



LIVER-STAGE VACCINES FOR MALARIA

Thesis submitted for the degree of Doctor of Philosophy

Trinity Term 2013

Rhea Longley

Magdalen College

Word count: $\approx$ 50 000 (excluding References, Appendices, Tables and Diagrams)

LIVER-STAGE VACCINES FOR MALARIA

Rhea Longley, Magdalen College, University of Oxford

Thesis submitted for the degree of Doctor of Philosophy, Trinity Term 2013

Abstract

The development of an efficacious *P. falciparum* malaria vaccine remains a top priority. Pre-erythrocytic vaccine efforts have traditionally focussed on two well-known antigens, CSP and TRAP, yet thousands of antigens are expressed throughout the liver-stage. The work described in this thesis aimed to assess the ability of other pre-erythrocytic antigens to induce an immune response and provide protective efficacy against transgenic parasites in a mouse model. Research undertaken in our laboratory has demonstrated the ability of prime-boost viral vectored sub-unit vaccination regimens to elicit high levels of antigen-specific T cells. Eight candidate antigens were therefore expressed individually in the viral vectors ChAd63 and MVA. Two antigens, PflSA1 and PflSAP2, were identified that confer greater protective efficacy in inbred mice than either CSP or TRAP. PflSA1 was also able to induce almost complete sterile efficacy in outbred mice, suggesting this vaccine should be assessed in a clinical trial. Immune responses to the candidate antigens were also assessed in human volunteers following their first exposure to controlled malaria infection. The antigen TRAP was further characterised by epitope mapping in volunteers vaccinated with ChAd63-MVA ME-TRAP. However, no functional T cell assay exists to measure inhibition of *P. falciparum* liver-stage parasites. An improved murine *in vitro* T cell killing assay was developed, and preliminary experiments were conducted that demonstrate the potential and promise of a *P. falciparum* T cell killing assay. Such assays will not only allow mechanistic studies to be undertaken, but could also change the way we screen pre-clinical liver-stage vaccines.

Acknowledgments

First and foremost, thank you to my three fantastic supervisors Dr. Alex Spencer, Dr. Katie Ewer and Prof. Adrian Hill. Together you have given me so much guidance and encouragement over the past three years. You have challenged me, extended my knowledge, and acted as great role models for my future in science. This thesis would not have been possible without your assistance and excellent supervision.

Thank you to all members of the Jenner Institute; in particular the technical help of Matt Cottingham, Karolis Bauza, Julie Furze, Andrew Worth, Andrew Williams, Sara Zakutansky, Vera Unwin, Sumi Biswas, Rebecca Hillson, and all members of the Vector Core. Thanks also to Viv Clark, Heather Gray and all staff at the FGF, as well as Karla Watson at the RRI. A huge thanks also goes to Jono and the team at IMSU for saving my thesis on multiple occasions.

Special thanks to fellow DPhil students Caitlin Mullarkey, David Llewellyn, Katharine Collins, Magali Matsumiya and Sean Elias, as well as Carly Bliss and Louisa Rose, my time at the Jenner would not have been the same without you and I am very lucky to have gained such wonderful friends. Thanks in particular to Kat and Cait for help during the writing process.

I would also like to thank Dr. Valerie Soulard and Prof. Dominique Mazier for welcoming me into their laboratory. My month in Paris was definitely a highlight of my DPhil and I'm very grateful to have been afforded the opportunity. Thanks also to our collaborators at Leiden, Ahmed Salman, Dr. Chris Janse and Dr. Shahid Khan,

for the generation of transgenic parasites, which proved crucial for a number of experiments.

Thank you to the Rhodes Trust, particularly former Warden Don Markwell and Mary Eaton, for enabling me to study at Oxford and providing a wealth of support, knowledge and entertainment.

Finally, I would like to thank my friends and family in Oxford and Australia for their constant emotional support and belief in my capabilities, including Kira and Tom for not only reading my thesis but also helping when my mac crashed in the final days. Last, but definitely not least, the biggest thanks to my fiancé Tim, who not only has put up with all of my demands but has also read each chapter at least five times.

Table of Contents

Abstract.....	II
Acknowledgments	III
Table of Contents.....	V
Table of Figures	IX
List of Tables	XIII
List of Abbreviations	XIV
Papers published from these studies	XVIII
1 Introduction.....	2
1.1 Malaria.....	2
1.1.1 The need for a malaria vaccine	3
1.1.2 <i>Plasmodium</i> life-cycle	5
1.1.3 Malaria pathogenesis	9
1.2 Naturally acquired immunity to malaria.....	10
1.2.1 The innate immune system	11
1.2.2 The adaptive immune system	13
1.3 Vaccination strategies for malaria	19
1.3.1 Whole sporozoite vaccination strategies	19
1.3.2 Sub-unit vaccination strategies	22
1.4 Current status of malaria vaccine development.....	27
1.4.1 Pre-erythrocytic vaccines.....	28
1.4.2 Blood-stage vaccines	33
1.4.3 Transmission-blocking vaccines.....	34
1.4.4 Multi-component vaccines.....	36
1.4.5 Immune correlates of protection	36
1.5 Thesis aims and outlines	38
1.5.1 Aims.....	39
1.5.2 Outline	40
2 Materials and Methods	42
2.1 Materials	42
2.1.1 Reagents.....	42
2.1.2 Solutions and buffers	50
2.2 Molecular biology and cloning	53
2.2.1 Antigen inserts	53
2.2.2 Polymerase chain reaction (PCR).....	55
2.2.3 Agarose gel electrophoresis	57
2.2.4 Restriction endonuclease digests	57
2.2.5 DNA ligation	58
2.2.6 Bacterial transformation	58
2.2.7 Plasmid DNA preparation.....	59

2.2.8	Restriction cloning into ChAd63	59
2.2.9	Restriction cloning into MVA	62
2.2.10	Generation of protein lysate.....	62
2.2.11	Genotyping of HHD mice.....	67
2.3	Virology	69
2.3.1	Adenovirus transfection and purification	70
2.3.2	MVA transfection and purification.....	70
2.4	Animals and immunisations	71
2.4.1	Mice	71
2.4.2	Immunisations and injections	72
2.4.3	Vaccines	72
2.5	Collection and preparation of cells and sera	73
2.5.1	Isolation of splenocytes	73
2.5.2	Isolation of peripheral blood mononuclear cells (PBMCs)	73
2.5.3	Isolation of liver mononuclear cells	74
2.5.4	Isolation and culture of primary murine hepatocytes	74
2.5.5	Collection of mouse sera	75
2.6	Immunological assays.....	75
2.6.1	Peptides.....	75
2.6.2	Intracellular cytokine staining (ICS)	77
2.6.3	Mouse <i>ex vivo</i> spleen IFN γ enzyme-linked immunosorbent spot (ELISpot) assay	80
2.6.4	Isolation and adoptive transfer of CD4 ⁺ and CD8 ⁺ T cells	81
2.6.5	Enrichment of CD8 ⁺ T cells.....	82
2.6.6	<i>In vivo</i> CD4 ⁺ or CD8 ⁺ T cell depletion.....	83
2.6.7	Luminescence immunoprecipitation system (LIPS).....	84
2.6.8	Enzyme-linked immunosorbent assay (ELISA)	86
2.6.9	Whole IgG passive transfer	87
2.7	Parasitology	88
2.7.1	Parasite strains	88
2.7.2	Preparation of thin blood smears	89
2.7.3	Sporozoite production (<i>P. berghei</i>)	89
2.7.4	Cryopreservation of parasitized RBCs	91
2.7.5	Sporozoite challenge.....	92
2.7.6	<i>In vivo</i> imaging using the IVIS system.....	92
2.7.7	Immunofluorescence antibody test (IFAT)	93
2.7.8	Murine <i>in vitro</i> T cell killing assay.....	93
2.8	Clinical trials.....	95
2.8.1	Overview of ME-TRAP clinical trials	96
2.8.2	Overview of Vac49 clinical trial objectives and study plan	97
2.8.3	Isolation of human PBMCs, plasma and serum.....	97
2.8.4	Freezing and thawing of PBMCs.....	98
2.8.5	Sorting of CD8 ⁺ and CD4 ⁺ T cells	99

2.8.6	Human <i>ex vivo</i> IFN γ PBMC ELISpot assay.....	100
2.8.7	Human cultured IFN γ PBMC ELISpot assay.....	101
2.8.8	Human <i>in vitro</i> T cell killing assay.....	102
2.9	Statistical analysis.....	104
3	Selection and design of candidate liver-stage antigens for a <i>Plasmodium falciparum</i> vaccine.....	106
3.1	Introduction.....	106
3.2	Results.....	109
3.2.1	Identification of candidate liver-stage antigens for a malaria vaccine .	109
3.2.2	Generation of ChAd63 and MVA molecular constructs	112
3.2.3	T cell immunogenicity of ChAd63 and MVA Pf[antigen] vaccines	116
3.3	Discussion.....	122
4	Assessment of immunogenicity and efficacy of eight candidate <i>Plasmodium falciparum</i> vaccines in mice	128
4.1	Introduction.....	128
4.2	Results.....	131
4.2.1	Cellular immunogenicity of the candidate vaccines delivered in a heterologous prime-boost regimen	131
4.2.2	CD4 ⁺ and CD8 ⁺ cytokine responses induced by the candidate vaccines	134
4.2.3	Antibody responses induced by the candidate vaccines	140
4.2.4	Assessment of protective efficacy of PfCelTOS, PFI0580c and PfUIS3 against heterologous <i>P. berghei</i> challenge	147
4.2.5	Assessment of protective efficacy of the eight <i>P. falciparum</i> candidate vaccines using transgenic <i>P. berghei</i> sporozoites expressing the cognate <i>P. falciparum</i> antigen	152
4.3	Discussion.....	162
5	Assessment of PfUIS3, PflSA1 and PflSAP2 as candidates for a liver-stage malaria vaccine	171
5.1	Introduction.....	171
5.2	Results.....	175
5.2.1	Further assessment of ChAd63-MVA PfUIS3	175
5.2.2	Further assessment of ChAd63-MVA PflSA1	194
5.2.3	Further assessment of ChAd63-MVA PflSAP2	205
5.2.4	Comparison of the protective efficacy induced by PfUIS3, PflSA1 and PflSAP2 vaccination and assessment of competition between vaccines	216
5.3	Discussion.....	220

6	Cellular immune responses to <i>Plasmodium falciparum</i> antigens in human volunteers.....	229
6.1	Introduction.....	229
6.2	Results.....	233
6.2.1	Epitope mapping of <i>P. falciparum</i> TRAP in volunteer 012	233
6.2.2	Confirmation of identified epitopes in further volunteers	239
6.2.3	Identification and re-assessment of minimal epitopes in <i>P. falciparum</i> T9/96 TRAP	241
6.2.4	HLA restriction of identified epitopes	246
6.2.5	Assessment of cellular responses to multiple <i>P. falciparum</i> antigens in volunteers experimentally challenged with <i>P. falciparum</i> malaria	249
6.2.6	Assessment of summed responses to multiple <i>P. falciparum</i> antigens over time	253
6.2.7	Responses to the <i>P. falciparum</i> pre-erythrocytic antigen Exp1	255
6.2.8	Assessment of cellular memory responses to multiple <i>P. falciparum</i> antigens in volunteers experimentally challenged with <i>P. falciparum</i> malaria	257
6.3	Discussion.....	259
7	Development of an <i>in vitro</i> T cell killing assay for the liver-stage of malaria ...	266
7.1	Introduction.....	266
7.2	Results.....	270
7.2.1	Development of the infrastructure for an <i>in vitro</i> T cell killing assay..	270
7.2.2	Infection of the hepatoma cell lines with <i>P. berghei</i> GFP	274
7.2.3	The effect of cytokines and naïve T cells on <i>P. berghei</i> GFP infected Hepa1-6 cells	278
7.2.4	Assessment of inhibition of <i>P. berghei</i> GFP infected Hepa1-6 cells by <i>P. berghei</i> TRAP-specific splenocytes	281
7.2.5	Development of a human <i>in vitro</i> T cell killing assay	287
7.3	Discussion.....	295
8	Discussion.....	305
8.1	Overview.....	305
8.2	Conclusions and future directions	307
8.2.1	Comparative assessment of pre-erythrocytic vaccines and identification of novel candidates	307
8.2.2	T cell-mediated killing of liver-stage parasites	313
8.2.3	Naturally acquired immunity versus vaccine-induced immunity	315
8.2.4	Identifying correlates of protection	316
8.2.5	PfLSA1 as a vaccine candidate.....	317
8.3	Final remarks	318
	References.....	321
	Appendices	368

Table of Figures

Figure 1.1. Percentage of population at risk from malaria in 2011.	3
Figure 1.2. <i>Plasmodium falciparum</i> life-cycle.	6
Figure 1.3. The interaction of liver-stage parasites with the host immune system. ...	17
Figure 2.1 Cloning scheme for insertion of liver-stage malaria antigens into the viral vectors ChAd63 and MVA.	61
Figure 2.2. Cloning scheme to generate plasmids containing the liver-stage malaria antigens upstream of the <i>Renilla</i> luciferase tag.	64
Figure 2.3. HHD (HLA-A2 transgenic) mice elicit an immune response to the HLA-A2-restricted Influenza A epitope.	69
Figure 2.4. Example of the ICS gating strategy used in all experiments.....	79
Figure 2.5. Schematic representation of the LIPS assay.	85
Figure 2.6. Relative antibody measurements calculated by LIPS correlate significantly with the same samples measured by ELISA.....	86
Figure 2.7. Comparison of different experimental protocols for the production of sporozoites.	91
Figure 3.1. Schematic representation of the expression of the candidate antigens throughout the parasite's life-cycle.	111
Figure 3.2. T cell immunogenicity of ChAd63-[antigen] and MVA-[antigen] vaccines.....	119
Figure 3.3. T cell immunogenicity to predicted epitopes of the candidate antigens.	121
Figure 4.1. Cellular immunogenicity of the eight candidate <i>P. falciparum</i> vaccines administered in a prime-boost eight-week interval regimen.	133
Figure 4.2. CD8 ⁺ and CD4 ⁺ cytokine responses in BALB/c mice in the blood following heterologous prime-boost vaccination with the <i>P. falciparum</i> candidate liver-stage antigens.....	135
Figure 4.3. CD8 ⁺ and CD4 ⁺ cytokine responses in BALB/c mice in the spleen following heterologous prime-boost vaccination with the <i>P. falciparum</i> candidate liver-stage antigens.....	137
Figure 4.4. CD8 ⁺ and CD4 ⁺ cytokine responses in C57BL/6 mice in the spleen following heterologous prime-boost vaccination with the <i>P. falciparum</i> candidate liver-stage antigens.....	139
Figure 4.5. Assessment of antibody responses in BALB/c mice following heterologous prime-boost vaccination with the <i>P. falciparum</i> candidate liver-stage antigens.....	142
Figure 4.6. Assessment of antibody responses in C57BL/6 mice following heterologous prime-boost vaccination with the <i>P. falciparum</i> candidate liver-stage antigens.....	144
Figure 4.7. Fold change in the antibody level from background to two weeks post-MVA boost in BALB/c and C57BL/6 mice.	146
Figure 4.8. Heterologous challenge with <i>P. berghei</i> sporozoites in C57BL/6 and BALB/c mice vaccinated with ChAd63-MVA PfCelTOS.....	149

Figure 4.9. Heterologous challenge with <i>P. berghei</i> sporozoites in BALB/c mice vaccinated with ChAd63-MVA PFI0580c.	150
Figure 4.10. Heterologous challenge with <i>P. berghei</i> sporozoites in BALB/c mice vaccinated with ChAd63-MVA PfUIS3.	151
Figure 4.11. Protective efficacy after ChAd63-MVA vaccination with the <i>P. falciparum</i> candidate antigens and challenge with transgenic <i>P. berghei</i> sporozoites expressing the cognate <i>P. falciparum</i> antigen.	153
Figure 4.12. Protective efficacy after ChAd63-MVA vaccination with <i>P. falciparum</i> CSP or TRAP and challenge with transgenic <i>P. berghei</i> sporozoites expressing the cognate <i>P. falciparum</i> antigen.	156
Figure 4.13. Median delay in time to 1% parasitaemia following challenge with transgenic <i>P. berghei</i> expressing the cognate <i>P. falciparum</i> antigen in mice vaccinated with ChAd63-MVA Pf[antigen].	159
Figure 5.1. Confirmation of protection in BALB/c mice induced by PfUIS3 vaccination.	177
Figure 5.2. The time to 1% parasitaemia correlates with the bioluminescence of transgenic PbPfUIS3 liver-stage parasites.	178
Figure 5.3. Depletion of CD8 ⁺ T cells abolishes the protection induced by ChAd63-MVA PfUIS3 vaccination in BALB/c mice.	180
Figure 5.4. Adoptive transfer of splenocytes from ChAd63-MVA PfUIS3 vaccinated mice to naïve mice does not provide the same level of protection from sporozoite challenge as direct vaccination.	182
Figure 5.5. Polyfunctionality of CD8 ⁺ T cells induced by ChAd63-MVA PfUIS3 vaccination in BALB/c mice.	184
Figure 5.6. ChAd63-MVA PfUIS3 vaccination induces protection against sporozoite challenge in C57BL/6 mice.	186
Figure 5.7. ChAd63-MVA PfUIS3 vaccination does not induce protection against sporozoite challenge in CD-1 outbred mice.	188
Figure 5.8. Epitope mapping of PfUIS3 in BALB/c, C57BL/6 and HHD mice.	190
Figure 5.9. PfUIS3-specific cells were observed in both the liver and spleen of mice after ChAd63-MVA vaccination.	192
Figure 5.10. PfUIS3 vaccination induces predominantly antigen-specific cells of the effector memory subset.	193
Figure 5.11. Confirmation of pre-erythrocytic protection in BALB/c mice induced by PfLSA1 vaccination.	195
Figure 5.12. The time to 1% parasitaemia correlates with the bioluminescence of transgenic PbPfLSA1 liver-stage parasites.	196
Figure 5.13. The percentage of PfLSA1-specific CD8 ⁺ T cells producing IFN γ in the blood does not correlate with the time to 1% parasitaemia after sporozoite challenge.	197
Figure 5.14. Polyfunctionality of CD8 ⁺ T cells induced by ChAd63-MVA PfLSA1 vaccination in BALB/c mice.	198

Figure 5.15. Depletion of CD8 ⁺ T cells abolishes the protection induced by ChAd63-MVA PflSA1 vaccination in BALB/c mice.....	200
Figure 5.16. ChAd63-MVA PflSA1 vaccination does not induce protection against sporozoite challenge in C57BL/6 mice.....	201
Figure 5.17. ChAd63-MVA PflSA1 vaccination induces protection against sporozoite challenge in CD-1 outbred mice.	202
Figure 5.18. Epitope mapping of PflSA1 in BALB/c mice.....	203
Figure 5.19. ChAd63-MVA PflSA1 vaccination in BALB/c mice induces a low magnitude antigen-specific cellular response in the liver.....	204
Figure 5.20. Confirmation of protection in BALB/c mice induced by PflSAP2 vaccination.....	206
Figure 5.21. The time to 1% parasitaemia correlates weakly with the bioluminescence of transgenic PbPflSAP2 liver-stage parasites.	208
Figure 5.22. Polyfunctionality of CD8 ⁺ T cells induced by ChAd63-MVA PflSAP2 vaccination in BALB/c mice.	210
Figure 5.23. ChAd63-MVA PflSAP2 vaccination does not induce protection against sporozoite challenge in C57BL/6 mice.....	211
Figure 5.24. Epitope mapping of PflSAP2 in BALB/c, C57BL/6 and HHD mice.	213
Figure 5.25. ChAd63-MVA PflSAP2 vaccination induces an antigen-specific cellular response in the liver.	215
Figure 5.26. Vaccination with combinations of PfUIS3 and PflSAP2 with ME-TRAP, or with each other, does not result in reduced cellular immunogenicity in C57BL/6 mice compared to each vaccine given alone.....	218
Figure 5.27. Vaccination with both PflSA1 and TRIP does not result in reduced cellular immunogenicity in BALB/c mice compared to vaccination with either alone.....	219
Figure 6.1. Epitope mapping of <i>P. falciparum</i> T9/96 TRAP peptides 1-30 (aa1-310) in eighteen volunteers vaccinated with ChAd63-MVA ME-TRAP.....	232
Figure 6.2. Epitope mapping of <i>P. falciparum</i> T9/96 TRAP peptides 1-30 (aa1-310) in volunteer 012.	235
Figure 6.3. Minimal epitope mapping of the six dominant peptides identified for volunteer 012.	236
Figure 6.4. Classification of CD4 ⁺ or CD8 ⁺ dependent responses for the epitopes identified in volunteer 012.....	237
Figure 6.5. Confirmation of epitopes within <i>P. falciparum</i> T9/96 TRAP peptides 1-30 (aa1-310).....	240
Figure 6.6. Confirmation of epitopes within the <i>P. falciparum</i> T9/96 TRAP peptides 2 and 12.....	242
Figure 6.7. Minimal epitope mapping for the <i>P. falciparum</i> T9/96 TRAP peptides 2 and 24.....	242
Figure 6.8. Identification of epitopes within the <i>P. falciparum</i> T9/96 TRAP peptides 6, 13, 15 and 16.....	244

Figure 6.9. <i>P. falciparum</i> T9/96 TRAP epitope mapping in HLA-A3 volunteers vaccinated with ChAd63-MVA ME-TRAP, compared to previously obtained data from non-HLA-A3 vaccinated volunteers.	248
Figure 6.10. Cellular immune responses to sixteen malaria antigens in volunteers challenged with <i>P. falciparum</i> by injection.	251
Figure 6.11. Summed responses to sixteen malaria antigens in volunteers challenged with <i>P. falciparum</i> by injection.	252
Figure 6.12. Combined cellular responses from volunteers challenged with three different doses of sporozoites indicates there are no significant differences between time-points post-challenge and no correlation between the magnitude of the response and the day of diagnosis with malaria.	254
Figure 6.13. Cellular immune responses to the pre-erythrocytic antigen PfExp1 in volunteers challenged with <i>P. falciparum</i> sporozoites by injection.	256
Figure 6.14. Cellular memory responses to six pre-erythrocytic antigens in volunteers challenged with <i>P. falciparum</i> sporozoites by injection.	258
Figure 7.1. Infection of the hepatoma cell lines Hepa1-6 and HepG2 with serial dilutions of AdHu5-GFP.	272
Figure 7.2. Effect on AdHu5-GFP infected Hepa1-6 and HepG2 cells by addition of GFP-specific splenocytes.	273
Figure 7.3. <i>P. berghei</i> GFP infection in the hepatoma cell line Hepa1-6.	274
Figure 7.4. An example of the gating strategy used to calculate the percentage of <i>P. berghei</i> GFP infected hepatoma cells.	275
Figure 7.5. Infection of Hepa1-6 and HepG2 cells with dilutions of <i>P. berghei</i> GFP sporozoites.	276
Figure 7.6. Infectivity of Hepa1-6 cells with 40 000 <i>P. berghei</i> sporozoites with or without centrifugation.	277
Figure 7.7. Effect of IFN γ and TNF α on <i>P. berghei</i> GFP infected Hepa1-6 cells.	278
Figure 7.8. Effect of splenocytes from naïve mice on <i>P. berghei</i> GFP infected Hepa1-6 cells.	279
Figure 7.9. Effect of <i>P. berghei</i> GFP infection of Hepa1-6 cells on MHC Class II expression.	280
Figure 7.10 A schematic representation of the murine <i>in vitro</i> T cell killing assay.	281
Figure 7.11. Cellular immunogenicity induced by ChAd63-MVA PbTRAP vaccination in C57BL/6 mice.	282
Figure 7.12. CD8 ⁺ enriched splenocytes from PbTRAP vaccinated mice inhibit <i>P. berghei</i> GFP infected Hepa1-6 cells in a dose dependent manner.	284
Figure 7.13. Assessment of the mechanism of inhibition observed by PbTRAP-specific splenocytes on <i>P. berghei</i> GFP infected Hepa1-6 cells.	286
Figure 7.14. Inhibition of <i>P. falciparum</i> infected primary human hepatocytes by the addition of PBMCs from volunteers vaccinated with ME-TRAP.	294

List of Tables

Table 2.1. Commercially available reagents used throughout this thesis.....	42
Table 2.2. Commercially available antibodies used throughout this thesis.....	49
Table 2.3. Primers used to isolate the liver-stage malaria antigen sequences from the entry vectors.....	65
Table 2.4. Primers used to genotype HHD mice.	68
Table 2.5. <i>P. falciparum</i> T9/96 TRAP 20mer peptides used to generate 9mers.	77
Table 3.1. List of identified <i>P. falciparum</i> candidate antigens and the rationale for their inclusion.	110
Table 3.2. Sequence construct design for the selected <i>P. falciparum</i> candidate antigens.	115
Table 3.3. Predicted T cell epitopes in the <i>P. falciparum</i> candidate antigens.....	116
Table 3.4. The final eight successfully immunogenic <i>P. falciparum</i> liver-stage vaccine candidates.	126
Table 4.1. Sterile protection from transgenic <i>P. berghei</i> sporozoite challenge after vaccination with the eight <i>P. falciparum</i> candidate antigens: comparison with PfCSP or PfTRAP vaccination.	157
Table 4.2. Summary of the immunogenicity and protective efficacy of the eight candidate <i>P. falciparum</i> vaccines in BALB/c mice.....	161
Table 5.1. Comparison of protective efficacy induced by PfUIS3, PfLSA1 and PfLSAP2 in three different strains of mice.....	216
Table 6.1. Summary of minimal epitope mapping in volunteer 012.	238
Table 6.2. Summary of the dominant minimal epitopes identified.	245
Table 6.3. Identified peptides, minimal epitopes and potential HLA restrictions....	247
Table 7.1. Assessment of the best method for measurement of green (GFP) or red (mCherry) fluorescence.	272
Table 7.2. Screening of 22 volunteers vaccinated with ME-TRAP for high cultured IFN γ ELISpot responses.....	289
Table 8.1. Summary of immunogenicity and efficacy of the protective vaccines identified in this thesis.	309

List of Abbreviations

aa	Amino acid
ACK	Ammonium Chloride Potassium
AdHu	Human adenovirus
AMA1	Apical membrane antigen 1
ANN	Artificial neural network
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen-presenting cells
ATCC	American Type Culture Collection
Av	Average
β2M	Beta-2-microglobulin
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BGH	Bovine growth hormone
BSA	Bovine serum albumin
C+1	Challenge plus one day
CBF	Clinical bio-manufacturing facility
CD	Cluster of differentiation
CEF	Chicken embryo fibroblasts
CellTOS	Cell-traversal protein for ookinetes and sporozoites
ChAd63	Chimpanzee adenovirus serotype 63
CHMI	Controlled human malaria infection
CMV	Cytomegalovirus
conA	Concanamycin A
CPE	Cytopathic effect
CR1	Complement receptor 1
CSP	Circumsporozoite protein
CTL	Cytotoxic lymphocytes
CytoF	Cytometry by time-of-flight
D	Day
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
dH ₂ O	Deionised water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
D-PBS	Dulbecco's Phosphate Buffered Saline
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
EMP1	Erythrocyte membrane protein 1
EP	Electroporation
ER	Endoplasmic reticulum
E:T	Effector to target ratio
Exp1	Exported protein 1
FACS	Fluorescence activated cell sorting

FCS	Fetal calf serum
FGF	Functional genetics facility
FITC	Fluorescein isothiocyanate
FP	Fowlpox virus
GAP	Genetically attenuated parasites
GFP	Green fluorescent protein
GIA	Growth inhibitory activity
GMP	Good manufacturing practice
GPI	Glycosylphosphatidylinositol
GSK	GlaxoSmithKline
HBsAg	Hepatitis B surface antigen
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP70	Heat-shock protein 70
HSPGS	Heparan sulfate proteoglycans
ICAM	Intracellular adhesion molecule
ICS	Intracellular cytokine staining
i.d.	Intradermal
IEDB	Immune epitope database
IFAT	Immunofluorescence antibody test
IFN γ	Interferon-gamma
ifu	Infectious units
Ig	Immunoglobulin
IL	Interleukin
ILSDA	Inhibition of liver-stage development assay
i.m.	Intramuscular
i.p.	Intraperitoneal
IRS	Indoor-residual spraying
ISI	Inhibition of sporozoite invasion
ispz	Irradiated sporozoites
ITN	Insecticide treated bed-nets
i.v.	Intravenous
LB	Luria Bertani
L-FABP	Liver-fatty acid binding protein
LIPS	Luminescence immunoprecipitation system
LN ₂	Liquid nitrogen
LSA	Liver-stage antigen
LSAP	Liver-stage associated protein
LSECs	Liver sinusoidal endothelial cells
LU	Light units
M1	Matrix protein 1
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
malERA	Malaria eradication research agenda
ME	Multi-epitope string
MEM	Minimum Essential Medium Eagle
MIF	Migration inhibitory factor

MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MSP	Merozoite surface protein
MVA	Modified vaccinia virus Ankara
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NK	Natural killer cell
NOD	Non-obese diabetic
NP	Nucleoprotein
OD	Optical density
PABA	P-amino benzoic acid
Pb	<i>Plasmodium berghei</i>
PbPf[antigen]	Transgenic <i>P. berghei</i> expressing the <i>P. falciparum</i> antigen
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline/Tween
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pf	<i>P. falciparum</i>
pfu	Plaque forming units
PHA/SEB	Phytohemagglutinin/Staphylococcal enterotoxin B
Phz	Phenylhydrazine
pNPP	<i>Para</i> -Nitrophenylphosphate
pRBC	Parasitized red blood cell
PRRs	Pattern recognition receptors
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
Py	<i>P. yoelii</i>
RBC	Red blood cell
RESA	Ring-infected erythrocyte surface antigen
RH5	Reticulocyte binding protein homolog 5
RFP	Red fluorescent protein
SAP1	Sporozoite asparagine-rich protein 1
s.c.	Sub-cutaneous
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFC	Spot forming cells
SFU	Spot forming units
SOC	Super optimal broth
STARP	Sporozoite threonine and asparagine-rich protein
TAE	Tris-acetate-EDTA
TD	Transient dominant
TK	Thymidine kinase
TLR	Toll-like receptor
T _m	Melting point
TNF α	Tumor necrosis factor-alpha

tPA	Tissue plasminogen activator
TRAP	Thrombospondin-related adhesion protein
tt1%	Time to 1% parasitaemia
UIS3	Upregulated in sporozoites
VC	Vector control
VIMT	Vaccines that interrupt malaria transmission
vp	Viral particles
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

Papers published from these studies

The following papers include work presented in this thesis:

Sheehy SH, Spencer AJ, Douglas AD, Sim BK, **Longley RJ**, Edwards NJ, Poulton ID, Kimani D, Williams AR, Anagnostou NA, Roberts R, Kerridge S, Voysey M, James ER, Billingsley PF, Gunasekera A, Lawrie AM, Hoffman SL, Hill AV. (2013). *Optimising Controlled Human Malaria Infection studies using cryopreserved P. falciparum parasites administered by needle and syringe*. PLoS One. 8(6); e65960

Ewer KJ, O'Hara GA, Duncan JA, Collins KA, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD, **Longley RJ**, Rowland R, Poulton ID, Draper SJ, Blagborough AM, Berrie E, Moyle S, Williams N, Siani L, Folgori A, Colloca S, Sinden RE, Lawrie AM, Cortese R, Gilbert SC, Nicosia A, Hill AVS. (2013). *Protective CD8⁺ T cell Immunity to Human Malaria Induced by Chimpanzee Adenovirus-MVA Immunisation*. Nature Communications. 4: 2836.

Chapter 1: Introduction

1 Introduction

1.1 Malaria

Malaria is one of the most problematic infectious diseases affecting the world today. The disease is caused by the parasite *Plasmodium* and transmitted by female *Anopheles* mosquitoes. Five species of *Plasmodium* are known to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. The World Health Organization (WHO) estimated there were between 154-289 million cases of malaria in 2010, with 660 000 to 836 000 deaths [1]. However, there is uncertainty in these measures [2, 3], and efforts need to be made to improve diagnostic testing and surveillance in malaria endemic countries. Whilst there is increasing recognition of non-falciparum malaria [4-8], *P. falciparum* still accounted for 90% of the estimated cases in 2010 [1], and the greatest burden is still within sub-Saharan Africa (Figure 1.1). Apart from the tremendous level of morbidity and mortality associated with malaria, the disease also places an extreme economic [9-12] and social burden [9, 13] on endemic countries.

Current effective preventative measures include insecticide-treated bed-nets (ITN) and indoor-residual spraying (IRS) [14], and their reported use has been increasing [1]. Chemoprophylaxis is still used for non-immune individuals travelling to malaria endemic areas, and whilst intermittent preventive therapy can be effective for high-risk groups living in endemic areas (such as pregnant women and infants, see [15]), the high cost, increased risk of drug resistance, short half-life of most malaria drugs and the potential delay in development of naturally acquired immunity has prevented its widespread use. Treatment of malaria has traditionally been with anti-malaria

drugs such as quinine, chloroquine and primaquine (the only known licensed drug to eliminate hypnozoites [16]), but more recently, the first line of defense has been artemisinin-based combination therapies due to resistance of parasites to essentially all anti-malarial drugs given alone [17-19]. Unfortunately, even primaquine cannot be used in much of South East Asia due to high frequencies of glucose-6-phosphate dehydrogenase deficiency, and the associated haemolysis in these patients when given primaquine [20].

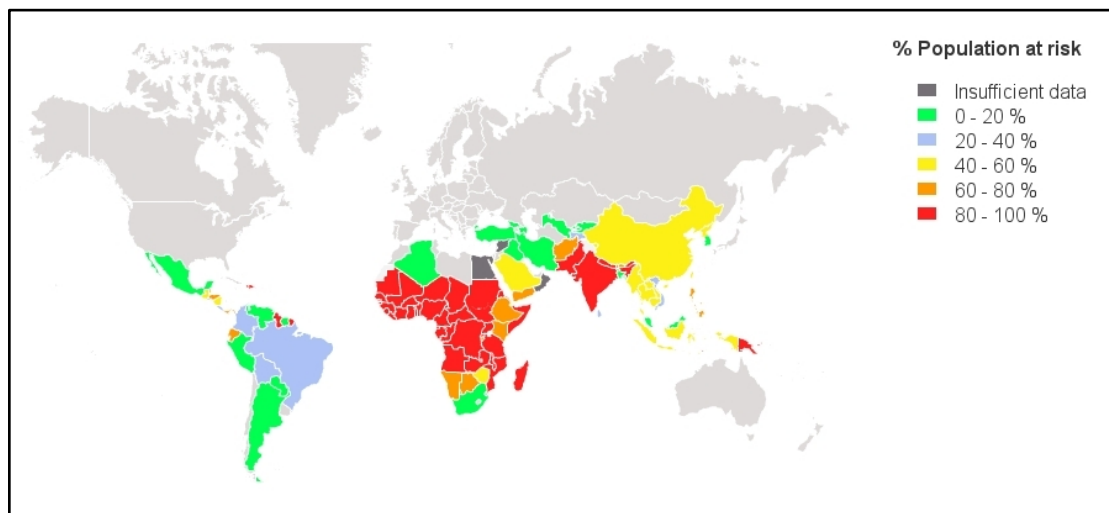


Figure 1.1. Percentage of population at risk from malaria in 2011.

The percentage of population at risk from malaria, created using the Global Malaria Mapper, based on the WHO World Malaria Report 2012. The Global Malaria Mapper was created by the Malaria for Medicines Venture and the WHO Global Malaria Program. © 2013 Global Malaria Mapper.

