibody response following immunisation with a mixture of antigens could have three possible causes and these are discussed below. First, PbMSP9 may bind to PbMSP1$_{42}$. During infection MSP9 binds to MSP1$_{19}$ or MSP1$_{42}$ forming a co-ligand complex which binds to erythrocyte band 3 on the erythrocyte surface and facilitates sialic acid-independent erythrocyte invasion [110]. *In vitro* studies have shown that PfMSP1$_{19}$ can bind directly to a PfMSP9 fragment consisting of PfMSP9 αα 77-235 [110]. The *P. berghei* homologue of this fragment is included in the PbMSP9 viral vectored vaccines used here, and it is possible that binding to the MSP1$_{42}$ vaccine antigen is occurring *in vivo* post-immunisation and somehow interfering with the development of an MSP1$_{19}$-specific immune response. Second, PbMSP9 protease activity [38, 114] may be disrupting the antigens. The PbMSP9 vaccine antigen used here contains an αα substitution to remove the active serine protease site and therefore should not theoretically have protease activity, however this has not yet been directly assessed. Third, the lack of an MSP1$_{19}$

antibody response may indicate an immunodominance hierarchy. Studies in which RTS,S was mixed with PfMSP1$_{42}$ and PfAMA1 recombinant protein in rhesus macaques found that there was no interference between RTS,S and MSP1$_{42}$-specific antibody responses, but that MSP1$_{42}$ interfered with development of AMA1-specific antibodies [202]. It is possible that MSP9 is immunodominant in comparison to MSP1.

Although the induction of antigen-specific T cell responses by vaccination was not assessed in these experiments, AdHu5_MVA immunisation regimes expressing a variety of *Plasmodium* antigens are known to induce both CD4$^+$ and CD8$^+$ T cell responses in mice, macaques and humans [158], and it is likely that T cell responses are also induced here.

5.3.2 Efficacy of viral vectored vaccines against wild-type *P. berghei* ANKA and *P. berghei* NK65

Despite induction of PbMSP1$_{19}$-specific antibodies detectable by ELISA and capable of binding to *P. berghei* ANKA schizonts, it had previously been observed that AdHu5_MVA-PbMSP1$_{42}$ does not protect against *P. berghei* ANKA pRBC challenge. Similarly, this study found that AdHu5_MVA expressing PbAMA1 or PbMSP9 could not protect against blood-stage *P. berghei* ANKA.

All the published examples of successful subunit vaccination against *P. berghei* have tested efficacy against the Pb NK65 clone, rather than Pb ANKA [37-39]. It was therefore hypothesised that the immunisation regimes tested here may be capable of inducing protection against Pb NK65. This parasite is less synchronous and free

merozoites are in the bloodstream for longer periods of time as compared to Pb ANKA, which may lead to a greater likelihood of antibodies successfully binding and preventing merozoite invasion of erythrocytes at any one time. However, this did not appear to be the case - protection against this parasite was not observed following AdHu5_MVA immunisation with any of the antigens tested, nor with a mixture of all three antigens (for which the immunogenicity has not been established).

One possible reason for the failure to protect against Pb NK65 could be differences in homology between the vaccine antigens and challenge strain. The PbMSP1$_{42}$ and PbAMA1 sequences expressed in the vaccines were based on *P. berghei* ANKA rather than NK65. The full length sequence of Pb NK65 MSP1 is not available, however an 144 αα fragment has been sequenced and only contains 6 αα changes as compared to Pb ANKA, suggesting that the sequences are not likely to be hugely divergent. Furthermore, a PbMSP1 fusion protein based on blocks 3 and 4 of PbMSP1 ANKA expressed in *Salmonella* can partially protect against Pb NK65 [37]. PbAMA1 NK65 has not been sequenced, therefore sequence diversity for this antigen between the two strains is not known. However, IFATs are currently underway to assess whether the Pb ANKA MSP1$_{42}$ and AMA1 antigens expressed by AdHu5 and MVA vectors are capable of inducing antibodies that can bind to Pb NK65 schizonts.

The PbMSP9 vaccine antigen used here was derived from the *P. berghei* NK65 strain and the *P. berghei* ANKA homologue has not yet been sequenced. However, antibodies induced by AdHu5_MVA-PbMSP9 vaccination were capable of binding *P. berghei* ANKA schizonts in an IFAT, indicating that the sequence must be largely conserved between strains. Nevertheless, despite previously published efficacy with a

recombinant protein vaccine [38], protection against Pb NK65 or Pb ANKA pRBC was not observed here. One possible factor is that the PbMSP9 vaccine antigen contained six αα substitutions to prevent N-glycosylation and to remove an active serine protease site. These changes may have affected the immunogenicity of the antigen, however induction of antibodies that bound to Pb ANKA schizonts indicates that the vaccines are immunogenic and induce antibodies that are capable of recognising native parasite antigen.

It is possible that the magnitude of the antibody responses induced by the vaccines tested here were insufficient to protect. However, previous attempts to further increase Ad_MVA induced IgG responses by a second boost with protein-in-adjuvant did not enhance $PbMSP1_{19}$ or $PfMSP1_{19}$-specific IgG, nor enhance protection (Dr Anna Goodman, DPhil thesis). This is in contrast to studies with other antigens which found that protein boosting could enhance Ad_MVA induced antigen-specific antibody titres [281, 282]. Assessing whether higher titre antibodies can protect in this model by passive immunisation with pooled high titre sera or IgG, or perhaps a high titre mAb, could be another option to test this hypothesis.

Although an identical vaccination approach can protect against blood-stage challenge in the *P. yoelii* and *P. chabaudi* rodent malaria models [48, 51], it remains possible that the lack of protective efficacy against *P. berghei* is related to antigen selection. A PbMSP1 fusion protein based on blocks 3 and 4 of PbMSP1 expressed in *Salmonella* protected 27% of BALB/c mice against *P. berghei* NK65 despite no detectable antibodies [37]. Unlike $PyMSP1_{42}$, it may be that the $PbMSP1_{42}$ antigen alone is insufficient for protection in this model, or that T cell epitopes outside $MSP1_{33}$ are

required for immunity. Studies with the attenuated parasite *P. berghei* ΔPM4 demonstrated that the spleen is essential for antibody-mediated clearance of this parasite [291]. The antigenic targets of protective antibodies against *P. berghei* ΔPM4 were not investigated in that study, however protection is likely to be due to Fc-mediated phagocytosis by splenic macrophages [291]. Therefore inducing antibodies that target antigens involved in merozoite invasion of erythrocytes (such as those used here) are unlikely to be effective in this model.

5.3.3 The role of FcγRIIb (CD32b) in antibody-mediated protection against *P. berghei*

Disappointingly, protection induced by AdHu5_MVA-PbMSP1$_{42}$ against *P. berghei* was not improved by immunisation of CD32b$^{-/-}$ mice, which lack the inhibitory FcγR. Immunisation of CD32b$^{-/-}$ mice might be expected to enhance immune responses to the vaccine due to removal of the regulatory/inhibitory FcγR at the time of immunisation. This has been reported in studies for antibodies [301, 302], tumour-specific CD8^{+} T cells [303] and in the *P. chabaudi* model [304]. Wild-type and CD32b$^{-/-}$ mice were not compared directly in this study. However, in comparison to historical data PbMSP1$_{19}$-specific IgG responses appeared to be lower than in wild-type C57BL/6 mice. In future studies which directly compare wild-type and knock-out mice it will be important to establish the impact of the lack of CD32b on both priming and boosting.

At the time of challenge enhanced efficacy of IgG was expected due to absence of the inhibitory FcγRIIb signalling. This would be more likely to enhance the action of IgG1 (due to the greater inhibition of this isotype and low A:I ratio) but could still

also enhance the action of IgG2a and IgG2b induced by immunisation. However, in the experiment performed here enhanced efficacy against *P. berghei* in CD32b$^{-/-}$ mice was not observed. This is in contrast to studies using non-lethal *P. chabaudi* infection that found reduced parasitaemia in CD32b$^{-/-}$ mice [304].

5.3.4 Why is protection against blood-stage *P. berghei* so difficult?

There could be a number of possible reasons for the difficulty in protecting against *P. berghei* including sequestration of the parasite, immune suppression or avoidance, and antigenic variation. There are discussed in more detail below.

Unlike other rodent malarias, *P.* berghei infected erythrocytes sequester at the schizont-stage in lungs and adipose tissue [305]. The gene SMAC (Schizont Membrane Associated Cytoadherence) has recently been identified as the cause of sequestration in *P. berghei* ANKA, and schizonts lacking this protein (Pb ΔSMAC) are found mainly in the circulation or spleen as opposed to lung or adipose tissue [306]. It is possible that sequestration of *P. berghei* is an immune evasion mechanism. First, sequestering in the lung may prevent clearance by splenic macrophages, which are thought to play a role in clearance of blood-stage *P. berghei* [291]. Second, sequestering may bring infected RBC closer to non-infected RBC, leading to faster re-invasion and less time for antibodies to inhibit merozoite invasion. In a recent study at the Jenner Institute mice were immunised with AdHu5_MVA expressing PbMSP1$_{42}$, PbAMA1, PbMSP9, or all three antigens, and challenged with Pb ΔSMAC pRBC (kindly provided by Dr. Franke-Fayard at Leiden University). However, no protective efficacy was observed (Dr. A. Douglas, unpublished data).

These data do not rule out a role for sequestration in *P. berghei* immune evasion, but demonstrate that sequestration is not the only cause of the failure of Ad_MVA vaccines to protect against blood-stage *P. berghei.*

Another possible cause for the difficulty in protecting against blood-stage *P. berghei* could be immune suppression during infection. Blood-stage *P. berghei* can rapidly suppress MHC class I and class II presentation of both malarial and other antigens by DCs within 4 days of infection [307]. This is thought to be due to rapid activation of DCs, leading to an associated decrease in phagocytosis of antigen by DC. A role for soluble innate signals which reach the DC prior to antigen encounter has been suggested [307].

P. falciparum merozoites are capable of invading erythrocytes by both sialic acid-dependent and -independent pathways [308]. Similar pathways have not been identified in rodent malarias, but the multiplicity of merozoite surface proteins in *P. berghei* suggests alternative pathways of invasion [41], which may limit the efficacy of vaccines targeting invasion.

5.3.5 Blood-stage protection against Pb-PfM19

In the second half of this chapter protection against blood-stage transgenic *P. berghei* expressing *P. falciparum* $MSP1_{19}$ was assessed. This parasite was developed as an *in vivo* model to test the efficacy of vaccines targeting $PfMSP1_{19}$, however, protection against this parasite by active immunisation of mice with a subunit vaccine has so far not been achieved (Prof. Brendan Crabb, personal communication). However, passive transfer of $PfMSP1_{19}$-specific rabbit IgG induced by vaccination has twice been

demonstrated to protect against challenge with blood-stage Pb-PfM19 parasites [56, 294]. These parasites were not identical and were developed independently, which adds weight to the credibility of the data. However, despite the encouraging published data, in the study presented here polyclonal purified IgG, from $PfMSP1_{19}$ immunised rabbits, could not protect against Pb-PfM19. The failure of these experiments could be technical, for example the IgG was not checked post-purification for functional activity by ELISA, IFAT or GIA. It could also be that insufficient IgG was transferred, however previous studies used 0.3-1mg/dose, therefore it would be expected that 0.5mg/dose (i.e. 1.5mg in total over three days) would be sufficient. Nevertheless, it may be worth repeating these studies with a higher dose of IgG. Ideally, future studies to assess efficacy against PbPfM19 challenge should also include a positive control rabbit IgG sample from the authors of the published studies [56, 294]. Variability between animal facilities is a common problem [266] and including a positive control would confirm that protection against this parasite is achievable at the Jenner Institute. Furthermore, the parasite used in these studies was developed by de Koning-Ward *et al*. [57], and does not include the first 4 αα of $PfMSP1_{19}$, but rather expresses the first 4 αα of $PbMSP1_{19}$. Although protection against this parasite by transfer of $PfMSP1_{42}$-specific IgG has been observed [56], the independently developed Pb-PfM19 parasite developed by Cao *et al*. [294], expresses full length $PfMSP1_{19}$ and therefore would be a better parasite to use in these assays in future.

It is possible that the antibodies induced against $PfMSP1_{19}$, by the PfM128 or PfM115 immunogens, do not target the protective epitopes within $PfMSP1_{19}$. It is known that antibodies against $PfMSP1_{19}$ may be inhibitory (inhibit invasion),

blocking (block the inhibitory antibodies) or neutral, and antibodies targeting certain epitopes within PfMSP1$_{19}$ have been associated with protection in the field [138, 140]. Mapping the epitopes induced by PfM128 vaccination of mice, rabbits and humans, and comparing these to those induced by natural exposure or protein immunisation, would be an extremely interesting and important study and might explain the negative result observed here.

Interestingly, variable non-specific protection was observed following transfer of rabbit serum, perhaps due to activation of non-specific innate immunity or an anti-parasitic effect of a component of the sera. One possible component could be complement, which is known to have a non-specific anti-parasitic effect in GIA assays and is the reason that IgG is purified prior to assessment to GIA. The role of complement activity in non-specific protection by rabbit sera against Pb-PfM19 could be tested by heat inactivation of sera prior to transfer. However, an anti-parasite effect of complement cannot be a strong normal protective mechanism *in vivo*, otherwise malaria infection would not occur.

5.3.6 Summary

The data presented in this chapter and discussed above demonstrate that it is substantially harder to protect against blood-stage *P. berghei* as opposed to *P. yoelii* or *P. chabaudi* by either subunit immunisation or by whole parasite approaches. A variety of clinical trials targeting PfAMA1 or PfMSP1 by immunisation have also failed to show any significant effect against blood-stage *P. falciparum* in humans [200]. Given the relative ease with which vaccines targeting MSP1 or AMA1 can protect in the *P. yoelii* or *P. chabaudi* mouse models, it may be that *P. berghei* (against which protection mediated by antibodies targeting these antigens is much

harder to induce), is a better predictor of efficacy of a vaccine strategy against *P. falciparum*. Continued attempts to understand the immune mechanisms responsible for protection, or failure of protection, against blood-stage *P. berghei* could be crucial to the development of an efficacious vaccine against *P. falciparum*.

