

TOWARDS IMPROVED BLOOD-STAGE MALARIA VACCINES: CHARACTERISING THE UNDERLYING IMMUNOGENICITY OF VACCINE ADJUVANTS AND VECTORS

Thesis submitted for the degree of Doctor of Philosophy

Trinity Term 2011

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Word count: ≈49,800 (excl. Bibliography, Appendix, Tables and Diagrams)

Towards Improved Blood-Stage Malaria Vaccines: Characterising the Underlying Immunogenicity of Vaccine Adjuvants and Vectors

Simone de Cassan, Lincoln College, University of Oxford
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Abstract

The vaccine field generally accepts that the induction of high-titre antibodies will be necessary to prevent the invasion of erythrocytes utilising vaccines targeting the blood-stage of malaria. Protein-in-adjuvant formulations are commonly used to achieve such responses. However, their clinical development can be limited by the reactogenicity of some of the most potent pre-clinical adjuvants and the cost and complexity of licensing new adjuvants for human use. Work in this thesis describes the comparative assessment of classical adjuvants such as alum to new pre-clinical adjuvants and adjuvants in clinical development for the induction of high and sustained antibody and T cells responses. These adjuvants induced a broad range of antibody responses when used in a three-shot protein-in-adjuvant regime using the model antigen ovalbumin and leading blood-stage malaria vaccine candidate antigens. Surprisingly, this range of antibody immunogenicity was greatly reduced and antibody responses were of a comparably high magnitude when a protein-in-adjuvant vaccine was used to boost antibody responses primed by an AdHu5 vaccine recombinant for the same antigen. This adenovirus prime, protein boost regime also induced a more cytophilic antibody response and demonstrated improved efficacy of merozoite surface protein-1 protein vaccines against a high-dose *Plasmodium yoelii* blood-stage challenge. Investigating the underlying differences between the vaccine platforms used suggests the improved boosting observed following an adenovirus prime is not due to differences in antigenic dose exposed to the immune system or extended immunisation intervals between adenovirus and protein vaccines, but rather appears to be inherent to the adenoviral vector, which induces more germinal centre cells and T follicular helper cells. Attempts to further enhance the immunogenicity of viral vectors through the direct addition of the adjuvants chloroquine and Adju-Phos® was not successful.

Acknowledgements

Firstly I would like to thank Dr. Simon Draper for his guidance, support and friendship throughout this thesis. Without his encouragement and advice this thesis would not have been possible - I count myself as very lucky to have had him as my supervisor.

I would also like to thank Prof. Adrian Hill for affording me the opportunity of carrying out this project and for his supervision. His scientific input and comments throughout the process and writing of this thesis have been greatly valued.

A special thank you goes to Sumi Biswas, Emily Forbes, Alexandra Spencer and Matthew Dicks for their constant advice and for giving up their time to help me at various stages throughout this project. Your support (both scientific and emotional) has been invaluable.

I would also like to thank all the other members of the Jenner Institute past and present for their help, in particular: Frances Pearson, Karolis Bauza, Anna Checkley, Leanne Marsay, Katharine Collins, Teresa Lambe, Anna Goodman, Caitlin Mullarkey as well as all of 'Team Blood-stage' - especially Sandy Douglas and David Llewellyn. Thank you also to Julie Furze, Andrew Worth, all members of the Vector Core Team, the Adjuvant Bank,

Reg Boone, Gary Strickland, Carol Williams, Viv Clark and the staff at the FgF. Thank you for your technical support and for making the lab such an enjoyable place to work.

I would also like to thank my collaborators Chetan Chitnis and Rushdi Shakri at the ICGEB for their experimental input and for the provision of reagents.

Another special thanks goes to my friends near and far for keeping me sane throughout the duration of this thesis.

And finally I would like to thank my family, without which I would not be where I am today. To Mum, Dad, Philipp and my grandparents: thank you for all your support and unconditional love throughout the years; I could not have done this without you. Thank you again to my brother Philipp whose generous gift afforded me with the technical tool to write up this thesis from home.

This work was funded by the PhD Programme of the European Malaria Vaccine Development Association, a European Commission Framework Programme 6-funded consortium (Grant LSHP-CT-2007-037506).

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Abbreviations

αα	Amino acid
Ab	Antibody
ACK	Ammonium chloride potassium
ADCC	Antibody-dependent cellular cytotoxicity
ADCI	Antibody-dependent cellular inhibition
AdHu5	Human adenovirus serotype 5
ADRB	Antibody-dependent respiratory burst
AGP	Aminoalkyl glucosaminide 4-phosphate
AID	Activation induced cytidine deaminse
AM	Adenoviral prime, MVA boost regime (8 week interval)
AMA-1	Apical Membrane Antigen 1
AP	Adenoviral prime, protein boost regime (8 week interval)
APC	Antigen presenting cells
AS	Adjuvant System
ASC	Antibody secreting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
C4bp	C4 binding protein
CD	Cluster of differentiation
CI	Confidence interval
ChAd63	Simian adenovirus Ch63
CLR	C-type lectin receptor
CMI	Cell-mediated immunity
CQ	Chloroquine
CSP	Circumsporozoite surface protein
CSR	Class-switch recombination
CXCR	Chemokine receptor
DBP	Duffy Binding Antigen
DC	Dendritic cells
DMSO	Dimethyl Sulfoxide
DT	Diphtheria-tetanus
EBA	Erythrocyte binding antigen
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISpot	Enzyme-linked immunosorbent spot assay
ERAP1	Endoplasmic reticulum aminopeptidase-1
FcR	Fc Receptor
FDC	Follicular DC
FP9	Fowlpox strain virus FP9

FSL-1	Synthetic lipoprotein
g	The acceleration due to gravity
GAVI	Global Alliance for Vaccines and Immunisations
GC	Germinal Centre
GFP	Green fluorescent protein
GIA	Growth Inhibitory Activity
GLA	Glucopyranosyl Lipid A
GSK	Glaxo Smith Kline
GST	Glutathione S-transferase
h	Hour
HBsAg	Hepatitis B surface antigen
HEL	Hen egg lysozyme
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
i.d.	Intra-dermal
i.m.	Intra-muscular
i.p.	Intra-peritoneal
i.v.	Intra-venous
IC	Immune complex
ICGEB	International Centre for Genetic Engineering and Biotechnology
ICS	Intracellular cytokine staining
IDRI	Infectious Disease Research Institute
IFA	Immunoflourescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
iu	Infectious units
kDa	Kilodalton
KO	Knock-out
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALP2	Macrophage-activating lipopeptide
mBC	Memory B cell
ME	Multi epitope string
MEM	Minimum essentials medium
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MSP	Merozoite surface protein

MVA	Modified vaccinia virus Ankara
NLR	NOD-like receptors
NYVAC	New York vaccinia virus
O/W	Oil-in-water
OD	Optical density
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
P.f.	*Plasmodium falciparum*
P.v.	*Plasmodium vivax*
P.y.	*Plasmodium yoelii*
Pam3CSK4	Pam3-Cys-Ser-Lys4 - synthetic bacterial lipopeptide
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	0.05% Tween 20 in phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
pfu	Plaque forming units
Poly (I:C)	Polyinosinic–polycytidylic acid
PPP	Three shot protein-in-adjuvant regime
pRBC	Parasitized red blood cell
PRR	Pattern recognition receptors
QKNG	FVO allelic variant of *P. falciparum* MSP-119
R848	Resiquimod
RBC	Red blood cell
RLR	Rig-like receptor
RNA	Ribonucleic acid
RPM	Revolutions per minute
RSV	respiratory syncytial virus
RT	Room temperature
RTS,S	CSP based malaria vaccine co-administered with HB**s**Ag polypeptide from GSK
s.c.	Subcutaneous
SCS	Subcapsular sinus
SE	Stable emulsion
SFC	Spot forming cell
SFU	Spot forming unit
ss	Single-stranded
TAP	Transporter associated with antigen processing
TB	Tuberculosis
TCR	T-cell Receptor

T_{FH}	T-follicular helper cell
Th	T helper cell
TIP	Synthetic polyepitope string
TLR	Toll-like receptor
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
TRAP	Thrombospondin-related adhesion protein
WT	Wild type

Papers Published from these Studies

de Cassan SC, Forbes EK, Douglas AD, Milicic A, Singh B, Gupta P, Chauhan VS, Chitnis CE, Gilbert SC, Hill AV, Draper SJ. (2011). *The requirement for potent adjuvants to enhance the immunogenicity and protective efficacy of protein vaccines can be overcome by prior immunization with a recombinant adenovirus*. Journal of Immunology. 187(5):2602-16.

Chapter 1

Introduction

1 Introduction

1.1 The need for vaccines

The use of vaccines has been instrumental in the prevention and control of many infectious diseases and remains one of the most cost-effective public health tools available to man. Throughout history there are several accounts of "vaccination" being used to control infectious diseases, such as variolation routinely practiced in Turkey during the 18th Century. However Edward Jenner was the first to formally demonstrate that inoculation with cowpox virus could confer protection against smallpox [1]. This led to the birth of routine vaccination and culminated in the successful world-wide eradication of smallpox in 1980 [2]. To date smallpox remains the only human infectious disease to be eradicated through vaccination, but the morbidity and mortality associated with several other infectious diseases such as diphtheria, measles, rubella and *Haemophilus influenzae* type B, is still controlled and reduced world-wide through vaccination [3,4]. Vaccination against polio for example has reduced the incidence of this disease by >99% [5]. However, despite these success stories there is still an urgent need for vaccines against diseases such as malaria, human immunodeficiency virus (HIV) and tuberculosis (TB), which still cause substantial morbidity and mortality around the world. The Global Alliance for Vaccines and Immunisations (GAVI) estimated that every

year 1.7 million children under 5 die of vaccine-preventable diseases underscoring the importance of continued vaccine development and deployment.

1.2 Vaccine Immunology

To generate vaccine-mediated protection the human immune system needs to be activated, with both the innate and adaptive arms of the immune system contributing to the establishment of a robust and effective immune response. Historically vaccines have been deployed empirically with a limited understanding of the immune effector mechanisms that directly contribute to their protective efficacy. Most licensed vaccines to date mediate protection through the induction of high-affinity immunoglobulin (Ig) G serum antibodies. However, for the partially effective Bacillus Calmette-Guérin (BCG) vaccine aimed at TB, T cells producing interferon (IFN)-γ appear to be the main effector mechanism. It is now becoming clear that for difficult disease such as malaria, HIV and TB it is likely that both the humoral and cellular arm of the immune system need to be targeted.

1.2.1 Innate Immune System

The first line of defence against invading pathogens, when barrier immunity is breached, and the first point of contact of vaccines with the human immune system is the innate immune system. The innate immune system provides a rapid, broadly specific response

that can act to clear or control pathogens as well as activate the adaptive immune system. It does not confer long-lasting immunological memory.

1.2.1.1 Complement System

The complement system consists of serum and membrane proteins that interact to trigger various effector functions. Proteolytic reactions generate active forms of latent complement components, which can be triggered by the lectin pathway (carbohydrate recognising soluble factors), the classical pathway (antibodies bound to antigen in immune complexes) or through the alternative pathway (directly at microbial surfaces) [6,7]. The production of C3 convertase is induced by all three complement pathways, and further proteolytic processing events by this protease result in the effector functions of complement. These include triggering an inflammatory response, promoting phagocytosis by opsonisation, formation of a membrane attack complex and the induction of antibody responses.

1.2.1.2 Innate Immune cells

Several leukocytes of the innate immune system patrol the human body. Natural killer (NK) cells, γδ T cells, mast cells, basophils and eosinophils are non-phagocytic cells. Mast cells, eosinophils and basophils protect the epithelial surfaces of the human body. Of these three cells eosinophils and basophils circulate and can be recruited from the blood. Upon pathogen encounter these cells release histamine, which is involved in

defence against parasites and also contributes to allergic and inflammatory responses. NK cells are cytotoxic cells which release perforin and granzyme upon activation through their NK receptors usually upon encounter of cells that are damaged or have down-regulated their major histocompatibility complex (MHC) class I molecule expression due to viral infection [8]. γδ T cells are innate-like cells with some properties of lymphocytes of the adaptive immune system. They undergo gene rearrangement of their T cell receptor (TCR), a feature of αβ T cells, but their receptor diversity is limited. They do not recognise antigens presented on MHC molecules but do release cytokines such as IFN-γ and can mediate cytotoxicity through perforin [9].

Neutrophils, macrophages and dendritic cells (DCs) represent the phagocytic cells of the innate immune system. They recognise and internalise pathogens upon interaction with various cell-surface receptors (DCs can also act as antigen-presenting cells, see section 1.2.1.3). This results in the formation of the phagolysosome, which contains antimicrobial peptides, proteases and reactive oxygen species and results in destruction of the phagocytosed pathogen. In addition to phagocytic properties neutrophils are also capable of degranulating and releasing reactive oxygen species and proteolytic enzymes [10].

1.2.1.3 Dendritic cells

DCs are professional antigen presenting cells (APCs), which not only provide defence against pathogens but also activate the cellular arm of the adaptive immune system. The activation of these cell sub-sets is therefore essential to generate a protective immune response. DCs circulate in peripheral tissues where they capture antigen, process it for display on MHC molecules and migrate to secondary lymphoid organs where they display antigen to T cells [11]. In addition to T cells, DCs have also been shown to present antigen to B cells at the border of high endothelial venules (HEVs) [12]. DCs recognise pathogen-associated molecular patterns (PAMPs) and common structural motifs of pathogens through pattern-recognition receptors (PRRs), which lead to their activation and maturation and also result in the secretion of cytokines, which can further act to modulate the immune response induced. Several PRRs are expressed by DCs such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin-like receptors (CLRs) and RIG-like receptors (RLRs) [13].

1.2.2 Adaptive Immune System

If the innate immune system fails to clear a pathogen then the adaptive arm of the immune system gets activated (through the innate immune system) to create a specific, long-lasting immune response capable of memory. This arm of the immune system is the target of vaccination.

1.2.2.1 T cells

T lymphocytes comprise the cellular arm of the immune system and recognise antigen presented by APCs (DCs and B cells) on MHC molecules in the presence of other co-stimulatory signals. CD4+ T cells recognise exogenous antigen that is taken up by APCs and displayed on MHC class II molecules. CD8+ T cells recognise endogenous antigen (degraded by cells in their cytoplasm) that is displayed on MHC class I molecules. However the phenomenon of cross-presentation, in which exogenous antigen is loaded onto MHC class I molecules through cross-presentation allowing for the activation of CD8+ T cells in response to these antigens (see section 1.2.2.2), has also been discovered.

1.2.2.2 Antigen presentation on MHC molecules

APCs can present antigen displayed on MHC class I or II molecules to T cells through direct- or cross-presentation. Direct presentation of endogenous antigen occurs in a

transporter associated with antigen processing (TAP)-dependent manner (Figure 1.1) [14]. Foreign proteins, such as those present in the cytosol following the direct infection of APCs by viruses, are degraded by the proteasome into peptide fragments. These are then translocated into the ER lumen through TAP and may be trimmed by the ER aminopeptidase (ERAP)-1. The MHC class I α chain is synthesised in the ER and associates with β_2-microglobulin and is stabilised via chaperone proteins (including tapasin and calreticulin) until a stable MHC-peptide complex has formed in the ER. Once this occurs the chaperone proteins dissociate and the complex is released to migrate to the golgi complex and finally the cell surface.

Direct presentation of exogenous antigen on MHC class II molecules occurs in a TAP-independent manner (Figure 1.1) [14]. Peptides are generated from internalised particles in the endosomal-lysomal pathway of APCs. Enzymes such as cathepsins, which function in the acidic environment of the endosomal compartment, degrade internalised particles. MHC class II molecules are synthesised in the ER and are stabilised via the invariant chain. Once released from the ER the invariant chain directs the MHC class II molecule to the endosomal-lysosmal pathway where the non-classical MHC chaperone molecule DM stabilises the MHC class II molecule until a MHC-peptide complex is formed which then proceeds to the cell surface.

The exact mechanism mediating cross-presentation has not yet been elucidated. It has been suggested that cross-presentation occurs in a TAP-dependent manner, with the contents of the endosomal compartment escaping into the cytosol and thus being transported into the ER via TAP for processing on MHC class I molecules, or in a TAP-independent manner wherein peptides are generated in endosomes and are loaded onto MHC class I molecules recycling from the cell surface [15]. One recent model suggests that endocytic compartments fuse with the ER membrane to acquire ER molecules such as ERAP-1 and MHC class I molecules and thereby the ability to present exogenous antigens [16]. Though macrophages can also cross-present antigen, DCs are the principal APC that mediate efficient cross-presentation. Further research will be necessary to fully elucidate the mechanism of cross-presentation.

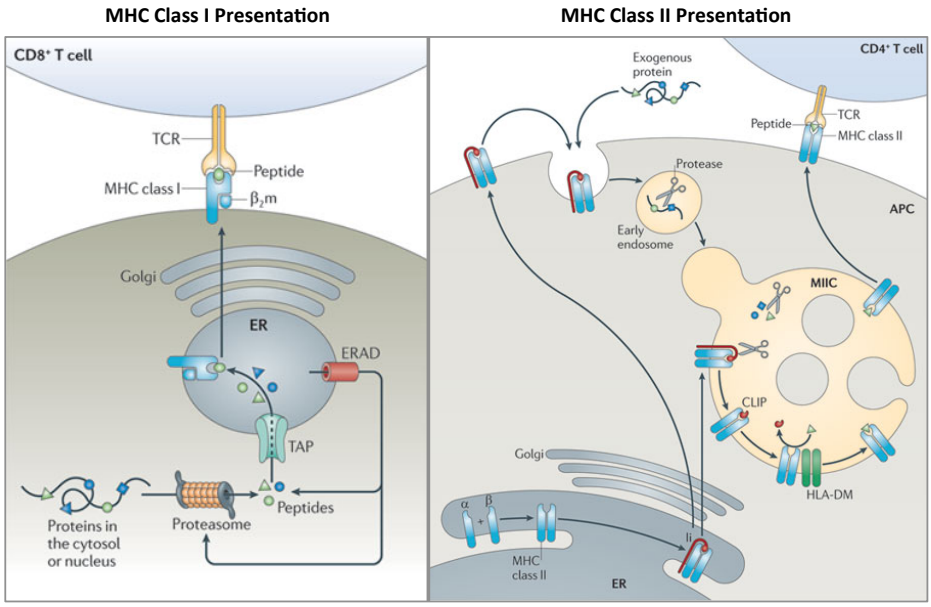

Figure 1.1 Direct Presentation on MHC class I and II molecules

MHC class I presentation: antigens are degraded by the proteasome. The resulting peptides are translocated via transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER) lumen and loaded onto MHC class I molecules. Peptide–MHC class I complexes are released from the ER and transported via the Golgi to the plasma membrane for antigen presentation to $CD8^+$ T cells. MHC class I presentation: MHC class II α- and β-chains assemble in the endoplasmic reticulum (ER) and form a complex with the invariant chain (Ii). The Ii–MHC class II molecule is transported through the Golgi to the MHC class II compartment (MIIC), either directly and/or via the plasma membrane. Endocytosed proteins and Ii are degraded by resident proteases in the MIIC. The class II-associated Ii peptide (CLIP) fragment of Ii remains in the peptide-binding groove of the MHC class II dimer and is exchanged for a peptide through HLA-DM. MHC class II molecules are then transported to the plasma membrane to present antigenic peptides to $CD4^+$ T cells. APC: antigen-presenting cell, β_2mL β_2-microglobulin, ERAD: ER-associated protein degradation, TCR: T cell receptor. Figure taken from and figure legend adapted from [14].

1.2.2.3 CD4+ T cells

CD4+ T cells act through the recruitment and activation of other cell types and the secretion of cytokines. CD4+ T cells can be divided into subsets of Th1, Th2, Th17, T regulatory cells and others, based on their cytokine expression profile and phenotype, although these divisions are not absolute. In the presence of IL-2 and IFN-γ, naïve CD4+ T cells differentiate into Th1 T cells through the activation of transcriptional regulator T-bet [17]. These cells act by secreting cytokines such as IFN-γ and tumour necrosis factor (TNF)-α, recruiting macrophages and NK cells, increasing survival of CD8+ T cells as well as promoting opsonising and complement-fixing antibody production and isotype class switching of B cells. Th2-type cells are activated in the presence of IL-4 through the activation of transcriptional regulator GATA-3 [18]. These cells secrete IL-4, IL-5, IL-9 and IL-13 and act to recruit histamine-releasing cells as well promoting the production of IgE and IgA, which do not primarily activate complement or induce phagocytosis.

Th17 cells are induced in the presence of TGF-β and IL-6 through the transcriptional regular ROR-γt and produce the pro-inflammatory cytokine IL-17A in large quantities [19]. Their effector functions are mostly ascribed to this cytokine which causes the release of cytokines such as IL-6, IL-8, IL-21, IL-22, GM-CSF, G-CSF, CXCL1 and CXCL10 from epithelial and stromal cells which can lead to bone marrow expansion and the recruitment of neutrophils and other mononuclear cells to sites of infection [20]. Th17 cells are mainly known for their role in mediating autoimmune pathology but have been

shown to help clear pathogens, which require robust tissue inflammation, such as bacterial and fungal infections. Mice with disruptions in IL-17 signalling have been shown to display increased susceptibility to extra-cellular bacterial infections such as *Klebsiella pneumoniae*, *Bordetella pertussis* and *Streptococcus pneumoniae* [21]. In this setting IL-17 signaling appears critical for the induction of a chemokine gradient resulting in the recruitment of effector cells to the site of infection. Intra-cellular bacteria, such as *Mycobacteria tuberculosis,* have also been shown to drive the production of IL-17 and mice deficient in IL-17 have been found to have reduced IFN-γ production by CD4+ T cells and impaired granuloma formation after BCG infection [22,23]. Following infection with *Candida albicans* mice deficient in the IL-17 receptor display an increased fungal burden and increased pathology due to the inadequate recruitment of neutrophils and succumb to infection [24].

T regulatory cells (Tregs) are a subset of CD4+ T cells which act to suppress T cell responses and thereby prevent pathology from chronic or excessive immune responses. This is thought to occur through cell contact dependent processes, such as direct cell lysis, or through the production of immunosuppressive cytokines such as IL-10 [25]. Forkhead box protein (Foxp)-3 is the key transcription factor necessary for Treg development [26]. All Tregs express Foxp3 and enforcing the expression of Foxp3 in conventional T cells leads to the induction of Treg function, as well as to the up-regulation of defining surface markers such as CD25, GITR protein and CTLA4 [27].

Follicular helper T cells (T_{FH}) are another form of CD4+ T cells, which reside in B cell follicles in secondary lymphoid organs and express CXCR5. Once activated by DCs these cells recognize peptide-MHC class II complexes on B cells and drive further differentiation of these cells through the engagement of CD40 on B cells and the induction of ICOS promoting the formation of germinal centres leading to the induction of high-affinity, isotype-switched antibody [28].

1.2.2.4 CD8+ T cells

The major role of CD8+ T cells is to directly kill infected target cells. Upon recognition of peptide displayed on MHC class I of an infected cell, CD8+ T cells secrete IFN-γ and TNFα. As discussed in the context of Th1 cells these are pro-inflammatory cytokines and recruit phagocytes to the site of infection. IFN-γ also up-regulates MHC class I expression and induces the immunoproteasome. These cytokines function as good markers for the effector functions of CD8+ T cells. Furthermore these cells induce the death of their target cells through the release of perforin and granzyme as well as through the activation of FasL which can bind to the death receptor Fas on the target cell [29]. Although CD8+ T cells can be activated by DCs, in some cases they require help from CD4+ T cells, which are thought to induce the expression of ligands for 4-1BB and other TNF-family costimulatory molecules on DCs, as well as induce IL-2 to further activate CD8+ T cells.

1.2.2.5 B cells and antibodies

B cells produce antibodies, which constitute the secreted form of the B-cell receptor (BCR) and can provide systemic humoral immunity throughout the body. Three types of B cells exist: B1 B cells, marginal zone (MZ) B cells and follicular B cells. B1 B cells and MZ B cells have 'innate-like' properties, as they do not circulate through lymph nodes. They also make natural antibodies of low-affinity and can be activated without T cell help. Follicular B cells require T cell help (from T_{FH} cells) for activation, which results in the clonal expansion and differentiation of these cells. All B cells make IgM (and IgD) initially but through signals received from T-helper cells and the surrounding cytokine milieu they can class-switch to produce other antibody isotypes including IgG, IgA, and IgE. B cells also undergo somatic hypermutation to produce high-affinity antibodies in the germinal centre reaction.

The general structure of immunoglobulin can be seen in Figure 1.2. Though the variable regions of antibodies determine antigen-specificity and mediate the binding of antibodies to their targets, the heavy-chain constant regions determine antibody effector functions. Antibodies have a variety of effector functions (see Table 1.1). A major role of antibodies is to mediate neutralisation and antibodies can also trigger the complement cascade through IgM and IgG binding to C1q. Antibodies can also mediate effector functions through Fc receptors (FcRs), which are expressed on a variety of cells and bind to the Fc region of antibodies [30]. This can cause the initiation of phagocytosis

in neutrophils and macrophages but can also lead to the induction of antibody-dependent cellular cytotoxicity (ADCC) through NK cells, antibody-dependent cellular inhibition (ADCI) via monocytes or antibody-dependent respiratory burst (ADRB) from neutrophils. Four different isotypes of IgG antibodies have been discovered. The size of the hinge region as well as the number of inter-chain disulphide bonds between the two heavy chains constitute the structural characteristics that differ between different IgG isotypes and determine differences in their functional activity. IgG isotypes fall into two functional groups. In humans IgG2 and IgG4 and IgG1 in mice do not activate complement well nor bind with high efficiency to Fc receptors mainly mediating neutralisation. IgG1 and IgG3 in humans and IgG2a, IgG2b and IgG3 in mice activate complement and bind to activating Fc receptors and thus mediate phagocytosis through opsonisation and promote inflammatory responses.

Both class-switching and somatic hypermutation are mediated by the enzyme activation induced cytidine deaminase (AID). This enzyme deaminates cytidine residues in the variable region of antibodies resulting in the introduction of point mutations for somatic hypermutation. For CSR AID deaminates cytidine residues in repetitive switch (S) regions found upstream of each heavy chain constant region gene (except for Cδ) leading to ds-DNA breakages and end-joining recombination through an intrachromosomal deletion event (reviewed in [31]). This process moves the variable region exon next to the heavy chain constant domain exon (Cμ-Cδ) of an antibody through end-joining recombination

between S regions resulting in a class-switched antibody. The precise mechanisms of AID targeting have not yet been elucidated but different cytokines have been shown to be involved in determining antibody subclass [32].

Contact with antigen in secondary lymphoid organs initiates T-dependent antibody responses causing B cells to migrate to the edge of the T cell zone, which is regulated by the expression of CCR7. Antigen bound to the BCR is taken up and processed and presented to T cells on MHC class II molecules [33]. T cells recognising this peptide-MHC complex secrete cytokines such as IL-6 and IL-21 and synthesise CD40L, which interacts with CD40 on the B cell to initiate the germinal centre reaction and a rapid burst of antibody. Activated B cells either produce short-lived plasma cells (SLPCs) or can enter the germinal centre reaction to produce long-lived plasma cells (LLPCs) and memory B cells (mBCs), which can differentiate back into plasma cells / antibody secreting cells (PCs/ASCs) upon antigen re-encounter as a key component of humoral immunological memory that provides long-term immunity.

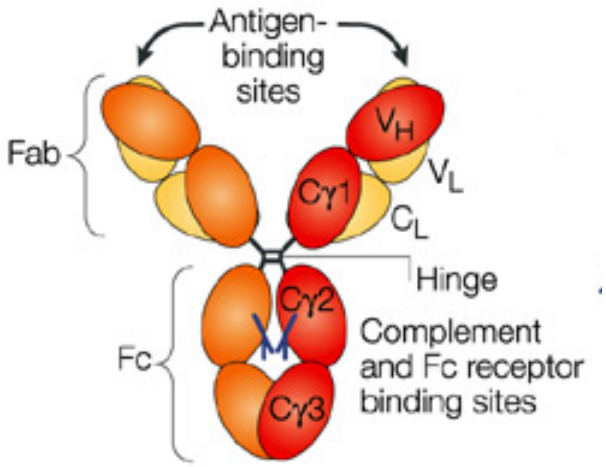

Figure 1.2 Immunoglobulin structure

Immunoglobulins are composed of two identical heavy chains and two identical light chains, linked by disulphide bridges. The class of an antibody is determined by its heavy chain (μ, γ, α, ε or δ), with all classes sharing the same light chains (λ or κ). Light chains fold into a variable domain (V_L) and a constant domain (C_L), whereas heavy chains are composed of one variable domain (V_H) and either three (in IgD, IgG and IgA) or four (in IgM and IgE) constant domains. The antigen-binding sites at the tip of the Fab regions are formed from the variable domains of both the heavy and light chains. The Fc region mediates interaction with effector molecules, such as complement and Fc receptors. Usually the Fab and Fc regions are separated by a flexible hinge region, which is not present in IgM or IgE. The heavy chain is shown in red, the other in orange, with light chains in yellow. The above structure represents human IgG. Figure taken from and legend adapted from [34].

Antibody Class	Major Functional Properties
IgM	Neutralisation, opsonisation, complement activation, antigen receptor of naïve and some memory B cells
IgG	IgG1: neutralisation, opsonisation IgG2a, IgG2b &IgG3: complement activation, induction of phagocytosis, ADCC, ADCI
IgA	Neutralisation, protection of mucosa, induction of phagocytosis, maternal antibodies
IgE	Activation of mast cells and basophils
IgD	Antigen receptor on naïve follicular B cells

Table 1.1 Effector functions of antibodies.

IgG isotypes are shown for murine immunoglobulins.

1.3 Subunit Vaccine Delivery Platforms

1.3.1 Protein-in-Adjuvant Vaccines

Several strategies have been employed towards the development of novel and effective vaccines. Traditionally live, attenuated or killed whole organism vaccines had been the focus of most vaccine development programmes before the isolation of protective and immunogenic non-infectious particles of the hepatitis B surface antigen (HBsAg) from infected individuals [35]. The concerns about a plasma-derived vaccine led to the expression of recombinant HBsAg in yeast and mammalian cell lines and paved the way for the production of recombinant protein subunit vaccines. Several subunit vaccines are now licensed such as those against hepatitis B (HBV), cholera and anthrax [36,37]. Despite their success the production of recombinant protein vaccines is complicated by the purification of correctly folded protein from prokaryotic and other expression systems. In addition to this, recombinant proteins are often not immunogenic in isolation and require formulation with adjuvants to facilitate their immunogenicity.

Moreover, once formulated protein-in-adjuvant vaccines often require multiple immunisations to reach protective antibody titres.

1.3.1.1 Adjuvants

Adjuvants have been described as an "immunologist's dirty little secret" [38] and are formulations of chemical compounds or molecules routinely added to recombinant protein vaccines to facilitate their immunogenicity. Adjuvants are added to these vaccines as they lack the intrinsic immunogenic properties associated with live, attenuated or killed whole organism vaccines. In addition to potentiating and directing the immune response towards target antigens, adjuvants have also been associated with a dose-sparing effect, prolonging immune responses induced by vaccination as well as increasing seroconversion rates in populations that respond poorly to vaccines such as the elderly [39,40,41]. They have also been shown to increase the breadth and quality of the immune responses that they induce [42,43,44,45]. To date only six adjuvants have been licensed for use in humans [46]. These adjuvants include aluminium salts/alum (aluminium-phosphate and aluminium-hydroxide), the oil-in-water emulsion MF59 (from Novartis), virosomes as well as the AS03 and AS04 adjuvant platform created by GlaxoSmithKline (see Table 1.2 for a list of all licensed vaccine adjuvants). Alum is the oldest and most widely used licensed adjuvant and the only adjuvant licensed for use in the USA. Like most other licensed adjuvants, alum predominantly induces a broad and reliable antibody response. Though the induction of

this immune profile has proven to be sufficient for the generation of successful vaccines such as those against HBV and diphtheria, it is now widely accepted that novel and even more potent adjuvants will likely be required for targeting diseases such as malaria, TB and HIV for which extremely high titre antibody responses and/or cellular immunity have been shown to contribute to protection [47,48,49,50]. These adjuvants will also be required to stimulate cross-presentation, needed for exogenous protein antigens to prime CD8+ T cells.

Several novel adjuvants are currently in development. However, despite encouraging pre-clinical results many experimental adjuvants have unacceptable safety profiles in humans [51,52,53,54], which almost certainly precludes their licensure in prophylactic applications. For example a 6.6 fold higher incidence of narcolepsy was found in children and adolescents following vaccination with the influenza vaccine Pandemrix formulated in AS03 (see section 1.5.1 for adjuvant composition) than in those not vaccinated in a retrospective study carried out by the Swedish Medical Products Agency investigating cases of narcolepsy during a defined period [55]. In addition to the generation of an acceptable safety profile, careful consideration must also be given to the formulation of adjuvants with their antigens because this affects the stability and immunogenicity of vaccines – as has been seen through the failure of incorrectly formulated vaccines [56]. Another limiting factor facing the development of novel adjuvants is that often information pertaining to formulation and access to components is limited, because

adjuvants are frequently developed by private sector companies. This is especially disruptive for diseases such as malaria and TB for which there is little commercial interest.

Recent insights into innate immunology through the discovery of PAMPs and PRRs has led to the partial dissection of the mode of action of several licensed adjuvants. In turn, this is leading to the more rational design of vaccine adjuvants, which were previously designed empirically. Adjuvants are often categorised according to their components or their proposed mechanism of action. Two routinely used subdivisions are: immunostimulants versus delivery systems. Immunostimulants are thought to directly initiate an innate immune response by signalling through PPRs or by activating DCs leading to the induction of cytokines. Delivery systems are thought to enhance the immune response by affecting antigen presentation and/or by causing a 'depot effect' leading to the controlled release of antigen(s). However recent evidence shows that often the mode of action of adjuvants is not clear-cut [57]. Table 1.3 summarises some of the experimental and licensed adjuvants (or their components) according to their proposed function.

Adjuvant	Company	Type	Indication	Area
Aluminium salts	Various	Mineral salts	HepA&B, Hib, Diphtheria and Tetanus	World-wide (excl. US)
MF59™	Novartis	O/W emulsion	Influenza	EU
AS03	GSK	O/W emulsion	Influenza	World-wide (excl. US)
AS04	GSK	MPL+ Alum	HBV and HPV	World-wide (excl. US)
Virosomes	Crucell	O/W emulsion	HBV and Flu	EU

Table 1.2 Adjuvants licensed for use in humans.

Hep: Hepatitis, O/W: Oil-in-water, EU: European Union, US: United States, GSK: GlaxoSmithKline, HBV: Hepatitis B virus & MPL: Monophosphoryl lipid A.

IMMUNOSTIMULANTS (TLR)	IMMUNOSTIMULANTS (other)	DELIVERY SYSTEMS
Lipopeptides (Pam3CSK4 - TLR 1)	Saponins (QS21, Quil-A)	Mineral salts (alum)
Bacterial lipoprotein, peptidoglycan (TLR2)	Cytokines (GM-CSF, IFN-γ, IL-2)	Emulsions (Montanide®, AS0 platform)
dsRNA (Poly (I:C) - TLR3)	Bacterial Toxins (cholera toxin, CTA1-DD)	Liposomes
LPS, MPL & GLA (TLR 4)		Virosomes
Flagellin (TLR 5)		ISCOM, ISCOMATRIX™
Viral ssRNA (imidazoquinolines -TLR7&8)		Microspheres
Unmethylated CpG (TLR9)		

Table 1.3 Experimental and Licensed vaccines.

Pam3CSK4: Pam3-Cys-Ser-Lys4 - synthetic bacterial lipopeptide, Poly (I:C): Polyinosinic:polycytidylic, LPS: Lipopolysaccharide, MPL: Monophosphoryl lipid A, GLA: Glucopyranosyl Lipid A. GM-CSF: Granulocyte macrophage colony-stimulating factor, CTA1-DD: cholera toxin-derived vaccine adjuvant.

1.3.1.1.1 Aluminium salts

Aluminium-based adjuvants are the oldest and most widely used vaccine adjuvants, and are the only adjuvants currently licensed for use in the USA. The adjuvant properties of aluminium were first discovered in 1926 [58] but its mode of action is only recently being dissected. Aluminium-based adjuvants are components of several licensed vaccines such as those against diphtheria-tetanus (DT), inactivated poliovirus, hepatitis A and the human papilloma virus (HPV). Aluminium-based adjuvants, commonly referred to as alum, are based on aluminium-hydroxide ($Al(OH)_3$), aluminium-phosphate ($Al(PO_4)_3$) and aluminium-potassium sulphate ($AlK(SO_4)_2$), the latter of which is rarely used. Aluminium-hydroxide forms stronger electrostatic interactions with negative proteins, whereas aluminium-phosphate forms stronger electrostatic interactions with positive proteins [59]. Alum predominantly induces moderate antibody responses as well as a classical "Th-2 type" response [60]. Originally the mode of action of alum-adjuvants was thought to be due to the slow release of antigen from insoluble salt particles at the site of injection, which is commonly referred to as the "depot effect". Owing to this belief, the adsorption of alum to its antigen was thought to be essential for adjuvant function, and the WHO recommends, for example, that 80% of the antigen in DT vaccines be adsorbed onto alum [61]. Several factors affect the adsorption of antigen to alum, which is predominantly driven by electrostatic forces. Physical conditions such as temperature and pH can also affect adsorption, and these should thus be considered during formulation [62]. However, despite careful formulation

designed for maximal adsorption of the vaccine, upon administration interstitial fluid can rapidly lead to the dissociation of antigens from alum-containing adjuvants as has been shown in *in vitro* studies [63,64]. These studies have suggested that the degree of adsorption of antigens in interstitial fluid is a better correlate of immunogenicity than the degree of adsorption following formulation [65]. However, immunopotentiation has also been seen with de-adsorbed antigens [66,67,68], indicating that adsorption of antigen to alum-containing adjuvants is not the sole mechanism of action.

Studies with MyD88 and TRIF-deficient mice, unable to signal through TLR receptors, showed no difference in antibody responses compared to control mice, indicating that alum is likely to function through a TLR-independent mechanism [69]. Recent studies have instead highlighted the contribution of the Nalp3 inflammasome to the action of alum. Nalp3 is a NOD-like receptor, which forms the inflammasome with caspase-1 and the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Alum induces uric acid, which is released by cells undergoing necrosis (which alum has been shown to induce) [70]. This in turn activates the inflammasome, potentially through destabilisation of the phagosome, leading to the production of pro-inflammatory cytokines IL-1β and IL-18 [71,72,73,74]. This leads to the recruitment of DCs, which can activate CD4+ T cells and lead to the production of antibody responses [72]. A role has also been suggested for IL-4 in the maintenance of a Th-2 type bias and the suppression of a Th-1 type response, though the contribution of this cytokine does

not appear to be absolute [75,76]. However, recently it has been shown that alum binds to plasma membrane lipids on DCs independent of the inflammasome suggesting another potential mechanism that contributes to its overall mode of action [57]. As alum has been shown to induce the production of several other cytokines and chemokines [77], it is likely that other factors contribute to the mode of action of this adjuvant. Overall, aluminium-based adjuvants have excellent safety profiles, are easy to formulate on a large-scale, and have been shown to successfully adjuvant antibody responses induced by several licensed vaccines. However, alum does not induce strong T cell responses or extremely high titre antibody responses, which are likely to be required for next generation vaccines against malaria, TB and HIV, thus likely limiting its further use as a sole adjuvant component.

1.3.1.1.2 Emulsions

Emulsion-based adjuvants were first used during the 1930s when a mixture of killed mycobacteria and paraffin oil (known as Freund's adjuvant) was found to successfully adjuvant immune responses [78]. Although shown to be a potent adjuvant in animal models, Freund's adjuvant is highly reactogenic, even in small animals. This unacceptable safety profile makes its use in humans unfeasible. Owing to this, efforts have been directed at making emulsions that are human-compatible using metabolisable oils instead of mineral oils. The Montanide® Adjuvant System (developed by Seppic) comprises various emulsion formulations (water-in-oil, W/O, and water-in-

oil-in-water, W/O/W) with Montanide®ISA720 being the furthest in the development pipeline. This adjuvant contains squalene (non-mineral metabolisable oil) and mannide oleate as a surfactant and is formulated as a W/O emulsion [79]. This adjuvant has shown promise with the induction of high-titre antibodies in both human and animal studies [80,81,82,83]. However, sterile abscesses have been observed in some volunteers in Phase Ia clinical trials [52,84] indicating that further development is required before Montanide®ISA720 can be licensed. In addition to this, the correct formulation of emulsion adjuvants is technically difficult and if done incorrectly can lead to the loss of potency of vaccines as has been seen in recent Phase I trials for malaria blood-stage vaccines [56,85]. In addition to this long-term storage of Montanide®ISA720 vaccines formulated with protein has also been shown to lead to protein degradation [85].

The pharmaceutical company GlaxoSmithKline (GSK) has developed the proprietary AS0 adjuvant system, of which the first generation adjuvants were based on emulsions. The various adjuvants developed by GSK encompass a variety of different formulations, which are summarised in Table 1.5. As mentioned previously, AS03 and AS04 are components of vaccines licensed in Europe. All AS0 adjuvants have also been tested extensively with the malaria vaccine candidate RTS,S [86], with varying results as discussed later in this chapter.

The only purely emulsion-based adjuvant currently licensed for use in humans is MF59, which is used in Europe in an influenza vaccine. MF59 is an oil-in-water submicron (<250nm droplets) emulsion consisting of squalene produced by Novartis [87]. It has shown an acceptable safety profile in several phase I trials [88] and has been shown to lead to a broader more balanced IgG1/IgG2a response in mice. In addition to this, MF59 can induce ≈1000 antigen-specific CD4+ T cell responses per 10^6 total CD4+ cells, which have been shown to predict persistence of protective antibody responses in a Phase I H5N1 influenza vaccine trial [89]. Like alum, this adjuvant was originally thought to function through an antigen depot effect, however, recent studies utilising microarrays as well as flow cytometry have shown that MF59 triggers the rapid recruitment of CD11b+ cells into the injected muscle, and also up-regulates genes associated with antigen presentation, leukocyte migration as well as cytokines [90]. MF59 has also been shown to recruit neutrophils and monocytes to the draining lymph node [91] and to act in an inflammasome-independent manner [92].

1.3.1.1.3 TLR agonists

PAMPs and common structural motifs of pathogens are recognised by PRRs, which lead to the activation of the innate immune system through distinct signalling pathways resulting in the secretion of cytokines. Although several PRRs are expressed by innate immune cells such as TLRs, NLRs, CLRs and RLRs; TLR agonists have been extensively used in adjuvant development [93]. Nine functional TLRs have been discovered in

humans and can be broadly classified according to their ligands (reviewed in [94]). TLRs 1, 2, 4 & 6 recognise lipid structures and are expressed on the cell surface of many cells of the immune system, whereas TLRs 3, 7, 8 & 9 recognise nucleic acid and are expressed in endosomal compartments. All TLRs apart from TLR 3 signal through the adaptor molecule MyD88, whereas TLR 3 signals through TRIF. TLR 4 is unique in its ability to signal through both adaptor molecules. Signalling leads to the activation of the transcription factors NF-κB and interferon regulatory factors (IRFs), which regulate the production of cytokines. Table 1.4 summarises the known TLRs and their proposed ligands.

Most work utilising TLR agonists as adjuvants has focussed on TLR 4 ligands, and to date monophosphoryl lipid A (MPL) is the only TLR agonist licensed for use in humans as part of the Fendrix HBV vaccine produced by GSK. MPL is a chemically modified analogue of the Lipid A moiety of Lipopolysaccharide (LPS), which is itself too toxic for use in humans [95,96], and has been shown to induce potent antibody and Th1-type immune responses. The correct formulation of this adjuvant has been shown to be important for generating effector T cell responses in addition to antibody responses. As part of the propriety AS0 adjuvant system developed by GSK [97], MPL formulated with alum (AS04) in an aqueous solution was shown to be less potent than when given in either an oil formulation (AS02) or with liposomes (AS01) (though both of these adjuvants also

include the saponin QS21) [98]. A synthetic analogue of MPL, glucopyranosyl lipid adjuvant (GLA), is now being developed [99].

Synthetic oligodeoxynucleotides (ODNs), containing unmethylated CpG motifs have also been explored as vaccine adjuvants and have been shown to induce strong antibody and effector CD8+ T cell responses, enhance the expansion of mBCs as well as increase the number of vaccine responders in animal and human studies [100,101,102,103,104,105,106]. They have also shown to enhance the proliferation and APC function of B cells [107]. The addition of CpG to Alhydrogel®, an aluminium hydroxide-based adjuvant, has also shown to enhance this adjuvant's potency [108]. However, the development of Wegener's granulomatosis in a HBV vaccine trial in 2008 has led to safety concerns surrounding this adjuvant, and these effects have also since emerged in some animal models [51,53]. As with other TLR agonists, the different distribution of TLR 9 on human and mouse cells, as well as differences in the phenotype of immune responses induced by different classes of synthetic ODNs, are further complicating the development of these agonists as adjuvants [109,110,111,112,113].

TLR 7/8 agonists have also been investigated for their adjuvant properties. Small-molecule nucleotide analogues imiquimod (R837) and resiquimod (R848) have been shown to have anti-viral activities through the induction of inflammatory cytokines, as well as the ability to adjuvant antibody and CD4+ and CD8+ T cell responses

[114,115,116,117]. However, they do not appear to be as potent as TLR 9 and TLR 4 agonists for the induction of T cell responses [118,119]. Furthermore, there are conflicting data about the functional status of TLR 8 in mice, which limits the utility of pre-clinical studies investigating TLR 8 vaccine adjuvants [120,121].

Other TLR agonists explored as vaccine adjuvants include TLR 3 and TLR 5 agonists. TLR 3 recognises dsRNA and the synthetic analogue poly(I:C) has been shown to adjuvant antibody and multi-functional CD4+ T cell responses when administered to non-human primates with a malaria liver-stage vaccine [122], and has also been shown to promote memory CD8+ T cell responses [123]. However, TLR 3 agonists again do not appear to be as potent as TLR 9 agonists [119]. Similarly although flagellin, a TLR 5 agonist, has been shown to lead to a substantial increase in antibody responses compared to an alum-based adjuvant when fused to an influenza matrix protein [124], this agonist is less potent than TLR 9 and TLR 4 in inducing cytotoxic T cell (CTL) responses [119]. As work on the development of TLR ligands as adjuvants continues, it is becoming evident that combinations of TLR agonists in vaccine formulations can act synergistically and could therefore further act to tailor and enhance the immune response induced in response to vaccination [125].

TLR	Pathogen-associated ligand	Synthetic ligand
TLR 1	Triacylated lipopeptides	Pam3CSK4
TLR 2	Triacylated lipopeptides Peptidoglycan, bacterial lipoprotein, GPI-anchor proteins, phospholipomannan	Pam3CSK4, FSL-1, MALP2
TLR 3	dsRNA	Poly (I:C)
TLR 4	LPS, F-protein (RSV)	MPL, AGP
TLR 5	Flagellin	Flagellin
TLR 6	Diacylated lipopeptides, zymosan	FSL-1, MALP2
TLR 7	Viral ssRNA	Imidazoquinolines
TLR 8	Viral ssRNA	Imidazoquinolines
TLR 9	Unmethylated CpG-DNA	CpG-oligonucleotides
TLR 10	no ligands found	n/a

Table 1.4 TLR receptors and their ligands.

Adapted from [126]. Pam3CSK4: Pam3-Cys-Ser-Lys4 - synthetic bacterial lipopeptide, FSL-1: synthetic lipoprotein, MALP2: macrophage-activating lipopeptide, Poly (I:C): Polyinosinic:polycytidylic, RSV: respiratory syncytial virus, MPL: monophosphoryl lipid A, AGP: aminoalkyl glucosaminide 4-phosphate.

1.3.1.1.4 Immunostimulatory complexes (ISCOMs)

Immunostimulatory complexes (ISCOMs) are a form of particulate antigen delivery system and several ISCOM and ISCOMATRIX^{TM} vaccines are currently being developed for the clinic [127]. ISCOMs are composed of cholesterol, phospholipid and saponin. The saponin and cholesterol molecules contained within ISCOMs form a cage-like structure that is approximately 40nm in diameter, with antigen trapped in this particulate

structure [128]. The hydrophobic interactions necessary for particulate formation and antigen incorporation limit the choice of antigens that can be incorporated into ISCOMs to hydrophobic antigens. The ISCOMATRIX™ adjuvant is based on the same components as ISCOMs, but consists of pre-formed particles which are co-administered with the antigen of choice allowing for a wider application [129]. Both ISCOM and ISCOMATRIX™ function as delivery system adjuvants but also contain saponin fractions, which have inherent adjuvant properties. Quil-A, as well as QS-21 which is used in the AS0 adjuvant system developed by GSK, are saponins isolated from the bark of the *Quillaja saponaria* tree. Quil-A is the most widely used saponin fraction and is composed of a heterogenous mixture of triterpene glycosides. Purified fractions of Quil-A vary widely in their adjuvanticity and toxicity, mostly caused by haemolysis, and considerable effort has gone into identifying fractions suitable for human use [130,131]. ISCOMs and ISCOMATRIX™ have been shown to lead to the induction of high-titre antibody responses as well as CD4+ and CD8+ effector T cell responses in human and animal studies with a range of candidate antigens [132,133,134,135]. Due to their particulate nature, ISCOMs are efficiently targeted to APCs and have been shown to lead to the up-regulation of MHC class I and II expression on DCs [136]. Antigens delivered by ISCOMs have been shown to be processed in the endosome for both MHC class I and II presentation [137], and the efficient induction of cross-presentation by ISCOMs due to destabilisation of the endosomal membrane is thought to account for induction of CTL

responses [138,139]. ISCOMs have also been shown to induce pro-inflammatory cytokines [132].

1.3.1.1.5 Adjuvant development

Several other adjuvants including liposomes, chitosan, cytokines and toxins are also currently being developed to enhance immune responses to vaccination, although these adjuvants remain far from licensure. As more is becoming known about the mode of action of adjuvants and the immune responses they induce, more emphasis is being placed on the rational design of adjuvants including the use of synergistic combinations of components. However, it is unlikely that one adjuvant or delivery system will be able to induce a protective immune response across a broad spectrum of diseases. Eventually it is hoped that adjuvants can be used to tailor the immune response induced by vaccination to match known correlates of protection for certain diseases. Several regulatory difficulties, related to the sourcing of adjuvant components and their inherent safety, are also faced in the licensure process making the successful development of a novel vaccine adjuvant for the clinic difficult.

1.3.2 Poxvirus Vectored Vaccines

In parallel to the development of novel adjuvants for use with recombinant protein vaccines, recombinant viral vectors have also been explored as another vaccine delivery

platform, particularly aimed at facilitating the induction of strong T cell responses through direct infection of cells leading to loading of target antigens onto MHC class I molecules or through the induction of cross-presentation of secreted antigens [140]. As mentioned previously, smallpox remains the only human disease to this day that has been eradicated. The original use of cowpox virus to vaccinate individuals against smallpox was possible due to the highly conserved nature of viruses within the Orthopoxvirus genus, thus allowing immune responses raised against cowpox to cross-protect against variola virus, the causative agent of smallpox. This cross-reactivity was further exploited when the closely related vaccinia virus was used to vaccinate millions during the WHO's campaign to eradicate smallpox. As vaccinia virus is a replication competent virus, safety concerns relating to its use in a small but significant number of contra-indicated groups, led to the development of a replication-incompetent vaccinia virus. Through the passage of vaccinia virus in chick embryo fibroblast cells (CEFs) over 570 times an attenuated version of vaccinia virus named modified vaccinia virus Ankara (MVA) was created. This virus has lost over 15% of its genome and is unable to replicate in almost all mammalian cells [141]. The use of MVA in the 1960s to immunise >100,000 people against smallpox, and the use of an MVA expressing the HIV-1 nef protein in HIV-1 infected individuals, demonstrated an excellent safety profile, thus paving the way for the use of this virus as a vaccine vector for other diseases. Orthopox viruses are large, enveloped viruses with dsDNA genomes (180-300kb) which replicate in the cytoplasm.

These viruses can accommodate large amounts of foreign DNA (>25kb), which makes them very attractive as vaccine vectors.

MVA vectors have now been successfully used to target cancer as well as many infectious diseases including malaria, HIV and TB [142,143,144]. MVA expressing the *Mycobacterium tuberculosis* antigen 85A, known as MVA85A, has been used in several Phase I/II clinical trials to boost responses induced by the BCG vaccine. MVA85A has been shown to be safe, induce potent CD4+ T cell responses in both pre-clinical and clinical studies, and protect in animal models of TB infection [145,146]. MVA has also been used to generate malaria vaccines targeting both the liver- and blood-stages of this disease leading to the induction of high frequencies of CD8+ T cells [147,148]. An alternative vaccinia virus (New York vaccinia, NYVAC) encoding seven malaria antigens, termed NYVAC7, attenuated through the deletion of 18 genes [149], was taken into Phase I/IIa clinical trial and led to the protection of 1 out of 35 individuals against an experimental malaria challenge [150]. MVA expressing the liver-stage malaria antigen multi-epitope strong (ME) - thrombospondin-related adhesion protein (TRAP), has also led to the protection of adult malaria-naïve volunteers when used in a prime-boost vaccination regime with an attenuated avipox virus – fowlpox strain FP9 [151]. MVA has now entered several Phase I/IIa trials as part of a prime-boost regime expressing various other malaria antigens at the Jenner Institute in Oxford and consistently demonstrates a good safety profile and an excellent ability to boost immune responses [152].

The use of viral vectors has been shown to induce strong immune responses to the antigen/transgene insert. However, immune responses are also induced to the viral vector itself. This has raised concerns about the feasibility of viral vector re-use due to the possibility of anti-vector immunity diminishing the response to subsequent re-vaccination. Immune responses induced to the antigen insert contained in the attenuated vaccinia virus NYVAC, were shown to be less immunogenic in vaccinia-exposed individuals than in vaccinia naïve individuals [150]. However, re-boosting malaria semi-immune individuals, who had 12 months previously received a dose of MVA expressing ME-TRAP, boosted the effector T cell population back to a level similar to or higher than that observed after the primary MVA immunisation in a DNA-MVA vaccination regime [153]. This indicates that vector-specific immune responses do not necessarily interfere with the ability of MVA to boost antigen-specific responses.

1.3.3 Adenovirus Vectored Vaccines

Adenoviruses are members of the Adenoviridae family and are dsDNA viruses (34-43kb). They are attractive to vaccine developers as they are well-characterised and easy to manipulate [154]. Furthermore, wild-type adenoviruses usually only cause mild symptoms and such infections are rarely life-threatening in immunocompetent individuals. To utilise these viruses as vaccine vectors, they have been modified to be replication incompetent in human cells. Replication-defective adenoviruses have been

created by deleting the E1 region, essential for initiation of viral replication [155]. To increase the insert capacity further modifications have included the deletion of the E3 region, involved in immunomodulation, allowing for the insertion of up to ≈6kb of foreign DNA [156].

Most adenovirus vaccines have been based on the human serotype 5 adenovirus (AdHu5). This vaccine vector has been shown to induce strong B and T cell responses encoding multiple different antigens that have also been shown to surpass responses induced by MVA following a single immunisation [157,158,159,160,161]. AdHu5 vaccines have also been shown to be safe in humans when administered in a Phase I multi-clade HIV-1 vaccine trial inducing strong CD8+ T cell responses [162]. The safety of adenoviral vectors was thrown into doubt following an HIV trial in which HIV sero-negative volunteers were immunised with an AdHu5 expressing HIV-1 antigens gag, pol and nef. A non-significant trend for an increased HIV infection rate in immunised volunteers who were sero-positive for AdHu5 before vaccination was found [163]. Further analysis encouragingly revealed no durable link between vaccination and increased HIV infection (unpublished data) [164]. These claims have however impacted the perception of the utility of this promising vaccine vector.

Although useful for screening vaccines in pre-clinical settings, the high prevalence of pre-existing immunity to AdHu5 within the human population has been shown to

decrease the immunogenicity of AdHu5-based vaccines thus substantially limiting their clinical use [162,163,165]. To overcome this hurdle rarer human adenovirus seroytpes such as AdHu35 have been explored as vaccine vectors, as well as adenoviruses from other species such as chimpanzees, which are not neutralised by human sera [166,167]. These non-human adenoviruses such as C6 and C7, as well as ChAd63, have been shown to induce strong cellular and humoral immune responses similar to AdHu5 in pre-clinical animal models and have been shown to be protective against murine malaria challenges [159,168,169]. Furthermore, recent Phase I/IIa clinical trials utilizing ChAd63 encoding ME-TRAP followed by MVA recombinant for the same insert, have shown this vectored vaccine regime to be safe, extremely immunogenic and capable of inducing sterile protection against a malaria sporozoite challenge (O'Hara *et al.*, unpublished).

1.3.4 Prime-Boost Regimes

Despite the development of several different vaccine delivery platforms, single immunisations or sequential immunisations utilising the same vaccine often do not induce immune responses of a protective magnitude. Owing to this, prime-boost regimes have been developed in which two vaccine vectors expressing the same transgene are administered sequentially [152]. This strategy has mainly been aimed at enhancing T cell responses induced by viral vectored vaccines, and was developed in an attempt to avoid anti-vector immunity induced against the priming vector, which can

prevent boosting of the primary immune responses induced to the insert following homologous administration of the same vector. Initially these regimes encompassed DNA-MVA, FP9-MVA as well as heterologous adenovirus vaccine regimes for a wide spectrum of diseases including malaria, HIV and TB [151,153,168,170,171,172,173]. However, adenovirus prime, MVA boost regimes (AM) have been progressively revealed to be the most potent regimes for the induction of high frequencies of antigen specific CD8+ T cells as well for the induction of high-titre antibodies [148,168,169,174,175]. Interestingly these studies have revealed a differential ability of viral vectors to prime and boost humoral and cellular immune responses. MVA fails to prime antibody responses but can efficiently boost adenovirus primed antibody responses, whereas adenovirus can effectively prime and boost antibody responses. It has also been found that the interval between immunisations can be crucial. Using an AdHu5 prime, attenuated recombinant vaccinia virus boost regime encoding the CSP antigen of *Plasmodium yoelii,* an extended immunisation interval was found to increase the frequencies of CD8+ and CD4+ T cells as well as increase the efficacy against a sporozoite challenge [176]. Similarly increasing the interval between AM boost regimes from 2 to 8 weeks was found to significantly increase antibody responses to the merozoite surface protein (MSP)-1 antigen insert as well as lead to enhanced protection against a blood-stage *P. yoelli* challenge [174].

Recently adenoviral prime – protein-in-adjvuant boost (AP) regimes, whereby the two leading subunit vaccine platforms are combined, have been shown to induce improved antibody responses compared to the use of either strategy on its own [177,178,179,180]. This AP regime is therefore attractive for the development of vaccines against HIV and the blood-stage of malaria, where antibodies have been shown to be involved in protection. Furthermore, the addition of a protein-in-adjuvant boost to an AM regime has been shown to induce both high titre antibodies as well as strong T cell responses [177], indicating that it may be possible to broadly tailor immune responses induced by subunit vaccination.

1.4 Malaria

Malaria is a parasitic disease caused by a protozoan parasite of the genus Plasmodium. The morbidity and mortality associated with this disease is immense with nearly 1 million deaths occurring annually [181]. The greatest burden of this disease is found in Africa, where 91% of all malaria-related deaths occur and where ≈250 million cases of febrile malaria occur per year in children under 5 (Figure 1.3) [182,183]. In addition to this, malaria also poses an immense socioeconomic burden and has been shown to slow down economic growth in affected countries [184]. Despite several control measures such as malaria chemoprophylaxis and insecticide treated bed-nets changing the face of malaria transmission in some regions [185], such measures are facing difficulties through emerging drug-resistance, non-compliance and difficulties in their distribution

[186,187]. A malaria vaccine would provide a cost-effective way of controlling this disease.

Malaria can be caused by five *Plasmodium* species in humans: *falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi* with *P. falciparum* being the causative agent in approximately 90% of all deaths. The *P. falciparum* life cycle is very complex involving both the human host and the parasite's vector: the *Anopheles mosquito*, with stage-specific expression of hundreds of parasite antigens. The parasite's life cycle can be divided into three stages (Figure 1.4).

Pre-erythrocytic stage: infection begins when a feeding female mosquito injects haploid sporozoites into the host. Within minutes, the sporozoites are carried through the blood to the liver where they invade hepatocytes. Over a course of 5-7 days the sporozoites undergo asexual replication within a parasitophorous vacuole resulting in approximately 20-30,000 merozoites. For *P. vivax* and *P. ovale* alone, dormant hypnozoites can develop at this stage, and in the cases of all species, at the end of hepatic development, merozoites are released from the infected hepatocytes ready to invade red blood cells. The liver stage of infection is asymptomatic.