1.1.1 The need for a malaria vaccine

The past ten to fifteen years saw a huge rise in the recognition of malaria as a major global health problem, largely through public campaigns by the Roll Back Malaria Partnership and the United Nations' Millennium Development Goals. These campaigns helped to significantly increase funding into malaria research and enabled

the widespread use of ITNs. However, whilst there were reports of reduction in malaria admissions and hospital deaths since these major preventative measures have been established [21, 22], there were also conflicting reports of increased admissions in other geographical areas [23], and causality has been difficult to establish. Furthermore, there have been recent reports of resistance of *P. falciparum* to artemisinin in Western Cambodia [24-28], the same location where both chloroquine and sulfadoxine-pyrimethamine resistance evolved [29, 30]. In addition, reports of resistance of mosquitoes to pyrethroids (the only class of insecticide approved for use in ITN) are also increasing [31, 32]. These reports have led to widespread agreement that a vaccine for malaria is urgently needed [33], with campaigns shifting their focus from control of malaria through preventative measures to eradication of malaria by vaccination. The goal of the Malaria Vaccine Technology Roadmap is to have a first generation licensed vaccine by 2015 that is greater than 50% effective and lasts at least a year, and this is also supported by the Malaria Eradication Research Agenda (malERA [34]). However, others suggest that due to the impact preventative measures have had on reducing the burden of disease, a vaccine needs to provide greater than 70% efficacy in order to be deployable and feasible [35, 36].

Vaccines are one of the most cost effective tools, and have been used in the prevention and control of many diseases, including small pox [37], polio [38], measles [39] and *Haemophilus influenzae* type B [40]. Development of a vaccine against the *Plasmodium* parasite has proved more difficult, largely because of its highly complex life-cycle and the parasite's immune evasion mechanisms. Furthermore, the pressure exerted by the human immune system has selected for extensive polymorphism in the immunodominant antigens expressed throughout the

life-cycle [41]. No vaccines against parasitic diseases are currently licensed for use in humans [42], but some have been licensed for parasitic livestock infections (e.g. poultry coccidiosis [43] and bovine babesiosis [44]). Additionally, protection against malaria in humans was first demonstrated in the 1970s through vaccination with irradiated parasites delivered by mosquito bite [45-49]. Whilst such an approach has not proved appropriate for deployment so far, though recent results are encouraging (see section 1.3.1 below) [50], it has demonstrated that a vaccine against malaria is an achievable goal.

1.1.2 *Plasmodium* life-cycle

This thesis focuses on *P. falciparum*, as it currently causes the highest levels of morbidity and mortality. Unfortunately, *P. falciparum* does not infect small animal models, raising difficulties in pre-clinical studies of malaria. However, a number of rodent malaria parasites have been established that are capable of infecting murine hosts: *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*. None of these murine parasites are able to completely mimic the infection and pathogenesis seen in humans. Both *P. berghei* and *P. yoelii* have traditionally been used in assessment of pre-clinical vaccination efficacy (e.g. [51-55]). However, *P. berghei* sporozoite production was better established and more commonly used in our laboratory, and hence was chosen as the murine model in this thesis to allow comparison with previous studies. Furthermore, *P. berghei* is more amenable to transfection procedures used in the generation of transgenic parasites. Whilst the life-cycles of all *Plasmodium* parasites are very similar, that of *P. falciparum* is specifically outlined below.

1.1.2.1 *Plasmodium falciparum* life-cycle

The life-cycle of *P. falciparum* can be divided into three main stages (Figure 1.2), as outlined below.

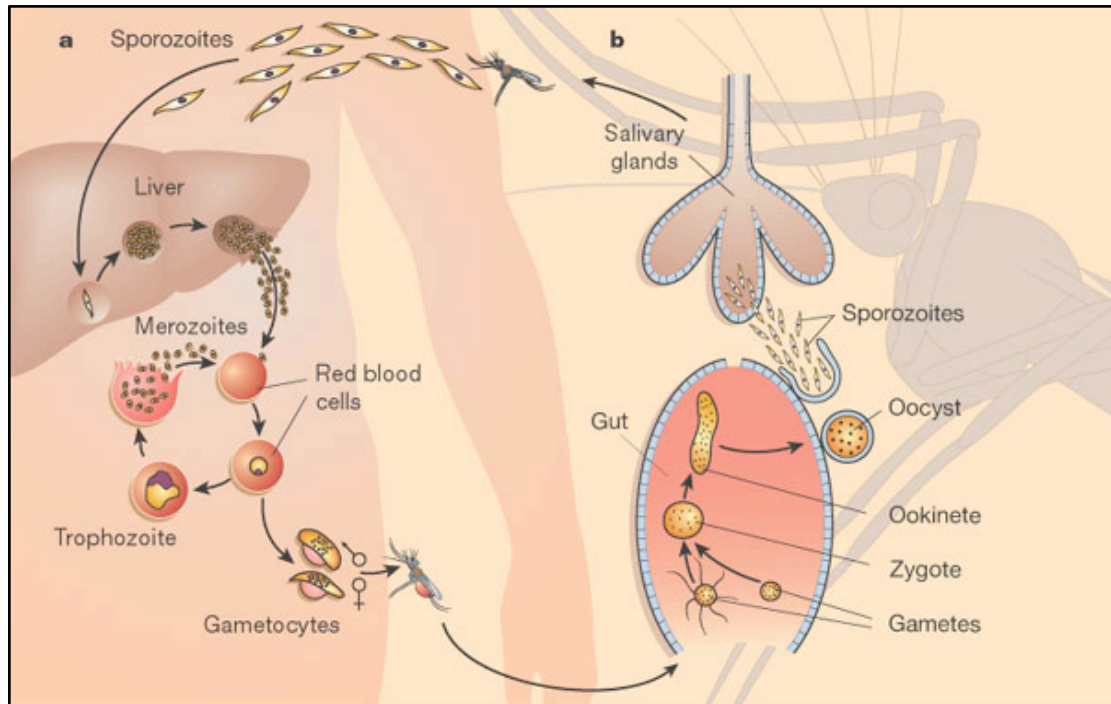


Figure 1.2. *Plasmodium falciparum* life-cycle.

Taken from [56]. The *P. falciparum* cycle in (a) the human host and (b) the *Anopheles* mosquito vector.

Pre-erythrocytic stage: The *P. falciparum* pre-erythrocytic stage has been difficult to study in humans due to the lack of a simple culture system replicating this stage *in vitro*; hence, most of what is known about this stage is extrapolated from findings in experimental murine models, as referenced below. The pre-erythrocytic stage begins when an infected *Anopheles* mosquito injects up to a few hundred sporozoites [57-59] into the skin of a human host. The majority of sporozoites then migrate quickly to the liver, likely taking between fifteen minutes to one hour [60, 61], whilst some remain in the skin or drain to lymph nodes and interact with the host immune system [62,

63]. Once in the liver, sporozoites migrate through the Kupffer (liver-resident macrophages) and liver sinusoidal endothelial cells (LSECs) to enter the space of Disse [64], then traverse several hepatocytes before settling in one cell for liver-stage development [65]. The liver-stage of *P. falciparum* lasts for approximately seven days, during which the sporozoites undergo asexual replication within a parasitophorous vacuole (PV) to yield tens of thousands of merozoites. At the end of hepatic development, the merozoites leave the liver cells in small vesicles called merosomes and enter the bloodstream [66]. The pre-erythrocytic stage of the life-cycle is asymptomatic.

Erythrocytic stage: Merozoites released from the liver invade uninfected red blood cells (RBCs). Inside the RBC, the merozoite undergoes asexual replication forming first a ring-stage trophozoite, followed by a mature trophozoite and eventually an erythrocytic schizont. Once the schizont ruptures the host RBC, it releases approximately twenty new merozoites capable of infecting other RBCs [67], beginning the cyclic nature of the erythrocytic stage (48 hours for *P. falciparum*). The erythrocytic stage of the life-cycle is responsible for all pathology associated with malaria.

Sexual stage: During the erythrocytic stage, some merozoites differentiate into gametocytes, initiating the sexual stage of the parasite's life-cycle. A mosquito feeding on an infected human host can then ingest the gametocytes, which fuse and fertilise to produce zygotes that imbed in the mosquito gut [68]. This zygote then forms a motile ookinete, which imbeds in the mid-gut wall and forms an oocyst. Sporozoites are formed within the oocyst, known as sporogony, and are eventually

released upon rupture of the oocyst. These sporozoites migrate to the salivary glands, ready to be injected into the next host. The mosquito remains infective for one to two months.

1.1.2.2 *Plasmodium berghei* as a pre-clinical model

The life-cycles of murine malaria species such as *P. berghei* are very similar to that of *P. falciparum*, with the major difference being the duration of the liver-stage. For all murine malaria species the liver-stage is much shorter than that of human species; for *P. berghei*, the liver-stage lasts approximately 48 to 52 hours. *P. berghei* has been used extensively as a model for *P. falciparum*, both to elucidate biological mechanisms that can then be confirmed in human parasite species and to assess vaccine candidates *in vivo*.

A major technological advancement in recent years has been the development of efficient transfection and targeted integration of DNA into the *Plasmodium* genome [69, 70], allowing, for example, the generation of fluorescent parasites [71, 72]. Since these early studies, transgenic murine malaria parasites that express specific *P. falciparum* antigens have now been developed, allowing research into biological functions of these antigens *in vivo* [73] in addition to assessment of *P. falciparum* (or *P. vivax*) vaccines in mice [74-79]. Transgenic parasites can be generated by either allelic replacement of the *P. berghei* gene with the *P. falciparum* homolog [80], or integration of the *P. falciparum* gene at a disposable point [81, 82] in the *P. berghei* genome by double homologous recombination. Such technology is paving the way for advancements in pre-clinical screening of *P. falciparum* vaccine constructs in a murine model.

1.1.3 Malaria pathogenesis

The outcome of a malarial infection can range from asymptomatic to fatal; the extent of the illness depends not only on the *Plasmodium* species but also on host factors, such as pre-existing immunity, age and genetics. Common symptoms include bouts of fever, headache, fatigue, nausea and vomiting that coincide with the rupture of infected erythrocytes, whilst severe malaria can lead to coma (cerebral malaria), anaemia and respiratory distress (due to metabolic acidosis). Many of these pathologies may not only be induced by host-parasite interactions, but also due to the reaction of the host immune system (immunopathology). Furthermore, the disease can also be immunosuppressive, leading to a greater risk of concurrent infections [83], which can contribute to both morbidity and mortality.

Disease due to host-parasite interactions is largely caused by the presence of knobs on parasitized RBCs (pRBCs), which are protrusions formed by adhesive proteins (e.g. PfEMP1 [84, 85]). These proteins allow adhesion to vessel walls, to each other or to pRBCs (known as rosetting [86, 87]), any of which can lead to sequestration in organs and the ultimate dysfunction of those organs [88, 89]. A number of host receptors have been implicated in binding the parasite adhesive proteins, including ICAM-1, CD36, CD31, CR1 and E-selectin [86, 90-93]. Sequestration is believed to provide a more suitable microaerophilic venous environment, as well as a defense mechanism allowing evasion of elimination in the spleen [94]. During the blood-stage, the spleen is the main organ involved in generating an immune response and eliminating pRBCs [95-97]. Activation of the vascular endothelium has also increasingly been recognized as an important host-parasite interaction contributing to severe malaria [98]. Activation can be caused by the release of pro-inflammatory

cytokines, mentioned below, or due to material released by pRBCs, and results in perfusion abnormalities and coagulation.

Immunopathology generally results from two separate functions: (1) increased splenic immune function removing both infected and uninfected RBCs (which can contribute to malarial anaemia) [99-101] and (2) the production of pro-inflammatory cytokines in response to schizont rupture in the blood, which can lead to fever and other pathological effects [102, 103]. The production of cytokines, such as tumour necrosis factor alpha (TNF α) and interferon gamma (IFN γ), has also been known to inhibit erythropoiesis [104] thereby contributing to malarial anaemia. Macrophages have also been implicated in malarial anemia, both by phagocytosing infected RBCs and through the release of MIF (migration inhibitory factor), which has also been shown to suppress erythropoiesis in a murine model [105].

1.2 Naturally acquired immunity to malaria

The host immune response to malarial infection is complex and not yet fully understood. To develop new strategies against malaria, a more thorough understanding of the immune response in endemic areas needs to be developed. Epidemiological evidence suggests naturally acquired immunity to malaria is slow to develop and requires exposure to multiple bouts of infection [106-108]. Immunity to death or severe disease, however, is quickly acquired and, evidence suggests, can be gained after one exposure to malaria [109]. There is little evidence that naturally acquired immunity is ever sterilizing, with adults in endemic regions still undergoing bouts of infection yet remaining asymptomatic [106, 110], which perhaps reflects disease tolerance [111]. Furthermore, despite pre-existing immunity, pregnant women become highly susceptible to life-threatening infection [112]. Whilst naturally

acquired immunity is likely anti-parasitic and reduces the load of the infection, it may also involve a regulatory response that reduces the clinical symptoms [113-115].

Most naturally acquired immunity is directed at the erythrocytic stage, with little evidence of naturally acquired immunity to the pre-erythrocytic stage [116, 117]. Research suggests this could be because parasite numbers are low prior to multiplication in the liver, and possibly because natural immune ligands are hidden from pattern recognition receptors (PRRs), leading to a minimal innate response [118]. Alternatively, it could be because the liver is an important organ, and as such, is programmed to minimize inflammation (and hence the innate system is not activated) [119]. This is also due to the fact that the liver receives blood coming from the gut, and hence if there was not an immune tolerant environment the liver would be in a constant state of inflammation [120]. It is important to understand naturally acquired immunity in order to better inform our vaccine efforts, yet this does not necessarily mean a vaccine should mimic natural infection – particularly given such immunity is non-sterilizing. Hence, the discussion below is based upon naturally acquired immunity observed to the blood-stage but also highlights how an immune response to the pre-erythrocytic stage could be an important component of an effective vaccine.

1.2.1 The innate immune system

The innate immune system is the first line of immune defense once the barrier system has been breached (e.g. the skin). This defense is very rapid and broadly specific, not only acting to control infections but also to activate and engage the adaptive immune system [121]. Innate immune recognition is mediated by PRRs, such as toll-like receptors (TLRs), and each receptor has broad specificities for general microorganism

characteristics [122]. The innate system includes both the complement system (with roles such as opsonization, triggering inflammation and induction of antibodies) and innate immune cells, such as natural killer (NK) cells and phagocytic cells (e.g. neutrophils, macrophages and dendritic cells (DCs)).

Whether an innate immune response is mounted against sporozoites is currently unknown [116], however, there are data to suggest a weak response is generated which the parasite is able to evade. The evidence supporting an interaction between the innate immune system and sporozoites is two-fold: first, DCs are able to capture sporozoites that remain in the lymphatic system [62]; second, there is evidence in murine models of an innate cellular infiltrate in the liver surrounding infected hepatocytes [123-125]. This infiltrate is not seen when heat-killed sporozoites are injected [124, 126], and a greater infiltrate is seen in BALB/c mice than C57BL/6 mice (which are more susceptible to malaria) [127]. Furthermore, the difference in virulence between *P. yoelii* (17X non-lethal) and *P. berghei* has been correlated with the innate cell infiltrate in the liver [124].

However, there is overriding evidence that the sporozoite can evade detection or action from the innate immune system. First, there is rapid migration of sporozoites to the liver [60, 61], leaving little time for the parasites to activate the innate system. Second, evidence has shown that sporozoites, likely through the major surface protein (circumsporozoite protein, CSP), can suppress Kupffer cell activation [116, 128]. Third, there is also evidence that the liver-stage blocks pro-inflammatory cytokine production, either through CSP indirectly interfering with the NF- κ B signaling pathway [129] or through the production of heme oxygenase-1 [123]. Finally, it has

also been demonstrated that liver-stage parasites inhibit host cell apoptosis [125, 130, 131], increasing the time available for development of merozoites.

Whilst the blood-stage of the parasite life-cycle has been well studied in nearly all regards (e.g. natural adaptive immunity and biology), our knowledge of the role of the innate immune system in recognizing *Plasmodium* blood-stages is still limited. Research has demonstrated that the innate system responds quickly to blood-stage infection by inducing a high amount of pro-inflammatory cytokines [132, 133], which contributes not only to the first line of defense but also to immunopathology. It is likely that many molecular host-parasite interactions instigate this response, however, the relevance of each is not yet known. Both the glycosylphosphatidylinositol (GPI) anchors on the parasite and the by-product haemozoin have been suggested as targets of recognition by innate cells, through PRRs such as TLR2, 4 and 9 [134-138]. Consequently, TLR ligands have more recently been exploited as adjuvants or targets for blood-stage vaccines (e.g. GPI [139], CpG DNA [140-142] and haemozoin [143]).

1.2.2 The adaptive immune system

The innate system can also activate the adaptive immune system. The adaptive system delivers specific, long-lasting immunity that is capable of memory and is the target of vaccination strategies. This system is composed of two interrelated components: cellular and humoral immunity. Cellular immunity is carried out by T lymphocytes that recognize antigen presented by antigen-presenting cells (APCs) on major histocompatibility molecules (MHC), in the presence of other co-stimulatory signals. T lymphocytes are defined by the surface expression of the markers CD4 and CD8, and the co-receptor CD3. CD4⁺ T cells primarily act through the recruitment

and activation of other cell types, and through the release of cytokines. CD4⁺ T cells are further divided into a number of subsets, including Th1, Th2, Th17 and T regulatory cells. CD8⁺ T cells are capable of directly killing infected target cells through the release of cytokines or cytotoxic granules. B cells producing antibodies generate humoral immunity. The functions of antibodies include neutralization, opsonization and activation of complement, as well as antibody-dependent cellular cytotoxicity (through NK cells). In malaria, antibodies can inhibit the migration, invasion and traversal activity of sporozoites, as well as inhibiting invasion of merozoites into RBCs and the subsequent intracellular growth.

1.2.2.1 Antigen presentation

In general, CD4⁺ T cells recognize exogenous antigen presented by APCs on MHC Class II molecules, whilst CD8⁺ T cells recognize endogenous antigen, processed and presented by APCs on MHC Class I, through the T cell receptor [144]. However, CD8⁺ T cells can also recognize exogenous antigen through cross-presentation onto MHC Class I molecules [145, 146]. In addition, endogenous peptides can also be presented on MHC Class II molecules when processed by autophagy [147]. The process of MHC Class I antigen presentation can be simplified into six steps: (1) receive peptide or protein, (2) tag for destruction by ubiquitylation, (3) proteolysis, (4) transport to the endoplasmic reticulum (ER), (5) bind to MHC Class I in the ER, and (6) transport of the peptide-MHC Class I complex to the cell surface [148]. MHC Class II molecules are also synthesized in the ER, but then delivered by vesicular transport to endolysosomal compartments that supply exogenous peptide antigens. After peptide loading, the MHC Class II complex is also transported to the cell surface. MHC Class II expression is restricted to professional APCs, whilst MHC Class I can be expressed on nearly all nucleated cells. B cells also process exogenous

antigen: the antigen is internalized (along with the B cell receptor), degraded and presented on the cell surface in association with MHC Class II. The peptide-MHC Class II complex is recognized by antigen-specific CD4⁺ helper T cells. This process stimulates the helper cells to secrete cytokines that in turn cause the B cells to proliferate and their progeny to differentiate into antibody-secreting cells [149].

Interestingly, the liver is an autonomous priming site for naïve CD8⁺ T cells and does not solely require professional APCs, as a number of cell types can present antigen. These include hepatic DCs, hepatocytes themselves, stellate cells, LSECs and Kupffer cells (essentially acting as professional intrahepatic APCs) [68, 120, 150, 151]. Most of these cell types express MHC Class I and II, as well as co-stimulatory molecules. However, hepatocytes themselves normally only express MHC Class I [120].

1.2.2.2 Cellular immunity against malaria

There is little scientific evidence that adaptive cellular immunity truly contributes to naturally acquired immunity against malaria in humans. Research suggests this is likely because of the small liver-load, similar evasion mechanisms as those for innate immunity and perhaps due to impairment of the liver-stage by concurrent blood-stage infections, as has recently been demonstrated in a murine model [152, 153]. Only a few associations between cellular immune responses to the liver-stage and protection from malaria have been identified. These include: cytokine responses and cytotoxic cells directed against the protein liver-stage antigen 1 (LSA1) [154-157]; memory responses against the thrombospondin-related adhesion protein (TRAP, also known as SSP2) [158]; and one account of CD4⁺ T cells directed against the CSP [159].

However, cell-mediated immunity has been consistently demonstrated as a major factor in vaccine-induced protection in both humans and animal models [160-164], and some of the aforementioned reasons make the pre-erythrocytic stage an excellent target, particularly since the extremely low numbers of parasites essentially represent a bottle-neck in the population. Whilst T cell mechanistic studies are technically challenging on human samples, murine research has shown that T cells can recognize parasite-derived peptides on the surface of hepatocytes [55, 165-168] (lymphocytes and hepatocytes can both project extensions through the fenestrations of LSECs [169]). These T cells could either be primed in the liver [170, 171], or primed in draining lymph nodes and the spleen, followed by homing to the liver [63]. The liver does contain populations of liver-resident lymphocytes, with a greater proportion of CD8⁺ to CD4⁺ T cells (the opposite to the blood) [172]. A simplified view of the liver-stage and its interaction with cells of the immune system is shown (Figure 1.3).

There is some evidence of a cell-mediated response to the blood-stage of infection in naturally acquired immunity [159, 173-176]. For example, Riley *et al.*, found a strong correlation between both antibody and lymphoproliferative responses to the blood-stage antigen merozoite surface protein 1 (MSP1) and resistance to severe fever in children in West Africa [176]. Immunity after experimental infection with parasitized RBCs was characterized by an absence of detectable antibodies, but the presence of a proliferative T cell response [175]. However, naturally acquired immunity directed towards the blood-stage has more often been associated with antibody-mediated protection.

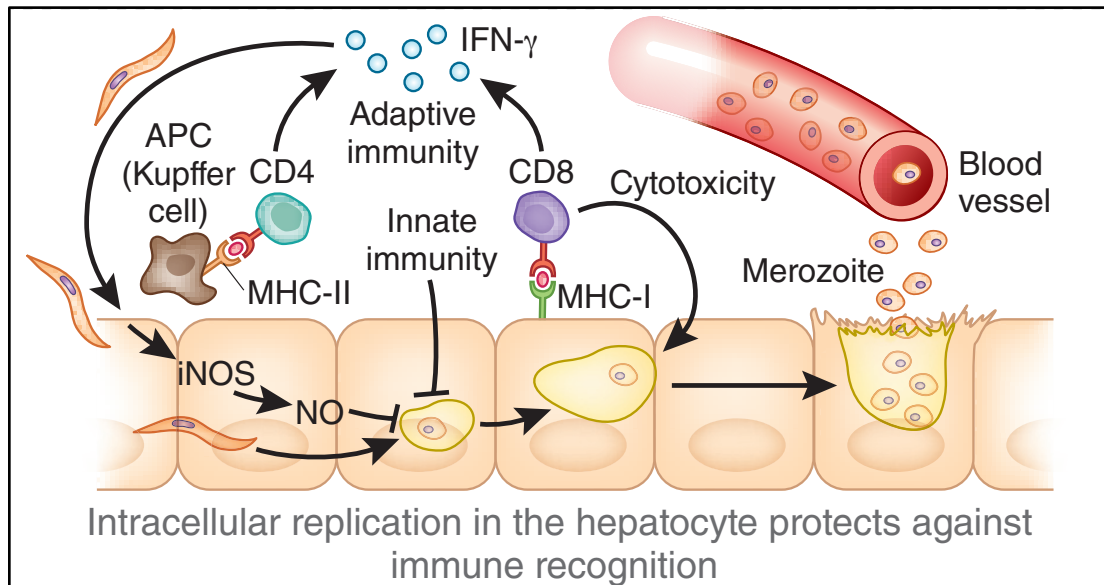


Figure 1.3. The interaction of liver-stage parasites with the host immune system.

Adapted from [118]. Sporozoites migrate from the skin to the liver, where they travel through LSECs and Kupffer cells and traverse multiple hepatocytes before beginning intracellular replication. This stage is not only clinically silent, but also considered to be ‘hidden’ from the innate immune system. However, hepatocytes can act as APCs and present peptide, in association with MHC Class I, to CD8⁺ T cells. CD8⁺ T cells are then able to inhibit liver-stage parasites, but the exact mechanism is not known. Inhibition is likely through a combination of both IFN γ and direct cytotoxicity of CD8⁺ cells. Any infected hepatocytes that are not inhibited will lead to the release of merozoites into the blood stream.

1.2.2.3 Humoral immunity against malaria

Antibody-mediated immunity has a known contribution to naturally acquired immunity at the blood-stage and, at a lesser extent, to the pre-erythrocytic stage. The most widely studied sporozoite antigen, CSP, can induce antibodies and high antibody levels have been associated with a decreased risk of malaria infection in Kenya [177, 178]. However, studies of the contribution of antibody-mediated immunity at the pre-erythrocytic stage are limited in natural infection. In a vaccine setting, the leading vaccine RTS,S (see section 1.4.1) is primarily protective through inhibition of sporozoite invasion into the liver, by inducing antibodies against CSP

[179]. However, there is only a short amount of time available for sporozoites and the immune system to interact, which may account for the sub-optimal efficacy induced by RTS,S. Nevertheless, both antibody and cell-mediated approaches for pre-erythrocytic vaccines are being actively pursued.

Antibody-mediated immunity against blood-stage malaria essentially encompasses what is classified as ‘naturally acquired immunity to malaria’. A pivotal study in 1961 demonstrated that transferring serum from malaria immune adults to children could protect against malarial infection, and was independently repeated thirty years later [180, 181]. Since then, multiple studies have continually demonstrated the protective effect of antibodies, directed at the blood-stage, in naturally acquired immunity [182-190]. However, the protective association is dependent on the antigen studied [191], and evidence in favour of particular antigens is often contradicted in other studies. Antibody-mediated effector functions include the inhibition of RBC invasion, neutralization, opsonization and antibody-dependent cellular inhibition [192]. Despite the knowledge that antibodies are responsible for naturally acquired immunity and their known inhibition of blood-stage parasites, an antibody-mediated vaccine against blood-stage malaria has not reached the same level of efficacy as demonstrated for pre-erythrocytic vaccines [193-195]. This is likely due in part to the limited understanding of the targets and the exact effector mechanism of the antibodies, the very high numbers of parasitized RBCs, the short transit time for merozoites between RBCs and because naturally acquired immunity is never sterilizing.

1.3 Vaccination strategies for malaria

Traditionally, most vaccines have been made from live, attenuated or killed organisms [196]. However, in the early 1980s a recombinant protein vaccine was developed for hepatitis B, based on the hepatitis B surface antigen (HBsAg) [197], paving the way for the development of a number of sub-unit vaccines that are now licensed, including those against anthrax [198] and human papillomavirus [199]. Whilst sub-unit vaccines represent a simpler strategy for malaria vaccination in terms of manufacture, only whole sporozoite vaccination regimens have demonstrated sterile protection from malaria infection. These different vaccination strategies for malaria are discussed below.

1.3.1 Whole sporozoite vaccination strategies

Whole sporozoite vaccination strategies first began in the early 1940s in chickens [200], followed in the late 1960s/early 1970s in both murine models and humans by the demonstration of complete sterile protection after delivery of irradiated sporozoites by mosquito bite [45-49, 53]. Irradiated sporozoites are capable of invading hepatocytes but development is arrested, providing a repertoire of antigens for the immune system to respond against whilst not producing a blood-stage (symptomatic) infection [201]. Protection by irradiated sporozoites is dependent upon CD8⁺T cells targeting the liver-stage [164, 165, 202-204]. However, over 1000 bites from mosquitoes were required to provide protection in these models, limiting the utility of this approach. Nevertheless, the biotechnology company Sanaria set out to make this a plausible vaccination strategy [205]. The company was able to successfully develop a technique to cryopreserve sporozoites, enabling such a vaccine to be delivered by injection rather than mosquito bite, and greatly increasing the feasibility of this approach. Recent studies have shown complete protection in six

volunteers when five doses of 1.35×10^5 irradiated sporozoites were delivered by intravenous injection [50], though poor efficacy when delivered by subcutaneous or intradermal injection (presumably because of reduced efficiency or viability resulting in substantially reduced numbers of sporozoites making it to the liver) [206]. Whilst these recent results are promising (despite small sample sizes), deployment of a vaccine that requires storage in liquid nitrogen (LN₂), intravenous administration and five doses will be very difficult in malaria endemic countries. Furthermore, not all the naïve controls were infected in this study, indicating it was a weak challenge, and heterologous strain protection has not yet been assessed.

Following the success of the irradiated sporozoite approach, another whole sporozoite vaccination strategy was developed: genetically attenuated parasites (GAPs). Rather than irradiating sporozoites, key antigens are genetically deleted or mutated resulting in the attenuation of the parasite in the liver-stage [52, 207-209]. To date, this strategy has only been tested in mice, but it has a suggested advantage over irradiated sporozoites because proteins expressed at the late liver-stage can be targeted, increasing the time parasites are present in the liver and thereby allowing a broader immune response to develop [210]. GAPs have shown comparable efficacy to irradiated sporozoites, and protection appears to be CD8⁺ T cell dependent [168, 211]. However, the main risk of this strategy is the potential for breakthrough infections and the parasite reaching the blood-stage, leading to disease as opposed to protection [212]. The difficulty will be in transferring this model from the pre-clinical stage to a regulated clinical product [213, 214]. Additionally, chemical attenuation of sporozoites has also demonstrated sterile protection in mice [215, 216].

A similar strategy, whilst not necessarily a vaccine, is the experimental infection of humans with sporozoites while undergoing anti-malarial drug treatment. Roestenberg *et al.*, infected ten volunteers under cover of the blood-stage acting drug chloroquine [217] with *P. falciparum* malaria, through the bites of twelve to fifteen infected mosquitoes once a month for three months [218]. All ten volunteers were protected from subsequent malaria challenge after chloroquine was cleared, and efficacy was associated with cellular immunity. Protective efficacy largely remained when the same volunteers were challenged two years later [219]. This trial was based on early murine studies showing protective efficacy after this regimen was comparable to that seen by irradiated sporozoites [220]. That so few mosquito bites were needed to provide sterile protection is surprising, but suggests that as the parasites progress until the blood-stage more immune targets are revealed (as for late liver-stage GAPs). A second study recently confirmed these findings and demonstrated convincingly that protection was due to pre-erythrocytic, rather than blood-stage, immunity [221]. The difficulty in this strategy will be ensuring that the anti-malarial drugs are taken in parallel to infection, and hence there is a risk of breakthrough infections, as for GAPs.

Interestingly, similar whole-parasite strategies have also been tested for efficacy against blood-stage malaria. Many examples exist in murine models (reviewed in [222]), and in 2002 Pombo *et al.*, infected human volunteers with low-levels of *P. falciparum* infected RBCs and found they were subsequently protected from further challenge with malaria [175]. However, it is unlikely this will become a clinically accepted vaccination strategy for malaria, given the small risk of infection in donated blood and the need to grow large numbers of blood-stage parasites [223].

1.3.2 Sub-unit vaccination strategies

The sterile efficacy of irradiated sporozoite vaccines demonstrated the feasibility of developing a pre-erythrocytic vaccine and, in particular, a vaccine inducing CD8⁺ T cells. Sub-unit vaccination regimens hence became the next generation in development. The difficulty has been choosing the protein to target in such a vaccine, given over 5000 genes are expressed by the parasite [224]. Efforts have traditionally focused on CSP, given it was one of the first antigens identified and was originally thought to mediate protection of irradiated sporozoite vaccines [225, 226]. However, a number of other antigens have also been assessed as vaccine candidates in clinical trials, both at the pre-erythrocytic stage (e.g. TRAP, LSA1, Exp1) and the blood-stage (e.g. MSP1, AMA1) [227]. The focus of this thesis is on pre-erythrocytic vaccines, and hence, candidate liver-stage antigens are discussed in more detail throughout the coming chapters. The following paragraphs introduce the various vaccination platforms that can be used to deliver malaria antigens in a sub-unit regimen, whilst section 1.4 discusses the current status of malaria vaccine development.

1.3.2.1 Protein-in-adjuvant

Protein vaccines are generally delivered with an adjuvant in order to increase the immunogenicity of the protein. Adjuvants essentially trigger danger signals that strongly activate the immune system. Adjuvants include aluminum salts, emulsions, liposomes and virosomes [228], and have been shown to greatly increase antibody responses to vaccine antigens. However, disadvantages include the difficulty in producing correctly folded proteins [229] (a requirement for B cell receptor antigen presentation), along with the need for multiple immunisations to induce a protective level of antibodies. Furthermore, protein-in-adjuvant vaccines are generally poorly

immunogenic for CD8⁺ T cells in humans, as has been shown in both the RTS,S vaccine [179, 230, 231] and a recombinant protein PflSA1 vaccine [232]. However, there is ongoing development of adjuvants that do increase cellular immunogenicity, for example TLR agonists [233, 234], immunostimulatory complexes [235, 236] and molecular adjuvants [237, 238]. Given the requirement of a cell-mediated response to protect during the liver-stage of malaria infection, protein-in-adjuvant platforms are currently not ideal for liver-stage targeted vaccines.

1.3.2.2 DNA

DNA and viral vectored approaches both work by delivering gene sequences rather than actual proteins. After being delivered into a host cell, the gene sequence is then transcribed and translated, leading to expression of the antigen within the cell. As an internal antigen, this method activates the endogenous antigen-processing pathway, leading to expression of the antigen on the cell surface in complex with MHC Class I molecules. For this reason, both methods are better at inducing CD8⁺-mediated immunogenicity than protein vaccines. The first malaria DNA vaccine tested was *P. yoelii* CSP; vaccination in mice resulted in strong CD8⁺-mediated immunogenicity and efficacy against homologous challenge [239, 240]. However, it quickly became clear that DNA vaccines were better at producing strong immune responses in mice than in humans [241-244]. Further research suggested the potential of DNA vaccines lay in a prime-boost regimen, with greater levels of immunogenicity reached when DNA was combined with a viral vectored approach [163, 245-250]. The prime-boost regimen is discussed in section 1.3.2.4. In summary, DNA vaccines are simple and stable, can be rapidly produced and can include multiple epitopes [251, 252], yet the weak immune response in humans has focused research on the prime-boost approach and viral vectored vaccines. Notably, a recent strategy using DNA vaccines combined

with *in vivo* electroporation (EP, to increase cellular uptake of the vaccine) produced cellular immunogenicity comparable to that induced by viral vectors in our laboratory [253]. EP has also previously been demonstrated to increase immunogenicity of DNA vaccines [254, 255], as have other strategies such as increasing the time between sequential vaccinations and the use of adjuvants.

1.3.2.3 Viral vectors

Viral vectors act in the same manner as DNA vaccines, by infecting cells and hence inducing a MHC Class I-dependent response. Viral vectors have so far shown an incredible ability to induce robust cellular immune responses [160], greater than seen for other vaccine platforms. The virus itself is also able to act as an adjuvant, not only a delivery system, enhancing the specific response [228]. Two major concerns have surrounded the use of viral vectors as vaccine platforms: first, the issue of safety, and second, the issue of pre-existing immunity. These concerns have been largely addressed, with the common viral vectors (poxvirus and adenovirus) displaying an excellent safety profile [160, 194, 245, 256-260], the pre-existing immunity being overcome by the use of rarer serotypes or simian adenoviruses [261, 262], or with the time between sequential vaccinations being increased in the case of poxviruses [263]. Poxviral and adenoviral vectors were utilized in this thesis and are discussed in more detail below, however work is also ongoing in the research community on the use of the lentivirus [264], measles virus [265], flavivirus [266] and cytomegalovirus (CMV) [267] as vaccine vectors.