Chapter 6

Modifying viral vectored vaccines to enhance immunogenicity

6 Modifying viral vectored vaccines to enhance immunogenicity

6.1 Introduction

New generation vaccine delivery platforms, primarily based on recombinant adenovirus-MVA prime-boost immunisation regimes, are capable of inducing T cell responses in non-human primates of a higher magnitude than ever reported before, as well as inducing potent antibody responses [281, 309, 310]. Nevertheless, further enhancing the immunogenicity of antigens expressed by viral vectored vaccines will likely be crucial to the ultimate development of highly effective vaccines against a variety of difficult infectious diseases and cancers.

One potential method of enhancing immunogenicity is to include 'molecular adjuvants' within viral vectored vaccines [311]. These are small molecules, usually members of the innate immune system, which can act to enhance immunogenicity in the absence of additional chemical formulations. These may be encoded as a fusion with the antigen, such as C3d (the final 300 αα degradation product of the complement protein C3b), which can enhance antigen-specific antibody responses when fused to a variety of antigens including influenza HA [312], HIV gp120 [313] and *P. yoelii* $MSP1_{19}$ [314]. They may also be supplied *in trans*, for example, DNA vaccines expressing the innate modulators *tak1* and *tram* when given simultaneously with DNA vaccines expressing a target antigen, can significantly enhance antigen-specific $CD8^+$ T cell responses [315]. In the first half of this chapter two candidate molecular adjuvants are investigated. First, the oligomerisation domain of the α-chain

of complement 4b-binding protein (C4bp), and second, the Fc region of murine IgG2a.

A second method of enhancing immunogenicity may be to alter the promoters used to express the antigens within viral vectored vaccines. Increased antigen expression by use of stronger promoters has been associated with enhanced immunogenicity from both adenoviral [244] and MVA [188, 316] vectored vaccines during primary immune responses, or following successive immunisation with stronger promoters. However, the impact of stronger promoters on secondary responses has not been widely investigated. The second half of this chapter investigates the promoters used to drive antigen expression within MVA vaccines and their impact on immunogenicity following both immunisation with MVA alone and, more importantly, following clinically relevant Ad_MVA prime-boost regimes.

6.1.1 Molecular adjuvants

The oligomerisation domain of murine C4bp α-chain (IMX108) fused to $PyMSP1_{19}$ recombinant protein significantly increased antigen-specific antibody titres and protection against blood-stage *P. yoelii* challenge [219]. Furthermore, IMX108 fused to $PyMSP1_{42}$ and expressed by viral vectors (deployed in an Ad_MVA vaccination regime), clearly enhances antibody responses [48]. Although $CD4^+$ T cell responses were also apparently increased in that study, this finding represented a preliminary observation at the time and required further investigation. Here, T cell immunogenicity was investigated in detail, using AdHu5 and MVA vectors alone, multiple candidate blood-stage malaria antigens, and IMX108 or the optimised version, IMX313. IMX313 has been optimised for minimal sequence homology with

human C4bp α-chain oligomerisation domain, is a candidate sequence for clinical development, and has potent adjuvant activity when fused to $PyMSP1_{19}$ protein [219].

The mechanism of C4bp α-chain oligomerisation domain adjuvanticity remains poorly understood. Ogun *et al.* have investigated adjuvant activity of a variety of different C4bp α-chain oligomerisation domain homologues when fused to $PyMSP1_{19}$ protein, and the majority led to significantly higher antibody titres than a non-related heptameric oligomerisation domain from *P. abyssi* [219]. This suggests that antigen oligomerisation is not the sole mechanism explaining the adjuvant activity of this structural motif. The central core domain of C4bp has been reported to contribute to binding CD40 on B cells [317], as well as to the pentraxins, serum amyloid P component (SAP) [318, 319] and C-reactive protein (CRP) [320]. We therefore hypothesised that adjuvant activity may occur due to binding of the oligomerisation domain of C4bp to these acute-phase proteins. CRP and SAP can function by binding Fcγ receptors (FcγR) on leukoctyes, resulting in enhanced FcγR-mediated phagocytosis and cytokine secretion [321], which may consequently lead to an enhanced adaptive immune response. An alternative hypothesis is that oligomerisation of the vaccine antigen may lead to formation of immune complexes following antibody induction, therefore enhancing activation of FcγR. It was thus investigated whether the observed adjuvant activity is dependent on FcγR.

A second potential molecular adjuvant is the Fc fragment of IgG. This has been shown to enhance immunogenicity when fused to a variety of antigens in protein or DNA vectored vaccines [228-230, 232]. In this study we developed an adenovirus vaccine expressing murine IgG2a Fc fragment fused to the PfAMA1 antigen. The

murine IgG2a Fc fragment is expected to preferentially bind to the activatory receptor, FcγRIV [295, 322, 323], which is highly expressed on the surface of cells of the myeloid lineage, including DCs, macrophages, monocytes and neutrophils, but not B cells, NK cells, T cells or mast cells [322]. By targeting antigen to these cell types we aim to increase uptake and presentation of antigen and therefore to enhance anti-AMA1 immunogenicity.

6.1.2 MVA promoters

The balance between antigen expression and the dose of MVA used is crucial for a number of reasons. Poxviruses are large and contain many viral epitopes against which immune responses may be generated. The immunodominance of the many poxviral epitopes, rather than antigen-specific epitopes, may be one of the reasons that MVA, in comparison to smaller adenoviruses, is a poor priming agent. Increasing the expression of the antigenic insert, by using a stronger promoter, may shift the balance in immunogenicity towards the antigenic insert. It has been suggested that MVA may adjuvant the antigen-specific immune responses induced [324, 325] and it may therefore be tempting to use a high dose of MVA to both increase the amount of antigen expressed and the adjuvanticity of the MVA. However, at very high doses MVA is reactogenic [105] and would also be more expensive to manufacture and deliver. Carefully choosing the promoter used could allow this balance between antigen expression, adjuvant activity and reactogenicity to be optimised.

An additional reason to investigate MVA promoters is in regard to the development of multivalent MVA vaccines, which express different antigenic inserts from different sites within the viral genomic backbone. Expressing different antigens under different

promoters is almost certainly necessary to prevent recombination between the homologous promoter DNA sequences, and therefore suitable promoters need to be identified.

In this study MVA vectors were generated that express the $PyMSP1_{42}$ antigen under the controls of three different poxviral promoters – the classical / widely used P7.5, the short synthetic promoter (SSP) and the modified H5 promoter (mH5). P7.5 is the promoter of a 7.5kDa polypeptide, was first identified in VV (strain WR) [326] and has both early and late promoter activity [327]. It is widely used for expression of foreign inserts from MVA, and all MVA vectors used elsewhere in this thesis are under the control of P7.5. SSP is 40bp long and has both strong early and late activity. It was rationally designed for maximal activity based on studies that mutated P7.5 [185, 245]. mH5 is derived from the promoter for the H5 gene of VV WR strain and contains two nucleotide substitutions to enhance promoter strength and remove a potential translation initiation codon [187]. It is a strong early and moderate late promoter [187]. A previous study which compared all the three promoters for *in vitro* expression of β-galactosidase from a recombinant VV expressing the reporter gene *lacZ*, either with or without cytosine arabinoside (Ara-C) (which prevents DNA replication, and thus late promoter activity), found that SSP had the greatest overall promoter activity, but that mH5 had three to five times greater early promoter activity than the other two promoters [187].

6.2 Results

6.2.1 IMX108 enhances T cell, but not antibody responses, following a single immunisation with AdHu5-PyMSP1$_{42}$

It has previously been demonstrated that following priming with AdHu5 and boosting eight weeks later with MVA, both expressing PyMSP1$_{42}$ fused to IMX108, antigen-specific IgG and CD4$^+$ IFN-γ$^+$ responses are significantly increased compared to the same immunisation regime with vectors expressing antigen without an IMX108 fusion [48]. CD8$^+$ IFN-γ$^+$ responses also tended towards being higher, but did not reach significance, and enhanced protection against blood-stage *P. yoelii* challenge was observed [48]. However, in these experiments immunogenicity after a single AdHu5 vaccination was not assessed. A previous study adjuvanting an AdHu35 vector with aluminium phosphate (AdjuPhos) showed a clear dose sparing effect of the adjuvant [274], and the adjuvanticity of IMX108 after a single immunisation was therefore investigated.

Adenoviral vectors may be titred by two different methods, either by counting viral particles (vp) or by assessing infectious units (ifu). The measure of infectious units includes only viable virus capable of infecting cells and producing antigen, whilst vp also includes non-viable packaged DNA. It has been shown that vaccine dosing based on ifu more accurately reflects immunogenicity (A. Spencer *et al.*, unpublished), although clinically adenoviral vaccines are still administered based on dosing in vp [216]. In order to confirm adjuvanticity of IMX108, immunogenicity following dosing based on both vp and ifu was investigated.

In the first experiment, BALB/c mice were immunised with two different doses (10^{10} vp or 10^8 vp) of AdHu5-PyMSP1$_{42}$±IMX108 (hereafter referred to as Ad42-IMX108 or Ad42-Nil) given i.m.. A further group of mice were immunised i.d. with 10^8 vp, to assess the impact of immunisation route on immunogenicity. Following a single immunisation, the magnitude of antigen-specific $CD8^+$ T cell responses at the lower dose, whether by the i.m. or i.d. route, were significantly increased in terms of production of IFN-γ, TNFα and IL-2 if the vaccine included IMX108. At the higher dose IFN-γ responses were significantly increased by IMX108, and TNFα production also increased in the presence of IMX108, although this was not significant (Figure 6.1). $CD4^+$ responses were not detectable above background (data not shown).

In order to determine whether the adjuvant effect was observed when mice were immunised based on a dose given in ifu, mice were immunised with either 1.5 x 10^9 ifu or 1.5 x 10^7 ifu Ad42-IMX108 or Ad42-Nil. In this case enhanced $CD8^+$ T cell responses were observed for all three cytokines at the higher dose, and for IFN-γ at the lower dose, with the other cytokines also tending towards higher responses in the presence of IMX108 (Figure 6.2A). These results confirm the previous findings based on vp dosing.

It has been suggested that the multi-functional "quality" of T cells in terms of the number of cytokines produced may be important for protection against *Leishmania major* [262] and liver-stage *P. berghei* malaria in mice [261]. Given an adjuvant may potentially change the functionality or phenotype of the T cells induced, cells were also analysed for their ability to co-produce the three cytokines tested. This revealed that the "quality" of the $CD8^+$ T cell response in terms of the proportion of cells

producing all three cytokines (3+, IFN-γ^+TNFα^+IL-2^+), two cytokines (2+, predominantly IFN-γ^+TNFα^+IL-2^-) or a single cytokine (1+, predominantly IFN-γ^+TNFα^-IL-2^-) was unaffected by fusion of IMX108 to the PyMSP1_{42} antigen (Figure 6.2A).

$CD4^+$ T cell responses also tended towards being higher with IMX108 at both doses, with significantly greater production of IFN-γ and TNFα at the high dose, and greater production of TNFα at the lower dose (Figure 6.2B). However, these $CD4^+$ responses were too low for accurate multi-functional analysis. Also, unlike an Ad_MVA prime-boost immunisation regime [48], following a single AdHu-PyMSP1_{42} vaccination total PyMSP1_{19}-specific IgG was not enhanced by IMX108 (Figure 6.3).

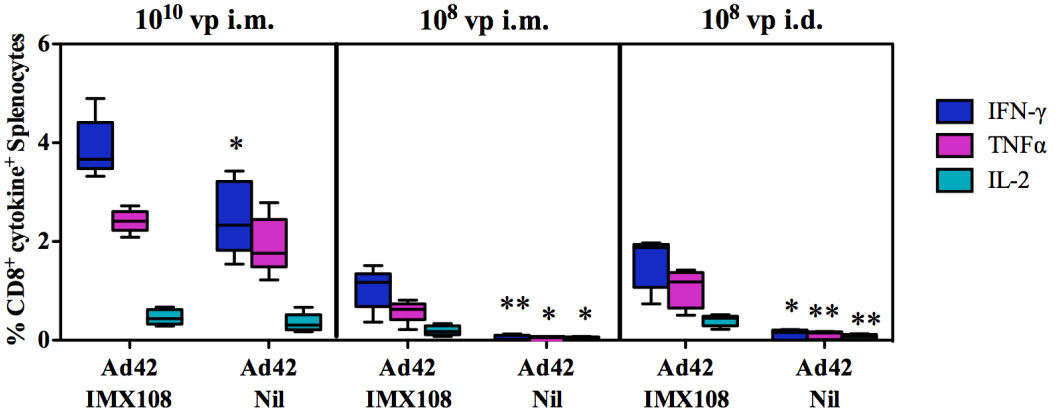


Figure 6.1: CD8$^+$ T cell responses following Ad42-IMX108 and Ad42-Nil immunisation

BALB/c mice were immunised with 1 x 10^{10} vp or 1 x 10^8 vp Ad42-IMX108 or Ad42-Nil by either the i.m. or i.d. route as indicated. Two weeks later PyMSP1$_{33}$-specific production of IFN-γ, TNFα and IL-2 from CD8$^+$ splenic T cells was measured by ICS. Box and whisker plots show median, IQR and range for n = 5 mice/group. ** $P < 0.01$, * $P < 0.05$ by Mann Whitney test comparing Ad42-Nil to Ad42-IMX108 given by the same dose and route.