Erythrocytic stage: merozoites released from the liver invade uninfected red blood cells (RBCs). Within the RBC, the invading merozoite undergoes morphological changes and

develops first into a “ring” trophozoite, followed by a mature trophozoite and finally a schizont. Each mature schizont-stage parasite contains approximately 16 new merozoites, which develop through asexual division. The schizont eventually ruptures the host RBC and releases its merozoites, which go on to invade new RBCs. This process of invasion, replication and rupture follows a 48h cycle for *P. falciparum*. This stage of the malaria life-cycle is responsible for the pathology associated with malaria.

Sexual stage: an unknown signal causes a subset of merozoites to develop into gametocytes within infected RBC. When released from infected RBCs these are then taken up by a feeding mosquito within the blood meal and fuse in the mid-gut of the mosquito. This forms a zygote, followed by a motile ookinete, which penetrates the mid-gut wall and forms an oocyst in which haploid sporozoites form via meiosis. Eventually the oocyst bursts and releases its sporozoites, which travel to the mosquito’s salivary gland ready for injection into a new host.

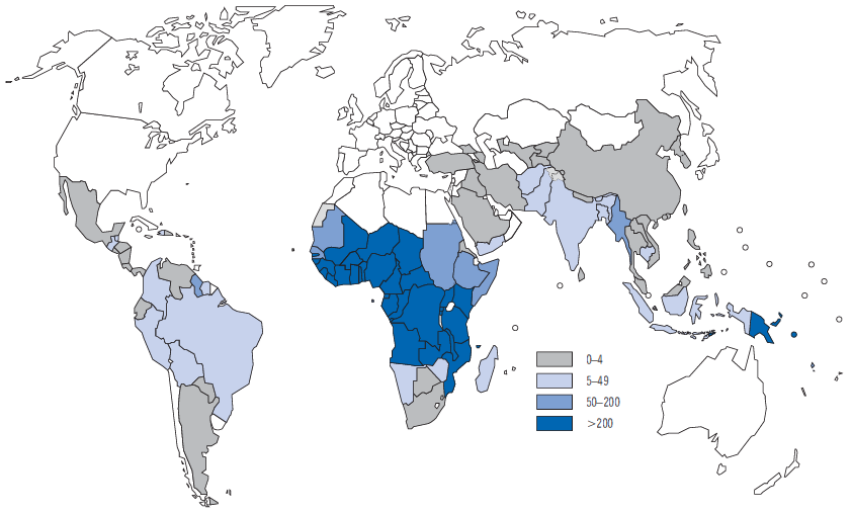

Figure 1.3 Estimated Incidence of Malaria in 2006.

Adapted from [188]. In 2006 there were an estimated 247 million malaria cases worldwide of which 91% were due to *P. falciparum*. 19 countries account for 90% of all cases in Africa including Nigeria, the Democratic Republic of the Congo and Ethiopia.

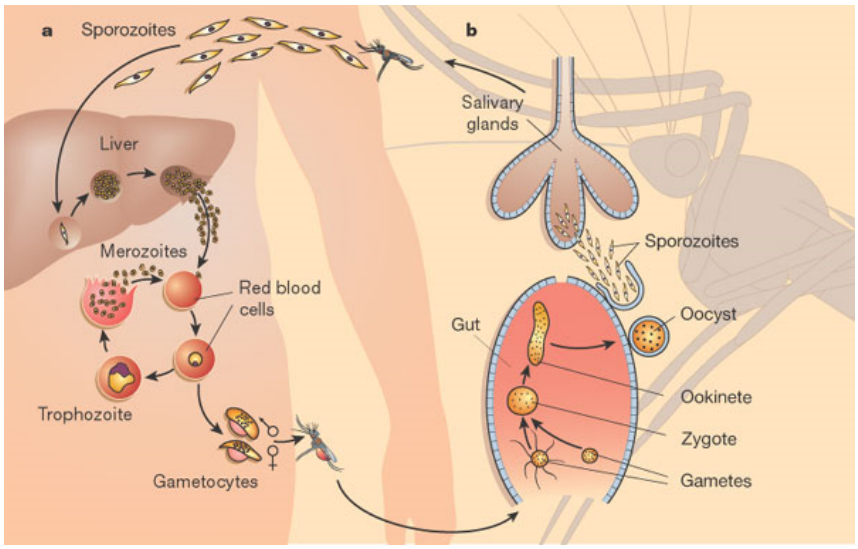

Figure 1.4 *P. falciparum* life-cycle.

Adapted from [189]. The *P. falciparum* lifecycle in the human host (a) and *Anopheles* vector (b).

1.4.1 Malaria and the immune system

1.4.1.1 Pre-erythrocytic immunity

Immune responses can develop to all major stages of the parasite lifecycle within the human host and involve both the innate and adaptive arms of the immune system. These immune responses can act to limit parasitaemia and prevent pathology, yet typically fail to clear the infection completely. The first point at which the immune system can react to malaria-related antigens is when sporozoites are injected into the host. However, these sporozoites are only exposed to the host's immune system for a very short time period (2-30 minutes) before they invade hepatocytes. Antibodies directed against this stage may act to opsonize sporozoites or induce their phagocytosis through Fc-mediated effector functions. It has been suggested that anti-CSP antibodies may be able to directly neutralise sporozoites by inhibiting gliding motility and cell traversal [86], which is thought to be the presumed action of the malaria vaccine RTS,S [190,191].

Another target for immunity during the pre-erythrocytic stage is the parasite within hepatocytes. As the parasite is intra-cellular throughout the liver-stage of its life-cycle, cellular mediated immunity (CMI) is implicated as being important and T cells have been shown to recognise parasite-derived peptides presented on the surface of infected hepatocytes. These T cells are thought to be primed in the lymph node by a small

number of sporozoites that drain from the inoculation site to the lymph node and are cross-presented by DCs [192]. This is in contrast to previous models suggesting hepatocytes act as APCs. Studies in mice have suggested that CD8+ effector T cells kill infected hepatocytes through the release of IFN-γ [193]. Mice challenged with sporozoites under chloroquine (CQ) cover were also found to develop protective immunity to subsequent sporozoite challenge, which was found to be dependent on CD8+ and CD4+ T cells producing IFN-γ and nitric oxide [194]. In a similar clinical study, volunteers were exposed to the bites of infectious mosquitoes under CQ cover before a subsequent live challenge. This vaccination protocol induced 100% sterile protection in humans, which was shown to be associated with multifunctional effector memory T cells [195]. CD4+ T cells have also been shown to contribute to protection in the *P. yoelii* rodent model of malaria whereby adoptively transferred CD4+ T cells directed against *P. yoelii* CSP have been shown to mediate protection [196]. However the protective mechanism induced by vaccination has been shown to be strain and vaccine dependent, highlighting the complexity of the contribution of T cells and CMI to pre-erythrocytic-stage malaria immunity [197].

1.4.1.2 Blood-stage immunity

Immunity against blood-stage malaria is mainly thought to be antibody-mediated as was suggested by early data from passive immunisation studies of children with sera from immune adults used to treat malaria [198,199]. At this stage of the parasite's life-cycle,

antibodies are thought to target both the infected red blood cell as well as free merozoites. Natural acquired immunity is thought to be mediated through the acquisition of antibodies against multiple parasite strains over time. In Kenyan children, antibodies to the RBC surface expressed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) have been shown to be associated with naturally acquired immunity [200]. Epidemiological studies have also discovered an association between antibodies to several other blood-stage antigens and protective outcome [201,202,203]. Similar correlations between antibodies and protective immunity have also been shown in pre-clinical models [174,204].

Antibody-mediated protection is thought to occur through a variety of effector functions [205]. Antibodies directed against merozoite surface proteins can block RBC invasion or opsonise merozoites for phagocytosis. Antibodies can also induce ADCI by binding to Fc receptors on various innate immune cells such as monocytes. Antibodies can also target the infected erythrocyte, where antibodies are again thought to opsonise infected RBCs and target them for phagocytosis or complement-mediated lysis. Antibodies may also inhibit sequestration of parasites. However, it has been shown that some antibodies induced to surface antigens such as MSP-1 can act to block protective antibodies [206].

There is some evidence for a protective role of T cell responses against blood-stage antigens during both the erythrocytic-stage infection as well in the preceding late-liver stage where these antigens are also expressed. CD8+ T cells directed against MSP-1 have been shown to be protective against a *P. yoelii* sporozoite challenge and CD4+ T cells have been shown to provide help for the development of B cell responses [148,207]. In the *P. chabaudi chabaudi* AS model CD4+ T cells have also been shown to control peak parasitaemia in the absence of antibodies [208,209]. The role of T cells in blood-stage immunity in humans is less clear. Repeated infection of volunteers with blood-stage parasites followed by drug-cure has been shown to induce protection against a blood-stage challenge which was not associated with antibodies [210]. This indicated that T cells may contribute to protection, however potential residual anti-malaria drugs present at the time of challenge complicate the interpretation of this result.

1.5 Malaria Vaccines

A malaria vaccine would provide a cost-effective way of controlling this disease. Though there is some compelling evidence that a malaria vaccine would be feasible, such as by the successful protection of volunteers exposed to radiation-attenuated sporozoites through the bites of infectious mosquitos [211], success has been limited so far. Even though this first proof–of-concept study provided important support for the development of a malaria vaccine, levels of protection achieved with this vaccination

strategy are variable and protection is only reliably achieved if over a thousand infectious bites are received by volunteers [212]. Regulatory and manufacturing issues are also faced by this attenuated whole organism approach [213]. In addition to this, natural immunity to malaria is only acquired after repeated infections and is not sterile but rather results in asymptomatic carriage, raising the question of whether a vaccine that prevents infection is feasible [214]. Other difficulties faced by vaccine developers are the complex parasite life-cycle with stage-specific antigen expression, antigenic variation as well as polymorphism [215]. Despite the odds stacked against vaccine developers, many malaria vaccine candidates have now entered clinical trials with some limited success.

1.5.1 Pre-erythrocytic Vaccines

Vaccines targeting the pre-erythrocytic stage of malaria aim to induce sterile protection by i) preventing the invasion of hepatocytes by sporozoites which constitutes the initial phase of infection or ii) eliminating all infected hepatocytes via CMI. Several vaccines targeting this stage of malaria infection have gone into the clinic with some showing promising efficacy. As previously mentioned, the demonstration that exposure to radiation-attenuated sporozoites through the bite of infectious mosquitos could induce sterile protection against *P. falciparum*, indicated that liver-stage vaccines could potentially provide long-lasting protection against malaria. These volunteers were

exposed to over a thousand bites by infectious mosquitoes carrying irradiated sporozoites, and then challenged with five infectious bites from mosquitoes carrying non-irradiated sporozoites. Upon challenge, 93% of volunteers were protected and a sub-set of those protected volunteers challenged again a total of 15 times were 100% protected [212]. The numbers of infectious bites needed for protection are thought to inoculate volunteers with 100,000 to 300,000 irradiated sporozoites, which constitutes a substantial number. Attempts to scale-up production and develop a viable malaria vaccine based on irradiated sporozoites, through the dissection of mosquitos and cryopreservation of these sporozoites has shown in mice, using the rodent malaria model *P. yoelii*, to require 2-4 times higher numbers of irradiated sporozoites to reach protective levels [216]. Several trials are now underway to explore the efficacy of cryopreserved irradiated sporozoites. However, it is likely that the route of administration is likely to affect the efficacy of this vaccine. In addition the development of such a vaccine and it's production to GMP standards as well as its storage, complicated through the need of cold-chain (liquid nitrogen) preservation, is unlikely to make this a successful strategy for the deployment of a malaria vaccine.

Another whole-organism approach has been the development of genetically attenuated parasites [217]. However, the concern of break-through blood-stage infections is currently limiting their development.

The most advanced malaria vaccine to date is RTS,S which is being developed by GSK. This subunit vaccine is based on the CSP antigen that is expressed on sporozoites and within infected hepatocytes [218]. 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 and the entire C-terminal region of the protein containing T cell epitopes. This is fused at the N-terminus to HBsAg, which can form virus-like particles (VLPs), and together this part of the vaccine is referred to as RTS. In order to stabilise the protein it is co-expressed in yeast with wild-type HBsAg, referred to as S. The final product is 20% RTS and 80% S antigen. This subunit vaccine has been tested with several of GSK's proprietary adjuvants from the AS0 adjuvant platform, and four of these have been taken into human trials (Table 1.4). Several clinical trials have been undertaken in malaria-naïve individuals with vaccine efficacy ranging from 0-87.5% [86]. These trials determined that AS01B was the most potent adjuvant and protected a higher percentage of malaria-naïve individuals from experimental sporozoite challenge [219].

RTS,S/AS02A has also been taken forward into several field trials with varied results. In Gambian adults vaccine efficacy was found to be 34%, with an initial prevention of infection of 71%. However, this protection waned by 6 months [220]. In a paediatric trial carried out in Mozambique, RTS,S/AS02D induced a vaccine efficacy of 45% and was also shown to have an impact on clinical disease [221]. Further trials in Tanzania have also shown enhanced efficacy using the AS01E formulation in a further paediatric trial [191].

Despite its partial efficacy, RTS,S is now in Phase III trials across Africa and its licensure is likely by 2015.

In parallel to RTS,S, viral vectored vaccines based on the liver-stage antigen TRAP fused to the ME string, containing multiple epitopes to other malaria antigens, has been developed [151]. When given to malaria naïve individuals in a heterologous prime-boost regime, ChAd63 and MVA vectors expressing ME-TRAP induced 21% sterile efficacy against *P. falciparum* sporozoite challenge (O'Hara *et al*., submitted). This regime is now being tested in Phase I/IIb trials in The Gambia and Kenya.

Adjuvant	**Components**
AS04	Alum + MPL
AS03	Oil-in-water (squalene based)
AS02A/D	Oil-in-water + MPL + QS21
AS01B/E	Liposomes + MPL + QS21

Table 1.5 AS0 adjuvants tested in human clinical trials.

The suffix D and E denote the paediatric formulations of the indicated AS0 adjuvants in which the dosing of active ingredients and administered volume differs to the adult dose denoted by the suffix A and B [222,223].

1.5.2 Blood-stage Vaccines

Vaccines targeting the blood-stage of malaria aim to stop the erythrocytic life-cycle of the parasite and therefore prevent the disease associated with malaria. Such a vaccine would be especially pertinent if immunity to a partially effective pre-erythrocytic vaccine wanes without individuals developing naturally acquired immunity making them susceptible to clinical malaria.

Several blood-stage malaria vaccines, mostly based on MSP-1 and apical membrane antigen-1 (AMA-1), have entered the clinic. These two antigens are the candidate antigens that have classically led the blood-stage malaria vaccine field. Few of these vaccines have assessed efficacy and those that have, have shown limited success. However, encouragingly recently an AMA-1 vaccine based on the 3D7 strain of *P. falciparum* formulated with AS02A was shown to have an efficacy of 64.3% against the vaccine parasite strain, though efficacy against the primary end point was a non-significant 17.4% [224].

Combination B is a recombinant protein vaccine encoding the ring–infected erythrocyte surface antigen (RESA), MSP-1 and MSP-2 formulated in Montanide®ISA720. In a Phase IIb trial in children in Papua New Guinea it was found to provide a 62% reduction in parasite density in one of the two groups studied. However, these results were shown to be vaccine allele specific for MSP-2 [225]. The vaccine SPf66, a multi-epitope multi-

peptide vaccine, underwent several Phase III trials but the lack of efficacy obtained meant that the vaccine did not warrant further development [226].

The candidate antigens MSP-1 and AMA-1 have also been taken forward into Phase I/IIa clinical trials utilising viral vectors. ChAd63 and MVA vectors expressing MSP-1 (termed PfM128 designed to address the allelic diversity seen to this antigen [169]) administered in a heterologous prime-boost immunisation regime were found to induce both high levels of antibodies as well as CD8+ T cell responses. Three volunteers were challenged with *P. falciparum* sporozoites and a significant delay in onset of patent parasitaemia was observed (Sheehy *et al.*, submitted). These initial results pave the way for further clinical testing of this vaccine and other blood-stage antigens such as AMA-1 using a viral vectored vaccine delivery platform.

1.5.3 Transmission-blocking Vaccines

Transmission blocking vaccines aim to induce antibodies to the sexual stages of the parasite and block transmission of the parasite. Such a vaccine would protect communities by breaking the cycle of transmission but would not protect individuals from infection or disease. One of the leading candidate antigens for such a vaccine is Pfs25, an ookinete surface protein, which has been shown to induce transmission blocking antibodies in both pre-clinical and clinical studies [227,228].

1.6 Thesis aims and outline

As outlined above an efficacious vaccine for malaria is still lacking. Although the precise immune mechanisms mediating protection *in vivo* in humans against the blood-stage of malaria are unclear, the vaccine field generally accepts that the induction of high-titre antibodies will be necessary to prevent the invasion of RBCs utilising vaccines based on blood-stage (merozoite) antigens. Classically protein-in-adjuvant vaccines have been used to achieve such responses, so far with disappointing results. In addition to this, comparative data on adjuvant performance and mechanism are lacking, given adjuvants are usually owned by private-sector companies, with information pertaining to formulation and access to component composition being limited. The recent use of adenoviral prime, protein boost regimes in pre-clinical models of malaria has shown that this vaccine regime can induce even higher antibody titres than standard protein-only vaccine regimes. When combined with novel adjuvants, it is likely that these regimes can be used to induce the high-titres of antibodies needed for protection against difficult diseases such as malaria. In addition to this, given viral vectors have been shown to differ in their ability to prime and boost immune responses, it is of timely interest to explore whether protein-in-adjuvant vaccines are subject to a similar effect and whether the differences in the underlying immunogenicity of these vaccine platforms can be utilised to more rationally design and deliver vaccine antigens.

1.6.1 Aims

- To comparatively asses a panel of leading and accessible adjuvants for the induction of humoral and cellular immunity using a model antigen as well as leading blood-stage malaria candidate antigens in both a three-shot protein-in-adjuvant regime as well as an adenoviral prime, protein boost regime.

- To investigate the mechanisms underlying the differential priming abilities of protein vaccines and viral vectored vaccines for the induction of antibody and B cell responses.

- To assess the efficacy of three-shot protein-in-adjuvant regimes as well as an adenoviral prime, protein boost regime in the mouse malaria model *P. yoelii* and determine the underlying protective mechanisms.

- To improve the immunogenicity of pre-existing vaccination regimes and viral vectors through the direct addition of adjuvants.

1.6.2 Outline

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

Chapter 3 investigates the comparative immunogenicity of a panel of adjuvants in AP and PPP regimes using the model antigen OVA.

Chapter 4 investigates the comparative immunogenicity of a panel of adjuvants in AP and PPP regimes using leading blood-stage malaria vaccine candidates.

Chapter 5 investigates the underlying immunological differences between protein and viral vectored vaccines and the impact on AP and PPP regime performance.

Chapter 6 describes the efficacy and investigates the protective mechanisms of AP and PPP MSP-1 based vaccines against a blood-stage *P. yoelii* challenge.

Chapter 7 assesses whether the immunogenicity of viral vectored vaccines can be enhanced through the direct addition of chemical adjuvants.

Chapter 8 summarises the results of this thesis and discusses future directions.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Table 2.1 details all commercially available reagents used in this study.

Material	Supplier	Catalogue Number
0.2µm syringe filter	Sartorius	16534
15mL centrifuge tubes	Fisher, Inc.	FB55950
2-β Mercaptoethanol [50µm]	Gibco	31350-010
50mL centrifuge tubes	Greiner	227261
60mm petri dishes	Corning	430166
6-well tissue culture plates	Fisher, Inc.	TKT-520-030T
Agarose	Sigma Aldrich, UK	A9539
Ampicilin	Sigma Aldrich, UK	A5354
Anti-mouse CD11b Alexa Fluor® 700	Ebioscience	56-0112-82
Anti-mouse Cd11C PE	Ebioscience	12-0114-83
Anti-mouse CD3ε APC	Ebioscience	17-0031
Anti-mouse CD3ε FITC	Ebioscience	11-0031-85
Anti-mouse CD4 Pacific Blue® clone RM4-5	Ebioscience	48-0042
Anti-mouse CD4 PE clone RM4-4	Ebioscience	12-0043

Anti-mouse CD4 Qdot 605	Invitrogen	Q10092
Anti-mouse CD8α PerCP-Cy5.5	Becton Dickinson Ltd	551162
Anti-mouse CD95	BD Biosciences	554258
Anti-mouse GL7	Ebioscience	51-5902-82
Anti-mouse IFN-γ APC	Ebioscience	17-7311
Anti-mouse IgG γ-chain specific	Caltag (Invitrogen)	M30100
Anti-mouse IgG1biotin	BD Biosciences	553441
Anti-mouse IgG2a biotin	BD Biosciences	553388
Anti-mouse interleukin-2 (IL-2) PE	Ebioscience	12-7021
Anti-mouse Ly-6C APC	Ebioscience	17-5932-82
Anti-mouse Ly-6G eFluor® 450	Ebioscience	48-5931-82
Anti-mouse MHC Class II (I-A/I-E) APC-eFluor® 780	Ebioscience	47-5321-82
Anti-mouse TNFα FITC	Ebioscience	11-7321
Anti-mouse/human B220 PeCy7	Ebioscience	25-0452-82
Antisedan™	Oxford University Veterinary Services	n/a
Benzonase ® Nuclease	Novagen	70664-3
BugBuster® GST•Bind™ purification kit	Novagen	70794
CD16/32 (Fc block)	Ebioscience	14-0161-82
CD4+ T cell Isolation Kit	Miltenyi BioTech	130-091-041
Cell strainers	BD Biosciences	352350
Chloramphenicol	Sigma Aldrich, UK	C7795
Chloroquine diphospahte salt	Sigma Aldrich, UK	C6628
Cytofix/Cytoperm™ fixation/permeabilization solution kit	Becton Dickinson Ltd	555028

DH5α sub-cloning efficiency cells	Invitrogen	18265-017
Diethanolamine buffer	Pierce	34064
Dimethyl suloxide (DMSO)	Sigma Aldrich, UK	D2650
Domitor™	Oxford University Veterinary Services	n/a
Dried skimmed milk powder	Tesco	n/a
Dulbecco's PBS	Sigma Aldrich, UK	D8537
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK	E7889
ELIspot colour development kit	BioRad	170-6432
Endotoxin Free water	Sigma Aldrich, UK	W3500
Ethanol	Ethanol	32221
ExtrAvidin	Sigma Aldrich, UK	E2636
Expand PCR High Fidelity Kit	Roche	11 732 641 001
Fœtal Calf Serum (FCS)	Sigma Aldrich, UK	F-2442
Gateway® LR Clonase® II enzyme mix	Invitrogen	11791-020
Giemsa	Sigma Aldrich, UK	GS1L
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich, UK	A-3562
GolgiPlug	Becton Dickinson Ltd	555028
Hydrochloric Acid (HCl) [1M]	Sigma Aldrich, UK	S-7653
Immersion Oil	Sigma-Aldrich, UK	10890
IPTG	Sigma Aldrich, UK	I5502
Isoflo	Oxford University Veterinary Services	n/a
Kanamycin	Sigma Aldrich, UK	K0254
Ketaset™	Oxford University Veterinary Services	n/a

$KHCO_3$ [1mM]	Sigma Aldrich, UK	P-9144
KlenTaq Polymerase	Invitrogen	D5062
L-glutamine [4mM]	Sigma Aldrich, UK	G-7513
Lipofectamine™	Invitrogen	11668-019
LIVE/DEAD® Aqua	Invitrogen	L34957
Low phosphate PBS	Gibco	10010-031
LS Columns	Miltenyi Biotech	130-042-401
MAIP ELIspot Plates	Millipore	MAIPS4510
MEM α-modification	Sigma Aldrich, UK	M-4526
Methanol	Sigma Aldrich, UK	32213
Micro-fine Insulin Syringe with needle 1mL 29gx12.7mm	Nu-care products	M4891
Microlance needle 26g x 5/8 inch	Nu-care products	M4300
Microvette tubes (CB300K2E)	Sarstedt	16.444
MinElute gel extraction kit	QIAgen	28606
Mouse IFN-γ ELISPOT kit (ALP)	Mabtech	3321-2A
Mouse IgG1 K Isotype Control Purified	Ebioscience	14-4714-85
Mouse IgG2a K Isotype Control Purified	Ebioscience	14-4724-85
Na_2EDTA	Sigma Aldrich, UK	ED2SS
NaSCN – Sodium Thiocyanate	Sigma Aldrich, UK	251410
Neutral buffered formalin	Sigma-Aldrich	HT501128
NH_4Cl [0.15M]	Sigma Aldrich, UK	A-4514
NUNC Maxisorp 96-well plates	Fischer, Inc.	DIS-971-030J
Ovalbumin (Grade VII)	Sigma Aldrich, UK	A7641

Phosphate Buffered Saline (PBS)	Sigma Aldrich, UK	D-8537
PBS/Tween Sachets	Sigma Aldrich, UK	P3563
Pen/strep [100U penicillin 100μg strep]	Sigma Aldrich, UK	P-0781
Plastipak syringe 1mL Luer slip	Nu-care products	M0013
pNPP (20mg tablets)	Sigma Aldrich, UK	N-2765
Proteinase K	Qiagen	1017738
Purified Rat IgG	Sigma Aldrich, UK	I4131
Qiagen plasmid midi kit	QIAgen	12143
QIAprep spin miniprep kit	QIAgen	27106
Restriction and Ligation Enzymes	New England Biolabs	Various
RPMI-1640	Sigma Aldrich, UK	R0883
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, UK	L4390
S.O.C. medium	Invitrogen	15544-034
Smart Ladder DNA marker	EurogenTec	MW-1700-02
S-methyl-thiorea (SMT)	Sigma Aldrich, UK	M8445
Sodium Azide	Sigma-Aldrich, UK	S2002
SYBR Safe in DMSO	Invitrogen	S33102
Terrific Broth	Sigma Aldrich, UK	T0918
Trizma® HCL buffer solution (Ph8)	Sigma Aldrich, UK	T2694
Tris-acetate-EDTA	Fischer, Inc.	BP1332-1
Trisodium citrate	Sigma Aldrich, UK	S1804
U bottom plates, with lid, 96-well	Jencons	734-0027
V bottom plates, 96-well	Fischer, Inc.	DIS-210-150L

Table 2.1 Reagents used throughout the study.

2.1.2 Solutions

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

❖ **Blood-Stage Culture Medium**: 96mL RPMI-1640 plus 11mL FCS, 4.2mL 5% $NaHCO_3$ solution, and 5.5mg neomycin.

❖ **Chloroquine**: an 8mg/mL solution of chloroquine was made by diluting 1g of chloroquine diphosphate salt in 125 mL of PBS. The solution was filter sterilised for use *in vivo* and kept in the dark.

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

❖ **Complete Medium (D10)**: Minimal Essential Media (MEM) α-modification was supplemented with 5mL L-glutamine (2mM), 5mL pen/strep (100U penicillin, 100μg strep), 500μl 2-Mercaptoethanol (50μm) and 50mL of heat inactivated foetal calf serum (FCS) (10%).

❖ **Diethanolamine Buffer**: A 5x stock was purchased and diluted with dH_2O before use.

❖ **FACS buffer:** 2.5g (0.5%) Bovine Serum Albumin (BSA) was added to 500mL PBS.

❖ **Giemsa:** 5% Giemsa (10mL Giemsa plus 190mL Milli-Q water).

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

❖ **Phosphate buffered saline (PBS)** 0.01M: NaCl 0.138M, KCl 0.0027M, pH 7.4: made by dissolving tablets in dH_2O.

❖ **PBS Formalin 1%:** 15mL of 4% formaldehyde was added to 45mL PBS.

❖ **PBS/Tween (PBS/T)** 0.01M: NaCl, 0.138M, KCl 0.0027M, pH 7.4 Tween-20 0.05%. Sachets are dissolved in dH_2O.

❖ **Red Blood Cell Counting Solution**: 10mL formalin, and 32g trisodium citrate: made up to 1L in dH_2O.

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

❖ **Terrific broth**: 12g bacto-tryptone, 24g bacto-yeast extract, 4mL glycerol made up to 900mL with distilled water and autoclaved. 100mL sterile solution of 0.17M KH_2PO_4, 0.72M K_2HPO_4 added before use

❖ **Tris buffer:** a stock solution of 1M Tris was diluted in dH_2O to a working concentration of 20mM. The pH was adjusted to 7.5 with HCl (1M) and the solution was filter sterilised for use *in vivo*.

2.1.3 Adjuvants

The adjuvants used throughout this study were obtained by the Jenner Institute Adjuvant Bank. The supplier and catalogue number are listed for adjuvants that are commercially available. Selected Adjuvants that were not purchased commercially were obtained through Material Transfer Agreements (MTA) with the relevant companies or were obtained as samples from the relevant companies.

- Adju-Phos® (Brenntag Biosector, Denmark). Clinical Grade. Lot no.: 9093 and 9142. Expire: Jul 2011 and Dec 2012 respectively. 2% Aluminium phosphate ($AlPO_4$), equivalent to 0.45% Al^{3+} (pH adjusted to 6.4).

- Alhydrogel® (Brenntag Biosector, Denmark). Clinical Grade. Lot no.: 4191 and 4565. Expire: Jul 2010 and Nov 2012 respectively. 3% Aluminium hydroxide ($Al(OH)_3$).

- Abisco®100 (Isconova, Sweden). Pre-clinical Grade. Lot no.: various. Suspension of nano-sized (40nm) cage-like particles in PBS consisting of purified saponins (from *Quillaja saponaria* Molina), cholesterol from lanolin and phosphatidyl choline from fresh egg yolk.

- CoVaccine HT™ (British Technology Group). Research Grade. Lot no.: various. Submicron emulsion of squalane-in-water containing sucrose fatty acid sulphate ester $S_1(C12)_7$, (SFASE): 40mg/mL, emulsified squalane oil (squalane is a saturated analogue of squalene, chemically more inert): 160mg/mL, Polysorbate 80 (surfactant): 40mg/mL, 0.1mg/mL thiomersal (Hg-containing preservative) in PBS [229].

- Complete Freund's Adjuvant (Sigma Aldrich, UK – cat. no. F 5881). Pre-clinical Grade. Non-metabolisable oils: paraffin oil and mannide monooleate. Complete

Freund's Adjuvant (CFA) also contains killed *Mycobacterium tuberculosis*. Used as water-in-oil (W/O) emulsion.

- Incomplete Freund's Adjuvant (Sigma Aldrich, UK – cat. no. F5506). Pre-clinical grade. Non-metabolisable oils: paraffin oil and mannide monooleate. Used as W/O emulsion.

- Montanide®ISA720 (Seppic, France). Clinical Grade. Lot no.: T74920. Squalene (non-mineral metabolisable oil) and refined emulsifier/surfactant based on mannide oleate. Designed to be used as W/O emulsion.

- Stable Emulsion-based Adjuvants (EM Adjuvants). (Infectious Diseases Research Institute, USA). Pre-clinical Grade. Lot. no.: various. Stable emulsion oil-in-water (O/W) (variant of MF59) with or without TLR agonists.

    - ❖ EM01 (SE): stable emulsion.

    - ❖ EM05 (SE + TLR4): stable emulsion plus GLA (glucopyranosyl lipid A, 1mg/mL).

    - ❖ EM012 (SE + TLR4&7): stable emulsion plus GLA and resiquimod R848 (both 1mg/mL).

- ❖ EM014 (SE + TLR4&9): stable emulsion plus GLA (1mg/mL) and CpG (ODN 1826, type B, mouse specific, 5'-tcc atg acg ttc ctg acg tt-3', at 1.25mg/mL).
- ❖ EM020 (SE + TLR4,7/8&9): stable emulsion plus GLA (1mg/mL), resiquimod R848 (1mg/mL) and CpG (1.25mg/mL).

2.2 Methods – Molecular Biology

2.2.1 Antigen insert

All viral vectored vaccines used throughout this study were made according to similar methods. The different vaccines used are outlined in Table 2.4 with references citing their production. The production of AdHu5 and MVA vectors expressing ovalbumin (OVA) is outlined here. The full 1188-bp coding sequence of Hen OVA (GenBank Accession ♯MN205152, http://www.ncbi.nlm.nih.gov/genbank/) was used. An N311D amino acid substitution was carried out to prevent N-linked glycosylation as described elsewhere [230]. The final construct was codon optimised for human expression and was synthesised by GeneArt (Regensburg, Germany). AdHu5 and MVA vectors expressing the *P. vivax* Duffy Binding Protein (DBP) Region 2 (RII) were made by Dr. A. Goodman according to the same methods. The native 984-bp coding sequence of

Region II of the Duffy Binding Protein (GenBank Accession ♯DQ156512, Sal I Sequence) was used [231]. This final construct was also codon optimised for human expression and was synthesised by GeneArt as previously.

2.2.2 Agarose Gels

DNA reaction products or PCR products were separated by 1% agarose gel electrophoresis (100V, 40min) in 1x Tris-acetate-EDTA (TAE) buffer stained using SYBR Safe. 4µL of 6x loading dye was added to each sample before visualisation on a transilluminator. The size of bands was estimated by comparison to the DNA marker smart ladder.

2.2.3 Restriction Digests

Digests were usually incubated for 2h at 37ºC, unless the suggested incubation temperature for enzymes differed. Calf intestinal phosphatase (CIP) (1h, 37ºC) was added to minimise vector religation where digestion would be expected to generate complimentary "sticky ends". 20µL reactions were performed as outlined below. Reactions were vortexed to ensure thorough mixing of reagents before being pulse spun to sediment reactions prior to incubation.

1 µL	Total NEB Restriction Enzyme(s) (10,000U/mL)*
2 µL	NEB restriction buffer (10x)
2 µL	BSA (10x)
x µL	Plasmid DNA (0.5-1µg)
→20 µL	sterile distilled H_2O

* Double-digests were performed with 0.5µL each enzyme

2.2.4 Ligation Reaction

Digested DNA fragments were excised from agarose gels and purified using QIAgen MinElute gel extraction kits. Ligation was then performed overnight at 16ºC using the reactions and controls outlined below and in Jenner Protocol (J) 98 (all relevant Jenner Protocols can be found in Appendix One). Ligation reactions were stored at 4ºC once completed.

Reagent	Uncut control	No Insert control	No ligase control	Ligation Reaction
dH_2O	16 µL	16 µL	14 µL	9 µL
10x ligation buffer	2 µL	2 µL	2 µL	2 µL
Plasmid DNA	1 µL uncut	1 µL	1 µL	2 µL
Insert DNA	-	-	3 µL	6 µL
T4 DNA ligase	1 µL	1 µL	-	1 µL

2.2.5 Restriction cloning into MVA

To generate an MVA expressing OVA, this antigen was cloned into the MVA-GFP-tPA-Rh5 vector kindly provided by Dr S. Draper and originally produced by Dr. A. Douglas. Within this construct the human tPA leader sequence (GenBank Accession #K03021) preceded the antigen as the tPA leader sequence has been shown to assist the efficacy and immunogenicity of DNA malaria vaccines and is thought to aid the secretion of antigens by targeting them to the endoplasmic reticulum [232,233,234,235]. Antigen sequences were extracted from the GeneArt plasmid by Bgl II and Xho I double-digest. The shuttle vector, MVA-GFP2-tPA-Rh5 was digested with Bgl II and Xho I to remove the Rh5 antigen as outlined in Figure 2.1. DNA fragments were separated on a 1% agarose gel and DNA extracted from the gel using a QIAgen MinElute gel extraction kit. DNA from MVA-GFP2-tPA backbone was ligated to the OVA insert DNA overnight. Insert presence was confirmed by an analytical restriction digest. MVA shuttle vectors were then transfected and virus purified as described in 2.3.1.

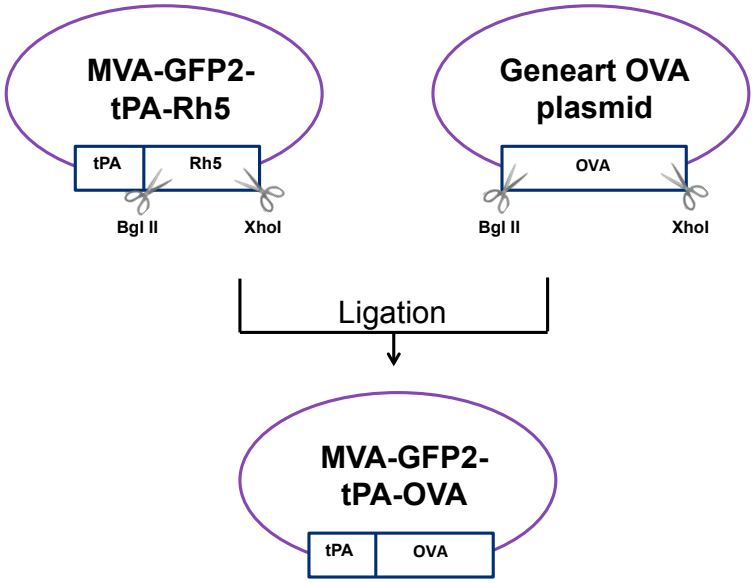

Figure 2.1 Cloning scheme for insertion of OVA antigen into MVA-GFP2-tPA.

2.2.6 Restriction cloning into AdHu5

The recombinant AdHu5 OVA was constructed using the ViraPower™ Adenoviral expression system from Invitrogen. This system uses the Gateway® technology to generate a recombinant adenovirus containing the gene of interest under the control of the promoter of choice. To generate an expression clone, a LR Clonase II mediated site-specific recombination reaction occurs between attachment L (attL) sites within an entry vector (containing the gene of interest) and attachment R (attR) sites within the destination vector (the adenovirus genome). The 1.9kbp CMV promoter (with regulatory element, enhancer and intron A), polylinker and BGH poly(A) transcription termination sequence from the DNA vaccine vector pSG2 were cloned into the entry vector

pENTR™4 by Dr S. Sridhar as previously described [236]. OVA was cloned into the pENTR™4.CMV.BGH entry vector from the MVA-GFP2-tPA-OVA shuttle vector by Kpn I and Eco RI double-digest. The entry vector was digested with Kpn I and Eco RI to allow insertion of the OVA antigen as outlined in Figure 2.2. DNA fragments were separated on a 1% agarose gel and DNA extracted from the gel using QIAgen MinElute gel extraction kits. DNA from the entry vector backbone was ligated to the OVA insert DNA overnight. The pENTR™4.CMV.BGH tPA-OVA entry vector was transformed into *E. coli* bacteria and the plasmid DNA prepared as described in 2.2.7 and 2.2.8.

The pENTR™4.CMV.BGH tPA-OVA entry vector was then recombined with the pAd/PL-DEST destination vector using site-specific LR Clonase II mediated recombination to construct the AdHu5 vaccine. The antigen construct was inserted into the 34.9kb E1- and E3-deleted adenovirus destination vector at the E1 site, between bases 519 and 2202 using the LR Clonase II enzyme reaction as per manufacturer's instructions outlined in J009. Reactions were terminated with proteinase K and transformed into *E. coli* bacteria and plasmid DNA prepared as described in 2.2.7 and 2.2.8. Insert presence was confirmed by an analytical restriction digest. Expression clones were then transfected and virus purified as described in 2.3.2.

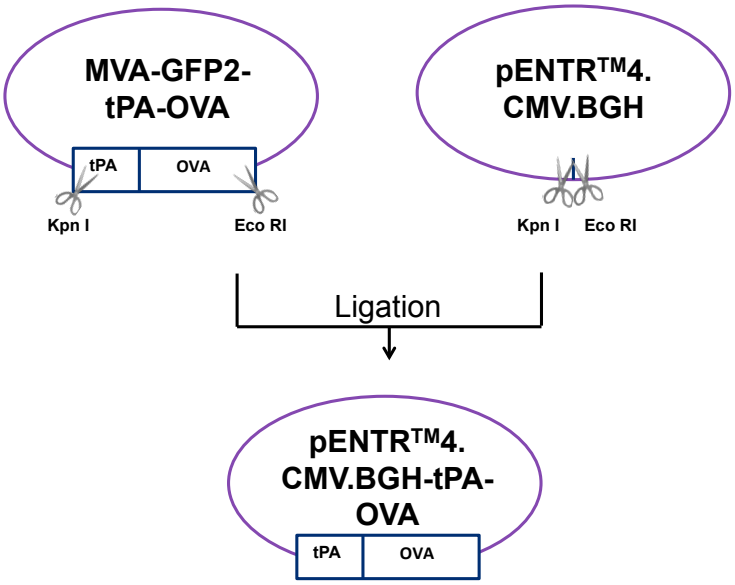

Figure 2.2 Cloning scheme for insertion of tPA-OVA into pENTR™4.CMV.BGH entry vector for site-specific recombination with the pAd/PL-DEST vector.

2.2.7 Bacterial transformation

50µl sub-cloning efficiency DH5α Escherichia coli were incubated on ice for 30 min with 2µl ligation reaction. Following this cells were heat-shocked at 42ºC for 45 seconds and incubated on ice for a further 2 min. Bacteria were plated onto LB agar containing selection antibiotics as required. For ampicillin resistance selection, bacteria were plated out immediately. For kanamycin or chloramphenicol resistance selection, bacteria were grown in 300µl S.O.C. medium at 37ºC (220rpm) for 1 hour prior to plating. Plates were incubated at 37ºC overnight before storage at 4ºC.

2.2.8 Plasmid DNA preparation

Individual colonies were selected and cultured overnight in 2-3mL LB medium plus selection antibiotics. The following day, plasmid constructs were purified by Qiagen QIAprep spin miniprep kits. The identity of DNA was confirmed using diagnostic restriction enzyme digests carried out as described in 2.2.3. Bacteria containing a correct version of each construct were amplified by incubation overnight in 150mL LB medium plus selection antibiotics. Following incubation a 1mL aliquot was removed. This was mixed with 1mL 50% glycerol in complete media to form a bacterial stock, which was stored at -80ºC for long-term use. DNA from the remaining 150mL culture was purified by Qiagen plasmid midi kits and stored at -20ºC. DNA concentrations were measured using a nanodrop.

2.2.9 GST protein purification

P. yoelii YM MSP-1_{19} was amplified by PCR and cloned into the glutathione S-transferase (GST)-fusion protein expression vector pGEX-2T (Amersham Biosciences, Bucks., UK) by Dr. S Draper as previously described [174]. This vector was transformed into Rosetta competent cells, which are designed to enhance the expression of proteins that use codons that are rare in *E. coli.* Following selection for chloramphenicol and ampicillin resistance, stocks were frozen at -80°C for long-term storage. To initiate protein production 2µL of the frozen Rosetta cell stock was added to a 100mL culture of terrific

broth plus chloramphenicol and ampicillin. This was then shaken overnight at 37ºC. The following day the culture was made up to 500mL with fresh antibiotic containing Terrific Broth and incubated as before for 1 hour. Protein expression was induced with by the addition of IPTG at a final concentration of 60µg/mL and cultures were incubated for a further 2 hours. Cells were harvested by centrifugation (6000g, 15min) and bacteria were lysed using BugBuster and Benzonase endonuclease as per manufacturer's instructions through vigorous vortexing. The suspension was centrifuged (15000g, 20min) and the pellet discarded. The lysate was ultracentrifuged (45000g, 1h, 4ºC) and the clarified extract was then loaded onto GST-affinity chromatography columns (GST-Bind Purification Kit) as per the manufacturer's instructions. Proteins were eluted using 10mM reduced glutathione, and collected in 1mL fractions. The protein concentration of fractions was measured using a nanodrop (protein A280) and fractions containing the majority of recombinant protein were stored at -20ºC. Proteins were identified by running an ELISA using positive serum.

2.2.10 Genotyping CD32b KO mice

To determine the genotype of CD32b KO mice (bred in-house) tail snips were collected from CD32b and WT (C57BL/6) mice. To extract DNA, 20µg of Proteinase K (Qiagen) diluted in digestion buffer (50mM Tris pH 8.0, 2mM NaCl, 10mM EDTA, 1% SDS) was added to each tail snip in a clean eppendorf tube and incubated at 55°C for 20 minutes. After vortexing samples thoroughly to break up the tail snip, tubes were incubated at

55°C for a further 20 minutes. 180µL of dH_2O was then added to each tube and samples were heated to 99°C to deactivate the Proteinase K for 5 min. After cooling samples were stored at -20°C until use. PCR reactions were carried out using the Expand High Fidelity PCR system from Roche in a final reaction volume of 50µL. Two separate mixtures of PCR reagents were prepared as outlined in Table 2.2 and scaled up for the desired number of samples. 25µL of Mix 1 and Mix 2 were immediately combined before thermal cycling in a G-storm Thermal Cycler at cycling conditions according to manufacturer's instructions. 5µL of extracted DNA was used in each PCR reaction. Primers were used as previously published [237] and are outlined in Table 2.3. A sample of known genotype was included as a positive control and a sample containing no template DNA was included as a negative control in each PCR run. PCR products were run as outlined in 2.2.2 using a 3% agarose gel. Expected band sizes were as follows: KO = 1 band of 232 bp, Heterozygote = 2 bands of 161bp + 232 bp and Wild type = 1 band at 161 bp. All animals used in experiments were determined to be KOs. PCRs were carried out by D. Llewellyn.

Mix 1	1 reaction
Deoxynucleotide mix, 10 mM of each dNTP	1µL
Primer 1-3	1.5µL of each
Template DNA	5µL
dH_2O	14.25 µL
Mix 2	1 reaction
Expand High Fidelity buffer, 10x conc. without $MgCl_2$	5µL
Expand High Fidelity enzyme mix	0.75µL
25mM $MgCl_2$ (final 4mM)	25µL
dH_2O	11.25 µL

Table 2.2 Components of each PCR reaction

Primer	Sequence
oIMR0618	CTC GTG CTT TAC GGT ATC GCC
oIMR0619	AAA CTC GAC CCC CCG TGG ATC
oIMR0620	TTG ACT GTG GCC TTA AAC GTG TAG

Table 2.3 Primers used for genotypic analysis of CD32b KO and WT C57BL/6 mice

2.3 Methods – Molecular Virology

All molecular virology was kindly carried out by the Jenner Institute Vector Core Facility under the supervision of Dr. A Turner and Dr. N Green by C. McCarthy, S. Elias, J. Matthews, A. Vaughan and C. Schultz according to Jenner Protocols (Appendix One).

2.3.1 MVA Transfection and Purification

Linearisation of the MVA shuttle vector was carried out according to J017. Chick Embryo Fibroblasts (CEFs) were maintained as described in J013. When the cells were approximately 90% confluent, they were infected with MVA expressing Red Fluorescent Protein (RFP) as outlined in J176. During this time a transfection mix containing the linearised shuttle vector was prepared and added to the CEFs containing MVA-RFP. This enabled recombination, whereby GFP replaces RFP at the TK locus in the MVA genome. Following incubation with virus for 2 days, cells were prepared for cell sorting as per J131. CEFs containing recombinants expressing GFP were separated from those infected with the original MVA-RFP by fluorescence using a MoFlo cell sorter operated by D. Worth, Manager of the Jenner Institute Flow Sorting Facility. The lysate generated by cell sorting was used to again infect CEFs as in J016 and successful recombinant MVAs

containing antigen were selected by repeated rounds of plaque picking as in J018. Once purified successful recombinants were obtained, the virus was bulked up as in J019 and an Identity and Purity analysis was carried out by PCR as detailed in J079. The virus titre in plaque forming units (pfu) was determined as in J111.

2.3.2 Adenovirus Transfection and Purification

Adenovirus transfection was carried out as described in J004. In brief, recombinant adenovirus plasmid (pAD) was linearised to expose 5' and 3' inverted terminal repeats using Pac I. OptiMEM was then added to the linearised plasmid. The remaining restriction enzyme mix was run on an agarose gel as outlined in 2.2.2 to confirm linearization. 300μl of 1:10 lipofectamine in OptiMEM was then prepared and mixed with an equal volume of linearised plasmid. The mixture was incubated for at least 20 minutes at room temperature (RT). This mixture was then added drop-wise onto pre-prepared 293 cell monolayers. 293 cells are permissive for the production and amplification of replication-incompetent adenovirus as they provide the E1 gene product *in trans*. Cells were incubated at 37°C, 5% CO_2 in a humidified incubator and monitored daily for cytopathic effect (CPE). Fresh media was added to cells every 2-3 days. Cells were harvested once complete CPE was evident. Recombinant adenovirus was purified by density centrifugation using a caesium chloride (CsCl) gradient as described in J005. Virus yield (virus particles in vp) was determined by both UV

spectrophotometry as in J106 and single-cell immunostaining (infectious units in ifu) as described in J006.

2.4 Methods – Immunology

2.4.1 Animals

All procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence (PPL 30/2414) and were approved by the University of Oxford Animal Care and Ethical Review Committee. All mice were housed in the Wellcome Trust Centre for Human Genetics Animal Facility (Functional Genomics Facility (FGF)) under Specific Pathogen Free (SPF) conditions. Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (Harlan Laboratories, Oxfordshire, UK) were 6-10 weeks old at the start of experiments and obtained from either Harlan (UK) or Charles River (UK). OT-II mice (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) (H-2^b) were obtained from Charles River (UK). CD32b KO mice (C57BL/6 background) were kindly provided by Prof. Richard Pleass (Liverpool School of Tropical Medicine, UK) and bred in the FGF by Dr. S. Draper. Both male and female knock-out mice were used for experiments depending on availability and were randomised into treatment and control groups as applicable.

2.4.2 Anaesthesia

Mice were anaesthetised before each immunisation using inhalation anaesthetic comprised of a mixture of isofluorane (2-3.5%) and oxygen (1-2l/min). Alternatively mice were anaesthetised using a mixture of 75mg/kg Ketaset™ (ketamine hydrochloride) plus 1mg/kg Domitor™ (medetomidine hydrochloride) diluted in endotoxin-free water administered intraperitoneally (i.p.). C57BL/6 mice were recovered from anaesthesia with 100µL i.p. of a 1:10 solution of Antisedan™ (atipamezole hydrochloride) diluted in endotoxin-free water.

2.4.3 Route and dose

❖Intramuscular (i.m.) vaccinations were administered in a volume of 50µL with vaccine divided equally into each *musculus tibialis* unless otherwise specified using 26G needles.

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

❖Intraperitoneal (i.p.) injections were administered in a volume of 100µL using a 28G needle.

❖Subcutaenous (s.c.) injections were administered into the scruff of the neck in a volume of 100µL using 26G needles.

Immunisation doses and intervals varied between experiments and are explained in the text and figure legends. The standard dose of vaccine was a total of 50µL of vaccine i.m. and 100µL of vaccine s.c. For experiments outlined in Chapter 4, due to the concentration of protein used, vaccine was administered in a volume of 100µL. Immune responses were assayed two weeks after each immunisation and before the protein vaccine boost in AP regimes in each experiment.

2.4.4 Viral Vectored Vaccines

Viral vectored vaccines were prepared in sterile, endotoxin free PBS. Co-administered viral vectored vaccines were made up in the same syringe. Viral vectors were dosed on vp. When using subsequent preparations of the same virus equivalent doses in ifu were administered. Replication deficient viral vectored vaccines (both AdHu5 and MVA) were made as outlined in the Molecular Biology and Molecular Virology section of this chapter. The production of AdHu5 OVA and MVA OVA is outlined in detail in the Molecular Biology section of this chapter. All other vaccines are published and the relevant references are listed in Table 2.4.

Construct	Transgene	Reference
AdHu5 & MVA	*P. yoelii* MSP-1_{42}	[174]
AdHu5	*P. yoelii* MSP-1_{33}	[174]
AdHu5 & MVA	Ovalbumin	Described here
AdHu5 & MVA	*P. falciparum* MSP-1 (PfM128)	[169]
AdHu5	*P. falciparum* EBA-175 Region F2 (PfEBA175_F2)	[238]
AdHu5 & MVA	*P. vivax* Duffy Binding Protein Region 2 (PvDBP_RII)	Described here

Table 2.4 Viral Vectored Vaccines used throughout this study.

2.4.5 Protein Vaccines

For protein vaccinations Grade VII Ovalbumin (≈45kDa) was obtained from Sigma Aldrich, UK. *P. yoelii* MSP-1_{19}-GST fusion protein was made as previously described using an *E. coli* expression system as in 2.2.9 [174]. *P. falciparum* EBA-175 (F2 region) and MSP-1_{19} protein as well as *P. vivax* DBP_RII were produced as previously described [83,239,240] and were a kind gift from Dr. C. Chitnis (ICGEB, India).

2.4.6 LAL assay

Endotoxin levels were measured for each protein using the Limulus amebocyte lysate (LAL) assay (Cambrex, UK) according to the manufacturer's instructions.

2.4.7 Adjuvant formulation

For protein vaccines the protein fraction was made up in low phosphate PBS (<5mM) and formulated with adjuvant as summarised in Table 2.5. In brief: Abisco®100 (Isconova, Sweden) (12µg/dose) was gently mixed with antigen in PBS. Adju-Phos® (Brenntag, Denmark) (75µg Al^{3+}/dose) and Alhydrogel® (Brenntag, Denmark) (75µg Al^{3+}/dose) were combined with antigen in PBS and spun at 4°C for 30 minutes before administration. CoVaccine HT™ (a novel proprietary vaccine adjuvant of Protherics Medicines Development, a BTG Company, London, UK) was mixed gently 1:1 with antigen in PBS (2mg sucrose fatty acid sulphate ester (SFASE)/dose). Complete and Incomplete Freund's Adjuvant (C/IFA) (Sigma, UK) were mixed vigorously through vortexing 1:1 with antigen in PBS. CFA was used only once and mice were subsequently boosted with IFA. Immunisations were administered s.c. for these adjuvants. Montanide®ISA720 (Seppic, France) and antigen in PBS were emulsified using a homogenizer under sterile conditions at 25,000rpm for 6 minutes keeping the sample on ice in a ratio of 3:7 (Antigen:Adjuvant). Adjuvants based on a stable emulsion (SE) with different TLR agonists incorporated into the emulsion (Infectious Disease Research Institute, USA) (20µg/dose) were mixed with antigen in PBS and vortexed for 30 seconds. All vaccines were kept on ice until administration. Where viral vectors were co-administered with adjuvants, the viral vector was diluted in the protein fraction of the

vaccine. Formulations for the various adjuvants were determined from the literature and manufacturer's instructions.

Adjuvant	Type	Formulation	Status
Adju-Phos®	Aluminium Phosphate	1.5mg/mL – rotated for 30 min at 4°C	L
Alhydrogel®	Aluminium Hydroxide	1.5mg/mL – rotated for 30 min at 4°C	L
Abisco®100	Phospholipid, cholesterol and saponin complex. Contains a mixture of Matrix A (QS7) and C (contains QS21) fractions which are purified from Quil A extracts	12µg/dose – mix by shaking	C
CoVaccine HT™*	Sucrose fatty acid sulphate esters (SFASE) immobilised on the oil droplets of a submicron emulsion of squalane in water	1:1 – gently mixed	C
EM01	Stable oil in water emulsion (SE)	20µg agonist/dose – vortex for 30 s	R
EM05	SE + TLR 4 (GLA)	20µg agonist/dose – vortex for 30 s	C
EM012	SE + TLR4/7/8 (GLA & Iziquimod)	20µg agonist/dose – vortex for 30 s	R
EM014	SE + TLR 4/9 (GLA & CpG ODN 1826)	20µg agonist/dose – vortex for 30 s	R
EM020	SE + TLR 4/7/8/9 (GLA & Iziqumod & CpG ODN 1826)	20µg agonist/dose – vortex for 30 s	R
Freund's adjuvant*	Non-metabolisable oils, Complete Freund's adjuvant contains mycobacterial derivatives.	1:1 – vortex thoroughly	E
Montanide® ISA720*	Squalene and refined emulsifier/surfactant based on mannide oleate	3 Ag:7 ISA, emulsified 6 min on ice using a T10 ULTRA-TURRAX® at 25,000rpm	C

Table 2.5 Formulation of Adjuvants used throughout this study.

* = Adjuvant dose dependent on final volume of vaccine. L: licensed, C: clinical development, R: pre-clinical/research stage, E: experimental

2.4.8 Adjuvant Safety

All adjuvants investigated were found to be safe and well tolerated at the doses used. Piloerection at the injection site was observed after using C/IFA which is a common reported occurrence following vaccination with this adjuvant. Swelling was also reported around the injection site following administration of CoVaccine HT™ in BALB/c mice in a final volume of 100µL. This swelling was transient and subsequent vaccinations were reduced to a total volume of 60µL.

2.4.9 Adjuvant-Antigen Adsorption

To determine the level of adsorption of recombinant protein to Alhydrogel® (aluminium hydroxide) and Adju-Phos® (aluminium phosphate) a protein specific capture ELISA was performed. A 0.5mL volume of recombinant protein vaccine formulated in Alhydrogel® and Adju-Phos® was prepared as previously described for each protein tested. A 0.5mL dose of recombinant protein vaccine formulated in low-phosphate PBS without Alhydrogel® and Adju-Phos® were also prepared as well as adjuvant-only aliquots. All vaccines were centrifuged at 4000rpm for 10 minutes. 96 well Nunc-Immuno Maxisorp plates were coated with 50µL of vaccine, protein and adjuvant supernatant and serially diluted three-fold down the plate and allowed to adsorb at RT overnight. The following day plates were washed in PBS/T and blocked with PBS/T containing 10% skimmed milk. Sera from mice positive for the recombinant protein of interest was diluted 1:100 and

50µL were added to each treatment well. Naïve serum was added to duplicate wells for each treatment. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) diluted 1:5000 in PBS/T. If adsorption of recombinant protein to aluminium hydroxide or aluminium phosphate occurs then centrifugation should sediment protein bound to aluminium-based adjuvants and antigen-specific antibody responses as detected by ELISA should consequently be lower than in the protein only vaccine control. Using this method OVA was found to adsorb to Alhydrogel® by 89%. OVA only adsorbed to Adju-Phos ® by 9% and *P. yoelii* MSP-1_{19}-GST adsorbed to Adju-Phos ® by 40% (data not shown).

2.4.10 Whole IgG ELISA

Blood was collected from tail veins into Microvette tubes, or by cardiac puncture into eppendorf tubes following cervical dislocation. It was allowed to clot at 4°C for > 12 hours and centrifuged at 13000rpm for 4min. Serum was removed, transferred into clean eppendorf tubes and stored at -20°C until use. Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated at a concentration of 2µg/mL with protein overnight at room temperature. Plates were washed with PBS/T and blocked with 10% skimmed milk powder in PBS/T. Sera were diluted 3-fold down the plate. Plates were incubated for 2 hours at RT and then washed as before. Goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma Aldrich, UK) was added for 1 hour at RT.

Following a final wash, *p*-nitrophenylphosphate at 1mg/mL diluted in diethanolamine buffer was used as a developing substrate (Pierce, UK). Optical density (OD) was read at 405nm using a Model 550 Microplate Reader (Bio-Rad, UK). Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD405 for serum from a naïve mouse. A standard positive serum sample and naïve serum sample were added as controls for each assay. Naïve mouse serum was negative for antigen-specific responses to all antigens. *P. yoelii* MSP-1_{19} specific antibodies, following immunisation of mice with GST-PyMSP-1_{19}, were measured using *P. yoelii* MSP-1_{19}-IMX108 protein which does not contain the GST-tag present in the protein used for immunisation [241]. Mouse serum is negative for responses to the IMX108 C4bp domain (data not shown). *P. falciparum* MSP-1_{19}-specific antibody responses were measured using *P. falciparum* MSP-1_{19}-GST (QKNG allele) made as described previously [169]. Responses to GST following viral vectored vaccines (which do not contain GST) have previously been shown to be negative [174]. Responses to GST following vaccination with *P. yoelii* MSP-1_{19}-GST are weakly positive (data not shown).

2.4.11 Isotype ELISA

To detect antigen-specific IgG1 and IgG2a responses, Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated at a concentration of 2µg/mL with protein

overnight at room temperature as before. A standard curve comprised of isotype purified IgG1 or IgG2a (eBioscience, UK) was added in duplicate to separate plates at a concentration of 20µg/mL and diluted 3-fold. A positive control of monoclonal antibody (mAb) at a dilution of 1:6075 for IgG1 and 1:2025 for IgG2a was also added to each separate plate. After blocking, serum diluted in PBS/T was added in duplicate to the plate for 2 hours at RT. Plates were then washed and either biotin anti-mouse IgG1 or IgG2a (BD biosciences) at a 1:5000 dilution were to the test plates. Following a 30 minute incubation with Extravidin Alkaline Phosphatase (Sigma Aldrich, UK) plates were developed using the same reagents as for total IgG ELISAs. Plates were developed until the mAb positive control reached an OD405 of 1.0. This point was defined as 1 Isotype unit (IU) and IUs were read off the standard curve similar to previously published methods [242]. Samples were diluted to fall on the linear part of the curve. Low titre samples from the experiments using OVA were diluted 1:100 and also developed until the mAb positive control reached an OD405 of 1.0. Isotype responses for these samples are reported as OD405.