Poxvirus vectors used in malaria vaccination strategies include modified vaccinia virus Ankara (MVA), a highly attenuated vaccinia denoted NYVAC and the fowlpox (FP) virus. A general feature of these viruses is that each can accommodate the

insertion of large pieces of foreign DNA. The MVA vector, used in this thesis, was derived by serial passaging of the chorioallantois vaccinia virus Ankara over 500 times in chicken embryo fibroblast (CEF) cells, resulting in a virus that was no longer replication competent [268]. Through this process, the MVA virus lost over fifteen percent of its genome [269], allowing accommodation of up to 25kb of foreign DNA. The MVA vector has now been used as a vaccine platform for a number of diseases, including the human immunodeficiency virus (HIV) [270, 271], hepatitis C [272], cancer [273] and tuberculosis [274, 275] in addition to malaria. In relation to malaria vaccines, the MVA vector was able to induce greater immunogenicity than DNA [276], however, prime-boost regimens elicited greater immunogenicity than either used alone [246]. The NYVAC vector [277] was used to express seven *P. falciparum* genes simultaneously, and in a clinical trial one of 35 vaccinated volunteers was protected from controlled human malaria infection (CHMI) [278]. Again, these authors moved to using a prime-boost regimen rather than the NYVAC vector alone, principally because they also wanted to induce antibodies. The fowlpox vector FP9 [279] has been tested extensively in Oxford in prime-boost regimens; it was found to act as a better prime vaccination than DNA, when followed by an MVA boost [276, 280]. In a CHMI trial, the FP9-MVA regimen was able to induce sterile efficacy in two of five volunteers when challenged two weeks post-final vaccination [259]; however, this did not translate to efficacy in the field [281, 282]. This sparked a move away from FP9 as the priming agent, towards use of adenoviral vectors.

Adenoviruses are approximately 38kb in size. Deletion of the E1 region renders the virus replication incompetent, whilst deletion of the E3 region increases the space available for the transgene insert to approximately 6-8kb. Human adenovirus

serotypes were initially tested as delivery vectors for malaria vaccines, with serotype 5 (AdHu5) being most common. However, the prevalence of pre-existing immunity to AdHu5 is over 35-40% in the United States and approximately 80% in African populations [262]. This led to the testing of rarer serotypes such as AdHu35 [283, 284] and simian adenoviruses [285], including the adenovirus used in this thesis, ChAd63 [160, 256]. However, the use of AdHu5 vectors still continues within malaria vaccine research efforts today [286-288]. Favourably, all the adenoviral vectors elicited potent cellular immune responses and, in particular, a greater CD8⁺ response than previously seen in other vectors and vaccine platforms [160, 256, 284, 289, 290].

1.3.2.4 Prime-boost regimen

As described above, whilst viral vectors were able to induce superior immunogenicity over DNA platforms, heterologous prime-boost regimens using combinations of DNA and viral vectors led to overall greater levels of immunogenicity and protective efficacy of sub-unit malaria vaccines. Furthermore, using heterologous vectors should avoid any anti-vector immunity in a prime-boost regimen. Malaria vaccines utilizing the prime-boost platform first began with DNA-MVA vectors [163, 245-249, 291], followed by FP9-MVA vectors after FP9 was shown to be more immunogenic than DNA [259, 263, 276, 281], and eventually to adenoviral-MVA regimens [160, 256-258, 292-297]. Many combinations have existed, but within our group a ChAd63 prime followed by a MVA boost has shown to be the most potent at producing CD8⁺ T cells [160, 256] and protective efficacy in the field (Ogwang *et al.*, 2013, submitted). Interestingly, both the order and length of interval have been crucially important in inducing a good response [54, 294, 297, 298]; MVA is not good at

priming, but is excellent at boosting, and an extended eight-week interval has shown the best results in our group so far.

Whilst CD8⁺ T cell responses are required for an effect at the liver-stage, and hence crucial for a pre-erythrocytic vaccine, antibodies are needed for a blood-stage effect. Whilst viral vectors can induce high levels of antibodies, prime-boost regimens incorporating a protein vaccine have also demonstrated potent antibody production [284, 299-302]. This may prove vital in a combination vaccine, targeting both the liver and blood or sporozoite stages, where both a high level of CD8⁺ T cells and antibodies will be required to exert the anticipated effect. Such a vaccine has been demonstrated pre-clinically with the combination of PbCSP delivered in an adenovirus prime followed by protein boost, and Ad-MVA encoding PbTRAP (Karolis Bauza, DPhil Thesis). Used alone, the adenovirus-protein vaccine regimen induced a high level of antibodies, but not T cells, and the Ad-MVA vaccine induced the opposite. Given together, the resulting vaccine induced both cellular and humoral immunogenicity and, importantly, increased the protective efficacy from approximately 0-40% using either vaccine alone to 100% in combination.

1.4 Current status of malaria vaccine development

Using the above platforms, malaria vaccines targeting each of the stages of the parasite's life-cycle are currently being tested pre-clinically and/or clinically [227]. The most advanced and successful vaccines are targeted towards the pre-erythrocytic stage, harnessing cellular or humoral arms of the immune system. Hence, the pre-erythrocytic stage is considered an excellent target for a malaria vaccine. Furthermore, as previously introduced, the successful whole sporozoite immunization regimens also provided protection at the pre-erythrocytic stage, and the liver-stage is

not only asymptomatic but also a bottle-neck in the parasite's life-cycle. An extremely efficacious pre-erythrocytic vaccine could also be transmission-blocking; if no parasites progress to the blood-stage, no gametocytes will be formed and the parasite will not be transmitted back to a mosquito. For these reasons, this thesis has focused on the development of novel liver-stage vaccines. However, the current status of blood-stage and transmission-blocking vaccines is also considered below.

1.4.1 Pre-erythrocytic vaccines

The aim of vaccines directed at the pre-erythrocytic stage is either to stop sporozoite invasion of hepatocytes or to eliminate parasite-infected hepatocytes. The current vaccines showing some success in clinical trials are irradiated sporozoites injected intravenously and a viral vectored approach expressing the antigen TRAP, both of which aim to eliminate infected hepatocytes, as well as the leading candidate, a protein-in-adjuvant approach targeting the antigen CSP that aims to stop sporozoite invasion.

The irradiated sporozoite approach was introduced in detail in section 1.3.1. Although this approach has consistently demonstrated sterile protection (through mosquito bite), albeit with small sample sizes, it is considered impractical for deployment in malaria endemic countries. The recent study of Seder *et al.*, demonstrated that intravenous injection of aseptic, cryopreserved sporozoites was able to mimic the early studies of the 1970s and provide 100% sterile protection, when given at a high enough dose [50]. Such a result is undoubtedly an excellent proof of concept that a vaccine providing sterile protection can be developed for malaria. However, the initial fears over the feasibility of this strategy have not been countered: the cryopreserved, irradiated sporozoite product is still required to be stored in liquid

nitrogen; it needs to be delivered intravenously; the dissecting process is still manual; it will take many technicians a long time to generate enough of the vaccine to be deployed in the field; it is unlikely to protect against heterologous strains and there are concerns of its durability.

The two pre-erythrocytic vaccines that are most advanced, with clinical trials undertaken in both malaria-naïve adults and semi-immune adults in malaria endemic countries, are both sub-unit vaccines: RTS,S [303], a particulate vaccine directed against the CSP which has undergone assessment in a Phase III trial, and ChAd63-MVA ME-TRAP [256], a viral vectored vaccine. CSP was the first sporozoite antigen identified and is the major coating protein on the sporozoite surface [304-306]. CSP contains a large central region of NANP repeats, surrounded by two conserved regions in the N and C terminal domains. CSP is involved in sporozoite motility [73, 307] and invasion of hepatocytes [308], through interaction with heparan sulfate proteoglycans (HSPGs) [309, 310], and as previously discussed is also able to downregulate the pro-inflammatory response through its indirect interaction with NF κ B [129]. CSP was initially chosen as a candidate antigen for a sub-unit vaccine based upon evidence suggesting immunity against CSP contributed to protection induced by irradiated sporozoite vaccination [225, 226], and through identification of antibodies against the NANP repeat region of CSP in immune African adults [311]. The first trials of CSP as a candidate antigen indicated it was poorly immunogenic when delivered as a protein vaccine and not protective in field studies [312, 313].

The turning point for a CSP-based vaccine came after development of the hepatitis B sub-unit vaccine based on the HBsAg, when it was theorized that using the HBsAg as

a carrier protein to generate a particle for CSP might result in increased immunogenicity, on the basis that particulate antigens were processed and presented in a more immunogenic manner than soluble proteins. This theory was proved correct, with the resulting vaccine RTS,S showing much greater immunogenicity and efficacy. RTS,S is a recombinant protein vaccine that fuses the NANP repeat and C-terminal regions of the CSP with HBsAg, and when expressed in yeast with additional non-recombinant HBsAg, these antigens form particulate structures of 23µm diameter. However, the increased immunogenicity and efficacy was also likely dependent on the improved adjuvants that were developed simultaneously [314]. Currently, the most successful formulations administer RTS,S in combination with a proprietary series of adjuvants from GlaxoSmithKline (GSK), AS01 or AS02 [315]. Both include the immune response enhancers monophosphoryl lipid A (MPL) [316] and a saponin derivative (QS21) [317]; AS01 is a liposomal adjuvant whilst AS02 is an oil-in-water emulsion.

RTS,S is currently the leading malaria vaccine and has progressed to Phase III trials in Africa. Initial CHMI trials identified AS02 as a leading adjuvant from an adjuvant screen [318], with two to three doses of RTS,S/AS02 protecting approximately half of volunteers tested [319]. RTS,S/AS02 was therefore taken into Phase I and IIb trials in endemic regions and initial studies demonstrated 34% vaccine efficacy in adults [320]. 29-35% vaccine efficacy was subsequently demonstrated in children of one to four years of age [321, 322], and importantly was maintained for 42 months [323]. Encouragingly, adjusted vaccine efficacy was even higher (65-66%) in two studies of children less than eighteen weeks of age, albeit with a short follow-up period (three to six months) [324, 325]. During the same period that RTS,S/AS02 was undergoing

assessment in endemic regions, AS01 was being developed pre-clinically. In a CHMI trial, RTS,S formulated with AS01 provided greater protective efficacy than RTS,S/AS02 [230], and resulted in higher antibody levels and similar efficacy in adults in endemic areas [326]. Phase IIb studies of RTS,S/AS01 in children resulted in 53% efficacy in both five to seventeen month and six to ten week old age-groups [327, 328], and was sustained up to fifteen to nineteen months [327, 329].

Due to the greater comparative efficacy of RTS,S formulated with AS01 over AS02, and the less reactogenic safety profile [230, 330, 331], AS01 was taken into a Phase III trial. The on-going phase III trial is being undertaken at eleven sites in seven countries, with 15 460 children. Two age groups are being assessed, five to seventeen months and six to twelve weeks of age. Vaccine efficacy at one year was 56% for the older children [332] and 31% for infants [333]. The eighteen-month follow-up was recently reported at the 6th Multilateral Initiative on Malaria Pan-African Conference in Durban, South Africa, in October 2013, and showed a relatively maintained level of vaccine efficacy (46% and 27%, respectively). The extended follow-up is of high interest, given recently published data of the four year follow-up of the five to seventeen month Phase IIb RTS,S/AS01 trial indicated vaccine efficacy had dropped to 16.8% [334]. RTS,S primarily works through the induction of high antibody titres against NANP, and to a lesser extent through CD4⁺ T cells [89, 231]. Despite its sub-optimal efficacy, RTS,S is likely to be licensed given its ability to reduce severe malaria in children [322]; GSK plans to submit a regulatory approval application in 2014 to the European Medicines Agency.

TRAP is a type 1 transmembrane protein first identified in the late 1980s/early 1990s [335, 336]. TRAP is essential for attachment and invasion of both the salivary gland and the liver, and is also important for the sporozoites gliding motility [310, 337-340]. TRAP was chosen as a potential candidate antigen given, like CSP, it was found to be an immunogenic target in volunteers vaccinated with irradiated sporozoites [341], and a *P. yoelii* homolog was shown to induce CD8⁺ T cell dependent protection in mice [342]. This suggested that TRAP might be the target of cell-mediated immunity at the liver-stage of infection. For this reason, the leading vaccine ChAd63-MVA ME-TRAP was designed to induce maximal cellular immunity, particularly CD8⁺ T cells. 'ME' is a multi-epitope string of cytotoxic lymphocyte (CTL) epitopes, T helper epitopes and an antibody epitope from various pre-erythrocytic antigens, designed to increase the breadth of the vaccine [247, 343].

First generation vaccines assessed ME-TRAP in a DNA prime-MVA boost regimen and demonstrated improved immunogenicity over either vector alone in both mice and humans [163, 247], leading to one out of eight (12.5%) volunteers being sterilely protected in a subsequent CHMI trial with a statistically significant effect on the Kaplan-Meier analysis of this group compared to controls [245]. The second generation assessed ME-TRAP in a FP9 prime-MVA boost regimen and demonstrated increased immunogenicity over DNA-MVA in mice [276] and similar overall efficacy in CHMI trials where two out of fifteen volunteers were sterilely protected [259]. However, these moderate levels of protection did not translate into the field, with reduced immunogenicity and no protection in an African field trial [281]. This led to the vaccine as it stands today: ChAd63-MVA ME-TRAP. This regimen demonstrated outstanding immunogenicity in both mice, non-human

primates and humans [256, 292, 296], with a recent study inducing 21% sterile protection in humans that was correlated with CD8⁺ T cells producing IFN γ [160]. Furthermore, very recent results suggest this protection has translated to field trials in Kenyan adults (Ogwang *et al.*, 2013, submitted). Despite the excellent immunogenicity of ChAd63-MVA ME-TRAP, the demonstrated efficacy is still sub-optimal, as for RTS,S.

1.4.2 Blood-stage vaccines

The aim of blood-stage vaccines is to inhibit parasites in the blood through induction of high levels of antigen-specific antibodies. In this way, such vaccines can prevent the morbidity associated with malaria infection, but they are unlikely to be completely effective at eliminating parasites. The progress of blood-stage vaccine development has been slow and relatively unsuccessful compared to pre-erythrocytic vaccines [227]. This is due to the need to express conformationally correct antigens, the limited level of antibody response achievable with existing adjuvants and need for very high antibody titres to achieve efficacy, extensive polymorphism of blood-stage candidates and the high rate of replication in this stage of the life-cycle [118, 223, 344]. Nevertheless, a successful multi-component malaria vaccine will benefit from inclusion of a blood-stage antigen target.

Most blood-stage vaccines have been protein-in-adjuvant designs based on the blood-stage antigens MSP1 and apical membrane 1 (AMA1). AMA1 administered with AS02 provided no efficacy against the primary endpoint of clinical malaria in a field trial in Mali, but did show some strain-specific protection [345]. A similar trial of MSP1, MSP2 and ring-infected erythrocyte surface antigen (RESA) demonstrated a modest decrease in parasite density in a field trial in Papua New Guinea. However,

this was again shown to be largely an allele-specific response [193]. A more recent CHMI trial assessed a combination vaccine of CSP and AMA1, delivered in a DNA prime-AdHu5 boost, and demonstrated 27% sterile protection associated with cell-mediated immunity to AMA1 [286]. Whilst this effect was statistically non-significant, the result reinforced the idea that cell-mediated immunity can contribute to blood-stage protection and suggests a viral vectored approach may be the most successful, given they can induce not only high levels of antibodies but also T cells. The antigen MSP3 is currently undergoing assessment as either a protein vaccine or long synthetic peptides in endemic areas, however, only safety and immunogenicity data has so far been reported [346-349]. Recently, a receptor-ligand pair, basigin and reticulocyte binding protein homolog 5 (Rh5), were identified as essential and non-redundant for erythrocyte invasion by the blood-stage parasite [350]. *P. falciparum* Rh5 is essential for the parasite [351, 352] and highly susceptible to cross-strain neutralizing vaccine-induced antibodies [353]. Rh5-specific IgG is induced in natural infection and has been shown to inhibit parasite growth *in vitro* [354, 355], and its presence was also associated with a longer time to subsequent infection [355]. Currently, only Rh5 has induced heterologous strain efficacy in non-human primates against *P. falciparum* blood-stage challenge (Douglas *et al.*, in preparation) and is thus probably the most promising blood-stage target, and hence future vaccine trials assessing this antigen will be of high interest.

1.4.3 Transmission-blocking vaccines

Transmission-blocking vaccines aim to induce antibodies to sexual stage or mosquito antigens and hence block transmission of the parasites to another individual. In this regard, transmission-blocking vaccines are unusual in that they would protect communities but would not prevent disease in individuals. Due to this community-

targeted protection, transmission-blocking vaccines have some clear disadvantages: the target population needs to be everyone in the community, requiring mass vaccination campaigns; this type of vaccine may suffer a more complex regulatory process; and the acceptability or up-take at the individual and community levels are unknown. Another difficulty is that many of the target antigens would not be expressed during the parasite's stages within the human host, and thus, any vaccine-induced responses would not be re-boosted following natural infection, as may be the case for pre-erythrocytic and blood-stage vaccines. Conversely, there are also many advantages to this vaccination strategy: if mosquito antigens are targeted, protection could feasibly be against multiple strains of malaria; transmission-blocking antigens are much less polymorphic than other stages [118, 356]; and in the renewed era of eliminating (rather than controlling) malaria, a transmission-blocking target will be an essential component of a malaria vaccine [34].

Despite the fact that the concept of transmission-blocking vaccines has existed since the 1970s [357], only one group has taken transmission-blocking candidates into Phase I clinical trials [358, 359]. These protein-in-adjuvant vaccines have been targeted at the ookinete surface antigens of *P. falciparum* and *P. vivax*, known as Pfs25 and Pvs25, and have induced humoral immune responses in vaccinated volunteers. Excitingly, the antibody titres correlated with transmission-blocking activity in a standardized membrane-feeding assay. This paves the way for other transmission-blocking candidates to be tested in the same manner, and if further studies share the same finding, they may alleviate the need for clinical efficacy trials in endemic areas. Other antigens currently undergoing assessment as targets in pre-clinical vaccines include Pfs230 and Pfs48/45. Recently, the malERA consultative

group introduced the concept of ‘vaccines that interrupt malaria transmission’ (VIMT) [34], which include not only the traditional transmission-blocking vaccines but also pre-erythrocytic and blood-stage vaccines that have an effect on transmission.

1.4.4 Multi-component vaccines

As discussed above, it is likely that an effective, deployable malaria vaccine will contain targets from multiple stages of the parasite’s life-cycle. Logically, if a CD8⁺ T cell inducing vaccine could reduce the number of parasites exiting the liver, the burden on the blood-stage would reduce and allow antibody-mediated blood-stage vaccines a better chance at eliminating the remaining parasites. However, the major drawback to a multi-component malaria vaccine is the increased manufacturing costs, unless antigens can be combined in a single technology (e.g. poxviral vectors or DNA with EP). This also demonstrates the need to identify the immunogenic portions of target antigens, as this could theoretically reduce the size of each component of the vaccine. Furthermore, it is not known how combining antigenic targets from different stages could positively or negatively affect the immunogenicity of each individual component; this will be an important part of pre-clinical testing of a multi-component vaccine.

1.4.5 Immune correlates of protection

For each of the leading vaccines targeting various stages of the parasite’s life-cycle, researchers have attempted to identify immune correlates of protection. As indicated above, various immune subsets or functions have been associated with protection; however, no vaccine trial has identified a true surrogate of protection (except for, perhaps, transmission-blocking vaccines). If a surrogate marker of protection could

be identified, it would enable an accelerated time frame for malaria vaccine development, potentially reducing the need for such large-scale efficacy trials. However, such markers are likely to be specific for each individual vaccine. Hand-in-hand with identifying correlates of protection goes the need for a detailed understanding of the protective mechanism.

Traditionally, vaccine-induced immune mechanisms have been classified by antibody immunogenicity, lymphoproliferation and the induction of cytokines, and they have been measured almost solely by the enzyme-linked immunosorbent assay (ELISA), intracellular cytokine staining (ICS) and the enzyme-linked immunosorbent spot (ELISpot) assay [360]. However, it is recognized by the vaccine community that this needs to improve (reviewed in [118, 361]), and suggested avenues include the assessment of polyfunctional cells, central and effector memory cells, transcriptomic profiling, mass spectrometry based cytometry by time-of-flight (CyTOF) analyses [362] and discrimination between quantitative and qualitative responses. Determining a qualitatively functional response is difficult for vaccines acting through cell-mediated immunity, as is the case for liver-stage malaria. There is currently no standardized assay that can measure whether the quantitative immunity (i.e. production of IFN γ secreting cells measured by ELISpot) is reflective of a functional response where such cells can inhibit liver-stage parasites. This is a gap not only in our understanding of vaccine-induced immunity, but also in our ability to screen vaccines pre-clinically before progressing to clinical trials. For the liver-stage vaccine field to grow, increasing the breadth of both our immunological pre-clinical screening and mechanistic understanding of protective vaccines needs to be addressed.

1.5 Thesis aims and outlines

As introduced above, whilst malaria vaccine efforts have been ongoing for almost forty years, a malaria vaccine of sufficient efficacy for realistic deployment in malaria endemic countries is still lacking. However, the irradiated sporozoite model has shown that an efficacious vaccine targeted at the liver-stage of infection is feasible. Furthermore, evidence is continually accumulating that the protection seen in this model is due to the action of CD8⁺ T cells inhibiting liver-stage parasites. Thus, there is a strong rationale for the development of a CD8⁺ T cell-inducing liver-stage vaccine. As outlined above, the viral vectored platform has so far given rise to the highest level of cellular immunogenicity seen in human volunteers compared to other platforms. Whilst the leading T cell-inducing vaccine, ChAd63-MVA ME-TRAP, has shown recent promise, new avenues need to be explored to increase efficacy. Of the 5000 or more proteins expressed by the parasite, only a few have been assessed as candidates for sub-unit vaccination. As has recently been shown in the blood-stage vaccine field, there are likely better targets in existence than those on which vaccine efforts have traditionally concentrated. For example, TRAP was not chosen as the “best” liver-stage antigen from a large selection; it was one of very few known antigens when first used and was even originally reported as a blood-stage antigen [335]. It is therefore of great value to assess other liver-stage antigens as vaccine targets. In addition to this, no true correlates of protection have been identified for pre-erythrocytic vaccines, so it is also of interest to expand upon our current pre-clinical models of vaccine assessment.

1.5.1 Aims

- To develop a number of novel viral vectored vaccine candidates targeting the liver-stage of malaria, based on antigens other than CSP and TRAP.
- To comparatively assess these novel liver-stage vaccines for the induction of both cellular and humoral immunogenicity and efficacy, utilizing newly available transgenic parasites.
- To assess the cellular immune response to the candidate antigens in naïve adult human volunteers after one episode of malaria.
- To characterize the immune response to ChAd63-MVA ME-TRAP through the identification of immunodominant epitopes.
- To develop a new approach for the pre-clinical assessment of liver-stage vaccines.

1.5.2 Outline

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

Chapter 3 describes the selection and development of eight novel liver-stage malaria vaccines.

Chapter 4 investigates the comparative immunogenicity and efficacy of the eight novel liver-stage malaria vaccines.

Chapter 5 investigates the mechanism of protection in the most promising novel liver-stage malaria vaccines.

Chapter 6 characterises the immune response to ChAd63-MVA ME-TRAP and investigates cellular immunogenicity to a panel of malaria antigens in human volunteers exposed to one malaria episode.

Chapter 7 describes the development of an assay to measure T cell-mediated inhibition of liver-stage parasites *in vitro*.

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

Chapter 2: Materials and Methods

2 Materials and Methods

Details of all materials and methods used in this thesis are provided below. In some instances, reference is also made to detailed Jenner Protocols (J#) provided in Appendix One.

2.1 Materials

2.1.1 Reagents

Details of all commercially available reagents used in this thesis are provided in Table 2.1 below. All commercially available antibodies used in this thesis are provided in Table 2.2.

Table 2.1. Commercially available reagents used throughout this thesis.

Material	Supplier	Catalogue Number
0.2µm syringe filter	Sartorius	16534
12-well tissue culture plates	Appleton Woods	BC011
15ml centrifuge tubes	Fisher Scientific	FB55950
18-gauge catheter 1.3x45mm	VWR International	TERUSR-DM1845PX
2-β Mercaptoethanol [50µm]	Gibco	31350-010
2ml syringes	Nu-Care Products	M0185
50ml centrifuge tubes	Greiner	227261
50ml syringes	Nu-Care Products	M0865
5ml polystyrene tubes	VWR International	734-0001
5ml syringes	Nu-Care Products	M2187
60mm petri dishes	Corning	430166
6-well tissue culture plates	Fisher Scientific	TKT-520-030T

70µm cell strainers	BD Biosciences	352350
8-well microscope slides	VWR International	631-0451
96-well collagen coated plates	BD Biosciences	356407
96-well U bottom plates	VWR International	734-0027
96-well V bottom plates	Fisher Scientific	DIS-210-150L
96-well flat bottom plates	Fisher Scientific	TKT-180-070U
Agarose	Sigma Aldrich	A9539
Ampicillin	Sigma Aldrich	A5354
Ammonium chloride (NH ₄ Cl) [0.15M]	Sigma Aldrich	A4514
Anti-Biotin MicroBeads	Miltenyi Biotec	130-090-485
Benzonase® Nuclease	Novagen	70664-3
Bovine Serum Albumin (BSA)	PAA Laboratories	K41-001
Carbonate-bicarbonate buffer	Sigma Aldrich	C3041
CD4 (L3T4) MicroBeads	Miltenyi Biotec	130-049-201
CD8 ⁺ T cell Isolation Kit, mouse	Miltenyi Biotec	130-095-236
CD8 ⁺ T cell Isolation Kit, human	Miltenyi Biotec	130-096-495
Cluster tubes	Corning	4401
Collagenase (Type IV)	Sigma Aldrich	C5138
Concanamycin A	Sigma Aldrich	C9705
Cryogenic vials	Star Lab	E3110-6122
Cytofix/Cytoperm™ fixation/permeabilization kit	BD Biosciences	555028
DAPI (4',6-diamidino-2-phenylindole)	Sigma Aldrich	101101353
DH5α maximum efficiency cells	Invitrogen	12297-016
Diethanolamine buffer	Pierce	34064

Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650
D-luciferin potassium salt	PerkinElmer	122796
Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich	D6546
Dulbecco's Phosphate Buffered Saline (D-PBS)	Sigma Aldrich	D8537
EasySep™ Human CD4 Positive Selection Kit	StemCell Technologies	18052
EasySep™ Human CD8 Positive Selection Kit	StemCell Technologies	18053
ELISpot colour development kit	BioRad	170-6432
ELISpot developer - BCIP/NBT (Plus) solution	Europa Bioproducts	M0711A
Endotoxin free water	Sigma Aldrich	W3500
Ethanol	Sigma Aldrich	32221
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E7889
Fetal calf serum (FCS)	Sigma Aldrich	F2442
Fructose	Sigma Aldrich	F0127
Fungizone®	Life Technologies	15290-018
Gateway® LR Clonase® II enzyme mix	Invitrogen	11791-020
Gentamicin solution	Sigma Aldrich	G1397
Giemsa	Sigma Aldrich	GS1L
Glycerol	Sigma Aldrich	G7893
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich	A3562
Golgi Plug	BD Biosciences	555028
Halt Protease Inhibitor	Fisher Scientific	PN78430
Hank's Balanced Salt Solution (HBSS)	Sigma Aldrich	H9394
HC-04 cell line	MR4	MRA-975
HEK 293A cell line	Invitrogen	R705-07

Hepa1-6 cell line	ECACC, through Sigma Aldrich	92110305
Heparin	Sigma Aldrich	H4784
HEPES	Sigma Aldrich	H3375
HepG2 cell line	ECACC, through Sigma Aldrich	85011430
Human IFN γ ELISpot kit	Mabtech	3420-2A
Hydrochloric acid (HCl) [1M]	Sigma Aldrich	S7653
Hydrochloric acid (HCl) [2M]	Fisher Scientific	J/4315/15
IgG from normal rat serum	Sigma Aldrich	14131
Immersion oil	Sigma Aldrich	10890
In-Fusion® HD Enzyme Premix	Clontech	638911
Isoflurane	Oxford University Veterinary Services	n/a
Kanamycin	Sigma Aldrich	K0254
KAPA2G Robust PCR Kit	Anachem	KK5700
Ketaset™ (ketamine)	Oxford University Veterinary Services	n/a
LB agar tablet	Sigma Aldrich	L7025-500TAB
LB broth tablet	Sigma Aldrich	L7275-500TAB
Leibovitz's L-15 medium	Life Technologies	11415-064
Leucosep tubes	VWR International	GREI227290U K
L-glutamine [4mM]	Sigma Aldrich	G7513
Lipofectamine™	Invitrogen	11668-019
LIVE/DEAD® Aqua	Invitrogen	L34957
LS Columns	Miltenyi Biotec	130-042-401
Lymphoprep	Aiere	NYC-1114547
Magnesium chloride (MgCl ₂)	Sigma Aldrich	M2393

MAIP ELISpot plates	Millipore	MAIPS4510
Methanol	Fisher Scientific	M/3900/17
Microcentrifuge tubes	Fisher Scientific	FB74031
Micro-fine Insulin Syringe with needle 0.5ml 29g x 12.7mm	Nu-Care Products	M4892
Micro-fine Insulin Syringe with needle 1ml 29g x 12.7mm	Nu-Care Products	M4891
Microlance needle 26g x 5/8inch	Nu-Care Products	M4300
Microscope slides	Fisher Scientific	MNJ-200-010H
Microvette tubes	Sarstedt	16.444
MinElute Gel Extraction Kit	QIAGEN	28606
Minimum Essential Medium Eagle (MEM) α - modification	Sigma Aldrich	M4526
Mouse IFN γ ELISpot Kit (ALP)	Mabtech	3321-2A
Mr. Frosty cryocontainer	Fisher Scientific	CRY-120-010T
MultiScreen HTS 96-well filter plates	Millipore	MSHVN4B50
Na ₂ EDTA	Sigma Aldrich	ED2SS
Neomycin sulfate	Invitrogen	21810-031
Neutral buffered formalin	Sigma Aldrich	HT501128
NUNC Maxisorp 96-well plates	Fisher Scientific	DIS-971-030J
Opti-MEM® I Reduced Serum Media	Invitrogen	11058-021
P-amino benzoic acid (PABA)	Sigma Aldrich	A9878
PBS	Sigma Aldrich	D8537
PBS/Tween sachets	Sigma Aldrich	P3563
PCR primers	Sigma Aldrich	n/a
Penicillin/Streptomycin [100U pen/ 100 μ g strep]	Sigma Aldrich	P0781
Percoll	Sigma Aldrich	P1644

Phenylhydrazine	Sigma Aldrich	P26252
Phusion® High-Fidelity DNA Polymerase	Fisher Scientific	FZF-531L
Phytohemagglutinin (PHA)	Sigma Aldrich	L1668
pNPP (20mg tablets)	Sigma Aldrich	N2765
Polypropylene columns	Pierce	29924
Polyvinyl alcohol 4-88	Fluka Analytical	81381
Potassium bicarbonate (KHCO ₃) [1mM]	Sigma Aldrich	P9144
Primaquine bisphosphate	Sigma Aldrich	160393
Propan-2-ol	Fisher Scientific	P/7490/17
Protein A/G UltraLink Resin	Fisher Scientific	PN53132
Proteinase K	QIAGEN	19131
Protein G agarose	Pierce	22852
QIAGEN MinElute DNA Purification Kit	QIAGEN	28604
QIAGEN Plasmid Midi Kit	QIAGEN	12143
QIAprep Spin MiniPrep Kit	QIAGEN	27106
Recombinant human IL-2	AbD Serotec	PHP042A
Recombinant mouse IFN γ	eBioscience	14-8311-63
Recombinant mouse TNF α	eBioscience	14-8321-62
Reddymix PCR Mastermix	Fisher Scientific	PCR-300-600G
<i>Renilla</i> Luciferase Assay System	Promega	E2820
Restriction and ligation enzymes	New England Biolabs	Various
Rompun® (Xylazine)	Oxford University Veterinary Services	n/a
RPMI-1640	Sigma Aldrich	R0883
Scalpel (surgical blade)	Swann Morton	0302

Silica gel desiccant	Sigma Aldrich	13767
SmartLadder DNA Marker	EurogenTec	MW-1700-02
SOC medium	Invitrogen	15544-034
Sodium azide (NaN_3)	Fluka Analytical	08591
Sodium bicarbonate (NaHCO_3)	Sigma Aldrich	S8761
Sodium chloride (NaCl)	Sigma Aldrich	S5886
Sodium dodecyl sulfate (SDS)	Sigma Aldrich	L4390
Staphylococcal enterotoxin B (SEB)	Sigma Aldrich	S4881
SYBR Safe	Invitrogen	S33102
Transfer pipettes	Fisher Scientific	FB55348
T-REx™ 293 cells	Life Technologies	R710-07
Tris-acetate-EDTA (TAE)	Fisher Scientific	BP1332-1
Triton™ X-100	Sigma Aldrich	X100
Trizma® HCl buffered solution (Ph8)	Sigma Aldrich	T2694
Trypan Blue	Sigma Aldrich	T8154
TrypLE™ Express	Invitrogen	12605010
Tubing	Fisher Scientific	PYH-700-020L
Vybrant® DiD cell-labelling solution	Life Technologies	V22887
William's E medium	Invitrogen	12551032
Xanthurenic acid	Sigma Aldrich	D102804

Table 2.2. Commercially available antibodies used throughout this thesis.

Antibody	Supplier	Catalogue Number
Alexa Fluor® 488 conjugated goat anti-mouse IgG	Life Technologies	A11008
Alexa Fluor® 488 conjugated goat anti-human IgG	Life Technologies	A11013
Anti-human CD8-APC clone OKT8	eBioscience	17-0086-73
Anti-human CD3-FITC clone OKT3	eBioscience	11-0037
Anti-human CD4-PeCy5.5 clone SK3	eBioscience	35-0048-71
Anti-human HLA-A2-FITC clone BB7.2	Abcam	Ab27728
Anti-human HLA-A2 Purified clone BB7.2	AbD Serotec	MCA2090EL
Anti-human HLA-A3 Purified clone 4i85	Abcam	Ab33640
Anti-IFN γ blocking antibody AN18	Mabtech	3321-3-1000
Anti-mouse CD107a-PE clone 1D4B	eBioscience	12-1071
Anti-mouse CD11b-Biotin clone M1/70	BioLegend	101204
Anti-mouse CD11c-Biotin clone N418	BioLegend	117304
Anti-mouse CD127-APCeFluor® 780 clone A7R34	eBioscience	47-1271-80
Anti-mouse CD19-Biotin clone MB19-1	BioLegend	101504
Anti-mouse CD16-32 Purified (Fc block) clone 93	eBioscience	14-0161-81
Anti-mouse CD3 ϵ -APC clone 145-2C11	eBioscience	17-0031
Anti-mouse CD45R (B220)-Biotin clone RA3-6B2	BioLegend	103204
Anti-mouse CD49b-Biotin clone DX5	BioLegend	108904
Anti-mouse CD4-Biotin clone GK1.5	BioLegend	100404
Anti-mouse CD4-eFluor® 450 clone RM4-5	eBioscience	48-0042-80
Anti-mouse CD4-eFluor® 650 clone GK1.5	eBioscience	95-0041-41
Anti-mouse CD4-FITC clone RM4-4	eBioscience	11-0043-81
Anti-mouse CD62L-PeCy7 clone MEL-14	eBioscience	25-0621-81

Anti-mouse CD8 α -FITC clone 53-6.7	eBioscience	11-0081
Anti-mouse CD8 α -PerCPCy5.5 clone 53-6.7	BD Biosciences	551162
Anti-mouse H-2K ^b Biotin clone AF6-88.5	BD Biosciences	553568
Anti-mouse IFN γ -APC clone XMG1.2	eBioscience	17-7311
Anti-mouse IFN γ -eFluor® 450 clone XMG1.2	eBioscience	48-7311
Anti-mouse IL-2-PeCy7 clone JES6-5H4	BD Biosciences	560538
Anti-mouse MHC Class II (I-A/I-E)-Biotin clone M5/114.15.2	BioLegend	107604
Anti-mouse MHC Class II (I-A/I-E)-PE clone M5/114.15.2	eBioscience	12-5321-82
Anti-mouse TNF α -FITC clone MP6-XT22	eBioscience	11-7321
Anti-TNF α blocking antibody clone 1F3F3D4	eBioscience	BMS177

2.1.2 Solutions and buffers

- **ACK Lysis Buffer:** 8.29g NH₄Cl (0.15M), 1g KHCO₃ (1mM), 37.2mg Na₂EDTA in 800ml dH₂O. pH adjusted to 7.2-7.4 with HCl (1M) before making a final solution up to 1L with dH₂O.
- **Buffer A:** 50mM Tris, 100mM NaCl, 5mM MgCl₂ and 1% Triton X-100 in dH₂O.
- **Cell Separation Medium:** 2% FCS and 1mM EDTA in D-PBS.
- **Coating Buffer:** 15mM sodium carbonate and 35mM sodium bicarbonate capsules were dissolved in dH₂O and autoclaved.
- **Complete α -MEM Medium:** 500ml MEM α -modification was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100 μ g streptomycin), 500 μ l 2mercaptoethanol (50 μ M) and 50ml of heat inactivated FCS (10%).