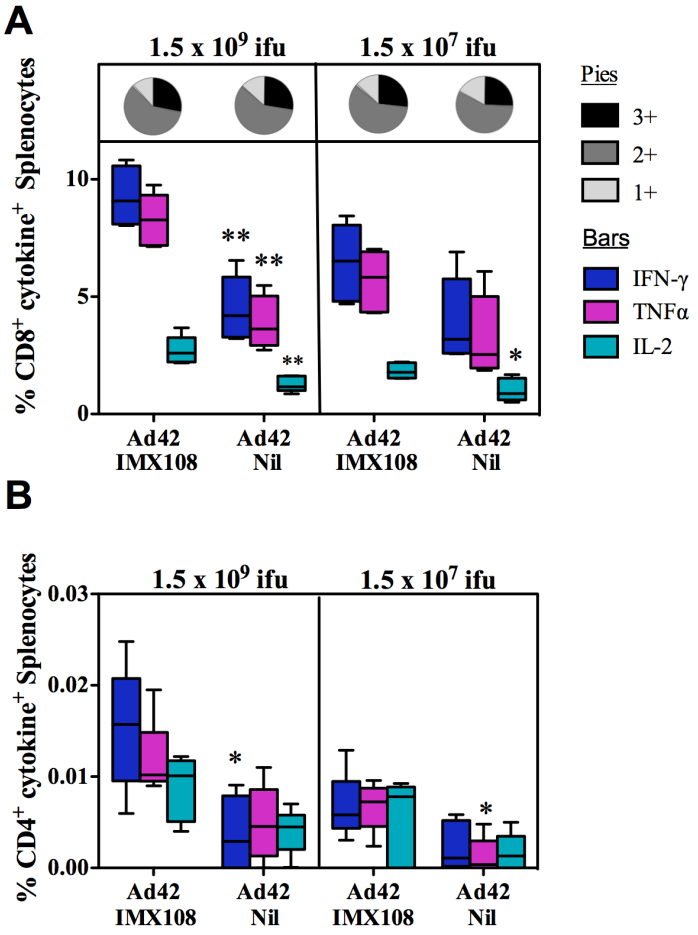


Figure 6.2: Cellular immune responses following Ad42-IMX108 and Ad42-Nil immunisation with dose given in ifu

BALB/c mice were immunised i.m. with 1.5 x 10^9 or 1.5 x 10^7 ifu Ad42-IMX108 or Ad42-Nil. Fourteen days post immunisation splenocytes were re-stimulated with PyMSP1$_{33}$ peptides and assessed for production of IFN-γ, IL-2, and TNFα from both $CD8^+$ (A) and $CD4^+$ (B) T cells by ICS. Box and whisker plots show median, IQR and range. The pie charts show the functional composition of the response and represent the proportion of PyMSP1$_{33}$-specific $CD8^+$ T cells producing 1, 2 or all 3 cytokines measured. n = 5 mice per group. ** $P < 0.01$, * $P < 0.05$ for Ad42-Nil compared to Ad42-IMX108 at the same dose by Mann Whitney test.

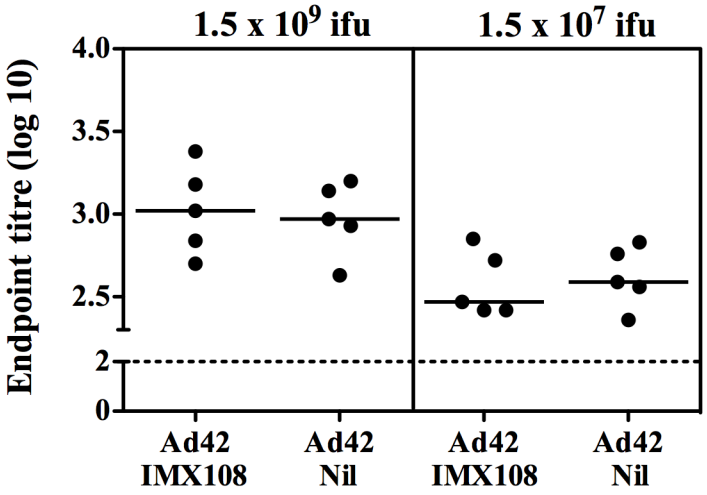


Figure 6.3: Total PyMSP1$_{19}$-specific IgG responses following Ad42-IMX108 and Ad42-Nil immunisation with dose given in ifu

BALB/c mice were immunised i.m. with 1.5 x 10^9 or 1.5 x 10^7 ifu AdHu5-PyMSP1$_{42}$±IMX108 (Ad42-IMX108 or Ad42-Nil). Fourteen days post-immunisation, total PyMSP1$_{19}$-specific IgG responses were measured by ELISA. Data points represent individual mice and bars indicate median titre. n = 5 mice per group.

6.2.2 Immunogenicity of AdHu5 vaccines expressing PfAMA1 and PfM128 with IMX313

A number of viral vectored vaccines encoding *P. falciparum* antigens are currently being tested in Phase I/IIa clinical trials in Oxford, UK. These include both adenoviral and MVA vectors expressing the *P. falciparum* antigens apical membrane antigen 1 (PfAMA1) [328] and an optimised composite version of MSP1 (PfMSP1), termed PfM128 [243]. Given the enhanced T cell immunogenicity observed with AdHu5 and MVA vectors expressing the rodent malaria antigen $PyMSP1_{42}$ with the murine form of the C4bp oligomerisation domain, IMX108, it was investigated whether immunogenicity of these candidate *P. falciparum* antigens could be similarly enhanced. AdHu5 vectors expressing each of these antigens fused to the hybrid chicken oligomerisation domain, IMX313, were kindly generated by Dr. Fergal Hill, Imaxio, France. When mice were immunised with AdHu5-PfAMA1±IMX313 (AdAMA1-IMX313 and AdAMA1-Nil respectively), production of antigen-specific IFN-γ and TNFα from both $CD8^+$ and $CD4^+$ T cells in the blood of mice immunised with AdAMA1-IMX313 was significantly increased (in comparison to AdAMA1-Nil), as measured by ICS three weeks post-immunisation (Figure 6.4A). Antigen-specific IL-2 from $CD4^+$ cells also tended towards being higher, although it was not significant. A similar result was observed when PfAMA1-specific responses were measured in the spleen by IFN-γ ELISPOT in an independent experiment (data not shown). Antibody titres were also assayed by ELISA, and similar to the previous experiments with Ad42-IMX108, total antigen-specific IgG was not increased following immunisation with a single dose of AdAMA1-IMX313 as compared to AdAMA1-Nil (Figure 6.4B). However, unlike PfAMA1, when mice were immunised

with AdHu5-PfM128±IMX313, $PfMSP1_{33}$-specific IFN-γ production was not enhanced by the presence of IMX313 in an ELISPOT assay of splenic T cell responses (Figure 6.5A). Furthermore, total $PfMSP1_{19}$-specific IgG was significantly lower if the vaccine contained IMX313 ($P = 0.0032$) (Figure 6.5B).

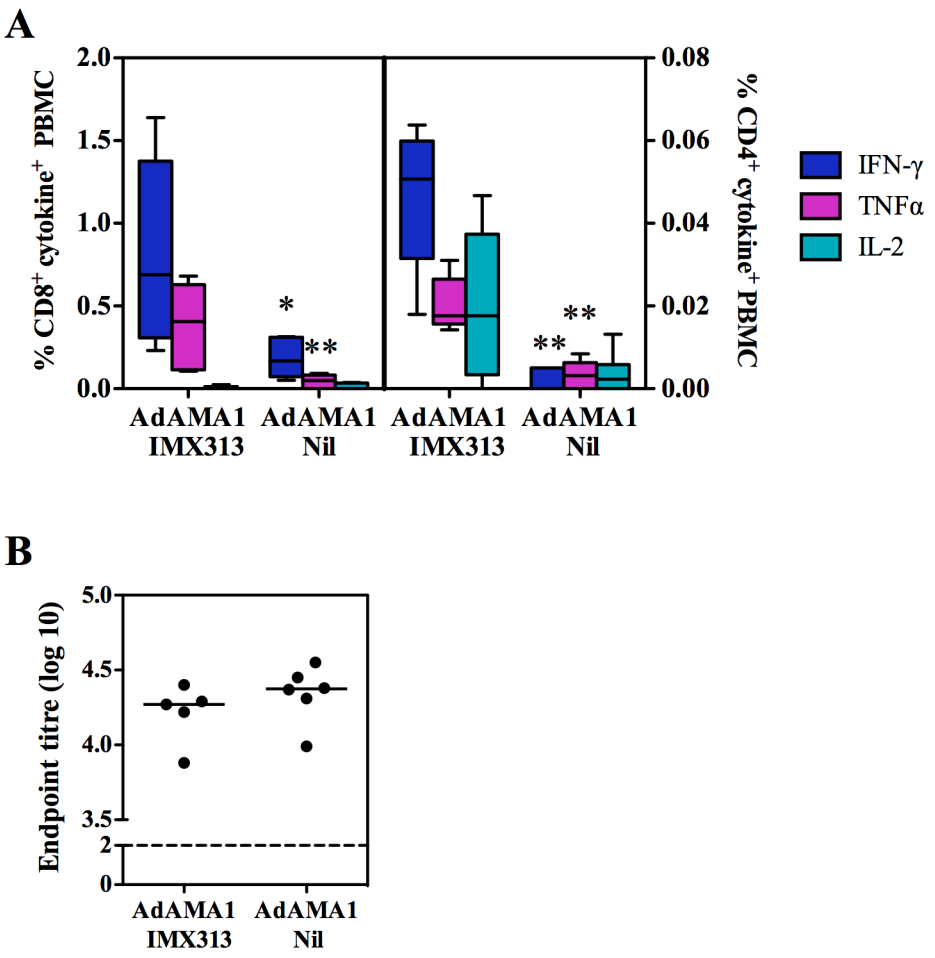


Figure 6.4: Immunogenicity of AdHu5-PfAMA1±IMX313

BALB/c mice were immunised i.m. with 1 x 10^9 ifu AdHu5-PfAMA1±IMX313 (AdAMA1-IMX313 and AdAMA1-Nil). Twenty-one days post-immunisation production of IFN-γ, TNFα and IL-2 from both $CD8^+$ and $CD4^+$ T cells were assessed in the blood by ICS following stimulation with PfAMA1 peptides (A). Box and whisker plots indicate median, IQR and range. Antigen-specific total IgG was assessed by ELISA (B). Points represent individual mice and bars indicate median responses (n = 5-6 mice per group). The dashed line indicates the limit of detection. ** $P < 0.01$, * $P < 0.05$ comparing AdAMA1-Nil to AdAMA1-IMX313 by Mann Whitney test.

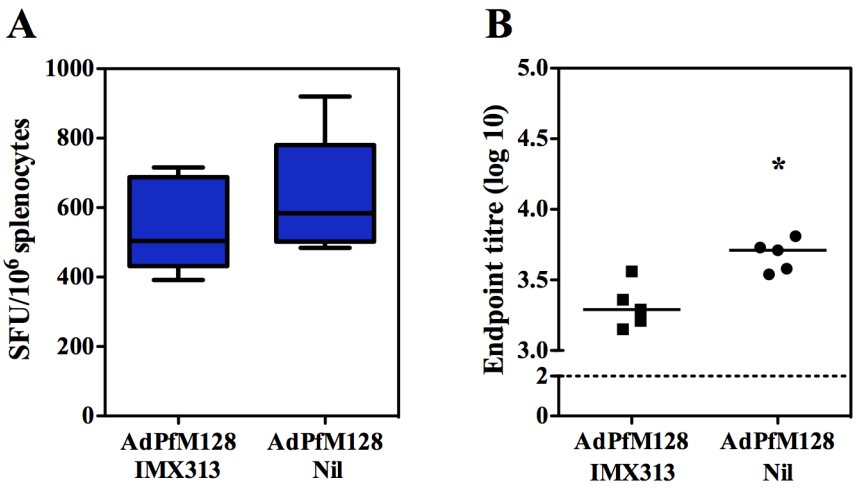


Figure 6.5: Immunogenicity of AdHu5-PfM128±IMX313

BALB/c mice were immunised i.m. with 1 x 10^7 ifu AdHu5-PfM128±IMX313 (AdPfM128-IMX313 and AdPfM128-Nil). Fourteen days later mice were culled and $PfMSP1_{33}$-specific production of IFN-γ from splenocytes was measured by ELISPOT. Data shown are spot forming units (SFU) per 10^6 splenocytes (A). Box and whisker plots indicate median, IQR and range for n = 5 mice per group. $PfMSP1_{19}$-specific total IgG was assessed by ELISA (B). Bars indicate median responses. The dashed line indicates the limit of detection. * $P < 0.05$ comparing AdPfM128-Nil to AdPfM128-IMX313 by Mann Whitney test.

6.2.3 Oligomerisation of the antigen may be necessary but not sufficient for *in vivo* adjuvantation

Given the significant enhancement of T cell responses following AdHu5 vaccination with PyMSP1$_{42}$ and PfAMA1 if the constructs included the oligomerisation domain, but lack of enhanced responses with PfM128-IMX313, oligomerisation of both PfAMA1 and PfM128 by addition of the C4bp domain was assessed *in vitro*. The oligomerisation of the C4bp core domain is dependent on disulphide bonds [221], and therefore the size of expressed protein was assessed in both non-reducing and reducing (to break the disulphide bonds) conditions by western blot (Figure 6.6). For PfAMA1 under reducing conditions monomers were observed. These were slightly smaller than the expected size of 83kDa for the full ectodomain [329], but larger than the band observed with the control 66kDa PfAMA1 ectodomain protein. The PfAMA1-IMX313 construct produced a slightly larger band due to the addition of the IMX313 sequence, which is approximately 5.5kDa. Under non-reducing conditions, larger bands were observed for all PfAMA1 proteins tested, possibly due to dimer formation. In the case of PfAMA1-IMX313, a much larger species was observed at the top of the molecular weight range indicating that multimeric antigen was also present. Although it is impossible to tell whether this species was precisely heptameric, these multimers were lost in reducing conditions, in agreement with data suggesting that oligomerisation of the C4bp core domain is dependent on disulphide bonds [221]. Interestingly, for the PfM128 antigen, a protein of the expected size (128kDa) was observed in both reducing and non-reducing conditions. However, under non-reducing conditions this band was relatively weak and a large amount of protein in excess of 230 kDa was observed, suggesting the presence of multimers.

Under reducing conditions this large protein was no longer observed, and both monomers and dimers were present in greater quantities. These data therefore suggest that multimers are formed if the construct for both AdHu5-PfAMA1 and AdHu5-PfM128 includes IMX313. However, given the differences in adjuvantation seen for these two antigens, this property of the expressed antigen observed *in vitro* does not necessarily predict the ability of the motif to lead to enhanced T cell immunogenicity *in vivo*.