2.4.12 Avidity ELISA

Antibody avidity was assessed using a sodium thiocyanate (NaSCN)-displacement ELISA. Whole IgG titres were determined for each sample and sera were individually diluted to give a titre of 1:100 and plated at 50 µL/well in 16 wells of a 96 well plate. Following incubation and washing, an ascending concentration of the chaotropic agent NaSCN was

added down the plate (0–7 M NaSCN). Plates were incubated for 15 min at RT before washing and development as for total IgG. The intercept of the OD405 curve for each sample with the line of 50% reduction of the OD_{405} in the NaSCN-free well for each sample (i.e. the concentration of NaSCN required to reduce the OD_{405} to 50% of that without NaSCN) was used as a measure of avidity.

2.4.13 Peptides

Peptides used in cellular assays were commercially synthesised by Protein Peptide Research or Sigma Aldrich, UK and dissolved in DMSO at a concentration of 20mg/mL and stored at -20°C. Working stocks were further diluted to 1mg/mL in PBS and stored at -20°C. Pools of peptides were made from 20mg/mL stocks and stored at a final concentration per peptide of 1mg/mL. The peptides used through this study can be found in Table 2.6. T cell responses to P. vivax Duffy Binding Protein (Region 2) (DBP_RII) were assessed by stimulation with recombinant protein (peptides were not available for this antigen) used at the same final concentration as peptides.

Epitope	Relevant Antigen(s)	Mouse strain (H2)	CD8/ CD4	Peptide Sequence	Refs
OVA (αα257-264)	OVA	BALB/c (H-2^b)	CD8	SIINFEKL	[243]
OVA (αα323-339)	OVA	BALB/c (H-2^b)	CD4	ISQAVHAAHAEINEAGR	[243]
OVA (αα265-280)	OVA	C57/BL6 (H-2^b)	CD4	TEWTSSNVMEERKIKV	[244]
Py MSP-1 $_{19}$ OLP	MSP-1	C57/BL6 (H-2^b)	CD4	15mers, overlapping by 10αα, which corresponded to PyMSP1 αα 1654-1757 – 19 peptides in total	[174]
Py MSP-1_{33} OLP	MSP-1	BALB/c (H-2^d)	CD8 & CD4	15mers, overlapping by 10αα, which corresponded to PyMSP1 αα 1394-1663 – 52 peptides in total	[174]
B80 (αα1473-1481)	Py MSP-1_{33}	BALB/c (H-2^d) and C57/BL6 (H-2^b)	CD8	YVIRNPYQL	[236]
M90 or M91 (αα 1354-1373)	PfM128	BALB/c (H-2^d)	CD8	NKEKRD**KFLSSYNYI** D**KFLSSYNYI**KDSID	[169]
M188 (αα1550-1564)	PfM128	C57BL/6 (H-2^b)	CD4	DKIDLFKNPYDFEAI	[169]
M100 or M101 (αα1404-1423)	PfM128	C57BL/6 (H-2^b)	CD8	INDKQG**ENEKYLPF**L GENE**KYLPFLNN**IET	[169]
M149 (αα1355-1369)	PfM128	C57BL/6 (H-2^b)	CD8	YRSLKKQIEKNIFTF	[169]
M86 (αα1334-1349)	PfM128	C57BL/6 (H-2^b)	CD8	IPYKDL**TSSNYVVK**D	[169]

Table 2.6 Peptides used throughout this study.

Predicted minimal epitopes are shown in bold.

2.4.14 Splenocyte preparation

Mice were sacrificed by cervical dislocation and spleens removed by dissection. Spleens were crushed in PBS, passed through a sterile 70μm cell strainer and centrifuged (500g, 5min). Supernatants were discarded and pellets resuspended in ACK lysis buffer for 5 min before addition of 25mL PBS. The mixture was immediately centrifuged again (500g, 5min) and resuspended in 5mL complete medium (D10). Splenocytes were counted using a CASY counter (Schärfe Systems, Germany) and resuspended at appropriate concentrations in complete medium

2.4.15 Peripheral Blood Mononuclaer cell (PBMC) preparation

8-10 drops of blood were collected from the tail vein of mice into 200μL 10mM EDTA. 1mL of ACK lysis buffer was added and cells were centrifuged for 4 min (4000rpm). Supernatants were discarded and pellets were again resuspended in 1mL ACK lysis buffer and centrifuged as before. Supernatants were removed and the cell pellet was re-suspended in approximately 500μL of D10 (exact volume dependent on subsequent protocol used).

2.4.16 Draining Lymph Node preparation

Mice were sacrificed and the draining inguinal lymph nodes (LNs) were excised. Lymph nodes were collected into 2mL PBS and subsequently crushed in a 6-well plate. Cells were recovered by passage through a 70µm strainer (Corning) and centrifuging at 1350 rpm for 5 minutes. Supernatants were discarded and pellets resuspended in FACS buffer for staining.

2.4.17 IFN-γ ELISpots

MAIP ELISpot plates were coated with anti-mouse-IFN-γ mAb at 5µg/mL in carbonate-bicarbonate buffer. Plates were blocked with D10. PBMCs were isolated from peripheral blood and spleens as described previously. 50µL of PBMCs harvested from the blood were plated into duplicate wells. 50µL peptide diluted in medium plus 0.25 x 10^6 naïve splenocytes were added to test wells. Medium and naïve splenocytes only were plated into the negative control wells. Spleen cells were re-suspended at 1x10^7cells/mL and 50µL of cells were plated in duplicate. 50µL peptide diluted in complete medium was added to test wells and complete medium alone was added to control wells. Peptides were added at a final concentration of 5µg/mL. Plates were incubated at 37°C, 5% CO_2 in a humidified incubator for ~18h. Plates were then washed 6 times with PBS and incubated for 2 hours with biotinylated anti-mouse-IFN-γ mAb (Mabtech, UK) diluted to 1µg/mL in PBS, followed by an incubation with a streptavidin alkaline phosphatase

polymer diluted to 1µg/mL in PBS. Spots were developed by addition of colour development buffer, and counted using ELISpot software (Autoimmun Diagnostika, Germany). Results are expressed as spot forming cells (SFC) per million cells. Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

2.4.18 Intracellular Cytokine Staining (ICS)

To analyse the percentage of cytokine producing peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes, cells were collected from the spleen and blood as previously described. Cells were restimulated for 5 hours at 37°C in the presence of Brefeldin A and peptides at a final concentration of 1µg/mL per peptide. Following restimulation, plates were stored at 4°C overnight. Cells were transferred to V-bottom plates and centrifuged (500g, 5 min). Supernatants were discarded and cells resuspended in anti-mouse CD16/CD32 (Fc block) for 15 minutes in the dark. Following a wash in FACS buffer, cells were surface stained with anti-CD8α PerCP-Cy5.5 and anti-CD4 PB for 30 minutes in the dark. After permeabilisation using Cytofix/Cytoperm, cells were washed and stained intracellularly with anti-IFN-γ APC, anti-TNF-α FITC and anti-IL-2 PE for 30 minutes in the dark. Samples were resuspended in 1% PBS Formalin and acquired on a LSRII and analysed using FloJo (TreeStar Inc.). Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis. To assess polyfunctionality

the Boolean gate platform in FlowJo was used with individual function gates to create all possible combinations of response patterns. Pestle version 1.3 (Mario Roederer, National Institutes of Health) was used to prepare data for the subsequent analysis of response profiles. The data analysis programme, simplified presentation of incredibly complex evaluations (SPICE) (Mario Roederer, National Institute of Health) was used to analyse data and to generate graphical representations of functional T cell responses using background-deducted data.

2.4.19 Germinal Centre Stain

To stain for Germinal Centres in the draining (inguinal) LNs and spleen, these organs were processed as described in 2.4.14 and 2.4.16. Cells were transferred to V-bottom plates and centrifuged (500g, 5 min). Supernatants were discarded and cells were surface stained with anti-CD95 PE, anti-B220 PeCy7 and anti-GL-7 AlexaFluor 647 for 30 minutes in the dark. Following this incubation, cells were washed twice and resuspended in 1% PBS Formalin and acquired on a LSRII and analysed using FloJo (TreeStar Inc.).

2.4.20 CXCR5+ Stain

To stain for CXCR5+ cells, the draining (inguinal) LNs were excised and processed as described in 2.4.16. Cells were transferred to V-bottom plates and centrifuged (500g, 5

min). Supernatants were discarded and cells resuspended in Fc block for 15 minutes in the dark. Following a wash in FACS buffer, cells were surface stained with anti-CD4 PB, anti-CD8α PerCP-Cy5.5 and anti-CXCR5 PE. Following this incubation, cells were washed twice and resuspended in 1% PBS Formalin and acquired on a LSRII and analysed using FloJo (TreeStar Inc.). The CXCR5+ gate was set using a Fluorescence Minus One (FMO) control, which contained anti-CD4 and anti-CD8 antibodies, but not the anti-CXCR5 antibody.

2.4.21 CD4+ T cell Depletion

$CD4^+$ T cells were depleted using an anti-CD4 GK1.5 (rat IgG2a) mAb purified using protein G affinity chromatography from hybridoma culture supernatants as previously described [174]. Mice were injected i.p. with 200µg of mAb diluted in PBS on days -2, -1 and 0 with respect to day of challenge on day 0. Control mice were given IgG purified from normal rat serum. The degree of $CD4^+$ T cell depletion was assessed by flow cytometry using anti-CD4 PB (clone RM5.4), anti-CD3ε APC and anti-CD8α PerCP-Cy5.5 in the PBMCs of vaccinated depleted mice and unvaccinated control mice on day +1 with respect to day of challenge. Depletion was assessed on CD3ε+ cells.

2.4.22 Antibody secreting cell assay

Cells isolated from the spleen and bone marrow were tested for the presence of antibody secreting cells (ASCs) as described previously [245]. Spleens were processed as described 2.4.14. Bone marrow was flushed from dissected femurs with media using a 26G needle and passed through a sterile 70µm cell strainer. Nitrocellulose bottomed 96-well MAIP ELISpot plates were coated with 5µg/mL of protein antigen of interest and incubated overnight at 4°C. Plates were washed twice with PBS and blocked for one hour at 37°C, 5% CO_2 with D10. 0.5 x 10^6 cells were plated onto the pre-coated ELISpot plate per replicate well and serially diluted. Plates were incubated for 5 hours at 37°C, 5% CO_2. Following incubation plates were washed twice with PBS and incubated overnight at 4°C with biotinylated anti-mouse γ-chain specific IgG antibody. Assays were developed using colour developing agents as in 2.4.17 that were filtered through a 0.2µm syringe filter. ELISpot plates were counted using the AID plate reader software (Autoimmun Diagnostika, Germany) and counts were visually confirmed. Antibody forming spots were relatively large, spherical in size with "fuzzy" granular edges.

2.5 Parasitology

2.5.1 Parasite Strains

Lethal YM *P. yoelii* parasites were originally provided by Dr. G. A. Butcher, Imperial College London. pRBC were stored by cryopreservation of parasite-infected blood with an equal volume of ice-cold 20% DMSO in blood-stage culture medium. 100µL aliquots were snap frozen in liquid nitrogen and stored at -196°C for later use.

2.5.2 Preparing Thin-blood Smears

Thin-blood smears were prepared on glass slides from a drop of blood obtained from a tail snip. Blood was air-dried, fixed in methanol for 30 seconds and stained in 5% Giemsa diluted in water for at least 30min. Slides were then allowed to air-dry.

2.5.3 Quantifying Blood-stage Parasitaemia

Thin-blood smears were examined on a light microscope at 100x under oil immersion. The percentage of parasitised RBCs (pRBCs) was calculated at a monolayer region of the thin blood smear. The number of total RBC and the number of pRBCs were counted and the % of pRBCs (parasitaemia) was calculated. The number of fields of view counted was dependent on the % parasitaemia. The field of view counted had approximately 500

RBCs. If the parasitaemia was around 1%, one field of view was counted. If the parasitaemia was < 1% and > 0.1%, five fields of view were counted; and if the parasitaemia was < 0.1%, 50 fields were counted. If the mice were anaemic and a field of 500 parasites could not be found, then several fields were counted.

2.5.4 Parasitised Red Blood Cell Challenge

4-5 days prior to challenge, a frozen aliquot of parasite-infected blood was thawed and injected immediately i.p. into a donor mouse. On the day of challenge, 5μl donor mouse blood was collected into 995μl counting solution and a further drop of donor mouse blood was used to make a thin blood smear as in 2.5.2 and the parasitaemia quantified as described in 2.5.3. The concentration of RBCs in the counting solution was calculated using an Improved Neubauer Haemocytometer counting 5 central squares. Using these calculations, the number of pRBCs/mL of donor blood was determined. Blood from the donor mouse was diluted accordingly in sterile PBS to give the required number of pRBCs/mL usually $1x10^6$ pRBC/mL (unless otherwise stated in the results section). Immunised and naïve control mice were infected i.v. with 100μl i.e. 10^5 pRBC. Mice were ear-tagged to enable identification. Their health status was monitored twice daily and slides were taken from day 3 onwards. The parasitaemia of challenged mice was determined as outlined in 2.5.3. When mice reached >70% blood-stage parasitaemia mice were sacrificed.

2.5.5 *P. vivax* Binding Inhibition Assay

A binding inhibition assay was carried out to study the ability of antibodies raised in mice against the *P. vivax* Duffy Binding Protein (PvDBP_RII) to inhibit the binding of PvDBP_RII to its receptor, the Duffy antigen receptor for chemokines (DARC). This assay was carried out by R. Shakri (ICGEB, India), with serum generated from the work described in this thesis. Each serum sample was tested at dilutions of 1:1000, 1:2000 and 1:4000. Sera were pre-incubated with 0.025µg/mL of recombinant PvDBP_RII (Sal I strain sequence) for 1h at RT. Serum was then applied to ELISA plates pre-coated with recombinant DARC (nDARC-Fc; N-terminal extracellular 60 amino acids of DARC fused to Fc region of human IgG) protein at 1µg/mL in sodium bicarbonate buffer overnight at 4°C. Bound PvDBP_RII was detected with rabbit polyclonal anti-PvDBP_RII sera (1:2500 dilution) and HRP-conjugated goat anti-rabbit IgG antibodies (1:5000 dilution) [239]. A standard curve, generated using PvDBP_RII at a maximum concentration of 0.025µg/mL, was used to convert OD values into amount of protein bound in each well, thus giving a read-out of binding inhibition. A positive control of serum raised in mice against PvDBP_RII using Freund's adjuvant and a negative control of naïve mouse serum samples were included in each run.

2.6 Statistics

All statistical analyses were carried out using Prism version 5 (Graphpad, USA). Normality of data distribution was assessed using the Kolmogorov Smirnov normality test. All ELISA titres were $\log_{10}$ transformed prior to analysis. For non-parametric data a Kruskal-Wallis test with Dunn's multiple comparison post-test was used to compare more than two groups. A one-way ANOVA was used for multiple comparisons of parametric data with Bonferroni's multiple comparison post-test for comparison of specific groups. An un-paired t-test was used to compare the means of two groups for parametric data and a Mann-Whitney test was used for non-parametric data. A two-way ANOVA with Bonferroni's multiple comparison post-test was used to explore the effect of two variables. Correlations were tested using Spearman's rank correlation. Where appropriate, fold changes in ELISA titre, with 95% CI of the mean, are reported. Area under the curve (AUC) analyses were performed on parasite density data. $p<0.05$ was considered significant (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Chapter 3

Comparative Screening of Adjuvants using the Model Antigen Ovalbumin

3 Comparative Screening of Adjuvants Utilising the Model Antigen Ovalbumin

3.1 Introduction

The use of vaccines has been instrumental in the prevention and control of many infectious diseases. Despite the creation of several efficacious vaccines such as those against smallpox and yellow fever, highly effective vaccines are still lacking for diseases such as malaria and TB which cause substantial morbidity and mortality each year [181]. Several strategies have been employed towards the development of novel vaccines aimed at these diseases with most focus being placed on subunit vaccines, particularly for vaccines targeting the blood-stage of malaria [246]. These subunit vaccines are often aimed at inducing antibody responses and have traditionally comprised recombinant proteins formulated with adjuvants to improve their immunogenicity. However, despite encouraging pre-clinical results many experimental adjuvants have unacceptable safety profiles in clinical trials [51,52,53,54] and to date only six adjuvants have been licensed for use in humans. These adjuvants include aluminium salts/alum (aluminium phosphate and aluminium hydroxide), the oil-in-water emulsion MF59 (from Novartis), virosomes, as well as the AS03 and AS04 adjuvant platform created by GlaxoSmithKline [46]. Most currently licensed adjuvants predominantly induce the humoral arm of the immune response, and may therefore be of limited use for diseases, such as TB and malaria,

where cellular immunity may be required as an important contributor to protective immunity [47,48]. Similarly, the lack of access to many promising adjuvants developed by some companies has had an adverse effect on vaccine development for difficult diseases, such as TB and malaria, where there is limited commercial interest and very strong immune responses are required for protection. This lack of accessibility and knowledge about the formulation of such adjuvants means that the development of effective human-compatible adjuvants for such diseases remains an urgent priority. Numerous experimental adjuvants are thus being developed that are aimed at inducing strong antibody and T cell responses including TLR agonists and novel emulsions [247]. However, it is unclear whether these adjuvants will still demonstrate reactogenicity profiles that are acceptable for vaccine licensure.

Viral vectored vaccines, although not without their own developmental and regulatory challenges, have been explored as another avenue to generate potent immune responses through subunit vaccination [248]. For example, sequential immunisations of recombinant adenovirus human serotype 5 (AdHu5) and modified vaccinia virus Ankara (MVA) vectors, encoding the malaria blood-stage antigen merozoite surface protein-1 42-kDa region (MSP-1_{42}), have been shown to generate strong T cell responses as well as high-titre antibodies that are protective against both a lethal *P. yoelii* sporozoite and blood-stage challenge [148,174]. The ability of viral vectors to induce strongly both the

humoral and cellular arms of the immune system has led to their use in various heterologous prime-boost strategies [168,169,177,178,249].

Adenoviral prime – protein boost (AP) regimes, whereby the two leading subunit vaccine platforms are combined, have more recently been shown to induce improved antibody responses compared to the use of either strategy on its own. Douglas *et al.* have demonstrated in mice that this AP immunisation strategy can lead to improved antibody responses, with a moderate T cell response induced by the adenovirus, when using *P. falciparum* MSP-1 vaccines [177]. These antibody responses were found to be more consistently primed by an adenoviral vector and also induced a more cytophilic antibody response dominated by IgG2a. In agreement with these murine data, non-human primate studies of similar regimes, for candidate malaria and HIV-1 vaccines, have also shown particular promise [178,179,180].

The studies described in this chapter initially sought to compare the potency of several promising adjuvants (both pre-clinical and clinically tested/approved) in a head-to-head manner, in order to provide useful comparative immunogenicity data on leading adjuvant formulations when administered three times with a protein (PPP regimes). This work was extended to carry out a detailed characterisation of the immunogenicity of AP regimes in comparison to three shot protein-in-adjuvant (PPP) regimes. The model antigen ovalbumin (OVA) was initially used to assess the immunogenicity of adjuvants in

these two regimes. This antigen was chosen because it is well characterised, easily available as a pure protein and allows for the assessment of both antibody and T cell responses in C57BL/6 mice, which have both CD8+ and CD4+ T cell epitopes [243,244]. To this effect OVA has been extensively used in the world of adjuvant development [91,250,251,252].

The adjuvants selected for investigation comprise both pre-clinical and clinically/tested approved adjuvants that are relatively readily available and easily accessible to the infectious disease vaccine field and are outlined in chapter 1. In brief, aluminium-based adjuvants Adju-Phos® (based on aluminium phosphate) and Alhydrogel® (based on aluminium hydroxide) comprise clinically licensed adjuvants which are components of several licensed vaccines. Though they have an excellent safety profile they induce predominantly Th2-type antibody responses [75]. Freund's adjuvant, Montanide®ISA720 and Stable emulsion (SE) are emulsion adjuvants based on mineral or metabolisable oils. Montanide®ISA720, a water-in-oil emulsion, is in clinical development and has been used in several Phase I trials, where it induced potent antibody responses [80,81]. However, significant and unacceptable levels of reactogenicity have also been observed with this adjuvant [52,84]. The SE adjuvants, based on an oil-in-water emulsion, contain TLR agonists, which act as innate immune modulators. Of those GLA[1], a synthetic

[1] Glucopyranosyl lipid A (GLA) = TLR 4 agonist

analogue of MPL contained in SE + GLA, is the furthest in the clinical development pipeline and is currently being tested in Phase I trials after showing promise in pre-clinical studies [99]. CoVaccine HT^{TM} is a sucrose fatty acid sulphate ester (SFASE) immobilised on the oil droplets of a submicron emulsion of squalane in water and has induced very impressive antibody titres in pre-clinical animal models [253,254,255]. Abisco®100 is a saponin, phospholipid and cholesterol complex that aids antigen delivery [128,256]. As stated earlier the potency of these promising adjuvants was first compared in a head-to-head manner when administered three times with a protein (PPP regimes). This work was then extended to carry out a detailed characterisation of the immunogenicity of AP regimes in comparison to PPP regimes.

3.2 Results

3.2.1 Antibody immunogenicity of protein vaccines can be improved by novel adjuvants

Though numerous adjuvants have been selectively tested with a diverse set of antigens [83,204,250,257,258], a comparative assessment of the immunogenicity of a leading panel of adjuvants is lacking. To address this short-coming, 11 adjuvants (outlined above) were assessed in a three shot protein-in-adjuvant regime (PPP regime) using the model antigen OVA. C57BL/6 mice were immunised three times with 20µg of OVA, two weeks apart, formulated with adjuvant as described in Table 2.5. Formulation and dosing was carried out according to manufacturer's instructions or based on the literature as described in 2.4.7. The adsorption of aluminium-based adjuvants to OVA was assessed as described in 2.4.9 as the adsorption of target antigens to aluminium-based adjuvants has been shown to affect their immunogenicity [59]. OVA was found to adsorb to Alhydrogel® by 89% and adsorbed to Adju-Phos® by 9% (data not shown).

Serum total IgG titres were assayed two weeks after each immunisation in response to OVA protein by ELISA. After one shot of protein-in-adjuvant only some mice immunized

with Abisco®100, SE+GLA+R848 and SE+GLA+R848+CpG[2] seroconverted (Figure 3.1A). After a second shot of protein-in-adjuvant, mice in all groups had detectable antibody responses. These IgG titres were significantly boosted by a third shot of protein-in-adjuvant for Abisco®100 and CoVaccine HT™ ($p<0.01$, two-way ANOVA with Bonferroni's multiple comparison post-test) (Figure 3.1A). Two weeks after the final immunisation a broad range of antibody responses was seen – with a 156-fold difference in median titres observed between the strongest and weakest responding groups (Figure 3.1B). All adjuvants induced significantly higher total IgG titres than OVA in PBS ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). CoVaccine HT™, Montanide®ISA720, SE+GLA+R848, and SE+GLA+R848+CpG induced the highest IgG titres. CoVaccine HT™ induced titres surpassing those induced with Freund's adjuvant and induced significantly higher IgG titres than the classical aluminium-based adjuvant Adju-Phos® ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). The remaining SE-based adjuvants induced IgG titres comparable to Freund's adjuvant, with Adju-Phos® (based on aluminum phosphate) inducing by far the lowest titres. Only two mice receiving OVA in PBS seroconverted after three immunisations, though these responses appeared to be transient as they returned to baseline when antibody titres were assayed six weeks after the final immunisation (data not shown).

[2] Resiquimod (R848) = TLR7/8 agonist, CpG = TLR9 agonist

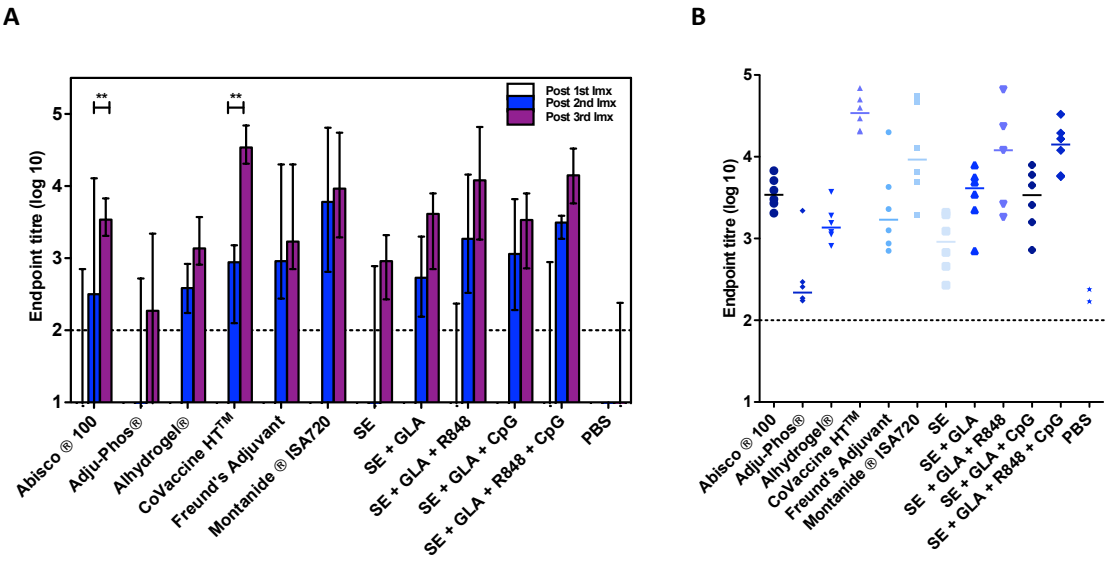

Figure 3.1 Novel adjuvants can improve the antibody immunogenicity of protein vaccines.

C57BL/6 mice (*n* = 6 / group) were immunised i.m. with 20µg of OVA formulated in adjuvant as described in materials and methods. Total IgG titres were measured in the serum in response to OVA protein by ELISA. (A) IgG titres measured two weeks after each immunisation (Imx). Median responses are shown with range. (B) IgG titres measured two weeks after the third immunisation. Median and individual data points are shown. ** $p<0.01$ by two-way ANOVA with Bonferroni's multiple comparison post-test. The dotted line indicates the threshold for responses above background.

3.2.2 T cell responses induced by protein vaccines can be improved using novel adjuvants

T cell responses were also assayed in the blood against the known H-2^b $CD8^+$ T cell epitope and pooled $CD4^+$ T cell epitopes present in OVA two weeks after the final immunisation. Though the hierarchy of T cell responses induced by the adjuvants differed to that of IgG, it was comparable for the $CD4^+$ T cell and $CD8^+$ T cell responses (Figure 3.2A and B). As previously shown, Alhydrogel® and Adju-Phos® were poor T cell inducers [250]. Emulsion based adjuvants such as Freund's adjuvant and Montanide®ISA720 induced a median of 431 and 692 $CD4^+$ SFU/10^6 PBMC respectively. SE adjuvants containing GLA and GLA+R848+CpG induced similar T cell responses, with the SE adjuvant containing the combination of GLA+CpG inducing the most potent T cell responses (median of 2402 $CD4^+$ SFU/10^6 PBMC, $p<0.05$ for $CD4^+$ and $CD8^+$ T cell responses by Kruskal-Wallis test with Dunn's multiple comparison post-test versus Adju-Phos®, Alhydrogel® and PBS). These data indicate that protein adjuvants not only induce T cell and antibody responses of different magnitudes, but that both the humoral and cellular immune responses induced by more classical protein vaccine adjuvants can be improved upon by using new experimental adjuvants.

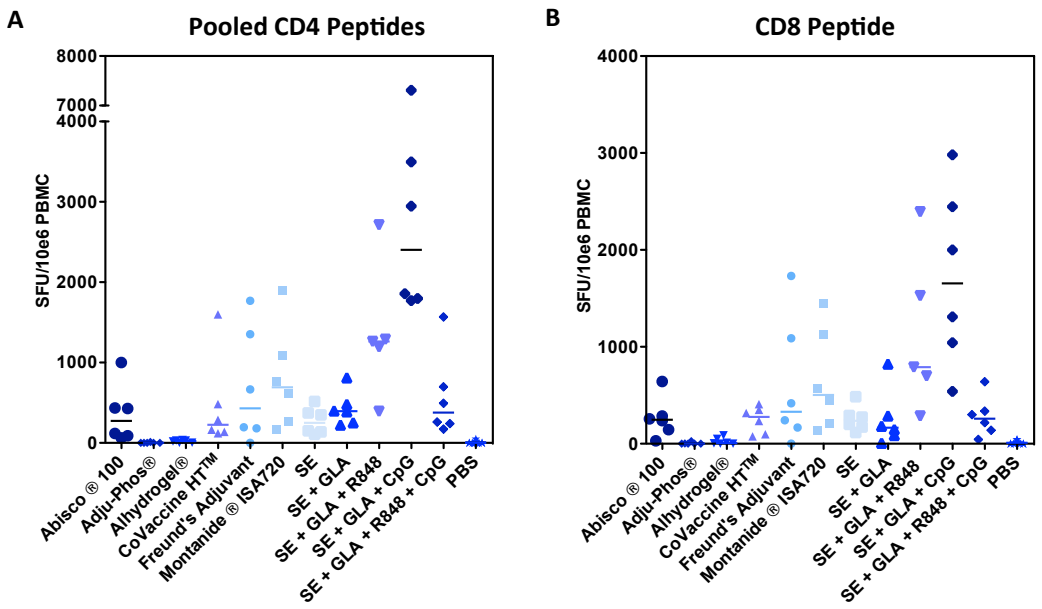

Figure 3.2 Novel adjuvants can improve the T cell immunogenicity of protein vaccines.

C57BL/6 mice (*n* = 6 / group) were immunised i.m. with 20µg of OVA formulated in adjuvant as described in materials and methods. T cell responses were assayed in the blood by *ex-vivo* IFN-γ ELISpot assay against the (A) pooled H-2^b $CD4^+$ T cell epitopes and (B) the H-2^b $CD8^+$ T cell epitope present in OVA. Median and individual data points are shown.

3.2.3 Boosting antibody responses with protein-in-adjuvant vaccines primed by a recombinant adenoviral vector induces comparably high antibody responses across the adjuvants tested

Recently the addition of a protein-in-adjuvant boost following a recombinant adenoviral prime (AP regime) has been shown to lead to enhanced antibody titres when compared to protein-in-adjuvant regimes on their own as well as recombinant adenoviral prime – MVA boost regimes (AM regime) [177,178]. C57BL/6 mice were thus primed with 1 x 10^{10} viral particles (vp) of a recombinant AdHu5 vector expressing OVA (AdHu5-OVA) and boosted eight weeks later with 20µg of OVA protein-in-adjuvant as prepared previously. The generation of the AdHu5-OVA viral vector is outlined in 2.2.1 and 2.2.6. The aim here was to assess whether a similar effect in terms of improved immunogenicity is seen over a range of different adjuvants. Three control groups primed with AdHu5-OVA and either not boosted, boosted with OVA in PBS or boosted with 1 x 10^7 plaque forming units (pfu) MVA-OVA were also included. The generation of MVA-OVA is outlined in 2.2.1 and 2.2.5. In the AdHu5-OVA only group, antibody responses peaked at week eight and then plateaued out to week ten, corresponding to two weeks post protein-in-adjuvant boost (Figure 3.3A). Interestingly, the IgG antibody titres induced by AdHu5-OVA alone at week ten were stronger than after three shots of OVA protein in Adju-Phos® (Figure 3.3B). Adenoviral primed antibody responses were significantly boosted by all protein-in-adjuvant vaccines, apart from in the

SE+GLA+R848+CpG group, as well as by MVA ($p<0.01$ by two-way ANOVA with Bonferroni's multiple comparison post-test) (Figure 3.3B). Analysis of the mean fold change from pre-boost to post-boost IgG titres in individual mice showed that some adjuvants boosted adenoviral primed responses more efficiently than others. SE+GLA+CpG induced a significantly higher mean fold change in IgG titre than Adju-Phos®, Alhydrogel® and CFA ($p<0.01$, by Kruskal-Wallis test with Dunn's multiple comparison post-test on fold change of significantly boosted groups). Abisco®100 also induced a higher mean fold change in titre than Alhydrogel® ($p<0.01$, by Kruskal-Wallis test with Dunn's multiple comparison post-test on fold change of significantly boosted groups). This indicates that some adjuvants can perform particularly well in the context of an AP immunisation regime. However, overall the differential immunogenicity observed following PPP immunisation was greatly reduced, with most responses across the different groups now being of a comparable and relatively high magnitude (Figure 3.3C). Only an 8-fold difference in median titres was observed within the AP groups that had showed significant boosting. This is also shown by the Spearman Rank Correlation of the median responses of the significantly boosted AP groups versus their comparator PPP groups, which was not significant ($r=0.045$, $p=0.89$; Figure 3.4A), i.e. the same "adjuvant hierarchy" was not maintained. Interestingly, using a recombinant adenoviral prime improved the boosting ability of the aluminium-based adjuvant Adju-Phos®, which now induced similar antibody responses to CoVaccine HT™, a potent inducer of antibody responses in the PPP experiments (8-fold increase in the mean, 95% CI: 2-35

for CoVaccine HT™ and 6-fold increase in the mean, 95% CI: 3-12 for Adju-Phos®; $p>0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). Protein-in-adjuvant boosting also surpassed boosting with MVA-OVA, in agreement with previously published data [177,178]. Surprisingly OVA in PBS also slightly boosted adenoviral primed IgG responses (2-fold increase in the mean, 95% CI: 0.5-10.9), a phenomenon seen with other antigens as well (see chapter 4). Overall, these data indicate that the differential immunogenicity of protein vaccines can be partially overcome when antibody responses are first primed with an adenoviral vector.

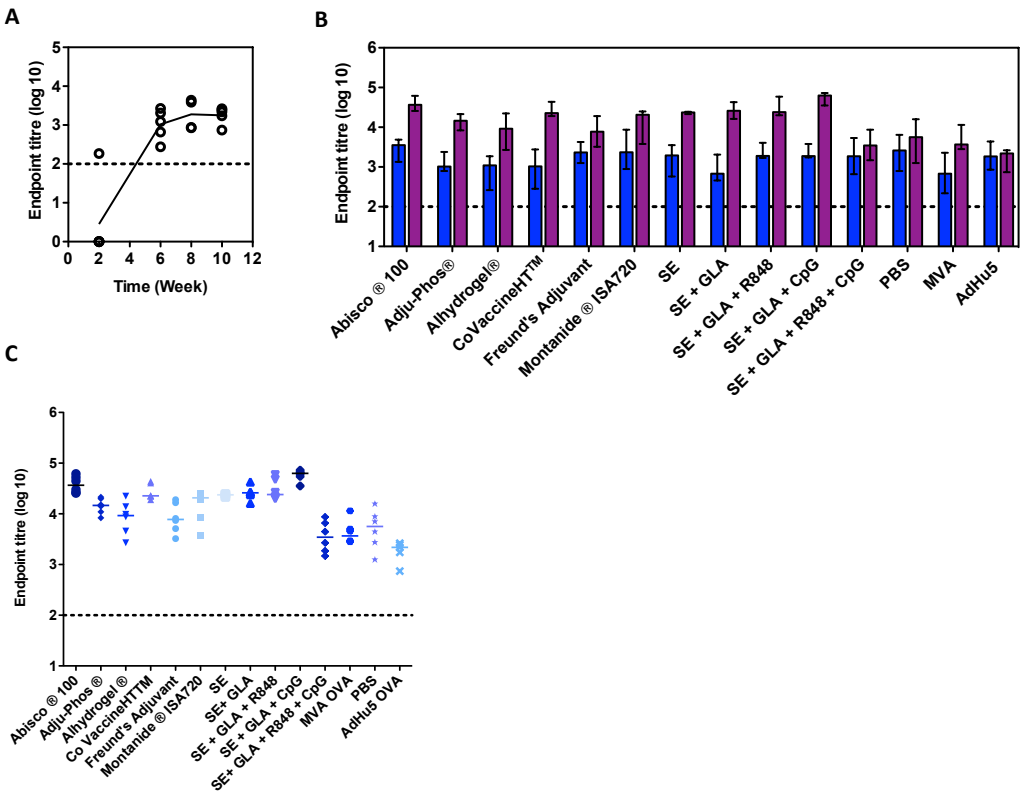

Figure 3.3 Boosting antibody responses with protein-in-adjuvant vaccines primed by a recombinant adenoviral vector induces comparably high antibody responses across the adjuvants tested.

C57BL/6 mice (*n* = 6 / group) were primed i.m. with $1x10^{10}$ vp of AdHu5-OVA and either boosted eight weeks later i.m. with OVA formulated in adjuvant as described in materials and methods or with 10^7 pfu MVA OVA or were not boosted. IgG titres were measured in the serum in response to OVA protein by ELISA. (A) IgG titres measured every two weeks after AdHu5-OVA. Median and individual data points are shown after each immunization. (B) IgG titres measured eight weeks after the AdHu5-OVA prime (■ pre-boost) and two weeks after the protein vaccine boost (■ post-boost). Median responses are shown with range. (C) IgG titres measured two weeks after the protein vaccine. Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background in (A) and (B).

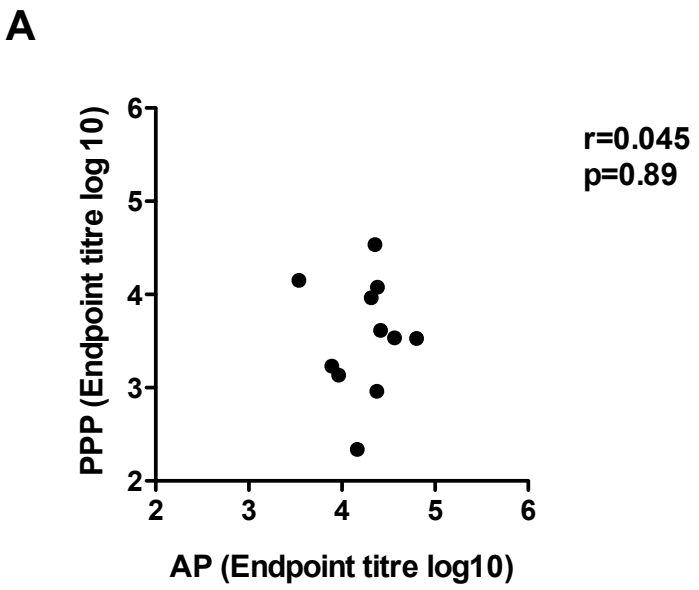

Figure 3.4 The rank order of AP and PPP group IgG median titres differs.

C57BL/6 mice (*n* = 6 / group) were immunised with PPP and AP regimes as described previously. Total IgG titres were measured in the serum in response to OVA protein by ELISA and median titres for each group were correlated. Spearman rank correlation coefficient is shown.

3.2.4 T cell immunogenicity of AP regimes

T cell responses were also assessed two weeks after the protein-in-adjuvant boost (Figure 3.5A&B). As seen with PPP regimes, TLR agonist containing adjuvants again induced robust T cell responses. Interestingly Abisco®100, in addition to inducing strong IgG titres (Figure 3.3A&B), induced a median of 6799 $CD4^+$ SFU/10^6 PBMC. These T cell responses are equivalent to those induced by the gold-standard AM regime, which has been optimised for T cell induction ($p>0.05$ for $CD4^+$ and $CD8^+$ T cell responses by Kruskal-Wallis test with Dunn's multiple comparison post-test for Abisco®100 versus MVA). This indicates that AP regimes can impact both the humoral and cellular arm of the immune system and that certain new adjuvants may be particularly suited for T cell as well as antibody induction.

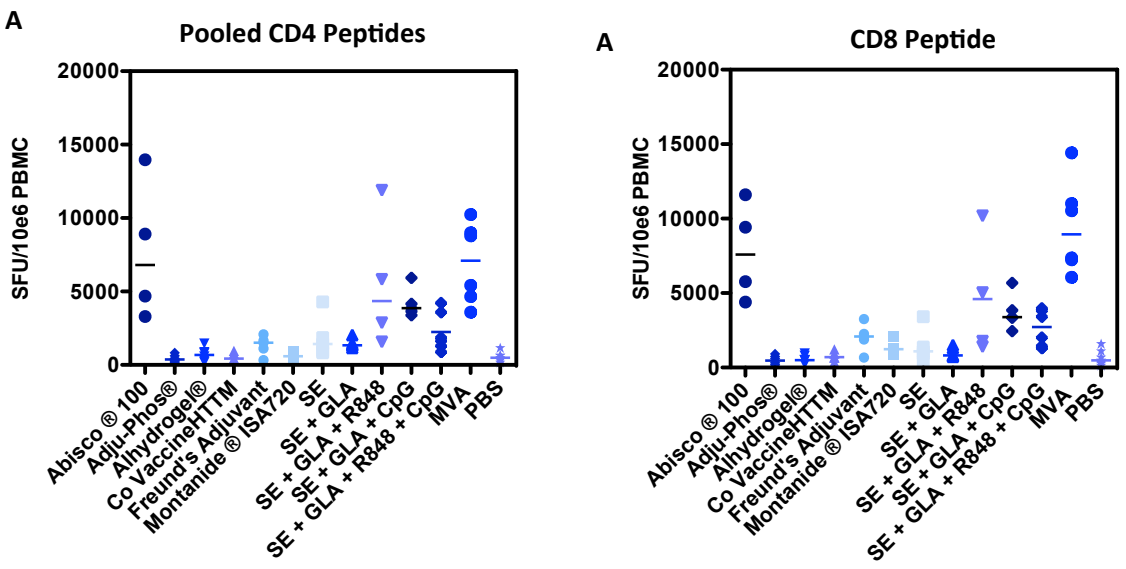

Figure 3.5 AP regimes can induce strong T cell responses.

C57BL/6 mice (*n* = 6 / group) were primed i.m. with $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with OVA formulated in adjuvant as described in materials and methods or with 10^7 pfu MVA OVA. T cell responses were assayed in the blood by *ex-vivo* IFN-γ ELISpot assay against the (A) pooled H-2^b $CD4^+$ T cell epitopes and (B) the H-2^b $CD8^+$ T cell epitope present in OVA. Median and individual data points are shown.

3.2.5 CD4+ T cell responses correlate with antibody responses

Given CD4+ T cells are essential for T-dependent antibody responses [259,260], CD4+ T cells two weeks after the last immunisation were correlated with IgG titres at the same time-point in each regime. Interestingly, CD4+ T cells positively correlated with IgG titres for both AP and PPP regimes (Figure 3.6A&B) indicating an association between the strength of CD4+ T cell induction and the magnitude of the antibody response post-boost. An association was also found between CD8+ T cell induction and antibodies for PPP regimes, but not for AP regimes (data not shown). This indicates that CD4+ T cell responses post-boost might be associated with IgG titres independently of overall immunogenicity.

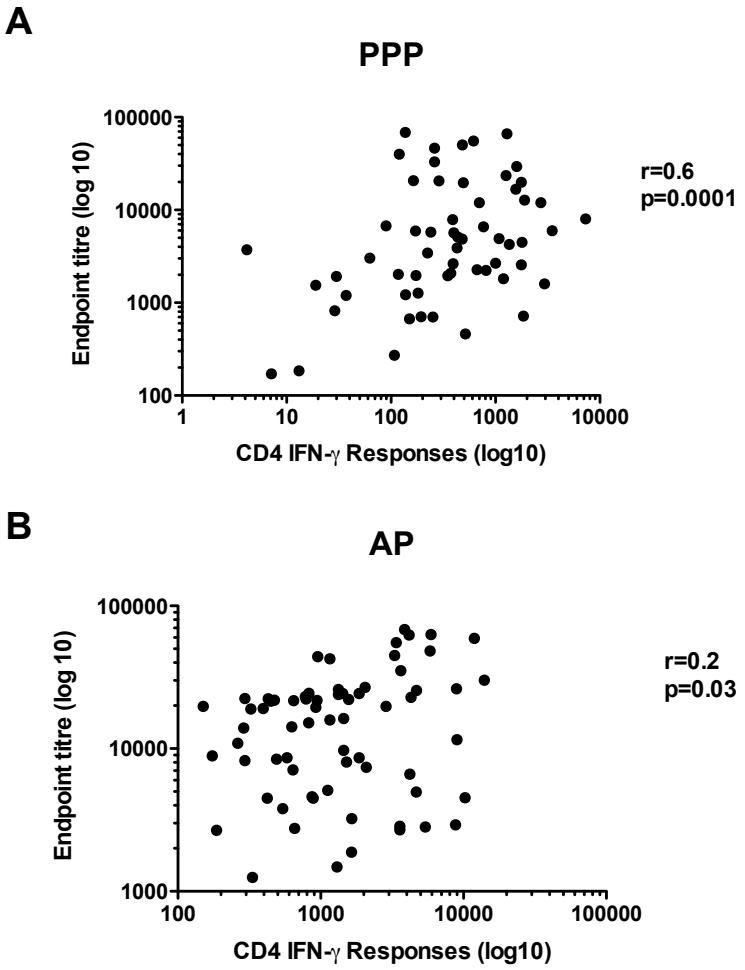

Figure 3.6 CD4+ T cells correlate with IgG titres.

C57BL6 mice (n=6 / group) were immunised with PPP and AP regimes as outlined previously. CD4+ T cell responses were correlated with IgG titres two weeks after the final immunisation. Spearman's rank correlation coefficients and p values are shown. Correlations were carried out on non-log transform data.

3.2.6 Longevity of vaccine-induced antibodies

The induction of not only high titre, but also sustained antibody responses is desirable for efficacious vaccines. The longevity of the antibody responses induced by the different adjuvants deployed in PPP and AP regimes were thus measured ten weeks after the final immunisation (Figure 3.7A&B). Antibody responses were generally higher ten weeks post vaccination in the AP groups compared to the PPP groups, which is most likely a reflection of initially higher titres eight weeks earlier. The reduction in log10 titres from the peak of the response to the late time-point in the OVA system was compared for the different adjuvants administered either in AP or PPP regimes (Figure 3.7C). There was a mean reduction of 0.3 and 0.5 log10 titres in AP and PPP regimes respectively. A significant difference in the reduction of log10 IgG titres over time between the two regimes was found for 3 of the 11 adjuvants tested (CoVaccine HT^{TM}, Freund's adjuvant and SE+GLA+CpG, $p<0.01$ two-way ANOVA with Bonferroni's multiple comparison post-test). However, in most cases there was no difference in the decline of antibody responses between AP and PPP regimes as has been previously found [177]. Thus almost all vaccine-induced IgG, irrespective of method of induction, seems to be subject to the same rate of decay / half-life over time.

The levels of plasma cells (antibody secreting cells, ASCs) in the spleen and bone marrow were also investigated to determine if there is a correlation between antibody titres and

plasma cells as has been suggested previously for other antigens [177,261,262]. However, in C57BL/6 mice immunized with OVA, ASCs were only detected above baseline in a few mice across all the different groups 10 weeks after the last immunisation in the bone marrow and spleen (data not shown), and so this assessment was not possible.

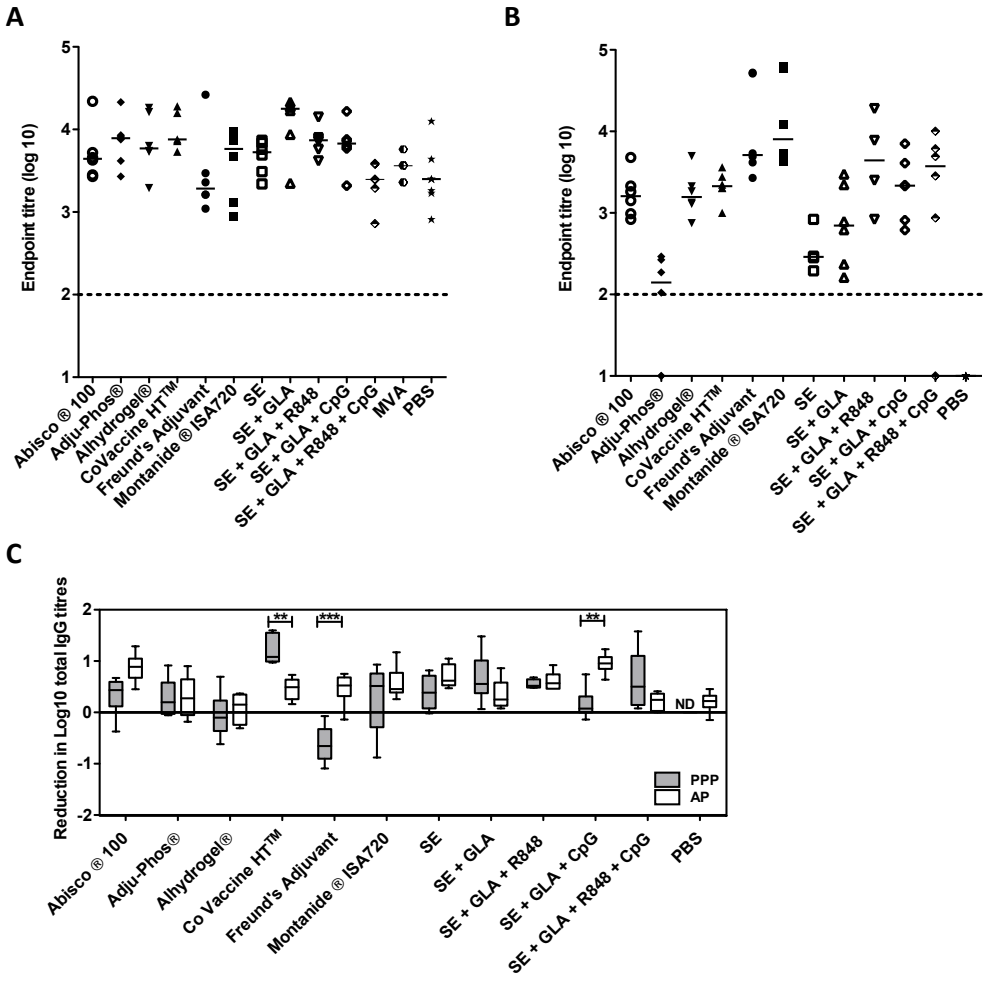

Figure 3.7 Longevity of vaccine-induced IgG responses.

C57BL/6 mice (*n* = 5-6 / group) were immunised as described previously. IgG titres were measured in the serum ten weeks after the final immunization in response to OVA protein by ELISA in mice immunized with (A) AP regimes and (B) PPP regimes. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. (C) The reduction in log10 titres was calculated from IgG titres measured two weeks after the final immunisation in each regime and IgG titres ten weeks after the final immunisation. Median responses are shown with range. ** p<0.01, *** p<0.001 by two-way ANOVA with Bonferroni's multiple comparison post-test.

3.2.7 An adenoviral prime skews adjuvants towards the induction of cytophilic antibody isotypes

Given adjuvants are known to skew the immune response towards either a Th1-type or Th2-type antibody response (dominated in mice by IgG2a or IgG1 respectively), and as it has previously been shown that viral vector containing regimes induce a more cytophilic antibody response that is maintained better over time [177,263], the induction of IgG antibody isotypes by AP regimes was compared to PPP regimes. IgG isotype ELISAs were carried out using the serum of mice immunized with PPP or AP regimes with OVA vaccines two weeks after the final vaccination. Moderate IgG1 and IgG2a antibodies were induced across the different adjuvants in response to OVA (Figure 3.8A). The effect of regime on the log isotype ratio was significant, consistent with previous published data where there was a trend towards a greater induction of IgG2a following a viral vector prime ($p<0.001$, two way-ANOVA with Bonferroni's multiple comparison post-test) (Figure 3.8B) [177]. Interestingly, the effect of adjuvant was also significant ($p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test), driven by adjuvants containing TLR agonists which induced a greater ratio of IgG2a:IgG1 in PPP as well as AP regimes.

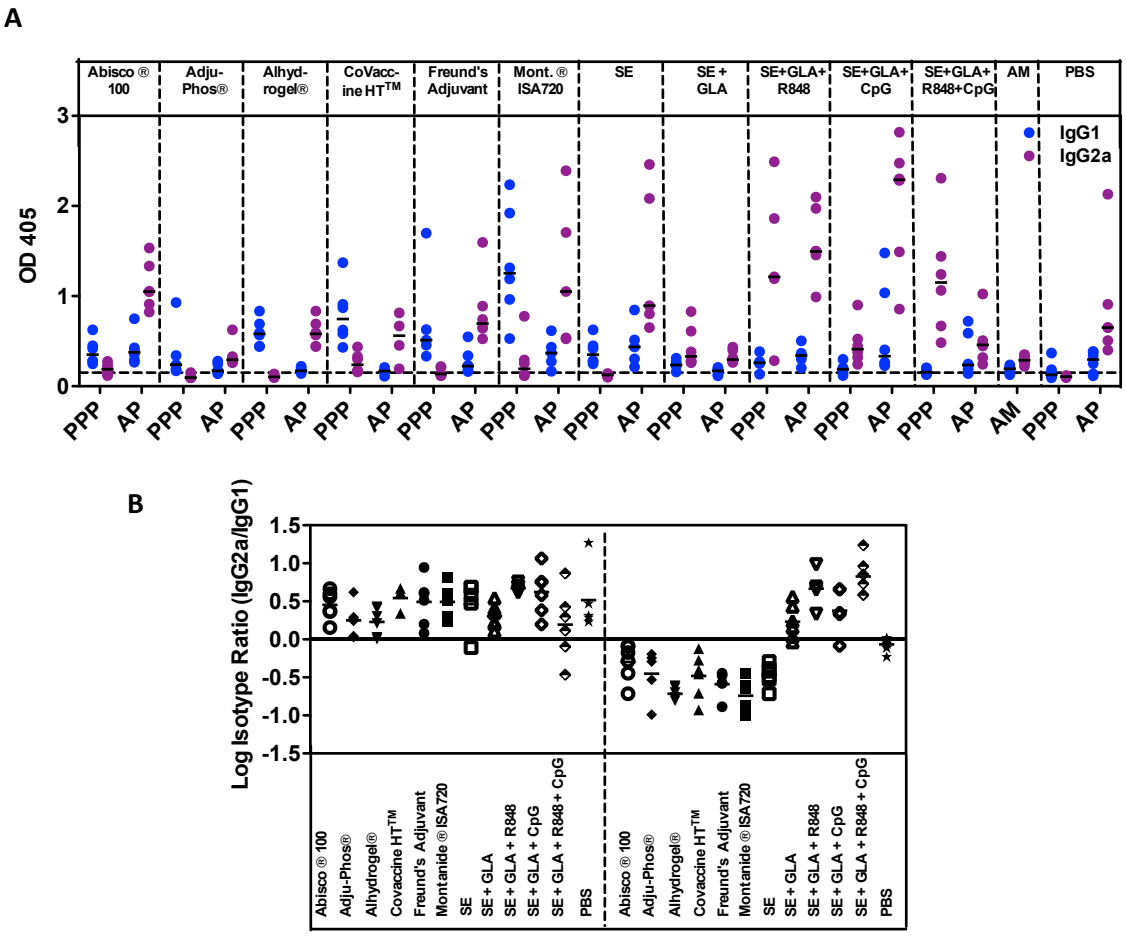

Figure 3.8 An adenoviral prime skews adjuvants towards the induction of cytophilic antibody isotypes.

C57BL/6 mice (*n* = 5-6 / group) were immunised as described previously with AP and PPP regimes. (A) IgG1 and IgG2a anti-OVA antibody responses were measured in the serum two weeks after the last immunization as described in materials and methods. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. (B) IgG2a:IgG1 ratios were calculated and log transformed. Mean and individual data points are shown.

3.3 Discussion

Viral vectored vaccines encoding blood-stage malaria antigens have repeatedly induced high-titre antibodies and strong T cell responses in pre-clinical models [148,169,177,178]. The antibody titres induced by these regimes have been shown to be further enhanced through the addition of protein-in-adjuvant vaccines to viral vectored regimes [177,178], although only a limited set of adjuvants have been tested in these studies. Given the comparative assessment of new vaccine adjuvants remains essential for subunit vaccine development, a panel of 11 leading and accessible vaccine adjuvants (both pre-clinical and clinically tested/approved) was assessed in a three shot protein-in-adjuvant regime and compared to an adenoviral prime – protein boost regime for the induction of high and sustained antibody responses using the model antigen Ovalbumin.

Novel adjuvants such as CoVaccine HT^{TM} and emulsion based adjuvants containing TLR agonists were found to induce strong antibody responses surpassing aluminium-based adjuvants and the classical reference adjuvant, Freund's adjuvant, when formulated with OVA in a PPP regime. Though most licensed adjuvants to date predominantly induce the humoral arm of the immune system, the SE+GLA+CpG emulsion adjuvant induced very strong $CD4^+$ and $CD8^+$ T cell responses with the soluble antigen OVA, as has been shown for TLR agonists administered with other antigens [264,265]. Interestingly, the differential immunogenicity of IgG titres induced by adjuvants in three-shot protein-in-adjuvant regimes was partially overcome when an AdHu5 vector was used to prime

antibody responses, although some adjuvants still boosted adenovirus-primed responses more efficiently than others. Importantly, this effect was observed for aluminium-based adjuvants such as Adju-Phos®, which performed poorly in a PPP regime, but which boosted antibody responses primed by an AdHu5 vector as efficiently as the most potent adjuvant CoVaccine HT™. Recently, a similar trend was reported when rhesus macaques, primed with the simian adenovirus ChAd63 vector expressing the blood-stage malaria apical membrane antigen (AMA)-1, were boosted either with AMA-1 protein formulated in Alhydrogel® or CoVaccine HT™. IgG titres were found to be only moderately and not significantly enhanced by CoVaccine HT™ in comparison to Alhydrogel® in this study [178]. The use of ChAd63 in this study also suggests that the results here with AdHu5 could potentially be extended to other adenovirus vectors, given the concerns surrounding pre-existing immunity to AdHu5 in humans and its use as a clinical vaccine vector [248,266]. AdHu5 was chosen as a model adenovirus in this study as it has no intellectual property restrictions and induces immunogenicity comparable to simian adenoviruses in pre-clinical models [169,249].

Although traditionally the adsorption of antigens to aluminium-based adjuvants is thought to be essential for adjuvanticity, anti-OVA IgG titres were boosted by protein in Adju-Phos® to high levels following an adenoviral prime despite limited adsorption of this antigen to the adjuvant. This is in agreement with some previous data showing that

adsorption is not essential for potentiating an immune response by aluminium-based adjuvants [65,66,68,267].

The two vaccine delivery platforms used in this study are inherently different and it is possible that the increased immunogenicity of protein vaccine adjuvants seen following an adenoviral prime is due to an adenoviral vector producing quantitatively more antigen *in vivo* at the time of priming than a given dose of protein vaccine. This "dose effect" could prime a quantitatively greater memory B cell response that can be subsequently boosted more effectively by protein-in-adjuvant vaccines. Alternatively, adenoviral vaccines may inherently prime a better quality of memory B cell response, likely related to the profile of innate sensors stimulated by adenoviruses [268,269], that can either be more effectively boosted by weak and potent adjuvants alike or which differ in their ability to recruit antigen presenting cells. This warrants further investigation and will be addressed in Chapter 5.

The induction of antibody isotypes by vaccine adjuvants was also explored in this study. Th-2 type responses (dominated by IgG1) are thought to function through neutralising antibodies whereas Th-1 type responses (dominated by IgG2a) are thought to activate complement and function through Fc receptors leading to antibody dependent cellular inhibition (ADCI), the release of protective pro-inflammatory cytokines as well as phagocytosis. It was found that when using OVA, a more Th-1 type antibody response is

induced following an AP regime in comparison to a PPP regime. This is in agreement with previous published mouse data where viral vector containing regimes induced more cytophilic antibody responses that were maintained better over time [177]. Interestingly adjuvants containing TLR agonists, particularly TLR 4 agonists such as GLA, were also able to induce a more Th-1 type antibody response in PPP regimes as has been previously shown [125,264]. Surprisingly CoVaccine HT^{TM} did not induce a skewed Th-1 response, despite evidence showing that some of its action is dependent on TLR 4 [254]. TLR agonist containing adjuvants also induced relatively strong T cell responses in the PPP OVA regime, which may account for the isotype switch as seen in T-dependent antibody responses. Furthermore, cytokines produced by T-follicular helper (T_{FH}) cells have been shown to shape the antibody repertoire through class-switching and somatic hypermutation of B cells, with IFN-γ leading to a Th-1 type antibody response [32]. As adjuvants have also been shown to influence the size of the T_{FH} cell compartment, with TLR agonists inducing a larger proportion of antigen-specific T_{FH} cells [270], it is possible that TLR agonists could be influencing T_{FH} cells in the germinal centre accounting for the observed enhancement of cytophilic antibody induction. The improved induction of $CD4^+$ T cell help (essential for T-dependent antibody responses [259,260]) by the adenovirus in the AP regimes could also be another explanation for improved B cell priming by adenoviral vectors and/or subsequent boosting by protein vaccines. A study by Galli *et al*. has recently demonstrated that the induction of $CD4^+$ T cells following an adjuvanted influenza vaccine predicted the persistence of antibody responses,

highlighting a potential link between these two cell types [89]. This was supported by a correlation between $CD4^+$ T cells and antibody responses seen here after the last immunisation. However, data using OVA-specific transgenic $CD4^+$ T cells has indicated that adoptively transferred transgenic T cells can only help antigen-experienced (and not naïve) B cells and that the size of the secondary antibody response is restricted by the amount of T cells present at the time of antigen re-exposure [271]. Further studies will thus be necessary to confirm whether the induction of better cellular immunity at the time of B cell priming is an important contributing factor and will be explored in Chapter 5.