- **Diethanolamine Buffer:** A 5x stock was diluted with dH₂O before use.
- **Digestion Solution:** 500ml DMEM was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin) and 1.7g HEPES (15mM). The solution was filtered prior to use. 1ml of 250mg/ml type IV collagenase was added just prior to use.
- **Ear Punch Buffer:** 5ml 1M Tris pH 8 (50mM), 40µl 5M NaCl (2mM), 2ml 0.5M EDTA (10mM) and 10ml 10% SDS (1%) were added to 82.96ml dH₂O.
- **Ex-flagellation Medium:** RPMI-1640 was supplemented with 25mM HEPES, 20% FCS, 10mM sodium bicarbonate and 50µM xanthurenic acid. pH was adjusted to 7.6.
- **FACS Buffer:** 1% FCS and 0.1% sodium azide in PBS.
- **Fructose/PABA Solution:** 80g fructose and 0.5g PABA were added to 1L of dH₂O. The solution was autoclaved prior to use.
- **Giemsa:** 5% Giemsa in dH₂O.
- **Hepa1-6 Medium:** 500ml DMEM was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin), 500µl 2mercaptoethanol (50µM) and 50ml of heat inactivated FCS (10%).
- **LB Agar/Broth:** Tablets were dissolved in dH₂O (1 tablet per 50ml). Antibiotics were added at the following working concentrations: ampicillin 100µg/ml, kanamycin 25µg/ml.
- **MACS Buffer:** 2.5g BSA (0.5%) and 2ml 0.5M EDTA (2mM) were added to 500ml D-PBS. The buffer was sterile filtered prior to use.
- **Mowiol:** 6g glycerol and 2.4g polyvinyl alcohol 4-88 were dissolved in 6ml dH₂O for two hours at 50°C with agitation. 12ml Tris pH 8.5 (0.2M) was added and the solution was dissolved for a further three hours at 50°C with

agitation. The solution was centrifuged at 5000xg for five minutes to remove any undissolved solids. DAPI was then added at a final concentration of 0.1µg/ml.

- **Perfusion Solution:** 5ml pen/strep (100U penicillin, 100µg streptomycin), 2.98g HEPES (25mM) and 200µl 0.5M EDTA were added to 500ml HBSS. The solution was sterile filtered prior to use.
- **Perm/Wash:** 10x Perm/Wash buffer was diluted in dH₂O prior to use.
- **PBS (0.1M):** 0.138M NaCl, 0.0027M KCl, pH 7.4; made by dissolving tablets in dH₂O according to the manufacturer's instructions.
- **PBS/Tween (PBS/T) (0.1M):** 0.138M NaCl, 0.0027M KCl, Tween 0.05%, pH 7.4; made by dissolving sachets in dH₂O.
- **PBS/BSA:** 2.5g BSA (0.5%) and 250µl sodium azide (0.05%) were added to 500ml D-PBS.
- ***Plasmodium berghei* Freezing Medium:** 11ml FCS, 4.2ml 5% NaHCO₃ and 5.5mg neomycin were added to 96ml RPMI-1640.
- **Primary Hepatocyte Culture Medium:** 500ml DMEM was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin) and 50ml of heat inactivated FCS (10%).
- **R0 Medium:** 500ml RPMI-1640 was supplemented with 5ml L-glutamine (2mM) and 5ml pen/strep (100U penicillin, 100µg streptomycin).
- **R10 Medium:** 500ml RPMI-1640 was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100µg streptomycin) and 50ml of heat inactivated FCS (10%).
- **TAE Buffer:** Made from 50x concentrate diluted in dH₂O.

2.2 Molecular biology and cloning

2.2.1 Antigen inserts

The selection of the liver-stage antigens used in this thesis for expression in virally vectored vaccines is detailed in Chapter 3. To create the constructs required for cloning into the viral vectors ChAd63 and MVA, the *P. falciparum* 3D7 sequence was obtained from PlasmoDB (<http://plasmodb.org/plasmo/>) and cross-referenced with NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The sequences were analysed using the SignalP 3.0 [363] and TMHMM Servers from the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/>) to generate a predicted structure. A number of modifications were made to the original sequences in order to aid production of the virally vectored vaccines, and to increase the insert expression and immunogenicity in mammalian cells. These modifications were the deletion of repetitive regions of sequence and the addition of the human tissue plasminogen activator (tPA) leader sequence (GenBank Accession K03021) [364] upstream. Genes were synthesized by GeneArt (Life Technologies, New York USA), with a number of further modifications requested. The antigen sequence, or tPA leader sequence, was preceded by the Kozak sequence to aid translation in mammalian cells [365] and the Kpn1 restriction enzyme site for cloning into the viral vectors. At the 3' DNA appendix, a STOP codon and the Not1 restriction enzyme site were added. No sequences contained the *Vaccinia* virus early gene transcription termination signal 5'- TTTTNT -3' [366]. Finally, the sequences were codon optimized for expression in human cells. Details of the reasoning and modifications for each antigen are provided in Chapter 3.

A number of previously generated constructs were also used in this thesis; details are provided below.

***P. berghei* TRAP (PbTRAP)**

The PbTRAP sequence (NCBI AAB63302.1) was synthesized by GeneArt and cloned into ChAd63 and MVA vectors by Dr. Karolis Bauza (DPhil Thesis). The sequence had two modifications, the addition of the tPA leader sequence and removal of the transmembrane domain by addition of two stop codons.

***P. falciparum* CSP (PfCSP)**

The CSP sequence (PlasmoDB PF3D7_0304600) was synthesized by GeneArt and cloned into ChAd63 and MVA vectors by Dr. Sue Morris, Dr. Matthew Cottingham and Nick Edwards [367]. The sequence had two modifications, the addition of the tPA leader sequence and removal of 26 of the NANP repeats from the central region.

***P. falciparum* ME-TRAP (ME-TRAP)**

The ME-TRAP construct has previously been described [247, 343]; the ME string contains known CD4 and CD8 epitopes from pre-erythrocytic *P. falciparum* antigens and the TRAP sequence is from *P. falciparum* T9/96 [335]. The ME string was codon optimized for expression in human cells, whilst TRAP was not. Fifteen amino acids were deleted from the T9/96 TRAP sequence (five repeats of PNP) and it contains its own signal peptide. Staff at the Clinical Bio-manufacturing Facility (CBF), within the Jenner Institute, cloned the ME-TRAP construct into ChAd63 and MVA vectors.

***P. falciparum* TRAP (TRIP)**

The TRIP construct is based on *P. falciparum* 3D7 TRAP (PlasmoDB PF3D7_1335900). It was codon optimized for expression in human cells, contains the Kozak sequence and also had the same fifteen amino acids deleted as for ME-TRAP. The predicted transmembrane helix and cytoplasmic domains were also deleted. Dr. Matthew Cottingham designed the construct and cloned it into ChAd63 and MVA vectors.

Luciferase

The *Photinus* luciferase gene (NCBI M15077) was sub-cloned from an existing plasmid into ChAd63 by Dr. Nicky Greene and into MVA by Chris Schultz, Aisling Vaughn and Dr. Alison Turner. The gene was confirmed to contain a Kozak sequence and absence of *Vaccinia* virus early gene transcription termination signals.

MVA-NP+M1

MVA expressing the nucleoprotein (NP) and matrix protein 1 (M1) from Influenza A was generated as previously described [368].

2.2.2 Polymerase chain reaction (PCR)

A standard PCR reaction was used for various applications throughout this thesis, unless otherwise stated. The standard PCR mix used, based on KAPA2G Robust Polymerase, was as follows for one reaction (overleaf):

5µl Reaction Buffer B (5x)
 1.25µl Primer 1 (10µM)
 1.25µl Primer 2 (10µM)
 0.5µl dNTPs (equal mix, 10nM each)
 0.2µl KAPA2G Robust Polymerase (5U/µl)
 5µl sample template
 15.8µl sterile H₂O

PCR was then performed according to the following program:

Primary denature	95°C	5 minutes	
Denature	95°C	30 seconds	} x29
Anneal	45-68°C	30 seconds	
Extension	72°C	30 seconds/kb	
End	10°C		

When PCR products were to be used for down-stream cloning, the Phusion® High-Fidelity DNA Polymerase was used. A typical PCR mix for one reaction was as follows:

25µl Phusion High-Fidelity master mix
 0.5µl Primer 1 (10µM)
 0.5µl Primer 2 (10µM)
 10ng sample template
 to 50µl with sterile H₂O

PCR was then performed according to the following program, where T_m refers to the melting point of the lower primer using an online calculator (<http://www.thermoscientificbio.com/webtools/tmc/>):

Primary denature	98°C	30 seconds	
Denature	95°C	10 seconds	} x25
Anneal	$T_m+3^{\circ}\text{C}$	30 seconds	
Extension	72°C	30 seconds/kb	
Final Extension	72°C	5 minutes	
End	10°C		

2.2.3 Agarose gel electrophoresis

DNA samples were analysed by 1% agarose gel electrophoresis (100V 60 minutes) in 1x TAE buffer, with DNA stained using SYBR Safe. Products were visualized on a transilluminator, and sized according to comparison with the molecular weight marker SmartLadder. DNA fragments required for cloning or sequencing applications were cut out and purified using the QIAgen MinElute DNA purification kit, following the manufacturer's instructions.

2.2.4 Restriction endonuclease digests

Restriction endonuclease digests were performed using New England Biolabs (NEB) endonucleases and the appropriate NEB buffer with or without BSA, according to the manufacturer. Digests were incubated at 37°C for two hours. A standard 20µl reaction was as follows (overleaf):

1µl NEB Restriction Enzyme (10 000U/ml)

2µl NEB Restriction Buffer (10x)

(2µl BSA (10x) when required)

xµl DNA (0.5-1µg)

xµl sterile dH₂O (to total volume 20µl)

2.2.5 DNA ligation

DNA fragments were inserted into the plasmids using NEB T4 DNA ligase. A vector to insert ratio of approximately 1:3 was used. Ligations were incubated at 16°C overnight. A standard 20µl reaction was as follows:

13µl sterile dH₂O

2µl NEB Ligation Buffer (10x)

1µl Plasmid DNA

3µl Insert DNA

1µl T4 DNA Ligase

Further details can be found in the Jenner Protocol J098 located in Appendix One.

2.2.6 Bacterial transformation

Ligation products were transformed into 50µl maximum efficiency DH5α *Escherichia coli* according to the manufacturer's instructions. In brief, 5µl of DNA was added to *E. coli* cells and incubated on ice for 30 minutes. Cells were subsequently heat-shocked at 42°C for 40 seconds then placed back on ice for two minutes. When using kanamycin resistant selection, the bacterial cells were pre-incubated in 250µl SOC medium for one hour at 37°C with 225rpm shaking before

being plated onto a LB agar plate containing kanamycin. When using ampicillin resistant selection, bacterial cells were plated immediately without the addition of SOC medium. Agar plates were incubated overnight at 37°C.

2.2.7 Plasmid DNA preparation

Single colonies were selected from agar plates and incubated overnight in 2ml LB broth containing selection antibiotics. Plasmid DNA was then extracted using a QIAprep Spin MiniPrep Kit according to the manufacturer's instructions. Purified DNA was quantified using a Nanodrop spectrophotometer and identity confirmed via analytical restriction digest. To purify greater quantities of DNA, 1ml of incubated broth was transferred to 50ml broth and incubated overnight at 37°C. Plasmid DNA was extracted using a QIAGEN Plasmid Midi Kit according to the manufacturer's instructions. To make bacterial stocks, 800µl of plasmid-containing LB broth was mixed with 800µl of 50% glycerol and stored at -80°C.

When the number of colonies on agar plates was not significantly greater than the number on negative control plates, colonies were screened by PCR prior to incubation in LB broth and plasmid extraction. A PCR mastermix was prepared as described in 2.2.2. A 10µl pipette tip was used to select a single colony and dip it in the appropriate PCR tube; this same tip was used to streak the colony onto an agar plate to grow the clone. This was done for ten colonies before the PCR was performed as previously described.

2.2.8 Restriction cloning into ChAd63

The recombinant ChAd63-[antigen] vaccines were constructed using a novel Gateway system developed by Dr. Matthew Cottingham at the Jenner Institute,

Oxford. This system uses the Gateway® technology to generate a recombinant adenovirus containing the gene of interest under the control of a promoter of choice. To generate such clones, a LR Clonase™ II mediated site-specific recombination 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) (Figure 2.1).

The entry vector used was pENTR™ 4-Mono, which contains the human CMV immediate-early promoter used to drive transcription and the bovine growth hormone (BGH) poly(A) transcription termination sequence. To avoid deletions during production, this entry vector contains a non-splicing CMV promoter without intron A. The antigen sequences provided by GeneArt, and pENTR™ 4-Mono, were digested with Acc65I and NotI and the resulting DNA fragments were separated on a 1% agarose gel as described in 2.2.3. The DNA bands of correct size were extracted from the gel using QIAgen MinElute extraction kits and the antigen insert was then ligated into the entry vector backbone overnight as described in 2.2.5. The pENTR™ 4-Mono-[antigen] entry vector was then transformed into *E. coli* bacteria and plasmid DNA prepared as per 2.2.6 and 2.2.7. Insert presence was confirmed by analytical restriction enzyme digest using PstI.

The pENTR™ 4-Mono-[antigen] entry vector was subsequently directionally inserted into the E1 and E3-deleted adenoviral genome at the E1 locus by site-specific recombination using the LR Clonase™ II enzyme mix, as outlined in J160. Reactions were terminated with proteinase K, transformed into *E. coli* bacteria and plasmid DNA prepared as previously described in 2.2.6 and 2.2.7. To confirm insert presence,

both analytical restriction enzyme digest using KpnI and sequencing (Gene Service, Oxford) were performed. Following confirmation of the correct sequence the expression clone was linearized with PmeI as per J004, prior to transfection and purification as described in 2.3.1.

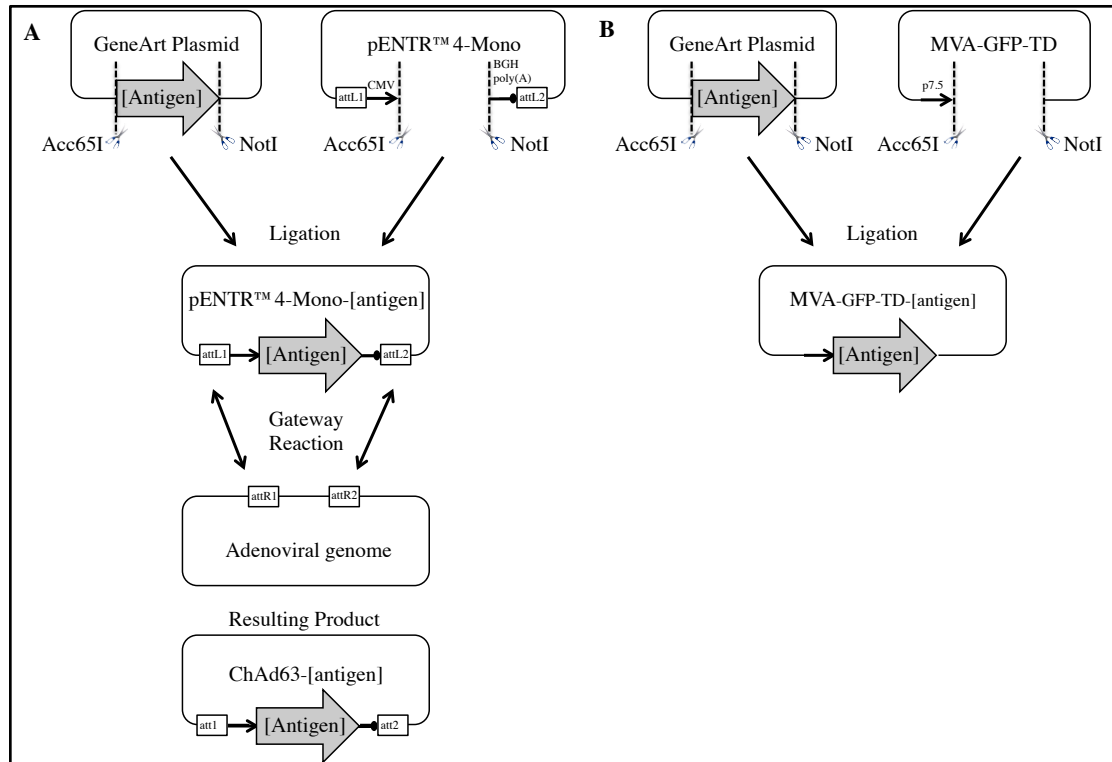


Figure 2.1 Cloning scheme for insertion of liver-stage malaria antigens into the viral vectors ChAd63 and MVA.

(A) To create ChAd63-[antigen] vaccines, the antigen of interest was first cloned into the entry vector pENTR™ 4-Mono by ligation, after digestion with the restriction enzymes Acc65I and NotI. The entry vector was then inserted into the ChAd63 genome through site-specific recombination using the Gateway® method. (B) To create MVA-[antigen] vaccines, a one-step cloning method was used. The antigen of interest was cloned into the markerless MVA genome by ligation, after digestion with the restriction enzymes Acc65I and NotI.

2.2.9 Restriction cloning into MVA

To generate recombinant MVAs the antigen of interest was cloned into the markerless MVA plasmid MVA-GFP-TD (Figure 2.1, above). The gene insertion site is at the thymidine kinase (TK) locus with the antigen under control of the p7.5 promoter. The antigen sequences were extracted from the plasmids provided by GeneArt by digestion with *Acc65I* and *NotI* as described in 2.2.4. The MVA-GFP-TD plasmid was also digested with the same enzymes, after alkaline phosphatase treatment as per J090. The DNA fragments were separated on a 1% agarose gel and extracted using QIAgen MinElute gel extraction kits. The antigen insert was then ligated into the MVA-GFP-TD plasmid overnight as described in 2.2.5. The MVA-GFP-TD-[antigen] vector was then transformed into *E. coli* bacteria and plasmid DNA prepared as per 2.2.6 and 2.2.7. To confirm insert presence, both analytical restriction enzyme digest using *PvuI* and sequencing (Gene Service, Oxford) were performed. The MVA-GFP-TD-[antigen] vectors were then transfected and purified as outlined in 2.3.2.

2.2.10 Generation of protein lysate

In order to detect the presence of antibodies in serum (see 2.6.7), protein lysate was generated for each of the antigens that were developed into virally vectored vaccines. This entailed In-Fusion® cloning to generate new constructs with the luciferase tag, transfection of HEK293 cells and harvest of the cellular lysate, as detailed below.

2.2.10.1 Generation of pMono2-[antigen]-rLuc8 constructs

In order to generate the lysate, a new construct containing the antigen upstream of the *Renilla* luciferase gene was generated by In-Fusion® cloning of the antigen into an existing destination plasmid pMono2-FliC-rLuc8 (Figure 2.2). The existing

destination plasmid was kindly provided by Dr. David Wylie, and contained the FliC gene upstream of the luciferase tag. This destination plasmid was digested with HindIII and BamHI to remove the FliC sequence; the DNA fragments were run on a 1% agarose gel and purified using the QIAgen MinElute gel extraction kit.

To obtain insert DNA, PCR primers were designed to cut out the antigen sequence of interest (without tPA leader sequence and STOP codon) from the entry vectors previously generated (see 2.2.8). These primers also contained fifteen base-pair overhangs matching the entry site of the destination plasmid, containing the HindIII and BamHI restriction sites (Table 2.3). The PCR was performed with Phusion® DNA Polymerase as described in 2.2.2. The PCR insert DNA was then entered into the digested destination vector using the 5x In-Fusion® HD Enzyme Premix according to the manufacturer's instructions, based on a 1:2 insert to vector ratio calculated using the In-Fusion® Molar Ratio Calculator. The resultant product, pMono2-[antigen]-rluc8, was transformed into *E. coli* bacteria and plasmid DNA prepared as per 2.2.6 and 2.2.7. The plasmids were sequenced to confirm correct antigen insert.

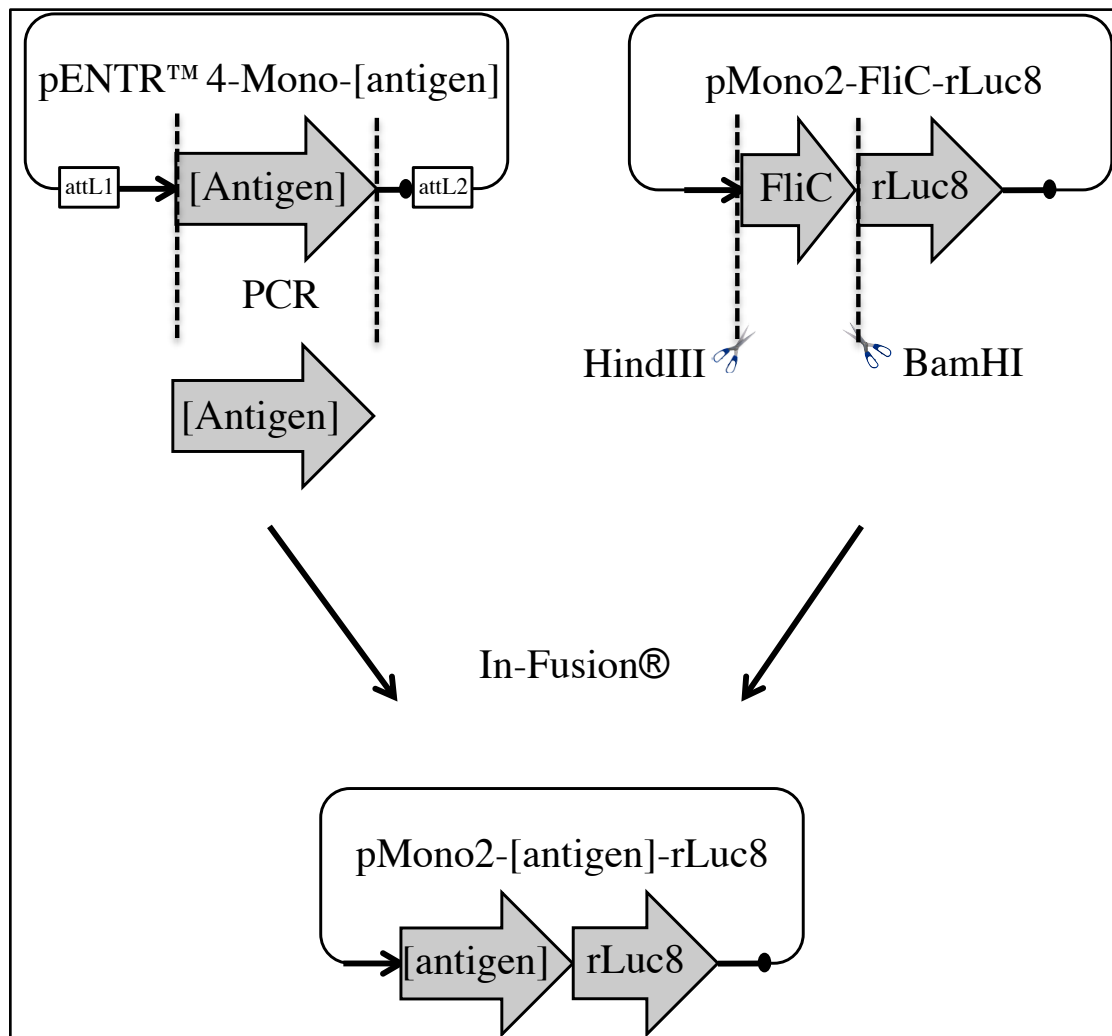


Figure 2.2. Cloning scheme to generate plasmids containing the liver-stage malaria antigens upstream of the *Renilla* luciferase tag.

The antigen sequence was isolated from an existing plasmid by PCR and the destination plasmid was prepared by digesting out the existing antigen with HindIII and BamHI. The malaria antigen sequence was then inserted into the digested destination plasmid using In-Fusion® cloning.

Table 2.3. Primers used to isolate the liver-stage malaria antigen sequences from the entry vectors.

The fifteen base-pair overhangs are highlighted in bold.

Primer	Sequence
PfCelTOS Forward	GCCAACATGAAGCTT ATGAACGCCCTGCGGCGGCTGCCTGTG
PfCelTOS Reverse	CCCGGGCCCCGGATCC GTCTGAAGAAATCGTCGCTCAGGCTTTCTCTCGC
PfE1590w Forward	GCCAACATGAAGCTT ATGCGGTTCAAGCAAGGTGTTCAGC
PfE1590w Reverse	CCCGGGCCCCGGATCC CTGCTCTTTCTTGGGTTCCTCAGTTTTC
PfExp1 Forward	GCCAACATGAAGCTT ATGAAGATCCTGTCCGTGTTCCTTCTGGCCCTG
PfExp1 Reverse	CCCGGGCCCCGGATCC GTGCTCGGTGCCGGACACCAAGTTGTTG
PfI0580c Forward	GCCAACATGAAGCTT ATGAACCTGCTGGTGTTCCTCTGC
PfI0580c Reverse	CCCGGGCCCCGGATCC CTGCACGTTCAAGTCCACGATCCG
PfLSA1 Forward	GCCAACATGAAGCTT ATGAAGCACATCCTGTACATCAGCTTCTACTTC
PfLSA1 Reverse	CCCGGGCCCCGGATCC CAGCTTCATGAAGTACTTGGTGATGTCC
PfLSA3 Forward	GCCAACATGAAGCTT ATGACCAACAGCAACTACAAAGAGCAACAACAAG
PfLSA3 Reverse	CCCGGGCCCCGGATCC TTTGCTTTTCTGTGTCCGGCTCTTTTTTGGC
PfLSAP1 Forward	GCCAACATGAAGCTT ATGAAGACCATCATCATCGTGACCC
PfLSAP1 Reverse	CCCGGGCCCCGGATCC TTCCACCATGTAGAAGTCGGCGTCC
PfLSAP2 Forward	GCCAACATGAAGCTT ATGTGGCTGTGCAAGCGGGGCCTG
PfLSAP2 Reverse	CCCGGGCCCCGGATCC GTTGGTCACGTTCCGCATGTTGTTTTCC
PfUIS3 Forward	GCCAACATGAAGCTT ATGAAGGTGTCCAAGCTGGTGCTGTTCG
PfUIS3 Reverse	CCCGGGCCCCGGATCC GTTCTCTTCCTGGCTCAGGTAGTTGGCG

2.2.10.2 Transfection of HEK 293A cells with pMono2-[antigen]-rLuc8

The transfection reagent was first prepared; 10µl lipofectamine was mixed with 250µl Opti-MEM® per sample and incubated for five minutes at room temperature. Meanwhile, 3µg pMono2-[antigen]-rLuc8 plasmid was mixed with 1µg green fluorescent protein (GFP) expressing plasmid in 250µl Opti-MEM®. The DNA and lipofectamine solutions were then mixed together and incubated for twenty minutes at room temperature. 300µl Opti-MEM® was then added per sample to bring the total volume to 800µl. The media was then removed from pre-prepared HEK 293A cells in a 6-well plate and the 800µl mix was added slowly to avoid disturbing the cells. The transfected cells were incubated overnight at 37°C 5% CO₂ in a humidified incubator. The transfection was then confirmed by the expression of GFP in the cells. HEK 293A cells were kindly maintained and provided by the Viral Vector Core Facility (part of the Jenner Institute).

2.2.10.3 Harvest of cellular lysate

Lysis buffer provided with the *Renilla* luciferase assay system was prepared by adding protease inhibitor (100x) immediately prior to harvesting the cellular lysate. The transfected cells were placed on ice and the medium was carefully removed and discarded. 1.4ml of lysis buffer was added per well and cells were mobilized through the use of a cell scraper. The lysate was transferred into pre-cooled microcentrifuge tubes and sonicated for fifteen seconds. The lysate was then clarified by centrifugation at 10 000xg for four minutes. The luciferase activity (light units, LU) of the lysate was quantified on a luminometer (Thermo Scientific Varioskan® Flash) by the addition of 1/100 *Renilla* luciferase assay substrate.

2.2.11 Genotyping of HHD mice

To determine the genotype of the HLA-A2 transgenic mice bred in-house, known as HHDs [369], ear punches were collected in sterile microcentrifuge tubes. To extract DNA, 20µl of ear punch buffer containing 1mg/ml proteinase K was added to each ear punch and incubated for twenty minutes at 55°C. The sample was then vortexed to help break up the tissue, followed by a further twenty minutes of incubation. 180µl dH₂O was then added to each tube and samples were heated to 99°C for five minutes to deactivate the proteinase K. After cooling samples were stored at -20°C until further use. PCR was then performed using the Reddymix PCR Master Mix (22.5µl Reddymix, 1.25µl each primer and 1µl DNA) and run on a PCR machine using the following program:

Primary denature	94°C	2 minutes	
Denature	94°C	10 seconds	} x35
Anneal	57°C	30 seconds	
Extension	72°C	1 minute	
End	72°C	1 minute	

Primers were designed using the online software program Primer3 [370] for HLA-A2, H-2D, human and mouse beta-2 microglobulin (β2m) (Table 2.4). Control DNA was collected from the HepG2 cell line (HLA-A2) and C57BL/6 mice (H-2D^b). HHD mice should contain human β2m, human HLA-A2 (α1 and α2 domains) and mouse H-2D^b (α3, transmembrane and cytoplasmic domains). However, the genotyping results indicated that whilst they do contain HLA-A2, they actually contain mouse β2m and not human β2m. Flow cytometry staining confirmed lack of expression of

H-2^b compared to C57BL/6 mice, and a low level expression of HLA-A2 using the antibodies to H-2K^b (AF6.88.5.5.3) and HLA-A2 (BB7.2). This also confirmed the finding that HHD mice contain mouse rather than human β 2m, as β 2m is essential for cell surface expression of MHC molecules. Nevertheless, these mice were able to generate HLA-A2-specific responses with an Influenza A HLA-A2-restricted epitope (Figure 2.3).

Table 2.4. Primers used to genotype HHD mice.

Primer Name	Sequence	Product Size
H-2D ^b Forward	GCGGAGAATCCGAGATATGA	157bp
H-2D ^b Reverse	CCGCGCTCTGGTTGTAGTAG	
HLA-A2 Forward	ACCGTCCAGAGGATGTATGG	202bp
HLA-A2 Reverse	CCAGGTAGGCTCTCAACTGC	
Human β 2m Forward	TGGCACCTGCTGAGATACTG	713bp
Human β 2m Reverse	CAGTTCCTTTGCCCTCTCTG	
Mouse β 2m Forward	CTTGGACCCTTGGTACCTCA	249bp
Mouse β 2m Reverse	AAGTCCAGTGTTGGGTCAGG	

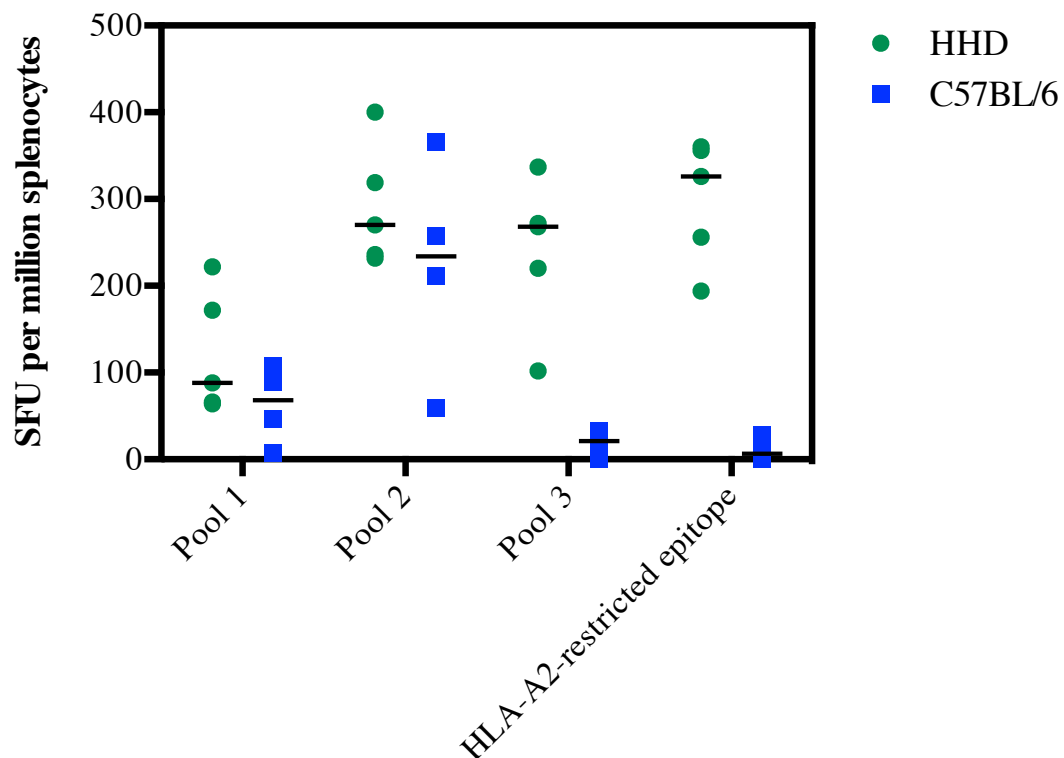


Figure 2.3. HHD (HLA-A2 transgenic) mice elicit an immune response to the HLA-A2-restricted Influenza A epitope not seen in the background C57BL/6 strain.

Female HHD and C57BL/6 mice (n=4 per strain) were vaccinated intramuscularly with 1×10^6 pfu MVA-NP+M1 and sacrificed twelve days later. Immune responses to the Influenza A virus were measured by *ex vivo* spleen IFN γ ELISpot. Splenocytes were stimulated with either overlapping peptides to NP+M1 split into three pools (80 peptides total, pool 1 1-26, pool 2 27-52, pool 3 54-80) or the HLA-A2-restricted epitope (located in M1, amino acids 58-66 [371, 372]). Results are expressed as spot forming units (SFU) per million splenocytes. Median and individual data points are shown.

2.3 Virology

Purification of recombinant viruses was kindly carried out by the Viral Vector Core Facility, led by Dr. Alison Turner. Rhea Longley carried out identity and purity PCRs. A brief overview of the methods used is provided below, with full protocols given in Appendix One as indicated.

2.3.1 Adenovirus transfection and purification

Adenovirus transfection was carried out as described in J004. In brief, 85µl linearised recombinant adenoviral plasmid (see 2.2.8) was mixed with 215µl Opti-MEM®. 300µl of 1:10 lipofectamine in Opti-MEM® was then prepared and mixed with the 300µl of linearised plasmid, followed by incubation of the resulting mixture for at least twenty minutes at room temperature. The mixture was then added to flasks of pre-prepared T-REx™ 293 cells. 293 cells are immortalized lines of primary human embryonic kidney cells transformed by sheared human adenovirus 5 DNA. They therefore provide the E1 gene product, *in trans*, for the replication-incompetent adenovirus. T-Rex™ cells have tetracycline-regulated expression, to enable silencing of the insert gene during virus production. This is achievable due to two tetracycline repressor-operator complexes within the CMV promoter. As the cell line expresses the tetracycline repressor protein, the protein can bind the operator elements and inhibit the gene expression [373, 374]. Cells were incubated at 37°C 5% CO₂ in a humidified incubator and monitored daily for cytopathic effect (CPE, morphological changes caused by virus infection). Cells were harvested once optimal CPE was evident. Recombinant adenovirus was purified by density centrifugation over a caesium chloride gradient as described in J005. Virus yield (infectious units) was determined by plaque immunostaining as described in J006. Identity was confirmed by PCR as described in J245.