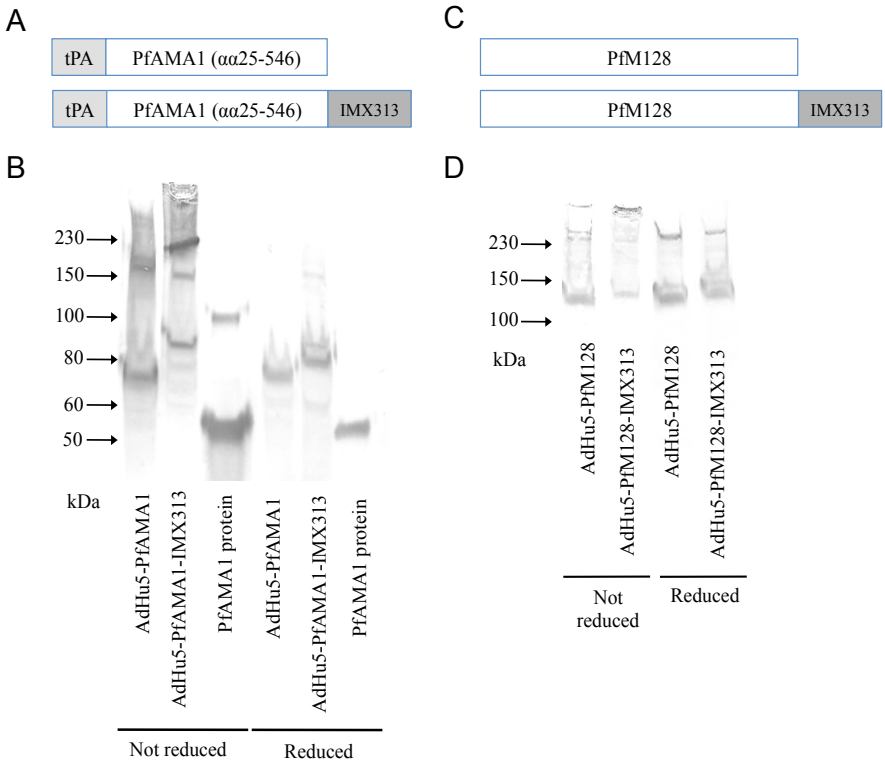


Figure 6.6: Schematic representation of PfAMA1±IMX313 and PfM128±IMX313 and assessment of antigen oligomerisation by western blotting

Schematic representation of PfAMA1±IMX313 (A) and PfM128±IMX313 (B) constructs are shown; tissue plasminogen activator signal peptide (tPA) (30αα), PfAMA1 (521αα), PfM128 (1111αα) and IMX313 (55αα). Western blots were performed by infection of 293 cells with AdHu5 vaccines expressing the antigen of interest at an MOI of five. Lysate was isolated 30h post-infection, washed and separated by SDS-PAGE. Western blots were probed with mouse anti-PfAMA1 polyclonal antibody (B) and mouse anti-PfMSP1$_{19}$ polyclonal antibody (D) under both reducing and non-reducing conditions. PfAMA1 3D7 protein was included as a positive control on blots. Control AdHu5 infected lysate without the antigen of interest was also included and no bands were observed (data not shown).

6.2.4 Adjuvant activity of IMX108/IMX313 in homologous prime-boost regimes

Given the lack of adjuvant effect of IMX108 on IgG responses after a single shot of AdHu5-PyMSP1$_{42}$, but the clear adjuvant effect on IgG observed after an Ad_MVA prime-boost regime (Ad_M42) [48], it was investigated whether a boost, per se, was needed for enhanced IgG, or whether boost vaccination with MVA specifically was required. Mice were immunised with two shots of AdHu5-PyMSP1$_{42}$±IMX108, eight weeks apart (Ad_Ad42). Fourteen days after the second immunisation mice were culled and T cell and antibody responses assayed. Similar to a single immunisation with Ad42, or Ad_M42 vaccination, CD4$^+$ and CD8$^+$ T cell responses tended towards being stronger in the mice receiving antigen fused to IMX108, with IFN-γ from both CD8$^+$ and CD4$^+$ T cells being significantly higher (Figure 6.7). However, unlike prime-boost vaccination with Ad_M42, following Ad_Ad42 immunisation total PyMSP1$_{19}$-specific IgG was not affected by addition of IMX108 (Figure 6.7). Similar experiments were also carried out with AdHu5-PfAMA1. Mice were immunised twice (8 weeks apart) with 1 x10^9 ifu AdHu5-PfAMA1±IMX313 i.m. and T cell responses in the spleen were assayed by ICS two weeks after the boost. Production of IFN-γ and TNFα from CD8$^+$ T cells was significantly higher when IMX313 was fused to PfAMA1, although IL-2 was almost undetectable even in the IMX313 group (Figure 6.8). Production of all three cytokines from CD4$^+$ T cells was significantly higher if antigen was fused to IMX313. Again, however, antigen-specific total IgG was not enhanced by IMX313 inclusion (Figure 6.8).

To investigate the role of MVA in enhanced antibody responses, mice were immunised with two-shots of MVA-PyMSP1$_{42}$±IMX108 eight weeks apart (M_M42) by Dr. Simon Draper at the Jenner Institute. Following the first immunisation T cell and antibody responses were not detectable (data not shown). However, following two immunisations, weak splenic CD8$^+$ IFN-γ$^+$ responses could be detected by ICS and were significantly higher if the vaccine included IMX108 (P = 0.0286) (Figure 6.9). Furthermore, PyMSP1$_{19}$-specific IgG was detectable following vaccination with MVA expressing PyMSP1$_{42}$ fused to IMX108, but not without (Figure 6.9).

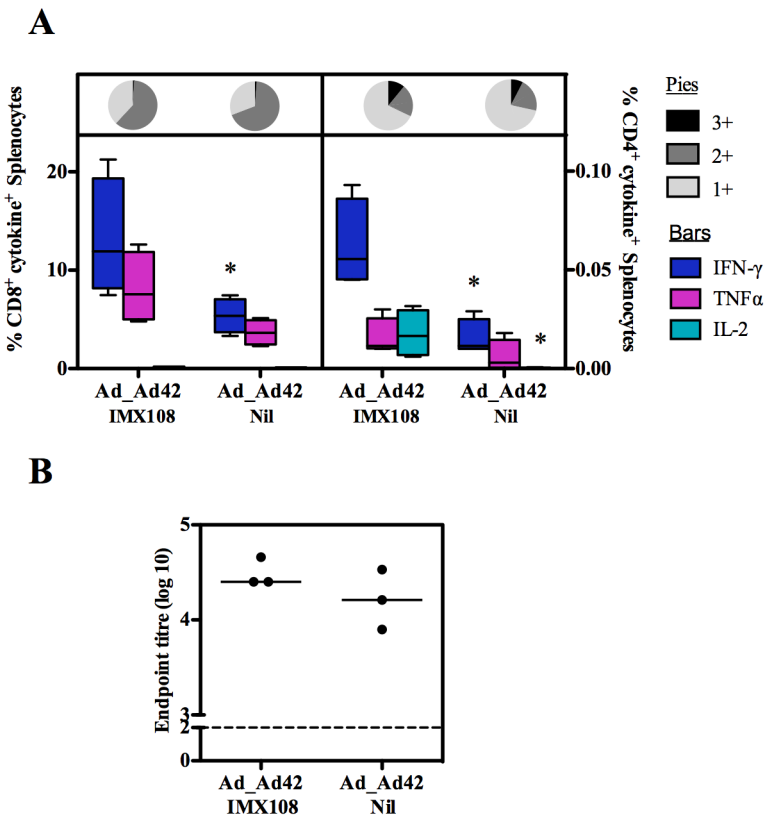


Figure 6.7: Immunogenicity following two immunisations with AdHu5-PyMSP1$_{42}$±IMX108

Antigen-specific production of IFN-γ, TNFα and IL-2 from CD4$^+$ and CD8$^+$ T cells in the spleen of BALB/c mice were measured by ICS fourteen days post-boost following two immunisations i.d. with 5 x 10^{10} vp AdHu5-PyMSP1$_{42}$±IMX108 (Ad_Ad42+IMX108 and Ad_Ad42-Nil respectively) (A). Immunisations were given eight weeks apart. Box and whisker plots show median, IQR and range and pies show the proportion of cells producing 1, 2 or all 3 cytokines. Antigen-specific total IgG responses were also measured in the serum fourteen days after boost by ELISA (B). Points represent individual mice and bars indicate median titre. The dashed line indicates the limit of detection. * $P < 0.05$ compared to the same cytokine and cell type following immunisation with Ad_Ad42-IMX108 by Mann Whitney test.

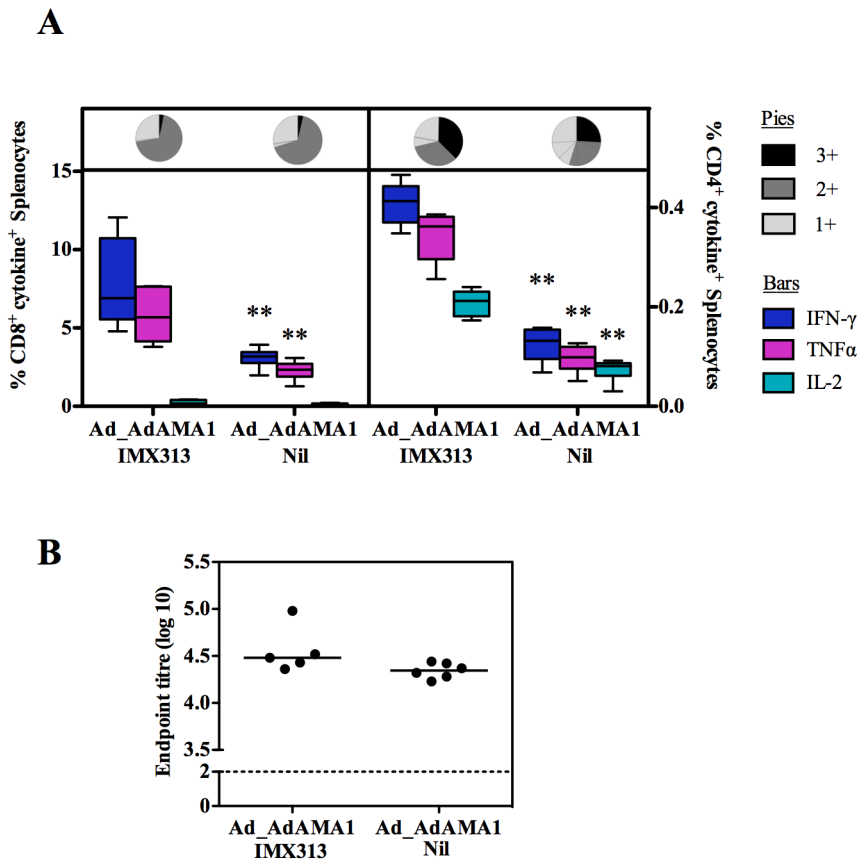


Figure 6.8: Immunogenicity following two immunisations with AdHu5-PfAMA1±IMX313

BALB/c mice were immunised twice with 1 x10^9 ifu AdHu5-PfAMA1±IMX313 i.m. (Ad_AdAMA1+IMX313 and Ad_AdAMA1-Nil) eight weeks apart. Antigen-specific production of IFN-γ, TNFα and IL-2 from $CD4^+$ and $CD8^+$ T cells in the spleen was measured by ICS fourteen days post-boost (A). Box and whisker plots show median, IQR and range and pies show the proportion of cells producing 1, 2 or all 3 cytokines. Antigen-specific total IgG responses were also measured in the serum fourteen days after boost by ELISA (B). Points represent individual mice and bars indicate median titre. The dashed line indicates the limit of detection. ** $P < 0.01$ compared to the same cytokine and cell type following immunisation with Ad_AdPfAMA1-IMX313 by Mann Whitney test.

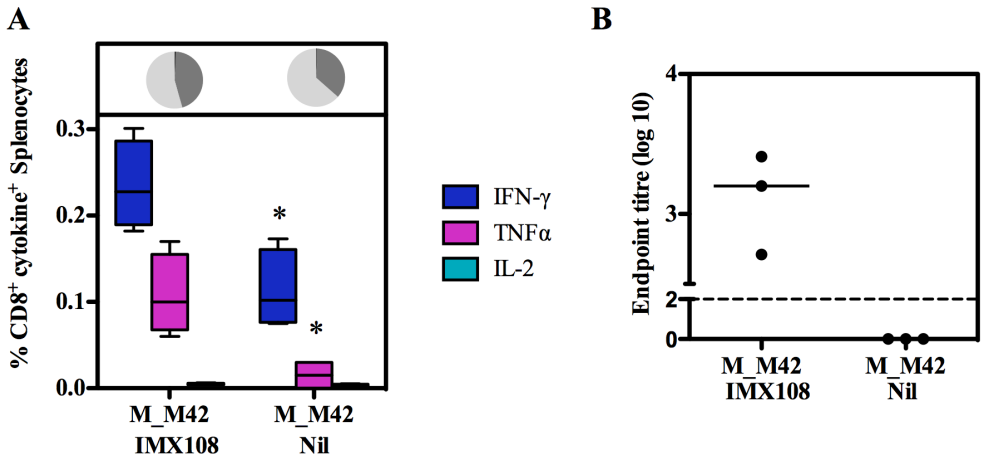


Figure 6.9: Immunogenicity following two immunisations with MVA-PyMSP1$_{42}$±IMX108

Antigen-specific production of IFN-γ, TNFα and IL-2 from CD8$^+$ T cells in the spleen was measured by ICS fourteen days post-boost following two immunisations with 5 x 10^7 pfu MVA-PyMSP1$_{42}$±IMX108 i.d. (M-M42+IMX108 and M-M42-Nil) (A). Immunisations were given eight weeks apart to BALB/c mice. Box and whisker plots show median, IQR and range and pies show the proportion of cells producing 1, 2 or all 3 cytokines. Antigen-specific total IgG responses were also measured in the serum fourteen days after boost by ELISA (B). Points represent individual mice and bars indicate median titre. The dashed line indicates the limit of detection. * $P < 0.05$, for the same cytokine following immunisation with M-M42 with IMX108 as compared to M-M42-Nil by Mann Whitney test.