Following immunisation with either the AP or PPP regimes, there appeared to be no difference in the rate of decline of IgG titres as has previously been suggested [177]. This contrasts to reports using the outer-membrane protein complex (OMPC) of *Neisseria meningitidis* as an adjuvant, which has been shown to lead to improved maintenance of antibody responses, thereby apparently influencing the rate of decline of IgG titres [272]. The effects mediating this phenomenon are unclear and it has been suggested that for chemical conjugates of protein vaccine to OMPC the resulting increased molecular antigen size, increased epitope density through multimerisation and/or increased T cell help by conjugation of the antigen to this adjuvant may influence the maintenance of the induced antibody responses. Exploring these avenues to generate a more sustained antibody response would be of significant further interest;

especially given this phenomenon was not observed here using a panel of 11 leading alternatives.

A difference in the rate of decline has been suggested for some IgG isotypes, with IgG2a being shown to be better maintained over time compared to IgG1 [177,273]. It would be interesting to investigate whether this phenomenon is also evident when comparing the different vaccination regimes and adjuvants used here. However, total IgG titres were not evidently better maintained over time in the case of AP regimes, despite the enhanced cytophilic IgG2a antibody response in this system.

ASC levels for OVA were very weak following both regimes 10 weeks after the final immunisation. Therefore it is hard to draw conclusions about which cell compartment (i.e. long-lived plasma cells or memory B cells) is contributing to or associated with the maintenance of the serum antibody titres. Given virally vectored vaccination regimes, as well as AP regimes, have been shown to induce memory B cells [178], the contribution of this cell type to antibody responses cannot be ruled out. It remains possible that a greater induction of memory B cells could account for the better boostability of IgG titres seen here and also as shown previously in human infants [274], and this warrants further investigation, as memory B cells were not explored in this study.

In summary this chapter shows that novel emulsion based adjuvants as well as adjuvants containing TLR agonists can induce both strong humoral and cellular immune responses in a classical subunit vaccination approach. More importantly, it was found that the differential immunogenicity of these protein vaccine adjuvants can be overcome through an adenoviral priming immunisation. This adenoviral prime leads to readily boosted antibody responses as well as a more cytophilic antibody response. When formulated with Abisco®100, a protein vaccine deployed in an AP regime also induces high level T cell responses which equal those induced by AdHu5-MVA immunisation, a viral vectored regime optimised for T cell induction. These data indicate that the differential immunogenicity of adjuvants may be largely minimised by utilising AP immunisation regimes. This approach could therefore enhance the clinical immunogenicity and utility of adjuvants that are traditionally considered poorly immunogenic, and circumvent the need for more potent and experimental chemical adjuvants that are currently required to deliver candidate protein vaccines for difficult diseases such as blood-stage malaria.

Chapter 4

Comparative Screening of Adjuvants utilising Malaria Blood-stage Candidate Antigens

4 Comparative Screening of Adjuvants Utilising Malaria Blood-stage Candidate Antigens

4.1 Introduction

Malaria is a parasitic disease caused by a protozoan parasite of the genus Plasmodium and as stated previously the morbidity and mortality associated with this disease is immense with nearly 1 million deaths occurring annually [275]. Although *P. falciparum* malaria accounts for 90% of all deaths, *P. vivax* is the most geographically wide-spread parasite and accounts for between 72-80 million cases each year with the greatest burden of disease located in South and South-east Asia followed by South America [276]. Traditionally thought to cause a benign form of malarial disease, it is now thought that the clinical impact and socio-economic burden associated with this parasite has been underestimated. An effective control strategy for malaria should therefore also address this plasmodium species.

A malaria vaccine would provide a cost-effective way of controlling this disease. Several stages of the malaria life cycle can be targeted by vaccination with blood-stage malaria vaccines aiming to stop the erythrocytic stage of the parasite's life cycle, therefore preventing the disease associated with infection [246]. Multiple blood-stage malaria vaccines are being developed with most focus being placed on a few candidate antigens

such as *P. falciparum* MSP-1 and erythrocyte binding antigen (EBA)-175 as well as *P. vivax* Duffy binding protein (DBP) (a homologoue of EBA-175), which are involved in erythrocyte invasion [246,277]. MSP-1 and EBA-175-based vaccines have entered clinical trials [175,278,279,280,281]

Erythrocyte invasion is a complex, multi-step process involving several receptor-ligand interactions between the parasite and host's RBC surface [282]. Invasion of RBCs by *P. vixax* appears to depend on the interaction of DBP with the Duffy antigen receptor for chemokines (DARC). This is supported by epidemiological data showing that the Duffy negative blood group, which confers resistance to *P. vivax*, predominates in West Africa, and also arose in Papua New Guinea, where other forms of malaria are rife [283]. Furthermore, studies focussing on *P. knowlesi*, a Plasmodium species that infects new-world monkeys, have shown that if the DBP gene is deleted these knockout parasites cannot complete invasion [284]. The receptor-binding domain of this antigen lies within the conserved, extracellular, cysteine-rich region known as region II (RII), on which most vaccines are based [285]. High titre antibodies can be induced against this antigen using recombinant DBP_RII protein/adjuvant vaccines [286,287] and antibodies raised against the *P. knowlesi* DBPα homologue, used to infect human and rhesus RBCs, can block human and rhesus erythrocyte invasion of *P. knowlesi in vitro* [288]. Furthermore, naturally acquired high titre binding inhibitory antibodies against PvDBP_RII have been

shown to be associated with reduced risk of *P. vivax* infection as well as lower *P. vivax* parasite densities following infection [289].

Unlike *P. viviax*, *P. falciparum* uses multiple redundant receptors to invade RBCs [290]. One of these, EBA-175, is a homologue of PvDBP and binds to sialic acid residues on glycophorin A on the surface of erythrocytes and can mediate invasion by malaria parasites [291]. EBA-175 contains Duffy-binding-like (DBL) domain repeats within region II referred to as F1 and F2. Antibodies induced against EBA-175_F2 have been shown to inhibit *P. falciparum* invasion of erythrocytes *in vitro* [83].

MSP-1 is the most abundant protein on the merozoite surface and has been the focus of most blood-stage vaccine development. MSP-1 is thought to be involved in mediating initial contact with RBCs and is thought to bind to band 3, although its precise ligand is not known [290]. MSP-1 is synthesised as a large precursor which undergoes a series of proteolytic cleavage events to produce an N-terminal 83-kDa fragment and a C-terminal 42-kDa fragment (MSP-1_{42}, which contains the GPI anchor) [292]. Upon RBC invasion, MSP-1_{42} is further processed into a 33-kDa (MSP-1_{33}) and 19-kDa (MSP-1_{19}) fragment [293]. Only MSP-1_{19} remains on the merozoite surface and is taken into the invaded RBC [294]. Immunising with MSP-1_{42} and MSP-1_{19} can protect Aotus monkeys against a blood-stage challenge [295,296]. Antibody responses directed against MSP-1_{19}, but not MSP-1_{33}, have been shown to be protective against blood-stage malaria [148,174,297].

However, a role has also been reported for MSP-1-specific $CD8^+$ T cells against liver-stage parasites, as MSP-1 is also expressed during late liver-stage infection [148,207]. Furthermore, MSP-1_{33}-specific $CD4^+$ T cells have been shown to provide help for B cells and aid the development of *de novo* antibody responses [148].

Although the precise immune mechanisms mediating protection *in vivo* in humans against the blood-stage of malaria are unclear [298], the vaccine field generally accepts that the induction of high-titre antibodies will be necessary to prevent the invasion of RBCs utilising vaccines based on blood-stage antigens such as those described above. Work in the previous chapter focused on comparatively assessing a panel of leading and accessible adjuvants in varying stages of clinical development for their ability to prime and boost high and sustained antibody responses using the model antigen ovalbumin in AP and PPP regimes. In this chapter this work is extended to further characterise the immunogenicity of adjuvants using PvDBP_RII, EBA-175_F2 and MSP-1 vaccines in a different mouse strain to assess whether the effects seen with OVA are antigen and/or mouse-strain specific.

4.2 Results

4.2.1 Antibody immunogenicity of *P. falciparum* MSP-1 and EBA-175 vaccines in a PPP vaccination regime

To further investigate the immunogenicity of selected adjuvants and to assess whether the effects seen with OVA are antigen and/or mouse strain specific, the leading *P. falciparum* blood-stage malaria antigens EBA-175_F2 (hereafter referred to as EBA_175) and MSP-1 were also assessed in BALB/c mice immunized with PPP regimes. A range of potent adjuvants were taken forward into this screen including Abisco®100, Montanide ®ISA720, SE+GLA and CoVaccine HT™, selected to induce maximal IgG titres. Here these two antigens were given as a mixture (10μg of each) formulated with adjuvant as described in materials and methods. Immunisations were administered three weeks apart in the PPP regime. All mice in the PPP groups seroconverted in response to EBA-175 after one immunization (Figure 4.1A) and followed the same hierarchy as seen after two shots of OVA (with Montanide®ISA720 and CoVaccine HT™ inducing the strongest IgG titres, see Figure 3.1B). IgG titres were significantly boosted by a second immunisation of protein-in-adjuvant ($p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test) but not by a third, after which all adjuvants induced comparable antibody responses to EBA-175 indicating that the antibody responses had reached a plateau ($p>0.05$, one-way ANOVA with Bonferroni's multiple comparison

post-test). Although mice immunised with EBA-175 in PBS had detectable antibody responses, IgG titres in the adjuvant groups were significantly higher ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test, Figure 4.1B). Endotoxin levels measured for this protein by the LAL assay were found to be 21 EU/25 µg (data not shown), which is within the range of endotoxin levels measured for other pre-clinical vaccines [299]. In agreement with previous data, mice immunised with *P. falciparum* MSP-1_{19} in Montanide®ISA720 had detectable IgG titres to MSP-1_{19} protein [177], however no antibody responses were detected with other adjuvants (Figure 4.1C).

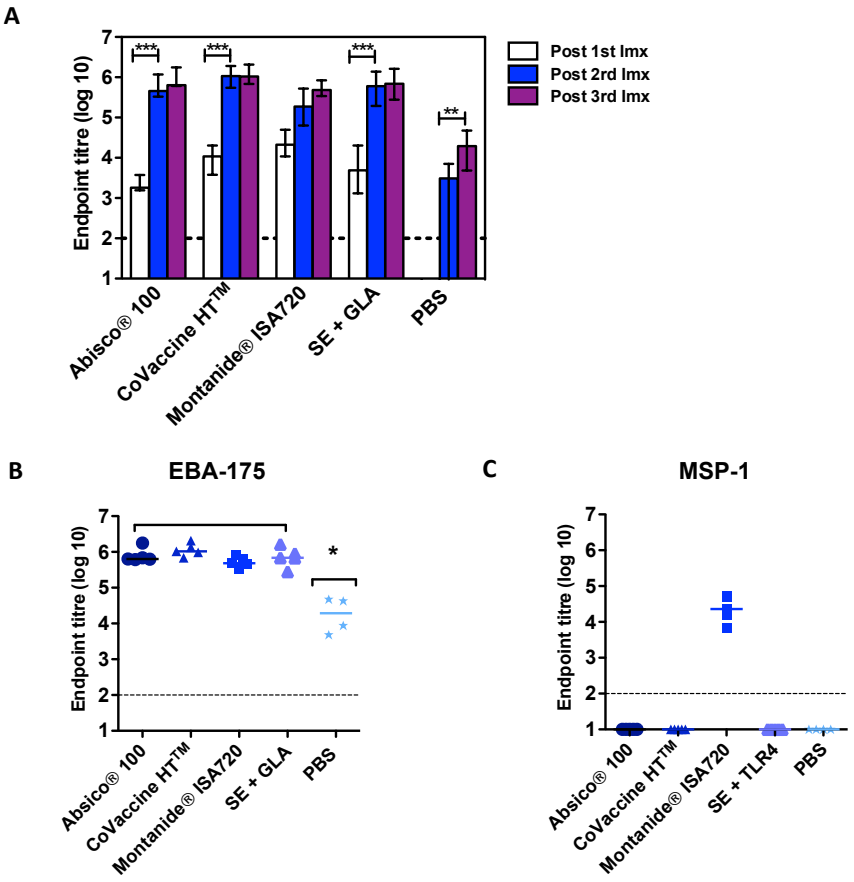

Figure 4.1 Antibody immunogenicity of MSP-1 and EBA-175 in the PPP regime.

BALB/c mice (n = 5 / group) were immunized thrice i.m. three weeks apart with a mixture of 10µg of *P. falciparum* EBA-175 protein and 10µg of *P. falciparum* MSP-1_{19} protein. IgG titres were measured in the serum to (A) EBA-175 protein two weeks after each immunisation (Imx). Median responses are shown with range. *** $p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test. IgG titres were measured in the serum in response to (B) EBA-175 protein and (C) MSP-1_{19} (QKNG allele) protein two weeks after the final immunisation. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. * $p<0.05$ by one-way ANOVA with Bonferroni's multiple comparison post-test.

4.2.2 Antibody immunogenicity of *P. falciparum* MSP-1 and EBA-175 vaccines in an AP regime

As described in Chapter 3, the broad range of antibody responses induced by different adjuvants can be largely overcome by a priming immunisation of a recombinant AdHu5 expressing the same antigen. To further test this hypothesis, mice were immunised with 1 x 10^9 vp of AdHu5-MSP-1 and 1 x 10^9 vp of AdHu5-EBA-175, also administered as a mixture, and boosted with protein vaccines eight weeks later as described in the previous chapter. The AdHu5 MSP-1 vaccine was generated as previously described [169]. This vaccine termed PfM128 contains both conserved and dimorphic regions of the 42-kDa C-terminal region of 3D7 and Wellcome strains of *P. falciparum* MSP-1. The AdHu5 EBA-175 vaccine contains the F2 region of EBA-175 from the Camp strain [300]. IgG titres were again assessed by ELISA two weeks after each immunisation as well as before the protein boost in this AP regime.

In contrast to the PPP regime, mice in the AP groups seroconverted two weeks after the adenoviral prime in response to both antigens (Figure 4.2A&B). Following a protein immunisation, total IgG titres were significantly boosted in response to both antigens by >1 log in all adjuvant groups comparing pre and post-boost titres (16-fold increase in the mean across all adjuvant groups, 95% CI: 13-20 for EBA-175 and 31-fold increase in the mean across all adjuvant groups, 95% CI: 23-41 for MSP-1_{19}; $p<0.001$, two-way ANOVA

with Bonferroni's multiple comparison post-test). Interestingly, although mice only responded to MSP-1_{19} in Montanide®ISA720 following three immunisations with protein vaccine, antibody responses to MSP-1_{19} in mice primed with the adenoviral vector were successfully boosted to high levels by each protein-in-adjuvant vaccine when administered in the AP regime (Figure 4.2A). There was no significant difference in the mean fold change from pre-boost to post-boost IgG titres in relation to the use of a particular adjuvant with the two different antigens (data not shown). Also, as seen with OVA, adenoviral primed responses were also boosted by protein in PBS comparing pre and post-boost titres (4-fold increase in the mean, 95% CI: 3-7 for EBA-175 and 13-fold increase in the mean, 95% CI: 5-35 for MSP-1_{19}; $p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test). Taken all together, these data indicate that despite some minor differences, the improved boostability of IgG responses observed following a recombinant adenoviral prime is consistent and can be observed in different mouse strains and when using different antigens.

The levels of plasma cells (antibody secreting cells, ASCs) in the spleen and bone marrow were also investigated to determine if there is a correlation between antibody titres and plasma cells as has been suggested previously [177,261]. Although OVA specific ASCs were only detected in a few mice across different groups (see Chapter 3), strong MSP-1_{19} specific ASC responses were detected in the bone marrow (Figure 4.3A) and spleens (Figure 4.3B) of BALB/c mice immunised with the AP regime ten weeks after the last

immunisation, with a trend towards stronger ASC responses with Abisco®100. As expected, ASC responses were stronger in the bone marrow where long-lived plasma cells are thought to be present in survival niches [301]. These ASC levels correlated with peak serum antibody titres (Figure 4.3C) as well as antibody levels at the late time point (Figure 4.3D) as previously reported for this antigen. ASC levels were not explored in PPP vaccinated mice because antibody responses were only detectable in the Montanide®ISA720 group for this antigen.

Antibodies induced by the viral vectors and protein vaccines used in the above regimes have previously been assessed for their ability to recognise native parasite antigen by an indirect fluorescence assay test (IFAT). Both native MSP-1 and EBA-175 were recognised by antibodies induced through vaccination indicating that antibodies induced are capable of recognising conformational parasite antigen [83,302,303].

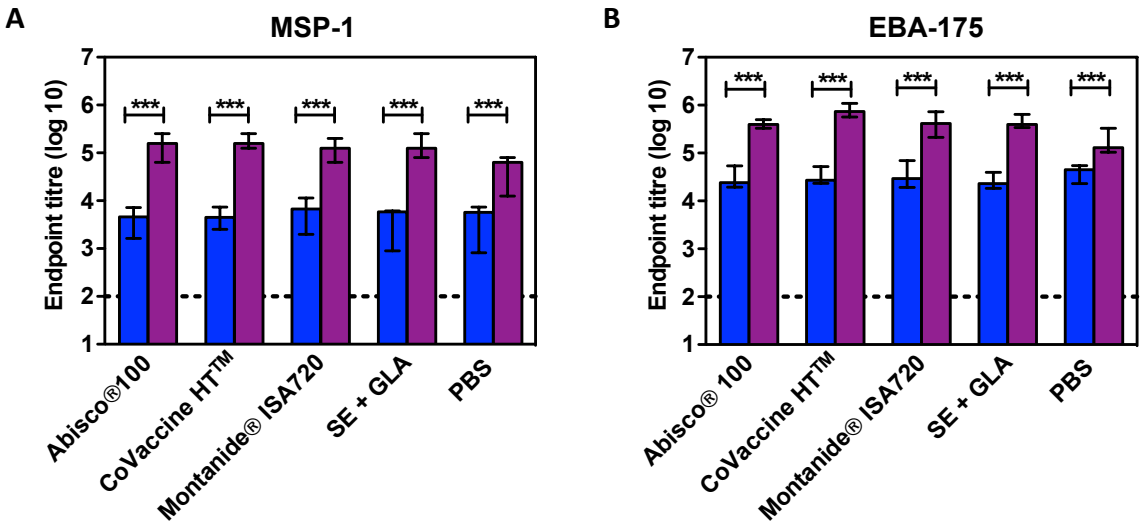

Figure 4.2 Antibody immunogenicity of MSP-1 and EBA-175 in AP regimes.

BALB/c mice (n= 5 / group) were primed i.m. with a mixture of $1x10^9$ vp of AdHu5-MSP-1 and AdHu5-EBA-175 and boosted i.m. eight weeks later with a mixture of 10µg of *P. falciparum* EBA-175 protein and 10µg of *P. falciparum* MSP-1_{19} protein. IgG titres were measured in the serum in response to (A) MSP-1_{19} (QKNG allele) protein and (B) EBA-175 protein eight weeks after the prime (■ pre-boost) and two weeks after the protein vaccine boost (■ post-boost). Median responses are shown with range. The dotted line indicates the threshold for responses above background. ***p<0.001 by two-way ANOVA with Bonferroni's multiple comparison post-test.

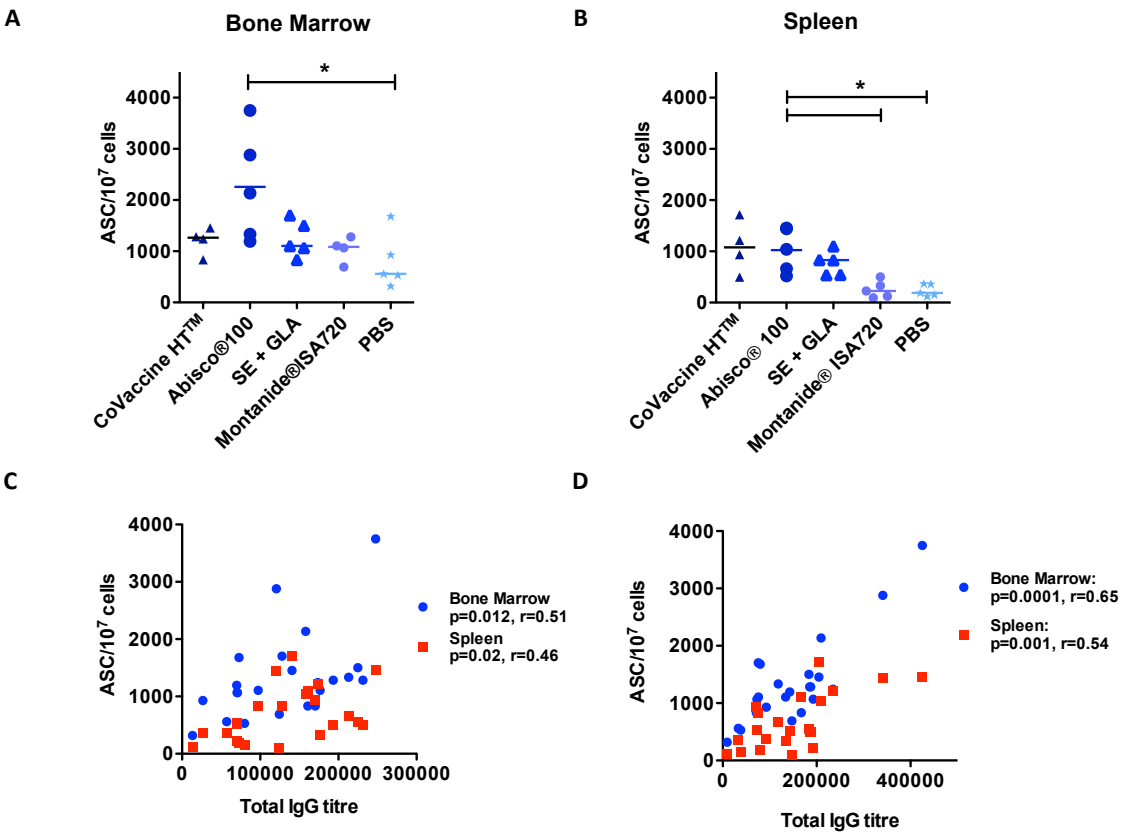

Figure 4.3 ASC responses correlate with antibody titres.

BALB/c mice (*n* = 5 / group) were immunised as described previously. Antibody secreting cells (ASC) per 10^7 cells in mice immunised with AP regimes were quantified in the (A) bone marrow and (B) spleen ten weeks after the last immunisation in response to MSP-1_{19} protein. * $p<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison post-test. Median and individual data points are shown. IgG titres two weeks after the last immunisation (C) and ten weeks after the last immunisation (D) were correlated with ASCs measured in the spleen (■) and bone marrow (●) to MSP-1_{19} protein for AP regimes. Spearman's rank correlation coefficients and p-values are shown.

4.2.3 Antibody isotypes induced by AP and PPP regimes

As adjuvants are known to skew the immune response towards either a Th1-type or Th2-type antibody response (dominated in mice by IgG2a or IgG1 respectively), and as it has previously been shown that viral vector containing regimes induce a more cytophilic antibody response that is maintained better over time [177], the induction of IgG antibody isotypes by AP regimes was compared to PPP regimes for *P. falciparum* antigens. As shown for PPP and AP regimes using the antigen OVA, there was again a trend towards a greater induction of IgG2a antibodies with AP regimes (Figure 4.4A&B). Overall, the adjuvants induced comparable isotype responses to both antigens, and SE +GLA again induced a greater ratio of IgG2a:IgG1 (as seen in the OVA system) in response to EBA-175 PPP vaccination (Figure 4.5A). The effect of regime, but not adjuvant, on the log isotype ratio was again significant for EBA-175 ($p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test) indicating a skew towards cytophilic antibodies after AP immunisation. Comparing IgG1 and IgG2a responses across the different adjuvants (Figure 4.5B&C), there was an increase in IgG2a isotype units with AP regimes compared to PPP regimes, apart from with SE+GLA. There was also a trend towards more IgG1 with PPP regimes. This was not investigated for MSP-1 vaccines because only mice immunised with Montanide®ISA720 in a PPP regime had detectable antibody responses.

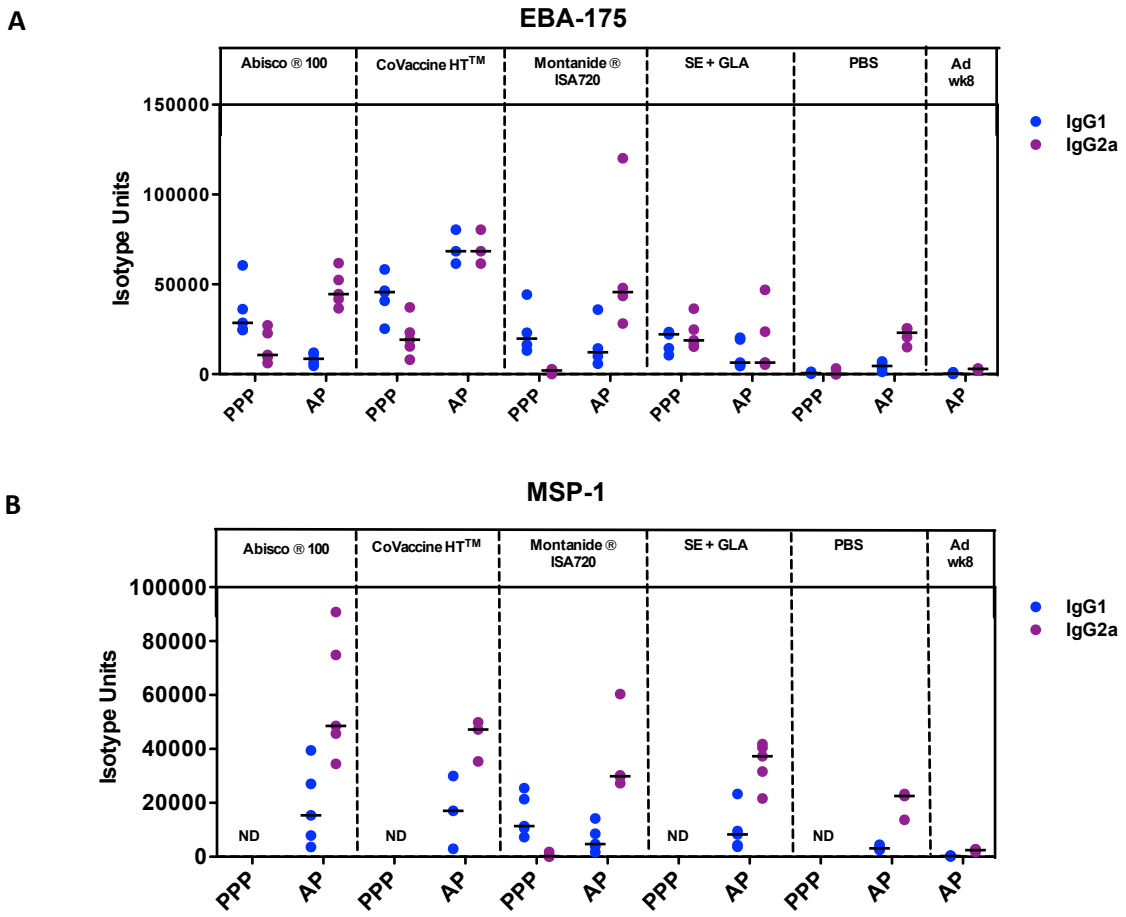

Figure 4.4 Isotype responses following AP and PPP regimes.

BALB/c mice (*n* = 5 / group) were immunised as described previously with AP and PPP regimes. IgG1 and IgG2a antibody responses were measured in the serum two weeks after the last immunisation in response to (A) EBA-175 and (B) MSP-1_{19} (QKNG allele) protein as described in materials and methods. Median and individual responses are shown. ND = no data.

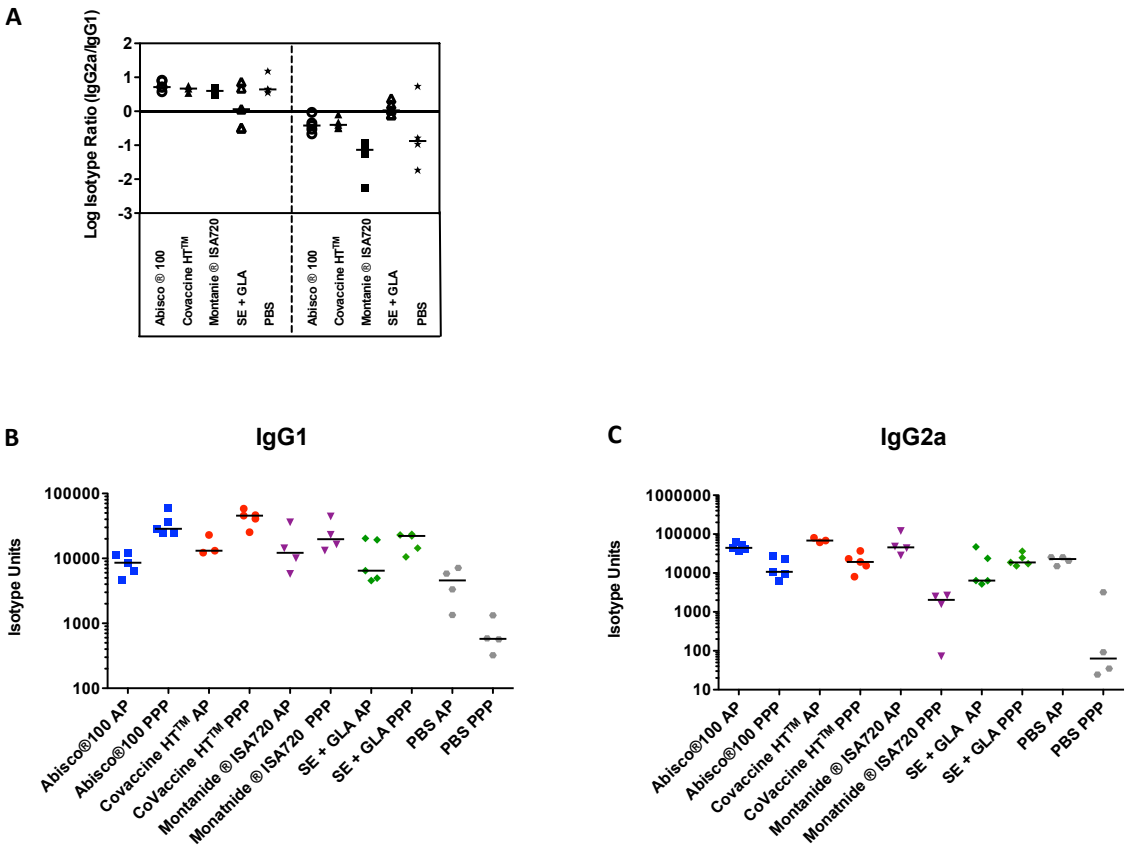

Figure 4.5 AP regimes induce a more cytophilic antibody response.

BALB/c mice (*n* = 5 / group) were immunised as described previously with AP and PPP regimes. IgG2a:IgG1 ratios were calculated for (A) EBA-175 and log transformed. Median and individual data points are shown. (B) IgG1 and (C) IgG2a antibody responses are shown across different regimes and adjuvants. Median responses and individual data points are shown.

4.2.4 Antibody immunogenicity of PvDBP_RII vaccines

To further assess the immunogenicity of adjuvants in a single antigen regime on a different genetic background, BALB/c mice were immunised with PPP and AP regimes using the PvDBP_RII antigen. The AdHu5 PvDBP_RII vaccine contains the native RII binding region from the Sal I strain of *P. vivax* [288]. 10µg of PvDBP_RII were administered three weeks apart formulated in adjuvant as described previously. In parallel mice were also primed with $1x10^{10}$vp of AdHu5 PvDBP_RII, and boosted eight weeks later with protein vaccine as described previously. The adjuvants taken forward in this study included Abisco®100, Montanide®ISA720, SE + GLA, SE + GLA and CpG and CoVaccine HT™. In addition to this, mice were also immunised in the PPP regime experiment using MVA PvDBP_RII as an adjuvant. This group was included as the MVA vector itself has previously been shown to exhibit adjuvant properties [304]. IgG titres were again assessed by ELISA two weeks after each immunisation and before the protein boost in the AP regime.

After one immunisation all mice in all groups seroconverted to PvDBP_RII (Figure 4.6A). As seen with MSP-1 & EBA175 as well as with OVA, CoVaccineHT™ again induced the most potent IgG titres. As seen with other *Plasmodium* antigens tested in this chapter, mice also seroconverted to PvDBP_RII in PBS indicating that this antigen is very immunogenic. As measured by the LAL assay outlined in materials and methods, the

endotoxin levels of this protein were 22 EU/25 μg of protein which is within the range of endotoxin levels reported for other pre-clinical vaccines [299]. IgG titres in the chemically adjuvanted groups (excluding MVA as an adjuvant) were significantly different from responses in the PBS group ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparisons post-test) (Figure 4.6A). Interestingly, despite published data indicating MVA can function as an adjuvant, responses induced in the PvDBP_RII protein + MVA PvDBP_RII group were not significantly different from those induced with PBS ($p>0.05$, un-paired t-test). Antibody responses were significantly boosted by a second immunisation of protein in adjuvant comparing pre and post-boost titres ($p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test) but not by a third shot, as IgG titres appeared to have reached a plateau (Figure 4.6B). After three immunisations of protein in adjuvant, all adjuvants induced comparable IgG titres that were significantly different from those induced by PvDBP_RII in PBS and co-administered with recombinant MVA ($p<0.05$, one-way ANOVA with Bonferroni's Multiple Comparisons post-test).

Consistent with previous data, all mice in the AP groups seroconverted in response to AdHu5 PvDBP_RII, with antibody titres reaching similar levels to those following one immunisation of PvDBP_RII protein in adjuvant (Figure 4.6C). IgG titres were only assessed for select groups following the AdHu5 PvDBP_RII prime given each group received the same priming vaccine and therefore served as an internal replicate. IgG

titres were not significantly different two weeks after the adenovirus prime across the groups tested ($p>0.05$, one-way ANOVA with Bonferroni's Multiple Comparisons Test). Antibody responses were boosted by >1 log in all adjuvant groups comparing pre and post-boost titres (13-fold increase in the mean across all adjuvant groups, 95% CI: 10-16; $p<0.001$, two-way ANOVA with Bonferroni's multiple comparison post-test). There was no significant difference in the mean fold change from pre-boost to post-boost IgG titres in relation to the use of a particular adjuvant to boost IgG titres following an adenoviral prime as seen previously for EBA-175 and MSP-1 (data not shown).

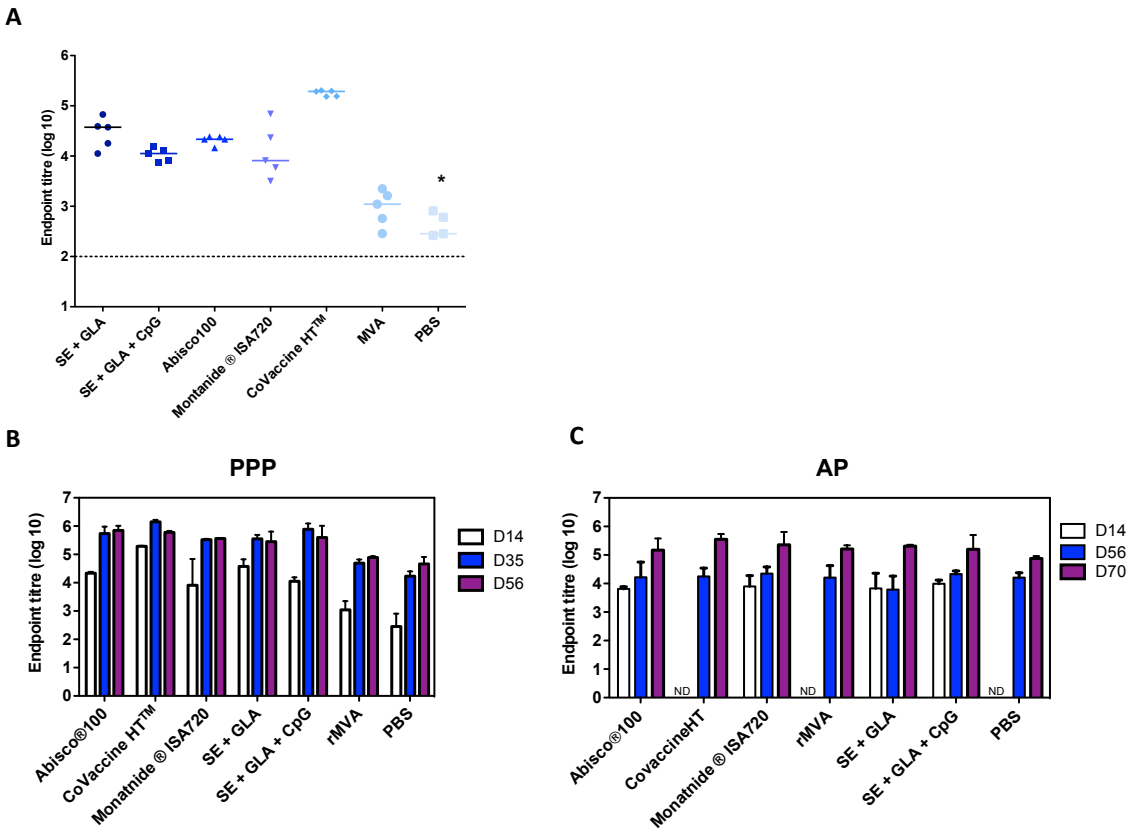

Figure 4.6 Antibody immunogenicity of PvDBP_RII vaccines.

BALB/c mice (*n* = 5 / group) were immunised thrice i.m. three weeks apart with 10µg of *P. vivax* DBP_RII protein. IgG titres were measured in the serum to (A) PvDBP_RII protein two weeks after the first immunisation. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. * p<0.05 by One-way ANOVA with Bonferroni's multiple comparisons post-test against PBS. IgG titres in the serum were also measured two weeks after each immunisation in response to PvDBP_RII protein by ELISA (B). BALB/c mice (n= 5 / group) were primed i.m. with $1x10^{10}$ vp of AdHu5 PvDBP_RII and boosted i.m. eight weeks later with 10µg of *P. vivax* DBP_RII protein. IgG titres were measured in the serum in response to PvDBP_RII protein by ELISA two weeks after each immunisation and before the protein boost (C). Median responses are shown with range.

4.2.5 T cell immunogenicity of PvDBP_RII vaccines

T cell responses were also assessed in BALB/c mice immunised with PvDBP_RII in AP and PPP regimes at the last time point, 10 weeks after the final immunisation (Figure 4.7). Splenocytes were re-stimulated with recombinant (r) PvDBP_RII protein, as no peptides were available for this antigen. T cell responses were induced with all adjuvants in the PPP regime (Figure 4.7A) with SE+GLA+CpG inducing the strongest T cells responses, with a median of 1068 SFU/10e6 splenocytes, as seen with OVA. SE+GLA and CoVaccine HT^{TM} induced moderate T cell responses with a median of 568 and 528 SFU/10e6 splenocytes respectively. The T cell responses induced with TLR containing adjuvants were significantly higher than those induced in the PBS group ($p<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison post-test). In contrast T cell responses induced in the AP regimes were weaker than in the comparator PPP groups (Figure 4.7B). Although PBS responses were higher following an AP regime than a PPP regime, probably due to T cells induced by the AdHu5 PvDBP_RII prime, T cell responses in the adjuvant groups appeared to be lower or of equal magnitude in the AP groups compared to their comparator groups in a PPP regime. This was surprising, given that in the OVA regime potent T cells were induced using Abisco®100 in an AP regime (see chapter 3). This is probably due to the nature of T cell re-stimulation used as rPvDBP_RII protein will probably not be cross-presented into the MHC class I pathway during the re-stimulation period of the ELISpot assay. Consequently, the potentially strong CD8+ T cell responses

typically induced by adenoviral vaccine-containing regimes are very likely to have remained undetected.

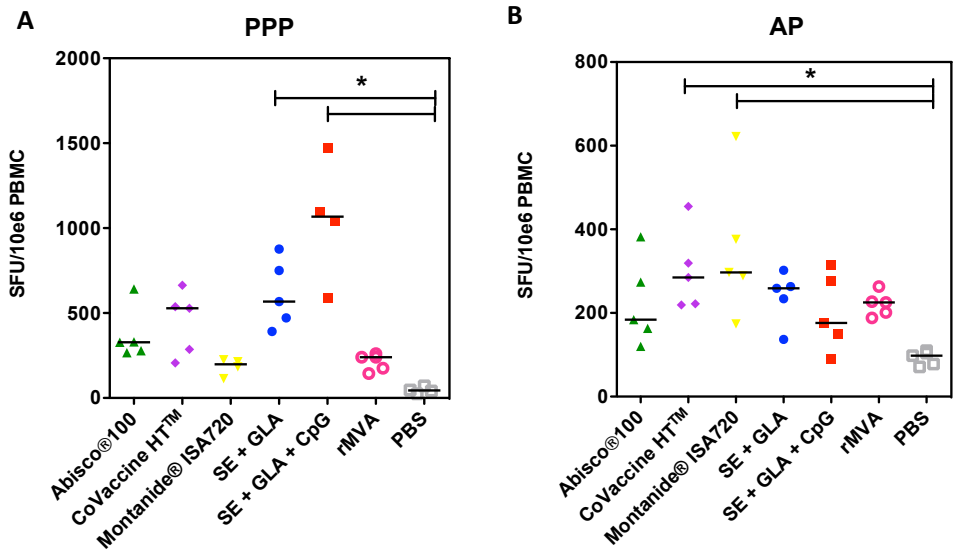

Figure 4.7 T cell immunogenicity of PvDBP_RII vaccines.

BALB/c mice (*n* = 5 / group) were immunised with PvDBP_RII PPP and AP regimes as described previously. Ten weeks after the last immunisation, splenic T cell responses were measured by re-stimulation with recombinant PvDBP_RII protein in (A) PPP and (B) AP immunised mice. Median and individual data points are shown. *p<0.05 by Kruskal-Wallis with Dunn's multiple comparison test.

4.2.6 Inhibition of DARC binding by PvDBP_RII vaccine-induced antibodies

The rationale behind a PvDBP-based vaccine relies on the induction of high-titre antibodies that inhibit RBC invasion by preventing the binding of DBP to its natural ligand, the DARC. To measure the ability of antibodies raised in mice against DBP to inhibit the binding of DBP to its receptor, an *in vitro* binding inhibition assay has been developed. This assay was kindly performed by R. Shakri (ICGEB, India) with sera generated in the experiments outlined in this chapter as described in 2.5.5. In brief, sera diluted to different concentrations are pre-incubated with recombinant PvDBP_RII protein and applied to ELISA plates pre-coated with recombinant DARC. PvDBP_RII antigen bound to DARC is detected using anti-rabbit polyclonal anti-PvDBP_RII sera and an anti-rabbit IgG-HRP conjugated antibody and developed in a similar manner to an ELISA. A standard curve generated using PvDBP_RII protein is used to convert OD values into amount of protein bound in each well, thus giving a read-out of % binding inhibition. This binding inhibition assay was carried out using serum samples from various vaccination regimes. Serum from mice immunised with AP and PPP SE + GLA, Abisco®100 and Montanide®ISA720 were tested two weeks after the final immunisation as well as sera from mice 8 weeks after a priming immunisation with AdHu5 PvDBP_RII (i.e. post-prime and pre-boost). In addition to this, serum from mice immunised with an AM regime ($1x10^{10}$ vp AdHu5 PvDBP_RII prime, $1x10^{7}$pfu MVA PvDBP_RII boost 8 weeks

later) was also tested. Sera from mice immunised with the AM regime was kindly provided by Dr. S Draper. Although immunised separately, the antibody responses induced followed a similar IgG kinetic to IgG titres following other AM regimes in mice (data not shown).

The binding inhibition of sera was tested at three different dilutions: 1:1000, 1:2000 and 1:4000 (Figure 4.8). High levels of inhibition >90% were achieved with sera from mice immunised with AP and AM regimes two weeks after the final immunisation at a dilution of 1:1000 (Figure 4.8A). A similar pattern of binding inhibition across the different regimes was also evident at 1:2000 and 1:4000 dilutions (Figure 4.8B&C). However, binding inhibition did appear to titre out at a 1:4000 dilution for the AP Montanide®ISA720, PPP SE+GLA, PPP Montanide®ISA720 and AM regimes. Binding inhibition appeared to be consistently high in AP and PPP Abisco®100 regimes across the different dilutions.

At a dilution of 1:4000 binding inhibition with sera from AM immunised mice was significantly lower than that achieved with AP Abisco®100 and AP SE+GLA when comparing two-shot viral vector containing regimes (AP and AM) ($p<0.05$, Kruskal-Wallis with Dunn's multiple Comparison post-test) (Figure 4.9). In the PPP regimes, immunisation with PPP Abisco®100 induced a significantly higher level of inhibition than PPP SE+GLA and PPP Montanide®ISA720 ($p<0.05$, Kruskal-Wallis with Dunn's Multiple

Comparison post-test). This indicates that the highest titres of antibodies, capable of binding inhibition, can be achieved in mice with AP regimes and protein vaccines formulated in Abisco®100. Binding inhibition correlated with IgG titres at all dilutions and was shown to follow a sigmoidal relationship as has been seen for other *in vitro* antibody assays measuring the functional activity of antibodies such as the growth inhibition assay [178,305] (Figure 4.10).

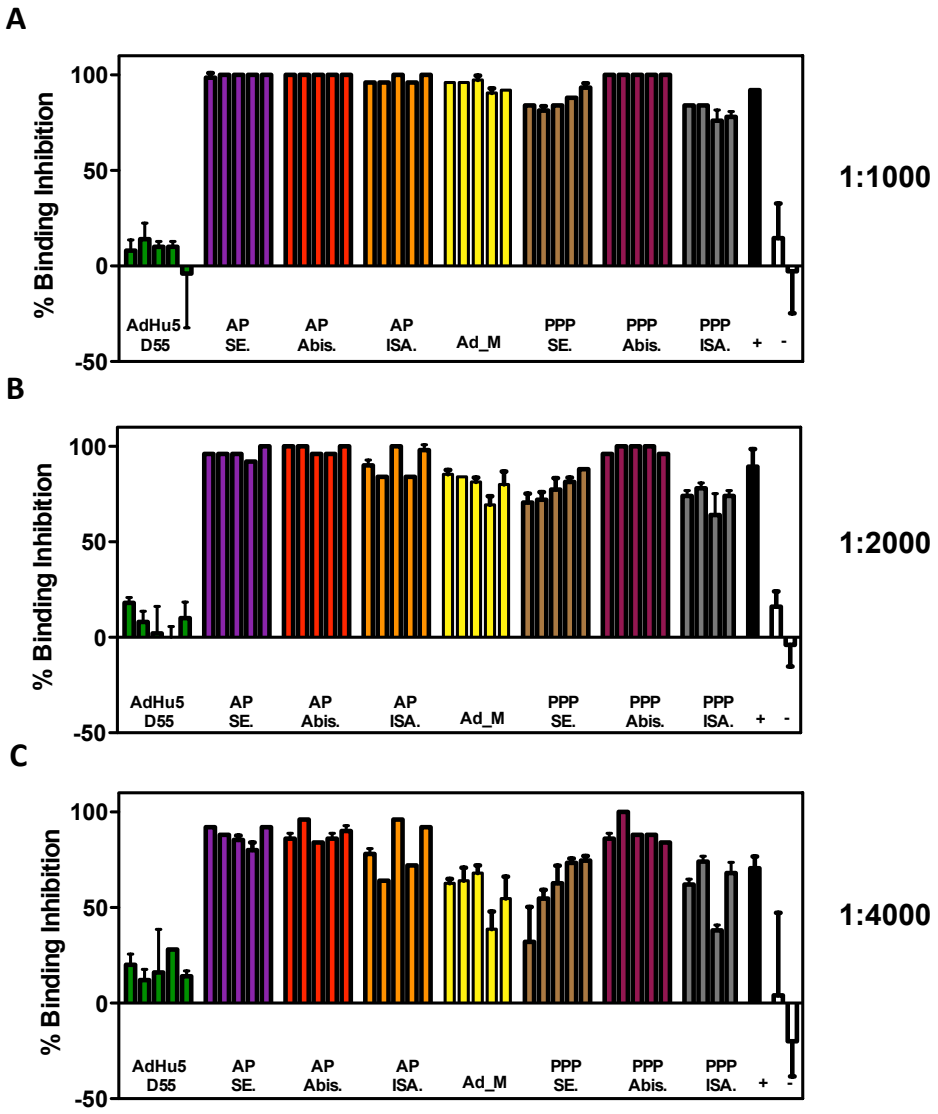

Figure 4.8 Binding inhibition of sera from PPP, AP and AM vaccinated mice.

BALB/c mice (*n* = 5 / group) were immunised with AP, PPP and AM regimes as described. Binding inhibition assays were carried out as described in materials and methods at a serum dilution of (A) 1:1000, (B) 1:2000 and (C) 1:4000. The mean and SD of the percentage binding inhibition for each sample run in duplicate/triplicate are shown. - = Negative control, % inhibition in 2 naïve mice. + = Positive control, % inhibition of reference serum raised in mice against PvDBP_RII using Freund's Adjuvant.

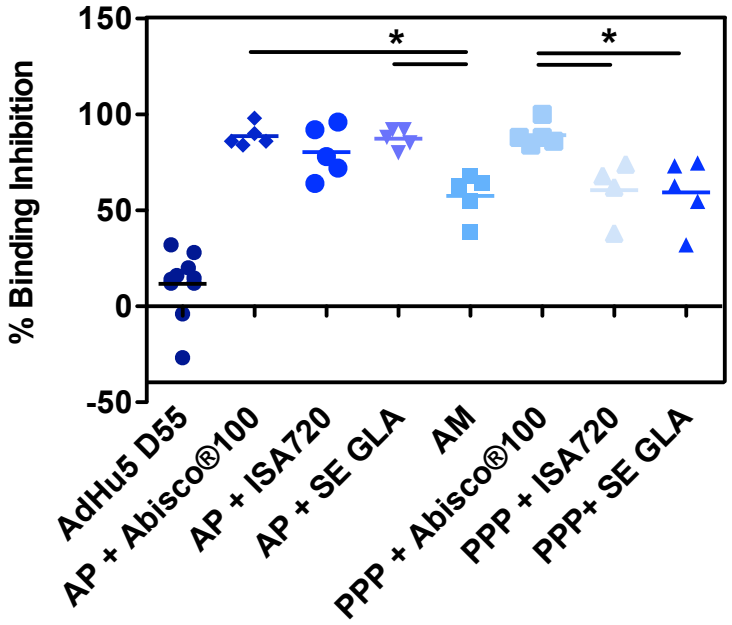

Figure 4.9 Binding inhibition at a 1:4000 dilution.

BALB/c mice (*n* = 5 / group) were immunised with AP, PPP and AM regimes as described. The percentage binding inhibition of each group was assessed at a 1:4000 serum dilution. Mean and individual data points are shown. * $p<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison post-test analysing viral vector containing regimes and protein only regimes separately.

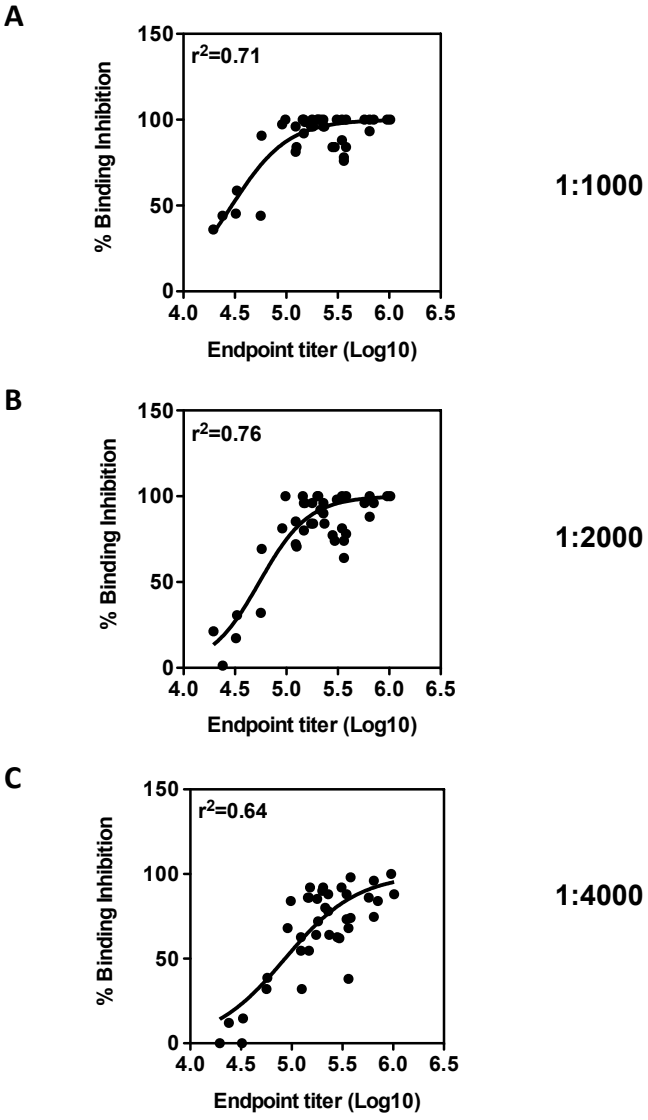

Figure 4.10 IgG titres correlate with binding inhibition.

BALB/c mice (*n* = 5 / group) were immunised with AP, PPP and AM regimes as described. Binding inhibition assays and IgG ELISAs were carried out with the sera from the groups at the time-points outlined previously. Relationship of binding inhibition with IgG titres at (A) 1:1000, (B) 1:2000 and (C) 1:4000 dilution. Non-linear regression co-efficient is shown.

4.3 Discussion

This chapter sought to further explore the immunogenicity of a range of adjuvants in clinical and pre-clinical development and to address whether the improved immunogenicity of adjuvants following an adenoviral prime found using the model antigen OVA can be translated to other antigens tested on a different genetic background. The blood-stage vaccine candidate antigens MSP-1, EBA-175 and DBP were used here to address these questions.

Initially MSP-1 and EBA-175 were administered as a mixture in AP and PPP regimes. The most potent adjuvants from the OVA screen were taken forward to assess their use alongside a candidate clinical vaccine. Although interference has been reported for some multi-antigen vaccines [306,307,308], initial studies using DNA vaccines in Aotus monkeys have shown an absence of competition between these two antigens [309], though this cannot be formally concluded here; so far both of these antigens have been used individually in pre-clinical vaccine studies [83,148,169,303]. Strong IgG titres were induced by both of these antigens in AP regimes when administered as a mixture. Strong IgG titres were also induced by EBA-175 in PPP regimes, but only against MSP-1 using Montanide®ISA720. A similar hierarchy in antibody immunogenicity as seen with OVA was observed after the first immunisation of EBA-175 in a PPP regime. However, after subsequent booster immunisations, IgG titres appeared to reach a plateau. This was likely due to the inherent immunogenicity of EBA-175 protein, which induced

antibody responses in PBS only, but had acceptable endotoxin levels as measured by the standard LAL assay. Antibody responses were only detectable to MSP-1_{19} in Montanide®ISA720 following a PPP regime as has been previously found [177]. It is possible that the absence of antibody responses to *P. falciparum* MSP-1_{19} with adjuvants other than with Montanide®ISA720 could be due to the fact that MSP-1_{19} has been shown to be refractory to antigen processing [310]. The observation that this problem can be overcome specifically by Montanide®ISA720 remains unexplained and warrants further investigation.

Following an adenoviral prime, IgG titres were boosted by over a log in all adjuvanted groups and similar to the OVA system there was no difference in the fold change (pre- and post-boost) across the different adjuvants. In contrast to the PPP regime, all mice receiving the AP regime seroconverted to MSP-1_{19} after the AdHu5 prime and antibody responses were boosted by protein in adjuvant for all adjuvants tested. There was no significant difference in the fold-change of IgG titres pre and post-boost supporting results seen with OVA in chapter 3. As previously described, MSP-1 is proteolytically processed upon RBC invasion to produce an N-terminal 83-kDa fragment and a C-terminal 42-kDa fragment (which contains the GPI anchor) [292]. Upon RBC invasion, MSP-1_{42} is further processed into a 33-kDa (MSP-1_{33}) and 19-kDa (MSP-1_{19}) fragment [293], with T cells directed against the MSP-1_{33} fragment and protective antibodies directed against the MSP-1_{19} fragment. MSP-1_{33}-specific $CD4^+$ T cells have been shown

to provide help for MSP-1_{19}-specific B cells and aid the development of *de novo* antibody responses following *P. yoelii* malaria challenge [148]. The MSP-1_{33} fragment of MSP-1 is contained within the AdHu5 vaccine expressing the clinical MSP1 transgene (PfM128) [169], but is absent from the MSP-1_{19} protein vaccine. It is therefore possible that this difference in the likely priming of MSP-1_{33}-specific CD4+ T cell help between the vectored and protein vaccines is mediating the difference in antibody induction (priming) seen between the two regimes for MSP-1. The role of MSP-1_{33} specific T cells is further explored in Chapter 6.

A more cytophilic antibody response was also again induced by AP regimes compared to PPP regimes which confirmed the results seen with the model antigen OVA. For MSP-1_{19} a correlation of ELISA titres with ASC levels in the spleen and bone marrow for the peak and late time-points was observed, as has previously been shown for this antigen [261]. This implies that serum antibody titres may be maintained by long-lived plasma cells (LLPCs), as has been suggested for other antigens [262,311,312]. However, although not explored in this study, serum antibody titres have also been shown to correlate with memory B cell levels, as measured by a cultured ELISpot assay, for some acute infections and vaccines [262,274,313]. This suggests that the maintenance of serological antibody titres may be under differential control by these two cell populations. As virally vectored vaccination regimes, as well as AP regimes, have been shown to induce memory B cells in rhesus macaques [178], the contribution of this cell type to antibody responses

cannot be ruled out either in mice. Furthermore, although the correlation between LLPCs and serum antibody titres indicates these cells may be maintaining serum antibody levels, it is possible that a greater induction of memory B cells could account for the better boostability of IgG titres seen here and also shown previously [274] and this warrants further investigation.

The ability of AP regimes and PPP in Montanide®ISA720 to induce high-titre antibodies to *P. falciparum* MSP-1_{19} is encouraging as antibodies directed towards MSP-1_{19} have been shown to correlate with protection in animal models and to be associated with protection from clinical disease in humans [204,314,315,316,317]. The protective capacity of the antibodies induced to *P. falciparum* MSP-1_{19} here cannot be assessed in rodent animal models, unless transgenic parasites are used. A transgenic parasite based on the rodent malaria parasite *P. berghei* expressing the *P. falciparum* form of MSP-1_{19} has been created by allelic replacement [318]. This transgenic parasite has been used to show the importance of inhibitory antibodies to MSP-1_{19}. However, despite the induction of high-titre MSP-1_{19} IgG titres through vaccination, convincing protection against infection with this transgenic parasite in rodents is lacking (A. Goodman, DPhil thesis 2009).

Antibodies induced to MSP-1_{19} have also been shown to inhibit the growth of *P. falciparum* parasites *in vitro* in the standardised assay of growth inhibition activity (GIA)

[169,296]. GIA assays assess the biological activity of antibodies and are carried out by mixing purified sera (from naturally immune individuals or vaccinated subjects) with a blood culture of *P. falciparum*. The growth of the parasite is measured in culture throughout one invasion cycle and the GIA of the purified IgG is assessed and compared to a control culture receiving control IgG. This assay can give useful information on the functional capacity of the antibodies induced. Carrying out GIA assays was not possible here, given the limited amounts of serum obtainable from mice. Recently an AP regime using AMA-1 in CoVaccine HT™ in rhesus monkeys has been shown to induce high-level GIA, surpassing that of an AM regime, which is promising given the high antibody titres induced here with AP regimes [178].