2.3.2 MVA transfection and purification

As described in 2.2.9, antigens were cloned into the markerless MVA plasmid (MVA-TD-GFP) where the GFP gene is present outside the TK locus. Chick Embryo Fibroblasts (CEFs) (obtained from the Pirbright Institute, Compton, UK) were maintained as described in J013 and infected with MVA expressing red fluorescent

protein (RFP) as outlined in J176. These cells were then transfected with the MVA-TD-GFP-[antigen] plasmid 90 minutes later, which enables homologous recombination to occur between the MVA virus and the plasmid. As the plasmid is circular, a single crossover event occurs resulting in a large unstable intermediate product containing the entire plasmid and MVA parental genome. This unstable product then resolves into either the recombinant markerless MVA or the parental MVA containing RFP. After incubation of the plasmid with the virus in CEFs, cells were sorted using a MoFlo cell sorter operated by Andrew Worth at the Jenner Institute Flow Sorting Facility, as described in J131. The unstable intermediate products expressing both GFP and RFP were collected and the lysate used to infect CEFs again. Successful recombinant MVAs containing the antigen were selected by repeated rounds of plaque picking as in J018, initially selecting GFP and RFP double positive cells followed later by the selection of colourless plaques. The virus was then bulked up and purified as per J019, followed by PCR analysis as described in J079 and titration (pfu) as per J111.

2.4 Animals and immunisations

2.4.1 Mice

All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Oxford Animal Care and Ethical Review Committee for use under Project License PPL 30/2414 or 30/2889. All mice were housed under Specific Pathogen Free (SPF) conditions in the Wellcome Trust Centre for Human Genetics Functional Genetics Facility (FGF), or temporarily in the Radiobiology Research Institute when used in imaging studies.

Five to six week old female C57BL/6J (H-2^b), BALB/c (H-2^d), TO (outbred) or CD-1 (outbred) mice were obtained from Harlan (UK). HHD (HLA-A2 transgenic) mice [369] were kindly provided by Professor Vincenzo Cerundolo (University of Oxford) and bred in the FGF by the facility's staff, assisted by Dr. Alex Spencer, Rhea Longley and Rebecca Hillson.

2.4.2 Immunisations and injections

All immunisations were carried out under inhalation anaesthesia, using 3.5% isoflurane carried by oxygen (2l/minute). Immunisations were administered intramuscular (i.m.) in a volume of 50µl into the musculus tibialis using 26-gauge needles.

Intravenous (i.v.) injections were administered in a volume of 100µl into the lateral tail vein using a 28-gauge needle. Prior to injection, mice were warmed for approximately ten minutes at 38°C to encourage vasodilation.

Intraperitoneal (i.p.) injections were administered in a volume of 100-300µl using a 28-gauge needle.

Subcutaneous (s.c.) injections were administered into the scruff of the neck in a volume of 50µl using 26-gauge needles.

2.4.3 Vaccines

All vaccines were formulated in endotoxin free D-PBS to a total volume of 50µl per mouse and administered i.m. Adenoviral vectored vaccines were given at a dose of 1x10⁶ or 1x10⁸ infectious units (ifu), whilst MVA vectored vaccines were given at

either 1×10^6 or 1×10^7 plaque forming units (pfu) as stated in the relevant text and figure legends.

2.5 Collection and preparation of cells and sera

The current and following section (2.6) refer to work carried out in murine models. Any assays and protocols used in human models are detailed in section 2.8. However, peptide information is relevant for both murine and human studies.

2.5.1 Isolation of splenocytes

Mice were sacrificed by cervical dislocation and spleens were dissected and removed into sterile D-PBS. Individual spleens were subsequently crushed in 5ml PBS using the flat end of a 5ml syringe in a 6-well plate. Single cell suspensions were prepared by passaging splenocytes through a 70 μ m cell strainer into a 50ml tube prior to centrifugation at 300xg for five minutes. To remove erythrocytes, supernatants were discarded and cell pellets resuspended in 5ml ACK lysis buffer for four minutes before addition of 25ml PBS to stop the reaction. Splenocytes were immediately centrifuged again and the resulting cell pellets resuspended in 5ml complete α -MEM. Splenocytes were counted using a CASY counter (Schärfe Systems, Germany) and diluted to the required concentration in complete α -MEM.

2.5.2 Isolation of peripheral blood mononuclear cells (PBMCs)

Five to six drops of blood were collected from the lateral tail vein into 200 μ l 10mM EDTA in PBS. Prior to bleeding mice were warmed for approximately ten minutes at 38°C to encourage vasodilation. Approximately 1ml of ACK lysis buffer was added to the blood, followed immediately by thorough vortexing and centrifugation at 400xg for four minutes. The cell pellet was resuspended in 1ml ACK lysis buffer and again centrifuged prior to resuspending the pellet in 320 μ l complete α -MEM.

2.5.3 Isolation of liver mononuclear cells

Mice were sacrificed by cervical dislocation and the liver was exposed. A 25-gauge butterfly needle attached to a 50ml syringe was used to flush the circulating blood from the liver with sterile D-PBS, by insertion into the hepatic portal vein. The liver was subsequently dissected and mashed through a 70 μ m cell strainer into a petri dish, with the flat end of a 2ml syringe. The cell strainer and petri dish were flushed with PBS and all cells were collected into a 15ml tube. The cells were centrifuged for seven minutes at 480xg, the supernatant discarded and the cell pellet resuspended in 10ml of 33% isotonic percoll solution. The cells were then centrifuged at 693xg for twelve minutes with the brakes off. The resulting upper layers were carefully removed with a transfer pipette and the cell pellet was resuspended in 1ml of ACK lysis buffer. The cells were incubated in the lysis buffer for four minutes at room temperature then 10ml complete α -MEM was added and the cells were spun for five minutes at 480xg. The final pellet was resuspended in 500 μ l complete α -MEM.

2.5.4 Isolation and culture of primary murine hepatocytes

The isolation and culture of primary murine hepatocytes was undergoing development and optimization as part of this thesis. The procedure described is based upon that of Prof. David Tosh at the University of Bath [375], with various modifications to suit the technical set-up available at the University of Oxford. To comply with the project license, mice were sacrificed by cervical dislocation prior to the procedure commencing. Mice were quickly dissected to expose the liver, moving all other organs to the side. An 18-gauge catheter was inserted into the vena cava, the needle removed and tubing connected to the solutions attached. The hepatic portal vein was cut; instantaneous blanching of the liver indicated successful insertion of the cannula. The liver was then perfused for ten minutes with the perfusion solution kept

at 37°C in a water bath and delivered at a constant rate (approximately 5ml/minute) through the use of a mechanical pump. Following adequate perfusion, the liver was digested for ten minutes with a constant rate of digestion solution at 37°C.

Subsequently, the liver was carefully dissected from the mouse and removed into a petri dish containing digest solution. The liver was gently teased apart with a pair of forceps, releasing the cells into the dish. The cell suspension was passed through a 70µm strainer into a 50ml tube. The cell suspension was then spun three times at 50xg for two minutes, with resuspension in primary hepatocyte culture medium. Cells were diluted in trypan blue to determine the number and viability of cells using a haemocytometer. Cells were resuspended at 5×10^6 cells/ml and 100µl were added per well of a 96-well collagen coated plate.

2.5.5 Collection of mouse sera

Mouse sera was obtained from either five to six drops of blood from the lateral tail vein collected in a microvette tube, or via cardiac puncture. Cardiac puncture was performed under anaesthetic (3.5% isoflurane, 2l/minute oxygen), using a 26-gauge needle to withdraw blood from the heart. Collected blood was stored at 4°C overnight to allow clotting. The following day blood was spun at 10 000xg for four minutes to separate the sera from the RBCs. Sera was removed into a clean microcentrifuge tube and stored at -20°C until required.

2.6 Immunological assays

2.6.1 Peptides

Peptides used in the cellular assays were commercially synthesized by Neo Group Inc., USA, Mimotopes, UK or Thermo Fisher Scientific, USA. Crude 20mer peptides

overlapping by ten amino acids were synthesized for the entire sequence used in the vaccine constructs for: *P. falciparum* 3D7 CSP, Exp1, LSA1, LSA3, LSAP1, LSAP2, PFE1590w, PFI0580c, TRAP and UIS3, *P. falciparum* T9/96 TRAP and *P. berghei* TRAP. Crude 15mer peptides overlapping by ten amino acids for the entire sequence of *P. falciparum* 3D7 AMA1, CelTOS, MSP1, MSP2, Pfs16 and STARP were kindly provided by Dr. Simon Draper. Crude 20mer peptides overlapping by ten amino acids for the Influenza A NP and M1 antigens, including the HLA-A2-restricted epitope in M1, were kindly provided by Dr. Teresa Lambe (University of Oxford). Peptides were reconstituted in DMSO at a concentration of 50 to 100mg/ml depending on the solubility. Peptides were subsequently combined into sub-pools of up to twenty peptides, before the sub-pools were combined into a total mega-pool for use in the cellular assays. These peptides were used for both murine and human cellular assays. Peptide sequences are provided in Appendix Two.

Peptide epitope mapping was conducted for *P. falciparum* LSA1, LSAP2, TRAP and UIS3. In these experiments single peptides were used in the cellular assays. For TRAP, after initial screening, crude 9mer peptides overlapping by eight amino acids were synthesized for peptides 2, 3, 6, 9, 12, 13, 15, 16, 19 and 24 to identify the immunogenic epitopes (Table 2.5). These peptides were reconstituted as stated above. Predicted epitopes were calculated for the *P. falciparum* antigens Exp1, CelTOS, LSA1, LSA3, LSAP1, LSAP2, PFE1590w, PFI0580c and UIS3 using the servers IEDB [376] (http://tools.immuneepitope.org/main/html/tcell_tools.html) and SYPEITHI [377] (<http://www.syfpeithi.de/index.html>). In some cases the epitopes already existed as individual peptides, and in others they were ordered separately and synthesized as above. Details of these epitopes are provided in Chapter 3.

Table 2.5. *P. falciparum* T9/96 TRAP 20mer peptides used to generate 9mers.

Crude 9mer peptides overlapping by eight amino acids were synthesized for the peptides 2, 3, 6, 9, 12, 13, 15, 16, 19 and 24; the original peptide sequence is shown in this table.

Peptide Name	Amino Acid Sequence
<i>P. falciparum</i> TRAP Peptide 2	VIVFLIFFDLFLVNGRDVQN
<i>P. falciparum</i> TRAP Peptide 3	FLVNGRDVQNNIVDEIKYSE
<i>P. falciparum</i> TRAP Peptide 6	LLMDCSGSIRRHNVNHAVP
<i>P. falciparum</i> TRAP Peptide 9	LNDNAIHLYVNVFSNNAKEI
<i>P. falciparum</i> TRAP Peptide 12	KEKALIIIRSLLSTNLPYGR
<i>P. falciparum</i> TRAP Peptide 13	LLSTNLPYGRTNLTDALLQV
<i>P. falciparum</i> TRAP Peptide 15	RKHLNDRINRENANQLVVIL
<i>P. falciparum</i> TRAP Peptide 16	ENANQLVVILTDGIPDSIQD
<i>P. falciparum</i> TRAP Peptide 19	RGVKIAVFGIGQGINVAFNR
<i>P. falciparum</i> TRAP Peptide 24	MKAVCVEVEKTASCGVWDEW

2.6.2 Intracellular cytokine staining (ICS)

Cellular immune responses were assayed in splenocytes, PBMCs and liver mononuclear cells via ICS. Isolated cells were plated at 150µl cells with 50µl stimulated (+peptide) or unstimulated (-peptide) mixes in a 96-well U bottom plate for six hours at 37°C 5% CO₂ in a humidified incubator. Mixes contained 1/1000 Brefeldin A (golgi plug) per well, 1/400 anti-mouse CD107a-PE +/- 5µg/ml peptide (final concentrations) in complete α-MEM. Plates were then stored at 4°C overnight or stained that day.

Plates were centrifuged at 600xg for three minutes to pellet cells, which were then washed in 100µl PBS/BSA and centrifuged again. For standard ICS, cells were then surface stained with 50µl per well of 1/50 anti-mouse CD16/32 (Fc block), 1/100 anti-mouse CD4-eFluor® 450 and 1/200 anti-mouse CD8α-PerCPCy5.5 diluted in PBS/BSA for 30 minutes at 4°C. Cells were subsequently washed once and fixed by incubation for five minutes at 4°C with 4% paraformaldehyde (10% neutral buffered formalin). Cells were washed once in Perm/Wash followed by intracellular staining with 50µl per well of 1/100 anti-mouse TNFα-FITC, 1/100 anti-mouse IL-2-PeCy7 and 1/200 anti-mouse IFNγ-APC diluted in Perm/Wash for 30 minutes at 4°C. Finally, cells were washed three times in Perm/Wash and once in PBS/BSA with final resuspension in 80µl PBS/BSA.

To stain for memory cell markers, the first layer compromised 1/50 anti-mouse CD16/32 (Fc block), 1/200 anti-mouse CD8α-PerCPCy5.5, 1/50 anti-mouse CD4-eFluor® 650, 1/50 anti-mouse CD62l-PeCy7, 1/50 anti-mouse CD127-APCeFluor® 780 and 1/200 Live/Dead Aqua diluted in PBS/BSA. The second layer compromised 1/100 anti-mouse TNFα-FITC and 1/100 anti-mouse IFNγ-eFluor® 450 diluted in PERM/WASH. All other steps were identical as for the standard ICS detailed above.

Samples were acquired on a LSRII (BD Biosciences) flow cytometer and analysis was performed using FlowJo (Tree Star Inc., USA). An example of the gating strategy used for all ICS experiments is shown (Figure 2.4); background responses in unstimulated wells were subtracted from the stimulated responses. In some experiments polyfunctionality of T cells was analysed using the Boolean gate platform in FlowJo followed by subsequent preparation of data in Pestle (Mario

Roederer, National Institutes of Health) for final analysis and graphical representation in SPICE (simplified presentation of incredibly complex evaluations, Mario Roederer [378]).

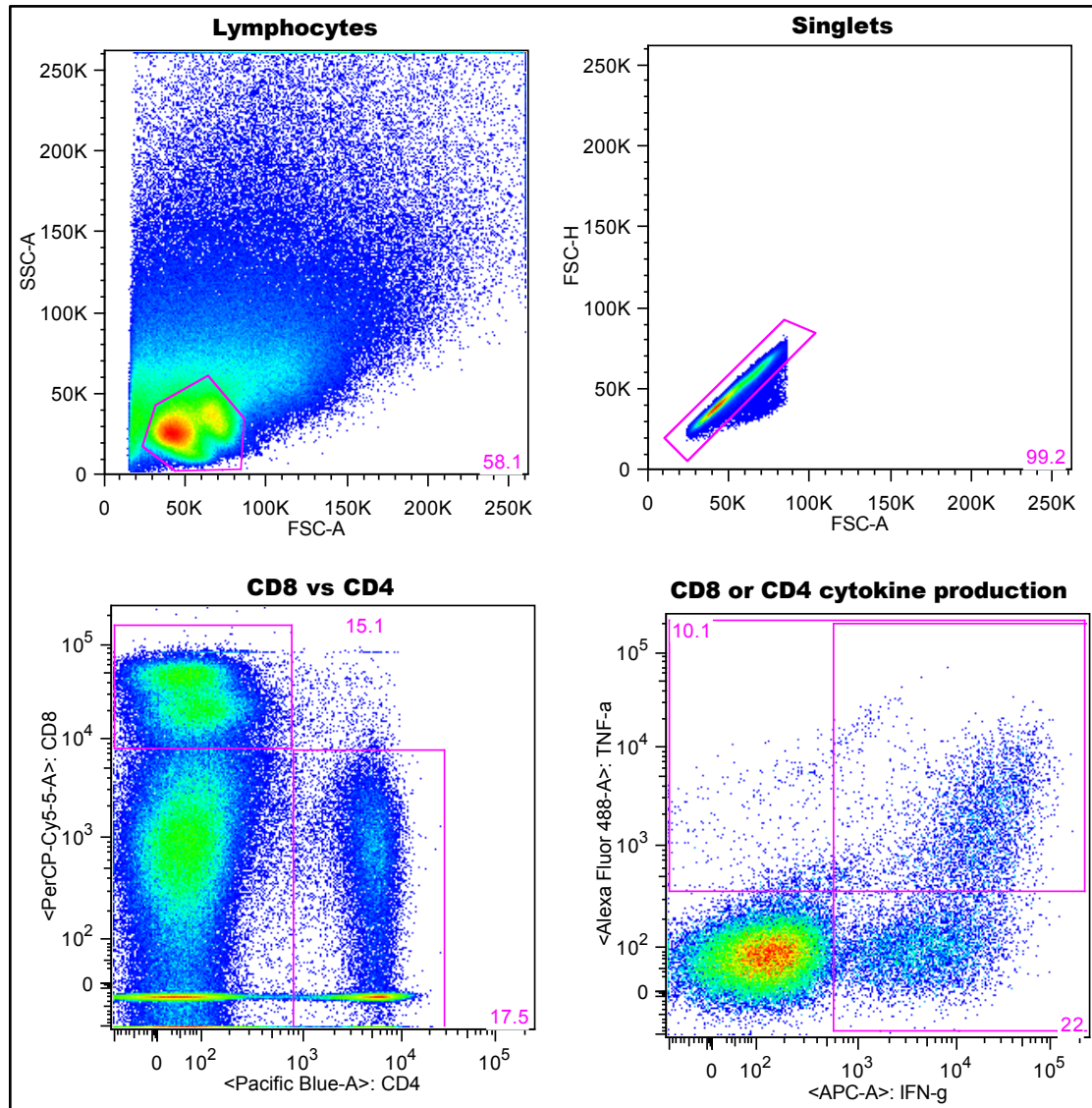


Figure 2.4. Example of the ICS gating strategy used in all experiments.

Splenocytes, liver mononuclear cells or PBMCs were first gated by size, followed by singlet cells. The cells were then separated into CD4 or CD8 positive subsets, and then cytokines gated from within those subsets. Gates show the percentage of the parent.

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

All ELISpot reagents were supplied in a mouse IFN γ ELISpot kit from Mabtech. ELISpot plates were coated with 50 μ l per well of 5 μ g/ml anti-IFN γ purified monoclonal antibody AN18 in coating buffer and incubated at 4°C overnight. Plates were then blocked for at least one hour at room temperature with 100 μ l complete α -MEM. Mouse splenocytes were prepared as described in 2.5.1 and diluted to an optimal starting concentration (most commonly 10x10⁶ cells/ml, dependent on expected/observed response). 50 μ l splenocytes were added per well in duplicate and serially diluted two-fold down the blocked plates. Peptides were diluted to 2 μ g/ml and 50 μ l was added per test well (final concentration of 1 μ g/ml); complete α -MEM alone was added to control wells. Plates were incubated for eighteen to twenty hours at 37°C 5% CO₂ in a humidified incubator.

Following incubation, plates were washed six times with PBS using an automated plate washer (Dynex Technologies, USA) then incubated with 50 μ l per well of 1 μ g/ml biotinylated rat anti-mouse IFN γ diluted in PBS for two hours at room temperature. Plates were subsequently washed again and incubated with 50 μ l per well of 1 μ g/ml streptavidin alkaline phosphatase polymer diluted in PBS for one hour at room temperature. Plates were washed again and finally incubated with 50 μ l per well of BioRad AP conjugate development buffer for approximately five to ten minutes at room temperature until spots developed. Washing the plates with tap water stopped the reaction and once plates were dry spots were enumerated using an AID ELISpot plate counter (Strassberg, Germany). Responses were expressed as spot forming units

(SFU) per million splenocytes. Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

2.6.4 Isolation and adoptive transfer of CD4⁺ and CD8⁺ T cells

Splenocytes were prepared and counted as per 2.5.1 followed by sequential isolation of CD4⁺ then CD8⁺ T cells, using the MACs CD4 (L3T4) MicroBeads (positive selection) and CD8⁺ T Cell Isolation Kit (negative selection) as per the manufacturer's instructions. All centrifugation steps were performed at 4°C, all incubation steps at 2-8°C and all solutions used were pre-cooled.

2.6.4.1 Positive selection of CD4⁺ T cells

Briefly, splenocytes were centrifuged at 300xg for ten minutes then resuspended in 90µl MACS buffer and 3.5µl CD4 (L3T4) MicroBeads per 10⁷ cells. Samples were mixed well then incubated for fifteen minutes followed by washing in 1-2ml MACS buffer per 10⁷ cells and centrifugation at 480xg for eight minutes. Cells were resuspended in 500µl MACS buffer for up to 10⁸ cells and separated using a MACS Separator and LS Column. The column was prepared by placing within the magnet and rinsing with 3ml MACS buffer. The cell suspension was then applied to the column and washed through three times with 3ml MACS buffer; the collected effluent was the unlabelled fraction. The column was removed from the Separator and placed on a 15ml tube; 5ml MACS buffer was added and the labelled cells were flushed out by firmly applying the provided plunger. The positive fraction (CD4⁺ T cells) was set-aside on ice and the unlabelled fraction was used to isolate CD8⁺ T cells.

2.6.4.2 Negative selection of CD8⁺ T cells

Briefly, the unlabelled fraction from the CD4⁺ selection was centrifuged at 300xg for ten minutes then resuspended in 40µl MACS buffer and 2.8µl Biotin-Antibody cocktail per 10⁷ cells. The Biotin-Antibody cocktail contained monoclonal antibodies (mAbs) against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHC Class II and Ter-119. Samples were mixed well then incubated for ten minutes, followed by addition of 30µl MACS buffer and 5.7µl Anti-Biotin MicroBeads per 10⁷ cells and further incubation for fifteen minutes. Cells were then washed in 1-2ml MACS buffer per 10⁷ cells and centrifuged at 480xg for eight minutes. Cells were resuspended in 500µl MACS buffer for up to 10⁸ cells and separated using a MACS Separator and LS Column. The column was prepared by placing within the magnet and rinsing with 3ml MACS buffer. The cell suspension was then applied to the column and washed through three times with 3ml MACS buffer; the collected effluent was the unlabelled fraction containing the CD8⁺ T cells.

The CD4⁺ and CD8⁺ T cell fractions were centrifuged and cell numbers determined. For injection into mice, the cells were again centrifuged and resuspended in RPMI-1640 with 10% FCS at the required concentration. The cells were injected i.v. in 100µl two days prior to challenge with *Plasmodium* parasites. The purity of the fractions was analysed by flow cytometry using 1/100 anti-CD4-eFlour® 450, 1/200 anti-CD8-PerCPCy5.5 and 1/50 anti-CD3ε-APC.

2.6.5 Enrichment of CD8⁺ T cells

Splenocytes were prepared and counted as per 2.5.1, then enriched for CD8⁺ cells by negative depletion using an in-house biotin-antibody cocktail and MACS anti-Biotin MicroBeads. The protocol was essentially as described in 2.6.4.2, with some minor

modifications. Briefly, splenocytes were centrifuged at 300xg for ten minutes then resuspended in 40µl MACS buffer and 1µl biotin-antibody cocktail per 10⁷ cells. The biotin-antibody cocktail contained mAbs against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5) and MHC Class II diluted 1/100 in MACS buffer and sterile filtered. Samples were mixed well then incubated for ten minutes, followed by addition of 30µl MACS buffer and 20µl Anti-Biotin MicroBeads per 10⁷ cells and further incubation for fifteen minutes. Cells were then washed in 1-2ml MACS buffer per 10⁷ cells and centrifuged at 300xg for ten minutes. Cells were resuspended in 500µl MACS buffer for up to 10⁸ cells and separated using a MACS Separator and LD Column. The column was prepared by placing within the magnet and rinsing with 2ml MACS buffer. The cell suspension was then applied to the column and washed through twice with 1ml MACS buffer; the collected effluent was the unlabelled fraction containing the CD8⁺ T cells. The column was removed from the Separator and placed on a 15ml tube; 3ml MACS buffer was added and the labelled cells were flushed out by firmly applying the provided plunger. The purity of the fractions was analysed by flow cytometry by staining with 1/100 anti-CD8α-FITC.

2.6.6 *In vivo* CD4⁺ or CD8⁺ T cell depletion

To determine the contribution of T cells in protection from malaria, subsets of T cells were depleted using the monoclonal antibodies anti-CD4 GK1.5 (rat IgG2a) or anti-CD8 2.43 (rat IgG2a) purified using protein G affinity chromatography from hybridoma culture supernatants. IgG from normal rat serum was purchased and purified using the same method. Purifications were kindly performed by Julie Furze. The optimal dose of depleting mAbs was determined experimentally as 100µg by dose titration.

Mice were injected i.p. with 100µg of mAb diluted in PBS on days -2, -1 and 0 (with respect to challenge with *Plasmodium* parasites on day 0). Control mice were treated in the same way. The degree of *in vivo* CD4⁺ or CD8⁺ T cell depletion was assessed by flow cytometry using 1/100 anti-CD4-FITC clone RM4-4, 1/200 anti-CD8-PerCPCy5.5 clone 53-6.7 and 1/50 anti-CD3ε-APC on day +4 with respect to day of challenge.

2.6.7 Luminescence immunoprecipitation system (LIPS)

The LIPS assay was used to detect antigen-specific antibody in sera from immunized subjects. Burbelo and colleagues developed this assay in 2005 [379, 380]; it is useful when purified recombinant proteins needed for ELISA are not available. The assay relies on the generation of plasmid constructs containing the antigen of interest fused to the *Renilla* luciferase sequence (which will emit light in the presence of the appropriate substrate). These plasmids are subsequently transiently transfected into cells and the cellular lysate harvested. Details of the constructs, transient transfection and harvest of cellular lysates can be found in 2.2.10. An overview of the assay is provided (Figure 2.5).

50µl of 1/100 sera diluted in Buffer A was mixed with 50µl of 2x10⁸ LU/ml of [antigen]-rluc8 lysate in a 96-well V bottom plate for one hour at room temperature on a rotary shaker. A 30% suspension of protein A/G beads in PBS was prepared, 5µl was added per well to a 96-well filter MultiScreen HTS plate and the 100µl sera-lysate mix was transferred to this plate and incubated for a further hour at room temperature on a rotary shaker. Plates were developed using the Promega Renilla luciferase assay system. The plates were first washed eight times with 100µl Buffer A, followed two times with PBS and finally left in 50µl PBS to prevent the

membrane from drying out. A 1/100 dilution of *Renilla* luciferase assay substrate was prepared in the provided buffer and 50µl was added per well. Plates were read immediately on a luminometer (Thermo Scientific Varioskan® Flash) and each well was subsequently quenched with 2M HCl to prevent cross talk between wells. The background level of luminescence was calculated using six replicates of naïve sera: two times the standard deviation plus the average. Where available, positive control sera or monoclonal antibodies were also included. The LIPS was validated by correlation with ELISA readings for the antigens PfTRAP and PfCelTOS (Spearman $r=0.7115$, $p<0.001$ and $r=0.61$, $p=0.0043$, respectively) (Figure 2.6).

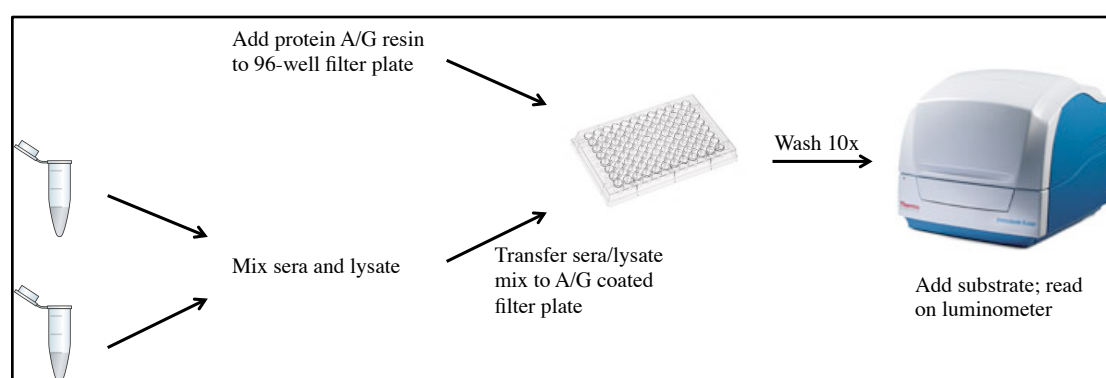


Figure 2.5. Schematic representation of the LIPS assay.

Sera collected from vaccinated mice was mixed with [antigen]-rLuc8 lysate for one hour at room temperature on a rotary shaker, to enable the antibodies in the serum to bind with the protein in the lysate. This mix was subsequently transferred to a filter plate pre-coated with protein A/G resin and incubated for one hour at room temperature on a rotary shaker. The protein A/G resin was capable of binding antibodies in the serum. After incubation, the plate was washed ten times to remove any unbound contaminants, as the bound antibodies will remain attached to the plate by the resin. Subsequently, the luciferase substrate was added which can react with the luciferase tag attached to the antigen in the lysate and give a relative reading of the amount of antibody present.

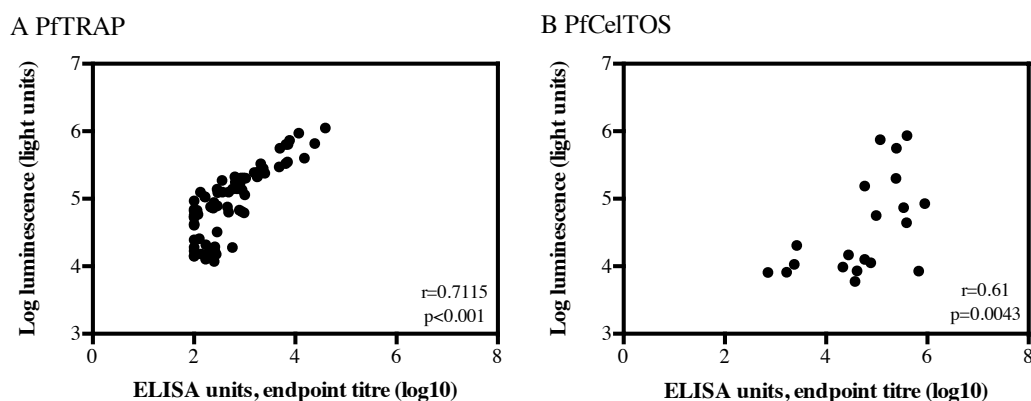


Figure 2.6. Relative antibody measurements calculated by LIPS correlate significantly with the same samples measured by ELISA.

LIPS and ELISA data were correlated using two different antigens and three different species. (A) Macaque and human serum samples ($n=84$) from studies where subjects were vaccinated with viral vectors encoding ME-TRAP were tested for antibodies to TRIP by both LIPS and ELISA. The correlation between the two measurements was analysed using Spearman's rank correlation coefficient, $r=0.7115$, $p<0.001$. Data was log transformed prior to analysis. Median data points are shown. (B) C57BL/6 and BALB/c mice were immunized with viral vectors encoding PfCelTOS and serum samples were tested for antibodies by both LIPS and ELISA. The correlation between the two measurements was analysed using Spearman's rank correlation coefficient, $r=0.61$, $p=0.0043$. Data was log transformed prior to analysis. Median data points are shown. TRAP ELISA data was kindly provided by Katharine Collins for comparison.

2.6.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to detect antibodies when recombinant purified protein was available, to provide a measure to test the accuracy of the LIPS assay. DPhil student Katharine Collins had previously obtained the TRAP ELISA data shown in Figure 2.6. PfCelTOS protein was obtained from Dr. Matt Higgins (Biochemistry, University of Oxford) to perform PfCelTOS ELISAs on sera from vaccinated mice.

NUNC Maxisorp 96-well flat bottom plates were coated with 50 μ l per well of 2 μ g/ml PfCelTOS protein diluted in carbonate-bicarbonate buffer and incubated at 4°C overnight. Plates were washed six times with PBS-0.05% Tween (PBS/T) then

blocked with 200µl 1% BSA in PBS/T per well for one hour at 37°C. Serum samples taken after a single shot of ChAd63-[antigen] were diluted 1/100 in PBS/T, samples taken after ChAd63-[antigen] with MVA-[antigen] boost were diluted 1/500. Samples were added to wells in duplicate and serially diluted three-fold down the plate. Plates were incubated for two hours at room temperature then washed six times with PBS/T.

Bound antibodies were detected by the addition of 50µl per well of 1/5000 goat anti-mouse whole IgG alkaline phosphatase conjugate diluted in PBS/T and incubated for one hour at room temperature. Plates were washed six times in PBS/T then developed with 100µl per well of 1mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate in diethanolamine buffer. Plates were read when the positive controls gave an optical density (OD)₄₀₅ of approximately one. The endpoint titres were taken as the dilution at which the OD of the sample reached the background plus three times the standard deviation calculated from naïve samples.

2.6.9 Whole IgG passive transfer

Serum was collected from anaesthetized mice as previously described in 2.5.5. Sera were pooled between groups and IgG purified using Pierce polypropylene columns pre-packed with 2ml protein G resin as per the manufacturer's instructions, for further details see J135. Approximately 1.5mg of purified whole IgG was obtained, and 173µg was injected i.v. in 100µl into each naïve mouse. Those mice were subsequently challenged with malaria sporozoites approximately six hours later.

2.7 Parasitology

2.7.1 Parasite strains

Plasmodium parasite strains were kindly provided by collaborators, as detailed below.

***P. berghei* ANKA GFP** (wild-type expressing GFP – referred to as *P. berghei* GFP in this thesis) was provided by Prof. Robert Sinden at Imperial College, London [381].

***P. berghei* transgenic parasites** containing an additional copy of the *P. falciparum* version of a particular gene inserted at the 230p locus under control of the *P. berghei* UIS4 promoter were provided by Leiden University, the Netherlands. All of these parasites also expressed a GFP/luciferase fusion gene under the *P. berghei* EF1 α promoter. Generation was through the ‘gene insertion/marker out’ technology, as previously described [382]. DPhil student Ahmed Salman performed cloning and Dr. Shahid Khan and Dr. Chris Janse performed transfections. Transgenic parasites were generated for the following *P. falciparum* antigens: CelTOS, LSA1, LSA3, LSAP1, LSAP2, UIS3, PFI0580c, PFE1590w, TRAP and CSP. In this thesis, they are referred to as PbPf[antigen], for example, PbPfCelTOS.

***P. falciparum* 3D7** was provided by Walter Reed Army Institute of Research (WRAIR), USA, and ***P. falciparum* NF54** by Radboud University Nijmegen, the Netherlands.

2.7.2 Preparation of thin blood smears

To monitor parasitaemia of infected mice, thin blood smears were prepared by snipping the end of the mouse's tail and collecting a single drop of blood onto a glass slide. The smear was air-dried, fixed in 100% methanol for one minute then stained in 5% Giemsa diluted in dH₂O for one hour. The slide was viewed on a light microscope at 100x under oil immersion. The percentage of pRBCs was counted at a monolayer region of the thin blood smear, where there were always approximately 500 RBCs per field of view. The number of fields of view counted depended on the parasitaemia. If the parasitaemia was above 1% five fields of view were counted, if it was between 0.1% and 1% ten fields of view were counted and if it was below 0.1% 40 fields of view were counted.