6.2.5 Ad-AdAMA1-IMX313 immunisation of rabbit does not lead to enhanced antigen-specific IgG induction

$CD4^+$ T cells were adjuvanted in mice with AdAMA1-IMX313. These $CD4^+$ T cell responses may provide T cell help, and although enhanced IgG wasn't seen in mice, the impact of IMX313 on antibodies in a second species was tested. New Zealand white rabbits were vaccinated i.m. with 1 x 10^9 or 5 x 10^7 ifu AdHu5-PfAMA1±IMX313 and boosted with the same vaccine 8 weeks later (Ad-AdAMA1-IMX313 or Ad-AdAMA1-Nil). The vaccines induced PfAMA1-specific IgG, but IMX313 did not significantly enhance antibody responses at any time-point at either the higher or the lower dose (Figure 6.10A). Purified IgG from sera taken on day 84 post priming immunisation was tested for growth inhibitory activity (GIA) at the GIA reference centre, National Institutes of Health, USA. At a lower than standard concentration of total rabbit IgG used in the assay (2.5mg/mL), the majority of rabbits showed moderate to high levels of GIA, with the exception of one rabbit in each of the low dose groups, and one rabbit that had the vaccine without IMX313 at the high dose (Figure 6.10B). Similar to previous studies [330], the GIA showed a sigmoidal relationship with ELISA titre of the purified IgG. Therefore, in agreement with the ELISA data there was no significant difference in the GIA of sera from rabbits that had been immunised with vaccines including IMX313 and those which had not. These data indicate that IMX313 did not alter the titre nor the functional activity of the antibodies induced. Higher levels of GIA were observed when IgG was used at 5mg/mL in the assay, however, again no significant differences between vaccines was observed (data not shown).

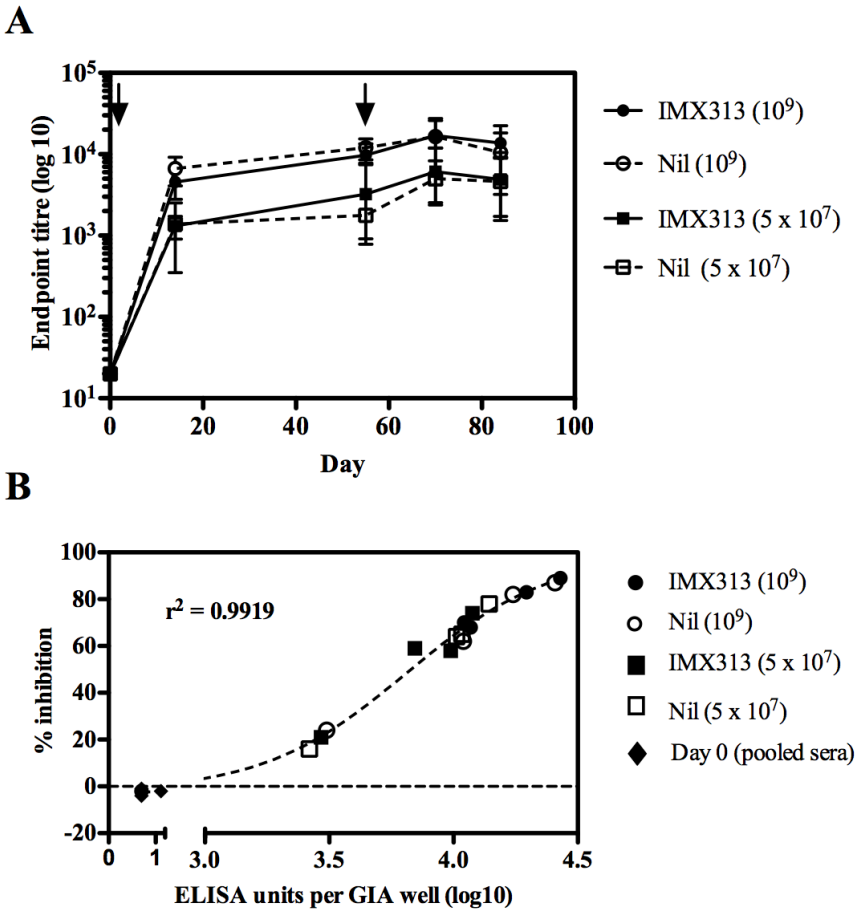


Figure 6.10: Antibody induction and GIA following immunisation of rabbits with AdHu5-PfAMA1±IMX313

New Zealand white rabbits were immunised i.m. with 1 x 10^9 or 5 x 10^7 ifu AdHu5-PfAMA1±IMX313, and 56 days later they were given a homologous boost. Serum was taken on days 0, 14, 55, 70 and 84 and IgG titres were measured by ELISA (A). Median titre and range for n = 4 rabbits/group are shown. The arrows indicate the time of immunisation. Purified IgG from day 84 post-immunisation was assayed for GIA (B). Data presented are percentage inhibition for individual rabbits relative to the parasite growth observed without sera using purified IgG at 2.5mg/mL. The non-linear regression curve is shown.

6.2.6 Adjuvant activity of the C4bp oligomerisation domain is not dependent on the FcR common γ-chain

Given the hypothesis that CRP and SAP binding to the oligomerisation domain and then to FcγR may be the mechanism of adjuvant activity, it was investigated whether an adjuvant effect could still be observed in mice that were deficient in the FcR γ-chain ($FcR\gamma^{-/-}$) which is common to the FcγRI, FcγRIII, FcγRIV and FcεRI [331, 332] in mice. $FcR\gamma^{-/-}$ mice were immunised with AdHu5-$PyMSP1_{42}$±IMX108. Two weeks later the mice were culled and $PyMSP1_{33}$-specific T cell responses in the spleen were assayed by ICS. This showed that enhanced T cell responses due to IMX108 also occurred in the FcR common γ-chain knockout mice (Figure 6.11A), indicating that FcγR that utilise the common γ-chain do not play a role in the T cell adjuvant activity of the C4bp oligomerisation domain. Furthermore, similarly to wild-type mice, antibody responses were not altered by IMX108 in FcγR knockout mice (Figure 6.11B).

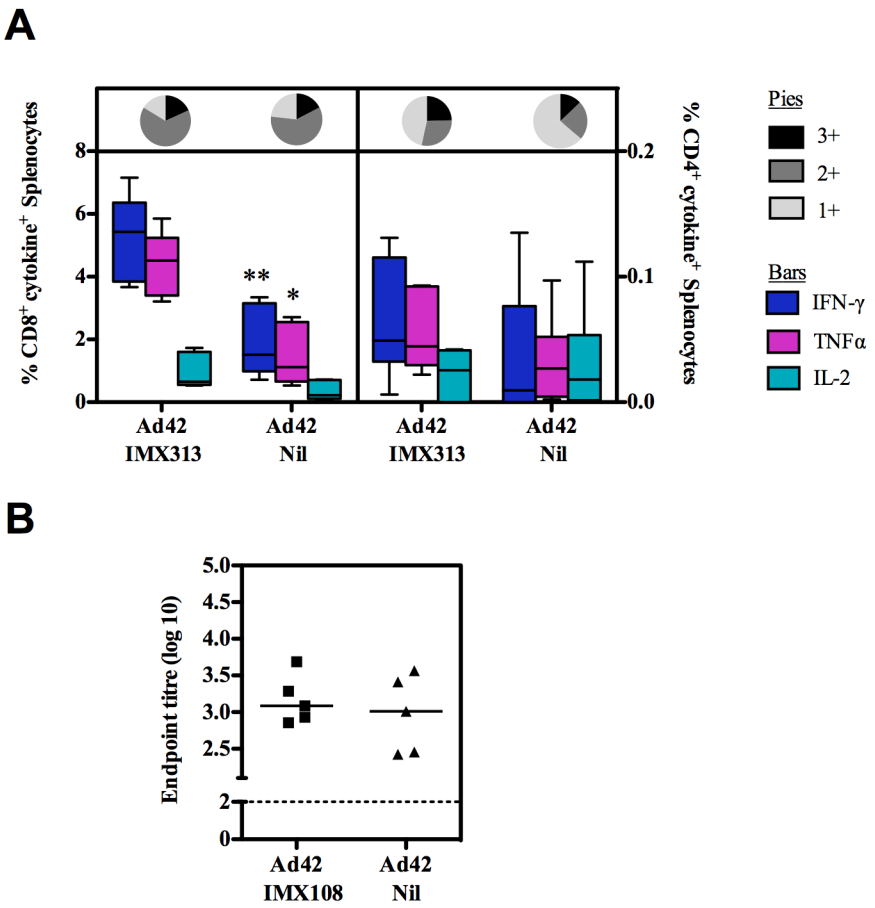


Figure 6.11: Adjuvanticity of IMX108 in FcγR$^{-/-}$ mice

FcRγ$^{-/-}$ mice were immunised i.m. with 1.5 x 10^9 ifu Ad42-IMX108 or Ad42-Nil. Two weeks later PyMSP1$_{33}$-specific production of IFN-γ, TNFα and IL-2 from CD8$^+$ and CD4$^+$ splenic T cells was measured by ICS (A). Box and whisker plots show median, IQR and range. Pies show the proportion of cells producing 1, 2 or all 3 cytokines. PyMSP1$_{19}$-specific total IgG was measured by ELISA (B). Points indicate individual mice and bars indicate median titre for n = 5 mice/group. ** $P < 0.01$, * $P < 0.05$ by Mann Whitney test.

6.2.7 Construction and immunogenicity of AdCh63-PfAMA1±Fc vaccines

A second potential molecular adjuvant – the Fc region of murine IgG2a, was assessed. Adenovirus chimpanzee serotype 63 (AdCh63) vectors expressing *P. falciparum* AMA1 (FVO strain) either with or without murine IgG2a Fc (Fc) fused at the C-terminus of the antigen were generated as described in Section 2.2.8.

BALB/c mice were immunised i.m. with AdCh63-PfAMA1±Fc. Two weeks later, T cell responses in the spleen were assessed by ELISPOT assay using peptides spanning the entire length of the antigen. This revealed that responses were significantly lower if the construct included the Fc fusion (P = 0.0043 by Mann Whitney test) (Figure 6.12A). Stimulation with two peptides known to contain $CD4^+$ epitopes ($PfAMA1_{151-165}$ and $PfAMA1_{471-485}$) and analysis by ICS showed that these responses were low and not significantly different, but tended towards being lower if the construct included the Fc region (Figure 6.12B). An anti-PfAMA1 FVO ELISA also showed that total IgG antibody responses also tended towards being lower if the construct included the Fc region (Figure 6.13).

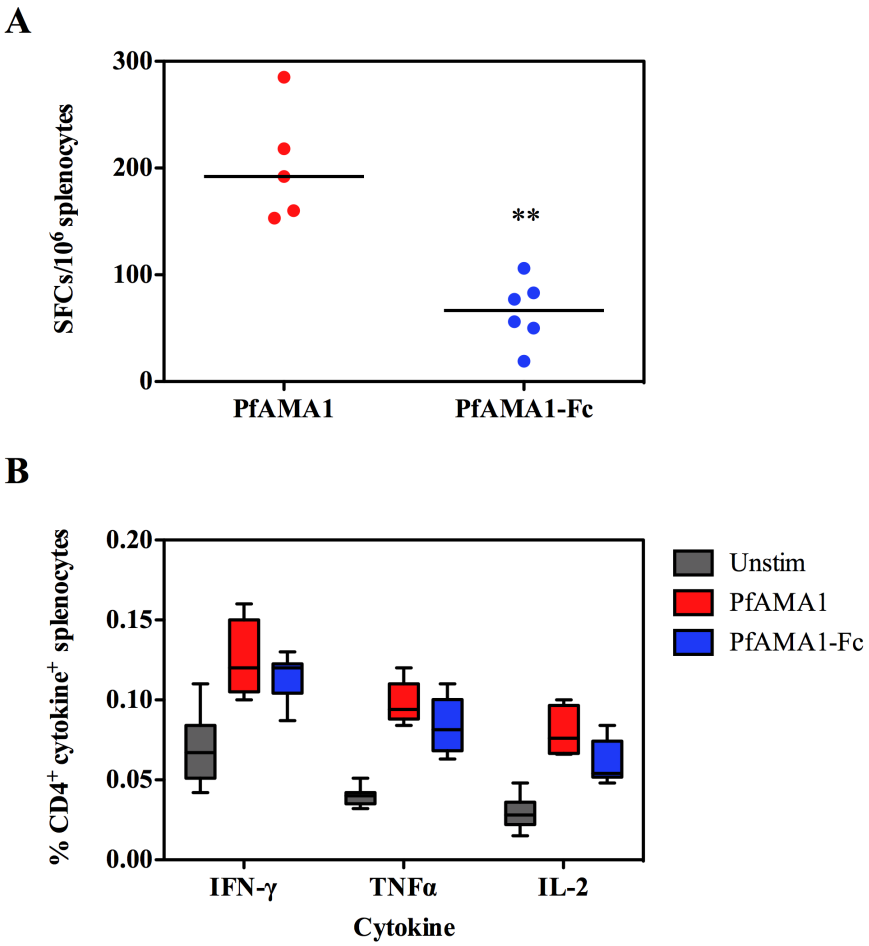


Figure 6.12: Cellular immune responses following AdCh63-PfAMA1±Fc immunisation

5-6 female BALB/c mice were immunised i.m. with 5 x10^7 ifu AdCh63-PfAMA1 or AdCh63-PfAMA1-Fc. Two weeks later the mice were culled and responses assayed in the spleen by ex-vivo IFN-γ ELISPOT using peptides against the entire PfAMA1 FVO antigen (A). Results are presented as number of spot forming cells per million PBMC (SFC/10^6 PBMC). Lines indicate median response, ** P = 0.0043 by Mann Whitney test. Background responses (< 30 SFC/10^6 PBMC) were subtracted. Spleen IFN-γ, TNFα and IL-2 responses against the two known CD4+ epitopes were also assayed by ICS (B). Box and whisker plots represent median, IQR and range. Background responses were not subtracted, but instead are shown (unstim).