However, despite the induction of antibodies to MSP-1_{19} in animal models, which are capable of GIA, Phase Ia clinical trials based on MSP-1 vaccines have been disappointing so far [175,279,280,281]. A study carried out by Miura *et al.* also showed that IgG titres needed to mediate 50% GIA vary across different antigens and in different species [319] and it has been argued that ultimately the concentration of antibody is the determinant of protection against *P. falciparum* [320]. It is therefore likely that even higher antibody titres than those induced in previous clinical trials may be needed for protection, which makes AP regimes an attractive vaccine delivery platform. Based on the data described in this thesis, a Phase Ia clinical trial investigating a ChAd63-AMA1 prime, AMA1-C1 Alhydrogel±CPG 7909 regime [321] is now being trialled at the Jenner Institute in Oxford

with the aim of further enhancing antibody responses in humans following subunit vaccination (ClinicalTrials.gov NCT01351948).

High-titre antibodies were also induced against EBA-175 using AP and PPP regimes. Increasing evidence suggests that invasion ligands such as EBA-175 would make promising candidate vaccine antigens. In pre-clinical animal models antibodies induced to EBA-175 following protein vaccination have been shown to inhibit parasite invasion *in vitro*, even against field isolates [83,300]. This invasion inhibition appeared to correlate with antibody titres. Similarly vaccinating Aotus monkeys with three shots of EBA-175 plasmid DNA followed by one shot of recombinant EBA-175 protein in Montanide®ISA720 resulted in the protection of 3 out of 4 Aotus monkeys following a parasitised RBC challenge [322]. In a recent Phase I trial EBA-175 expressed from *Pichia pastoris* was given in Adju-Phos® in a three-shot vaccination regime over a period of 6 months [278]. Moderate levels of GIA were seen after the third immunisation against the homologous parasite strain to the vaccine, which correlated with antibody titres. Quantifying the invasion inhibitory capacity of the antibodies induced here by AP and PPP regimes would be of further interest to continue exploring EBA-175 as a potential vaccine candidate.

The immunogenicity of selected adjuvants was also explored in AP and PPP regimes using the antigen PvDBP_RII. As with MSP-1 and EBA-175, high IgG titres were induced

by all adjuvants in both AP and PPP regimes. This is likely due to the most potent adjuvants from the initial OVA screen being taken forward, with the aim of inducing maximal antibody responses, as well as the inherent immunogenicity of PvDBP_RII protein used for vaccination. In hindsight, including one group immunised with an aluminium-based adjuvant such as Adju-Phos® would have allowed for a more direct comparison of the immunogenicity of the vaccine regimes. T cell responses 10 weeks after the last immunisation in each regime were also assessed. Moderate T cell responses were induced by both AP and PPP immunisation regimes that were of a similar magnitude as reported before following vaccination of rhesus macaques with two shots of PvDBP_RII in Montanide®ISA720 and AS02A [323]. Interestingly, when using the adjuvant SE+GLA+CpG in a PPP regime, strong antigen-specific T cell responses were induced surpassing those seen previously [323]. This is likely due to the TLR agonists, which have shown to be potent T cell inducers (see Chapter 3). Surprisingly, T cell responses induced by the AP regimes were lower than in the PPP immunised mice, which contrasts with previous reports comparing these vaccination platforms [177]. Typically stronger CD4+ than CD8+ responses are induced by protein regimes compared to viral vectored regimes, with the reverse seen following viral vectors [247,248]. It is likely that the recombinant protein used for re-stimulation here, unlike peptides, cannot bind directly to the MHC class molecules displayed on the surface of APCs but rather needs to be processed as exogenous antigen, and therefore is most likely subsequently loaded onto MHC class II molecules. Re-stimulation with recombinant antigen may thus

prevent detection of potentially strong CD8+ T cell responses usually induced by adenoviruses [324]. In a Phase I trial assessing the immunogenicity of an AM MSP-1 (PfM128) vaccine regime by re-stimulating PBMC with cryopreserved infected RBCs compared to peptides maintained comparable levels of CD4+ T cell responses, but unlike following peptide re-stimulation, CD8+ T cell responses were undetectable [175]. However, as shown in chapter 3 T cell induction following protein regimes is adjuvant dependent and therefore the nature of T cell re-stimulation may not fully explain this effect. The MVA vector itself was also explored as an adjuvant due to previous reports showing MVA can enhance both antibody and T cell responses when mixed with protein vaccines [304], however such an adjuvant effect was not seen here. A higher dose of MVA was used here than previously and it is possible that an adjuvant effect is only evident at lower doses of MVA. Co-administering an MVA expressing the influenza antigens nucleoprotein and matrixprotein 1 with seasonal flu vaccine TIV was only found to only adjuvant antibody responses at intermediate doses (A. van Laarhoven, personal communication).

An *in vitro* binding assay has been developed to assess the ability of antibodies induced by vaccination to inhibit the binding of PvDBP_RII to its receptor DARC [231,286]. Using the in vitro binding assay, high levels of binding inhibition >84% were achieved with AP and PPP regimes containing Abisco®100 at a serum dilution of 1:4000, surpassing previous studies assessing binding inhibition following vaccination in human compatible

adjuvants. In these studies, 50% binding inhibition was achieved at a serum dilution 1:900 in rhesus macaques following a two-shot Montanide®ISA720 protein regime and at a dilution of 1:1081 in mice following 3 shots of protein in Montanide®ISA720 [239,323]. Higher 50% inhibition titres have only been reported using Freund's adjuvant, which is not suitable for use in humans [325]. Binding inhibition correlated with IgG titres at all dilutions and was shown to follow a sigmoidal relationship as has been seen for other *in vitro* antibody assays measuring the functional activity of antibodies such as GIA [178,305]. A correlation between IgG titres and binding inhibition has previously been reported for DBP [323].

Unlike *P. falciparum*, developing a standardised GIA assay to assess the biological activity of antibodies on parasite growth for *P. vivax* has proven difficult. *P. vivax* merozoites require reticulocytes for propagation of their life-cycle which are a minor and short-lived population in peripheral blood making the maintenance of this parasite in culture difficult [326]. Furthermore, using blood from infected donors for these assays poses the problem of low-density infections from which meaningful observations are difficult to make. Recently efforts are underway to develop a standardised GIA assay using enriched reticulocytes from cord blood and *P. vixax* schizonts concentrated from clinical isolates [326]. These recent advances in culturing *P. vivax in vitro* could therefore allow for the assessment of inhibition by sera of parasite invasion into human erythrocytes against field isolates [287]. Exploring the binding inhibition and antibody

titres against heterologous alleles of DBP is also of interest to explore whether this vaccine candidate based on the SalI strain of *P. vivax* can induce cross-reactive antibodies. IFATs are now also underway to assess whether the antibodies induced through vaccination can bind to native parasite antigen. Furthermore, the use of TLR agonists with this PvDBP_RII vaccine has been shown to increase the breadth of the antibody repertoire, which also warrants further investigation [45]. Overall, despite the recent finding of cases of *P. vivax* in Duffy Negative individuals [327] this antigen remains the only rational and attractive target for vaccination against blood-stage *P. vivax*. The use of this candidate antigen in an AP regime in humans could lead to the induction of high titre antibodies capable of invasion inhibition. Vectored and protein PvDBP_RII vaccines are currently undergoing GMP manufacture, and such an approach should enter Phase Ia clinical testing in 2012/13.

Taken all together, these data indicate that despite some minor differences, the improved boostability of IgG responses observed following a recombinant adenoviral prime is consistent and can be observed in different mouse strains and when using different / clinically-relevant antigens. These data further support the assessment of this vaccination regime in clinical trials.

Chapter 5

Investigation the Immunological Mechanisms mediating Differential Priming between Adenoviral and Protein Vaccines

5 Investigating the Immunological Mechanisms Mediating Differential Priming Between Adenoviral and Protein Vaccines

5.1 Introduction

Work in the previous chapters of this thesis has explored the differential ability of protein vaccines to prime and boost immune responses in a PPP regime as compared to an AP regime with a range of candidate antigens. These results indicated that the differential immunogenicity of protein vaccines in a three-shot protein/adjuvant regime could be partially overcome using the same protein vaccines to boost adenoviral-primed responses. This AP immunisation regime appeared to show more consistent priming of humoral responses and also induced a more cytophilic antibody response dominated by IgG2a. Given adenoviral vaccines and protein vaccines are inherently different, this chapter explores underlying differences between these vaccine platforms in priming antibody responses.

Contact with antigen in secondary lymphoid organs causes B cells to migrate to the edge of the T cell zone due to the expression of CCR7. Antigen bound to the BCR is taken up and processed and presented to T cells on MHC class II molecules. T cells recognising this peptide-MHC complex secrete cytokines such as IL-6 and IL-21 and synthesise

CD40L, which interacts with CD40 on the B cell, to initiate the germinal centre (GC) reaction. These activated B cells subsequently migrate into primary follicles in the B cell zone where the GC reaction is established (Figure 5.1). Within the dark zone these B cells are termed centroblasts and undergo somatic hypermuation and class-switch recombination [328]. High-affinity B cells are then selected in the light zone of the GC by competition for antigen on FDCs. T follicular helper ($T_{FH)}$) cells which home into the primary follicles due to expression of CXCR5, provide survival signals and help shape the antibody repertoire within the light zone [32]. These high-affinity B cells then produce long-lived plasma cells and memory B cells.

Although ultimately T-dependent antibody responses are believed to be generated to antigens as described above, few studies have addressed whether there are differences in the initiation and magnitude of GCs or in the cell types involved in priming antibody responses following different vaccine platforms. Considering the inherent differences between viral vectors and soluble protein antigens, it is likely that underlying differences in i) the amount of antigen exposed to the immune system, ii) the induction of T cell help, iii) GC formation and iv) arrival of antigen in the lymph node (LN) could be accounting for differences between these vaccine platforms. Exploring these differences could give insights that aid the more rationale design of antibody inducing vaccines.

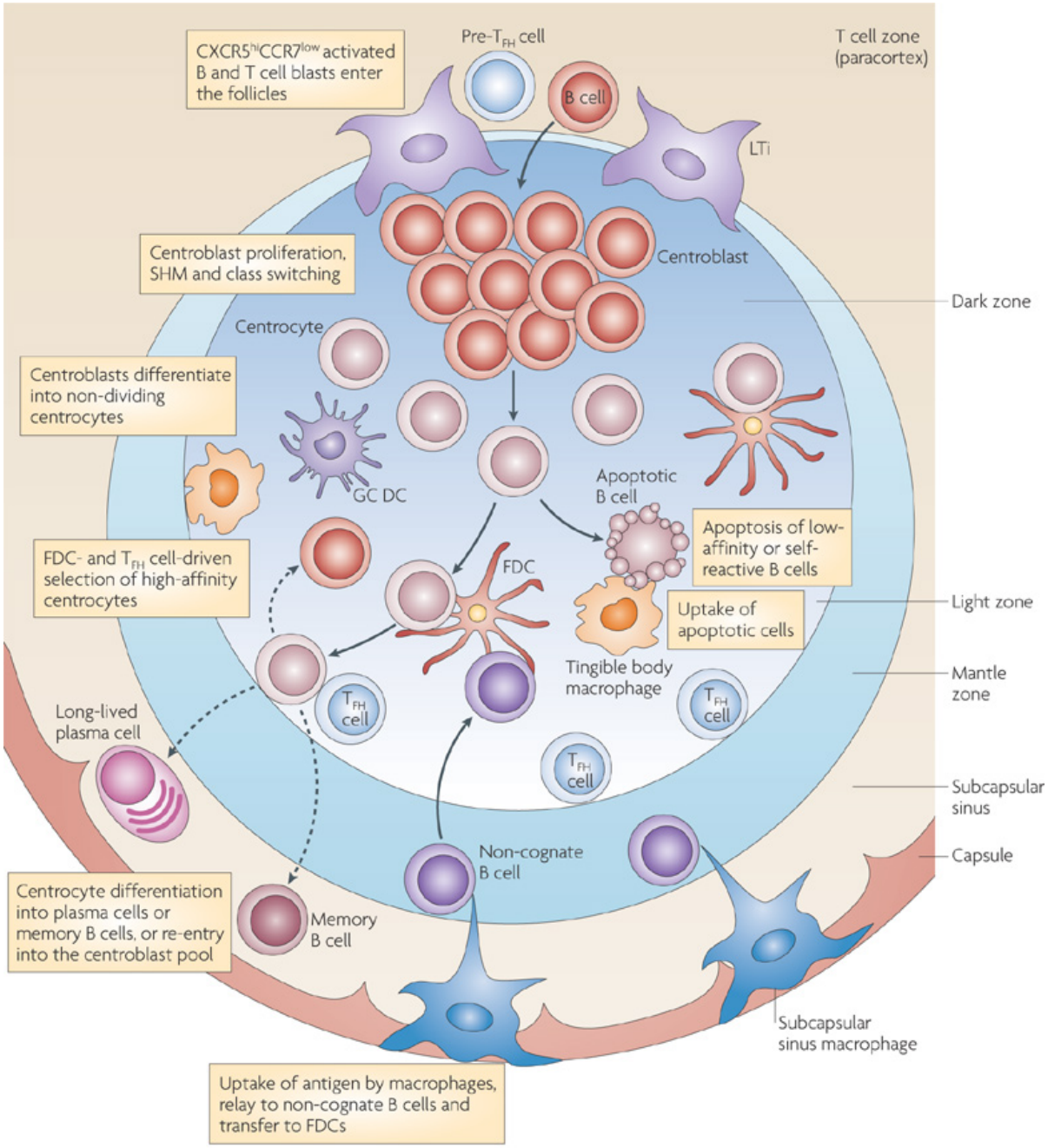

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Figure 5.1 The Germinal Centre Reaction

Antigen-activated B cells enter primary follicles and initiate the germinal centre (GC) reaction. T follicular helper (T_{FH}) cells that have been activated at the border of the T cell zone also enter the GC. Survival and growth signals may be provided to these cells by lymphoid tissue inducers (LTi). In the dark zone antigen-activated B cells differentiate into centroblasts that undergo clonal expansion. Through somatic hypermutation (SHM) base-pair changes are introduced into the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy and light chains. Centroblasts then differentiate into centrocytes and move to the light zone. The light zone is rich in follicular dendritic cells (FDCs) that have antigen bound on their surface. Antigen can be picked up by subcapsular sinus (SCS) macrophages from the lymph and delivered to non-cognate B cells that transfer the antigen to follicular dendritic cells (FDCs). FDCs bind the antigen in the form of immune complexes. Centrocytes, are selected after a successful interaction with FDCs, uptake of antigen through the BCR and presentation of this antigen to T_{FH} cells. Selection can lead to terminal differentiation into long-lived plasma cells or memory B cells or can induce centrocytes to re-enter the cell cycle and undergo further rounds of SHM. Failure to receive these survival signals leads to apoptosis *in situ*. Apoptotic cells are cleared by tingible body macrophages. Chemokine gradients control the movements of cells in the GC. Figure taken from and legend adapted from [329].

5.2 Results

5.2.1 The effect of protein dose and immunisation interval on antibody responses

As reported in the previous chapters, an adenoviral priming immunisation mediates improved boosting of IgG titres by protein-in-adjuvant vaccines. Since these vaccine delivery platforms are inherently different, it is possible that a difference in the dose of antigen exposed to the immune system at the time of priming (between protein vaccines and adenoviral vectors), and/or extended immunisation intervals (used in the AP regime) might be mediating this effect. Previously, an effect has been seen on IgG titres following an extended immunisation interval using viral vectored vaccines [174]. In order to address these questions, C57BL/6 mice were immunized with either 5 or 20 μg OVA in selected adjuvants at an interval of 8 weeks or immunized with 100μg OVA 2 weeks apart. Serum antibody responses were assessed two weeks after the second immunisation and compared to responses seen after two immunisations with 20μg OVA (see Figure 3.1). Antibody responses to OVA in PBS were negative at all doses and antibody responses pre-boost in mice receiving two vaccinations eight weeks apart were also negative (data not shown). For the adjuvants investigated, there was no enhancement of antibody responses with an increased dose of 100μg of OVA given two weeks apart in comparison to 20μg of OVA 2 weeks apart (Figure 5. 2A-D). There was a trend for an improvement of antibody responses using the standard 20μg dose with an

extended immunisation interval of eight weeks (versus 2), although this was only significant for Abisco®100 and SE+GLA+CpG ($p = 0.02$ for Abisco®100 and $p = 0.013$ for SE+GLA+CpG, by Mann-Whitney test) (Figure 5.2B&D). These data indicate that a prolonged time interval between immunisations, rather than dose of antigen, may improve antibody induction by a subset of adjuvants when used in protein only immunisation regimes. However, IgG titres induced by the above regimes are not as strong as those induced by AP regimes, indicating an additional improvement of IgG titres, beyond extended time-intervals, is mediated through the adenoviral prime.

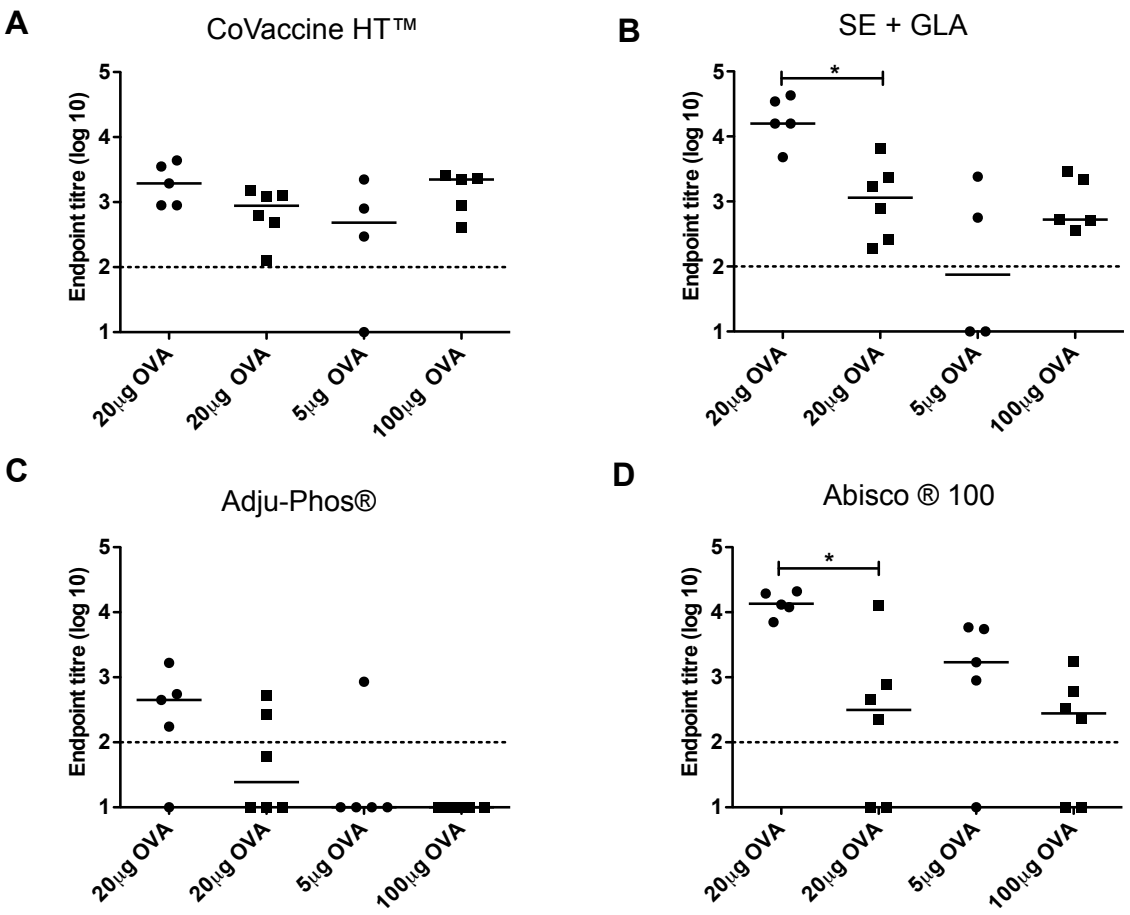

Figure 5.2 The effect of protein dose and immunisation interval on antibody responses.

C57BL/6 mice (*n* = 5-6 / group) were immunized i.m. twice with 5μg or 20μg of OVA protein eight weeks (●) apart or twice with 20μg or 100μg of OVA two weeks (■) apart. Protein was formulated in (A) CoVaccine HT^{TM}, (B) SE+GLA, (C) Adju-Phos® and (D) Abisco®100. IgG titres were measured in the serum two weeks after the final immunisation in response to OVA protein by ELISA. * $p<0.05$, by Mann Whitney test. Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background.

5.2.2 The effect of different doses of adenovirus at the prime on antibody responses

To further characterise the effect of dosing on the improved boostability of protein vaccines following an adenoviral prime, C57BL/6 mice were primed with decreasing doses of AdHu5 OVA and boosted with 20μg of OVA in Abisco®100 or Adju-Phos® as previously. One group of mice received decreasing doses of AdHu5 OVA and was not boosted. The AdHu5 OVA dose was de-escalated over 2 logs with the standard dose of 10^{10} vp used as well as two lower doses of 10^{9} vp and 10^{8} vp. As before ELISA titres were measured two weeks after the final immunisation. IgG titres pre-boost were negative at the two lower doses of AdHu5 OVA (data not shown) unlike for the 10^{10} vp dose.

A dose-response curve was clearly evident at week 10 in the not boosted AdHu5 OVA control groups (this time-point corresponds to two weeks post-protein boost in the AP immunised mice), with the highest IgG titres seen following 10^{10} vp of AdHu5 OVA (Figure 5.3). Following a protein-in-adjuvant boost, IgG titres in the AP groups were significantly higher than in the AdHu5 OVA only control group ($p>0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). Interestingly, although IgG titres following 10^{10} vp of AdHu5 OVA and 20μg of OVA in Abisco®100 were significantly higher than following a 10^{8} vp priming dose of AdHu5 OVA and the same protein vaccine ($p<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test), IgG titres

following Abisco®100 were not significantly different following 10^9 vp or 10^{10} vp of AdHu5 OVA. Following an AP Adju-Phos® regime, IgG titres were not significantly different after the boost following the different priming doses of AdHu5 OVA ($p>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test). These results indicate that the relatively high priming dose of AdHu5 (used in the previous studies in Chapters 3&4) is unlikely to be mediating the differences observed between protein/adjuvant vaccine performance following boosting in AP and PPP regimes.

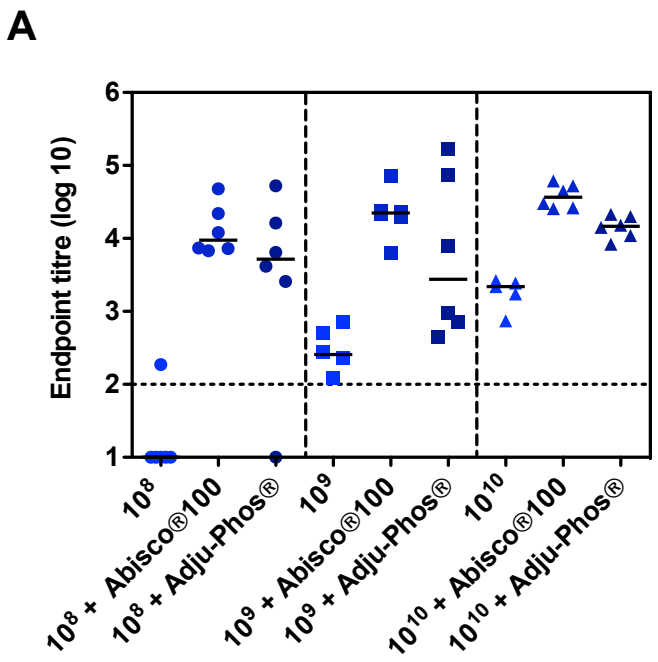

Figure 5.3 The effect of different doses of AdHu5 vaccine at the prime on antibody responses following a protein/adjuvant boost.

C57BL/6 mice (*n* = 5 / group) were primed with either 10^8vp, 10^9vp or 10^{10}vp of AdHu5 OVA. Eight weeks later mice were boosted with 20µg of OVA protein in either Abisco®100 or Adju-Phos® or not boosted. IgG titres were measured in the serum two weeks after the final immunisation in response to OVA protein by ELISA. Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background.

5.2.3 The effect of differential adjuvant priming on antibody responses

Although a prolonged time-interval may improve antibody induction by a subset of adjuvants in a PP immunisation regime, the data shown in 5.2.1 are insufficient to explain why adjuvants such as Adju-Phos® are better able to boost IgG responses in the context of an AP immunisation regime (as described in chapters 3). To investigate whether this was an adenovirus vector-specific phenomenon, mice were primed with 20µg of OVA in CoVaccine HT™ (the best primer of antibody responses in PPP regimes) followed by a boost 8 weeks later with 20µg of OVA formulated in Adju-Phos®. Interestingly this did not result in improved antibody responses (i.e. comparable to those seen with the AP regime) (Figure 5.4). This suggests that the improved boosting of IgG responses, seen with most adjuvants in the context of an AP immunisation regime, appears to be inherent to the adenoviral prime, rather than due to differences in immunisation schedules and/or antigen dosing, and even the best currently available chemical adjuvant (CoVaccine HT™) could not perform comparably to recombinant AdHu5.

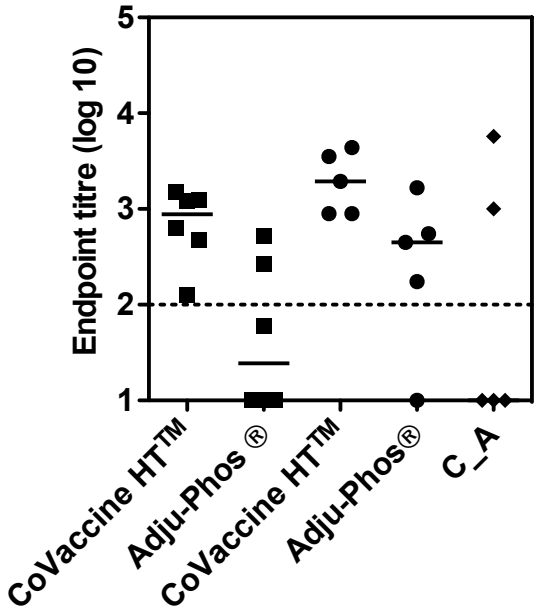

Figure 5.4 The effect of differential adjuvant priming on antibody responses.

C57BL/6 mice (*n* = 5-6 / group) were immunised twice i.m. with 20µg OVA protein eight weeks (●) apart or with 20µg OVA two weeks (■) apart formulated in either CoVaccine HT™ or Adju-Phos®. C57BL/6 mice were also immunized with 20µg OVA in CoVaccine HT™ and boosted eight weeks later with 20µg OVA in Adju-Phos® (C_A ▲). IgG titres were measured in the serum two weeks after the final immunisation in response to OVA protein by ELISA. Median responses and individual data points are shown. The dotted line indicates the threshold for responses above background.

5.2.4 A non-recombinant adenovirus can enhance secondary T cell and antibody responses when administered with a protein priming immunisation

The previous data in this chapter indicate that the improved boosting of antibody responses following a protein/adjuvant vaccine appears to be inherent to the adenovirus prime rather than a difference in dose between viral vectored and protein vaccines. Furthermore, viral vectors that are not recombinant for their transgene have also been reported to adjuvant both humoral and cellular immune responses to protein vaccines [304], although this finding could not be reproduced with MVA in the PvDBP_RII experiments in Chapter 4. To explore whether it is the innate immune "profile" induced by an adenovirus that leads to better priming of antibody responses, C57BL/6 mice were primed with either 20μg OVA protein in PBS (OVA only), 20μg of OVA protein in PBS plus 10^{10} vp non-recombinant AdHu5 empty vector (nrAd + OVA) or 10^{10} vp nrAd alone (nrAd only). IgG titres measured in the serum 2 and 8 weeks after the priming immunisation in response to OVA protein by ELISA were negative (data not shown). All groups were then boosted with 20μg of OVA in Abisco®100 at week 8 to explore whether the non-recombinant adenovirus prime can impact secondary immune responses.

T cell responses were assayed in the spleen against the two known H-2^b $CD4^+$ T cell epitopes present in OVA 2 weeks after the final immunisation (Figure 5.5A). Interestingly despite receiving the same boosting vaccine, CD4+ T cells responses to the CD4+ T cell epitope TEWT [244] were significantly higher in the group that received a nrAd+OVA priming immunisation than in the nrAd only group ($p<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test), with a trend towards higher responses than in the OVA only group. Responses to the CD4+ ISQA epitope were also present, yet these induced high background responses in the *ex vivo* ELISpot assay and are not shown. CD8 + T cell responses to the known H-2^b $CD8^+$ T cell epitope present in OVA were not explored due to the link between CD4+ T cells and antibody responses [259,260,330] which was the main focal point of this experiment.

IgG titres were also measured in the serum 2 weeks after the protein boost in response to OVA protein by ELISA (Figure 5.5B). As seen with CD4 + T cell responses, IgG titres were significantly higher in the nrAd+OVA group than in the nrAd only group ($p<0.05$, Kruskal-Wallis test with Dunn's multiple comparison test), as expected in the absence of an OVA-prime. There was also a trend towards enhanced IgG titres in the nrAd+OVA group compared to the OVA only group, however this was not significant ($p>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test). Overall these data indicate that nrAd is mediating an effect at the time of the prime, which affects the secondary recall response.

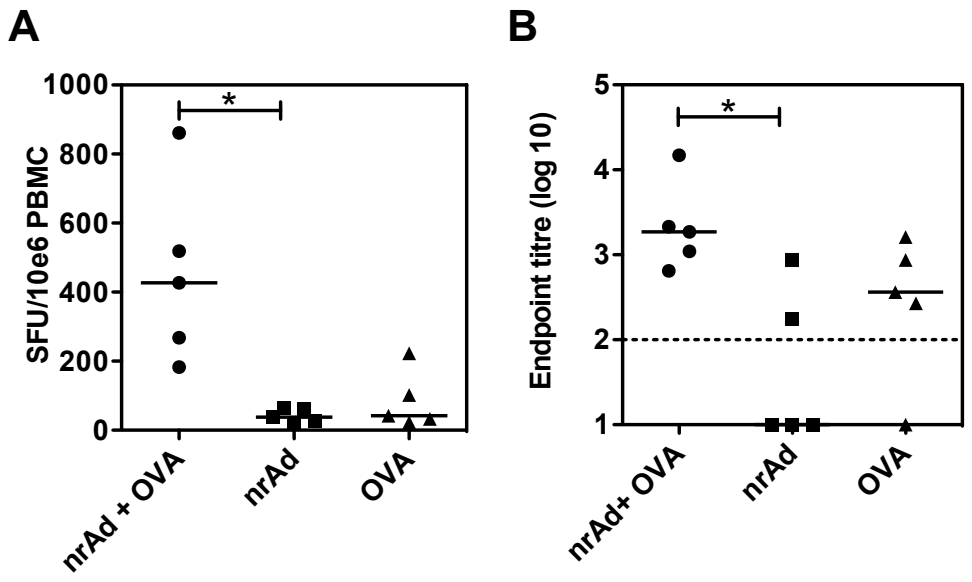

Figure 5.5 A non-recombinant adenovirus can enhance secondary T cell and antibody responses when administered with a protein priming immunisation.

C57BL/6 mice (*n* = 5 / group) were immunised i.m. with either 10^{10} vp non-recombinant AdHu5 + 20μg OVA protein in PBS (nrAd+OVA), 10^{10} vp nrAd, or 20μg OVA protein in PBS (OVA). Eight weeks later all groups were boosted i.m. with 20μg OVA protein in Abisco®100. Two weeks after the last immunisation T cell responses were assayed in the blood against the H-2^b $CD4^+$ T cell epitope TEWT (A). IgG titres were measured in the serum in response to protein by ELISA (B). Median responses and individual data points are shown. The dotted line indicates the threshold for responses above background in (B). * $p<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison test.

5.2.5 Differences in GC formation following different vaccine platforms

To further tease apart the differences in priming between adenoviral vaccines and protein vaccines that may be mediating differences in boosting, the induction of germinal centres by these vaccines was investigated. Within GCs, high-affinity and class-switched antibody (AB) is produced and the induction of GCs has been shown to correlate with AB titres [328,331]. Initially a time-course experiment was undertaken to investigate the induction kinetic of GCs following an adenoviral vaccine versus a protein vaccine. C57BL/6 mice were thus vaccinated i.m. with either 10^{10} vp AdHu5 OVA or with 20μg of OVA in Abisco®100. The draining inguinal LNs and spleen were harvested from representative mice at days 7, 9 and 11 post-vaccination and surface stained as described in 2.4.19. Given GC cells express the B and T cell activation marker (GL7) as well as Fas (CD95) at high levels, resulting in their killing via Fas-FasL interaction in the absence of survival signals from T follicular helper cells [28], GL7+CD95+B220+ cells were defined as GC cells. The gating strategy used to identify these cells can be seen in Figure 5.6. Given this GC stain is not antigen-specific, responses in naïve or PBS immunised control mice were also investigated.

GC cells were present at all time-points investigated following both vaccines in the draining LNs. Interestingly, the highest percentage of GL7+CD95+B220+ cells was found in mice immunised with AdHu5 OVA, which peaked at D9 (Figure 5.7A). Although

induced at a lower level, GL7+CD95+B220+ cells were still induced above background following a protein vaccine in the draining LN. A similar trend was also seen in the spleen, although as expected (given the mice were immunised i.m.) responses were lower than in the draining LN (Figure 5.7B).

The above pilot experiment was then repeated to investigate the induction of GC cells across different vaccines with greater power. C57BL/6 mice were immunised with AdHu5 OVA, non-recombinant AdHu5, MVA OVA, OVA protein in Abisco®100 and OVA protein in Montanide®ISA720. Given viral vectors provide antigenic targets other than the expressed transgene (which soluble protein vaccines lack), the nrAdHu5 control was included to tease apart how much of the GC formation was due to the AdHu5 component of the viral vectored vaccine itself versus the transgene. An MVA OVA vaccinated group was included given MVA vaccines have been shown to be poor inducers of antibodies when used as a priming vaccine. A Montanide®ISA720 group was included to investigate whether the effects on GCs seen with Abisco®100 were adjuvant-specific. Mice were immunised as previously and inguinal LNs and spleens were harvested at D9.

As seen before, the percentage of GL7+CD95+B220+ cells was higher in the AdHu5 OVA group than either protein/adjuvant vaccine group or the PBS control group ($p<0.05$, Kruskal-Wallis Test with Dunn's multiple comparison post-test, AdHu5 OVA versus PBS)

(Figure 5.8A). Interestingly, the responses in the AdHu5 OVA group were significantly higher than those induced in the nrAdHu5 group ($p=0.02$, Mann-Whitney test), which indicates that although some GC formation appears to be due to adenoviral vector-specific antigens, most appears to be due to the transgene. This is further corroborated looking at the percentage of GL7+CD95+B220+ cells induced by MVA OVA, which is a much larger enveloped virus and yet induces GCs at a magnitude comparable to nrAdHu5. The percentages of GL7+CD95+B220+ cells induced by the two protein vaccines tested were comparable, although there was a slight trend towards higher responses in the Montanide®ISA720 group. Overall, responses were again higher in the draining LN than in the spleen (Figure 5.8B). These results indicate that there is a greater induction of GCs following delivery of antigen using a recombinant AdHu5 vaccine rather than following an equivalent protein vaccine or MVA vaccine.

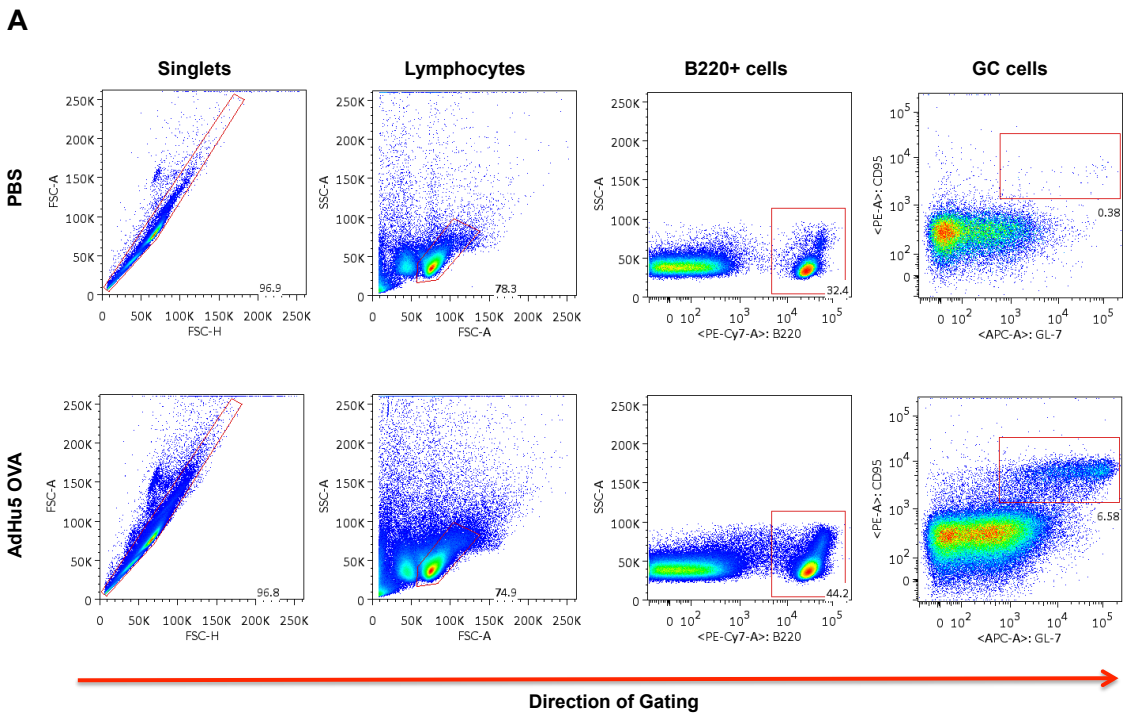

Figure 5.6 Gating strategy to identify GC cells.

C57BL/6 (n = 2 / group) were immunised i.m. with 10^{10} vp AdHu5 OVA or with the same volume of PBS. Nine days later the draining inguinal LNs were harvested and surface stained as outlined in 2.4.19. The sequential gating strategy is shown. Gates show the percentage of parent.

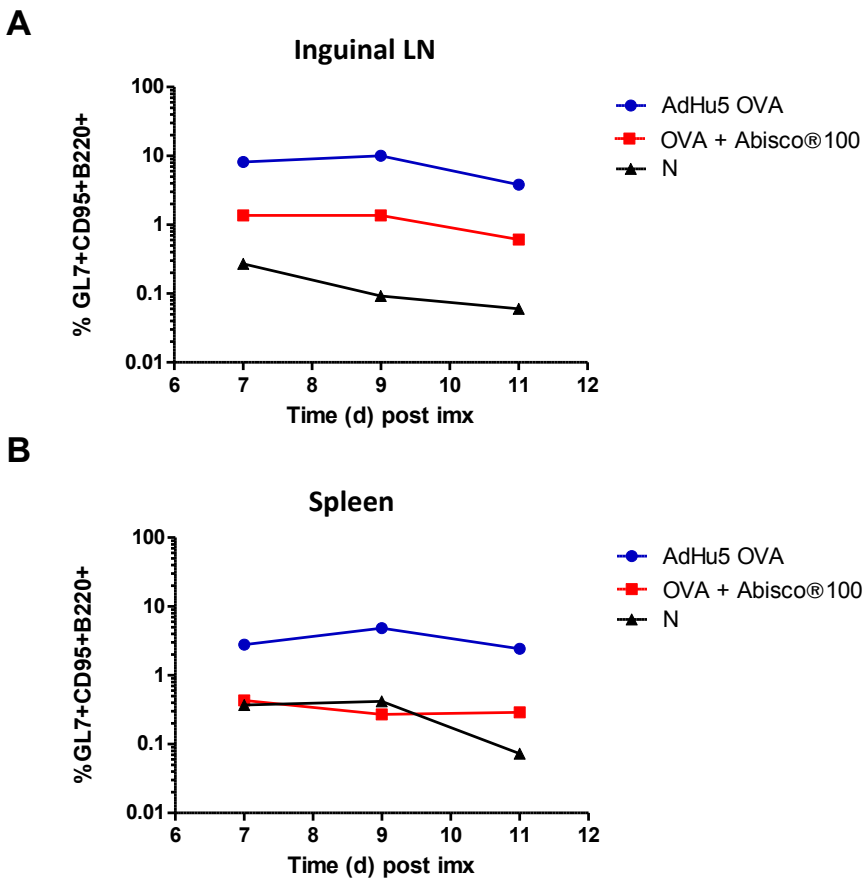

Figure 5.7 GC cells peak at day 9 in the draining LN post-vaccination.

C57BL/6 (n = 2 / group) were immunised i.m. with 10^{10} vp AdHu5 OVA, 20µg of OVA in Abisco®100 or with the same volume of PBS. Seven, 9 or 11 days later the draining inguinal LNs (A) and spleens (B) were harvested and surface stained as outlined in 2.4.19. The median % of GL7+CD95+B220+ cells are shown. N = naïve, imx = immunisation.

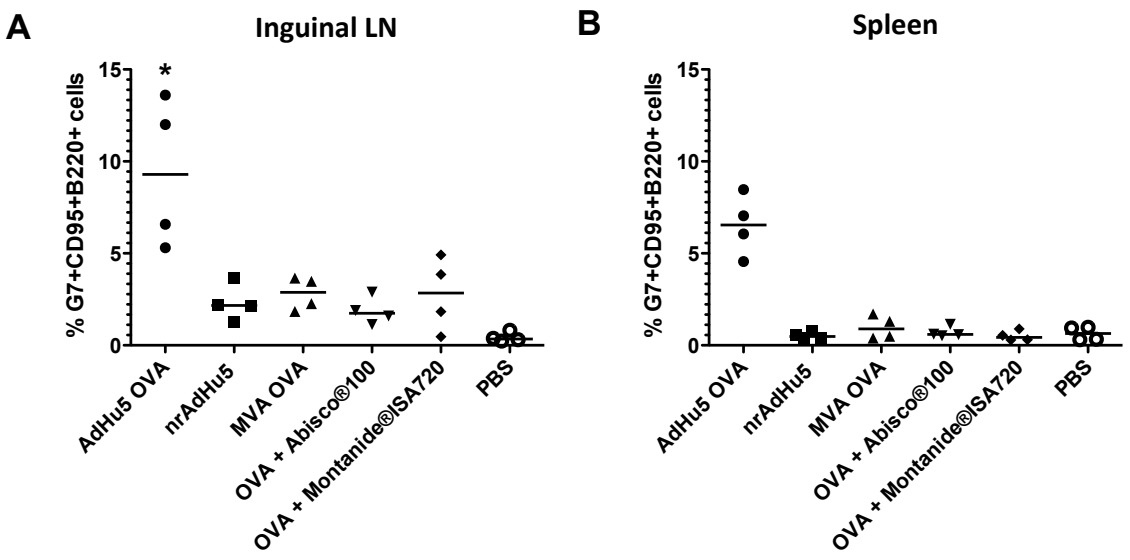

Figure 5.8 GC cells induction across different vaccine platforms.

C57BL/6 (n = 4 / group) were immunised i.m. with either 10^{10} vp AdHu5 OVA, 10^{10} vp nrAdHu5, 20µg OVA in Abisco®100 or Montanide®ISA720, 10^{7} pfu MVA OVA or with the same volume of PBS. Nine days later the draining inguinal LNs (A) and spleens (B) were harvested and surface stained as outlined in 2.4.19. The % of GL7+CD95+B220+ cells is shown. Median and individual data points are shown. * $p<0.05$, Mann-Whitney test versus nrAdHu5.

5.2.6 Differences in the induction of CXCR5+ CD4+ T cells following antigen delivery by different vaccine platforms

Follicular helper T cells (T_{FH}) express CXC chemokine receptor 5 (CXCR5), localise to B cell follicles, and support immunoglobulin production by supporting germinal centre formation [332]. Adjuvants have been shown to influence the size of the T_{FH} cell compartment, with TLR agonists inducing a larger proportion of antigen-specific T_{FH} cells [270]. Cytokines produced by T_{FH} cells have been shown to shape the antibody repertoire through class-switching and somatic hypermutation of B cells [32]. As seen in Chapters 3 and 4, adenoviral vectors appear to prime antibody responses more consistently and a skew towards cytophilic antibody responses was also seen following AP regimes and PPP regimes containing TLR adjuvants. To address the role of T_{FH} cells following viral vectored and protein vaccines, the induction of these cell types post-prime was investigated.

C57BL/6 mice were vaccinated as outlined in 5.2.5 and the draining inguinal LNs were harvested at day 9 post-vaccination. Given the expression of CXCR5 defines follicular homing T cells with B cell helper function [333], LN cells were stained with anti-CD4, anti-CD8 and anti-CXCR5 antibodies as outlined in 2.4.20. CD4+CD8-CXCR5+ cells were defined as T cells with a T_{FH} phenotype and gated as shown in Figure 5.9. Low levels of T_{FH} cells were induced in the draining LNs 9 days post-prime (Figure 5.10A). As seen with

GC cells (Figure 5.8A), the highest levels of T_{FH} cells were induced in mice immunised with AdHu5 OVA. These responses were significantly higher than responses seen in PBS immunised mice ($p<0.05$, by Kruskal-Wallis test with Dunn's Multiple Comparison post-test, AdHu5 OVA versus PBS). Although responses above background (PBS group) were also detected in the nrAdHu5 group, CD4+CXCR5+ cells were significantly higher in the AdHu5 OVA group than in the nrAdHu5 group ($p=0.02$, by Mann-Whitney test). The levels of CD4+CXCR5+ cells induced in mice immunised with MVA OVA and OVA protein in adjuvant were at background levels.

As CD8+ T cells expressing CXCR5 have also been reported [334], CD8+CD4-CXCR5+ cells were also investigated and found to be at background levels for all vaccinated groups (Figure 5.10B). This indicates the CD4+CD8-CXCR5+ cells reflect a defined cell population in the LN D9 post-immunisation. The levels of CD4+CXCR5+ cells were also found to correlate with GC cells ($p<0.0001$, $r=0.74$ by Spearman Rank Correlation) (Figure 5.10C).

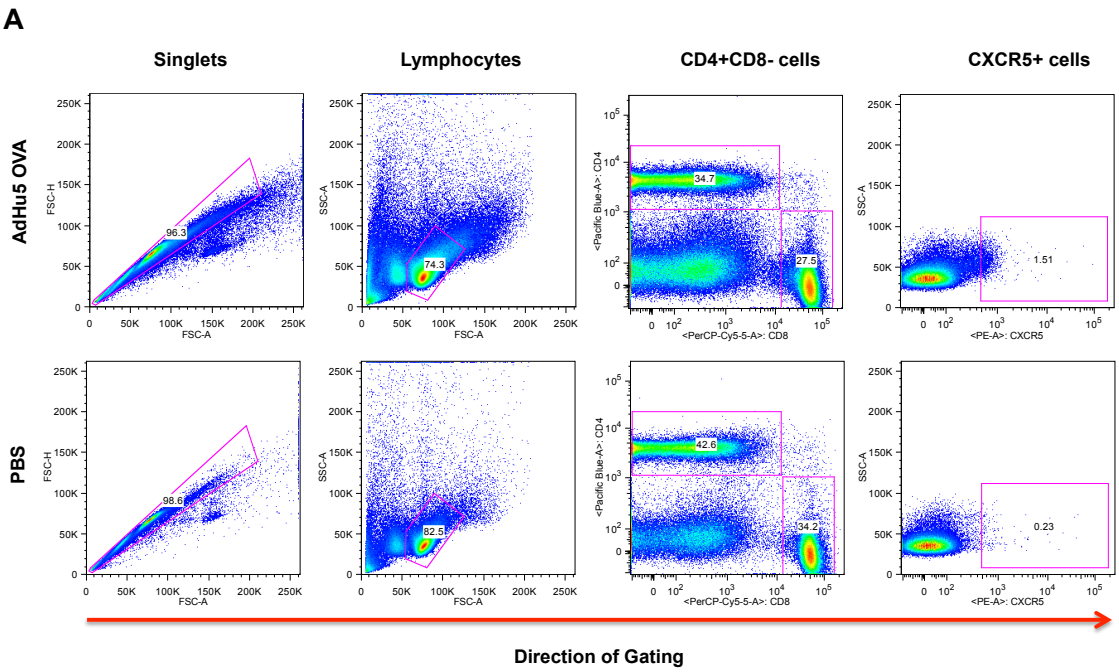

Figure 5.9 Gating strategy to identify T_{FH} cells.

C57BL/6 (n = 4 / group) were immunised i.m. with either 10^{10} vp AdHu5 OVA or with the same volume of PBS. Nine days later the draining inguinal LNs were harvested and surface stained as outlined in 2.4.20. The sequential gating strategy is shown. Gates show the percentage of parent.

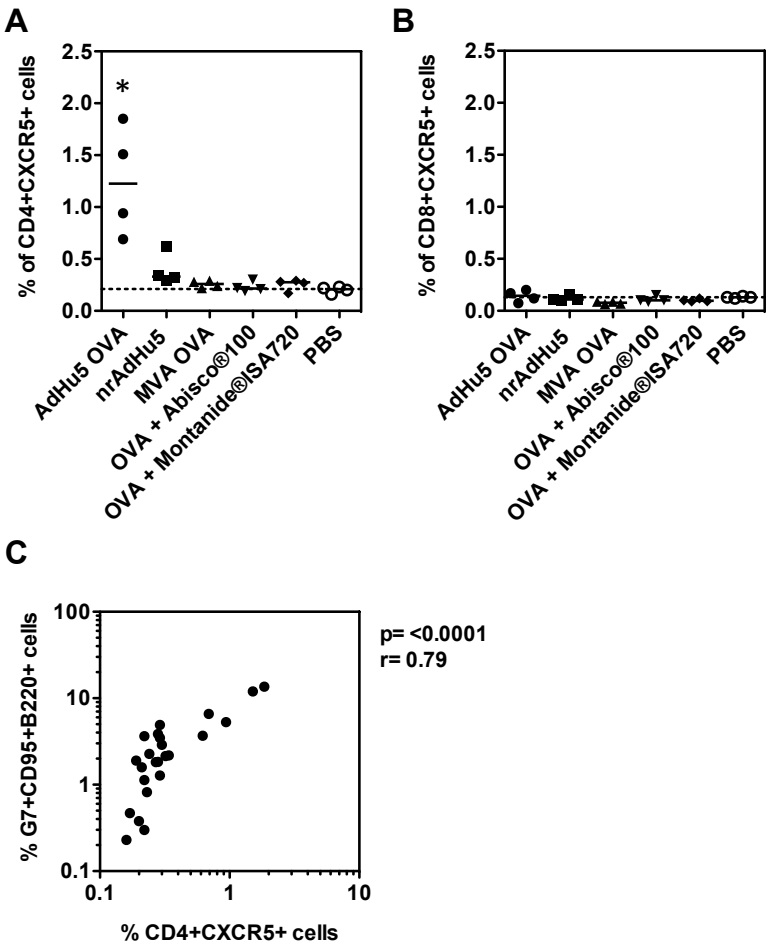

Figure 5.10 T_{FH} cell induction across different vaccine platforms.

C57BL/6 (n = 4 / group) were immunised i.m. with either 10^{10} vp AdHu5 OVA, 10^{10} vp nrAdHu5, 20µg OVA in Abisco®100 or Montanide®ISA720, 10^{7} pfu MVA OVA or with the same volume of PBS. Nine days later the draining inguinal LNs were harvested and surface stained as outlined in 2.4.20. The median and individual % of CD4+CD8-CXCR5+ (A) and CD8+CD4-CXCR5+ (B) cells are shown. * $p<0.05$ by Mann-Whitney test versus nrAdHu5. The % of GC cells (GL7+CD95+B220+) and T_{FH} cells (CD4+CD8-CXCR5+) correlate in the draining LN 9 (C). Spearman rank coefficient with p-value is shown.

5.2.7 The role of T cell help at the time of the prime and its effect on antibody responses

Another explanation, discussed in Chapter 3, to account for more comparable antibody titres seen following an AP regime versus a PPP regime, could be an increased level of CD4+ T cell help induced by the viral vector at the time of priming as compared to protein vaccines. Interestingly, a link between CD4+ T cells and IgG titres was seen by a correlation of peak CD4+ T cell responses and antibody titres post-boost in the OVA model system (Figure 3.6). Furthermore, the depletion of CD4+ T cells before a priming immunisation of OVA has been shown to inhibit the induction of IgG titres [330]. Therefore the role of CD4+ T cells at the time of the prime was investigated using the model antigen OVA again.

C57BL6 mice were primed with 20μg of OVA in Adju-phos®. This protein vaccine was chosen given it was shown to be a poor inducer of T cells and antibody responses in a PPP regime (Figures 3.1&3.2). One day before the priming immunisation, mice were adoptively transferred with CD4+ T cells i.v. isolated using the Miltenyi CD4+ T cell isolation kit according to manufacturer's instructions. These CD4+ T cells were either CD4+ T cells isolated from transgenic OT-II mice, AdHu5 OVA primed mice, nrAdHu5 (vector control) primed mice or naïve mice. OT-II mice express the mouse α-chain and β-chain T cell receptor (αβTCR) that pairs with the CD4 co-receptor and is specific for

chicken ovalbumin αα323-339 (ISQA epitope) in the context of I-A^b (C57BL/6 background). These mice were generated as previously described [335] and purchased from Charles River, UK. Given only male OT-II mice were available, OT-II cells were transferred into C57BL/6 males. The number of CD4 + T cells adoptively transferred from each treatment is described in Table 5.1.

OT-II and naïve cells were transferred at a "physiological number" of $2x10^4$ CD4+ T cells and at a high-dose of $1x10^6$ CD4+ T cells. The physiological number of CD4+ T cells was determined as the median number of antigen-specific CD4+ T cells two-weeks after a single immunisation of 10^{10} vp AdHu5 OVA as measured by an IFN-γ ELISpot in the spleen (Figure 5.11). Given antigen-specific cells following an AdHu5 OVA vaccination represent such a small number of total spleen cells, and cannot be isolated directly in a practical manner, a 'spleen-to-spleen' transfer constituting the number of total CD4+ T cells in the spleen was transferred into mice receiving CD4+ T cells from AdHu5 OVA or vector control immunised mice. The purity of the transferred CD4+ T cell fraction was assessed and found to be >90% pure for each treatment (Figure 5.12). Although stained with anti-CD3 antibodies, distinguishing CD3+ T cells in all fractions following isolation was difficult, possibly to due the fact that CD3 is down-regulated when it is bound by the beads in the isolation kit [336].

The presence of transferred T cells was assessed in the blood of mice one day after transfer (day of prime i.e. protein vaccine immunisation). An ex vivo IFN-γ ELISpot was carried out by re-stimulating PBMC to try and identify OVA specific cells with the ISQA-specific T cell peptide and vector-specific cells using AdHu5 lysate. Despite these efforts, no responses were seen (data not shown), most likely due to the low numbers of antigen-specific cells transferred which are probably trafficking to the spleen due to homing makers and are therefore not measurable in the periphery as has previously been reported (S. Biswas, DPhil thesis 2009).

Anti-OVA IgM titres were assessed in the serum by ELISA 7 days after the prime to investigate whether CD4+ T cells influence the early primary antibody response. IgG titres were assessed two weeks after the prime as usual; however, at both time-points antibody responses to OVA were negative (data not shown). As before, mice immunised with AdHu5 OVA seroconverted to OVA 8 weeks after the priming immunisation (Figure 5.13A). Select mice in the other groups also seroconverted, although there was no trend towards improved IgG titres in mice receiving CD4+ T cells compared to the PP only regime. This suggests that in this system CD4+ T cells transferred at the prime do not impact antibody responses.

All groups were then boosted at week 8 with 20μg OVA protein in Adju-Phos® to see whether an effect of T cell help at the time of the prime, leading to greater mBC

induction, could be measured through a secondary recall responses, as the recall response to OVA has previously been shown to be influenced by the number of antigen-specific CD4+ T cells present at the time of re-exposure [271]. IgG titres were measured in the serum 14 days after the boost (Figure 5.13B). As seen before, the AP regime induced significantly higher titres than the PP regime ($p=0.032$, by Mann-Whitney test). Titres in the PP groups here were higher than those seen previously with this regime (see 5.2.1). However, secondary IgG titres were not significantly enhanced through the transfer of either naïve, OT-II, vector control or AdHu5 OVA primed CD4+ T cells at the time of the priming immunisation ($p>0.05$, by Kruskal-Wallis test with Dunn's multiple comparison post-test versus PP). Surprisingly there was a trend towards reduced IgG titres in mice receiving both a 'physiological' and high-dose of OT-II cells, with 3 mice not seroconverting post-boost, although this was not significant compared to IgG titres in PP immunised mice. There was also no significant difference in IgG titres between mice receiving vector control or AdHu5 OVA primed CD4+ T cells ($p>0.05$, by Mann-Whitney test). Overall, these data indicate that the adoptive transfer of naïve or primed antigen-specific CD4+ T cells does not markedly increase antibody responses to protein vaccines post-prime, nor do they appear to markedly affect mBC induction which could account for improved secondary responses after a subsequent homologous booster vaccination.

Group	Prime (wk0)	Boost (wk8)	Sex	Cell nr. transferred	Phenotype of cells transferred
A	20µg OVA+Adju-Phos®	20µg OVA + Adju-Phos®	F	$2x10^4$	Naïve
B	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	F	$1x10^6$	Naïve
C	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	M	$2x10^4$	OT-II
D	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	M	$1x10^6$	OT-II
E	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	F	$1x10^7$	AdHu5 OVA primed
F	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	F	$1x10^7$	nrAdHu5 primed
G	AdHu5 OVA	20µg OVA + Adju-Phos®	F	n/a	n/a
H	20µg OVA + Adju-Phos®	20µg OVA + Adju-Phos®	F	n/a	n/a

Table 5.1 Number and phenotype of cells adoptively transferred.

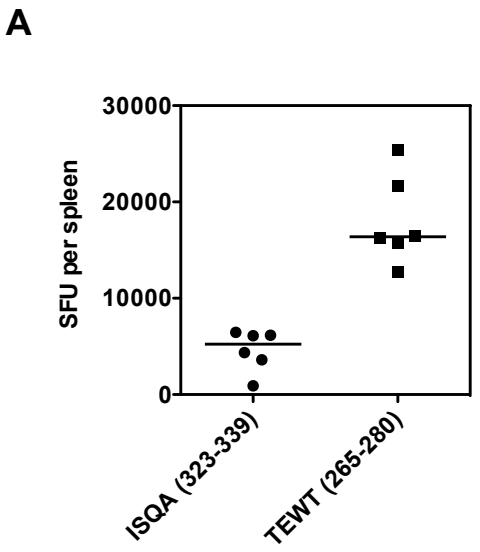

Figure 5.11 T cell responses to the known H-2^b CD4+ T cell epitopes following AdHu5 OVA immunisation.

C57BL/6 (n = 6) were immunised i.m. with 10^{10} vp AdHu5 OVA. T cell responses were assayed in spleen against the known H-2^b $CD4^+$ T cell epitopes TEWT and ISQA 14 days after the priming immunisation by ex vivo IFN-γ ELISpot. Median responses and individual data points are shown.