2.7.3 Sporozoite production (*P. berghei*)

Frozen *P. berghei* pRBC were thawed and 100-300µl was injected i.p. into a naïve TO donor mouse. Four days later the parasitaemia of the donor mouse was analysed as described in 2.7.2. The donor mouse was then cardiac bled as per 2.5.5, however in this case the syringe was lined with 300U/ml heparin to prevent the blood clotting. The blood was diluted to 1% parasitaemia and 100µl (approximately 10⁷ pRBCs) was injected i.p. into two recipient mice. Three days after the recipient mice had been inoculated they were anaesthetized with 50-100µl i.m. of a mix of 2% Rompun solution (20mg/ml xylazine), 100mg/ml Ketaset (ketamine) and PBS in a ratio of 1:2:3 and fed to a pot of starved four to seven day old female *Anopheles stephensi* mosquitos for approximately ten minutes. During the feed a drop of blood was taken to determine parasitaemia, and another drop to determine exflagellation. Exflagellation was measured by adding one drop of room temperature exflagellation

medium to the blood, covering with a cover slip and viewing under a light microscope at 40x.

Mosquitoes infected with *P. berghei* were maintained at 19-21°C in a humidified incubator on a twelve-hour day-night cycle and fed on Fructose/PABA solution. Mosquitoes were reared and maintained by staff at the Jenner Insectary. At approximately 21 days post-feed mosquito salivary glands were dissected to obtain infectious sporozoites (21 days is the peak time-point for sporozoite viability, however infectious sporozoites can be obtained from 18 to 28 days post-feed).

During the course of this DPhil a number of changes were made in order to increase the yield of sporozoites obtained from mosquitoes (Figure 2.7). In initial experiments the recipient mice were pre-treated three days prior to infection with 100µl phenylhydrazine, however, it was subsequently found that whilst this treatment increased the parasitaemia of the recipient mouse the final number of sporozoites obtained was comparable to un-treated recipient mice. In later experiments, mosquitoes were treated with the antibiotic gentamicin for the week prior to feeding to clear the mosquitoes of any infections that could detrimentally affect sporozoite production. A new recipe for the fructose solution was also tested (increased to 2g PABA, plus addition of 1g methyl 4-hydroxybenzoate), however, results suggested it was detrimental to the sporozoite yield and hence it was not taken further. Finally, the addition of a second uninfected blood meal one-week post the infected feed was also tested, however, due to the extra use of mice this was not taken further.

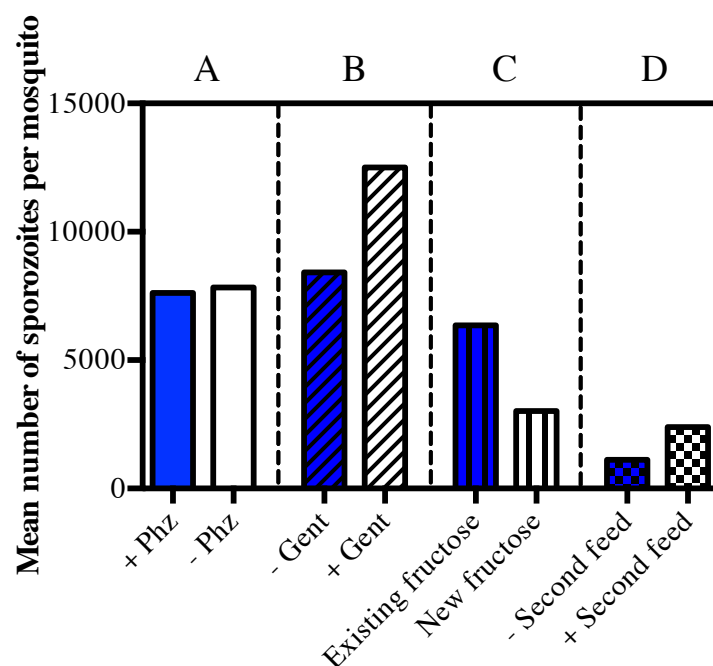


Figure 2.7. Comparison of different experimental protocols for the production of sporozoites.

Sporozoite production was carried out as previously described, with minor modifications. The blue bars indicate the standard experimental procedure prior to the comparison. The arithmetic mean is shown; a minimum of fifteen mosquitoes were dissected per treatment. (A) Sporozoite yield was compared between recipient mice pre-treated or not with phenylhydrazine (Phz). (B) Sporozoite yield was compared when the mosquitoes were pre-treated with gentamicin (Gent) to clear any existing infections. (C) Sporozoite yield was compared with a new recipe for the fructose solution. (D) Sporozoite yield was compared when the mosquitoes were given a second uninfected blood feed.

2.7.4 Cryopreservation of parasitized RBCs

To allow continued use of the same parasite strain, stocks of pRBCs were cryopreserved. Mosquitoes were fed on parasite-infected mice and the salivary glands were dissected 21 days post-feed using a dissecting microscope and two 1ml insulin syringes. The salivary glands were gently dissociated using a tissue homogenizer with RPMI-1640 to release the sporozoites. The sporozoites were then counted using a haemocytometer and diluted to 10 000 sporozoites/ml in RPMI-1640. To infect mice, 1000 sporozoites (100µl) were injected i.v. into the lateral tail vein. Mice were

monitored from six days after injection via thin blood films and once parasitaemia was between 5-10% mice were cardiac bled with 300U/ml heparin to prevent clotting. The blood was then mixed with an equal volume of *P. berghei* freezing medium containing 20% DMSO and aliquoted into vials which were subsequently snap-frozen in liquid phase LN₂. Stocks were stored in vapour phase LN₂.

2.7.5 Sporozoite challenge

To test the efficacy of liver-stage vaccines, vaccinated and naïve control mice were infected with 1000 sporozoites i.v. into the lateral tail vein as in 2.7.4. Mice were monitored from four or five days post-injection, dependent on mouse and parasite strain, via thin blood films. Once parasite positive blood films had been confirmed on three consecutive days, mice were sacrificed via cervical dislocation. The parasitaemia levels from three blood smears also allowed the calculation of the time to 0.5 or 1% parasitaemia via linear regression, dependent on the spread of data collected. If thin blood films were negative fourteen days post-infection mice were classed as ‘protected’ and were sacrificed by cervical dislocation.

2.7.6 *In vivo* imaging using the IVIS system

In certain challenge experiments, *in vivo* imaging of mice was also performed using the IVIS 200 imaging system as previously described [383]. When the transgenic parasites contained the luciferase reporter gene, mice were imaged 44 hours post-infection to assay the level of liver-stage burden via bioluminescence of the parasites. Mice were firstly shaved over the area of the liver, then anaesthetised (3.5% isoflurane, 2l/minute oxygen) and injected with 50µl 50mg/ml D-luciferin substrate s.c. into the scruff of the neck. Eight minutes after the injection of luciferin, mice were imaged for two minutes with the following settings: binning medium, F/stop 1,

excitation filter blocked and emission filter open. Quantification of the bioluminescence signal was performed using the Living Image 4.2 image analysis software program. A region of interest was created around the area of the liver and kept constant for all animals. The measurements were expressed as the total flux of photons emitted per second of exposure time.

2.7.7 Immunofluorescence antibody test (IFAT)

P. falciparum 3D7 sporozoites were isolated from the salivary glands of infected mosquitoes as per 2.7.4 with a minor modification; dissection was performed in PBS containing azide to kill the sporozoites. Sporozoites were counted and diluted to 2×10^5 sporozoites/ml with 100µl added to each well in an 8-well microscope slide. Slides were then air dried, wrapped in foil and stored in a sealed bag with desiccant at -80°C until further use. For the IFAT, all steps were performed in the dark at room temperature. Wells were initially blocked for two hours with 1% BSA in PBS/T, washed three times with PBS then serum samples were added at a dilution of 1/100 in PBS. Slides and sera were incubated together for one hour, washed three times followed by the addition of 1/200 Alexa Fluor® 488 conjugated goat anti-mouse IgG secondary antibody in 1% BSA PBS/T. Slides were incubated for 30 minutes, washed three times then mounted with Mowiol and a coverslip. Slides were dried at room temperature overnight in the dark.

2.7.8 Murine *in vitro* T cell killing assay

The *in vitro* T cell killing assay was developed as part of this thesis. Further details on its initial development and previous work undertaken in other laboratories can be found in Chapter 7, along with a diagrammatic overview of the assay.

2.7.8.1 *Preparation of hepatoma cells*

On Day -1 of the assay, the liver cell line Hepa1-6 was plated at 5×10^4 cells per well in a 96-well flat bottom plate. Prior to plating, the Hepa1-6 cells were labelled with the membrane dye Vybrant® DiD by incubating a suspension of cells (concentration 5×10^6 cells/ml in Hepa1-6 medium) with 10 μ l DiD per ml of cells for ten minutes at 37°C. Cells were subsequently washed twice in 15ml medium by centrifugation at 600xg for three minutes. Cells were counted using a haemocytometer, diluted to 5×10^5 cells/ml in Hepa1-6 medium and 100 μ l added per well of the 96-well flat bottom plate. The Hepa1-6 cells were left to form a monolayer overnight at 37°C 5% CO₂ in a humidified incubator.

2.7.8.2 *Infection of hepatoma cells with murine parasites*

On Day 0, *P. berghei* GFP sporozoites were dissected from the salivary glands of infected female *A. stephensi* mosquitoes as per 2.7.4. Sporozoites were counted using a haemocytometer and diluted to 4×10^5 sporozoites/ml in Hepa1-6 medium. Medium was removed from the Hepa1-6 liver cells previously prepared, and 40 000 sporozoites were added in 100 μ l Hepa1-6 medium per well. Plates were then spun at 500xg for five minutes and subsequently incubated at 37°C 5% CO₂ in a humidified incubator for a minimum of three hours to allow the sporozoites to invade the hepatocytes. To confirm only live sporozoites expressed GFP, sporozoites were heat-killed for twenty minutes at 95°C prior to addition in the assay.

2.7.8.3 *Addition of cytokines, drugs or splenocytes to the infected hepatoma cells*

Following the three-hour incubation, the medium was changed to reduce the chance of infection or experimental wells were initiated with the addition of cytokines, drugs or splenocytes. Experimental wells were performed in duplicate, or triplicate where

possible. Details on the addition of cytokines, drugs or splenocytes can be found in the figure legends in Chapter 7. When splenocytes were added, mice were sacrificed and spleens harvested. Enrichment of CD8⁺ cells was performed as described in 2.6.5. To inhibit the action of perforin-mediated cytotoxicity, enriched CD8⁺ splenocytes were pre-incubated with 10nM concanamycin A for twenty minutes at 37°C. To inhibit the action of cytokines such as IFN γ or TNF α , enriched CD8⁺ splenocytes were resuspended in medium containing blocking antibodies at various concentrations as detailed in the figure legends. The percentage of antigen-specific cells was calculated by setting up ICS (see 2.6.2) in parallel to the killing assay.

2.7.8.4 Assessment of infectivity by flow cytometry

24 hours after the addition of splenocytes, cytokines, drugs or fresh medium, cells were removed from plates by incubation for four minutes with 100 μ l trypsin. Cells were collected in 400 μ l 10% FCS in PBS in cluster tubes, then centrifuged at 200xg for three minutes. Cells were resuspended in 80 μ l 2% FCS in PBS. Immediately prior to running the cells on the flow cytometer (LSRII) 5 μ l of 1/1000 DAPI was added to stain dead cells. Infectivity was determined by the calculation of GFP⁺ Hepa1-6 cells. An example of the gating strategy is provided in Chapter 7.

Percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = 1 - (\text{test well} / \text{average of control wells}) \times 100$$

2.8 Clinical trials

Included in this thesis are the use of samples from the clinical trials known as Vac33, Mal34, Vac45 and Vac49. The NCT Numbers/Identifiers are NCT00890019, NCT00890760, NCT01623557 and NCT01465048, respectively (ClinicalTrials.gov).

Rhea Longley and Dr. Spencer performed all sample processing and *ex vivo* ELISpot assays for Vac49; Rhea Longley performed cultured ELISpots. Dr. Katie Ewer headed Vac33, Mal34 and Vac45, with sample preparation and initial *ex vivo* ELISpots performed by Research Assistants at the Jenner Institute. Rhea Longley performed all epitope mapping and cultured ELISpots presented in this thesis. For all trials, clinical staff at the Centre for Clinical Vaccinology and Tropical Medicine performed vaccination, challenge with *P. falciparum* and collection of blood.

2.8.1 Overview of ME-TRAP clinical trials

Volunteers in the ME-TRAP clinical trials followed the same vaccination regimen, unless otherwise stated; volunteers received 5×10^{10} viral particles (vp) ChAd63-ME-TRAP followed eight weeks later by 2×10^8 pfu MVA-ME-TRAP. In trials assessing efficacy, sporozoite challenge was performed approximately 21 days after the MVA-boost.

Vac33 was a Phase I study to assess the safety and immunogenicity of ChAd63-ME-TRAP alone and with MVA-ME-TRAP, using a prime-boost delivery schedule. As this was a dose escalation study, volunteers received either 1×10^8 , 1×10^9 , 1×10^{10} , 5×10^{10} or 2×10^{11} vp ChAd63-ME-TRAP. Not all volunteers received the MVA-boost.

Mal34 was a Phase I/IIa study to assess the protection against malaria by sporozoite challenge of healthy adults vaccinated with ChAd63-ME-TRAP and MVA-ME-TRAP.

Vac45 was a Phase I/IIa study to assess the protection against malaria by sporozoite challenge of healthy adults vaccinated with either of two vaccination regimens: ChAd63-ME-TRAP and MVA-ME-TRAP, or the same viral vectors encoding CSP.

2.8.2 Overview of Vac49 clinical trial objectives and study plan

In collaboration with the biotechnology company Sanaria (USA), a clinical trial was undertaken at the University of Oxford to determine the effectiveness of cryopreserved sporozoites to cause malarial infection in human volunteers. The primary objective was to determine whether cryopreserved sporozoites could result in 100% infectivity of human volunteers and hence provide a more consistent challenge model for vaccine efficacy studies than the current gold standard of sporozoites delivered by infectious mosquito bite. Secondary objectives were to look at the immune response to a panel of malaria antigens in volunteers after their first exposure to malaria.

Eighteen malaria-naïve healthy adults were challenged with cryopreserved *P. falciparum* sporozoites. Six volunteers received 2500 sporozoites i.d., six received 2500 sporozoites i.m. and six received 25 000 sporozoites i.m. Volunteers were monitored for malarial infection from six days post-challenge via blood smears and real-time PCR. Blood samples for immunological assays were taken on days C-1, C+1, C+7, C+9, C+11, C+14, Diagnosis, C+35 and C+90. *Ex vivo* IFN γ ELISpots were performed at C-1, C+7, C+11, C+35 and C+90.

2.8.3 Isolation of human PBMCs, plasma and serum

Blood received for serum collection was centrifuged at 600xg for five minutes. Serum was then collected and stored at -80°C until further use.

For the collection of PBMCs and plasma, leucosep tubes were prepared by the addition of 15ml lymphoprep to each tube and centrifugation for one minute at 1000xg. Approximately 15-30ml of blood was then added per tube and centrifuged for thirteen minutes at 1000xg without break at room temperature. At this stage plasma was collected from the tubes and stored at -80°C. Excess plasma was discarded and the PBMCs were collected from the interface and transferred into new tubes. Tubes containing PBMCs were topped to 40ml with R0 and centrifuged at 600xg for five minutes at room temperature. The supernatant was discarded, the pellet resuspended in 30ml R0 and centrifuged again. The resultant pellet was resuspended in 10ml R10 and counted using a CASY counter.

2.8.4 Freezing and thawing of PBMCs

Excess PBMCs were frozen at $5-10 \times 10^6$ cells per vial using Mr. Frosty Cryocontainers which were pre-chilled for at least 30 minutes prior to use. PBMCs were centrifuged at 600xg for five minutes at room temperature, the supernatant discarded and pellet resuspended in 0.5ml of FCS per vial to be frozen. PBMCs in FCS were then placed at 4°C for 30 minutes to begin the cooling process. Subsequently, an equal volume of 20% DMSO in FCS was added to the PBMCs, which were then aliquoted into cryovials and placed in the pre-cooled Mr. Frostys. The Mr. Frostys were then stored at -80°C overnight with vials transferred to LN₂ the following day.

Frozen PBMCs were thawed quickly by immersion in a 37°C water bath; once almost thawed cells were added drop-wise to 15ml warm R10 in a falcon tube. Cells were centrifuged at 600xg for five minutes and resuspended in 2ml R10 and 2µl benzonase

per ml of cells. Cells were subsequently incubated for at least two hours at 37°C 5% CO₂ in a humidified incubator prior to use.

2.8.5 Sorting of CD8⁺ and CD4⁺ T cells

To determine whether the IFN γ response was CD8⁺ or CD4⁺ dependent, these cell types were separated using Stem Cell Technologies Easy Sep positive selection beads, prior to the IFN γ ELISpot.

Cells were diluted to 1x10⁸ cells/ml in cell separation media and placed in a 5ml polystyrene tube. 100 μ l of positive selection cocktail was added per ml of cells and incubated at room temperature for fifteen minutes. The magnetic nanoparticles were mixed vigorously by pipetting, and then 50 μ l were added per ml of cells for CD4⁺ isolation and 100 μ l per ml of cells for CD8⁺ isolation. The cells were mixed well then incubated for fifteen minutes at room temperature. The volume was then increased to a total of 2.5ml per tube by the addition of further cell separation medium. To separate the cells, the tube was placed inside the provided magnet for ten minutes. The magnet and tube were then inverted for two to three seconds and the supernatant collected; labelled cells remained within the magnet. The tube was then removed from the magnet; 2.5ml cell separation medium was added and mixed by pipetting. The tube was subsequently placed back in the magnet for a further ten minutes. The previous steps were repeated for a total of three ten-minute separations.

Prior to using the depleted cell populations in an IFN γ ELISpot assay, the purity of the fractions was analysed by flow cytometry using 1/20 anti-human CD8-APC, 1/20 anti-human CD3-FITC and 1/20 anti-human CD4-PeCy5.5 in FACS buffer.

2.8.6 Human *ex vivo* IFN γ PBMC ELISpot assay

Peptides used are detailed in section 2.6.1. All ELISpot reagents were supplied in a human IFN γ ELISpot kit from Mabtech. ELISpot plates were coated with 50 μ l per well of 10 μ g/ml anti-IFN γ purified monoclonal antibody 1-D1K in coating buffer and incubated at 4°C overnight. Plates were then washed two times with 100 μ l PBS and subsequently blocked for one hour at room temperature with 100 μ l R10 medium. Human PBMCs were prepared as described in 2.8.3 or thawed as described in 2.8.4 and diluted to 5x10⁶ cells/ml. Blocking solution was then removed from plates, and 50 μ l positive control (10 μ g/ml PHA/0.02 μ g/ml SEB), negative control (R10) or peptides at 20 μ g/ml (200 μ g/ml when 9mers or lower were used, as they were crude peptides) were added per well, unless otherwise stated. 50 μ l PBMCs at 5x10⁶ cells/ml were then added to the appropriate wells in duplicate. Plates were incubated for 18-20 hours at 37°C 5% CO₂ in a humidified incubator.

Following incubation, plates were washed six times with PBS/T then incubated with 50 μ l per well of 1 μ g/ml biotinylated anti-human IFN γ mAb 7-B6-1 diluted in PBS for two hours at room temperature. Plates were subsequently washed again and incubated with 50 μ l per well of 1 μ g/ml streptavidin alkaline phosphatase polymer diluted in PBS for one hour at room temperature. Plates were washed again and finally incubated with 50 μ l per well of ELISpot development buffer (BCIP/NBT-Plus) for approximately three to five minutes at room temperature until spots developed. Washing the plates with tap water stopped the reaction and once plates were dry spots were counted using an AID ELISpot plate counter. Responses were expressed as spot forming cells (SFC) per million PBMCs. Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

2.8.7 Human cultured IFN γ PBMC ELISpot assay

Cells were thawed as per 2.8.4, counted and diluted to 5×10^6 cells/ml in R10. 1ml of cells was added to the middle wells of a 12-well plate; the outer wells contained 2ml R10 to avoid an edge effect. Peptide pools were prepared at $4 \mu\text{g/ml}$ in R10 and 1ml was added per well to the appropriate wells. Cells were incubated at 37°C 5% CO_2 in a humidified incubator for three days.

On Day 3, 1ml of media was carefully removed from the wells and replaced with 1ml R10 containing recombinant human IL-2 at a final concentration of 25IU/ml in each well. Cells were incubated for a further four days. On Day 7, IL-2 was added again as per Day 3 and cells were incubated for another two days.

On Day 9, cells were gently removed from the 12-well plates by pipetting and transferred into 15ml falcon tubes. Wells were washed five times with 2ml R10 to remove all cells. Cells were then centrifuged at $600 \times g$ for five minutes, and then washed three times with 10ml R10 to remove all IL-2. Cells were finally resuspended in 5ml R10 and incubated at 37°C 5% CO_2 in a humidified incubator overnight. ELISpot plates were coated as per 2.8.6.

On Day 10 ELISpot plates were washed and blocked as per 2.8.6. Cells were centrifuged and resuspended in 2.5ml ($=2 \times 10^6$ original cells/ml). Cells were counted for reference, and then cells and peptides were added to the ELISpot plates as per 2.8.6. Wells were performed in quadruplicate. Plates were incubated for 18-20 hours at 37°C 5% CO_2 in a humidified incubator. Plates were developed on Day 11 as per

2.8.6. Final analysis expressed the number of SFC per million original PBMCs (at Day 0).

When required, CD8⁺ T cells were sorted after ten days of stimulation using the Miltenyi Biotec CD8⁺ T cell isolation kit as per the manufacturer's instructions (for basic protocol see 2.6.4.2).

2.8.8 Human *in vitro* T cell killing assay

The human *in vitro* T cell killing assay was developed as part of this thesis. Development of the human assay was undertaken in collaboration with Prof. Dominique Mazier's laboratory at INSERM UMRS 945 (Paris, France), and builds upon work developed in the murine assay (see 2.7.8). This work was performed jointly by Rhea Longley and Dr. Valerie Soulard.

2.8.8.1 Inclusion criteria for PBMCs and human hepatocytes

To be used in the *in vitro* T cell assay, PBMCs were required to be HLA-A2 or A3, have a cultured IFN γ ELISpot response greater than 700 SFC per million original PBMCs to *P. falciparum* TRAP and greater than ten frozen vials of cells remaining. Human hepatocyte samples were included if they were HLA-A2 or A3 and plated well after thawing.

2.8.8.2 Culture of primary human hepatocytes

Research staff at INSERM cultured primary human hepatocytes, either from fresh healthy sections from human liver fragments collected from unrelated surgery, or from samples previously frozen. Cultures were maintained as previously described [384, 385]. Briefly, hepatocytes were obtained by collagenase perfusion of the liver sample followed by centrifugation over a 40% percoll gradient. 8×10^4 hepatocytes

were cultured per well of a 96-well collagen coated flat bottom plate in supplemented William's E medium. Frozen hepatocytes were thawed as previously described [385], then cultured as per fresh hepatocytes. Hepatocytes were HLA-A typed by Dr. Soulard using the SSP HLA-A low-resolution kit (Olerup, Austria), following the manufacturer's instructions. The collection, HLA typing and use of this material was approved by the Institutional Review Board (Comité de Protection des Personnes) of the Centre Hospitalo-Universitaire Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, France.

2.8.8.3 Infection of primary human hepatocytes and the addition of T cells

P. falciparum NF54 infected mosquitoes were dissected as per 2.7.4, with minor modifications. Mosquitoes were first decontaminated in 70% ethanol, followed by washing a further three times in Leibovitz's L15 medium containing 1% pen/strep and 1/1000 Fungizone®. Once salivary glands had been dissected and homogenised, they were passed through a 40µm filter prior to counting. Finally, they were centrifuged at 300xg for three minutes, resuspended and 40 000 sporozoites were added per well to the cultured liver cells. Infected liver cells were incubated for one hour at room temperature, followed by a further two hours at 37°C. The media was then changed, followed by further incubation for approximately four hours followed by the addition of re-stimulated TRAP-specific PBMCs (as per 2.8.7). The *in vitro* assay was incubated for approximately two days from infection.

2.8.8.4 Staining of intrahepatic parasites

Wells were fixed in cold methanol for five to ten minutes, washed in PBS then left in PBS containing azide overnight. The following day, the PBS/azide was removed and wells were washed two times in PBS/BSA (1%). Wells were stained for two hours at

room temperature with 1/2000 of polyclonal mouse serum directed against *P. falciparum* heat-shock protein 70 (HSP70) [386] diluted in PBS/BSA (1%). Wells were subsequently washed a further two times followed by incubation for 40-45 minutes at room temperature with 1/500 goat-anti human Alexa Fluor® 488 secondary antibody and 1/100 DAPI in PBS/BSA (1%). Wells were finally washed, and left in PBS/azide in the dark at 4°C until analysis by fluorescent microscopy as described in Chapter 7.

2.9 Statistical analysis

All statistical analyses were carried out using Prism version 5 (Graphpad, USA). Normality of data distribution was assessed using the D'Agostino-Pearson normality test. All LIPS data and ELISA titres were $\log_{10}$ transformed prior to analysis. A Mann Whitney test was used to compare the medians of two groups of non-parametric data, and the Kruskal-Wallis test with Dunn's Multiple Comparison post-test for comparing the medians between more than two groups. When groups represented repeated measures, the Friedman's test with Dunn's Multiple Comparisons post-test was used. Correlations were tested using Spearman's rank correlation on non-parametric data. A two-way ANOVA with Bonferroni post-test for multiple comparisons was used to explore the effect of two variables. Survival in challenge experiments was presented using Kaplan-Meier curves and significance tested using the Log-Rank (Mantel-Cox) Test. Significance was taken as $p < 0.05$, with the following notations: * $p < 0.05$ -0.01, ** $p < 0.01$ -0.001, *** $p < 0.001$ and **** $p < 0.0001$. Non-parametric data are shown as median $\pm$ inter-quartile range, or with individual data points plotted, unless otherwise indicated.

**Chapter 3: Selection and design of candidate
liver-stage antigens for a *Plasmodium*
falciparum vaccine**

3 Selection and design of candidate liver-stage antigens for a *Plasmodium falciparum* vaccine

3.1 Introduction

The two leading malaria vaccines currently in development, RTS,S and ChAd63-MVA ME-TRAP, are both sub-unit vaccines targeting the pre-erythrocytic stage of the parasite's life-cycle. The pre-erythrocytic stage is considered an excellent target for a malaria vaccine given it precedes development of clinical symptoms, lasts for an extended period of time in human hosts (approximately five to ten days) and represents a bottle-neck in the life-cycle before the number of parasites increase exponentially in the blood-stage. Furthermore, immunization with radiation-attenuated sporozoites (through the bite of infectious mosquitoes) has demonstrated that sterile protection is achievable with a vaccine directed at the pre-erythrocytic stage [45-49, 53]. Whilst such a vaccine is not yet plausible for large-scale delivery, it does provide a generally accepted “gold-standard” for the development of pre-erythrocytic malaria vaccines.

As introduced in Chapter 1, RTS,S is a recombinant protein vaccine that fuses the NANP repeat and C-terminal regions of CSP with HBsAg, and when expressed in yeast with additional non-recombinant HBsAg, these antigens form particulate structures. When administered in combination with a proprietary series of adjuvants from GSK, known as AS01 and AS02, efficacy in malaria-naive adults was approximately 40-50% [230, 319], however, reduced efficacy was seen with age de-escalation into endemic areas [320, 322, 328, 329]. This protective efficacy has been associated with high antibody titres against CSP, with limited to no production of

CD8⁺ T cells [179, 231, 387]. ChAd63-MVA ME-TRAP is a heterologous eight-week interval prime-boost approach, delivering the pre-erythrocytic antigen TRAP fused to a multi-epitope string (ME). This vaccination regimen has resulted in extremely high levels of T cell immunogenicity in humans [256], and moderate levels of protective efficacy, with 21% sterile protection in a recent Phase IIa CHMI trial [160].

However, both RTS,S and ChAd63-MVA ME-TRAP probably do not currently deliver adequate protective efficacy to progress to deployment in the field, although some maintain that RTS,S/AS01 may be deployable. Whilst CSP and TRAP have important functional roles in the onset of liver-stage malaria [73, 307-310, 337-340], and responses against these antigens have been demonstrated to contribute to protection induced by irradiated sporozoites [225, 226, 341], there is increasing evidence that other antigens are also involved. For example, vaccination with *P. berghei* irradiated sporozoites was able to induce sterile protection in the absence of *P. berghei* CSP in the challenge parasite (using a transgenic parasite with *P. berghei* CSP replaced by *P. falciparum* CSP) [388]. In a similar study, CSP transgenic mice were developed that were tolerant to CSP epitopes [389]. Upon vaccination with irradiated sporozoites the subsequent protection was greatly reduced, but not ablated, again suggesting a role for other antigens. Furthermore, researchers using bioinformatics predictions coupled with *in vitro* testing found that human immune responses to known antigens could not amount to the protection seen in the irradiated sporozoite vaccination model. They also saw no strict immunodominance and in fact found several new epitopes more antigenic than previously identified proteins such as CSP and TRAP [390]. More recently the same author suggests that the failure to

develop a malaria vaccine so far could be attributed to the historic focus on less than 0.5% of potential antigen candidates [391].

The liver-stage of malaria is thought to be the target of cell-mediated immunity, as the parasite resides within the hepatocyte and the immune system can only interact with antigenic targets transported to the surface of the cell. CD8⁺ T cells are likely the important effector cells, as shown through three main experimental findings: (1) CD8⁺ T cells from mice vaccinated with radiation or genetically attenuated sporozoites can eliminate *P. berghei* and *P. yoelii* liver-stage parasites *in vitro* [55, 165, 168], (2) adoptive transfer of CD8⁺ T cell clones specific for TRAP or CSP can induce protection against *P. berghei* and *P. yoelii* in naive mice [342, 392-394] and (3) depletion of CD8⁺ T cells completely abolishes the protection seen in radiation and genetically attenuated sporozoite models [164, 168, 202-204, 211, 395]. The adenoviral prime-MVA boost vaccination regimen is capable of producing extremely high levels of cellular immunogenicity, not only for liver-stage malaria vaccines, but also in a number of other disease settings (for example *Toxoplasma gondii* [396], HIV [397] and blood-stage malaria [257]).

Over 5000 different proteins are expressed throughout the *Plasmodium* life-cycle, leading to a high probability that a better target antigen than CSP or TRAP may exist, or a target antigen to be used alongside CSP or TRAP in a multi-component vaccination strategy. The results in this chapter describe the selection and development of a number of new *P. falciparum* liver-stage malaria vaccines, by searching the literature for potential candidate antigens and subsequently cloning the chosen antigens into the viral vectors ChAd63 and MVA. ChAd63 has become a

leading adenoviral vector due to its potency and the low levels of neutralizing antibodies observed in the general population [261], and is available for use in malaria vaccine studies in Oxford through an agreement with Okairos (Rome, Italy). These developed vaccines were then assayed for expression of the antigen insert by cellular immunogenicity in inbred mice.

3.2 Results

3.2.1 Identification of candidate liver-stage antigens for a malaria vaccine

A literature search was conducted to identify suitable antigens for a liver-stage malaria vaccine. The search was initially broad, and twelve antigens were chosen based on the following parameters: each was either well expressed at the liver-stage (in *P. falciparum* or rodent homologs), had been identified as a target of a T cell-mediated immune response in natural infection or during irradiated sporozoite immunization, or previous studies had shown a degree of protection using the antigen as a vaccine in mice (rodent *Plasmodium* homologs) or non-human primates. The twelve antigens were: PF14_0179, PfCelTOS, PFE1590w, PfExp1, PFI0580c, PflSA1, PflSA3, PflSAP1, PflSAP2, PfSAP1, PfUIS3 and PfUIS4. They ranged from recently identified antigens (PF14_0179, PFE1590w, PflSAP1 and PflSAP2) to well-known candidates that had previously been tested extensively in other vaccination regimens (PfExp1, PflSA1 and PflSA3). A summary of the selected candidate antigens and the rationale for their inclusion is outlined in Table 3.1.

Table 3.1. List of identified *P. falciparum* candidate antigens and the rationale for their inclusion.

Protection refers to the antigen being used as a vaccine in various platforms. Protection in non-human primates is using the *P. falciparum* gene, whereas protection in mice refers to a murine homolog. “ispz” refers to vaccination with irradiated sporozoites. Antigens localized to the parasitophorous vacuole membrane (PVM) were considered more likely to be in contact with the hepatocyte, and hence expressed as peptides on the cell surface.

Antigen	Reason for inclusion	Ref.
PF14_0179	Localised to the PVM; liver-stage specific; homolog of most abundant liver-stage <i>P. yoelii</i> transcript; <i>P. berghei</i> homolog important for parasite egress from hepatocytes	[398, 399]
PfCelTOS	Target of T cells in volunteers immunized with ispz; <i>P. falciparum</i> homolog was protective against <i>P. berghei</i> challenge in mice; highly conserved; potential for transmission-blocking effect as expressed at mosquito-stage	[51, 390, 400, 401]
PFE1590w	Interaction with apolipoprotein alleles that are associated with increased risk of malaria	[402]
PfExp1	Target of T cells in naturally exposed individuals; protection in mice (<i>P. yoelii</i> homolog) and monkeys; highly conserved	[403-406]
PFI0580c	Protection in mice (<i>P. yoelii</i> homolog) in combination with PyUIS3; <i>P. berghei</i> homolog inhibits parasite growth	[131, 407, 408]
PfLSA1	Target of T cells in naturally exposed individuals and volunteers immunized with ispz; liver-stage specific	[409-411]
PfLSA3	Target of T cells in naturally exposed individuals and volunteers immunized with ispz; protection in mice (<i>P. yoelii</i> homolog), chimpanzees and monkeys; highly conserved	[403, 412-415]
PfLSAP1	Upregulated during liver-stage infection in <i>P. falciparum</i> ; liver-stage specific	[416]
PfLSAP2	Upregulated during liver-stage infection in <i>P. falciparum</i>	[416]
PfSAP1	Essential for liver-stage development; protection in mice (<i>P. yoelii</i> homolog); <i>P. yoelii</i> SAP1 GAP immunization induced sterile protection	[417-419]
PfUIS3	Protection in mice in combination with PFI0580c (<i>P. yoelii</i> homologs); <i>P. yoelii</i> UIS3 GAP immunization induced sterile protection; transcript level was reduced by 53% in ispz	[52, 407, 420]
PfUIS4	Localized to the PVM (<i>P. yoelii</i> homolog); essential for liver-stage development	[208]

The chosen candidate antigens are expressed at various stages throughout the parasite's life-cycle, in addition to strong expression at the liver-stage. Only PfLSA1, PF14_0179 and PfLSAP1 are thought to be liver-stage specific [398, 410, 416]. A schematic representation of the expression of the antigens is given (Figure 3.1).

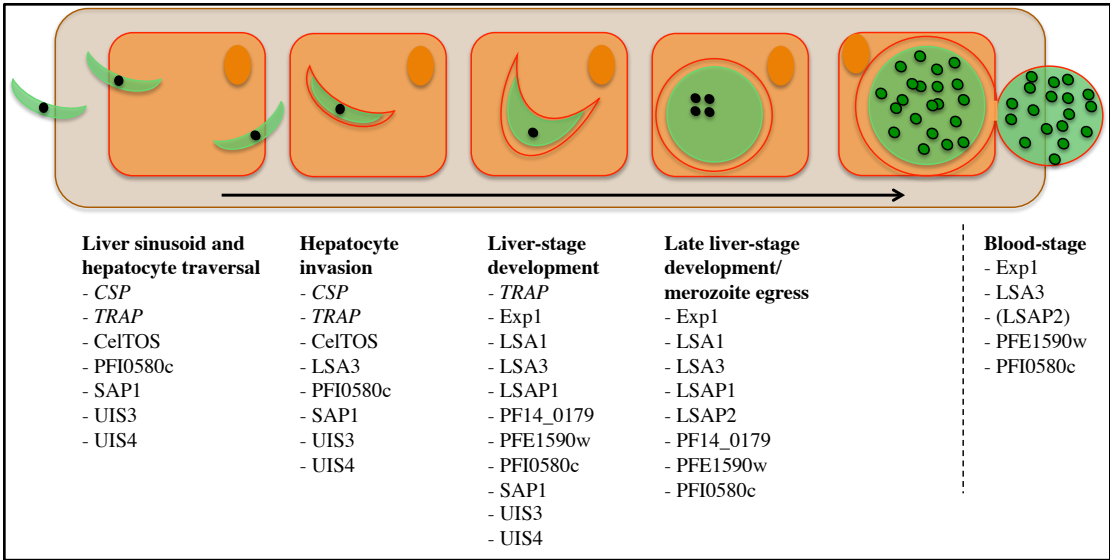


Figure 3.1. Schematic representation of the expression of the candidate antigens throughout the parasite's life-cycle.