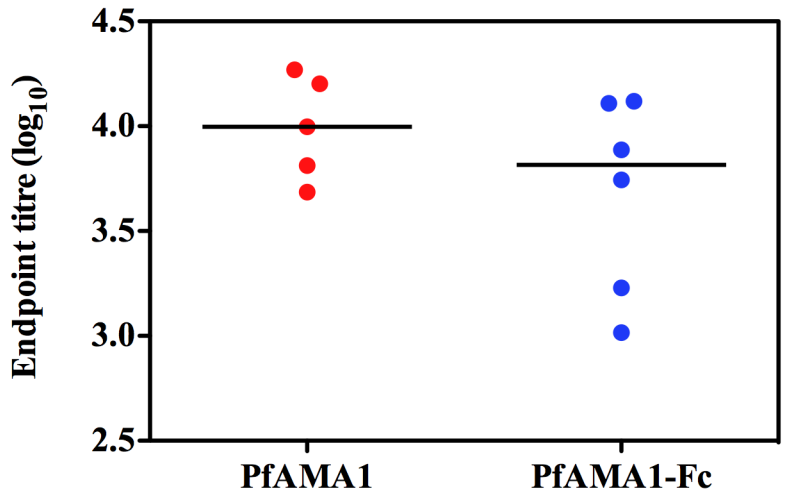


Figure 6.13: Humoral immune responses following AdCh63-PfAMA1±Fc immunisation

Sera were taken by cardiac puncture two weeks after i.m. immunisation of BALB/c mice with 5x 10^7 ifu AdCh63-PfAMA1±Fc. PfAMA1FVO specific total IgG responses were assayed by ELISA at a dilution of 1:100. Lines indicate the median response of log transformed responses.

6.2.8 Immunogenicity of MVA-PyMSP1$_{42}$ with three different promoters

In order to assess the impact of promoter strength on immunogenicity, MVA-PyMSP1$_{42}$ vectors were generated whereby antigen expression was under the control of the P7.5, SSP or mH5 promoters (as described in Section 2.2.8). To assess the immunogenicity of these new MVA vectors, BALB/c mice were immunised with a single dose of 1 x 10^7 pfu each MVA-PyMSP1$_{42}$ vector. A week later mice were culled and T cell responses in the spleen assayed by ICS following stimulation for five hours with a pool of PyMSP1$_{33}$ peptides. These data show that after a single MVA vaccination, CD8$^+$ T cell responses (in terms of production of IFN-γ, TNFα and IL-2) were all significantly higher if the antigen was under the control of mH5 compared to P7.5. Responses also tended towards being higher than P7.5 if the antigen was driven by SSP, however this did not reach significance (Figure 6.14A). CD4$^+$ T cell responses were barely detectable above background, as expected for a single dose of an MVA vaccine. IL-2 production from CD4$^+$ cells was significantly lower if the antigen was under control of mH5 than P7.5, however, given the low overall responses and high background one week after MVA, this result may not be meaningful (Figure 6.14B). Total PyMSP1$_{19}$-specific IgG responses were also significantly higher if antigen was driven by mH5 compared to P7.5. Five out of six mice sero-converted in this time, compared to 0/5 for P7.5. Similar to the CD8$^+$ T cell responses, the SSP promoter induced responses that tended towards being higher than P7.5, but also did not reach significance (Figure 6.14C). Given that responses were measured only one week after primary vaccination, IgM was also measured, however even at a low serum dilution of 1:50, IgM responses were not detectable in the sera (data not shown).

MVA has been identified as a suitable vaccine for boosting both antibody and T cell responses, particularly in Ad_MVA prime-boost regimes. We therefore assessed whether the three promoters would differ in their ability to boost responses primed by AdHu5-PyMSP1$_{42}$. In order to increase the likelihood of seeing differences between the promoters, a sub-maximal dose of both AdHu5 and MVA was used. Mice were primed with 1 x 10^8 vp AdHu5 and boosted with 1 x10^6 pfu MVA. All mice were primed with the same AdHu5-PyMSP1$_{42}$ vaccine and, as expected, pre-boost no significant differences between groups were observed (data not shown). Two weeks post-boost, no difference in either splenic $CD8^+$ or $CD4^+$ T cell responses was observed by ICS, nor were total IgG responses significantly different between groups (Figure 6.15).

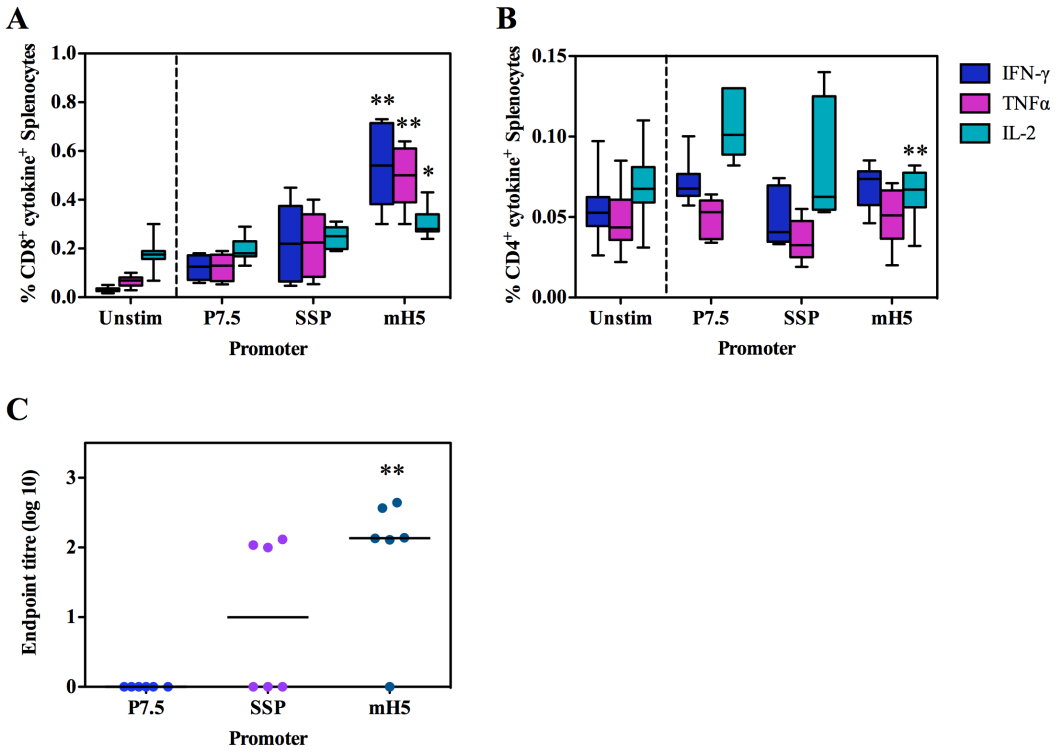


Figure 6.14: Immunogenicity of MVA-PyMSP1$_{42}$ with antigen expression under control of the P7.5, SSP or mH5 promoter

BALB/c mice were immunised i.d. with 1 x 10^7 pfu MVA-PyMSP1$_{42}$ with antigen expression under the control of three different promoters – P7.5, SSP or mH5. Seven days later mice were culled and antigen-specific $CD8^+$ (A) and $CD4^+$ (B) T cell responses in the spleen assayed by ICS. Box and whisker plots represent median, IQR and range for 6 mice/group. PyMSP1$_{19}$-specific total IgG was also measured (C). Points indicate individual mice and bars indicate median titre for n = 6 mice/group. ** $P < 0.01$, * $P < 0.05$ for production of the same cytokine from the same cell type, or production of total IgG, compared to mice immunised with PyMSP1$_{42}$ under the control of the P7.5 promoter by Dunn's multiple comparison test.

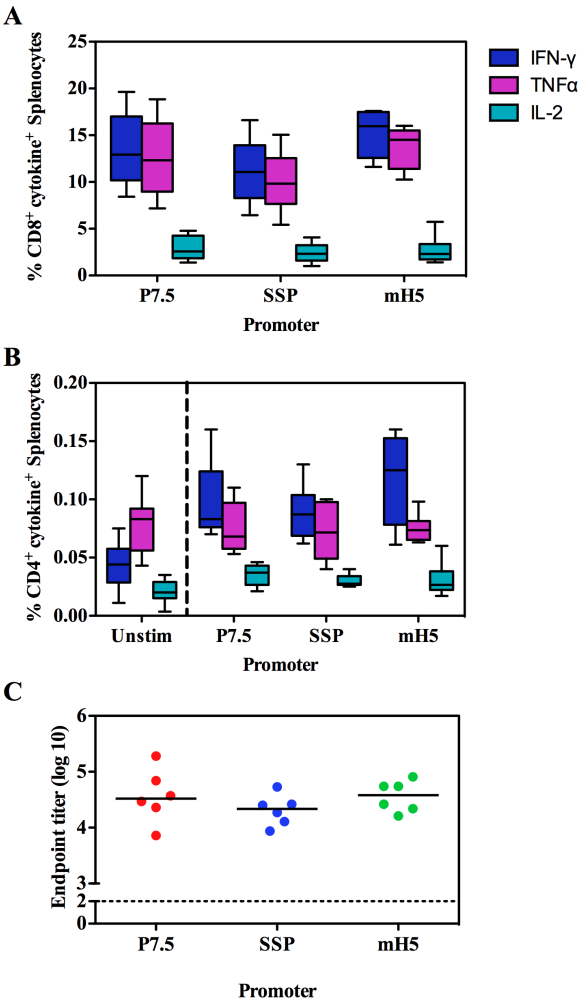


Figure 6.15: Immunogenicity of Ad_MVA PyMSP1$_{42}$ with MVA antigen expression under the control of the P7.5, SSP or mH5 promoter

BALB/c mice were immunised i.d. with 1 x 10^8 vp AdHu5-PyMSP1$_{42}$ and boosted with 1 x 10^6 pfu MVA-PyMSP1$_{42}$ with antigen expression from MVA under the control of three different promoters – P7.5, SSP or mH5. Two weeks post-boost mice were culled and antigen-specific $CD8^+$ (A) and $CD4^+$ (B) T cell responses in the spleen assayed by ICS. Box and whisker plots represent median, IQR and range for 6 mice/group. Total PyMSP1$_{19}$-specific IgG responses were measured in the sera two weeks post-boost (C). Points indicate individual mice and bars indicate median titre for n = 6 mice/group.

6.3 Discussion

6.3.1 C4bp α-chain oligomerisation domain

These data show that the C4bp α-chain oligomerisation domains can effectively adjuvant some, but not all, antigen-specific T cell responses when fused to antigen in an adenoviral vectored vaccine. This adjuvant activity was not exclusively dependent upon oligomerisation of the antigen as no adjuvant effect was observed with the PfM128-IMX313 construct, despite observing oligomerisation of the antigen *in vitro* by western blot. The domain led to enhanced production of antigen-specific IFN-γ, TNFα and IL-2 from both $CD8^+$ and $CD4^+$ cells following vaccination with AdHu5 vectors expressing $PyMSP1_{42}$ or PfAMA1. The adjuvant did not affect the multi-functional quality of the $CD8^+$ T cells induced in terms of the cytokines assessed, but rather increased the magnitude of all three cytokines proportionately. These data are in contrast to some classical chemical adjuvants, which skew responses towards Th1- or Th2-type cytokines [333], but in agreement with other observations using the IMX313 adjuvant fused to the *Mycobacterium tuberculosis* antigen 85A expressed from MVA where a similar T cell adjuvant effect was observed in mice and rhesus macaques [334].

One possible explanation of the failure of IMX313 to adjuvant T cells against the PfM128 antigen, may be that adjuvanticity requires not only oligomerisation of the antigen, but also efficient extracellular secretion of such oligomeric complexes. Studies with AdHu5-PfAMA1 vaccines have shown enhanced immunogenicity when the antigen construct is preceded by the tPA leader sequence, rather than the native AMA1 leader sequence [242] and tPA results in enhanced antigen secretion *in vitro*

as compared to the native leader sequence [242]. These data indicate that efficient secretion of antigen is vital to immunogenicity. Here, western blots were carried out using cell lysate and did not assess whether antigen was secreted. PfM128-IMX313 is 1165 αα in length, whilst PyMSP1$_{42}$–IMX108 is only 447 αα and PfAMA1-IMX313 is 624 αα. The large size of this PfM128-IMX313 in multimeric form may prevent it being efficiently secreted. Given that both cellular and humoral immune responses to this construct were lower than those observed with PfM128-Nil, it is possible that increasing the overall size of this large antigen even further was detrimental, rather than beneficial, to efficient transgene expression. These observations are also in agreement with the data for MVA expressing 85A-IMX313, which is 408 αα in length [334]. However, the finding that adjuvant activity did not occur for PfM128-IMX313, despite intracellular oligomerisation of the antigen, does not exclude the possibility that oligomerisation is a key factor in the adjuvanticity of this structural motif, provided that the protein is sufficiently secreted. The varying adjuvant activity observed with the C4bp α-chain oligomerisation domain from different species when fused to the PyMSP1$_{19}$ protein vaccine, or an unrelated oligomerisation domain [219], could be due to the variable stability of oligomers formed [335].

It was also hypothesised that adjuvant activity may occur due to the oligomerisation domain binding to CRP and SAP, which then bind to FcγR, leading to greater FcγR-mediated phagocytosis and consequently enhanced antigen presentation and cytokine secretion [321]. The data presented here suggest that the FcR common γ-chain is not required for adjuvant activity. However, it remains to be investigated whether antigens fused to IMX108/313 and expressed by the viral vectors bind to CRP and SAP at all, and therefore their possible role in adjuvant activity cannot be excluded.

Further studies will need to be carried out to determine whether binding of IMX313 to these soluble pentraxins occurs, in agreement with published data for the full-length C4bp molecule [318-320], and whether this is important in adjuvant activity.

Particulate antigens, such as oligomerised proteins, are known to be a better target for both cross-presentation [336, 337] and phagocytosis and this may potentially explain the ability of these domains to enhance antigen-specific T cell responses following both MVA and AdHu5 expression. However, greater uptake by phagocytosis might be expected to enhance antibody (as well as T cell) responses, but surprisingly, following a single or double AdHu5 vaccination, no differences in total IgG induction were observed for any antigen when fused to the oligomerisation domain either in BALB/c mice or rabbits. However, enhanced antigen-specific antibody responses were observed in mice following M_M $PyMSP1_{42}$, and have been reported previously following Ad_MVA [48] or protein [219] immunisation. This suggests that MVA or protein is necessary for enhanced antibody responses following immunisation with a vaccine containing IMX108 or IMX313. Whilst protein vaccines administered as heptameric particles may be more suited for antibody induction, differences in cellular tropism and/or innate immune responses to MVA versus AdHu5 may explain the ability of the oligomerised antigen to better stimulate B cell responses in the context of poxviral expression. These differences remain to be further investigated.