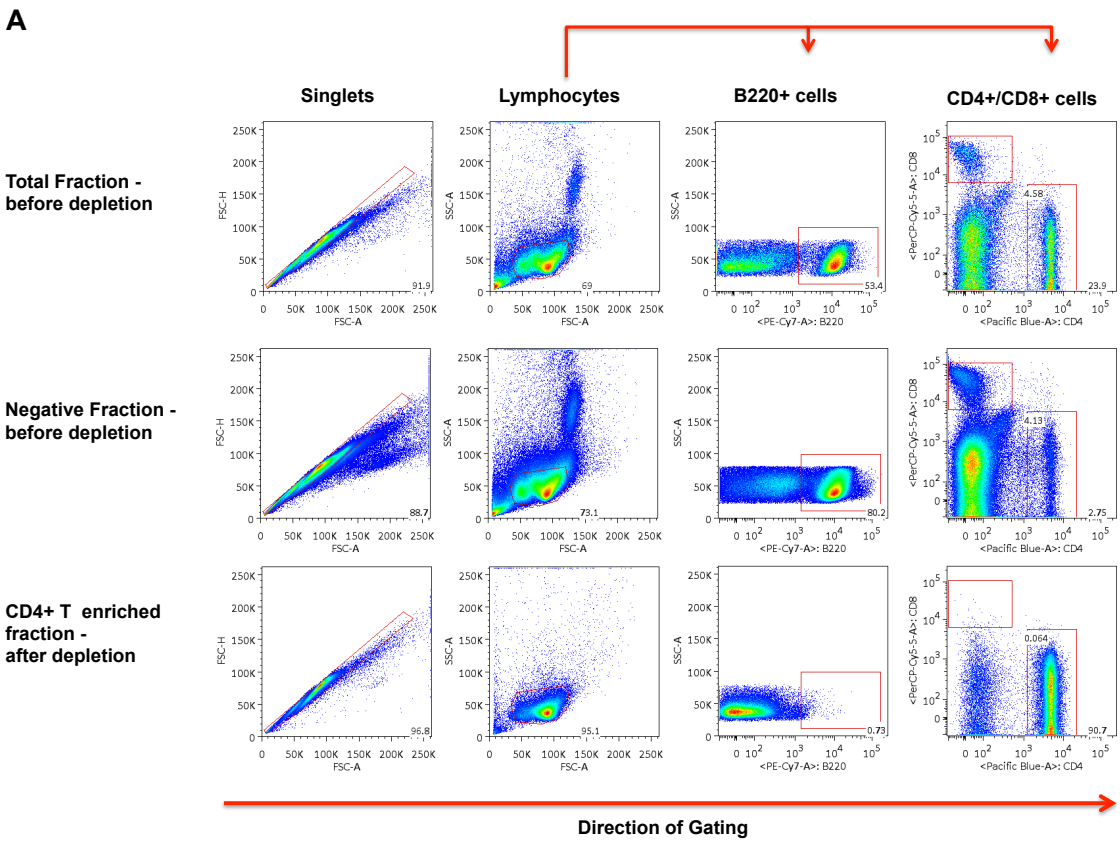

Figure 5.12 Purity of isolated CD4+ T cells.

CD4+ T cells were isolated from spleens of vaccinated, naïve and OT-II mice using the Miltenyi mouse CD4+ T cell isolation kit. The purity of the enriched CD4+ T cell fraction was determined. Splenocytes were stained with anti-CD4, anti-CD8 and anti-B220 antibodies and the gating strategy can be seen above for representative fractions from one OT-II mouse. The antibody clones used for staining did not block the mAbs in the depletion kit. The enriched CD4+ T cell fractions contained over >90% CD4+ T cells and below 1% CD8+ T cells or B220+ cells. Gates show the percentage of parent.

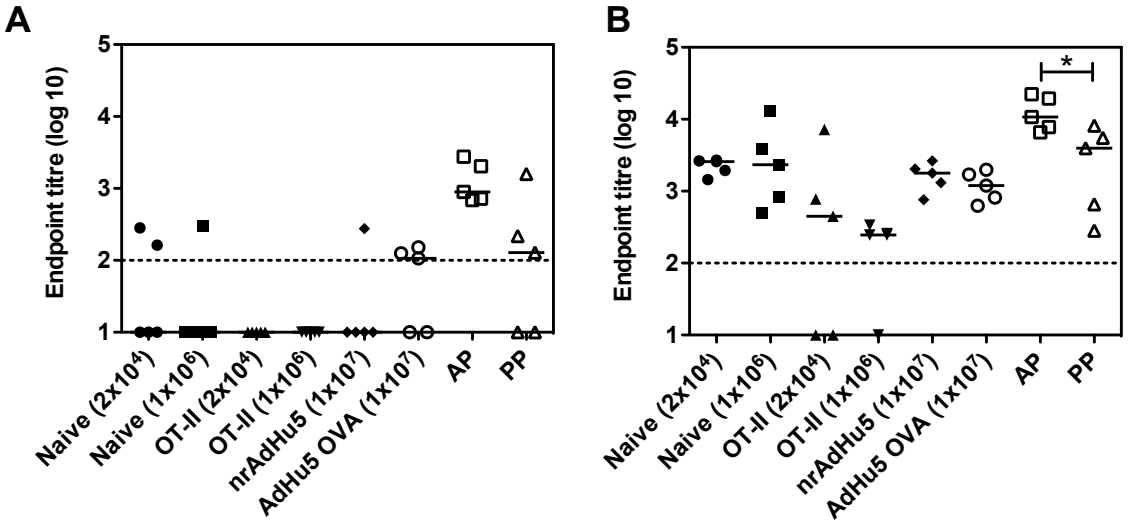

Figure 5.13 Adoptively transferred CD4+ T cells at the time of priming do not enhance primary or secondary antibody responses.

C57BL/6 mice (n = 5 / group) were primed i.m. with either 20µg OVA protein in Adju-Phos® (Groups A-F+H) or 10^{10} vp of AdHu5 OVA (Group G) (see Table 5.1). Eight weeks later all mice were boosted i.m. with 20µg OVA protein in Adju-Phos®. One day before the prime, mice were transferred i.v. with the indicated number of CD4+ T cells isolated from either AdHu5 OVA or nrAdHu5 primed mice or from OT-II transgenic or naïve wild-type mice. IgG titres were measured in the serum in response to protein by ELISA 55 days (A) after the priming immunisation and 14 days after the boost (B). Median responses and individual data points are shown. The dotted line indicates the threshold for responses above background. Groups are labelled according to the phenotype and number (in brackets) of CD4+ T cells transferred i.v.

5.2.8 The effect of CD32b on antibody responses and protective efficacy

Having addressed differences in dose/immunisation intervals, GC and T_{FH} cell induction as well as the role of CD4+ T cells in priming antibody responses between adenoviral vectors and protein vaccines; the role of B cell and DC regulators was investigated as another potential mechanisms affecting priming by these difference vaccine platforms. The activatory FcγRs present in mice are FcγRI, FcγRII and FcγRIII. There is only one inhibitory FcγR present in mice and humans: FcγRIIb (CD32b). In addition to expression on several innate immune cells, CD32b is the only FcγR expressed on B cells where it regulates activating signals through the BCR, by increasing the activation threshold of B cells, and thereby acts to restrain immune responses [30]. In mice CD32b can bind to IgG1, IgG2a and IgG2b. As expected, removal of CD32b has been shown to lead to enhanced antibody responses to T-dependent antigens in some studies [237,337,338]. Interestingly, a role for CD32b has also been suggested in processing antigen for presentation at the sub-capsular sinus (SCS) of LNs (Figure 5.1), and in recall responses to antigens in GCs. Follicular DCs express high levels of CD32b in GCs and are thought to trap immune complexes (ICs) in secondary immune responses. CD32b knockout (CD32b/-) mice were found to have reduced IC trapping in the LN and spleen and CD32b-/- mice reconstituted with wild-type (WT) lymphocytes were found to have significantly depressed recall responses [339]. This highlights a diversity of functions mediated by this receptor.

SCS macrophages express CD32b and this receptor has been shown to mediate internalisation of antigen into non-degradative compartments for later presentation [340]. These macrophages are thought to be responsible for the accumulation of larger antigens such as those found in bacteria and viruses [341]. As both adenoviruses and vaccinia viruses have been shown to be retained by SCS macrophages [51], it is possible that this receptor is involved in regulating immune responses induced to viral vectors.

To explore the role of CD32b in the priming and boosting of antibody responses following different vaccine modalities, CD32b-/- mice [237] were immunised with varying vaccine regimes. Initially wild-type (WT) C57BL/6 and CD32b-/- were primed with 10^{10} vp of AdHu5 expressing *P. yoelii* MSP-1_{42}. Eight weeks later mice were boosted with 10^7 pfu of MVA expressing the same transgene. IgG titres were measured in the serum in response to PyMSP-1_{19} protein by ELISA two weeks after each immunisation and before the boost. Two weeks after the priming immunisation mice in both groups seroconverted to the PyMSP-1_{19} antigen (Figure 5.14A). There was a trend towards lower IgG titres in the CD32b-/- vaccinated mice, however this was not significant. By week 8, IgG titres increased in the CD32b-/- mice reaching the same titres as those in the WT controls. Following the MVA vaccine, IgG titres were significantly boosted in the WT group ($p=0.031$, Wilcoxon-signed rank test), but not in the CD32b-/- group. Accordingly, IgG titres were significantly higher in WT mice compared to CD32b-/- mice post-boost ($p=0.04$, Mann-Whitney test). The fold change in log titres from pre- to post-

boost was calculated (Figure 5. 14B) and a trend towards a significantly lower fold change in CD32b-/- mice was found compared to WT controls although this did not reach significance (p=0.06, Mann-Whitney test). This indicates that although antibody responses to AM regimes are primed consistently in both WT and KO mice, secondary recall responses to MVA vaccines are depressed in CD32b-/- mice.

To investigate differences between a viral vector boost and a protein boost in the same context the model antigen OVA was again used in an AP regime and a two-shot PP regime. CD32b-/- and WT mice were primed with 10^{10} vp of AdHu5 OVA and boosted 8 weeks later with 20µg of OVA protein in Abisco®100 or primed with 20µg OVA protein in Abisco®100 and given a homologous boost 8 weeks later. As seen previously, IgG titres were negative 2 weeks post-prime following AdHu5 OVA and 8 weeks post-prime following a protein vaccine (Figure 5.15A). By week 8 mice seroconverted in the AdHu5 OVA groups and, as seen with PyMSP-1_{19}, there was no significant difference in IgG titres pre-boost ($p>0.05$, Mann-Whitney test). IgG titres were significantly boosted by a protein vaccine in both WT and CD32b-/- mice ($p=0.031$ and $p=0.036$, by Wilcoxon-signed rank test). There was also no significant difference in IgG titres post boost between the WT and CD32b-/- mice following an AP regime. Interestingly IgG titres were significantly higher in CD32b-/- mice compared to WT controls following a PP regime ($p=0.04$, Mann-Whitney test).

The fold change in log titres from pre- to post-boost was again calculated for AP regimes (as responses pre-boost for PP were negligible) (Figure 5.15B). There was a trend towards a higher fold change in CD32b-/- mice compared WT controls, although this was not significant ($p>0.05$, Mann-Whitney test). This is in contrast to results seen following an AM regime where there was a trend towards a reduction in the fold change pre- to post-boost in CD32b-/- mice compared to WT controls. Overall these data indicate that although CD32b does not seem to influence the priming of antibody responses by either AdHu5 or protein/adjuvant vaccines, its requirement for recall responses appears to differ between the MVA vector and a protein/adjuvant vaccine.

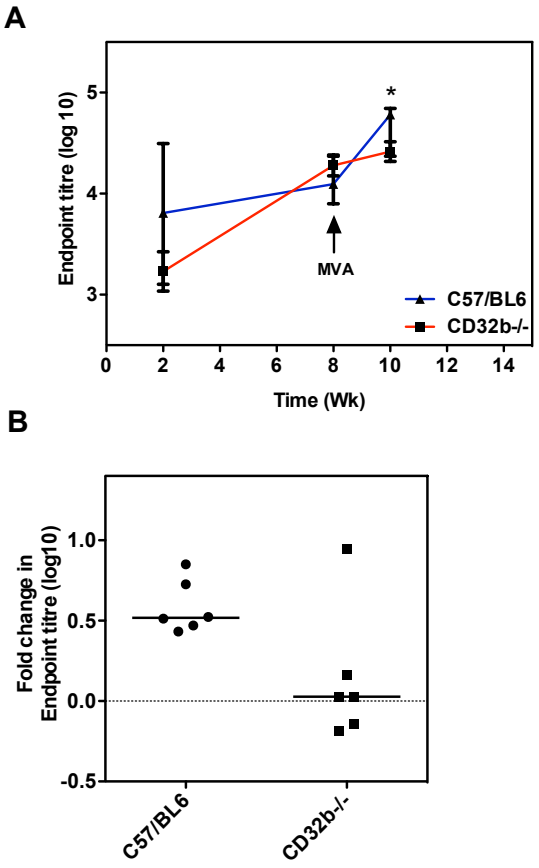

Figure 5.14 Antibody responses induced by a PyMSP-1_{42} AM regime in CD32b-/- mice.

C57BL/6 (n = 6 / group) were primed i.m. with 10^{10} vp of AdHu5 PyMSP-1_{42} and boosted 8 weeks later with 10^{7} pfu of MVA PyMSP-1_{42}. IgG titres were measured in the serum in response to PyMSP-1_{19}-GST protein by ELISA. (A) IgG titres measured 2 and 8 weeks after the AdHu5 prime and 2 weeks after the MVA boost. * $p<0.05$, by Mann Whitney test at week 10 between CD32b-/- and WT controls. Median and range are shown. (B) Fold change in log IgG titres measured pre- to post-boost. Median responses and individual data points are shown.

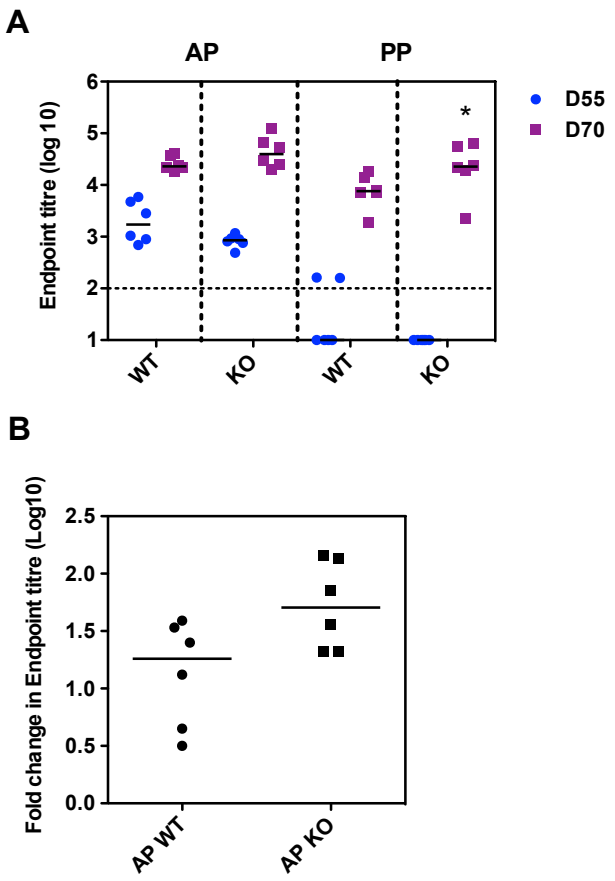

Figure 5.15 Antibody responses induced by AP and PP regimes in CD32b-/- mice.

C57BL/6 (n = 6 / group) were immunised i.m. with either i) 10^{10} vp AdHu5 OVA and boosted 8 weeks later with 20µg of OVA in Abisco®100 or ii) given two immunisations of 20µg of OVA in Abisco®100 8 weeks apart. IgG titres were measured in the serum in response to OVA protein by ELISA. (A) IgG titres were measured 8 weeks after the prime (D55) and 2 weeks after the protein boost (D70). (B) Fold change in log IgG titres measured pre- to post-boost. Median and individual data points are shown. * $p<0.05$, by Mann-Whitney test at week 10 between PP CD32b-/- and WT controls.

5.3 Discussion

Work in this chapter sought to address differences in the underlying immunogenicity of protein and viral vectored vaccines in an attempt to elucidate the mechanism underlying the differential boosting of protein vaccines following an adenoviral prime compared to a protein only regime. The role of protein and adenoviral dose and immunisation interval was first explored as a contributing factor to the differential priming of adenoviral vectors and protein vaccines. Subsequently the role of GC formation and T_{FH} cell induction was also explored. These studies were then extended to investigate the role of CD4+ T cell help for priming antibody responses and finally the role of the regulatory receptor CD32b and it's role in priming as well as secondary recall responses to protein vaccines and viral vectors was investigated. Each of these studies will be addressed in turn.

The two vaccine delivery platforms used in this study are inherently different and it is possible that the increased immunogenicity of protein vaccine adjuvants seen following an adenoviral prime is due to an adenoviral vector producing more antigen *in vivo* at the time of priming than a given dose of protein vaccine. This "dose effect" could prime a quantitatively greater memory B cell response that can be subsequently boosted more effectively. Alternatively, adenoviral vaccines may inherently prime a better quality of memory B cell response, likely related to the profile of innate sensors stimulated by adenoviruses [268,269] that can be more effectively boosted. Furthermore, adenoviral

vectors have been shown to persist in the muscle for a year following immunisation [342] whereas little work has addressed the persistence of protein-in-adjuvant vaccines. Viral vectors also differ in their ability to prime and boost antibody responses with adenoviruses being good at priming and MVA being good at boosting antibody responses. Several suggestions have been made to explain this phenomenon with some thinking it is the duration of antigen expression that may be mediating this difference. Adenoviruses are relatively non-cytotoxic to cells and therefore have a more prolonged period of antigen expression once cells are infected, whereas MVA vectors are thought to infect and kill cells very efficiently accounting for a shorter period of transgene expression (Reyes-Sandoval et al, unpublished) [343,344]. Given antibody responses appear to be dependent on transgene expression, this could explain why adenoviruses are better antibody inducers [345]. Alternatively, in MVA an *E3L*-mediated immune-evasion mechanism confers resistance to type I IFN [346]. As in some settings type I IFN has been shown to be essential for driving PC differentiation [347], a decreased type I IFN induction could also explain the weaker B cell priming seen with MVA.

Investigations into the amount of antigen produced by viral vectors are limited, although a study by Geiben-Lynn *et al.* utilising *in vivo* imaging of a recombinant AdHu5 expressing luciferase found 100µg of luciferase was expressed one day after an i.m. injection of 10^{10} vp AdHu5 in BALB/c mice (the dose of AdHu5-OVA used here) [343]. Dosing studies with OVA in adjuvant however revealed that there was no significant

enhancement of antibody priming after giving 100µg OVA versus 20µg at two week intervals, indicating that it is unlikely that the antigen dose accounted for the better priming ability of adenoviral vectors. Similarly in a recent Phase I trial of EBA-175 + Adju-Phos® given in a three-shot vaccination regime over a period of 6 months there was no appreciable increase in antibody titres between 5µg or 160µg of protein [278].

Interestingly, an effect was seen in terms of the immunisation interval (two weeks versus eight) for Abisco®100 and SE + TLR 4&9 (but not Adju-Phos® or CoVaccine HT™), where a prolonged interval led to improved responses. This effect has previously been reported for viral vectored immunisation regimes and indicates that prolonged prime-boost intervals may allow for optimal development of memory B cells [174,176]. However, priming mice with the potent adjuvant CoVaccine HT^{TM} and boosting mice eight weeks later with Adju-Phos® did not lead to increased IgG responses, which suggests that the priming effect is inherent to the adenovirus vector and that the prime-boost interval alone in the AP regime is unlikely to account for the improved boosting effect seen with weak adjuvants. In agreement with the data here, no significant differences were also observed in a Phase Ia clinical trial of an AMA1 vaccine formulated in Alhydrogel + CpG7909 using a 4 week versus 8 week immunisation interval [321]. It would thus appear that adenoviral primed antibody responses are more easily boosted, which has been suggested in a number of previous reports [177,178]. These data, suggesting antigen load is not the critical determining factor, were further supported by

administering different doses of AdHu5 OVA at the time of the prime and boosting with 20μg OVA protein in adjuvant. Interestingly, although antibody responses to the AdHu5 OVA vaccine appeared to be dose-dependent, the OVA protein/adjuvant vaccine was able to boost IgG titres to similar levels across a 2-log difference in AdHu5 priming dose. Similarly, the administration of a nrAdHu5 vector with OVA protein at the time of the prime (in the absence of an adjuvant) was able to partially enhance responses post-boost relative to a protein or nrAdHu5 only prime. This further supports that the improved boosting of antibody responses by protein vaccines is inherent to the priming ability of a recombinant adenovirus vector.

Next the induction of GCs was investigated in the draining LNs post-vaccination. Few studies have assessed the induction of GCs following different vaccine modalities, although there is evidence of adjuvants affecting the size and number of GCs with a cholera-toxin based adjuvant and TLR-containing adjuvants inducing more GCs than alum [125,331]. Here an AdHu5 vaccine recombinant for OVA induced significantly higher levels of GC B cells than a nrAdHu5 vaccine in the draining LNs 9 days post-boost. Furthermore, AdHu5 OVA was found to induce higher levels of GC cells than an MVA vaccine and representative protein-in-adjuvant vaccines. The GC B levels measured here were higher than those previously reported for an AdHu5 expressing LacZ 10 days post-vaccination [348], whereas the percentages of GC B cells induced following the protein vaccines were similar to levels previously reported [331]. However, in these published

studies, GC cells are defined as only B220+GL7+ and are not defined on co-expression of CD95. In a study by Benmark *et al.* GC induction using 4-hydroxy-3-nitrophenyl acetyl (NP)-chicken γ-globulin in CTA1-DD (a cholera toxin based adjuvant) correlated with persistent antibody responses and ASCs. This indicates that GC output can be associated with serum antibody levels. Whether mBC output is also associated with size of GCs is not known but warrants further investigation, especially since a higher mBC output from GCs following adenoviral vector immunisation could account for a more easily boosted recall response.

However, it must be kept in mind that this GC staining panel does not identify antigen-specific B cells, so it is possible that the increased GC cells seen following AdHu5 OVA could be due to responses against viral proteins. Nevertheless, both the nrAdHu5 and MVA group had lower levels of GC B cells than the AdHu5 OVA group, indicating that it is likely the increased GC cells are directed towards the transgene. Given MVA vaccines are known to be poor antibody primers, an absence of GC formation could also explain this phenomenon. Furthermore, to ascertain whether the innate profile induced by adenoviruses or the nature of antigen expression and arrival in the LN is key for the induction of higher levels of GCs, a nrAdHu5 vector could be administered with protein before analysis in the same way. Further dissecting the differences in GC induction and maintenance, as well as serum antibody titres between viral vectors and protein vaccines remains of great interest.

The induction of T_{FH} cells following vaccination with viral vectors or protein vaccines was also investigated as these cells have been shown to influence antibody isotypes through the secretion of cytokines and their number has also been shown to be influenced by adjuvants [32,270]. Only in AdHu5 OVA immunised mice were CD4+CXCR5+ cells induced above background levels. These CD4+CXCR5+ cells also correlated with GC cells. Although other markers of T_{FH} cells, such as PD-1 and ICOS [28], were not investigated here these CD4+CXCR5+ cells appeared to be a defined population. These results indicated that not only do AdHu5 vaccines induce greater levels of GC cells, but also greater levels of T_{FH} cells, which could contribute to the consistently primed and cytophilic antibody responses induced by AP regimes. In a study by Reinhardt *et al.*, T_{FH} cells with different cytokine profiles were isolated as conjugates with B cells undergoing cytokine-specific immunoglobulin class switching. IFN-γ produced by these cells induced a Th1-type antibody response and IL-4 induced a Th2-type antibody response as measured by post-switch and germline transcripts. Given viral vectors are known to induce CD4+ IFN-γ responses [148], the expression of this cytokine from T_{FH} cells in the LN could also be investigated. Furthermore, as TLR agonists have also been shown to induce a greater number of T_{FH} cells [270], when combined with the induction of relatively strong cellular immune responses, this could also explain the more cytophilic antibody response induced by TLR containing adjuvants (see chapter 3). Along these lines, it would be interesting to investigate whether blocking CXCR5 would result in a decrease of antibody responses / GC B cell numbers or alter the isotype profile of IgG

antibodies induced following adenoviral vaccines. Mice which lack the CXCR5 receptor on their T cells form fewer and smaller GCs and have reduced levels of isotype-switched ASCs and reduced serum IgG1 levels [349]. This suggests an important role for CXCR5 expressing T cells for the induction of high-affinity isotype switched antibody following vaccination and warrants further investigation.

Improved induction of $CD4^+$ T cell help (essential for T-dependent antibody responses [259,260]) by the adenovirus in the AP regime could be another explanation for improved B cell priming. A study by Galli *et al*. has recently demonstrated that the induction of $CD4^+$ T cells following an adjuvanted influenza vaccine predicted the persistence of antibody responses, highlighting a potential link between CD4+ T cells and IgG titres [89]. However, data using OVA-specific transgenic $CD4^+$ T cells have indicated that adoptively transferred OT-II cells can only help antigen-experienced (and not naïve) B cells, and that the size of the secondary antibody response is restricted by the amount of T cells present at the time of antigen re-exposure [271].

Adenoviral vectors are known to induce strong cellular immune responses, whereas the induction of T cells following protein vaccines is dependent on the adjuvant selected [247,248]. To confirm whether the induction of better cellular immunity at the time of B cell priming is an important contributing factor (that differs between protein vaccines and viral vectors) CD4+ T cell adoptive transfer studies were carried out here. To this

effect, OVA-specific CD4+ T cells (OT-II cells) and naïve CD4+ T cells were isolated from the spleen and transferred i.v. into mice which were subsequently primed with 20µg OVA protein in Adju-Phos®. A 'high' dose of $1x10^6$ and a 'low' dose of $2x10^4$ CD4+ T cells were transferred. Routinely in the literature $1x10^6$ T cells are transferred in such studies [350]. However, it has been argued that transferring such high number of antigen-specific cells is not a physiologically relevant reflection of the immune response. A study by Hataye *et al.* showed that transferred antigen-specific CD4+ T had an increased half-life and proliferative capacity inversely related to the number of cells transferred [351]. Due to these reports, the 'low' dose of $2x10^4$ CD4+ T cells was also transferred which corresponded to the number of IFN-γ producing T cells detected in an ELISpot assay 2 weeks after an immunisation with AdHu5 OVA. At the same time, 10^7 CD4+ T cells isolated from mice immunised with a nrAdHu5 (vector control) or AdHu5 OVA vaccine were also transferred in order to investigate whether adenoviral-primed versus antigen-specific CD4+ T cells impact priming. This number of cells constitutes a spleen-to-spleen CD4+ T cell transfer, given primed antigen-specific CD4+ T cells could not be isolated specifically. As seen before in the OVA system, IgG titres were negative 2 weeks after the prime with adenoviral-primed control mice and some protein vaccinated mice sero-converting by week 8 (pre-boost). The supplementation of antigen-specific T cells at the prime, either from OT-II mice or from AdHu5 OVA primed mice, did not enhance antibody responses to protein vaccination. This would suggest mechanisms other than total CD4+ T cell help are governing improved priming by AdHu5 vaccines. However, it

must be kept in mind that OT-II cells are specific for the 323-339 (ISQA) epitope whereas the AdHu5 OVA vector contains both CD4+ T cell epitopes present in OVA [243,244]. The 'physiological number' of cells transferred was also based on IFN-γ producing T cells in the spleen in response to both epitopes and it is likely that CD4+ T cell help may be defined by the secretion of other cytokines or by proliferative capacity [352]. CD4+ T cells isolated from AdHu5-primed mice were also primed, which contrasts to the naïve OT-II cells. In a study by Duffy *et al.*, naïve antigen-specific CD4+ T cells were able to help mBCs but not naïve B cells possibly accounting for the 3 non-responders seen in the OT-II group here [271]. Alternatively there could be competition between vaccine-induced and transferred CD4+ T cells. It would be of interest to see if CD4+ T cell depletion before priming with an AdHu5 vector would prevent the induction of antibody responses.

Finally the role of CD32b in priming and boosting antibody responses was also explored. Interestingly the priming of antibody responses was not enhanced in CD32b-/- mice compared to wild-type controls following an AdHu5 or protein vaccine prime. This differs from several studies where an enhancement of antibody titres was seen in CD32b-/- mice following *P. chabaudi* infection and immunisation with T-dependent antigens [237,337,338]. A divergent role for CD32b has also been suggesting in processing antigen for presentation at the SCS of LNs and in the recall responses to antigens in GCs. In one study, CD32b-/- mice were found to have reduced IC trapping in

the LN and spleen, and CD32b-/- mice reconstituted with WT lymphocytes were found to have significantly depressed recall responses to T-dependent antigens [339]. This argues for an effect of CD32b during a secondary recall response, given in naïve mice (at the time of the priming vaccination) there is no pre-existing antigen-specific IgG to bind CD32b. Along these lines, there was a reduction in the fold-change in IgG titres in CD32b-/- mice following an MVA boost but not a protein boost, indicating that although protein vaccines could boost adenoviral primed responses, MVA vaccines did not appear to be able too. This dampening of recall responses supports the study carried out by *Qin et al [339]*. However, as with the protein-vaccine boost here, *Takai et al.* saw an increase in IgG titres following homologous boosting of CD32b-/- mice with T-dependent antigens [237]. This indicates that the role CD32b-/- may differ between primary and secondary responses as well as between vaccine platforms. It would be of interest to investigate whether primary responses are differently affected to recall responses by investigating anti-MVA antibodies (constituting a priming immunisation) at the boost. Furthermore, using an MVA OVA to boost adenoviral primed responses, and thus excluding that an antigen-specific phenomenon underlies these results, could indicate that the difference between these two vaccine platforms at the time of the boost could therefore be due to the nature in which antigen arrives at the LN and encounters B cells and therefore utilises CD32b-/-.

Small soluble protein antigens (≈70kDa) can arrive directly in the LN through pores in the sinus floor or through the LN conduit system [353,354], whereas large antigens are thought to enter through a 'cell-mediated process' either through capturing by SCS macrophages or by being carried into the LN by DCs which can recycle antigen to their cell surface [355,356,357]. SCS macrophages and FDCs are thought to be responsible for the accumulation of larger antigens such as those found in bacteria and viruses [341] and express CD32b which has been shown to mediate internalisation of antigen into non-degradative compartments for later presentation [340]. This may explain the lack of boosting seen following MVA vaccines in CD32b-/- mice as this vaccine may be more dependent on CD32b-/- for subsequent presentation to cognate B cells. The exploration of cell types involved in antigen presentation to B cells following different vaccine platforms warrants further investigation.

Overall the results presented in this chapter sought to investigate underlying differences between viral vectored vaccines and protein vaccines in the priming of an antibody response, which could potentially explain the more efficient boosting of protein/adjuvant vaccines following an AdHu5 prime compared to a protein vaccine prime (Chapters 3&4). Differences in the dose of antigen exposed to the immune system between AdHu5 and protein vaccines did not appear to account for the difference in immunogenicity seen between AP and PPP regimes. Rather it appears that the priming ability of a recombinant AdHu5 vector is inherent to the viral vector itself, which induces

greater numbers of GC B cells as well as T_{FH} cells than a protein/adjuvant vaccine for the same antigen. Furthermore, a role for CD32b was not found in priming antibody responses but rather in recall responses to viral vectors but not protein vaccines, which indicates that the nature of antigen arrival in the LN and its subsequent encounter with B cells may explain differences between these vaccine platforms. Surprisingly the supplementation of CD4+ T cell help at the prime did not improve the antibody immunogenicity of protein vaccines. These results warrant further investigation given understanding the underlying immunogenicity of vaccine vectors and protein in adjuvant vaccines can aid the rationale design and engineering of next-generation vaccine delivery platforms for diseases such as malaria (at the erythrocytic-stage) and flu, which are likely to require high-titre antibodies for protective efficacy.

Chapter 6

In vivo efficacy of adenoviral and protein-in-adjuvant MSP-1 vaccines against lethal *P. yoelii* challenge

6 *In vivo* efficacy of adenoviral and protein-in-adjuvant MSP-1 vaccines against lethal *P. yoelii* challenge

6.1 Introduction

As stated previously, malaria is a parasitic disease caused by parasites of the genus Plasmodium and is responsible for a high degree of morbidity and mortality world-wide [181]. Several vaccines have been developed against all stages of the malaria life cycle including protein and viral vectored vaccine candidates [246,358]. Given *P. falciparum* does not infect mice, rodent models of malaria are commonly utilised to assess the efficacy of candidate vaccines and mechanisms of vaccine-induced protection. One of the models widely used to assess both blood-stage and liver-stage immunity is the *P. yoelii* rodent model of malaria, of which both the lethal (YM and 17XL) as well as non-lethal (17NXL) strains have been utilised. With the lethal YM strain, mice are typically infected with 10^4 parasitised (p)RBCs i.v. which results in >80% infected RBCs within 6 days in BALB/c mice [174,204,257].

In the case of blood-stage malaria vaccines, extensive work has been carried out characterising the mechanisms of blood-stage immunity induced by MSP-1 vaccines against lethal *P. yoelii* blood-stage challenges. *P. yoelii* MSP-1 has been shown to be processed similarly to *P. falciparum* MSP-1 [359]. Protein-in-adjuvant vaccines

comprising recombinant PyMSP-1_{42} protein, or its 19-kDa C-terminal processing product, have been shown to mediate protective efficacy against a blood-stage challenge through high-titre antibodies directed against the PyMSP-1_{19} fragment of this antigen, but not against PyMSP1_{33} [204,257,297,360,361]. The majority of these vaccines induce a Th2-type antibody response dominated by IgG1, which may act to neutralise the parasite. However, in addition to this, purified IgG2a has also been shown to exert a protective effect when adoptively transferred [362].

Draper *et al.* have explored the protective effect of PyMSP-1_{42} expressing viral vectored vaccines following an AM immunisation regime. High-titre antibody responses were induced to PyMSP-1_{19}, which were shown to be 76% protective against a 10^4 pRBC blood-stage challenge. In addition to this 100% of mice were also protected against a liver-stage challenge against 50 sporozoites, thought to be due to partial protection mediated by CD8+ T cells directed against PyMSP-1_{33}, as MSP-1 is also expressed during the late liver-stage [148]. In addition to this, a role has also been shown for $CD4^+$ T cells directed against PyMSP-1_{33}, which aid the development of *de novo* anti-parasite antibody responses [148]. $CD4^+$ T cells have also been shown to mediate some independent control of blood-stage parasitaemia [363,364,365], although most studies agree that the induction of high-titre neutralising antibodies directed against PyMSP-1_{19} are the major determinants of protective efficacy in this system.

This chapter sought to address whether the differential ability of protein vaccines to boost antibody responses following an adenoviral prime also translates to improved efficacy against a *P. yoelii* blood-stage challenge, which could not be addressed using *P. falciparum* antigens or the model antigen OVA. The immunological mechanisms of blood-stage protection are also explored.

6.2 Results

6.2.1 Immunogenicity of MSP-1 protein

To determine whether a differential ability of protein vaccines to boost adenoviral-primed responses also translates into a difference in efficacy against blood-stage challenge, mice were immunised with MSP-1 based *P. yoelii* vaccines. The MSP-1 protein vaccine corresponds to the 19kDa fragment of MSP-1 (MSP-1_{19}) that is left on the RBC surface after MSP-1 is proteolytically processed upon RBC invasion [294]. This *P. yoelii* protein was expressed as a GST-fusion protein using an *E. coli* expression system and purified using a GST-purification kit as previously described and outlined in 2.2.9 [174]. For the *in vivo* efficacy studies, Adju-Phos® and CoVaccine HT™ were selected because these adjuvants induced strikingly different IgG titres when used in a PPP regime, but boosted adenoviral primed IgG responses to equally high levels when using the model antigen OVA (see Chapter 3).

To determine the immunogenicity of PyMSP-1_{19}-GST protein (hereafter referred to as MSP-1_{19} protein), BALB/c mice were immunised twice i.m. with either 1.5µg, 5 µg or 20µg of MSP-1_{19} protein three weeks apart. *P. yoelii* MSP-1_{19} specific antibodies, following immunization of mice with GST-PyMSP-1_{19}, were measured using *P. yoelii* MSP-1_{19}-IMX108 protein [241] which does not contain the GST-tag present in the protein used for immunisation and would thus not lead to detection of anti-GST

antibodies. IgG titres after two immunisations are shown in Figure 6.1. A dose response curve was seen for MSP-1_{19} at the three doses tested within each treatment group, with responses plateauing at 20µg. As predicted by the OVA data, IgG titres induced by CoVaccine HT^{TM} were significantly higher than those induced by MSP-1_{19} in PBS at each dose ($p<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test). MSP-1_{19} was also immunogenic in PBS, as has previously been seen for other *P. falciparum* blood-stage antigens (see Chapter 4), but is likely due to the protein preparation used here. This protein vaccine was taken forward into two challenge studies at a low dose of 1.5µg and a high dose of 20µg to explore the protective efficacy of these protein vaccines at two very different doses.

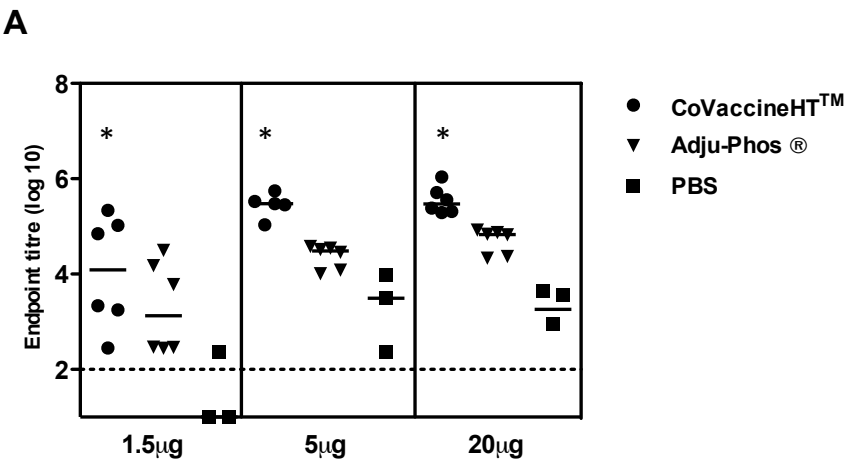

Figure 6.1 Antibody dose response of *P. yoelii* MSP-1_{19} protein vaccine.

BALB/c mice (n=3-6) were immunised twice i.m. with either 1.5μg, 5μg or 20μg of MSP-1_{19}-GST protein three weeks apart. IgG titres were measured in the serum in response to *P. yoelii* MSP-1_{19}-IMX108 protein (which does not contain the GST tag) 2 weeks after the final immunisation. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. *$p<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test against PBS within each dose.

6.2.2 Antibody immunogenicity of high-dose MSP-1 AP and PPP regimes

To investigate the immunogenicity of *P. yoelii* MSP-1 vaccines in PPP and AP regimes, BALB/c mice were immunised with the regimes outlined in Table 6.1 using a high-dose of MSP-1_{19} protein (20μg). The immunogenicity of low-dose MSP-1 vaccines is explored later in this chapter. IgG titres were measured as before – two weeks after each immunisation in the AP and AM regimes and after the third dose of protein in the PPP regime. Antibody titres were only measured at key time-points in the PPP regimes due to limited amounts of *P. yoelii* MSP-1_{19}-IMX108 protein available for ELISAs. Mice immunised with AdHu5 MSP-1_{42} sero-converted after the first immunisation (Figure 6.2A). IgG titres were significantly boosted ($p<0.05$, Wilcoxon-signed rank test) by >1 log by both protein vaccines and, as seen with OVA, there was no significant difference in the fold increase between the two adjuvants when used to boost adenovirus-primed responses (22-fold increase in the mean for CoVaccine HT™, 95% CI: 14-35; 12-fold increase in the mean for Adju-Phos®, 95% CI:5-30; $p=0.48$ by Mann Whitney). As seen previously, IgG titres induced by AP regimes were significantly higher than those induced by AM regimes (Figure 6.3, $p>0.05$, one-way ANOVA with Bonferroni's multiple comparisons post-test).

Strong IgG titres (surpassing those induced by a single immunisation with AdHu5 MSP-1_{42} as well as an AM regime) were induced by the MSP-1_{19} protein vaccines in CoVaccine

HT™ after two shots, which were not significantly boosted by a third shot as responses appeared to plateau ($p>0.05$, Wilcoxon-signed rank test) (Figure 6.2B). IgG titres induced by MSP-1_{19} in Adju-Phos® were lower than those seen after two shots of MSP-1_{19} in CoVaccine HT™ but were significantly boosted to similar levels by a third shot ($p=0.03$, Wilcoxon-signed rank test). Two weeks after the final immunisation, there was no significant difference in the IgG titres induced by these two adjuvants, which differs from previous results seen with OVA (Figure 6.3; $p>0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). As seen with other *P. falciparum* antigens and with this antigen in the dose de-escalation study, MSP-1_{19} in PBS also induced moderate IgG titres (Figure 6.2B). The high dose of protein used here probably accounts for the relatively high IgG titres induced by this protein in Adju-Phos®, as well the inherent immunogenicity of the protein preparation used for immunising. However, IgG titres in the AP, PPP and AM groups were still significantly higher two weeks after the final immunisation than both the PBS and AdHu5 only control groups (Figure 6.3, $p>0.05$, one-way ANOVA with Bonferroni's multiple comparisons post-test). Overall, these data indicate that the *P. yoelii* MSP-1 vaccines induce strong IgG titres when used in AP and PPP regimes, which surpass those induced by AM regimes when using a relatively high-dose of protein.

Regime & Adjuvant	Prime	Dose	Interval	n=
AP Adju-Phos®	AdHu5 MSP-1_{42} → MSP-1_{19} (GST tagged)	10^{10} vp 20µg	8 weeks	6
AP CoVaccine HT™	AdHu5 MSP-1_{42} → MSP-1_{19} (GST tagged)	10^{10} vp 20µg	8 weeks	6
PPP Adju-Phos®	MSP-1_{19} (GST tagged)	20µg	3 weeks apart (3 immunisations)	6
PPP CoVaccine HT™	MSP-1_{19} (GST tagged)	20µg	3 weeks apart (3 immunisations)	6
AM	AdHu5 MSP-1_{42} → MVA MSP-1_{42}	10^{10} vp 10^{7} pfu	8 weeks	
Ad only	AdHu5 MSP-1_{42}	10^{10} vp	n/a	6
PPP PBS	MSP-1_{19} (GST tagged)	20µg	3 weeks apart (3 immunisations)	4

Table 6.1 Immunisation schedule for high-dose MSP-1 vaccines.

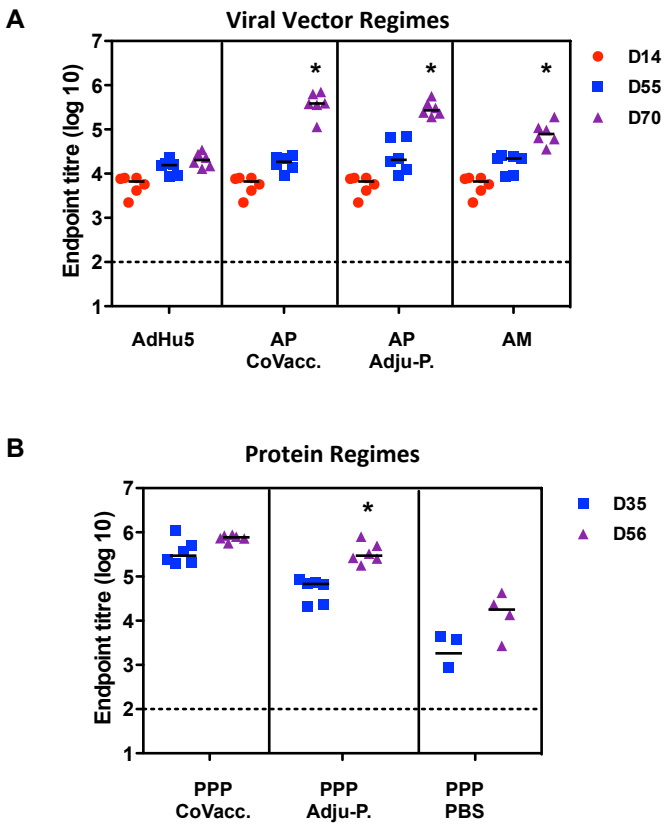

Figure 6.2 Immunogenicity of high-dose *P. yoelii* MSP-1 vaccines.

BALB/c (n=4-6) were immunised with the regimes outline in Table 6.1. IgG titres were measured at the indicated time-points in the serum in response to *P. yoelii* MSP-1_{19}-IMX108 protein. (A) IgG titres measured two weeks after each immunisation (D14 & D70) and before the boost (D55) in the viral vector containing regimes. (B) IgG titres measured two weeks after the second (D35) and third (56) protein vaccine in the protein vaccine regimes. Median and individual data points are shown. The dotted line indicates the threshold for responses above background. * $p<0.05$, Wilcoxon-signed rank test post-boost versus pre-boost titres.

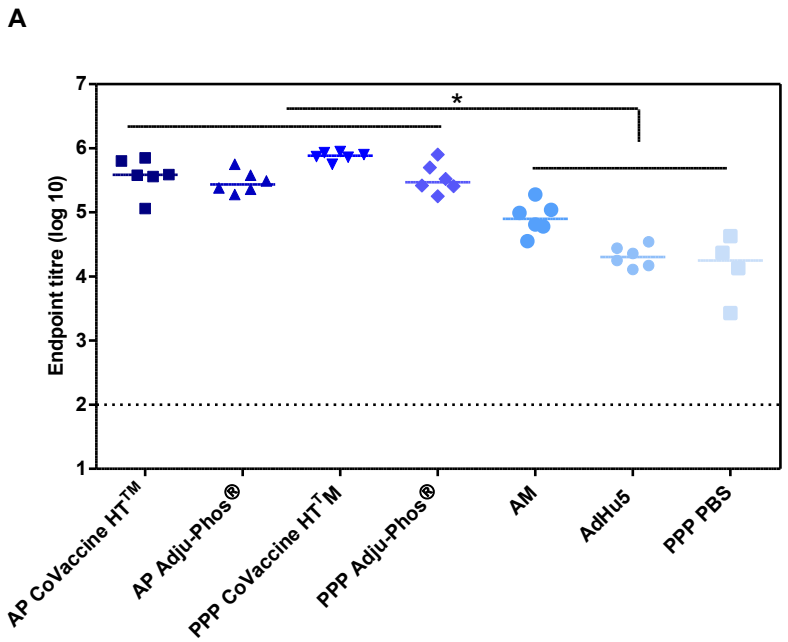

Figure 6.3 IgG titres two weeks after the final immunisation.

BALB/c mice (n=4-6) were immunised as outlined in Table 6.1. IgG titres were measured in the serum in response to *P. yoelii* MSP-1_{19}-IMX108 protein by ELISA two weeks after the final immunisation in each regime. Median and individual data points are shown. * $p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test (AM group also, significant versus AdHu5 and PPP PBS). The dotted line indicates the threshold for responses above background.

6.2.3 Protective efficacy of high-dose MSP-1 AP and PPP regimes against blood-stage challenge

To assess whether the AP and PPP regimes described in the last section could also confer protective efficacy, mice were challenged with 10^6 pRBC two weeks after the final immunization. This challenge dose is two-fold higher than routinely used in the literature [174,204,257,366]. Given IgG titres are known to correlate with protection in this challenge system, a high-dose challenge of 10^6 pRBC was used here to try and establish any inherent differences between the protective efficacy of these regimes that all induced relatively high and robust IgG responses. Peak parasitaemia and area under the curve (AUC) for mice that survived challenge were analysed to explore an effect at the blood-stage of infection.

All mice in the control groups succumbed to infection by day 5 post-challenge (Figure 6.4). All mice in the AP groups as well as the PPP CoVaccine HT™ group survived challenge (6/6), although no mice were sterilely protected. Interestingly, and despite comparably high IgG titres, only 50% (3/6) of mice survived in the PPP Adju-Phos® group. Previously these MSP-1 vaccines used in an AM regime were shown to be 76% protective against a 10^4 pRBC challenge. Here, against a more stringent challenge, only 33% (2/6) survived in the AM regime. An AUC analysis of parasitaemia on days 3-5 indicated a significant reduction in parasitaemia between the protein vaccine containing

regimes and the AM and control groups ($p<0.005$, One-way ANOVA with Bonferroni's multiple comparison post-test) (Figure 6.5A). Although survival in the AM regime was reduced compared to using the same dose of vaccines in a 'low-dose' blood-stage challenge [148,174], some control of parasitaemia was evident early post-challenge as indicated by a significant reduction in parasitaemia compared to the control groups ($p<0.005$, one-way ANOVA with Bonferroni's multiple comparison post-test) (Figure 6.5A). To examine the difference in survival between the AP and PPP Adju-Phos® group an AUC analysis between D3-16 was carried out, indicating a significant effect of an adenoviral prime on parasitaemia, but no effect of adjuvant ($p<0.05$, two-way ANOVA with Bonferroni's multiple comparison post-test) (Figure 6.5B). IgG titres were also found to inversely correlate with peak parasitaemia ($r=-0.56$, $p=0.001$) (Figure 6.6). Interestingly, despite inducing the same total IgG titres, there was a difference in survival between the AP and PPP Adju-Phos® regime. Potential reasons for this difference are explored later in this chapter.

Overall, these data indicate that the protective efficacy resulting in increased survival of protein-in-adjuvant vaccines against a high-dose pRBC challenge can be improved through the use of more potent adjuvants such as CoVaccine HT™ and through the use of an AP regime.

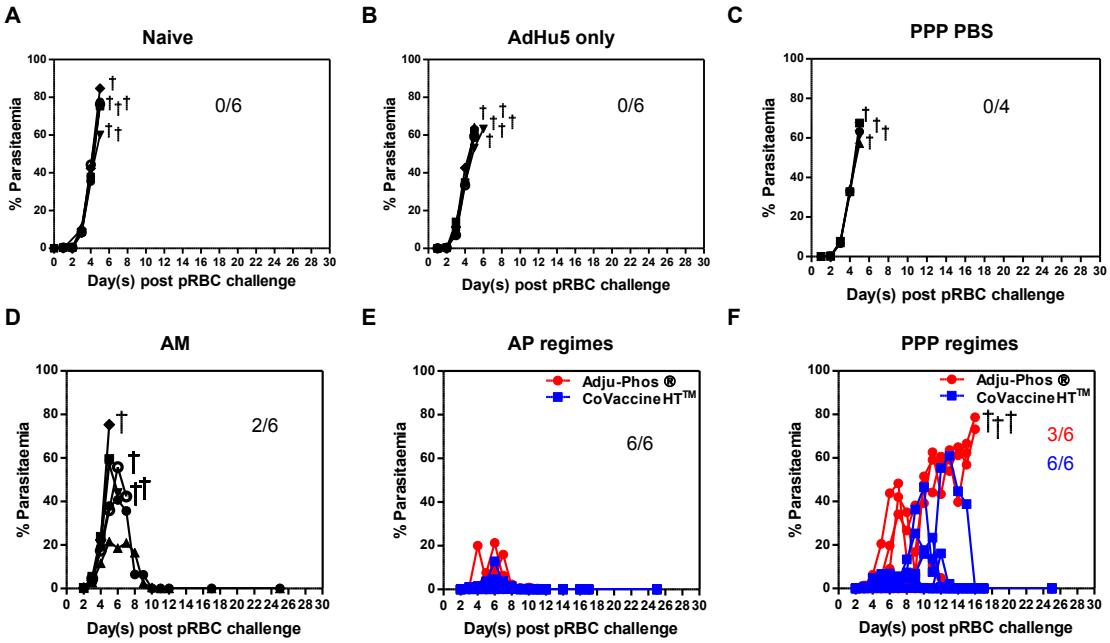

Figure 6.4 Survival following a high-dose blood stage challenge.

BALB/c mice (n=4-6) were challenged i.v. with 10^6 RBC two weeks after the final immunisation. Parasitaemia was measured as the percentage of infected red blood cells over time by Giemsa-stained thin-blood smear. Individual results are shown in (A) for naïve unimmunised, (B) for AdHu5 only immunised, (C) for PPP PBS immunised, (D) for AM immunised, (E) AP immunised and (F) PPP immunised mice. Crosses indicate when mice were sacrificed.

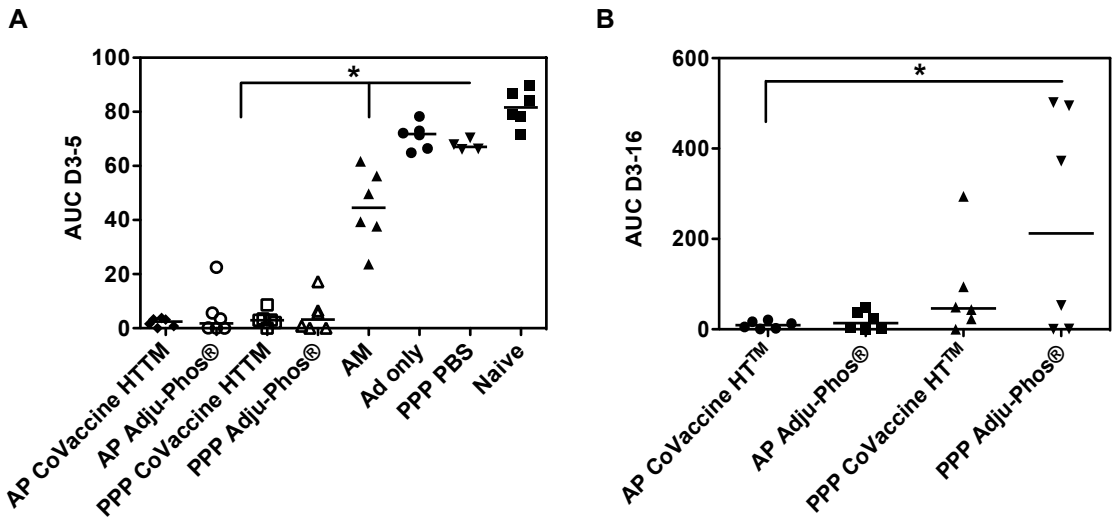

Figure 6.5 AUC analysis of parasitaemia.

Area under the curve (AUC) analysis of parasitaemia between days 3-5 (A) and days 3-16 (B). Median and individual data points are shown. * $p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test.

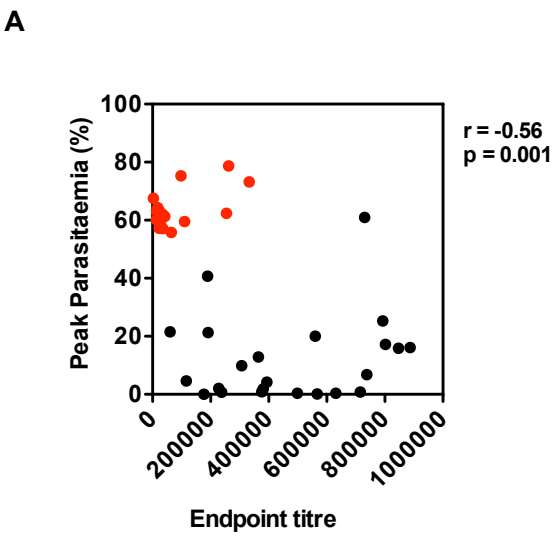

Figure 6.6 IgG titres correlate with peak parasitaemia.

IgG titres measured two weeks after the final immunization in each regime were correlated with percentage peak parasitaemia. Spearman's rank correlation coefficient and p value are shown. Mice that succumbed to infection are indicated by red data points, surviving mice by black data points.

6.2.4 Effect of challenge on antibody responses

To further characterise the protective antibody responses elicited, IgG titres were assessed in mice that had survived challenge 20 days after the blood-stage challenge (Figure 6.7). IgG titres were significantly decreased in the PPP CoVaccine HT™ group ($p=0.03$, Wilcoxon-signed rank test). Although IgG titres could only be measured in two surviving mice in each group, IgG titres also decreased in the PPP Adju-Phos group, but increased in the AM group by > 0.5 of a log. This latter increase could possibly be due to T cells induced by the viral vector more effectively boosting mBC responses, however as only two mice survived challenge this can not be formally concluded. In contrast to the PPP and AM regime, antibody titres in the AP groups appeared to be maintained stably at the pre- and post-challenge time-point tested.

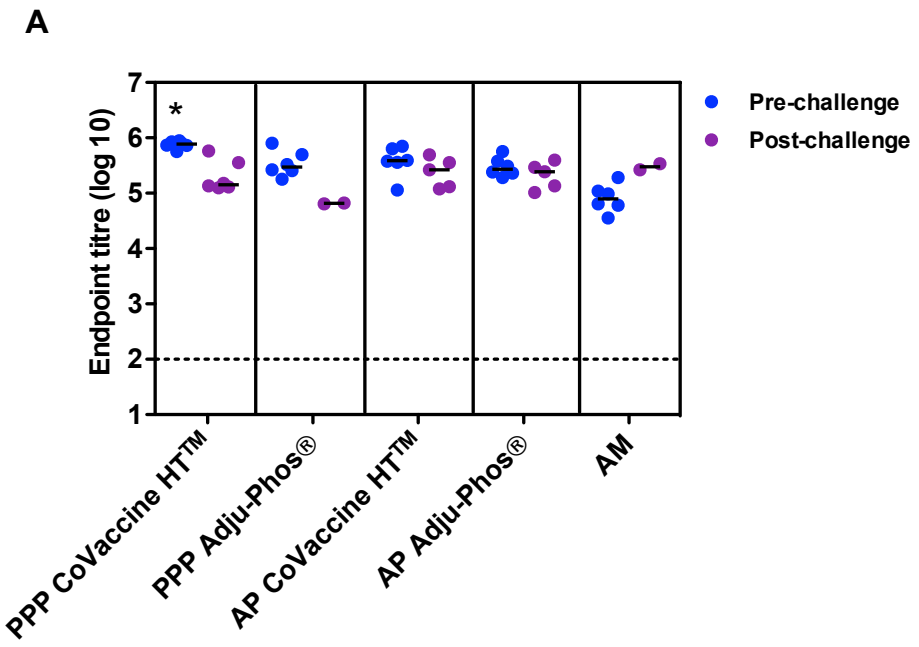

Figure 6.7 Effect of challenge on antibody responses to MSP-1.

IgG titres were measured in the serum in response to *P. yoelii* MSP-1_{19}-IMX108 protein by ELISA two weeks after the final immunisation (pre-challenge) and 20 days after the blood-stage challenge (post-challenge) in mice that survived. Median and individual data points are shown. The dotted line indicates responses above background. * p=0.03, Wilcoxon-signed rank test.

6.2.5 Antibody immunogenicity of low-dose MSP-1 AP and PPP regimes

To further investigate the immunogenicity and efficacy of these MSP-1 immunisation regimes in this system, the above experiments were repeated using a lower dose of 1.5μg of MSP-1_{19} protein formulated in adjuvant. BALB/c mice were immunised with the regimes outlined in Table 6.2. IgG titres were assessed by ELISA against MSP-1_{19} two weeks after the final immunisation in each regime (Figure 6.8). Now in agreement with studies of other antigens (and contrasting to studies with this protein at a higher dose), two weeks after the final immunisation IgG titres were significantly higher in mice immunized with AP regimes and the PPP CoVaccine HT™ regime compared to mice immunized with the PPP Adju-Phos® regime and control vaccines ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). These data confirm the previous observations that an adenoviral prime can improve the antibody immunogenicity of protein vaccines.

Regime (BALB/c)	Vaccine	Dose	Interval	n=
AP Adju-Phos®	AdHu5 MSP-1_{42} → MSP-1_{19} (GST tagged)	10^{10} vp 1.5µg	8 weeks	6
AP CoVaccine HT™	AdHu5 MSP-1_{42} → MSP-1_{19} (GST tagged)	10^{10} vp 1.5µg	8 weeks	6
PPP Adju-Phos®	MSP-1_{19} (GST tagged)	1.5µg	3 weeks apart (3 immunisations)	6
PPP CoVaccine HT™	MSP-1_{19} (GST tagged)	1.5µg	3 weeks apart (3 immunisations)	6
Ad only	AdHu5 MSP-1_{42}	10^{10} vp	n/a	6
PPP PBS	MSP-1_{19} (GST tagged)	1.5µg	3 weeks apart (3 immunisations)	4

Table 6.2. Immunisation schedule for low-dose MSP-1 vaccines.

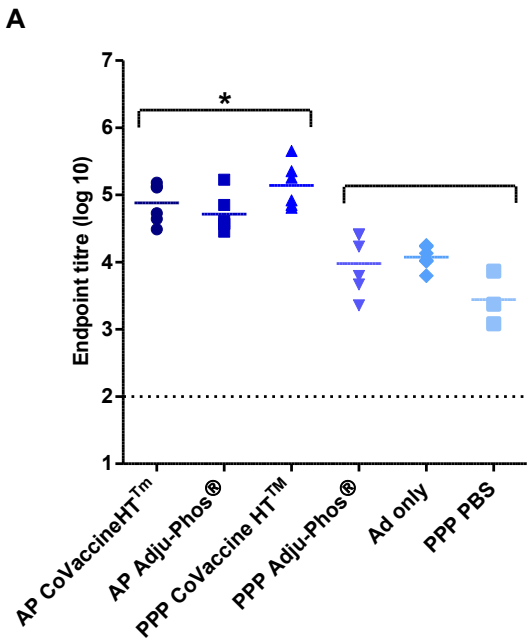

Figure 6.8 Immunogenicity of low-dose *P. yoelii* MSP-1 vaccines.