The diagram indicates where the antigens are likely expressed in comparison to the two leading candidates CSP and TRAP, but does not necessarily mean they are essential for that part of the life-cycle. The expression profile is based on evidence from *P. falciparum* where possible, but also from rodent homologs. LSAP2 expression has been suggested to extend into the blood-stages [421], but this has not been confirmed. CelTOS is the only antigen that expression has been demonstrated for during the mosquito-stages.

3.2.2 Generation of ChAd63 and MVA molecular constructs

The twelve candidate antigens were assessed for their suitability for expression in the viral vectors ChAd63 and MVA. First, *P. falciparum* homologs needed to be identified for those antigens for which research had only been on the murine homolog. The 3D7 strain was chosen as it has been fully sequenced [224] and is also the challenge strain used in CHMI clinical trials at the Jenner Institute. *P. falciparum* genes were identified for all candidates except for UIS4. UIS4 was chosen as a candidate antigen as it is required for liver-stage development (as indicated by UIS4 GAPs) and is also located in the PVM, which suggests it is likely to have a role interacting with the host cell [208]. A predicted *P. falciparum* ortholog was identified as PF10_0164 (30% identity) through chromosomal synteny searches [208], and it was also structurally similar to *P. berghei* and *P. yoelii* UIS4. However, further research indicated that PF10_0164 is unlikely to be a functional ortholog of UIS4. PF10_0164 was found to be essential for blood-stage development and was not able to complement *P. yoelii* UIS4 in a transgenic replacement model [422]. Therefore UIS4 was excluded from the *P. falciparum* candidate antigen screen.

Second, various online servers outlined in Chapter 2 were used to predict the structure of the eleven remaining candidates. This was to determine whether the antigen was likely to be secreted, contain transmembrane domains or repetitive sequences (Table 3.2). For both PfSAP1 and PF14_0179, it was discovered that these were very large antigens (greater than 2900 amino acids) and it was questionable as to whether they would fit into the ChAd63 vector, given E1 and E3 deleted adenoviruses can accommodate only up to 8kb (approximately 2600 amino acids) [423]. Both antigens contained repeat regions but even deletion of these sequences

did not substantially reduce the size, and given the increased cost of a larger sequence, both PfSAP1 and PF14_0179 were excluded from the *P. falciparum* candidate antigen screen.

The remaining nine candidate antigens were then subjected to various modifications prior to ordering the DNA sequence (Table 3.2), however with the overriding principle of keeping the sequence as native as possible given this was the first time most of these genes had been expressed in viral vectors. Most antigens had the tPA leader sequence [364] added upstream, to aid in secretion of the antigen and to potentially increase expression and thereby immunogenicity [424-426]. The exceptions were PflSA3, PflSAP1 and PflUIS3 as these antigens were predicted to contain two transmembrane domains (anchored in the membrane) and it was considered that adding a signal sequence could drastically alter the structure of the protein. The antigens PFE1590w, PflSA1 and PflSA3 contained repetitive regions of sequence; such regions are considered deleterious as they increase the overall size making expression in viruses more difficult, and they also increase the likelihood of homologous recombination events when inserted into viral vectors. For PFE1590w, the repeats were not removed, as the overall size of the antigen was only 181 amino acids. For PflSA1, it contained 43 degenerative seventeen amino acid repeats (in 3D7). The repeats were aligned in MegAlign (DNASTAR Lasergene suite, USA) via ClustalW and one copy of the most representative repeat (DLEGERLAKEKLWWR) and one copy of the most different repeat (DLERTKASTETLHEQQS) were kept, the others were deleted (see Appendix Three for alignment). PflSA3 contained numerous repeats and these were deleted to one copy (see Appendix Three for modified sequence). Lastly, all sequences were codon optimized for expression in

mammalian cells. Codon usage differs substantially between *P. falciparum* and mammalian cells; it has previously been shown that using human codon optimization can increase the expression and immunogenicity of *Plasmodium* antigens in eukaryote expression systems and vaccine models [427, 428], however the effect may be antigen-dependent [429].

The modified sequences were then ordered from GeneArt and cloned into the viral vector plasmids ChAd63 and MVA, as described in Chapter 2. Restriction enzyme digestion or DNA sequencing was used to screen and check the success of each cloning step. PflSA3 proved to be exceptionally difficult to clone into the plasmids, with the majority of clones not containing the insert. PCR screening was required to find clones that had successfully integrated the insert into the destination plasmid. Finally, each plasmid was then transfected into T-REx™ 293 cells or CEFs and purified as viruses, with purity and identity determined by PCR. The only problematic virus was ChAd63-PfExp1, which the Viral Vector Core Facility failed to produce after the initial preparation. The initial stock was of very low yield (3.1×10^9 ifu/ml) and only allowed one vaccination experiment, for which results are shown. Multiple attempts were made to produce the virus, but numerous problems were encountered including insufficient CPE, poor caesium chloride bands and very high P:I ratios (number of particles to infectious particles). After careful consideration, PfExp1 was excluded from the *P. falciparum* candidate antigen screen, given the likely difficulties in making a clinical grade vaccine.

Table 3.2. Sequence construct design for the selected *P. falciparum* candidate antigens.

Gene ID refers to the PlasmoDB Gene ID (<http://plasmodb.org/plasmo/>), size is given in amino acids and the predicted structure indicates whether the antigen had a predicted signal peptide, transmembrane domain or any repetitive sequences. The final two columns refer to modifications of the antigens: whether or not a tPA leader sequence was added and whether any repetitive sequences were removed.

Antigen	Gene ID	Size	Predicted Structure	tPA	Repeats
PF14_0179	PF14_0179	3597	Unclear; repeats	N/A	N/A
PfCelTOS	PFL0800c	182	Signal peptide	Yes	N/A
PFE1590w	PFE1590w	181	Signal peptide; transmembrane domain; repeats	Yes	Did not delete
PfExp1	PF11_0224	162	Signal peptide; transmembrane domain	Yes	N/A
PFI0580c	PFI0580c	413	Signal peptide	Yes	N/A
PfLSA1	PF10_0356	1162	Signal peptide; 17aa repeats	Yes	Deleted to two copies
PfLSA3	PFB0915w	1558	Non-secretory; two transmembrane domains; many repeats	No	Deleted to one copy
PfLSAP1	PFL0065w	106	Signal peptide, two transmembrane domains	No	N/A
PfLSAP2	PFB0105c	302	Non-secretory; transmembrane domain	Yes	N/A
PfSAP1	PF11_0480	2940	Unclear; repeats	N/A	N/A
PfUIS3	PF13_0012	229	Signal peptide; two transmembrane domains	No	N/A

3.2.3 T cell immunogenicity of ChAd63 and MVA Pf[antigen] vaccines

To confirm successful expression of the antigen from the viral vectors, mice were vaccinated and antigen-specific *ex vivo* spleen IFN γ ELISpots were performed to pools of overlapping peptides spanning the length of the antigen. All peptide sequences are provided in Appendix Two. This method of assessing expression was chosen given all antigens had predicted epitopes in BALB/c mice (Table 3.3). The predicted epitopes were compared to the predicted strength of the strong *P. berghei* CSP H-2^d-restricted epitope known as Pb9 [393].

Table 3.3. Predicted T cell epitopes in the *P. falciparum* candidate antigens (overleaf).

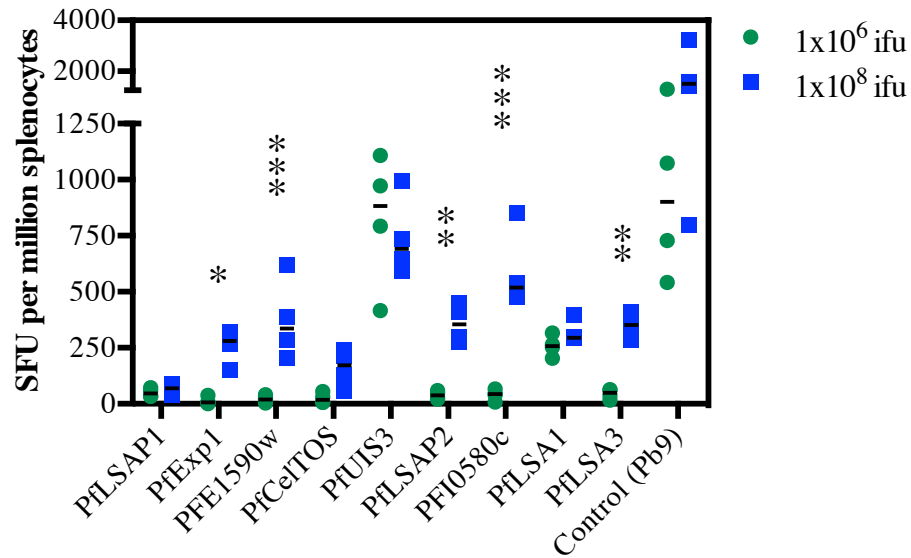
T cell epitopes were predicted using two predictions servers, SYFPEITHI and IEDB, using H-2^d for both MHC Class I and II. The H-2^d-restricted epitope Pb9, from *P. berghei* CSP, was used as a control for epitope strength. Using SYFPEITHI [377], the higher the score the greater the likelihood the peptide is processed and presented, based on binding motifs. Pb9 was given a score of 32. The IEDB prediction server uses two different methods for MHC Class I and II. IEDB employs the ANN method [430, 431] to determine the likely ability of the sequence to bind MHC Class I molecules and gives the result an IC50. IC50 less than 50nM were considered high; Pb9 was given an IC50 of 4.44. For predicted CD4⁺ epitopes IEDB uses a consensus approach [432, 433] to combine different methods and the score is given as a percentile rank, comparing the peptides average score of four methods against 5 million random 15mers selected from the SWISSPROT database. A small percentile rank indicates high affinity. For PfLSA1, a number of epitopes had previously been identified and were also included [434].

Table 3.3. Predicted T cell epitopes in the *P. falciparum* candidate antigens (legend on previous page).

Antigen	Epitope (score)	Sequence	Source
PfCelTOS	Peptide 30 (33)	NKVETAVGAKVSDEI	SYFPEITHI
	Peptide 2 (30)	NKVETAVGAKVSDEI	
	Peptide 18 (32)	ESSFDIKSEVKKHAK	
PFE1590w	Pred. 1 (10.10)	IGTALYASRKSSKAC	IEDB (cons)
	Peptide 11 (24)	SSSSSSPSASSSSSS	SYFPEITHI
	Peptide 12 (24)	SDDSKNASLDKIDEE	
PfExp1	Pred. 1 (28)	GSGVSSKKKNKKGSG	SYFPEITHI
	Pred. 2	KSKEYKLATSVLAGLLGVV	SYFPEITHI +
	(27/7.4/0.06)		IEDB
PFI0580c	Pred. 1 (10.12)	QLVGGSSMLISKIKP	IEDB (cons)
	Peptide 33 (28)	GFVWTL LGVHKKKPL	SYFPEITHI
	Peptide 35 (27)	SYVSPDISV	
	Peptide 30 (31.8)	KIISINSTV	IEDB (ANN)
PfLSA1	Dominant A	EKEKFIKSLFHIFDG	[434]
	Dominant B	DKSLYDEHIKKYKND	
	Peptide 17 (27)	KKEHGDVLAEDLYGR	SYFPEITHI
PfLSA3	Peptide 118 (31/2594)	APFISAVAAFASYVV	SYFPEITHI + IEDB (ANN)
PfLSAP1	Pred. 1 (30/1.59)	NTKTQLILSAVCASILLSL	SYFPEITHI + IEDB (cons)
	Peptide 1 (21)	LFILILNTI	IEDB (ANN)
PfLSAP2	Pred. 1 (28)	EHKL PFKIKMKKWWK	SYFPEITHI
	Peptide 6 (26)	KDFYMLFLSNKKEKI	
	Peptide 23 (4.4)	KFNKMKSSL	IEDB (ANN)
	Peptide 8 (1.12)	SKFMKLSISLLLLAL	IEDB (cons)
PfUIS3	Pred. 1 (32/1.24)	KLIYYSLIASGAIAS	SYFPEITHI + IEDB (cons)
	Pred. 2 (129.8)	KYQENKAVM	IEDB (ANN)

ELISpots were performed two weeks after single-shot vaccination, using two different doses for ChAd63-[antigen] and one dose for MVA-[antigen]. All vaccines, both ChAd63 and MVA, successfully induced an antigen-specific immune response (Figure 3.2). The greater dose of ChAd63 resulted in a higher IFN γ response ($p < 0.0001$, two-way ANOVA), except for PfLSAP1, PfCeITOS, PfUIS3 and PfLSA1 where the response was not statistically different (Figure 3.2 A). ChAd63-PfLSAP1, the smallest antigen in size, was the least immunogenic of the vaccines. Single-shot MVA vaccination resulted in lower immunogenicity than single-shot ChAd63 vaccination, with an overall lower number of SFU per million splenocytes observed (Figure 3.2 A compared to B). As the results in Figure 3.2 A were obtained over four experiments, the results from the control vaccine *P. falciparum* CSP (containing the Pb9 epitope) were also included to demonstrate the variability in ELISpot results between experiments.

A ChAd63-[antigen]



B MVA-[antigen]

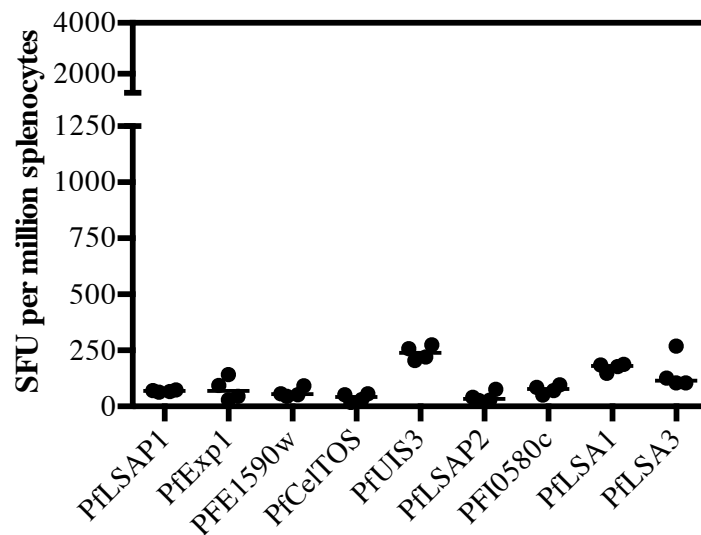


Figure 3.2. T cell immunogenicity of ChAd63-[antigen] and MVA-[antigen] vaccines.

BALB/c mice (n=4) were vaccinated i.m. with (A) ChAd63-[antigen] at a high dose of 1×10^8 ifu or a low dose of 1×10^6 ifu or (B) 1×10^7 pfu MVA-[antigen]. T cell immunogenicity was assessed two weeks post-vaccination by *ex vivo* spleen IFN γ ELISpot to a pool of overlapping peptides to the appropriate antigen (range 10 to 130 peptides per antigen). Median and individual data points are shown. The results in part (A) were performed over four separate experiments and compared to responses against the control vaccine *P. falciparum* CSP (which contains the Pb9 epitope). Each data point from the control represents the median result of CSP from one of the four different ELISpots performed. Statistical difference between the high and low doses was assessed using a two-way analysis of variance (ANOVA) with Bonferroni post-test, * $p=0.05-0.01$ ** $p=0.01-0.001$ *** $p<0.001$. All vaccines were deemed immunogenic with 1×10^8 ifu ChAd63 and 1×10^7 pfu MVA. The antigens are arranged in size order on the x-axis.

Whilst initial analysis of expression and immunogenicity was performed using overlapping peptides to the entire antigen sequence, *ex vivo* spleen IFN γ ELISpot was also performed using the individual predicted peptides (Table 3.3). When mice were vaccinated with the high dose of ChAd63-[antigen], the majority of animals did not respond to the predicted epitopes despite responding to the overlapping peptide pool (Figure 3.3). The exceptions were PfLSAP1, PfUIS3, PFI0580c and PfLSA1. For PfLSAP1, a low response was observed to the predicted epitopes; in contrast, the predicted epitope 2 for PfUIS3 had a response approximately double that of the peptide pool. Peptide 33 for PFI0580c was potentially a sub-dominant epitope with an IFN γ response approximately four fold lower than that of the total peptide pool. For PfLSA1, a response to the previously identified Dominant A epitope [434] was confirmed, however the Dominant B epitope was not recognized. The overall accuracy of the prediction servers was low, with SYFPEITHI predicting 13.3% (2/15) correctly, the IEDB ANN method predicting 40% (2/5) and the consensus method predicting 16.7% (1/6) correctly for an overall accuracy rate of 19%.

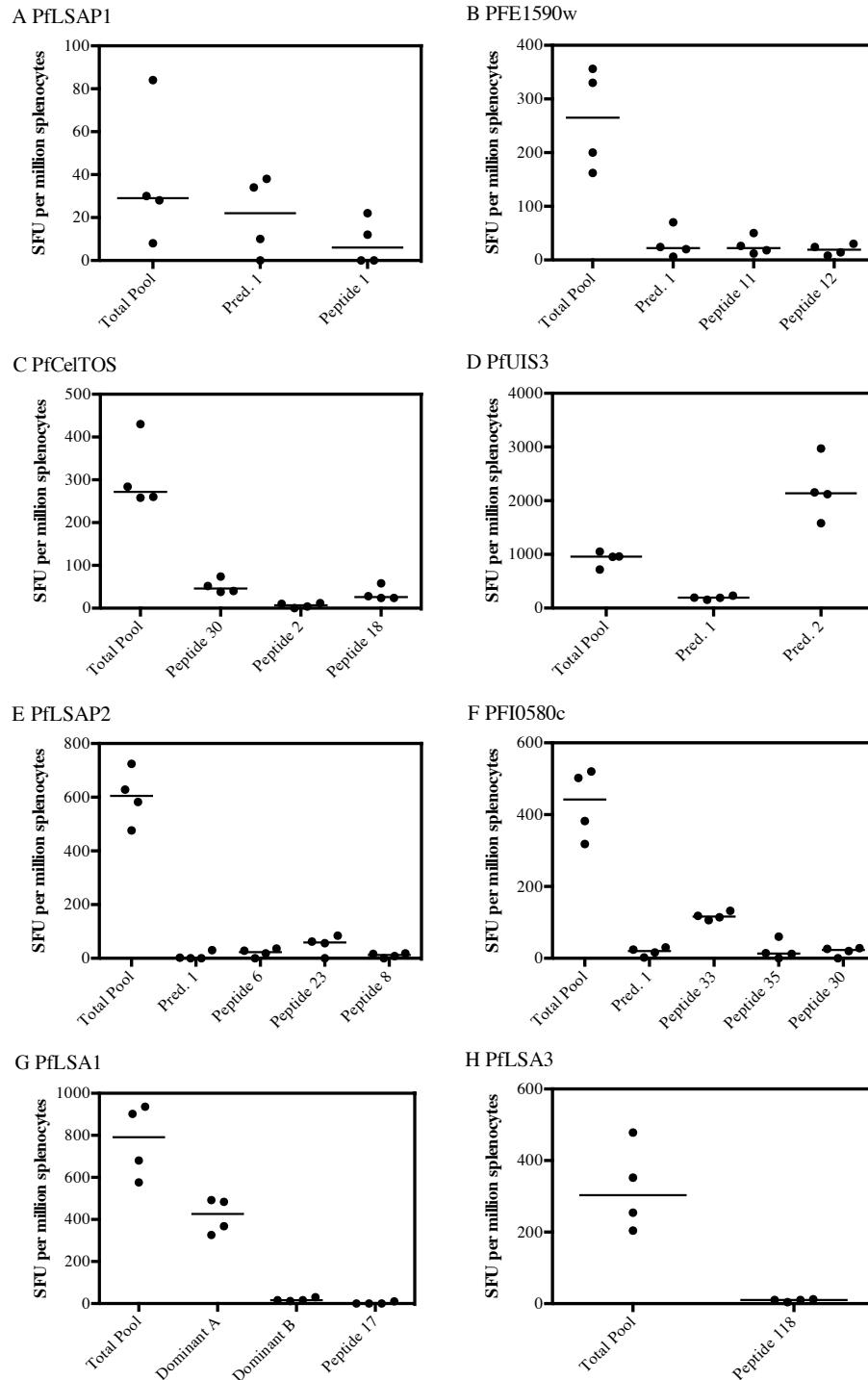


Figure 3.3. T cell immunogenicity to predicted epitopes of the candidate antigens.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] and an *ex vivo* spleen IFN γ ELISpot performed two weeks post-vaccination using a pool of overlapping peptides and the predicted epitopes. Each vaccine is presented on a separate graph: (A) PflSAP1 (B) PFE1590w (C) PfCelTOS (D) PfUIS3 (E) PflSAP2 (F) PFI0580c (G) PflSA1 and (H) PflSA3. Median and individual data points are shown.

3.3 Discussion

The two most advanced malaria vaccine candidates, RTS,S and ChAd63-MVA ME-TRAP, probably do not currently provide a high enough level of protective efficacy to warrant deployment in the field. New research avenues need to be explored and one that is currently gaining more interest is the use of alternative pre-erythrocytic antigens. To this end, a literature search was conducted to identify promising pre-erythrocytic antigenic targets. Antigens expressed at the liver-stage were prioritized as there is greater time for the immune system to mount an adequate response compared to the minutes to hours the parasite spends travelling from the skin to the liver. However, the majority of antigens were also expressed at either the sporozoite or blood-stage. Essentially, all known antigens expressed at the liver-stage at the onset of this DPhil thesis were included.

The ChAd63-MVA prime-boost vaccination regimen was chosen due to the superior level of antigen-specific CD8⁺ T cells generated compared to other platforms [160, 256, 292, 296]. For this reason, antigens that had previously been studied in vaccination trials with inferior vaccine platforms (e.g. DNA) were included [51, 232, 404, 412, 413, 415]. Twelve liver-stage antigens were initially identified, with one (UIS4) being excluded due to the lack of a known *P. falciparum* ortholog. The remaining eleven were assessed for their suitability to be cloned into the viral vectors, and at this point two more were excluded (PfSAP1 and PF14_0179) due to their size and the unlikely probability they would fit inside the ChAd63 vector. Validating this point, the only antigen that posed difficulties during the cloning process was PfLSA3, which at 4259 base pairs after modification was easily the largest antigen yet still smaller than the 7-8kb maximum insert size.

The selected candidate antigens were subsequently cloned into the ChAd63 and MVA plasmids, following minor modifications to the sequence as described. The sequences were left as close to native as possible, given this was the first time they had been expressed in viral vectors and the aim being an initial antigen screen, whilst any promising candidates could be modified in the future. Most antigens were preceded by the tPA signal sequence, to aid secretion from the cell by directing the protein to be translated at the endoplasmic reticulum. Whilst this might not be necessary for a CD8⁺-mediated immune response, because defective ribosomal translation products will leave a pool of unfolded proteins in the cytosol for Class I antigen-processing [435], there is evidence to suggest that a tPA signal sequence can increase cellular immunogenicity as it is likely important for a CD4⁺-mediated response [425, 426]. Repetitive sequences were deleted within PflSA1 and PflSA3 to reduce the risk of homologous recombination, which can cause difficulties in stability during manufacture [367], and to reduce the size of these large antigens.

The cloned plasmids were transfected and purified by the Viral Vector Core Facility at the Jenner Institute. All vaccines were successfully purified except for ChAd63-PfExp1. Surprisingly, the MVA-PfExp1 was purified without problem so it is unclear what specifically about the adenovirus made it difficult to produce. However, it was decided not to proceed with this antigen given good manufacturing practice (GMP) production for use in a clinical trial would likely face the same problems, and there was no reason to believe it was a higher priority candidate than other antigens. The viruses were then tested for expression of the transgene insert by measuring the cellular IFN γ response in mice. This method was chosen as each antigen had predicted H-2^d-restricted epitopes that were of comparable strength to the very

immunogenic BALB/c H-2^d-restricted epitope Pb9 from *P. berghei* CSP [393]. As PflSA1 was a well-studied candidate, a number of epitopes had also previously been mapped [434].

Ultimately, overlapping peptides to cover the entire antigen sequence were synthesized to be sure to include any dominant epitopes, given the potential unreliability of the prediction servers based on colleagues' previous experience within our group. All vaccines were immunogenic, confirming that each virus was expressing its transgenic insert. The magnitude of the immune response did vary between antigens, with PflSAP1 being the least immunogenic possibly because it was the smallest antigen and hence perhaps could not encompass as many T cell epitopes. The variation in magnitude was further explored in experiments described in Chapter 4. As expected, a single-shot of ChAd63 was more immunogenic than a single-shot of MVA, as it has been shown previously that MVA acts more effectively as a boosting agent [54, 298]. An alternative approach to assess transgene expression would have been the inclusion of a protein tag in the antigen sequence [253]; this would have allowed assessment by western blotting (with an antibody against the tag) rather than immunogenicity. In this study, all vaccines were immunogenic, however, a scenario could have arisen where the protein was expressed but was not immunogenic. Another limitation in using immunogenicity to assess expression is the use of mice, and hence future projects should consider the use of a tag.

To test the accuracy of the prediction servers, the immune response to the individual predicted epitopes was also assessed. Two prediction servers were used, SYFPEITHI and IEDB, using H-2^d restriction to enable screening in BALB/c mice. In some cases

the epitope had already been synthesized when generating the overlapping peptides, and hence is denoted 'Peptide 1' in Table 3.3. Others were ordered specifically and are hence denoted 'Pred. 1'. Perhaps unsurprisingly [436], the predictions were unsuccessful for most antigens. The exception was for PfUIS3, where the response to the predicted epitope exceeded that to the overlapping peptide pool. This antigen also generated the greatest response in terms of magnitude, which could perhaps influence the accuracy of the predictions. The IEDB ANN method was most successful, predicting 40% correctly. It predicts based on the likely ability of the sequence to bind MHC Class I. Previous research has indicated that the ANN method is one of the better predictors for Class I epitopes [437]. The IEDB consensus method for MHC Class II epitopes followed at 16.7%, with the SYFPEITHI prediction server only predicting 13.3% correctly. As this was only a study of nine antigens, the results are not conclusive but do show the risk in only using peptides generated by prediction servers. A better prediction accuracy would likely have been achieved if full epitope mapping was performed for each antigen, and if more than two prediction servers were used to create a more stringent definition of a predicted epitope (reviewed in [438]).

In summary, eight immunogenic new liver-stage *P. falciparum* virally vectored vaccines were designed and successfully made. The final vaccines, including the names they are referred to by in this thesis and final size, are provided in Table 3.4.

Table 3.4. The final eight successfully immunogenic *P. falciparum* liver-stage vaccine candidates.

Vaccine Name	Final Insert Size (base pairs)
PfCelTOS	665
PfE1590w	662
PfI0580c	1358
PfLSA1	1502
PfLSA3	4259
PfLSAP1	347
PfLSAP2	1025
PfUIS3	716

**Chapter 4: Assessment of immunogenicity and
efficacy of eight candidate *Plasmodium*
falciparum vaccines in mice**

4 Assessment of immunogenicity and efficacy of eight candidate *Plasmodium falciparum* vaccines in mice

4.1 Introduction

The two leading malaria vaccine candidates, RTS, S and ChAd63-MVA ME-TRAP, are both sub-unit vaccines targeting the pre-erythrocytic phase of malaria. Whilst neither vaccine currently provides optimal protective efficacy for deployment in endemic countries [160, 327, 333, 334], they both demonstrate the strength of targeting the pre-erythrocytic phase, as no blood-stage vaccine has progressed as far in clinical development [227]. Vaccination with irradiated sporozoites delivered by mosquito bite has been considered the “gold-standard” of malaria vaccines, as whilst it is impractical for deployment, this regimen has repeatedly shown sterile protection in vaccinated volunteers [45-50, 53]. The increased efficacy of irradiated sporozoite immunization over sub-unit vaccines is likely because immune responses are induced to a broad range of antigenic targets. However, perhaps not only multiple targets are needed to create an efficacious sub-unit vaccine, but also better targets than those traditionally focused on (e.g. CSP and TRAP). For these reasons, eight new pre-erythrocytic malaria vaccines were developed based on eight candidate liver-stage antigens as described in Chapter 3. The aim of the experiments described in this chapter was to comparatively screen both immunogenicity and efficacy of these vaccines in mice.

As previously discussed, the liver-stage of malaria is known to be the target of CD8⁺ T cells [55, 164, 165, 168, 202-204, 211, 342, 392-395], possibly through IFN γ [202, 203, 394, 439, 440]. For this reason, the *ex vivo* IFN γ ELISpot has been used as the

assay of choice to measure cellular immunogenicity in vaccine trials. However, we do not truly know what induces a protective response (reviewed in [118]), as most vaccine trials have failed to identify consistent correlates of protection [50, 160, 230, 259, 286, 333]. A multitude of factors could be involved and analysed, including alternative cytokine responses other than IFN γ , memory responses, chemokines and chemokine receptors as well as T cell trafficking to various organs. Assessing all such factors at once would require an immense amount of reagents and time, but multi-parameter flow cytometry offers the opportunity to detect multiple cytokines and markers from both CD8⁺ and CD4⁺ T cells. For the pre-erythrocytic antigen screen undertaken in this chapter, in addition to IFN γ , the cytokines TNF α and IL-2 were also assessed as well as the degranulation marker CD107a; this constitutes the standard panel of markers recommended for assessment [441]. TNF α has been shown to inhibit liver-stage malaria parasites [442], whilst IL-2 has been implicated in immunity induced by RTS,S vaccination [443, 444] or infected mosquito bites under chloroquine cover [219]. CD107a-expressing CD8⁺ T cells represent cells capable of cytotoxic killing in an antigen-specific manner [445]. As the majority of the candidate antigens are also expressed at either the sporozoite or blood-stage (apart from PflSA1 and PflSAP1, see Chapter 3), and these stages are known targets of antibody-mediated immunity [180, 230], relative antibody levels were also assessed.

Whilst it is important to determine that vaccines induce an immune response, the measures listed above do not necessarily indicate *functional* immunogenicity. That is, whether the T cells (or antibodies) induced by vaccination have the capability of inhibiting liver-stage malaria parasites (or other forms). It has been extremely difficult to assess functionality of immune responses directed at the *P. falciparum*

liver-stage, for a number of reasons. First, *P. falciparum* cannot infect commonly used small animal models such as mice, and whilst non-human primates can be infected with human malaria parasites they are not widely available and cost is a limiting factor. Species of malaria that infect rodents are commonly used to study the liver-stage of infection, such as *P. berghei* and *P. yoelii*, yet it is not clear how well studies using these models reflect *P. falciparum* infections in humans. Furthermore, many of the newly identified *P. falciparum* antigens do not have known rodent malaria homologs, making such studies currently impossible for those antigens. Second, unlike blood-stage vaccines where functional antibody responses can be tested *in vitro* using the growth inhibition assay (GIA), no such assay currently exists for the liver-stage of malaria. This is largely due to the difficulties in culturing human hepatocytes and the low rates of *P. falciparum* infection in these cells; such an assay was explored and the results are described in Chapter 7.

A new model that has been increasingly used over the last ten years is the generation of transgenic *P. berghei* parasites that express a particular *P. falciparum* (or *P. vivax*) gene. Two methods have commonly been used, either replacement of the endogenous *P. berghei* gene with the *P. falciparum* homolog under control of the relevant *P. berghei* promoter, or addition of the *P. falciparum* copy of the gene inserted at a different and dispensable point in the genome. For example, the 230p locus in both *P. berghei* and *P. yoelii* is considered “silent”, and replacement of this gene has no adverse effect on expression of other genes and no impact on parasite viability or behavior [69, 382, 446]. Such transgenic parasites allow assessment of efficacy of *P. falciparum* or *P. vivax* sub-unit vaccines in mice, using *P. berghei* expressing the appropriate human malaria antigen as the challenge agent. So far, transgenic parasites

have been developed and used for the study of transmission-blocking vaccines [76, 79], blood-stage vaccines [74] and in particular for CSP [73, 75, 77, 78].

Ten transgenic *P. berghei* parasites were developed expressing each of the eight candidate antigens studied in this thesis, as well as CSP and TRAP as controls. The addition strategy was employed: the *P. falciparum* antigens were placed under control of the *P. berghei* UIS4 promoter, given not all the candidates have *P. berghei* homologs, and inserted at the *P. berghei* 230p locus. *P. berghei* UIS4 is expressed at both the sporozoite and liver-stage [208], and hence antigens placed under control of this promoter will also be expressed at these stages regardless of their native expression profile [82]. This allowed the immune response to each antigen to be comparatively screened, given all targets would have the same expression level and profile. DPhil student Ahmed Salman, and his supervisors at the Leiden University Medical Centre, developed and provided the transgenic parasites. Ahmed Salman also provided assistance with the transgenic challenges performed in this chapter. For antigens with known *P. berghei* homologs, PfCelTOS, PFI0580c and PfUIS3, efficacy was initially assessed with a *P. berghei* wild-type challenge.

4.2 Results

4.2.1 Cellular immunogenicity of the candidate vaccines delivered in a heterologous prime-boost regimen

The viral vectored vaccines, generated and described in Chapter 3, were assessed for their relative levels of cellular immunogenicity by *ex vivo* spleen IFN γ ELISpot. The vaccines were delivered in an eight-week interval ChAd63 prime-MVA boost regimen, and the cellular immune response was measured two weeks after each

vaccination (representing peak time-points post-immunization [296, 297]). Immunogenicity was measured in two different strains of mice with different immune profiles: BALB/c, which preferentially produce Th2 cytokines (including IL-4, IL-5, IL-9 and IL-13), and C57BL/6, which preferentially produce Th1 cytokines (including IFN γ and TNF α) [447-450]. Each vaccine induced a measurable immune response in BALB/c mice (Figure 4.1 A), as expected given the single-shot results previously discussed in Chapter 3. The MVA boost was able to return the IFN γ response to at least the level seen after the priming vaccination. For both PfUIS3 and PfLSA1, the boost vaccination significantly increased the IFN γ response above that observed two weeks after the prime ($p < 0.0001$, two-way ANOVA). These two vaccines also induced the highest IFN γ ELISpot responses, with medians of 1200 and 868 SFU per million splenocytes post-boost, respectively. PfLSAP1 vaccination induced the lowest response, with a median of 43 SFU per million splenocytes. The antigens are listed on the x-axis in increasing size order, and as can be seen, there was no clear correlation between antigen size and the magnitude of the IFN γ response.

In C57BL/6 mice, no detectable response was observed after vaccination with either PfLSAP1 or PfLSA1 (Figure 4.1 B). MVA-PFE1590w was unable to boost the IFN γ response to the level observed two weeks after the prime. For the remaining antigens, the MVA vaccination was able to boost the response to at least the level observed two weeks after the prime, with PfCelTOS, PfUIS3 and PfLSA3 showing an increase in the median response post-boost compared to post-prime. The overall magnitude of the IFN γ responses was higher in C57BL/6 as compared to BALB/c (Figure 4.1 A compared to B), as was the variation between mice. PfUIS3 induced the highest response, with a median of 1852 SFU per million splenocytes. As all vaccines

induced a cellular response in BALB/c mice, this strain was chosen to compare the efficacy of the eight vaccines against *Plasmodium* challenge (see 4.2.5).