Overall, these data demonstrate that C4bp α-chain oligomerisation domains can effectively enhance both $CD8^+$ and $CD4^+$ T cell responses induced by AdHu5 vectored vaccines expressing certain antigens. It remains likely that this adjuvant activity is dependent on not only oligomerisation of the antigen, but also its efficient

secretion. Few adjuvants are known to significantly enhance T cell responses from viral vectored vaccines – a technology that is fast becoming a leading and clinically-relevant subunit vaccine delivery platform [158]. Given that maximal T cell responses are likely required to provide protective efficacy against cancers and difficult infectious diseases, IMX313, which has been optimized for use in humans, remains a strong candidate for inclusion in T cell inducing vaccine antigen constructs.

6.3.2 Murine IgG2a Fc

Disappointingly, these data show that in BALB/c mice the inclusion of the murine IgG2a Fc region into an AdCh63-PfAMA1 vaccine significantly decreased T cell responses against the PfAMA1 antigen and also resulted in antibody responses that were weak, although not significantly lower, than those induced without the Fc region. To confirm this result further studies should be carried out to determine the immunogenicity of this vaccine in other strains of mice.

Murine IgG2a binds preferentially to FcγRIV that is highly expressed on the surface of DCs, macrophages, monocytes and neutrophils [331]. However, it is possible that monomeric IgG2a Fc fusion antigen induced by vaccination is insufficient for inducing a signal from FcγRIV. Activation of FcγR is thought to require cross-linking of receptors by immune complexes (ICs), whilst monomeric mIgG2a is known to continuously bind to FcγRI [295]. In order to test whether multimeric antigen is required to activate a response, constructs that oligomerise at the N-terminus, perhaps using the IMX108/313 domain, could be developed. Natural ICs may also form if the construct was used as a boosting, rather than priming, agent due to aggregation of antigen by pre-existing anti-antigen IgG. As heterologous Ad_Ad regimes are known

to be extremely immunogenic, this could be tested by priming with AdHu5-PfAMA1 and boosting with AdCh63-PfAMA1-Fc.

The previously published studies demonstrating the adjuvanticity of the Fc domain used human IgG1 Fc as the adjuvant [228, 232], rather that murine IgG2a. Human IgG1 is similarly capable of binding to murine FcγRIV [331], however it is possible that the differing binding capacities of these Fc fragments for FcγR is the cause of the failure to observe adjuvanticity here. Further studies could aim to test Fc fragments from other isotypes of IgG, or from different species, provided the Fc fragment is known to bind to murine FcγR, and should test human IgG1 in the first instance.

A possible cause of the reduced T cell responses observed is that the Fc region of IgG can have an anti-inflammatory effect if the Fc fragment is sialylated [338]. A single N-linked glycan is located at Asn^{297} of the Fc domain and its core carbohydrate structure can be variably modified with fucose, galactose and terminal sialic acid moieties. Sialylated forms of antibody have a 5- to 10-fold reduction in binding affinity for their respective FcγR, as compared to asialylated forms [338] and sialylation of IgG has been suggested as a mechanism for ensuring that steady-state IgG antibodies maintain an anti-inflammatory response. One method to overcome any potential effects of sialylation might be to remove the glycosylation site. However, glycosylation is required to maintain the open conformation of the two Fc heavy chains [339] and is therefore essential for Fc binding to all FcγRs [340]. Deglycosylated IgG antibodies cannot mediate *in vivo* inflammatory responses [295]. Therefore whilst removing the N-glycosylation site in the Fc fragment might prevent

sialylation and thus an anti-inflammatory response, it is also likely to result in a failure of the Fc-fusion construct to bind to FcγR.

Murine IgG2a Fc is not currently an attractive adjuvant for enhancing viral vectored vaccine immune responses. However, further studies should be carried to test the ability of this adjuvant to boost responses and Fc fragments from other isotypes of IgG should be tested.

6.3.3 MVA promoters

These data demonstrate that using promoters of different strengths can alter the immunogenicity of single-administration MVA vaccines. mH5 which is known to have the greatest early promoter activity, although not the greatest overall activity [187], was the most immunogenic. This suggests that production of large amounts of antigen early in the viral life-cycle is important for inducing optimal immune responses when MVA is used as a priming agent.

MVA is known to preferentially infect professional antigen presenting cells such as DCs, and *in vitro* only early genes are expressed in infected murine or human DCs, and the life-cycle is arrested before late gene expression [341-343]. It might therefore be expected that antigens expressed early in viral infection would induce potent $CD8^+$ T cell responses by direct presentation on MHC class I molecules. This appears to be supported by the data presented here. However, despite the arrest of the MVA life-cycle prior to late gene expression in DCs, post-prime immune responses to late viral epitopes can be observed [278]. These are likely to be induced by infection of other

somatic cells, in which development is not arrested, and cross-presentation of antigen to DCs [344]. Nevertheless, direct presentation is likely to be the dominant mechanism, resulting in the enhanced immunogenicity observed with mH5. Competition between recombinant antigen and poxviral epitopes for immunodominance may also be a contributing factor – perhaps due to peptide epitopes competing for presentation on MHC class I, or for B and T cells competing spatially for rapid expansion of epitope-specific populations. An antigen which is expressed abundantly immediately upon cell infection would likely be more effective at competing with viral antigens in the context of either of these mechanisms.

Adenoviral vectors are thought to be good priming agents due to prolonged high-level expression of antigen [158, 159]. This is in contrast to classical MVA (with antigen expression driven by P7.5), which is considered a poor priming agent due to the shorter burst of antigen expression [158, 159]. Increased early antigen expression and associated enhanced immunogenicity by the use of stronger early promoters may make MVA a more attractive priming agent and should be further investigated.

When MVA is used as the boosting agent following adenovirus prime, strength of antigen expression did not apparently alter the magnitude of the response induced in terms of either antibodies or T cells. The T cell responses were equivalent to those observed using higher doses of AdHu5 and MVA expressing $PyMSP1_{42}$ (Section 3.2.4.2), indicating that maximal responses may have been induced. It would be thus be interesting to see if differences between groups could be observed by lowering the dose. Unlike T cell responses, antibody responses were not maximal. Higher titres have previously been observed by using higher doses and including IMX108 in the

construct (Section 3.2.3). These data suggest that the amount of antigen produced at the boost is less important than at the prime for determining immunogenicity and are in agreement with the finding that MVA or heterologous adenovirus vaccines can boost adenoviral primed antibody responses equally well [243], despite Ad inducing a greater amount of antigen than MVA [159]. These data are in contrast to studies of secondary immune responses following boosting with MVA with antigen expression driven exclusively by the late promoter, I1L [345]. Mice primed with AdHu5-PyCSP and boosted with MVA-PyCSP had significantly lower antibody and T cell responses if antigen expression from MVA was driven by I1L as opposed to P7.5 (Dr. S. Biswas, DPhil thesis). Similarly, a study in which the H3L gene of MVA (which is normally expressed as a late protein) was knocked out and replaced under the early/late P7.5 promoter, found that during boosting H3L responses were only detectable if this antigen was driven by the P7.5 promoter, but not by its natural promoter [278]. Taken together, these data suggest that early antigen expression is required for efficient boosting, but the resulting secondary immune response is independent of the amount of early antigen expressed.

The mechanism resulting in the failure of late antigens to induce a secondary immune response has not been directly assessed. However, it is unlikely to be due to arrested development of MVA in DC due to the capacity of memory T cells to respond to non-DCs [346]. The lack of a late antigen response is known to be dependent upon the presence of primed T cells [278] and could be partly due to rapid killing of MVA-infected cells by antigen-specific CTL, resulting in reduced late antigen expression [65, 278]. This theory is supported by the data presented in Chapter 4, which suggested that killing of MVA-infected cells by primed T cells may result in reduced

secondary responses. However, the MVA-PyMSP1$_{42}$ and MVA-PyCSP vaccines used in Chapters 3 and 4 both used P7.5 to drive antigen expression and therefore, given the conclusion above that any early antigen expression is sufficient for the development of a robust secondary response, cannot be the complete mechanism.

In this study the strength of antigen expression *in vitro* was not directly assessed, however studies are currently underway at the Jenner Institute to assess these promoters, as well as a variety of endogenous MVA promoters, for antigen expression using both luciferase and GFP, as well as by assessing immunogenicity.

Overall, these data suggest that stronger MVA promoters are not beneficial in enhancing post-MVA boost immunogenicity. Nevertheless, these promoters do induce potent post-boost immune responses and will therefore be useful for expression of antigen in multi-valent MVA vaccines. Furthermore, although MVA is not currently considered a useful priming agent, these data suggest that using stronger early promoters may increase the capacity of MVA to prime immune responses. The efficacy of MVA mH5 as a priming agent in prime-boost regimes should therefore be investigated.

Chapter 7

Concluding Remarks and Future Directions

7 Concluding remarks and future directions

7.1 Summary

Viral vectored vaccines are capable of inducing potent antibody and T cell responses and may be useful tools for the fight against a variety of difficult infectious diseases. This thesis describes investigation of viral vectored vaccines targeting the blood-stage of malaria infection and explores a number of ways in which immunogenicity and efficacy may be enhanced. A combination vaccine strategy targeting both the pre-erythrocytic and blood-stages of malaria infection in the *P. yoelii* mouse model was explored by co-administration of AdHu5 vaccines and boosting by co-administration of MVA vaccines expressing PyCSP and $PyMSP1_{42}$ as a mixture. Vaccine co-administration leads to maintained antibody responses and efficacy against blood-stage infection, but reduced secondary $CD8^+$ T cell responses against both antigens as well as reduced efficacy against liver-stage infection. $CD8^+$ T cell interference can be minimised by co-administering the MVA vaccines at separate sites, resulting in enhanced liver-stage efficacy in mice immunised against both antigens compared to just one. This study also found that despite the ability of viral vectors to protect against *P. yoelii* and *P. chabaudi* blood-stage infection, they were unable to protect against blood-stage *P. berghei*, or transgenic *P. berghei* expressing $PfMSP1_{19}$. Finally, T cell immunogenicity of some, but not all, blood-stage antigens tested, could be enhanced with the C4bp α-chain oligomerisation domain as a molecular adjuvant. However, the murine IgG2a Fc fragment could not enhance the immunogenicity of an AdCh63-PfAMA1 vaccine. Immunogenicity of antigens expressed by MVA vectors could also be enhanced by using stronger promoters to drive antigen expression,

however, in an Ad_MVA prime-boost regime this did not increase post-boost immunogenicity.

7.2 Conclusions

7.2.1 PyCSP vaccines.

AdHu5 and MVA vectors expressing PyCSP given in a prime-boost regime induce potent $CD8^+$ T cell and antibody responses that can mediate protection against *P. yoelii* sporozoite challenge. However, protection was variable and $CD4^+$ T cell responses were also low. The mechanisms of protection induced by the PyCSP vaccines tested here were not directly assessed, but given the reduced protection observed in a combination vaccine strategy when PyCSP-specific $CD8^+$ T cell responses were reduced, it is likely that protection is $CD8^+$ T cell mediated, in line with the majority of the published literature [249, 257].

7.2.2 Immunogenicity of an AdHu5_MVA combination vaccine strategy

Following immunisation with a mixture of AdHu5 vaccines and boosting with a mixture of MVA vaccines, secondary $CD8^+$ T cell responses to both antigens were reduced. $CD8^+$ T cell interference (following MVA co-administration as a mixture) may partly be caused by a lack of physiological space for high magnitude responses against multiple antigens, but is not caused by competition for presentation of antigen on MHC class I molecules, nor is it likely due to restricted T cell access to APCs presenting both antigens. Instead, enhanced killing of peptide-pulsed cells is observed in mice possessing pre-existing T cells against two antigens, in comparison to just one, suggesting priming against multiple antigens may in part reduce the potency of multi-antigen MVA vectors to stimulate secondary $CD8^+$ T cell responses.

7.2.3 Efficacy of a combination vaccine strategy.

A number of interesting and important observations were made regarding efficacy of a multi-stage viral vectored vaccine strategy. First, maintaining high-level $CD8^+$ T cell and antibody responses against both the PyCSP and $PyMSP1_{42}$ antigens, by immunisation at separate sites, was associated with the highest levels of efficacy against a stringent high dose challenge with *P. yoelii* sporozoites. Second, the antigenic target of a pre-erythrocytic vaccine is crucial to the efficacy of that vaccine. In *P. yoelii* a $CD8^+$ T cell response targeting $PyMSP1_{42}$ and PyCSP did not reduce the liver-parasite burden to the same degree as a $CD8^+$ T cell response of comparable magnitude targeting only PyCSP. This suggests that $CD8^+$ T cells targeting PyCSP are more efficient at killing infected hepatocytes than those targeting $PyMSP1_{42}$. This may be because PyCSP is expressed throughout most of the liver-stage, whilst PyMSP1 is only expressed during the late liver-stage [19, 86]. These data suggest that the antigens included in a multi-antigen, multi-stage candidate malaria vaccine must be chosen with care. Finally, in the *P. yoelii* mouse model, peak parasitaemia correlated inversely with $PyMSP1_{19}$-specific IgG titres, regardless of liver burden. This is in contrast to the proposed models of multi-stage vaccine induced immunity (Section 1.4.2.). It remains to be seen whether this is a feature of the *P. yoelii* mouse model, or would also be the case in naturally exposed humans who had previously been immunised with an efficacious multi-stage malaria vaccine.

7.2.4 *P. berghei* blood-stage vaccines.

Protection against blood-stage *P. berghei* has so far not been achieved using viral vectored vaccines targeting the blood-stage antigens $PbMSP1_{42}$, PbAMA1 or PbMSP9, which are well established as protective antigens in other mouse models.

The reasons for this remain unknown, but it is consistent with the paucity of published literature demonstrating protection against blood-stage *P. berghei* [37-39] and the recent publications indicating a failure to protect by sub-unit vaccination [41, 42]. It was hypothesised that differences in the clone used to challenge may be an important factor, but protection could not be achieved against either Pb ANKA or Pb NK65. It was further hypothesised that immune signalling through FcγRIIb may be inhibiting an effective anti-parasite antibody response, however, protection was not achieved in FcγRIIb$^{-/-}$ mice. Finally, Pb ANKA parasites that cannot sequester (SMAC knock-out), were tested at the Jenner Institute, but similarly protection was not observed.