BALB/c mice (*n*= 3-6 / group) were immunized i.m. with either i) 1.5 µg of *P. yoelii* MSP-1_{19}-GST protein in Adju-Phos® or CoVaccine HT™ or PBS three weeks apart (PPP); or ii) primed with 1 x 10^{10} vp of AdHu5 MSP-1_{42} and either not boosted (Ad only) or boosted eight weeks later with 1.5µg of *P. yoelii* MSP-1_{19}-GST protein in Adju-Phos® or CoVaccine HT™ (AP). IgG titres were measured in the serum in response to *P. yoelii* MSP-1_{19}-IMX108 protein two weeks after the final immunisation (day before challenge). * $p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test. Median and individual data points are shown. The dotted line indicates the threshold for responses above background.

6.2.6 Protective efficacy of low-dose MSP-1 AP and PPP regimes against blood-stage challenge

The efficacy of the antibody responses induced by the vaccine regimes above was again assessed by a lethal blood-stage challenge, using a reduced challenge dose of 10^5 pRBC. Following challenge, all mice immunised with control vaccines or the PPP Adju-Phos® regime succumbed to infection (Figure 6.9A). Survival was seen in four out of six mice (67%) immunised with both AP regimes and in two out of six mice (33%) immunised with the PPP CoVaccine HT™ regime (Figure 6.9B-D). There was a significant reduction in parasitaemia, as measured by an AUC analysis of parasitaemia between days 3 and 5, in both AP regimes and the PPP CoVaccine HT™ regime as compared to the PPP Adju-Phos® regime and mice immunised with control vaccines/regimes (Figure 6.10). Peak parasitaemia was again shown to inversely correlate with antigen specific total IgG titres (Figure 6.11; r=-0.56, p=0.007). This further highlights that the AP immunisation regime not only improves the immunogenicity but also the protective efficacy of MSP-1 protein vaccines following a *P. yoelii* blood-stage challenge.

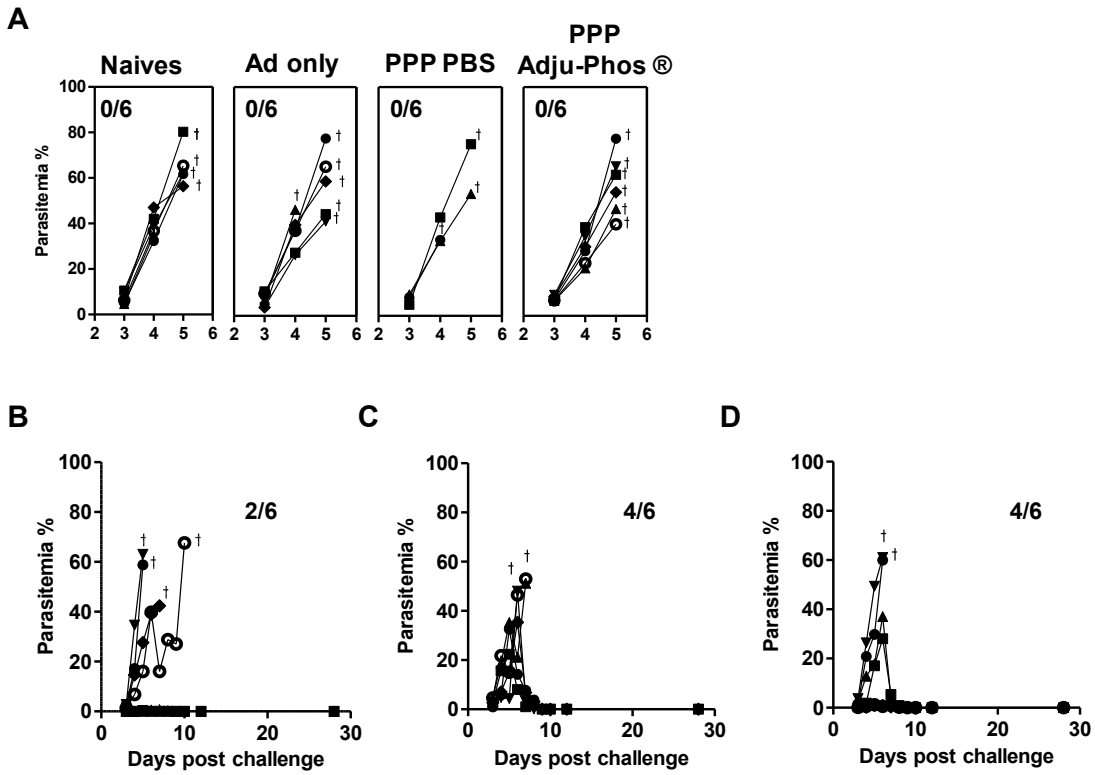

Figure 6.9 Survival following blood stage challenge.

BALB/c mice (n=4-6) were challenged with 10^5 pRBCs i.v. and parasitaemia was measured as the percentage of infected red blood cells over time. Results are shown in (A) for the naïve unimmunized, Ad only and PPP PBS control groups as well as the PPP Adju-Phos® group and in (B) for mice immunized with PPP in CoVaccine HT™; (C) mice immunized with AP Adju-Phos®; and (D) mice immunized with AP CoVaccine HT™. Crosses indicate when mice were sacrificed.

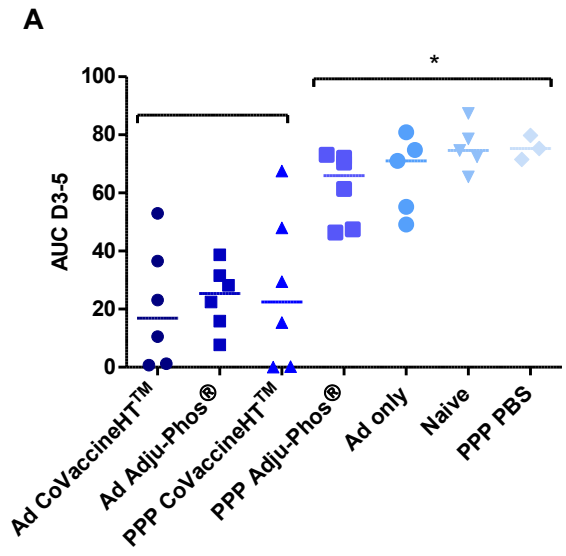

Figure 6.10 AP immunisation improves the efficacy of MSP-1 vaccines.

AUC analysis of parasitaemia on days 3-5. Median and individual data points are shown. * $p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test.

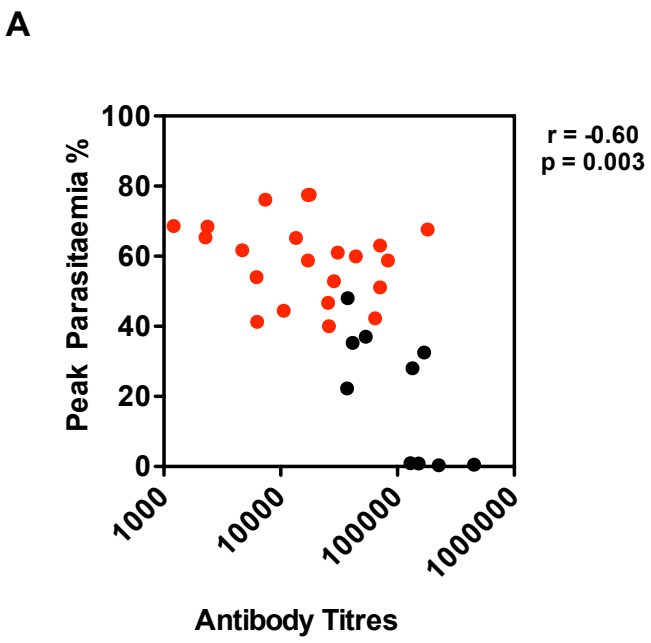

Figure 6.11 Peak parasitaemia correlates with IgG titres.

IgG titres measured two weeks after the final immunization (pre-challenge) in each regime were correlated with percentage peak parasitaemia. Spearman's rank correlation coefficient and p values are shown. Mice that succumbed to infection are indicated by red data points, surviving mice by black data points.

6.2.7 Role of CD4+ T cells in protection

As investigated above, antibody responses against *P. yoelii* MSP1$_{19}$ have been shown to associate with protection against blood-stage malaria infection [148,204]. However, a role has also been shown for CD4^{+} T cells directed against MSP-1$_{33}$, which can aid the development of *de novo* anti-parasite antibody responses [148], as well as mediate some independent control of blood-stage parasitaemia [363,364,365]. To rule out that the improved efficacy of the AP Adju-Phos® regime (compared to the comparator PPP regime for both the high and low protein doses) was not due to CD4+ T cells directed against the PyMSP-1$_{33}$ fragment included in the AdHu5-PyMSP-1$_{42}$ vaccine, (but absent from the PyMSP-1$_{19}$ protein vaccine), mice were immunised with the regimes outlined in Figure 6.12. CD4+ T cells were depleted prior to pRBC challenge in mice receiving an AP Adju-Phos® regime to explore the effect of PyMSP-1$_{33}$ specific CD4+ T cells in mediation of protection. CD4+ T cells were depleted by administering 200µg of GK1.5 mAb i.p. over three days as described previously with control mice receiving the same dose of normal rat IgG [174]. In a separate group, mice were primed with an AdHu5 expressing PyMSP-1$_{33}$ [148] and boosted with the previously used PPP Adju-Phos® regime in order to determine whether the supplementation of T cell help through the adenoviral prime could improve this regime (but in the absence of priming protective PyMSP-1$_{19}$-specific antibodies via use of AdHu5 expressing PyMSP-1$_{42}$). To confirm that the AdHu5 vectors expressing PyMSP-1$_{42}$ and PyMSP-1$_{33}$ induced comparable T cell responses, PBMC from

the blood of immunised mice were phenotyped two weeks post-prime in response to an overlapping peptide pool of PyMSP-1_{33}. CD8+ IFN-γ+ T cell responses measured two weeks after the prime in response to both vectors were not significantly different ($p>0.05$, Mann-Whitney test) (Figure 6.12A) suggesting comparable T cell immunogenicity of the two vectors (as has been reported before following AdHu5-MVA immunization) [148]. However, antigen-specific CD4+ T cell responses could not be detected after the single AdHu5 immunization, which was not surprising given these have been shown to be relatively low even after the AdHu5-MVA prime-boost PyMSP-1_{33} regime [148].

The depletion of $CD4^+$ T cells was also assessed 24 hours post-challenge in the PBMC of depleted mice by flow cytometry, using a CD4 mAb of a different clone to the depleting mAb, and was over 98% successful (Figure 6.12B). An AUC analysis of parasitaemia on days 3-5 revealed that the depletion of CD4+ T cells in mice receiving an AP Adju-Phos® regime resulted in no significant difference in comparison to mice receiving the same regime and control rat IgG ($p>0.05$, Mann-Whitney test, Figure 6.12C). The same analysis also confirmed a significant difference in the AUC between the AP immunized mice versus mice receiving an AdHu5 MSP-1_{33} priming immunization followed by a PPP Adju-Phos® regime, and the standard PPP Adju-Phos® regime ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test). Taken all together, these data confirm that the improved efficacy observed in AP regimes (when using the Adju-Phos®

adjuvant) compared to PPP regimes in this challenge system is not due to CD4+ T cells that are primed against PyMSP-1_{42} by the adenoviral vector.

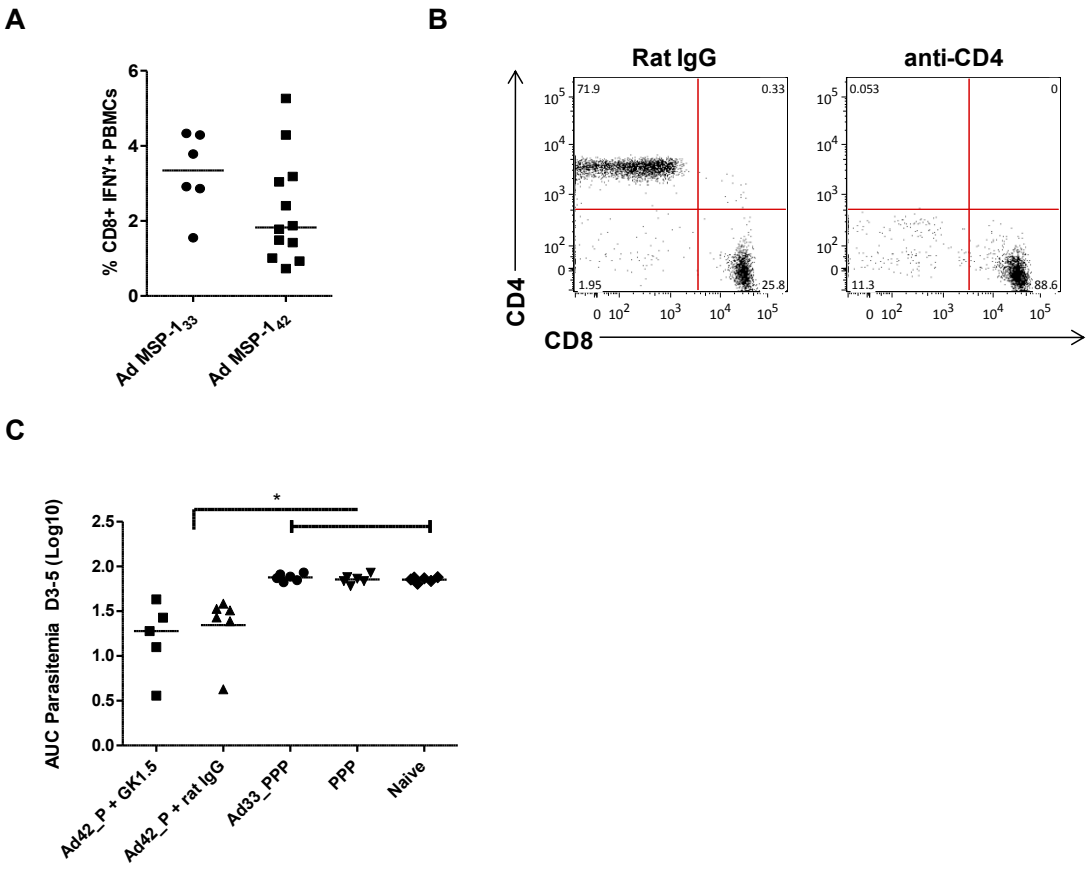

Figure 6.12 Improved efficacy of AP regimes following *P. yoelii* blood-stage challenge is not CD4$^+$ T cell dependent.

BALB/c mice (*n* = 6 / group) were immunized i.m. with either 1.5 µg of *P. yoelii* MSP-1_{19}-GST protein in Adju-Phos® three weeks apart (PPP), or primed with 1 x 10^{10} vp of AdHu5-PyMSP-1_{42} (Ad42) and boosted eight weeks later with 1.5 µg of *P. yoelii* MSP-1_{19}-GST protein in Adju-Phos®. One group of mice immunized with the AP Adju-Phos® regime was depleted of CD4+ T cells, the other received normal rat IgG as a control. Separately BALB/c mice (*n* = 6 / group) were primed i.m. with 1 x 10^{10} vp of AdHu5-PyMSP-1_{33} (Ad33) and boosted with three shots of 1.5 µg of *P. yoelii* MSP-1_{19}-GST protein in Adju-Phos® three weeks apart. All mice were challenged with 10^5 pRBCs i.v. two weeks after the final immunisation and parasitaemia was measured as the percentage of infected red blood cells over time. (A) The percentage of CD8$^+$ IFN-γ$^+$ T cells was measured by ICS in the PBMC of mice two weeks after the AdHu5

vaccinations. Median and individual data points are shown. (B) Representative flow plots from one depleted and control mouse showing the percentage of single and double CD3+ CD4+ and CD3+ CD8+ positive cells. (C) AUC analysis of parasitaemia. * $p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test. Median and individual data points are shown.

6.2.8 IgG2a but not IgG1 or IgG avidity correlate with protection

To further investigate how the differences in the vaccination regimes could account for the improved efficacy of MSP-1 protein vaccines, the protective antibody responses induced were further characterised. IgG1 and IgG2a responses were first assessed in the serum of mice receiving regimes containing high-dose MSP-1 protein vaccines two weeks after the final immunisation (pre-challenge). As seen with OVA, EBA-175 and *P. falciparum* MSP-1 more cytophilic IgG2a antibodies were again induced by AP regimes against *P. yoelii* MSP-1_{19} (Figure 6.13A). However, no correlation was found for IgG1 or IgG2a isotype units two weeks after the final immunisation with percentage peak parasitaemia (Figure 6.13B).

IgG1 and IgG2a responses were also assessed in the serum of mice receiving regimes containing low-dose MSP-1 protein vaccines two weeks after the final immunisation (pre-challenge). More cytophilic IgG2a antibodies were again induced by AP regimes against *P. yoelii* MSP-1_{19} (Figure 6.14A). An inverse correlation was found for IgG2a responses and peak parasitaemia (Figure 6.14B), but not for IgG1 (Figure 6.14C). Antibody avidity was also assessed at the time of challenge by a sodium thiocyanate displacement ELISA. There was a trend towards higher avidity in groups receiving an AP regime compared to a PPP regime, although AP regimes were only significant against PPP CoVaccine HT^{TM} ($p>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-

test, Figure 6.14D). There was no correlation between antibody avidity and total IgG titres or peak parasitaemia (Figure 6.15A&B). Overall this indicates that the improved efficacy seen in this model with the AP immunisation regime appears to be dependent on high IgG titres with a minimal contribution from IgG2a antibodies. Neither levels of IgG1 nor total IgG avidity were associated with protective outcome.

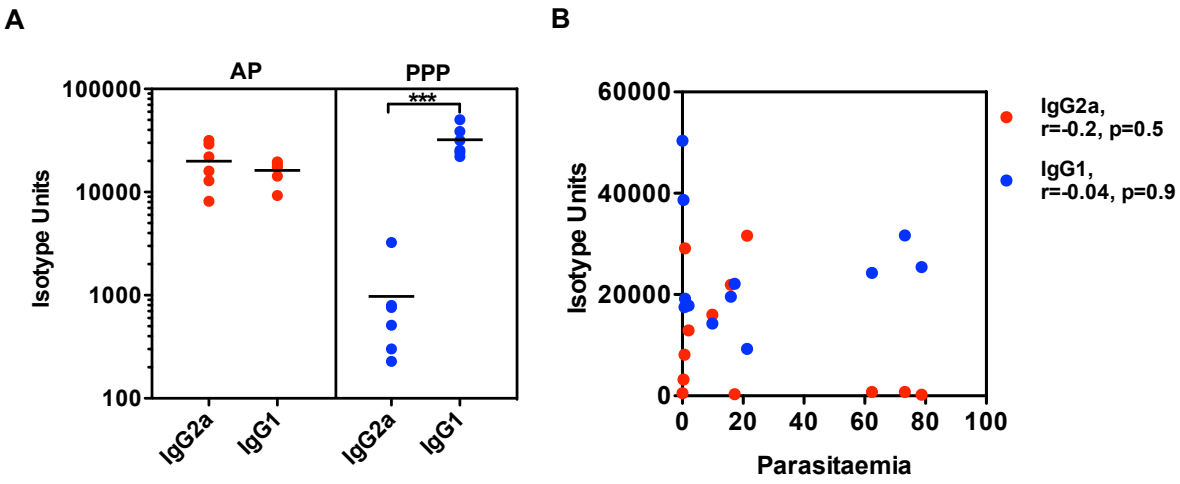

Figure 6.13 Isotype responses induced by high-dose MSP-1 vaccines.

BALB/c mice were immunized with the Adju-Phos® regimes outlined in Table 6.1. IgG1 and IgG2a antibody responses were measured in the serum two weeks after the last immunisation in response to PyMSP-1_{19}-IMX108 protein. (A) Median responses are shown for AP and PPP regimes. ***p<0.001 by paired t-test. (B) IgG1 and IgG2a isotype units measured two weeks after the final immunisation for AP and PPP regimes were correlated with percentage peak parasitaemia. The Spearman's rank correlation coefficient and p value are shown.

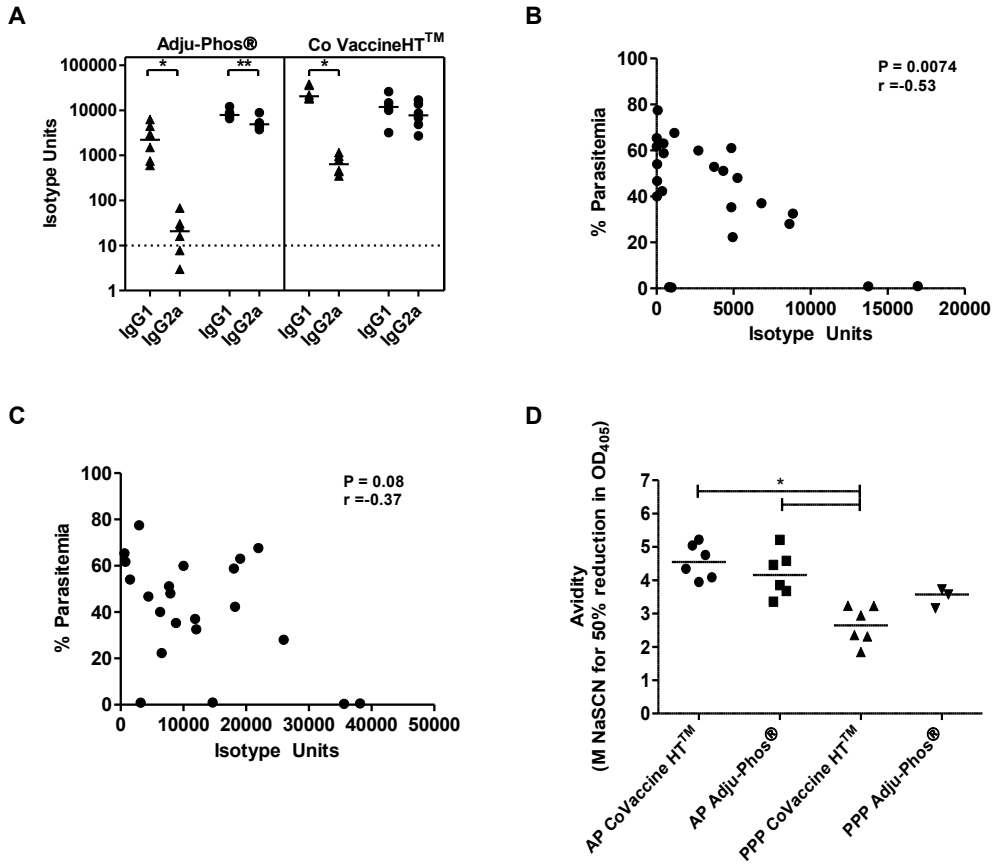

Figure 6.14 Cytophilic antibody responses correlate with protection.

BALB/c mice were immunised with the regimes outlined in Table 6.2. IgG1 and IgG2a antibody responses were measured in the serum two weeks after the last immunisation in response to PyMSP-1_{19}-IMX108 protein. (A) Median responses are shown for AP (●) and PPP (▲) regimes. *p<0.05 and **p<0.001 by paired t-test. (B) IgG2a isotype units measured two weeks after the final immunisation for AP and PPP regimes were correlated with percentage peak parasitaemia. (C) IgG1 isotype units measured two weeks after the final immunization for AP and PPP regimes were correlated with percentage peak parasitaemia. The Spearman's rank correlation coefficient and p value are shown. (D) Antibody avidity measured two weeks after the final immunisation for AP and PPP regimes. Only three samples from the PPP Adju-Phos® regime had total IgG titres high enough to allow for the assessment of antibody avidity. *p<0.05, Kruskal-Wallis test with Dunn's multiple comparison post-test.

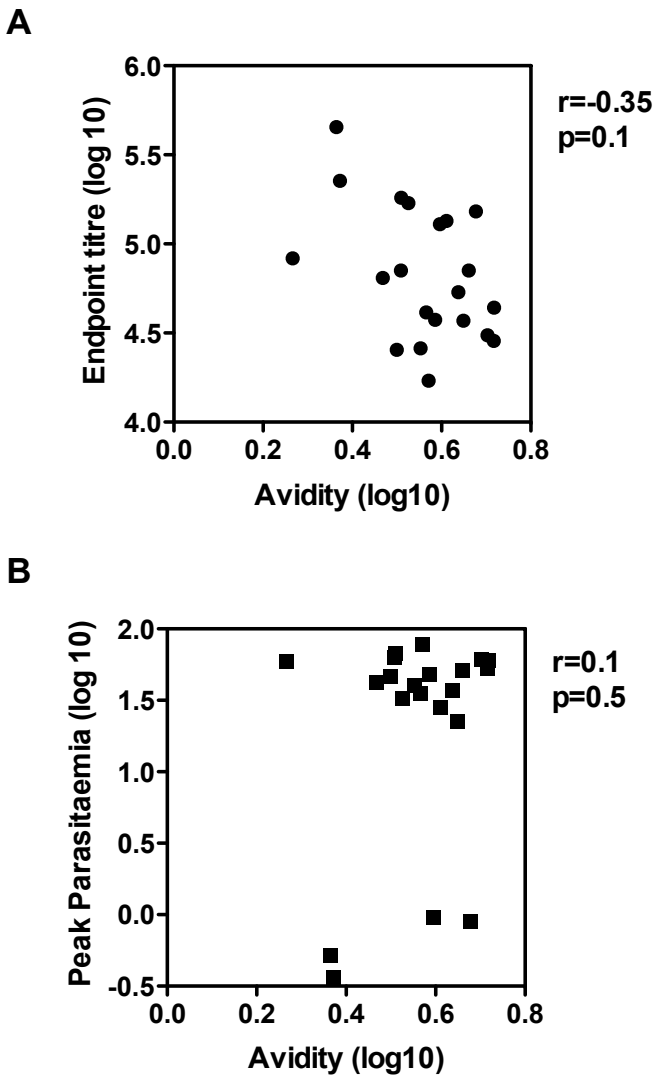

Figure 6.15 Antibody avidity does not correlate with IgG titres or peak parasitaemia.

Antibody avidity measured two weeks after the final immunisation for AP and PPP regimes was correlated with (A) IgG titres and (B) peak parasitaemia. The Spearman's rank correlation coefficient and p value are shown.

6.3 Discussion

The data presented in this chapter highlight that an adenoviral priming immunisation can not only improve the immunogenicity of protein vaccines, as has been shown with other antigens in previous chapters, but can also improve the protective efficacy of protein vaccines against a lethal *P. yoelii* blood-stage challenge. This protection was shown to correlate with MSP-1_{19} specific IgG and IgG2a, as has been previously shown [204,362] and was not dependent on MSP-1_{33} specific CD4+ T cells.

The immunogenicity of PyMSP-1_{19} protein was first assessed using the adjuvants Adju-Phos® and CoVaccine HT™. These adjuvants were selected for the experiments described in this chapter as they induced very different antibody immunogenicity profiles when used with OVA in a PPP regime, but boosted adenoviral primed antibody responses equally well. Therefore, they provide a good proof-of-concept choice for immunogenicity and efficacy studies.

PyMSP-1_{19} protein was immunogenic in PBS, even at low doses of 1.5µg, as has been shown for other *P. falciparum* antigens investigated in this thesis. However, after three shots of 1.5µg or 20µg of PyMPS-1_{19} in PBS, IgG titres in the adjuvanted groups were still significantly higher than those induced by protein in PBS. Furthermore, as shown by the efficacy studies and other groups [257], PyMSP-1_{19} in PBS was not protective against a blood-stage challenge, indicating that the protein preparation of PyMSP-1_{19} used here

does not lead to the induction of antibodies of a protective magnitude, compared to adjuvanted protein and viral vector containing regimes.

Both high- (20µg) and low-dose (1.5µg) PyMSP-1_{19} protein was taken forward into immunogenicity and efficacy studies. Using a high-dose of PyMSP-1_{19} protein, strong IgG titres were induced by both PPP and AP regimes using CoVaccine HT™ and Adju-Phos®. This differs to results seen with OVA, but are likely explained by the immunogenicity of the PyMSP-1_{19} protein preparation used, as previous results with OVA showed that even doses of 100µg of OVA in a PPP regime did not substantially improve antibody immunogenicity. It is also likely that a potential difference between the AP and PPP regimes is obscured due to antibody responses reaching the top of the dose-response curve. This is further supported by data investigating AP and PPP regimes using a lower dose of PyMSP-1_{19} protein. At a dose of 1.5µg, there was a significant difference in the IgG titres induced by Adju-Phos® in an AP and PPP regime, but no difference in IgG titres induced by CoVaccine HT™ as was previously seen. This further supports earlier observations that an adenoviral prime can improve the immunogenicity of protein vaccines formulated in relatively weak adjuvants.

The efficacy of the above vaccination regimes was then assessed against a lethal blood-stage challenge. Such a model was chosen as PyMSP-1_{19} antibodies have been shown to be a key determinant of protection in this system [174,204]. The peak parasitaemia and

area under the curve for mice that survived challenge was determined to explore an effect at the blood-stage of infection. Interestingly, following a high-dose blood-stage challenge of 10^6 pRBC, despite the induction of the same total IgG titres, a difference in protection was observed between 20µg of PyMSP-1_{19} protein in a PPP regime versus 20µg of PyMSP-1_{19} protein following an AdHu5 PyMSP-1_{42} prime using the adjuvant Adju-Phos® (AP regime). In addition to this, at a dose of 1.5µg of PyMSP-1_{19} protein there was a significant reduction in parasitaemia between D3-5 between the AP regimes and PPP CoVaccine HT™ regime compared to the PPP Adju-Phos® and control regimes. This indicates that an adenoviral prime can improve the protective efficacy of MSP-1 protein vaccines even following a pRBC challenge dose of 10^5 pRBC which is a log higher than that routinely used in the literature [174,204,257,366]. This is further supported by a two-way ANOVA of the high-dose MSP-1 vaccines indicating a significant effect of an adenoviral prime.

As mentioned previously, $CD4^+$ T cells directed against the PyMSP-1_{33} fragment, included in the adenoviral vector but not present in the protein vaccine, have been shown to be involved in protection and have been shown to lead to faster induction of antibody responses against MSP-1_{19} following exposure to native MSP-1_{19} upon challenge [148,207]. To exclude the possibility that enhanced $CD4^+$ T cell help induced by the adenoviral prime allowed for the induction of protective *de novo* antibody responses against MSP-1, AP Adju-Phos® immunised mice were depleted of CD4+ T cells

before challenge. An AUC analysis of parasitaemia showed that there was no significant effect of depletion, indicating that $CD4^+$ T cells directed against the PyMSP-1_{33} fragment are not mediating the difference in efficacy seen between the two regimes. This result was confirmed when mice were immunized with an AdHu5 vector expressing PyMSP-1_{33} followed by a PPP Adju-Phos® regime where, again, there was no decrease in the AUC compared to the standard PPP regime following a 10^5 pRBC challenge. Moreover, as whole IgG titres were shown to correlate with protection in this study, as has been reported previously for *P. yoelii* blood-stage infection [174,204], this indicates that the enhanced antibody titres induced by the AP regime are likely mediating the differences seen in protection. Another way to formally address this would be to utilise *P. yoelii* MSP-1_{42} protein in an AP and PPP regime, however as parasitaemia is almost certainly controlled by invasion-inhibitory neutralising antibodies it is likely a similar result would be seen.

In both challenge studies antigen-specific antibodies were shown to correlate with protection as previously shown [204]. However, as following high-dose MSP-1 vaccines there was a difference in survival despite the induction of the same total IgG titres, the antibody isotypes induced by vaccination were also explored. AP regimes again induced a more cytophilic antibody response and IgG2a antibodies were also shown to correlate with protection following low-dose MSP-1 vaccines (as has been previously shown for this subclass [362]). To formally confirm the role of IgG2a antibodies in protection, a

serum transfer study of vaccine-induced polyclonal IgG2a could be carried out, although isolating enough pure antigen-specific IgG2a antibodies of a protective magnitude could prove challenging. Furthermore, there is disagreement about the role of Fc-mediated protection against malaria. The γ-chain is common to FcγRI, FcγRIIIa, FcεRI, and FcαRI and is required for signal transduction and subsequently Fc-γ chain -/- mice are unable to mediate ADCC through these receptors [367,368]. The transfer of hyperimmune sera into FC-γ chain -/- chain mice and subsequent challenge with *P. yoelii* suggested that protection in this system is mediated through Fc-independent mechanisms [368]. However, it is possible that undefined FcRs or other signalling molecules might still be mediating Fc-dependent functions. IgG3 can also still elicit phagocytosis from Fc-γ chain -/- mice though the contribution of this subtype has also been ruled out [369,370]. Contrastingly, the passive transfer of immunoglobulin fractions into BALB/c and outbred mice prior to a lethal *P. yoelii* challenge revealed that only IgG2a antibodies led to an altered course of infection [362]. This in agreement with other data suggesting a protective effect mediated by cytophlic antibody isotypes [371,372]. These studies have also been extended to *P. falciparum* where humanised IgG1 antibodies against *P. falciparum* MSP-1_{19} were able to suppress parasitaemia against a *P. berghei* transgenic for *P. falciparum* MSP-1_{19} in mice transgenic for human FcγRI. Blocking studies revealed this suppression was dependent on FcγRI [373].

It would be of interest to explore the protective efficacy of AP and PPP regimes in Fc-γ chain KO mice further to investigate the role of cytophilic antibody responses, particularly following high-dose MSP-1 vaccines where the same total IgG titres were induced yet a skew towards cytophilic antibodies was evident.

The antibody avidity of the vaccine regimes was also explored. There was a trend towards higher avidity in mice receiving AP regimes compared to PPP regimes, which contrasts with previous data showing comparable avidity using Montanide®ISA720 in an AP and PP regime [177]. As shown previously, antibody avidity did not correlate with whole IgG [177] or with parasitaemia. This supports data by Lynch *et al.* who showed that neither isotype nor avidity correlate with protection against non-lethal *P. chabaudi* using AMA-1 and MSP-1 protein vaccines [374]. In the case of human *P. falciparum* malaria, it has been argued mathematically that antibody concentration and not avidity will determine the efficacy of antibodies against invading blood-stage merozoites [320]. If *P. yoelii* infection reflects that of *P. falciparum*, this argument would also suggest that antibody concentration and high titre responses are more important to protective efficacy, than the antibody binding affinity.

Overall work in this chapter shows that the improvement of antibody immunogenicity of protein vaccines following an adenoviral prime also translates to improved protection following a high-dose lethal blood-stage challenge using the aluminium-based adjuvant

Adju-Phos® which is traditionally considered to be poorly immunogenic. This protection is not dependent on T cells directed against PyMSP-1_{33} but is associated with high-titre PyMSP-1_{19} antibodies. These data set the stage for further clinical testing of this vaccination regime, especially for vaccines likely to require high-titre antibodies such as blood-stage malaria vaccines. A phase I clinical trial of an ChAd63 prime, AMA-1-based protein vaccine boost regime is now commencing in Oxford to explore whether the increased immunogenicity of AP regimes in pre-clinical models also translates to the clinic (ClinicalTrial.gov NCT01351948).

Chapter 7

Assessment of Viral Vectored Vaccine Adjuvantation

7 Assessment of Viral Vectored Vaccine Adjuvantation

7.1 Introduction

Viral vectored vaccines, such as recombinant adenovirus and MVA vaccines used in heterologous prime-boost regimes, have repeatedly been shown to induce high T cell responses as well as high-titre antibody responses in pre-clinical animal models and Phase I/II clinical trials [142,174,175,235,249]. However, for difficult diseases such as malaria and TB, which require potent immune responses for protection, it is likely that further enhancement of the immunogenicity of these vaccine platforms will remain essential for the development of next generation vaccines that are required to confer immediate and long-lasting immunity.

Several attempts have been made to improve the immunogenicity of these vaccine platforms in the past. One of these methods has been to include "molecular adjuvants" fused to the target antigen. These molecules are usually part of the innate immune system and can act to enhance transgene immunogenicity. One of these, the oligomerisation domain of the α-chain of complement 4b-binding protein (C4bp) termed IMX313 [241], has been shown to adjuvant CD8+ T cell responses when used in murine studies of malaria vaccines and non-human primate studies of a candidate TB vaccine expressing the antigen 85A [174] (Forbes *et al.*, submitted; Spencer *et al*., submitted). Members of the toll-like receptor signalling pathway, such as tak1 and tram,

have also been shown to enhance CD8+ T cells responses when co-expressed in the DNA backbone of DNA vaccines using a model antigen termed TIP (a synthetic polyepitope string) [375].

An alternative strategy for enhancing the immunogenicity of viral vectored vaccines is direct formulation with chemical adjuvants. Although viral vectors stimulate multiple signalling pathways of innate immunity [376,377], a variety of studies have addressed the potential for further enhancing responses through the direct addition of TLR agonists or more conventional chemical adjuvants. A study carried out by Goodman *et al.* using DNA, AdHu5 and MVA vaccines expressing TIP co-administered with various TLR agonists found that CD8+ IFN-γ+ responses were consistently decreased or not enhanced through the addition of adjuvants (A. Goodman, DPhil thesis 2009). However, other studies have had more success and have been able to demonstrate an enhancement of immunogenicity through the addition of adjuvants to viral vectors. 1μg of a glycolipid termed 7DW8-5, when co-administered with an AdHu5-based CSP vaccine in mice, was found to significantly enhance both anti-CSP antibody titres and IFN-γ secreting cells in the spleen and lead to enhanced protection against *P. yoelii* sporozoite challenge compared to AdHu5-CSP only vaccination [378]. Oil-in-water emulsions from the Montanide® series by Seppic and an oil-in-water emulsion produced by Mirel [379] have also been co-administered with adenovirus and poxvirus-based vaccines with an enhancement in antibody titres observed, however cellular responses were not

explored in these studies [380,381]. The addition of the polymer carbopol to a canarypox (ALVAC) vaccine targeting equine herpes virus found that this formulation increased antibody titres and protection against challenge in ponies [382]. Similarly, another study reporting the successful adjuvantation of a viral vectored vaccine was carried out by Ophorst *et al*. In this study an AdHu35 vaccine expressing PfCSP resulted in significantly higher CSP-specific T and B cell responses in mice, following either single administration or homologous prime-boost vaccination, when formulated with aluminium phosphate as compared to AdHu35-PfCSP immunisation alone [383]. In this case, a dose-sparing effect was observed, rather than an increase in maximal responses.

In parallel to these studies, isolated reports have also suggested that co-administration of the anti-malarial drug chloroquine (CQ) can enhance T cell responses in mice to soluble OVA antigen and increase T cell responses in humans vaccinated with a HBV vaccine [384,385]. This adjuvant effect is thought to be due to enhanced cross-presentation resulting from reduced acidification of the phagolysosome by CQ. This may also explain the results seen in a Phase I trial where volunteers were bitten by only 36-45 *P. falciparum* infected mosquitoes under CQ cover, which lead to their sterile protection following subsequent challenge. This protection was found to be associated with T cells and not antibodies [195]. One hypothesis put forward to explain this result is that CQ cover in conjunction with soluble sporozoite antigen may lead to enhanced

cross-presentation and therefore an extremely effective CD8+ T cell responses, though this remains to be proven.

Based on these studies, this chapter sought to explore whether the addition of CQ or aluminium phosphate to viral vectored vaccines (AdHu5, ChAd63 and MVA) could successfully adjuvant cell-mediated and/or humoral immune responses. These two adjuvants were chosen for investigation because these compounds are already licensed for human use and would therefore provide a sensible route for incorporation into clinical vaccination regimes if shown to successfully enhance immune responses in pre-clinical models.

7.2 Results

7.2.1 Chloroquine (CQ) as an adjuvant

7.2.1.1 CQ does not enhance CD8+ T cell responses to soluble protein vaccines in PP and AP regimes

As previous evidence pointed towards CQ being able to enhance CD8+ T cell responses following the administration of soluble proteins under CQ cover [384,385], the effect of CQ as an adjuvant was investigated using PP and AP regimes with the model antigen OVA. These regimes were chosen given the AP regime has been shown to induce high antibody titres with a range of CD8+ T cell responses induced depending on the adjuvant used (Chapter 3). Similarly PP regimes vary in their T cell induction based on which adjuvant is selected for formulation with the protein vaccine (Chapter 3). To investigate whether CQ could increase the utility of these regimes by enhancing T cell responses at the boost, C57BL/6 mice were immunised with either 40μg of OVA in Alyhdrogel® twice 8 weeks apart or primed with 10^{10} vp of AdHu5 OVA and boosted 8 weeks later with 40μg of OVA in Alhydrogel® formulated as outlined in Chapter 2. This protein dose was chosen as a similar AP regime has recently entered Phase I clinical trials in Oxford. Two hours before the boost and 6 hours after the boost mice were given 800μg of CQ i.p. or the same volume of PBS. T cell responses were assayed in the blood against the known H-2^b $CD8^+$ T cell epitope present in OVA 9 days after the final immunisation (Figure 7.1).

Mice immunized with an AM OVA regime, included as a positive control for T cell induction, had a median of 24,440 CD8+ SFU/10^6 PBMC following immunisation. Mice immunised with an AP regime under PBS cover (AP+PBS) showed a median of 10,740 SFU/10^6 PBMC. The addition of CQ cover at the boost did not significantly enhance CD8+ T cell responses compared to the AP+PBS group ($p>0.05$, Mann-Whitney test). As seen previously, the protein-only vaccination regime (PP) induced lower levels of CD8+ T cell responses, with a median of 315 SFU/10^6 PBMC in mice receiving a PP+PBS regime. Again the addition of CQ at the boost did not enhance CD8+ T cell responses ($p>0.05$, Mann-Whitney test).

T cell responses were also assessed in the spleen two weeks after the final immunisation and this time measured in response to both the known H-2^b $CD8^+$ T cell epitope (Figure 7.2A) and pooled $CD4^+$ T cell epitopes (Figure 7.2B) present in OVA. The hierarchy of the magnitude of the CD8+ T cell responses was the same in the spleen at this time-point as in the blood 5 days earlier. Again the addition of CQ did not significantly enhance CD8+ T cell responses compared to the same regime given with PBS ($p>0.05$, Mann-Whitney test). The addition of CQ did also not significantly decrease CD4+ T cell responses ($p>0.05$, Mann-Whitney test).

Antibody responses in mice receiving these regimes were also investigated (Figure 7.3). As seen previously, the AP regime induced higher IgG titres than a AM immunisation

[177,178]. Surprisingly, in this experiment, the PP regime formulated in Alhydrogel® induced responses comparable to the AM regimes. This could be explained by the increased dose used and extended immunization interval, which has been shown to enhance antibody responses in some instances (see Chapter 5). There was no significant difference in antibody response between groups receiving regimes under CQ or PBS cover ($p>0.05$ by Mann-Whitney test).

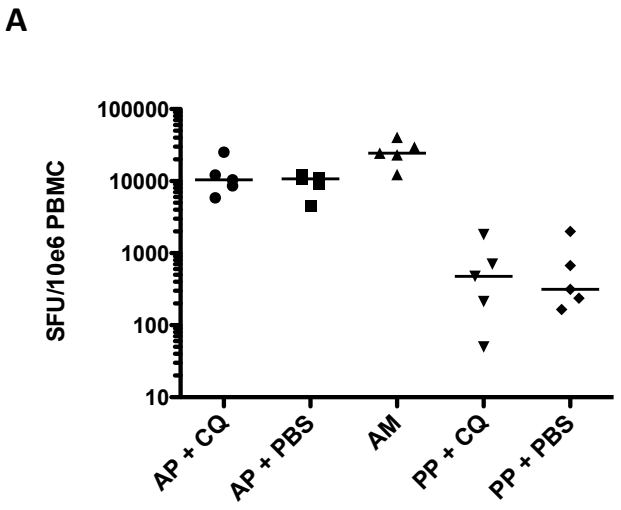

Figure 7.1 CQ does not enhance CD8+ T cell responses to soluble protein vaccines in PP and AP regimes.

C57BL/6 mice (n= 5 / group) were primed i.m. with either i) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 40µg of OVA formulated in Alhydrogel® (AP); ii) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 10^7 pfu of MVA-OVA (AM); or iii) immunised twice i.m. with 40µg of OVA formulated in Alhydrogel® as described in Chapter 2 eight weeks apart (PP). Two hours before and six hours after the boost mice receiving each AP and PP regime were immunised i.p. with either 800µg of CQ in 100µl or with 100µl of PBS. Nine days after the last immunisation, T cell responses were assayed in the blood against the known H-2^b $CD8^+$ T cell epitope present in OVA. Median responses are shown with individual data points.

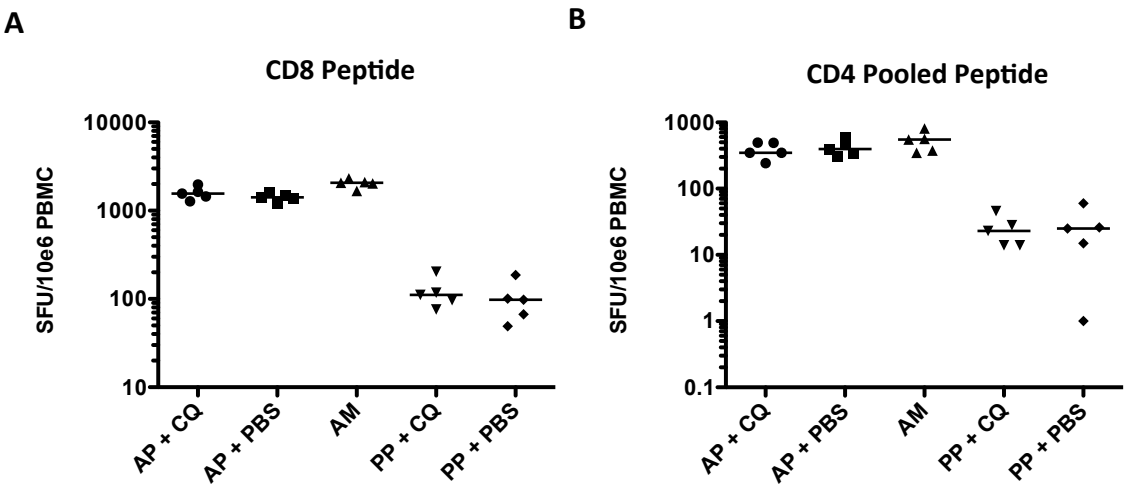

Figure 7.2 CQ does not enhance CD8+ or CD4+ T cell responses to soluble protein vaccines in PP and AP regimes.

C57BL/6 mice (n= 5 / group) were primed i.m. with either i) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 40μg of OVA formulated in Alhydrogel® (AP); ii) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 10^7 pfu of MVA-OVA (AM); or iii) immunised twice i.m. with 40μg of OVA formulated in Alhydrogel® as described in Chapter 2 eight weeks apart (PP). Two hours before and six hours after the boost mice receiving each AP and PP regime were immunised i.p. with either 800μg of CQ in 100μl or with 100μl of PBS. Two weeks after the last immunisation T cell responses were assayed in the spleen against the (A) H-2^b $CD8^+$ T cell epitope and (B) pooled H-2^b $CD4^+$T cell epitopes present in OVA. Median responses are shown with individual data points.

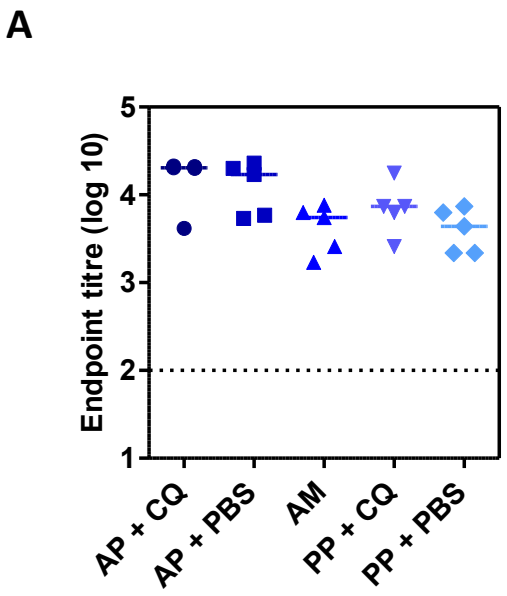

Figure 7.3 CQ does not enhance antibody responses to soluble protein vaccines in PP and AP regimes.

C57BL/6 mice (n= 5 / group) were primed i.m. with either i) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 40µg of OVA formulated in Alhydrogel® (AP); ii) $1x10^{10}$ vp of AdHu5-OVA and boosted eight weeks later i.m. with 10^7 pfu of MVA-OVA (AM); or iii) immunised twice i.m. with 40µg of OVA formulated in Alhydrogel® as described in Chapter 2 eight weeks apart (PP). Two hours before and six hours after the boost mice in each AP and PP regime were immunised i.p. with either 800µg of CQ in 100µl or with 100µl of PBS. Total IgG titers were measured in the serum in response to OVA protein by ELISA two weeks after the final immunisation. Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background.

7.2.1.2 CQ does not enhance CD8+ T cell responses to a single immunisation of recombinant viral vectored vaccines

To investigate whether CQ cover can enhance CD8+ T cell responses induced by viral vectors, C57BL/6 mice were immunised with either 10^{10} vp of AdHu5 PyMSP-1_{42} or with 10^7 pfu MVA PyMSP-1_{42}. These viral vectors express a soluble transgene that should be secreted due to presence of the mammalian tPA leader sequence [234,235,386]. In these experiments an extended period of CQ cover was given, because in mice administration of blood-stage parasites under prolonged CQ cover has been shown to induce protection following subsequent challenge [387]. Mice were thus primed with 800µg of CQ i.p. 1 day before vaccination (day 1) and then given 800µg of CQ i.p. daily for a further 9 days with vaccination occurring on day 2. Control mice received the same total volume of PBS. On day 10, 1 day after the final CQ administration and 8 days post-immunisation, the percentage of CD8+IFNγ+ cells were assessed in the blood in response to the overlapping peptide pool of PyMSP-1_{33}. A median of 1.5% and 0.9% of CD8+IFNγ+ cells were induced following AdHu5 PyMSP-1_{42} + CQ and AdHu5 Py MSP-1_{42} + PBS respectively (Figure 7.4A). Thus, as seen with soluble proteins, CQ did not significantly enhance CD8+ T cells following adenoviral-vectored vaccination ($p>0.05$, by Mann-Whitney). As seen previously, this MVA vaccine did not induce appreciable CD8+ T cell responses when used in a single-shot regime in C57BL/6 mice [236] (E. Forbes, DPhil thesis 2011). To further investigate whether CQ affected the proportion of cells secreting more than one cytokine, the polyfunctionality of antigen-specific T cells was

also assessed for AdHu5 immunised mice (Figure 7.5A). The biggest percentages of CD8+ responding cells were double-positive TNFα+IFNγ+ cells and single-positive IFNγ+ cells in both the PBS and CQ receiving mice. As seen in 7.2.1.1, CQ did also not enhance IgG titres measured in the serum in response to PyMSP-1_{19} protein by ELISA (Figure 7.6A).

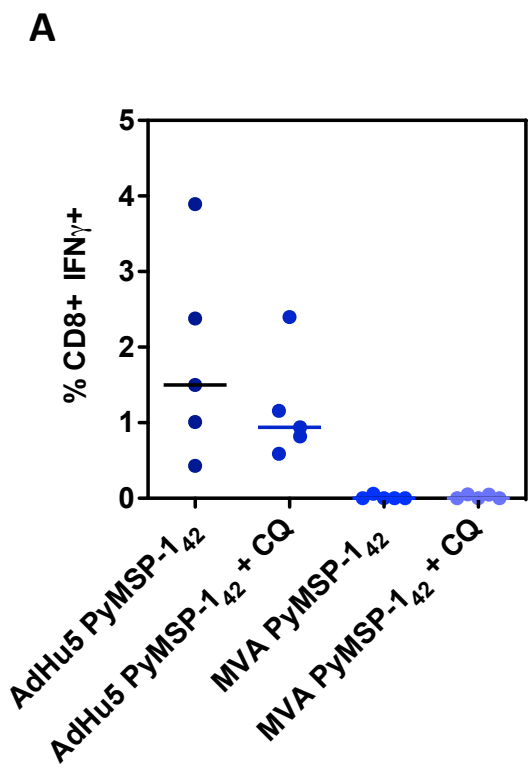

Figure 7.4 CQ does not enhance CD8+ T cell responses to a single immunisation with a recombinant AdHu5 vaccine.

C57BL/6 mice (n = 5 / group) were immunised i.m. with either 10^{10} vp of AdHu5 PyMSP-1_{42} or 10^{7} pfu of MVA PyMSP-1_{42}. One day before immunisation and for nine consecutive days thereafter mice were administered i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. Eight days after the priming immunisation the percentage of CD8+ IFNγ+ cells were assessed in the blood in response the overlapping peptide pool of PyMSP-1_{33}. Median responses with individual data points are shown.

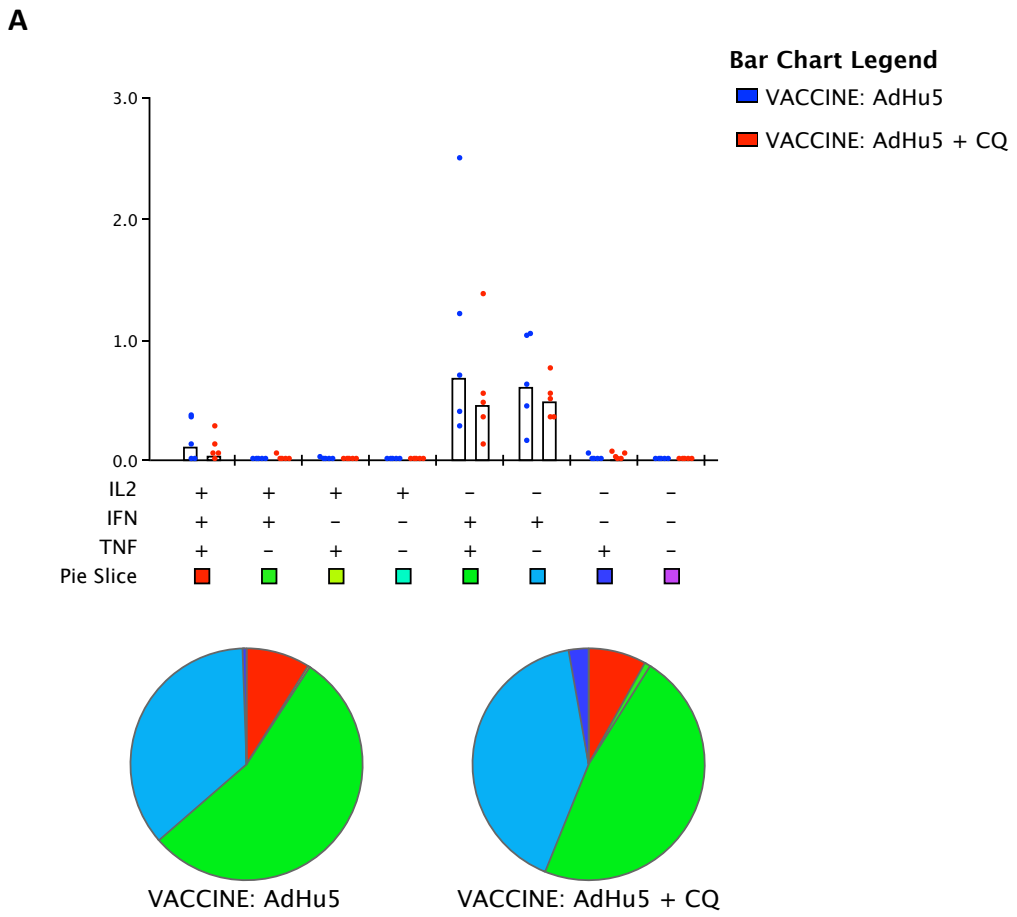

Figure 7.5 CQ does not alter the polyfunctionality of T cell responses following a single immunisation of a recombinant AdHu5 vaccine.

C57BL/6 mice (n = 5 / group) were immunised i.m. with either 10^{10} vp of AdHu5 PyMSP-1_{42} or 10^{7} pfu of MVA PyMSP-1_{42}. One day before immunisation and for nine consecutive days thereafter mice were administered i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. Eight days after the priming immunisation the percentage of CD8+ cells producing IFNγ+, IL-2 and TNFα were assessed in the blood in response the overlapping peptide pool of PyMSP-1_{33}. Antigen-specific production of IFN-γ, TNFα and IL-2 from CD8+ T cells is shown. Pie charts show the proportion of cells producing 1, 2 or all 3 cytokines.

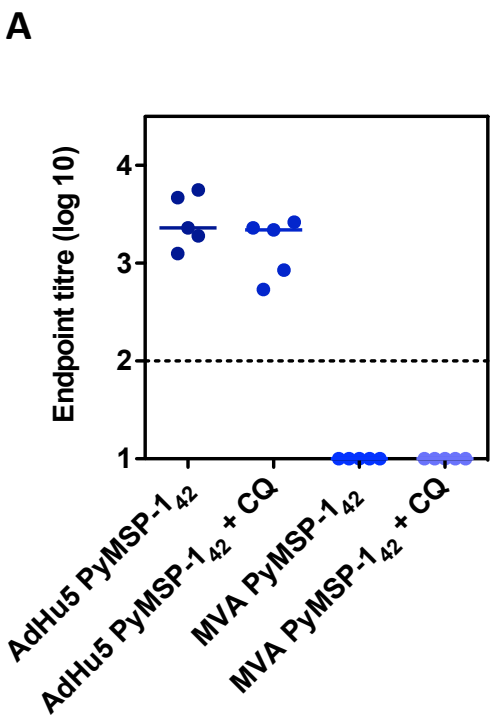

Figure 7.6 CQ does not enhance antibody responses to a single immunisation with a recombinant AdHu5 vaccine.

C57/BL6 mice (n = 5 / group) were immunised i.m. with either 10^{10} vp of AdHu5 Py MSP-1_{42} or 10^{7} pfu of MVA Py MSP-1_{19}. One day before immunisation and for nine consecutive days thereafter mice were administered i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. Total IgG titres were measured in the serum in response to OVA protein by ELISA eight days after the priming immunisation. Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background.

7.2.1.3 CQ does not enhance CD8+ T cell responses of an MVA vaccine following an adenoviral (AdHu5) prime

To investigate whether CQ could enhance CD8+ T cell responses boosted by a recombinant MVA vaccine, BALB/c mice were immunised with an AM prime-boost regime for the PyMSP-1_{42} antigen. Mice were primed with 10^{10} vp of AdHu5 PyMSP-1_{42} and boosted 8 weeks later with 10^{7} pfu of MVA PyMSP-$1_{42.}$ Mice were given 800µg of CQ i.p. 1 day before the MVA boost (day 1) and then given 800µg of CQ i.p. daily for 6 days thereafter with vaccination occurring on day 2. Control mice received the same total volume of PBS. The percentage of CD8+ IFNγ+ cells were assessed in the blood in response to the overlapping peptide pool of PyMSP-1_{33} 2 weeks after the prime (day 14) and before the MVA boost (day 55) to assess the kinetics of AdHu5-induced T cells and subsequent boosting under CQ cover. Following the MVA boost the percentage of CD8+ IFNγ+ cells were also assessed in the blood 7 (day 63) and 14 days (day 70) after vaccination. Low levels of CD8+IFNγ+ T cells were induced following an AdHu5 PyMSP-1_{42} prime (Figure 7.7). They were significantly boosted by the MVA PyMSP-1_{42} vaccine administration, as assessed 7 days post-boost ($p=0.007$ for both CQ and PBS comparing day 55 with day 70 by Mann-Whitney test). However, there was no significant difference in the percentage of CD8+IFNγ+ T cells in the blood following CQ or PBS cover 7 days or 14 days post-boost ($p>0.05$ by Mann-Whitney). The polyfunctionality of CD8+ T cells was

again assessed in the spleen 14 days post-boost (Figure 7.8). As before, double-positive TNFα+IFNγ+ T cells were the largest subset induced, followed by triple-positive and single IFNγ+ cells. There was no difference in the subsets induced between mice receiving CQ or PBS, with an apparent decrease of double-positive cells evident in the CQ group.

There was also no significant difference in IgG titres as measured in the serum in response to PyMSP-1_{19} protein by ELISA post-boost ($p>0.05$, Mann-Whitney test) (Figure 7.9A). Overall these data again indicate that CQ does not enhance viral vector vaccine-induced immune responses.