In recent years a number of *P. falciparum* vaccines targeting MSP1 and/or AMA1 have failed to demonstrate significant efficacy against blood-stage *P. falciparum* infection in humans [200], despite the ability of similar vaccines to protect in both the *P. yoelii* and *P. chabaudi* mouse models. These data suggest that *P. berghei* may be a better model for blood-stage *P. falciparum* infection, and that efforts should be made to understand immunity against blood-stage *P. berghei* and to develop vaccines that are efficacious against it. Immunity to blood-stage *P. berghei* should be investigated by a more detailed study of immunity following repeated exposure and drug-cure in the first instance. Adoptive transfer and depletion studies should be used to identify the important antibody isotypes and/or cell types, and antigen screening to identify targets of immunity.

7.2.5 C4bp α-chain oligomerisation domain

IMX108 had previously been shown to adjuvant antibody responses when included in an Ad_MVA regime expressing $PyMSP1_{42}$ [48]. Here it was found that that the C4bp α-chain oligomerisation domains can effectively adjuvant some, but not all, antigen-specific T cell responses when fused to antigen in an adenoviral vectored vaccine. However, after a single immunisation with recombinant adenovirus, antibody responses were not enhanced by IMX108/313. The mechanism of C4bp α-chain oligomerisation chain action remains to be elucidated, but does not appear to be purely dependent on oligomerisation of the antigen. Adjuvanticity may also depend upon efficient secretion of the expressed antigen and may possibly involve interaction with the pentraxins, CRP and SAP, although the data presented here in $Fc\gamma R^{-/-}$ mice suggested that this is not the case. An MVA vector expressing the TB antigen 85A fused to IMX313 and AdCh63 expressing an optimised version of *P. falciparum* CSP (termed CSN) fused to IMX313 are now being developed with the aim of testing IMX313 in the clinic.

7.2.6 Fc fusion constructs

The murine IgG2a Fc domain failed to enhance the immunogenicity of AdCh63-PfAMA1 when fused to the antigen as a molecular adjuvant. In fact, it reduced PfAMA1-specific T cell responses. One possible explanation for these reduced responses is that if sialic acid residues are present on the Fc fragment they can reduce Fc binding to FcγR, thereby preventing efficient induction of an immune response. However removal of the N-glycosylation site to prevent sialylation would also interefere with FcγR binding [338]. Murine IgG2a-Fc was chosen for these studies due to its high affinity for the activatory receptor FcγRIV [295]. However, signalling

from FcγRIV requires cross-linking of FcγRs by immune complexes [331], which are unlikely to be formed following a single immunisation with AdCh63-PfAMA1-Fc. Future studies should test the ability of the adjuvant to boost responses, because immune complexes may be formed by interaction of antigen-Fc with primed antibodies. Previously published studies have used the human IgG1 Fc fragment to enhance immunogenicity, which is also reported to bind to murine FcγRIV [331]. However, it is possible that differences in the affinities of these isotypes for FcγRs accounted for the failure to observe adjuvanticity here. It may be worth exploring adjuvanticity of human IgG1 Fc as an adjuvant of viral vectored vaccines in future. Fc fusion constructs could also be assessed with other antigens or viral vectors as a means of enhancing antigen-specific immunogenicity.

7.2.7 MVA promoters.

Following a single immunisation with MVA, the mH5 promoter induced greater antibody and T cell responses than the standard MVA promoter, P7.5. The use of this promoter may make MVA a more attractive priming agent and its ability to induce immune responses in heterologous prime-boost regimes should be assessed.

In an AdHu5_MVA prime boost regime, the promoters did not significantly alter post-boost immunogenicity. In humans it is known that responses can be increased post-boost by using a higher dose of MVA, however, higher doses are associated with increased reactogenicity [105]. In mice and rabbits heterologous Ad_Ad and Ad_MVA regimes induce remarkably consistent post-boost immunogenicity (Chapter 5 and [104]). However, in humans heterologous Ad_Ad immunisation against Hepatitis C was not as immunogenic as Ad_MVA (Prof. P. Klenerman, unpublished

observations). Therefore, the ability of MVA-mH5, to boost responses in higher species should be assessed. This may increase immunogenicity without the reactogenicity associated with a higher dose.

These promoters tested here, and others found within the MVA backbone, will also be useful in the development of a multivalent MVA expressing a variety of malaria antigens. Given the possibility of recombination, the same promoter cannot be used more than once within the same MVA vector.

7.2.8 Multi-functional quality of cells induced by viral vectored vaccines.

Throughout this thesis, T cell responses were primarily measured by intracellular cytokine staining for IFN-γ, TNFα and IL-2. The "quality" of the multi-functional response in terms of $CD8^+$ T cells expressing all three cytokines, two cytokines or a single cytokine was consistent throughout experiments, regardless of whether mice were immunised with more than one antigen simultaneously or whether the vaccine included a molecular adjuvant. Dose did not alter the functionality either, unless responses were extremely low. The response was consistently a mixture of IFN-γ-producing cells and IFN-γ- and TNFα-producing cells, with a small proportion of cells in the spleen producing all three cytokines simultaneously (and this proportion increased post-MVA boost). In the blood, IL-2 responses were barely detectable at any time-point. These data are in agreement with human clinical trials of AdCh63_MVA-PfM128 in which $CD8^+$ T cells in PBMC produced IFN-γ, TNFα, but very little IL-2 [105]. In mice IL-2 is primarily expressed by 'central memory' $CD8^+$ T cells, which are predominantly found in secondary lymphoid organs. 'Effector memory' $CD8^+$ T cells rarely produce IL-2 [270, 271]. These data suggest that

Ad_MVA vaccination regimes induce primarily effector memory $CD8^+$ T cells that circulate in the blood, which is in agreement with studies using Ad_MVA PbCSP which found that the largest proportion of the cells in the blood were effector memory, as opposed to effector cells or central memory cells [272]. In that study protection was associated with effector memory cells and adoptive transfer of PbCSP-specific effector memory $CD8^+$ T cells ($CD62L^-CD127^+$) could protect mice against *P. berghei* sporozoite challenge, whilst transfer of other $CD8^+$ T cell populations could not [272].

The importance of the multifunctionality of $CD8^+$ T cells in cell-mediated immunity against malaria has not been fully established, although both IFN-γ and TNFα are known to be important components of non-cytolytic pathways that underlie $CD8^+$ T cell mediated immunity [84]. Cells producing IFN-γ have also recently been associated with ME-TRAP-specific liver-stage immunity in mice [261].

7.3 Future directions

7.3.1 Multi-stage vaccine strategies

Immunological interference can be antigen dependent [202]. It will therefore be important to investigate whether the antigenic interference observed here with PyCSP and $PyMSP1_{42}$ is replicated with other antigens, particularly *P. falciparum* candidate vaccine antigens. Ideally the same *P. falciparum* antigens should be tested in both mice and humans to establish the validity of the mouse model for predicting antigenic interference in humans.

This study implied that antigenic interference may be prevented by immunisation at separate sites. However, in the field this strategy is unlikely to be either economically viable or practical to deliver. Establishing whether antigenic interference occurs in humans with viral vectored vaccines is therefore crucial and Phase I/IIa clinical trials are currently underway at the Jenner Institute. These will assess the efficacy of AdCh63 and MVA vectored vaccines expressing *P. falciparum* MSP1, AMA1 and ME-TRAP alone and in combination. Based on the pre-clinical work presented here, vaccines are being co-administered at separate sites in the first instance.

As a cheaper and more practical vaccine strategy, multivalent MVA vectors capable of expressing candidate pre-erythrocytic and blood-stage malaria antigens are being developed at the Jenner Institute. These may use the MVA promoters tested here, which were found to be equally as immunogenic as P7.5 (at boosting responses), as well as other new promoters that have been identified within the MVA backbone (T. Orubu, unpublished data). Establishing whether antigenic interference occurs with these MVA constructs will be vital to whether they are a success, or whether the less practical separate site immunisation strategy is necessary.

Given the smaller insert capacity of adenoviral vectors, development of multi-cistronic adenoviruses will be more difficult, although bicistronic adenoviruses are feasible and several different strategies are being developed. The most widely used approach is multi-antigen fusion proteins, however fusing multiple proteins can compromise their function and immunogenicity. A second approach is to encode internal ribosome entry sites (IRES) sequences between antigens. These are structural elements found in the untranslated region (5' UTR) of RNA viruses which mediate

translation of a downstream open reading frame via a cap independent mechanism [347]. The presence of an IRES sequence at an internal site within mRNA, will initiate translation from that site. However, expression levels of the transgene downstream of the IRES are reported to be consistently lower than that of the upstream transgene [348]. An alternative approach is to encode picornavirus 2A sequences between antigens [349-351]. These cause the ribosome to skip formation of a peptide bond between the 2A and downstream sequence during translation of mRNA, resulting in two proteins from a single reading frame [352]. Unlike IRES, it has been reported that this approach results in approximately equal expression of antigens upstream and downstream of the 2A sequence [352, 353]. AdHu5 vectors encoding multiple *Plasmodium* antigens separated by 2A sequences are now being developed at the Jenner Institute (Dr. A. Douglas, unpublished data). Establishing whether immunogenic interference between antigens occurs with any of these strategies could be vitally important to the development of a multi-antigen Ad_MVA vaccine strategy.

7.3.2 Enhancing blood-stage vaccines

This thesis has primarily focused on attempting to enhance blood-stage vaccines either by combination with a pre-erythrocytic component, or by increasing immunogenicity. However, it may well be that it is the quality rather than the quantity of the response induced by immunisation that is important for some antigens. Field studies have established that it is antibody fine specificity, rather than total antibodies against *P. falciparum* $MSP1_{19}$, that are associated with protection [138-140]. Despite this knowledge, the majority of vaccine trials continue to measure antibodies against total antigen, rather than against specific epitopes. $PfMSP1_{19}$-specific antibodies may be 'blocking' and interfere with beneficial antigen-specific invasion inhibitory

antibodies if they have overlapping specificities. Other PfMSP1$_{19}$ antibodies can be 'neutral' and have no impact on either inhibitory or blocking antibodies [137]. Competition ELISAs with a panel of monoclonal antibodies of known epitope specificity and invasion-inhibitory, blocking or neutral function can be used to identify epitopes that are associated with resistance to malaria [138-140] and these should therefore be carried out in future trials of PfMSP1$_{19}$ vaccines. In particular, the fine specificity of antibodies induced by viral vectored vaccines and protein vaccines as compared to antibodies observed in individuals with naturally acquired immunity should be tested. The importance of antibody fine-specificity against other blood-stage antigens also needs to be determined. This would allow a greater understanding of how blood-stage malaria vaccines might be improved to enhance efficacy in the field. Studies could also compare between antibodies induced in animal models and humans immunised with the same vaccination regime. It has already been shown that the fine specificity of anti-AMA1 and anti-Pfs25 antibodies induced by protein vaccination varies between species [354, 355]. This could have major implications for the relevance of animal models to human immunisation.

A greater understanding of the mechanisms of antibody-mediated protection is also needed to allow development of improved blood-stage vaccines. Antibody functionality in clinical trials is primarily assessed by inhibition of growth *in vitro* (GIA) or for invasion-inhibitory activity (IIA). Another less commonly used *in vitro* assay is the processing-inhibition assay (PIA) which measures the ability of antibody to inhibit proteolytic processing of antigens at the merozoite surface [329]. So far these assays have not been correlated with protection in the field [356-358]. However, in the absence of an efficacious vaccine as a positive control it is difficult to

determine whether this is because these assays are poor predictors of vaccine efficacy, or whether the vaccines tested to date have simply not reached the threshold required in these assays to predict protection.

In vitro assays have also been developed to measure the role of Fc-mediated cellular mechanisms, which are thought to be important in antibody-mediated protection against both human and rodent malarias [145, 290, 292]. The antibody-dependent cellular inhibition assay (ADCI) involves assessment of growth inhibition of parasites following incubation of pRBC with anti-malarial antibodies and monocytes [146] and may be a predictor of field efficacy [145]. However, the assay relies upon fresh monocytes from healthy donors and the results are notoriously variable. Standardising the assay has therefore proved extremely difficult [359]. To overcome this problem the assay has been adapted to use the human monocyte cell line, THP-1 [360]. However, this has not shown reproducible results and the THP-1 cell line lacks human FcγRIII [331, 361]. Recently an antibody-dependent respiratory burst (ADRB) assay has been developed to assess killing by neutrophils [147]. Efforts are now underway to standardise this assay and to establish it's relevance to field efficacy.

Chimeric parasites, such as Pb-PfM19, are being developed to assess the efficacy of *P. falciparum* vaccines pre-clinically, and these parasites, in combination with humanised mice, may also be used to develop an *in vivo* assay of Fc-mediated killing. A PfMSP1$_{19}$-specific human IgG1 mAb is capable of inducing protection against Pb-PfM19 only in human CD64 (FcγRI) transgenic mice [292]. Purified IgG from individuals immunised against PfMSP1 and from naturally-exposed adults from Kilifi, Kenya are currently being tested at the Jenner Institute, to see if vaccine- or

naturally-induced polyclonal antibodies can similarly protect against the Pb-PfM19 parasite in CD64 transgenic mice. This assay could then be extended to other antigens by the development of new chimeric parasite lines, and ideally will be extended to use mice transgenic for other human FcγR. It is hoped that this *in vivo* assay may be more reproducible and may be a better predictor of vaccine efficacy.

7.4 Final remarks

It is widely recognised that vaccines capable of inducing T cell responses will likely be crucial in the development of efficacious vaccines against a wide range of infectious diseases including malaria, TB and HIV. Viral vectored vaccines, in particular Ad_MVA or heterologous Ad_Ad prime-boost regimes, are capable of inducing extremely potent T cell and antibody responses and are currently the leading candidate vectors in this field. The work described in this thesis has contributed to our understanding of the feasibility of developing a multi-antigen malaria vaccine using these clinically relevant Ad and MVA viral vectors, as well as to enhancing the immunogenicity of viral vectored vaccines that are a promising approach to the development of an efficacious vaccine against *P. falciparum*.

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Appendix