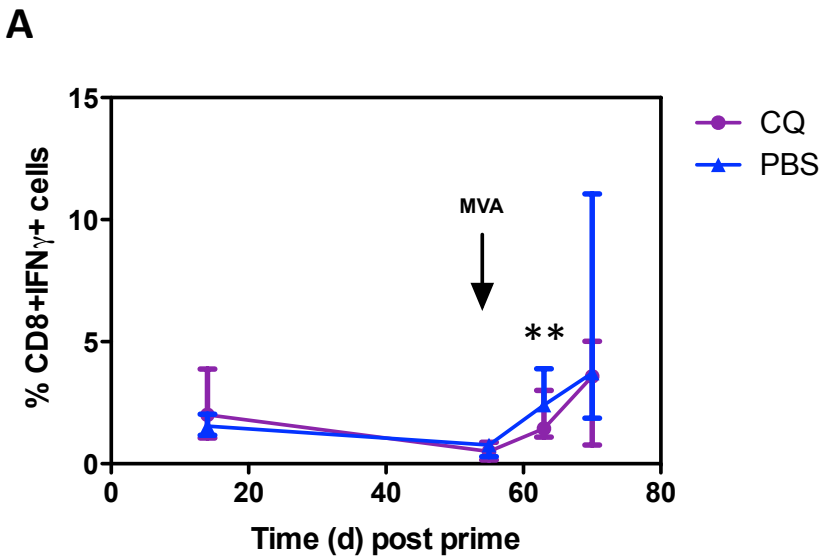

Figure 7.7 CQ does not enhance CD8+ T cell responses boosted by MVA vaccines following an adenoviral prime.

BALB/c mice (n = 5 / group) were primed i.m. with 10^{10} vp of AdHu5 PyMSP-1_{42} and boosted eight weeks later with 10^{7} pfu of MVA PyMSP-1_{42}. One day before, on the day of boosting and for 5 consecutive days thereafter mice were immunised i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. The percentage of CD8+IFNγ+ cells was assessed in the blood in response to the overlapping peptide pool of PyMSP-1_{33} two weeks after the prime (d14), one day before the boost (d55) as well as 7 (d63) and 14 days (d70) after the boost. Median responses with range are shown. ** $p<0.05$, Mann-Whitney test comparing day 55 and day 63 responses within both CQ and PBS regimes.

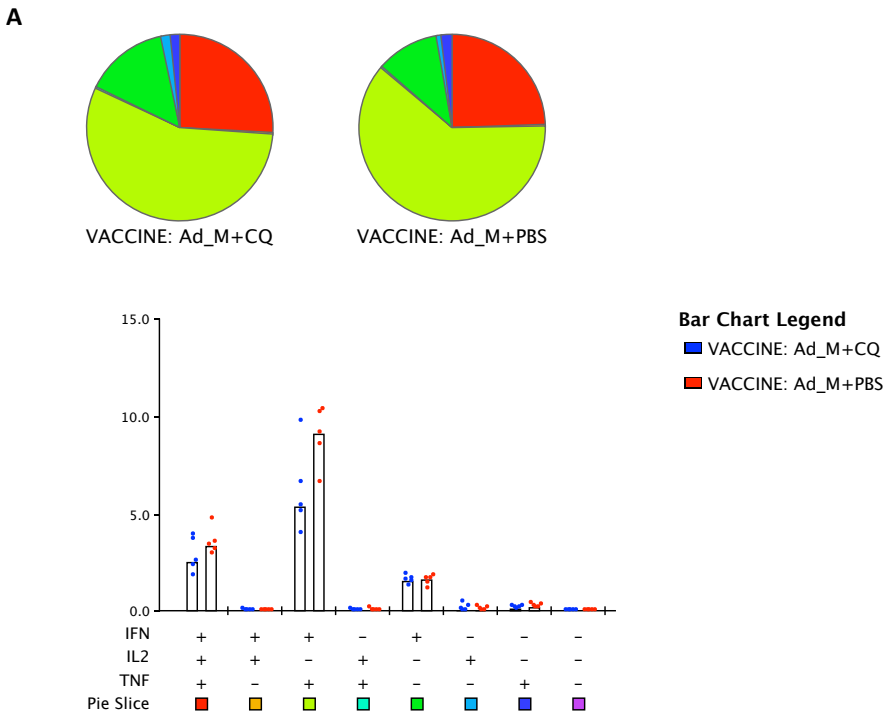

Figure 7.8 CQ does not alter the polyfunctionality of CD8+ T cell responses boosted by a recombinant MVA vaccine following an adenoviral (AdHu5) prime.

BALB/c mice (n = 5 / group) were primed i.m. with 10^{10} vp of AdHu5 PyMSP-1_{42} and boosted eight weeks later with 10^{7} pfu of MVA Py MSP-1_{42}. One day before, on the day of boosting and for 5 consecutive days thereafter mice were immunised i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. The percentages of cytokine-producing CD8+ T cells were assessed in the spleen 14 days after the final immunisation in response the overlapping peptide pool of PyMSP-1_{33}. Antigen-specific production of IFN-γ, TNFα and IL-2 from CD8+ T cells is shown. Pie charts show the proportion of cells producing 1, 2 or all 3 cytokines.

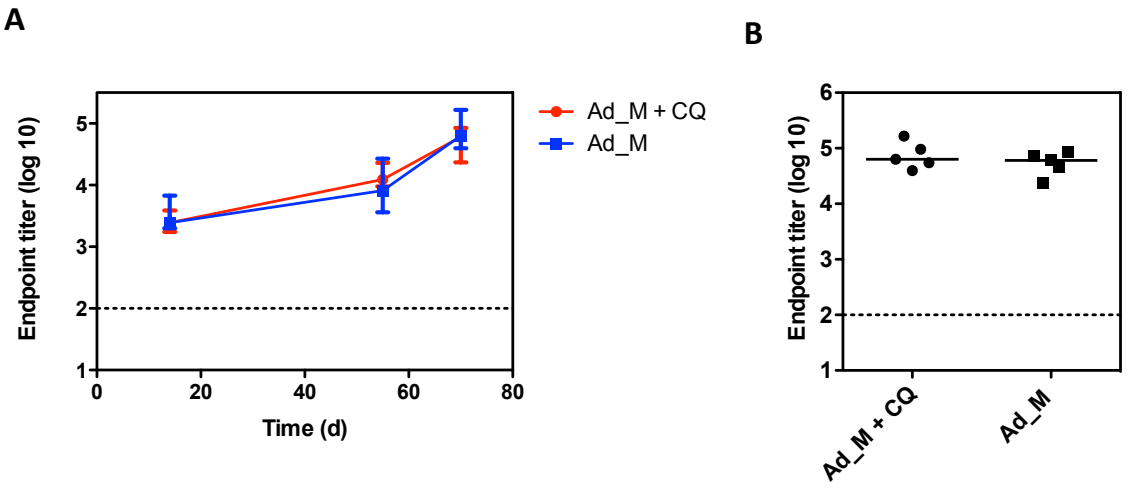

Figure 7.9 CQ does not enhance antibody responses boosted by a recombinant MVA vaccine following an adenoviral (AdHu5) prime.

BALB/c mice (n = 5 / group) were primed i.m. with 10^{10} vp of AdHu5 PyMSP-1_{42} and boosted eight weeks later with 10^{7} pfu of MVA Py MSP-1_{42}. One day before, on the day of boosting and for 5 consecutive days thereafter mice were immunised i.p. with either 800µg of CQ in 100µl PBS or with 100µl of PBS. Total IgG titres were assessed in the serum in response to PyMSP-1_{19} protein by ELISA two weeks after the prime (d14), one day before the boost (d55) and 14 days after the boost (d70). Median responses with range are shown (A). IgG titres two weeks after the final immunisation (d70). Median responses with individual data points are shown (B). The dotted line indicates the threshold for responses above background.

7.2.2 Adju-Phos® as an adjuvant

7.2.2.1 Adju-Phos® does not adjuvant ChAd63 expressing *P. falciparum* MSP-1

A study by Ophorst *et al*. has shown that an AdHu35 vaccine expressing the CSP antigen from *P. falciparum* could be successfully adjuvanted using the aluminium phosphate based adjuvant Adju-Phos® [383]. The second part of this chapter thus sought to assess whether similar results could be obtained with the more potent clinical simian adenoviral vaccine vector – ChAd63. C57BL/6 mice were immunised with $5x10^8$ vp of chimpanzee adenovirus ChAd63 expressing *P. falciparum* MSP-1 (termed PfM128 [169]). This intermediate dose of ChAd63 was chosen as the adjuvant effect of Adju-phos® appeared to be dose-dependent. As in the study by Ophorst *et al*. their vaccine was administered in Tris buffer, the ChAd63 PfM128 vaccine was given in this case in either Tris buffer versus in PBS as per usual. The ChAd63 vaccine was made up in half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® prepared in either Tris buffer or PBS, and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. It was found that the ChAd63 vaccine and Adju-Phos® did not adsorb (data not shown). T cell responses were assayed in the spleen two weeks after the priming immunisation against the known H-2^b $CD8^+$ T cell epitopes (M140, M149, M100 and M86) and the $CD4^+$ T cell epitope (M188) present in *P. falciparum* MSP-1. Moderate CD8+ T cell responses with a median of 370 SFU/10^6 splenocytes and CD4+ T cell responses of 47 SFU/10^6 splenocytes were induced by

ChAd63 PfM128 formulated in PBS (Figure 7.10). There was no effect of the use of Tris Buffer over PBS to formulate the vaccine ($p>0.05$, Mann-Whitney test comparing ChAd63 + PBS to ChAd63 + Tris for both CD4+ and CD8+ responses). However, neither CD8+ nor CD4+ T cell responses were significantly increased following the addition of Adju-Phos® to the viral vector as measured by ELISpot ($p>0.05$, Mann-Whitney test comparing adjuvanted and non-adjuvanted groups) (Figure 7.10A&B). However, there was a trend towards increased CD8+ T cells in the ChAd63 + Adju-Phos® + PBS group compared to the ChAd63 + PBS group, although this did not reach significance ($p=0.055$, Mann-Whitney test). This trend was also evident when looking at the percentage of CD8+ T cells producing IFN-γ by ICS ($p=0.056$, Mann-Whitney test) (Figure 7.11A).

Antibodies of a low magnitude were induced 2 weeks after the ChAd63 immunisation as measured in response to PfMSP-1_{19} protein (QKNG allele) in the serum by ELISA (Figure 7.11B). There was a trend towards increased IgG titres in mice immunised with ChAd63 + Adju-Phos® + PBS, although this was not significant against the relevant control group given no adjuvant ($p>0.05$, Mann-Whitney test).

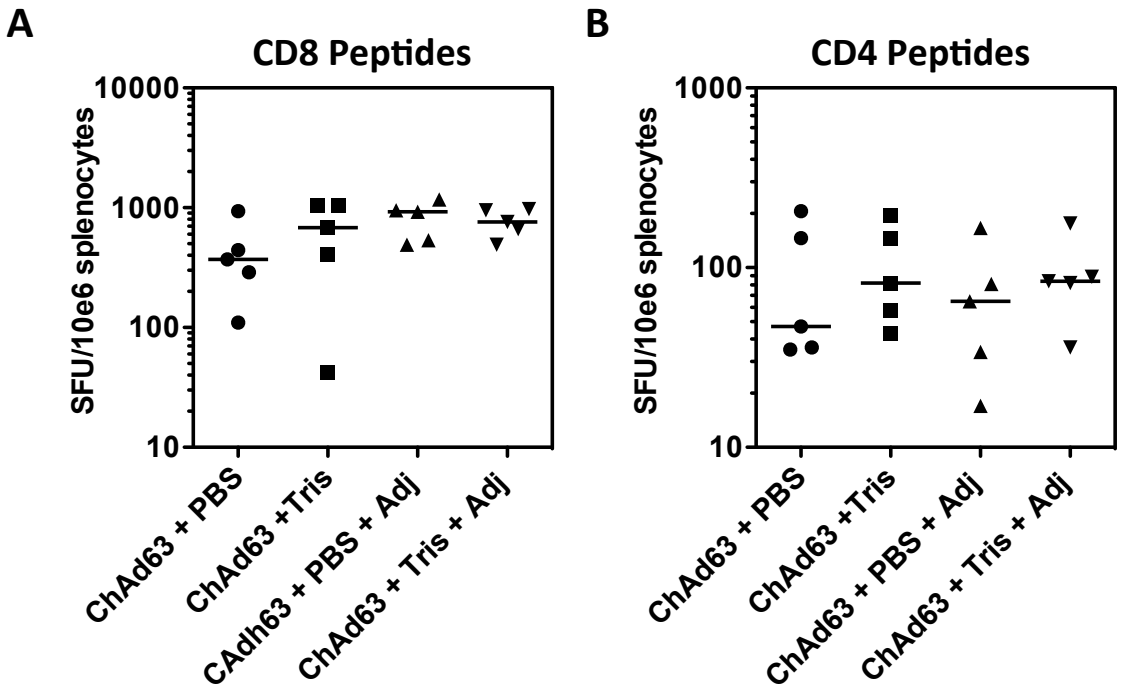

Figure 7.10 Adju-Phos® does not adjuvant ChAd63 expressing *P. falciparum* MSP-1.

C57BL/6 mice (n = 5 / group) were immunised i.m. with $5x10^8$ vp of ChAd63 PfM128 in either Tris buffer or PBS. The ChAd63 vaccine was made up in the half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® (Adj) made in either Tris buffer or PBS and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. Two weeks after the immunisation, T cell responses were assayed in the spleen against the known H-2^b $CD8^+$ (M140, M149, M100 and M86) and $CD4^+$ (M188) T cell epitopes present in *P. falciparum* MSP1. Median responses with individual data points are shown.

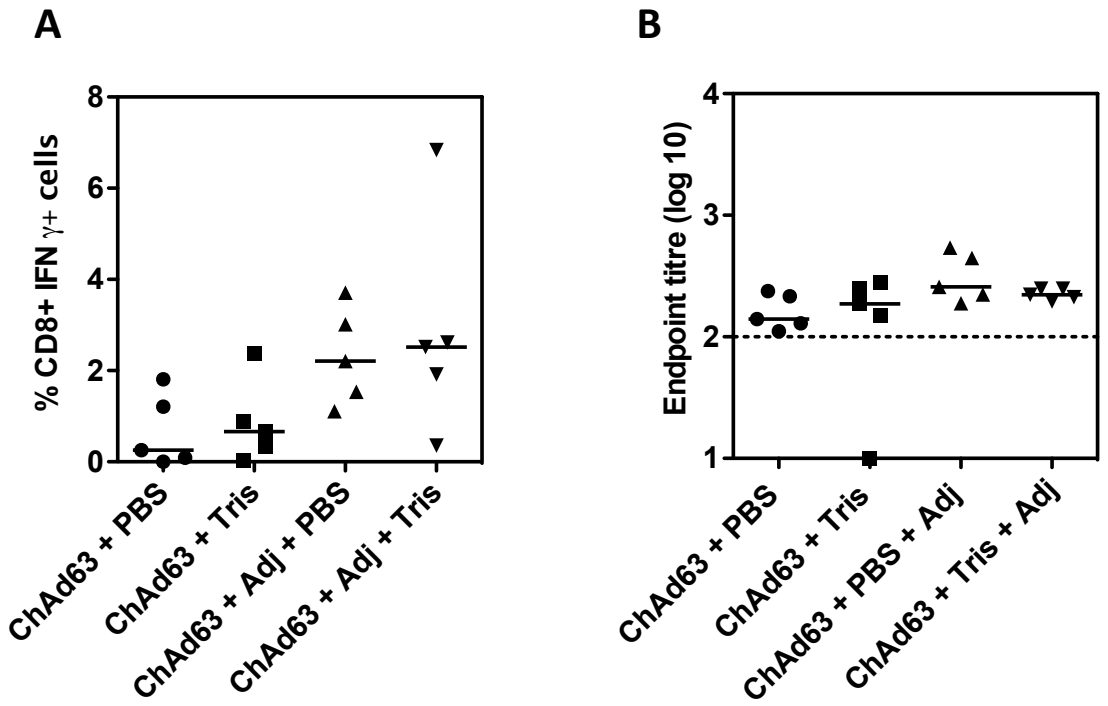

Figure 7.11 Adju-Phos® does not adjuvant ChAd63 expressing *P. falciparum* MSP-1.

C57BL/6 mice (n = 5 / group) were immunised i.m. with $5x10^8$ vp of ChAd63 PfM128 in either Tris buffer or PBS. The ChAd63 vaccine was made up in the half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® (Adj) made in either Tris buffer or PBS and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. The percentage of CD8+ IFNγ+ cells was assessed in the spleen two weeks after the immunisation against the known H-2^b $CD8^+$ (M140, M149, M100 and M86) epitopes present in *P. falciparum* MSP1 (A). Total IgG titres were measured in the serum in response to PfMSP-1_{19} protein by ELISA (QKNG allele) (B). Median responses with individual data points are shown. The dotted line indicates the threshold for responses above background in B.

7.2.2.2 Adju-Phos® does not adjuvant AdHu5 expressing *P. falciparum* MSP-1 in a homologous prime-boost regime

To investigate whether the lack of enhanced humoral and cellular immune responses was due to the use of ChAd63 and C57BL/6 mice in the previous study, BALB/c mice were immunised with an AdHu5 vaccine expressing PfM128. To investigate the effect of the AdHu5 dose, mice were immunised with either $5x10^8$ vp or $5x10^9$ vp of AdHu5 PfM128. As before the vaccine was given in Tris buffer and mixed 1:1 with 0.5mg/mL of Adju-Phos®. Similar to ChAd63, it was found that the AdHu5 vaccine and Adju-Phos® did not adsorb (data not shown). CD8+ T cells producing IFN-γ were measured in the blood in response to the known H-2^d $CD8^+$ epitope (M90/91) two weeks after the priming immunisation (Figure 7.12A). A dose effect of the AdHu5 vaccine was evident with 0.2% and 3.2% of CD8+IFNγ+ T cells induced at $5x10^8$ and $5x10^9$ vp respectively. CD8+IFNγ+ T cells were not significantly increased by Adju-Phos® at both vaccine doses ($p>0.05$, Mann-Whitney test). To see whether an adjuvant effect of Adju-Phos® could be evident following a recall response, mice were given a homologous boost of AdHu5 PfM128 4 weeks after the priming immunisation. At this time point, the AdHu5 vaccine was mixed 1:1 with 1mg/mL Adju-Phos®. The adjuvant dose was doubled to see if an increased dose of Adju-Phos® could successfully enhance immune responses. T cell responses were assayed in the spleen by ELISpot two weeks after the final vaccination. For both doses of AdHu5 PfM128, there was no significant enhancement of CD8+IFN-γ producing T cells through the addition of Adju-Phos® after a homologous prime-boost regime

(Figure 12B). Furthermore, responses in the $5x10^8$ vp AdHu5 PfM128 group were in fact significantly decreased by the addition of Adju-Phos® ($p=0.03$, Mann-Whitney test).

IgG titres were again assessed in the serum of mice 2 weeks after each immunisation against PfMSP-1_{19} protein by ELISA (Figure 7.13A&B). At both time-points there were no significant differences in antibodies induced in the Tris only versus Adju-Phos® groups ($p>0.05$, Mann-Whitney test).

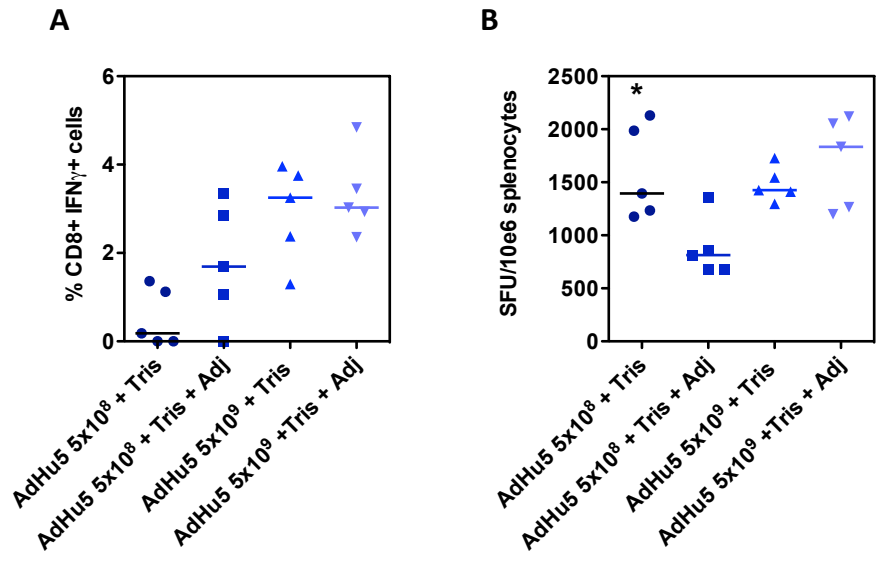

Figure 7.12 Adju-Phos® does not adjuvant T cell induction by AdHu5 expressing *P. falciparum* MSP-1 in a homologous prime-boost regime.

BALB/c mice (n = 5 / group) were primed with either $5x10^8$ vp or $5x10^9$ vp of AdHu5 PfM128 in Tris. The AdHu5 vaccine was made up in the half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® (Adj) made in Tris buffer and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. CD8+ T cells producing IFNγ were measured in the blood in response to the known H-2^d $CD8^+$ epitope (M90/91) two weeks after the prime (A). Four weeks later mice were given a homologous boost. At the boost, the AdHu5 vaccine was made up in the half the required volume and then mixed 1:1 with 1mg/mL of Adju-Phos® (Adj) made in Tris buffer and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. Two weeks after the second immunisation T cell responses were assayed in the spleen in response to the known H-2^d $CD8^+$ epitope (M90/91) present in MSP-1 (B). Median responses with individual data points are shown. *$p<0.05$, Mann-Whitney test comparing AdHu5 $5x10^8$ + Tris versus AdHu5 $5x10^8$ + Tris + Adj.

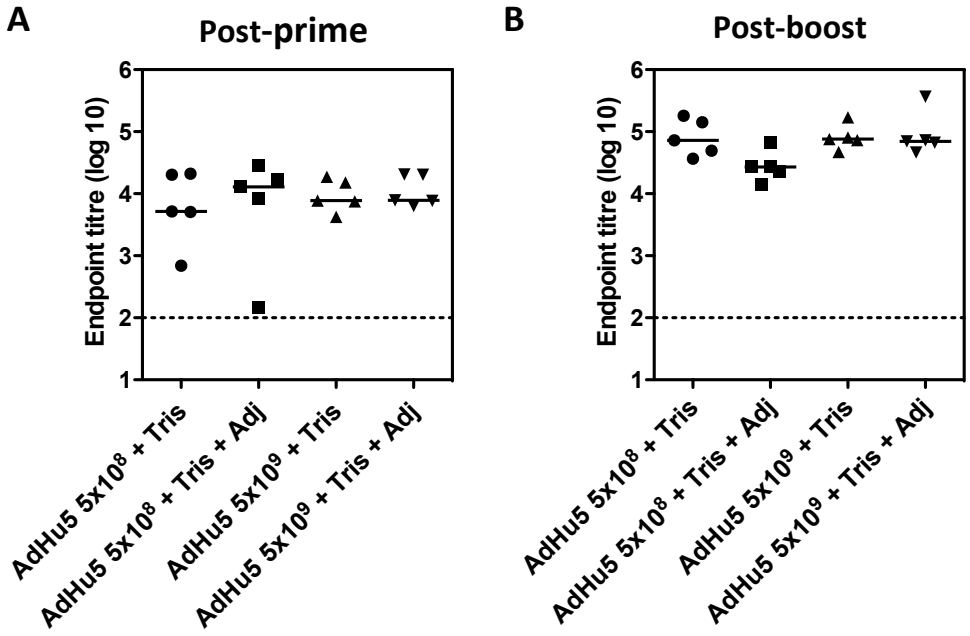

Figure 7.13 Adju-Phos® does not adjuvant IgG induction by AdHu5 expressing *P. falciparum* MSP-1 in a homologous prime-boost regime.

BALB/c mice (n = 5 / group) were primed with either $5x10^8$ vp or $5x10^9$ vp of AdHu5 PfM128 in Tris. The AdHu5 vaccine was made up in the half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® (Adj) made in Tris buffer and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. Four weeks later mice were given a homologous boost. At the boost the AdHu5 vaccine was made up in the half the required volume and then mixed 1:1 with 1mg/mL of Adju-Phos® (Adj) made in Tris buffer and adsorbed for 30 minutes at 4°C. The final vaccine was administered i.m. in a total of 50µL. Total IgG titres were measured in the serum in response to PfMSP-1_{19} protein (QKNG allele) by ELISA two weeks after the prime (A) and two weeks after the homologous boost (B). Median responses with individual data points are shown. The dotted line indicates the threshold for responses above background.

7.2.2.3 Adju-Phos® does not adjuvant AdHu5 or MVA vaccines expressing *P. falciparum* MSP-1

To determine whether Adju-Phos® could adjuvant MVA, BALB/c mice were immunised again with the AdHu5 PfM128 vaccine at a dose of $5x10^8$ vp mixed either 1:1 with 0.5mg/ml of Adju-Phos® in Tris or with Tris or PBS only. Mice were also primed with 10^6 pfu of a MVA vaccine expressing the same transgene with or without Adju-Phos® formulated as above. CD8+ T cells producing IFN-γ were measured in the blood in response to the known H-2^d $CD8^+$ epitope (M90/91) two weeks after the immunisation (Figure 7.14A). CD8+IFNγ+ cells were again induced by the AdHu5 PfM128 vaccine. There was no significant difference in responses induced by vaccines formulated either in PBS or Tris ($p>0.05$, Mann-Whitney test). Interestingly there was a significant enhancement of CD8+ T cells in the Ad + Tris + Adju-Phos® group ($p=0.03$, Mann-Whitney test compared to Ad + Tris), indicating a marginal dose-sparing effect. T cells induced by MVA PfM128 were of a lower magnitude, as routinely seen with MVA vaccines after a single immunisation [236] (E. Forbes, DPhil thesis 2011), and no enhancement of CD8+IFN-γ+ T cell responses was seen.

Antibody responses were also assessed in the serum in response to PfMSP-1_{19} protein by ELISA two weeks after the immunisation. Although there was a trend towards improved antibody responses by the addition of Adju-Phos®, this did not reach significance ($p=0.055$, Mann-Whitney test). Also, as routinely seen with MVA vaccines,

IgG titres were lower in the MVA vaccinated mice than in the AdHu5 vaccinated mice. There were no significant differences in the induction of IgG titres with or without Adju-Phos® in mice vaccinated with Tris (p>0.05, Mann-Whitney test)

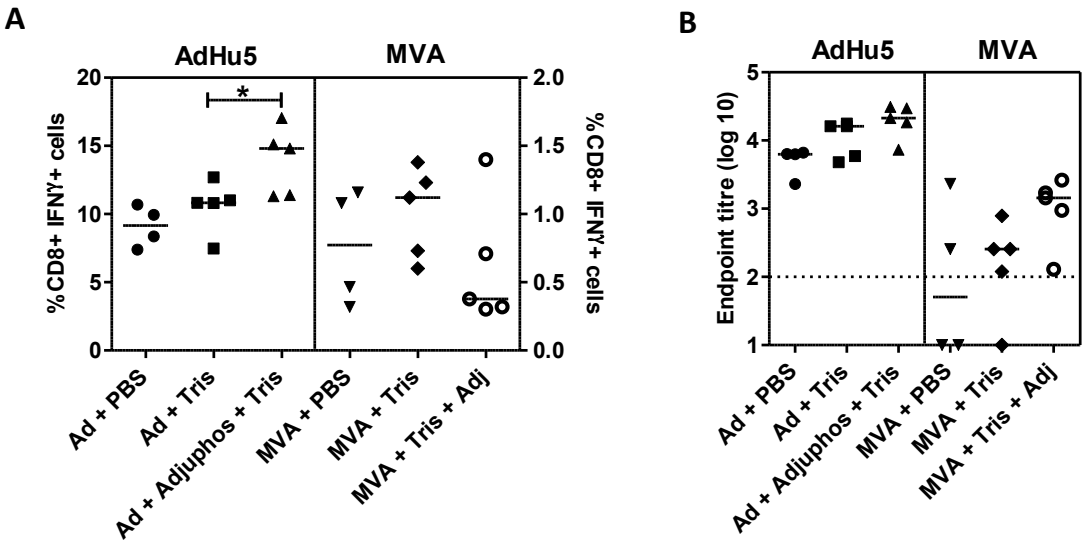

Figure 7.14 Adju-Phos® induces a minor dose-sparing effect for CD8+ T cells following AdHu5 vaccines expressing *P. falciparum* MSP-1.

BALB/c mice (n = 5 / group) were vaccinated with $5x10^8$ vp of AdHu5 PfM128 (Ad) or 10^6 of MVA PfM128 formulated in Tris buffer or PBS. The viral vaccines were made up in half the required volume and then mixed 1:1 with 0.5mg/mL of Adju-Phos® (Adj) made in either Tris buffer or PBS and adsorbed for 30 minutes at 4°C. CD8+ T cells producing IFNγ were measured in the blood in response to the known H-2^d $CD8^+$ epitope (M90/91) two weeks later. * p<0.05, Mann-Whitney test comparing Ad + Tris and Ad + Tris + Adju-Phos® (A). Total IgG titres were measured in the serum in response to PfMSP-1_{19} protein (QKNG allele) by ELISA at day 14 (B). Median responses are shown with individual data points. The dotted line indicates the threshold for responses above background in B.

7.3 Discussion

Viral vectored vaccines have been shown to induce strong cellular and humoral immune responses when used in prime-boost regimes. The work described here aimed to assess whether immune responses induced by MVA and/or adenoviral vaccines could be further enhanced by co-administration with chemical adjuvants. Disappointingly neither Adju-Phos® nor CQ were able to significantly and consistently enhance immune responses following vaccination, though there was minor towards a dose-sparing effect for CD8+ T cells following AdHu5 PfM128 + Adju-Phos® in BALB/c mice.

Two reports have shown that CQ can enhance cross-presentation and increase CD8+ T cell responses to soluble antigens [384,385]. Garulli *et al.* have shown that CD8+ T cell responses are increased if mice are treated with CQ during a primary immunisation of OVA protein formulated in aluminium hydroxide (Alhydrogel®) or with OVA-pulsed DCs [385]. Although not directly investigated, this study assumed that the enhanced responses were due to an inhibition of endosomal acidification of DCs leading to increased cross-presentation. Similarly Accapezzato *et al.* showed an enhancement of CD8+ T cell responses following a HBV booster vaccine under CQ cover in humans [384]. *In vitro* studies showed that CQ increased the export of soluble antigen into the cytosol for processing onto MHC class I molecules. This is thought to occur when internalised antigens are less efficiently processed if the lysosome is not acidified in the presence of

CQ. This leads to their accumulation in the endosome and therefore leads to a more efficient export (cross-presentation) of non-degraded protein antigens into the cytosol.

Despite these two proof-of-concept studies, these effects could not be replicated in the work described in this chapter. An enhancement of CD8+ T cell responses was not found when 40µg of OVA protein was administered with Alhydrogel® as a booster vaccine under CQ cover following a priming immunisation of either 40µg of OVA or 10^{10} vp of AdHu5 OVA 8 weeks earlier. Given CQ was seen in the previously described studies to adjuvant secondary immune responses, this result was surprising. The adjuvant effect of CQ was found to be dose dependent for OVA *in vivo* [385], and it is therefore possible that the dose of OVA protein used here could account for the lack of adjuvant effect seen (because not enough protein accumulates in the endosome for efficient cross-presentation). However, the 40µg of OVA protein used here is broadly equivalent to the dose used in the previous study for which an adjuvant effect was seen [385]. Some studies have also shown a decrease in CD4+ T cell responses during CQ administration [388,389,390], possibly due to antigen being preferentially cross-presented to MHC class I molecules instead of loaded onto MHC class II molecules, although this effect was not evident here. As CD8+ T cell responses as well as antibody responses have been shown to be dependent on CD4+ T cells [330,391], this stable CD4+ T cell population could account for the similar levels of IgG titres and CD8+ T cells seen under both treatment regimes.

The ability of CQ to adjuvant viral vectored vaccines was also explored and disappointingly CQ did not enhance CD8+ T cells induced by either MVA or AdHu5 vaccines expressing PyMSP-1_{42} following single-shot administration or at the time of the boost in an AM regime. This transgene is soluble and also contains the mammalian tPA leader sequence which should aid it's secretion by targeting it to the endoplasmic reticulum as has been shown for other target antigens [234,235,386]. Given the effects showing an adjuvant effect of CQ to soluble antigens were quite marginal (0.6 fold increase in CD8+ T cell proliferative responses for OVA and an expansion of antigen-specific effector CD8+ T cells in 6/9 individuals receiving HBV+CQ [384,385]), it is possible that due to the high CD8+ T cell responses induced by viral vectored vaccines [159,174,175] an improvement of responses was not evident. Immunising mice with a range of viral vector vaccines doses might reveal an adjuvant or dose sparing effect. However, a Grand Challenges in Global Health Project at the Jenner Institute is trying to improve the immunogenicity of viral vectored vaccines through genetic adjuvants. Despite extensive library screens of TLR pathway signalling molecules few molecules have been found that successfully adjuvant viral vectored vaccines [375]. This indicates that the successful adjuvantation of viral vectored vaccines may be difficult to achieve. Several studies have also shown that T cell induction following MVA infection is far more likely to occur via cross rather than direct presentation [140], therefore showing a further enhancement of CD8+ T cells induced via the cross-presentation pathway might be physiologically unlikely. CQ is also known to have anti-viral properties and inhibit

TNFα and IL-6 production from viruses [392] and yet despite these observations, no significant decreases in T cell responses were observed either following CQ and viral vector co-administration (compared to PBS control groups). Furthermore, it is possible that the prolonged CQ cover of 10 days, given with the viral vectors, could also explain the lack of an observed adjuvantation effect, given prolonged CQ administration is used in some settings as an immunosuppressive drug [393]. However, as previously stated, no significant decrease in T cell responses was seen following CQ co-administration.

A recent Phase I clinical trial, in which volunteers who were bitten by malaria infected mosquitoes under CQ cover were sterilely protected from subsequent sporozoite challenge, found an association between protection and cellular-mediated immunity [195,394]. This suggested that enhanced CD8+ T cells generated through co-administration of CQ at the time of live-sporozoite vaccination might be mediating the observed protective effect. Given CQ kills blood-stage parasites, which are thought to exert immune evasion properties and induce Tregs [395], it is thought that under CQ cover and in the absence of this immune-suppressive parasitic environment, an improved protective CD8 + T cell response can be established to a wide-array of late liver-stage antigens that are also present in the blood-stage parasite [396]. It is therefore possible that in the context of malaria vaccination, where CQ cover has also been shown to mediate protection in animal models [194,387], it is not the adjuvant properties of CQ *per se* that are of interest, but instead the anti-malarial effect allowing

for the improved induction of a protective immune response against late liver-stage target antigens at the blood-stage [397]. To clarify whether it is the adjuvant properties or anti-malarial properties of CQ mediating this protective effect, it would be interesting to explore whether similar protection can be achieved with other anti-malarial drugs [398]. Nevertheless, taken all together, using CQ as an adjuvant for protein vaccines and/or viral vectors does not appear promising in the context of enhancing CD8+ T cell responses.

The ability of Adju-Phos® to adjuvant viral vectors was explored after aluminium phosphate was shown to adjuvant an AdHu35 PfCSP-based vaccine [383]. Disappointingly here CD8+ T cell responses were not consistently adjuvanted by Adju-Phos®, although there was a marginal but significant effect in one experiment using AdHu5 PfM128 formulated in Tris buffer (Figure 7.14A). Given the viral vectors in this study, as reported with AdHu35-PfCSP, were not found to adsorb to Adju-Phos® it is likely that this adjuvant effect is mediated by mechanisms other than the formation of an adenovirus-adjuvant complex. This may occur through increasing the local inflammatory responses via the addition of alum resulting in the increased recruitment of APCs [399].

The lack of adsorption between Adju-Phos® and adenoviral vectors is not surprising given that the external capsids of both AdHu5 and ChAd63 are negatively charged [400],

leading to repulsive electrostatic forces between these viruses and the negatively charged aluminium phosphate [59,399]. It would be interesting to explore whether Alhydrogel®, based on aluminium hydroxide which forms stronger electrostatic interactions with negative proteins [59,399], could act as a better adjuvant allowing adenovirus-adjuvant complexes to form. However, it is also possible that association between viral vectors and alum could interfere with cell entry and receptor recognition thereby actually decreasing the immune response. However, in a study carried out by Shayakhmetov *et al.* pre-incubation of 293 cells with polybrene, a polycation, or a cationic lipid has been shown to increase binding of adenoviruses to the CAR receptor used for cell entry, presumably by masking surface charges on target cells [401]. The effect on immunogenicity was not explored in this study. An adjuvant effect has also been shown co-administering the glycolipid 7DW8-5 with an AdHu5-PyCSP vaccine [378]. However, this study did not address association between the adenovirus and adjuvant. Therefore, whether the positive charge is enhancing the binding of adenovirus to the CAR receptor, or the administration of lipids is disrupting cell membranes enhancing infection, and what effect this may have on adjuvant properties is not known.

Also in their study, Ophorst *et al.* used a recombinant AdHu35-based vaccine. This adenovirus is a group B adenovirus, which, unlike AdHu5 and ChAd63, does not bind CAR instead requiring interaction with receptor CD46 to gain cell entry. CD46 is widely expressed on human cells, but the murine CD46 homologue is scarcely distributed with

expression in mice confined mainly to the testes [402,403,404]. AdHu5 and ChAd63 vaccines induce stronger T cell responses than AdHu35 vaccines in mice [405], although a direct head-to-head comparison is difficult due to the differing tissue tropism between these viruses. It is likely however that due to the lower levels of immunogenicity of the AdHu35 virus in mice, co-administering Adju-Phos® can more effectively enhance the immune response to the transgene.

In conclusion, no enhancement of humoral or cellular immune responses was seen using CQ or Adju-Phos® to adjuvant protein and/or viral vectored vaccines, despite previous studies indicating the use of these compounds as adjuvants. Further investigations into the use of adjuvants to enhance immune responses to viral vectors nevertheless remains essential, for the improvement of the next generation viral vectored vaccines.

Chapter 8

Discussion and Future Directions

8 Discussion and Future Directions

8.1 Summary

A central goal in vaccinology is the induction of high and sustained antibody responses. Protein-in-adjuvant formulations are commonly used to achieve such responses. However, their clinical development can be limited by the reactogenicity of some of the most potent experimental adjuvants and the cost and complexity of licensing new adjuvants for human use. Also, few adjuvants induce strong cellular immunity, which is important for protection against many diseases, such as malaria. Work in this thesis described the comparative assessment of classical adjuvants such as aluminium salts to new experimental adjuvants and adjuvants in clinical development such as Abisco®100, CoVaccine HT™, Montanide®ISA720 and SE-GLA for the induction of high and sustained antibody and T cells responses. These adjuvants induced a broad range of antibody responses when used in a three-shot protein-in-adjuvant regime using the model antigen ovalbumin and leading blood-stage malaria vaccine candidate antigens. Surprisingly, this range of antibody immunogenicity was greatly reduced and antibody responses were of a comparably high magnitude when a protein-in-adjuvant vaccine was used to boost antibody responses primed by an AdHu5 vaccine recombinant for the same antigen. This AP regime also induced a more cytophilic antibody response and demonstrated improved efficacy of MSP-1 protein vaccines against a high-dose

Plasmodium yoelii blood-stage challenge. Investigating the underlying differences between the vaccine platforms used in these regimes suggests the improved boosting observed following an AdHu5 prime is not due to differences in antigenic dose exposed to the immune system or extended immunisation intervals between AdHu5 and protein vaccines, but rather appears to be inherent to the adenoviral vaccine which induces more GC B cells and T_{FH} cells. Interestingly, attempts to further enhance the immunogenicity of viral vectors through the direct addition of the adjuvants CQ and Adju-Phos® were not successful.

8.2 Conclusions

8.2.1 Comparative assessment of chemical adjuvants in AP and PPP regimes

A panel of 11 clinically tested/approved and experimental adjuvants was assessed in a three shot protein-in-adjuvant regime for the induction of high and sustained antibody titres. A broad range of IgG titres was induced when adjuvants were administered three times with OVA protein. CoVaccine HT™ and emulsion based adjuvants containing TLR agonists were found to induce strong antibody responses surpassing aluminium-based adjuvants and the classical reference adjuvant, Freund's adjuvant. The induction of T cell responses following a PPP regime also appeared to be adjuvant-dependent, with TLR4

agonist containing adjuvants, inducing strong CD4+ and CD8+ T cell responses as has previously been reported for TLR-based adjuvants [264,265]. This study was then extended to assess the adjuvants in an AP regime – given sequential immunisations with an adenovirus followed by a protein/adjuvant vaccine has been shown to induce strong antibody titres surpassing those achieved with PPP and AM regimes [177,178,179,180]. Remarkably, the broad range of antibody titres seen in the PPP regime was overcome using an adenoviral prime. This was particularly evident for typically 'weak' adjuvants such as those based on aluminium salts. Similarly, in a non-human primate study by Draper *et al.*, IgG titres following an AP regime using either Alhydrogel® or CoVaccine HT™ to adjuvant AMA-1 were only moderately and not significantly enhanced by CoVaccine HT™ in comparison to Alhydrogel® [178]. The use of ChAd63 in this study also suggests that the results here with AdHu5 could potentially be extended to other adenovirus vectors, given the concerns surrounding pre-existing immunity to AdHu5 in humans and its use as a clinical vaccine vector [248,266]. Interestingly strong IgG and T cell responses were induced by Abisco®100 in an AP regime, surpassing those obtained with an AM regime which has been optimised for T cell induction.

These results were further supported by comparative assessments of the most potent adjuvants in AP and PPP regimes using leading blood-stage candidate antigens PfMSP-1 and PfEBA-175_F2 (in combination) as well as PvDBP_RII. Strong IgG titres were again induced by adjuvants tested in an AP regime with no significant difference in the fold

change seen in each test group between pre- and post-boost titres. Interestingly IgG titres induced by PPP regimes against PfEBA-175_F2 and PvDBP_RII were of an equal magnitude to those induced following an AP regime. It is likely that the inherent immunogenicity of these proteins, which induced moderate IgG titres in PBS, is accounting for this. Furthermore, the most potent adjuvants from the initial OVA screen were taken forward in order to induce maximal antibody responses, the induction of which are thought to be essential for a successful blood-stage vaccine. In hindsight a group immunised with an aluminium-based adjuvant would have allowed for a more direct comparison of vaccine regimes and a direct replication of results in the OVA system. IgG titres following a PfMSP-1_{19} PPP regime were only induced by Montanide®ISA720 which has been used in a previous study [177] and contrasts to the induction of IgG titres with all adjuvants following an AP regime. MSP-1_{19} has been shown to be refractory to antigen presentation, due to the presence of several disulphide bonds [310], and it is likely that inclusion of the MSP-1_{33} fragment in the AdHu5 vector (not contained in the protein vaccine) accounts for this difference [148,169].

The ability of antibodies induced by vaccination to inhibit the binding of PvDBP_RII to its receptor DARC in an *in vitro* assay was also investigated and high levels of binding inhibition of >84% were achieved with both AP and PPP regimes containing Abisco®100. These levels of binding inhibition were found to correlate with IgG titres and surpassed

levels achieved in previous published studies assessing binding inhibition following vaccination of rhesus macaques with PvDBP_RII in human compatible adjuvants [323]. This is encouraging as naturally acquired high-titre binding inhibitory antibodies against PvDBP_RII have been shown to be associated with reduced risk of *P. vivax* infection as well as lower *P. vivax* parasite densities following infection [289]. Importantly, these vectors have now entered GMP manufacture, with Phase Ia clinical testing scheduled to commence in 2012.

8.2.2 Underlying characteristics of viral vector and protein vaccines

An adenoviral prime was shown to mediate improved boosting of IgG titres by protein-in-adjuvant vaccines throughout this thesis. Investigations into differences in protein dosing and immunisation intervals found that there was no significant enhancement of antibody priming after giving 100µg of OVA versus 20µg at 2-week intervals. Dose de-escalating the AdHu5 OVA prime also did not impact the boostability of IgG titres by protein vaccines. Interestingly, an effect was seen in terms of the immunisation interval for some adjuvants, where a prolonged interval led to an enhancement of responses. This effect has previously been reported for immunisations with viral vectored vaccines and indicates that prolonged prime-boost intervals may allow for optimal development of memory B cells [174,176,406]. This suggests that it is not a larger dose of antigen produced *in vivo* at the time of prime from the adenovirus (compared to a given dose of

protein vaccine), which is priming a quantitatively greater memory B cell response and which may be more effectively boost subsequently by weak adjuvants. Furthermore, priming mice with the potent adjuvant CoVaccineHT™ and boosting them with OVA formulated in the weak adjuvant Adju-Phos® did not lead to enhanced antibody responses indicating that the improved boosting following an adenoviral prime is adjuvant-independent and rather inherent to the adenovirus. This was supported by mixing a nrAdHu5 vector with OVA protein at the time of the prime, which resulted in partially enhanced IgG titres and T cell responses post-boost compared to OVA in PBS. The adjuvantation of protein vaccines through non-recombinant adenoviruses has also previously been reported [304]. However, given a recombinant AdHu5 vaccine is still better at inducing antibody responses, the innate profile induced is not the only governing factor.

Further teasing apart the induction of antibody responses at the prime revealed that the AdHu5 vector expressing the transgene OVA induced a greater number of GC B cells and T_{FH} cells in the draining LN than either MVA or protein/adjuvant vaccines. These responses could account for the superior priming ability of this vector, given GC B cells have been found to be associated with serum IgG titres [125,331]. As cytokines such as IL-6 and IL-21 have been shown to affect the differentiation of T_{FH} cells through the induction of Bcl6 it is possible that the cytokine profile induced by these different

viruses may be mediating a greater induction of T_{FH} which in turn supports more GC cells.

A potential difference in the interaction of protein vaccines and MVA vaccines with CD32b (shown to be involved in secondary recall responses) at the time of the boost was also found, possibly due to the fact that viral vectors may be more reliant on cell-mediated processes for the arrival of antigen in the LN.

Adoptively transferring CD4+ T cells into WT mice pre-prime (to supplement T cell help) in the form of antigen-specific cells or AdHu5 OVA primed CD4+ T cells, did not lead to enhanced IgG titres following OVA protein vaccination, either post-prime or post homologous boost. This was surprising given that CD4+ T cell help is important for the induction of antibody responses to T cell-dependent antigens [259,260,330] but supports data obtained in a study by Duffy *et al.* in which naïve antigen-specific CD4+ T cells were able to help mBCs induce antibody, but not naïve B cells.

8.2.3 Protective efficacy of MSP-1 AP and PPP regimes against a lethal *P. yoelii* blood-stage challenge

In addition to improving antibody immunogenicity, the AP regime also led to improved protective efficacy following a high-dose lethal *P. yoelii* pRBC challenge. At a dose of 1.5µg, there was a significant difference in the IgG titres induced by Adju-Phos® in an AP

and PPP regime, but no difference in IgG titres induced by CoVaccineHT™, further supporting earlier observations that an adenoviral prime can improve the immunogenicity of protein vaccines formulated in relatively weak adjuvants. This protection was shown to correlate with PyMSP-1_{19} specific IgG and IgG2a as has been previously shown [204,362], but not with PyMSP-1_{19} specific IgG1 or antibody avidity, and was not dependent on PyMSP-1_{33} specific CD4+ T cells.

Using 20µg of PyMSP-1_{19} protein, strong IgG titres were induced by both PPP and AP regimes using CoVaccineHT™ and Adju-Phos®. This differs to results seen with OVA, but is likely explained by the inherent immunogenicity of the PyMSP-1_{19} protein preparation used. Previous results with OVA showed that even doses of 100µg of OVA in a PPP regime did not substantially improve antibody immunogenicity. Interestingly, despite the induction of equivalent IgG titres in mice immunised with AP and PPP Adju-Phos®, a difference in survival was seen between these two groups following a 10^6 pRBC challenge. As seen with all other AP regimes tested, a more cytophilic antibody response was again induced, although this was surprisingly found not to correlate with protection.

8.2.4 Adjuvanting viral vectors

CQ and Adju-Phos® both failed to consistently adjuvant cellular and humoral immune responses following vaccination with viral vectors expressing blood-stage malaria

antigens based on MSP-1. Despite results in mice and humans indicating that CQ cover can enhance CD8+ T cells to soluble proteins such as OVA and HBV surface antigen [384,385], an OVA-based protein vaccine in Alhydrogel® given under CQ cover following either an AdHu5 OVA prime (AP regime) or OVA in Alhydrogel® prime (PP regime) failed to increase CD8+ T cells. Prolonged CQ cover also did not adjuvant cellular immune responses to AdHu5 or MVA vaccines expressing secreted soluble PyMSP-1_{42} antigen in single-shot or prime-boost regimes. The adjuvant effect of CQ, ascribed to an inhibition of endosomal acidification in DCs leading to increase cross-presentation, is thought to be antigen dose-dependent. However, the doses of protein used in the preceding experiments are broadly equivalent to those used in studies reporting an enhancement via CQ [384,385]. Given viral vaccine vectors are known to induce strong T cell responses which, for MVA, are efficiently cross-primed [140], it may be that enhancing such pathways further leads to no overall improvement in immune response induction, even when sub-optimal doses are used.

Similarly Adju-Phos® was not found to adjuvant AdHu5, ChAd63 or MVA vaccines consistently despite a report of Adju-Phos® enhancing both the cellular and humoral immunogenicity of an AdHu35-based CSP vaccine [383]. In one experiment, $5x10^8$ vp of AdHu5 PfM128 administered with Adju-Phos® in Tris buffer was found to have significantly enhanced CD8+IFN-γ+ T cells as measured by ICS. This result could not be repeated in other strains of mice or using ChAd63-based vaccines. In the study by

Ophorst *et al.,* an adjuvant effect of Adju-Phos® was seen with an AdHu35 vector. This vector has a different tissue tropism to AdHu5 and ChAd63 [402,403,404], and is inherently less immunogenic than AdHu5, which may account for the differences seen here [402,403,404,405]. Furthermore, co-administration of MVA85A with EPI vaccines formulated with aluminium-based adjuvants have been shown to dampen immune responses to the viral vectored vaccine, possibly due to the Th2 responses induced by this adjuvant [407].

8.3 Future Directions

8.3.1 Further assessment of regimes and underlying vaccine immunogenicity

Antibody responses appear to be more readily primed by an adenoviral vaccine than by a comparative protein vaccine, resulting in the improved boostability of protein vaccines. Although the induction of GC B cells and CD4+ T_{FH} cell help following these vaccines has been investigated, the role of mBCs was not assessed. Serum antibody titres have been shown to correlate with long-lived plasma cells for some antigens [262,311,312], and yet serum antibody titres have also been shown to correlate with memory B cell levels, as measured by a cultured ELISpot assay, for some acute infections and vaccines [262,274,313]. This suggests that the maintenance of serological antibody

titres may be under differential control by these two cell populations. Given viral vectored vaccination regimes, as well as AP regimes, have been shown to induce memory B cells in rhesus macaques [178] and humans (Elias *et al.*, unpublished), the contribution of this cell type to antibody responses cannot be ruled out. It remains possible that a greater induction of memory B cells could account for the better boostability of IgG titres seen here and as shown previously [274]. Attempts to standardise a cultured mBC assay (data not shown) to explore vaccine-induced mBCs in mice was not successful despite the publication by others of such assays [245,261]. The establishment of a cultured mBC assay to assess vaccine-induced murine mBCs remains essential and it is possible that previously published assays, which focus on models of infection, are not suited to study vaccine-induced mBCs, which are likely to be of a low-frequency. An assay recently published investigating vaccine-induced mBCs following DTPa vaccines through the re-stimulation of murine cells with CpG7909 and human rIL-2 could be explored [408].

Further exploring the induction of mBCs would also be interesting in the context of a greater induction of GC B cells following AdHu5 rather than protein vaccination. The fate of naïve follicular B cells has been shown to be associated with the affinity of the BCR for antigen. Naïve B cells with high affinity for antigen lose the ability to form GC B cells and become short-lived PCs. Moderate affinity between the BCR and antigen leads to the induction of GC reactions and the formation of LLPCs whereas lower affinity

interactions lead to the induction of mBCs [409,410,411]. It is possible that AdHu5 vaccines induce antigens of an affinity that steer B cells towards the induction of a larger GC response and/or mBC pool. This could be explored by assessing the avidity of antibodies early on in an immune response through a sodium thiocyanate displacement ELISA. Previously the avidity of antibodies was only assessed post-boost at which point the recall response is likely affecting the avidity of antibody responses.

Greater numbers of GC cells following an adenoviral prime expressing the transgene OVA compared to a non-recombinant adenovirus or protein vaccine also suggests that the production, presentation and processing of antigen (rather than the innate profile alone) from adenovirus vaccines appears to be a crucial determinant in the priming of antibody responses. To formally conclude this a nrAdHu5 could be administered with protein and the induction of GCs assessed.

Similarly the induction of T_{FH} cells following adenoviral vaccines could be further investigated by staining for other described T_{FH} cell markers such as PD-1 [28] and by looking at the cytokines produced by these cells, as T_{FH} cells producing IFN-γ associated with B cells in the LN have been found to induce a more cytophilic antibody response [32]. TLR agonists have also been shown to influence the magnitude of T_{FH} cells and the persistence of GCs [125,270], so studies assessing T_{FH} induction following TLR agonist-containing adjuvants would be also of interest.

Though both protein and adenovirus dosing studies revealed that is it unlikely that antigen dose is accounting for the better priming ability of adenoviral vectors, it would be of interest to more formally conclude this through the use of *in vivo* imaging. Utilising an AdHu5 expressing luciferase as well as luciferase protein-in-adjuvant the biodistribution and expression duration (through an AUC analysis) of these different vaccine platforms, as well as the initial antigenic dose, could be more directly compared.

There may also be a difference in the arrival of antigen in the LN between viral vectored vaccines and protein vaccines. In addition to directly infecting cells such as DCs viral vectors can also traffic to the LNs. Free antigen can also be released from adenovirus-infected cells at the site of injection that are apoptosing or by being actively secreted (if the tPA leader sequence is present). Depending on the nature of arrival in the LN it is likely that different cells are contributing to the establishment of an immune response. To this end the cell infiltrate in the LN could be assessed post-vaccination or different cell types could be isolated post-vaccination with an AdHu5 expressing hen egg lysozyme (HEL) or HEL protein-in-adjuvant and used to present antigen to HEL-transgenic B cells [412]. Furthermore, to assess whether 'free antigen' is essential for priming an antibody responses an adenovirus recombinant for a non-secreted transgene could be utilised and the induction of GCs investigated. However, antigen could still be released from dying cells, as the tissue-tropism of such a virus is not restricted.

8.3.2 Further characterising antibody responses induced by vaccination

Recently efforts have focussed on not only assessing the overall magnitude but also the fine-specificity of the antibody response induced by vaccination. This is of interest for diseases such as malaria and influenza for which highly conserved epitopes, which can be targeted by vaccination, are lacking and for which the induction of cross-strain specific antibodies is necessary. Several studies have investigated the broadening of the immune response through adjuvants [42,45,413,414], with cross-clade protection seen following a H5N1 vaccine in MF59™ attributed to this phenomenon [413]. The analysis of fine-specificity in these studies is usually investigated by looking at the diversity of BCR sequences through isolation of plasma cells and subsequent sequencing of their heavy and light chains. This allows an assessment of their clonality and of their somatic mutation frequency as a surrogate of affinity maturation. However, these studies rely on the isolation of what is assumed to be antigen-specific plasma cells at low frequencies at defined time-points post-vaccination (when these cell types are assumed to be in the blood). Monoclonal antibodies against isolated sequences must be generated, in the absence of a B cell 'tetramer', to assure the original cells represent the antigen-specific population – a labour intensive process [414]. However, this method can allow for the identification of protective epitopes [414]. Alternatively the specificity of antibody responses induced and the association of specific epitopes with protective immunity can also be investigated using peptide arrays or phage display libraries

[42,45,415]. For blood-stage malaria, field studies have also identified epitopes associated with protection [416,417]. Investigation of the induction of these epitopes, in addition to the overall magnitude of responses, is therefore essential. Furthermore, fine-specificity induced between animal models and humans as well as between vaccine platforms is of interest, given the fine-specificity of AMA-1 protein vaccines has been shown to differ between species [418]. The investigation of the impact of both adjuvants and adenoviral priming on the breath of the antibody response remains of interest, although these studies would have to be carefully designed – delineating quantity and quality is a difficult task in immunological studies, especially given both parameters most often vary simultaneously.

8.3.3 *P. vivax* blood-stage malaria vaccines

High-titre antibodies capable of impressive levels of *in vitro* receptor binding inhibition were also generated with AP and PPP regimes targeting the *P. vivax* antigen DBP_RII. This assay assesses the ability of antibodies induced by vaccination to inhibit the binding of PvDBP_RII to its receptor DARC [231,286]. As inhibition titres of >84% were induced following certain AP and PPP regimes, even at a dilution of 1:4000, assessing the $_{IC}50$ of antibodies, as is used for GIA against *P. falciparum* [418], may be a more meaningful output. This would quantify the dilution which gives 50% binding inhibition and allow for a more direct comparison of the binding inhibition obtained with other vaccines [323].

Exploring the binding inhibition and antibody titres against heterologous alleles of PvDBP_RII is also of interest to explore whether this vaccine candidate based on the SalI strain of *P. vivax* can induce cross-reactive antibodies. It is also essential to confirm the antibodies induced through vaccination can bind to native parasite antigen through IFATs against field isolates, which is now underway. Recent advances in culturing *P. vivax in vitro* through the use of concentrated shizont preparations from clinical isolates as well as enriched reticulocytes from cord blood [326] would allow for the assessment of the inhibition of invasion of human erythrocytes by *P. vivax* by test sera, which would provide useful and much-needed functional data.

8.3.4 Mechanisms of vaccine-mediated protection against *P. yoelii*

High-titre antibodies have been shown to correlate with protection for *P. yoelii* [204]. These antibodies are thought to be neutralising and act against merozoite target antigens inhibiting erythrocyte invasion by the parasite. However, antibodies can also act through cell-mediated mechanisms. Interaction of the Fc region of antibodies with Fc receptors on innate immune cells can reportedly inhibit parasite replication through antibody-dependent cellular inhibition (ADCI) by monocytes [419,420] or through antibody-dependent respiratory burst (ADRB) activity mediated by neutrophils [421]. These cell-mediated responses are thought to be mediated by cytophilic antibodies responses although there is some controversy about their contribution to protection

and this may be malaria species / host dependent [368,370,371,372,422]. To explore the role of cytophilic antibody responses, specific "isotype-dominated" serum from mice immunised with high dose PyMSP-1_{19} vaccines in AP and PPP regimes, which have the same total IgG responses yet a skew towards IgG2a in the former, could be transferred into mice to assess protective efficacy. Furthermore, Fc common γ chain KO mice could be directly immunised with these regimes. The γ-chain is common to FcγRI, FcγRIIIa, FcεRI, and FcαRI and is required for signal transduction. Consequently Fc-γ chain -/- mice are unable to mediate ADCC through these receptors [367].

Recently, an ADRB assay has been developed to assess parasite opsonisation and activation of respiratory burst activity by neutrophils. Clinical protection from natural malaria infection in the field has been shown to correlate with neutrophil respiratory burst activity against merozoites opsonised with human sera [421]. Serum from vaccinated mice could also be assessed in this assay to investigate whether protection is associated with this specific Fc-dependent activity of neutrophils. Alternatively neutrophils could be depleted using an anti-granulocyte mAb [423].

Antibody responses were also boosted against PyMSP-1_{19} following *P. yoelii* challenge in AM groups but not in PPP vaccinated mice. Although the number of surviving mice was small, making a formal conclusion difficult, this supports data seen following *P. knowlsei* challenge with an AMA-1 protein vaccine in rhesus macaques [255] and AM regimes in

human Phase IIa trials against PfMSP-1 (S. Draper, personal communication). It is possible that enhanced CD4+ T cells induced by the MVA boost allow for better boosting of MSP-1_{19} responses upon parasite exposure. To assess this CD4+ T cells could be depleted prior to challenge. Alternatively a lower challenge dose or a non-lethal *P. yoelii* challenge could also be used to increase survival in AM groups allowing for a further investigation of this boosting phenomenon.

8.4 Final Remarks

An efficacious vaccine for malaria is still urgently needed. For blood-stage malaria vaccines it is believed that the induction of high-titre antibodies will be necessary to prevent the invasion of RBCs and subsequent clinical disease. Work in this thesis described the utility of AP regimes for the induction of high-titre antibodies and explored the mechanistic basis of these findings. Most importantly, adenoviral vaccines were able to consistently reduce the differential immunogenicity of protein/adjuvant vaccines. These results are highly encouraging, given this approach could enhance the clinical immunogenicity and utility of adjuvants that are traditionally considered poorly immunogenic (but which are readily available), and circumvent the need for more potent and experimental chemical adjuvants that are currently required to deliver candidate protein vaccines for difficult diseases. A Phase Ia clinical trial of a ChAd63 prime – AMA-1-based protein vaccine boost regime (ClinicalTrial.gov ID: NCT01351948)

is currently underway at the Jenner Institute in Oxford to explore whether the increased immunogenicity of AP regimes in pre-clinical models also translates to humans.

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Appendix One