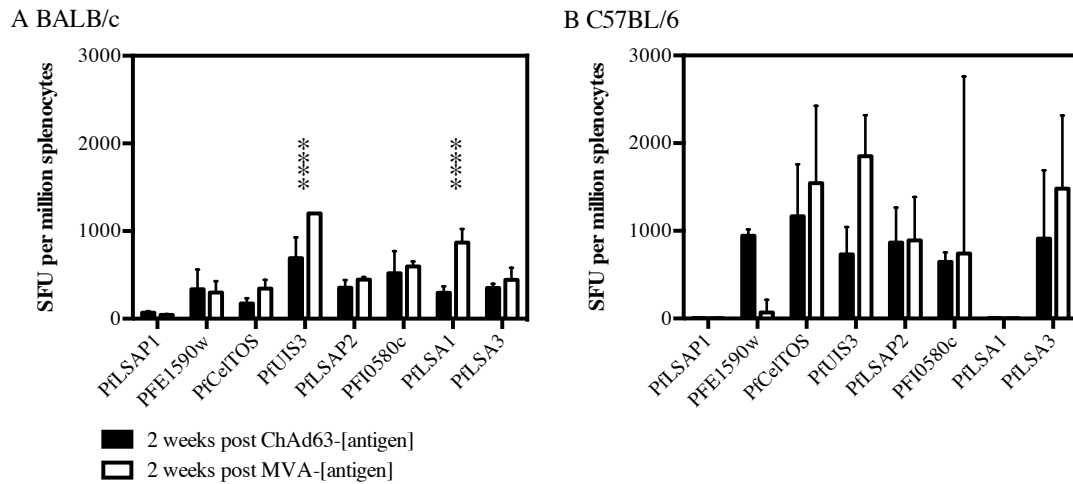


Figure 4.1. Cellular immunogenicity of the eight candidate *P. falciparum* vaccines administered in a prime-boost eight-week interval regimen.

Mice (n=4 per time-point per strain) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] followed eight weeks later by MVA-[antigen]. Spleens were harvested two weeks after each vaccination to assess T cell immunogenicity by *ex vivo* spleen IFN γ ELISpot to a pool of overlapping peptides from the appropriate antigen. Vaccines were tested in two strains of mice: (A) BALB/c, where the MVA dose was 1×10^7 pfu and (B) C57BL/6, where the MVA dose was 1×10^6 pfu. For BALB/c mice, the post-prime data was obtained from the experiment described in Chapter 3. Results are expressed as the median SFU per million splenocytes; error bars indicate the interquartile range. Statistical difference was assessed using a two-way ANOVA with Bonferroni post-test, **** p<0.0001. For the antigen PFUIS3 two weeks post-MVA boost in BALB/c mice, the number of spots seen was at a maximum level counted by the ELISpot reader; therefore, an arbitrary value of 1200 SFU per million splenocytes was assigned. Antigens are listed on the x-axis in increasing size order.

4.2.2 CD4⁺ and CD8⁺ cytokine responses induced by the candidate vaccines

ICS was performed to determine whether the response was mediated predominantly by CD8⁺ or CD4⁺ T cells and whether other cytokines were also secreted in response to *ex vivo* antigen stimulation. Two time-points were assessed in the blood, two weeks post-prime and one-week post-boost, in addition to two weeks post-boost in the spleen. Samples were taken from the same mice vaccinated in section 4.2.1. In addition to IFN γ , cells were stained for production of TNF α and IL-2, and the cell surface localisation of the degranulation marker CD107a. The responses two weeks post-prime were just above the limit of detection and as such the data are not shown. For all vaccines in both the blood and the spleen post-boost, in both strains of mice, it was found that the response was predominantly CD8⁺ T cells producing IFN γ or TNF α or expressing CD107a, with minimal levels of IL-2 secretion, as detailed below.

In BALB/c mice, the cytokine profiles were quite different in the blood compared to the spleen. In the blood the greatest CD8⁺ IFN γ ⁺ responses were to antigens PflSA1, PflSA3 and PflUIS3 (medians of 6.3%, 4.8% and 4.2%, respectively) (Figure 4.2). PflSA1 and PflSA3 also showed the highest CD8⁺ TNF α ⁺ responses (medians of 7.3% and 6.4%, respectively) and CD8⁺ CD107a⁺ responses (medians of 17.1% and 12.1%, respectively). The CD4⁺ responses were much lower than the CD8⁺ responses for all antigens, at less than 1% for each cytokine. In summary, in BALB/c mice in the blood one-week post-boost, the response was predominantly CD8⁺ T cells producing IFN γ , TNF α or expressing CD107a with the highest magnitude for PflSA1 and PflSA3.

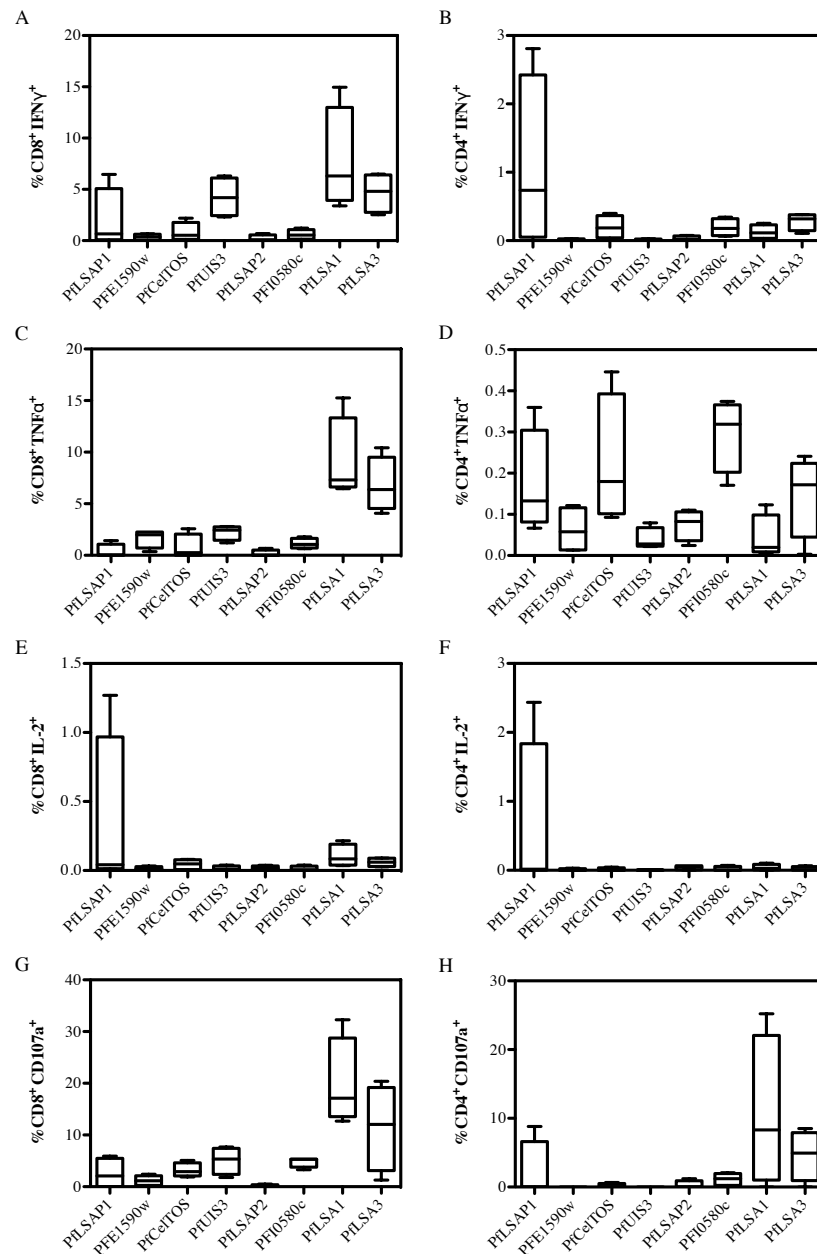


Figure 4.2. CD8⁺ and CD4⁺ cytokine responses in BALB/c mice in the blood following heterologous prime-boost vaccination with the *P. falciparum* candidate liver-stage antigens.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] followed eight weeks later by 1×10^7 pfu MVA-[antigen]. Blood was taken one-week after the final vaccination to assess CD8⁺ and CD4⁺ cytokine responses by ICS, after stimulation for six hours with a pool of overlapping peptides from the appropriate antigen. Results are expressed as the percentage of CD8⁺ (left hand side panel) or CD4⁺ (right hand side panel) T cells expressing the cytokines, with box plots indicating the median response and the whiskers showing the minimum and maximum responses. Antigens are listed on the x-axis in increasing size order. Four different markers were assessed: (A+B) IFN γ , (C+D) TNF α , (E+F) IL-2 and (G+H) the degranulation marker CD107a.

In the spleen, the highest cytokine responses were observed for the antigens PfUIS3 and PfLSA1 (Figure 4.3). This was the case for CD8⁺ IFN γ ⁺ (14% and 5.8%, respectively), CD8⁺ TNF α ⁺ (11.4% and 5.3%, respectively) and CD8⁺ CD107a⁺ (13.9% and 6.6%, respectively). The CD4⁺ response was slightly higher than that seen one-week earlier in the blood, with the majority of responses less than 2%. The highest CD4⁺ response was CD4⁺ IFN γ ⁺ cells with a median of 1.4% for PfLSA3. There was also some detectable CD4⁺ IL-2⁺, approximately 0.5% for most antigens, with the response trending towards being dependent on antigen size (antigens are listed in order of increasing size on the x-axis). In summary, in BALB/c mice in the spleen two weeks post-boost, the response was predominantly CD8⁺ T cells producing IFN γ , TNF α or expressing CD107a with the highest magnitude for PfUIS3 and PfLSA1 in each case. Considering both the responses in the blood and the spleen, the antigens PfUIS3, PfLSA1 and PfLSA3 were the most immunogenic in BALB/c mice.

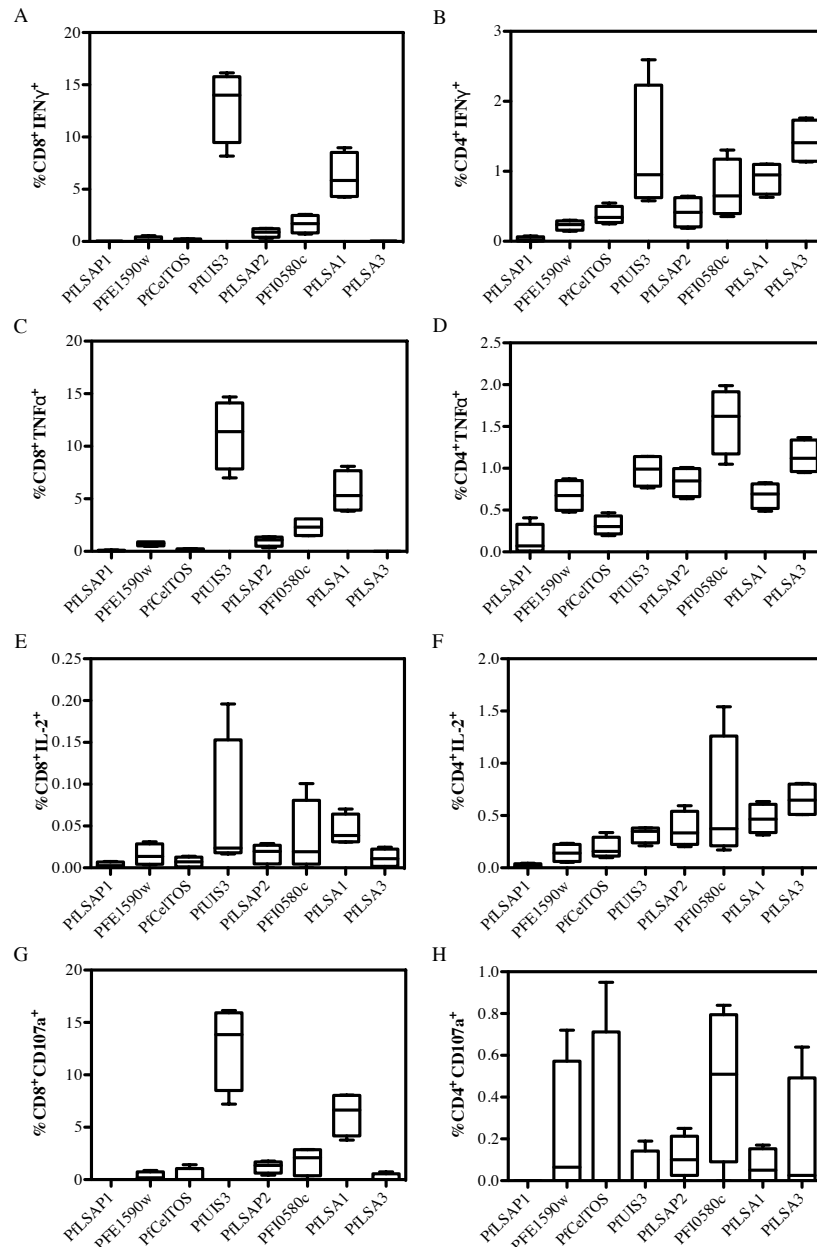


Figure 4.3. CD8⁺ and CD4⁺ cytokine responses in BALB/c mice in the spleen following heterologous prime-boost vaccination with the *P. falciparum* candidate liver-stage antigens.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] followed eight weeks later by 1×10^7 pfu MVA-[antigen]. Spleens were harvested two weeks after the final vaccination to assess CD8⁺ and CD4⁺ cytokine responses by ICS, after stimulation for six hours with a pool of overlapping peptides from the appropriate antigen. Results are expressed as the percentage of CD8⁺ (left hand side panel) or CD4⁺ (right hand side panel) T cells expressing the cytokines, with box plots indicating the median response and the whiskers showing the minimum and maximum responses. Antigens are listed on the x-axis in increasing size order. Four different markers were assessed: (A+B) IFN γ , (C+D) TNF α , (E+F) IL-2 and (G+H) the degranulation marker CD107a.

In C57BL/6 mice, the cytokine profile was similar between the blood and spleen post-boost, and hence results are shown for the spleen only. The cytokine staining confirmed the results obtained by IFN γ ELISpot that there were no T cell epitopes for the antigens PfLSAP1 and PfLSA1 in C57BL/6 mice (Figure 4.4). Compared to BALB/c mice, the median responses were generally higher in C57BL/6 mice and a greater number of antigens had strong responses, consistent with data obtained by IFN γ ELISpot. PfUIS3, PfLSA3, PfCelTOS and PFI0580c demonstrated the highest CD8 $^{+}$ response measured by IFN γ^{+} (medians of 6-14.16%), TNF α^{+} (medians of 4.18-13.19%) or CD107a $^{+}$ (medians of 7.65-15%). The IL-2 $^{+}$ responses for both CD8 $^{+}$ and CD4 $^{+}$ were low, as were the CD4 $^{+}$ responses in general (less than 2%), but in each case the pattern of antigens responding with the highest magnitude was essentially the same. In summary, for C57BL/6 mice in both the blood and the spleen, the response was predominantly CD8 $^{+}$ T cells producing IFN γ , TNF α or expressing CD107a with the highest magnitude for the antigens PfUIS3, PfLSA3, PfCelTOS and PFI0580c.

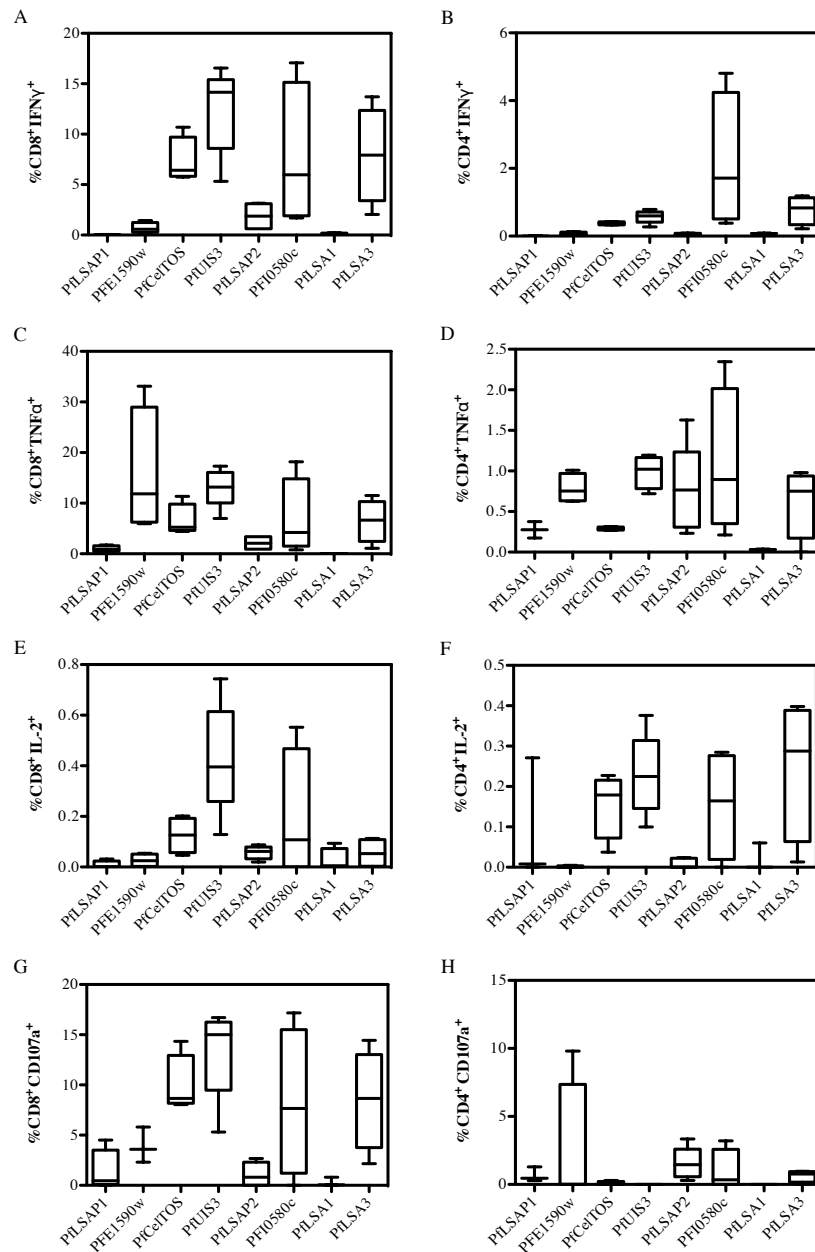


Figure 4.4. CD8⁺ and CD4⁺ cytokine responses in C57BL/6 mice in the spleen following heterologous prime-boost vaccination with the *P. falciparum* candidate liver-stage antigens.

C57BL/6 mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] followed eight weeks later by 1×10^6 pfu MVA-[antigen]. Spleens were harvested two weeks after the final vaccination to assess CD8⁺ and CD4⁺ cytokine responses by ICS, after stimulation for six hours with a pool of overlapping peptides from the appropriate antigen. Results are expressed as the percentage of CD8⁺ (left hand side panel) or CD4⁺ (right hand side panel) T cells expressing the cytokines, with box plots indicating the median response and the whiskers showing the minimum and maximum responses. Antigens are listed on the x-axis in increasing size order. Four different markers were assessed: (A+B) IFN γ , (C+D) TNF α , (E+F) IL-2 and (G+H) the degranulation marker CD107a.

4.2.3 Antibody responses induced by the candidate vaccines

Given most of the candidate liver-stage antigens are also expressed at either the sporozoite or the blood-stage, it is plausible that vaccination with these antigens could provide some degree of protective efficacy through an antibody-mediated effect. Therefore antibody levels in serum samples were measured using the LIPS assay, as previously described in Chapter 2. In brief, this system allows the measurement of antibody when protein samples are not available to perform standard ELISAs. Instead, genetic constructs are designed that fuse the antigen of interest to the *Renilla* luciferase reporter gene. The expressed construct can then be used to measure antibody in the LIPS assay, with luminescence (light units) as the read-out. Constructs were designed, cloned and transfected for each of the eight candidate antigens.

Serum samples were collected from the experiments outlined in section 4.2.1, and post-prime for some of the challenge experiments outlined in the following sections. All samples for each antigen were measured at the same time. Antibody levels were assessed at both five to six weeks post-prime (D35-42) and two weeks post-boost (D70), representing peak time-points for antibody production post-vaccination [294, 451]. In BALB/c mice, only vaccination with the antigens PfUIS3, PflSAP2 and PFI0580c generated a detectable antibody response after the ChAd63 prime vaccination (Figure 4.5). No antibody responses above background levels were detected against PflSAP1 at any time-point measured. All other vaccines generated a detectable antibody response after the MVA boost; this represented a small increase from the antibody level at D35-42 for PflSA3 ($p=0.028$, Mann Whitney test) and a significant increase for all other antigens ($p=0.01-0.001$, Mann Whitney test), except

PfCelTOS. In all cases, the increase was approximately one unit, which may not represent a physiologically significant difference.

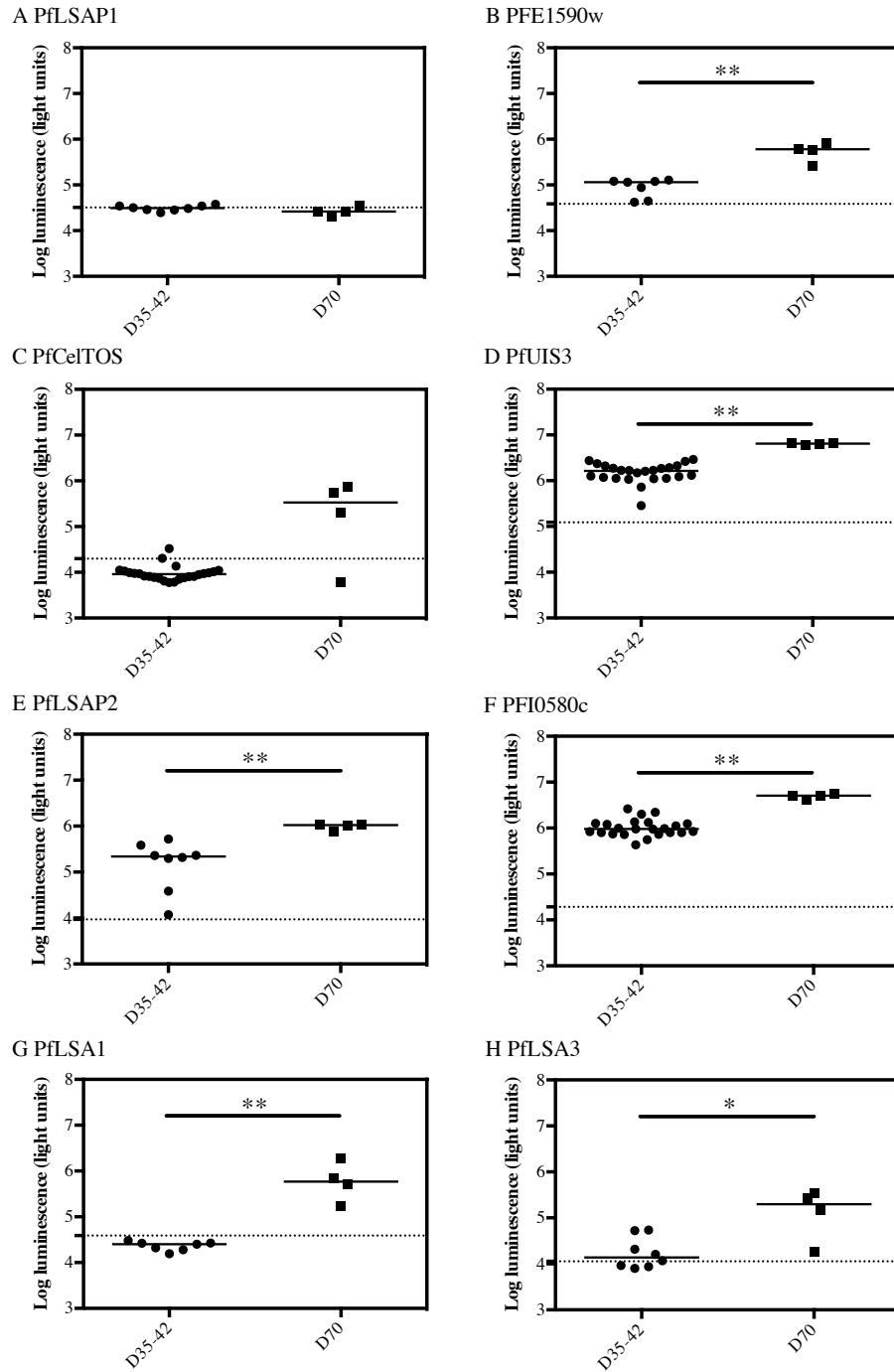


Figure 4.5. Assessment of antibody responses in BALB/c mice following heterologous prime-boost vaccination with the *P. falciparum* candidate liver-stage antigens.

In the experiment described in 4.2.1 and efficacy experiments using the same vaccination regimen, sera was collected at five to six weeks post-prime (D35-42) and two weeks post-boost (D70) and antibody levels measured by the LIPS assay (n=4-24 BALB/c mice). The background response to each antigen is indicated by the dotted line, and is equal to the average of six naïve replicates plus two times the standard deviation. Raw data was log-transformed prior to analysis; results are expressed as the log luminescence (light units) measured. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, *p=0.05-0.01 ** p=0.01-0.001.

The pattern of antibody responses observed in C57BL/6 was different to that observed in BALB/c mice. No antibody responses were detected at any time-point for PfLSAP1, PFE1590w, PfLSAP2 and PfLSA1 (Figure 4.6). After the prime vaccination, antibody responses were only detected to PFI0580c. However, after the boost vaccination antibody responses were detectable against PfCelTOS, PfUIS3 and PfLSA3, in addition to PFI0580c. This represented a significant increase from the antibody levels at D42 for both PfUIS3 ($p=0.0112$, Mann Whitney test) and PfLSA3 ($p=0.0286$, Mann Whitney test), of approximately one unit. Whilst there was a trend towards an increase at D70 for PfCelTOS and PFI0580c, this was not statistically significant.

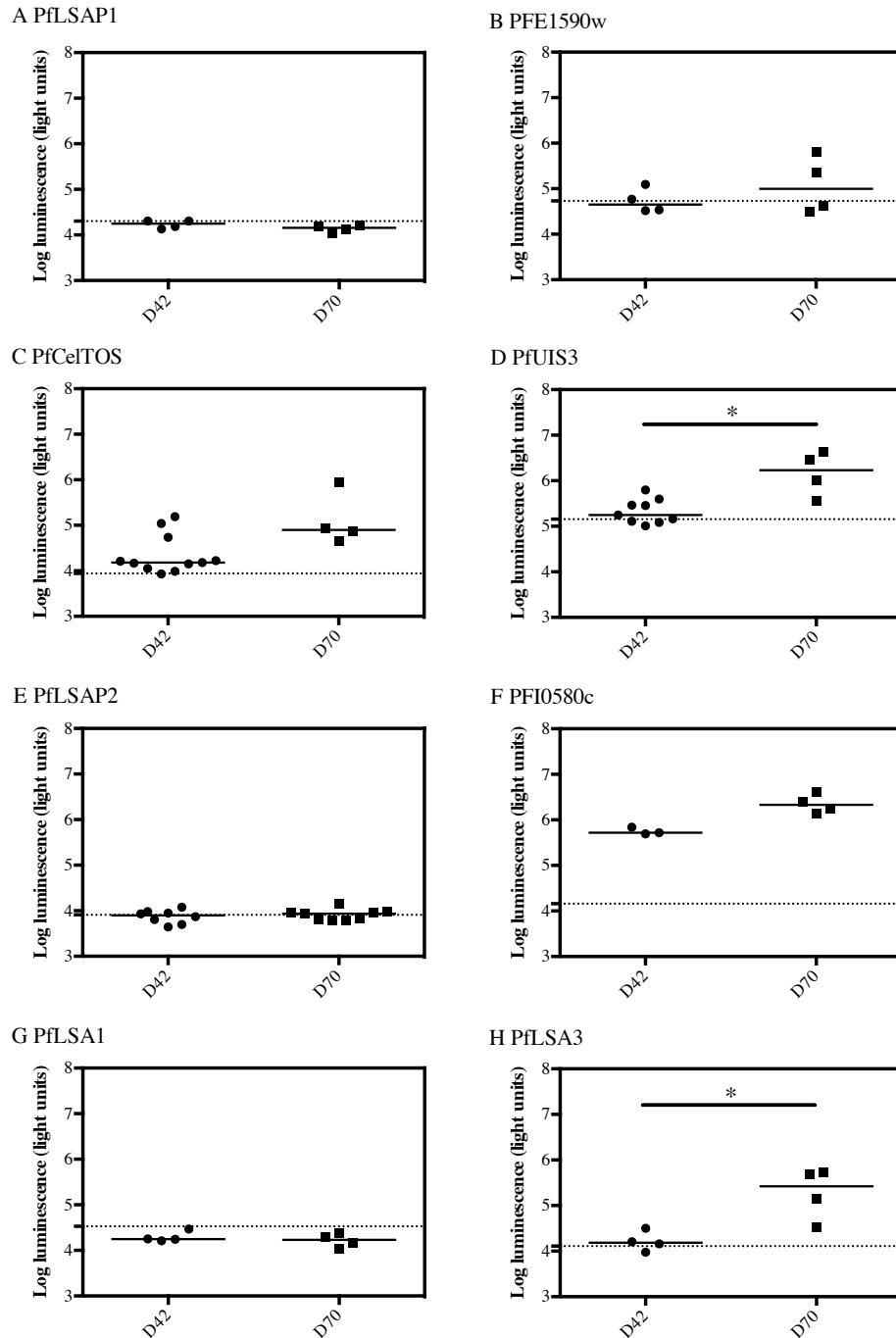


Figure 4.6. Assessment of antibody responses in C57BL/6 mice following heterologous prime-boost vaccination with the *P. falciparum* candidate liver-stage antigens.

In the experiments described in 4.2.1 and efficacy experiments using the same vaccination regimen, sera was collected at six weeks post-prime (D42) and two weeks post-boost (D70) and antibody levels measured by the LIPS assay (n=3-11 C57BL/6 mice). The background response to each antigen is indicated by the dotted line, and is equal to the average of six naïve replicates plus two times the standard deviation. Raw data was log-transformed prior to analysis; results are expressed as the log luminescence (light units) measured. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, *p=0.05-0.01.

As each antigen measured in the LIPS assay generated a different background response, analysis was then performed to measure the fold change from the naïve (background) antibody level to the level reached after the boost vaccination (D70). This enabled the antigens to be compared side-by-side (Figure 4.7). In BALB/c mice, vaccination with PFI0580c and PflSAP2 generated the highest levels of antibodies (fold change of 1.6 and 1.5, respectively). The remaining antigens were all comparable, with a fold change from background of 1.3 (excluding PflSAP1). In C57BL/6 mice, vaccination with PFI0580c resulted in the highest level of antibody production, with a fold change from background of 1.5. The next highest levels were seen for PflSA3 (1.3), PflCelTOS and PflUIS3 (both 1.2). In summary, vaccination with PFI0580c generated the highest antigen-specific antibody response in both strains of mice, with the relative antibody levels comparable between C57BL/6 and BALB/c mice.

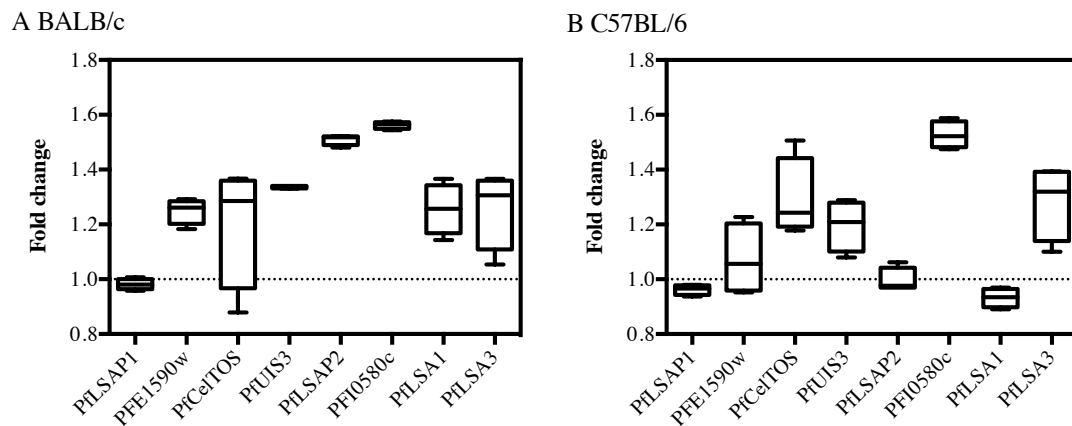


Figure 4.7. Fold change in the antibody level from background to two weeks post-MVA boost in BALB/c and C57BL/6 mice.

The fold change from the background response to the antibody level post-boost in (A) BALB/c and (B) C57BL/6 mice was calculated for each antigen (post-boost response divided by the background response), from the data shown in Figure 4.5 and Figure 4.6. The background response was calculated as the average of six naïve replicates plus two times the standard deviation. Data was log transformed prior to analysis. Box plots represent the median with the whiskers indicating the maximum and minimum values. The dotted line represents no change in antibody level from the background value (=fold change of 1).

4.2.4 Assessment of protective efficacy of PfCelTOS, PFI0580c and PfUIS3 against heterologous *P. berghei* challenge

After measuring the relative immunogenicity elicited by each candidate antigen in a prime-boost vaccination regimen, the ability of these vaccines to protect against *Plasmodium* was then assessed. Since *P. falciparum* does not infect mice, other challenge models were investigated. According to PlasmoDB, *P. berghei* homologs only exist for the *P. falciparum* candidate antigens PfCelTOS, PFI0580c and PfUIS3. A relatively high sequence similarity exists between the *P. falciparum* and *P. berghei* protein sequences; 65% similarity for CelTOS, 54% for UIS3 and 52% for PFI0580c, calculated using the European Bioinformatics Institute EMBOSS needle pair-wise protein sequence alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). For this reason, it was assumed that if any epitopes fell in the regions of similarity it may be possible to see cross-species protection after vaccination with the *P. falciparum* antigen and challenge with *P. berghei* sporozoites.

Protective efficacy of ChAd63-MVA PfCelTOS vaccination was assessed in both BALB/c and C57BL/6 mice, given previous studies had demonstrated cross-species protection with PfCelTOS protein vaccination [51, 400]. Mice were vaccinated with the standard eight-week prime-boost regimen, with blood collected six days after MVA boost to check cellular immunogenicity via ICS prior to challenge two days later (eight days post-boost) with 1000 *P. berghei* wild-type sporozoites injected intravenously. Despite a good cellular immune response observed in C57BL/6 mice (median 8.8% CD8⁺ IFN γ ⁺), vaccination with ChAd63-MVA PfCelTOS failed to protect against challenge with *P. berghei* sporozoites (Figure 4.8 A and B). ICS results are only shown for CD8⁺ T cells producing IFN γ or TNF α , or expressing the

degranulation marker CD107a, as there were minimal responses from CD4⁺ T cells and minimal IL-2 production. Antibodies were not measured in this experiment, but previous data indicated that post-boost PfCelTOS vaccination resulted in a comparatively strong antigen-specific antibody response in C57BL/6 mice (Figure 4.6). Given PfCelTOS vaccination in BALB/c mice resulted in low level cellular immunogenicity in the blood as measured by ICS (negligible cytokine secretion, median 5.9% CD8⁺ CD107a⁺), it was not surprising that such vaccination was not protective against *P. berghei* challenge (Figure 4.8 C and D). Antibodies were not assessed in this experiment but previous data indicated there was little to no antigen-specific antibody production after PfCelTOS vaccination in BALB/c mice. This experiment was repeated (in BALB/c mice only) and the same result was found.

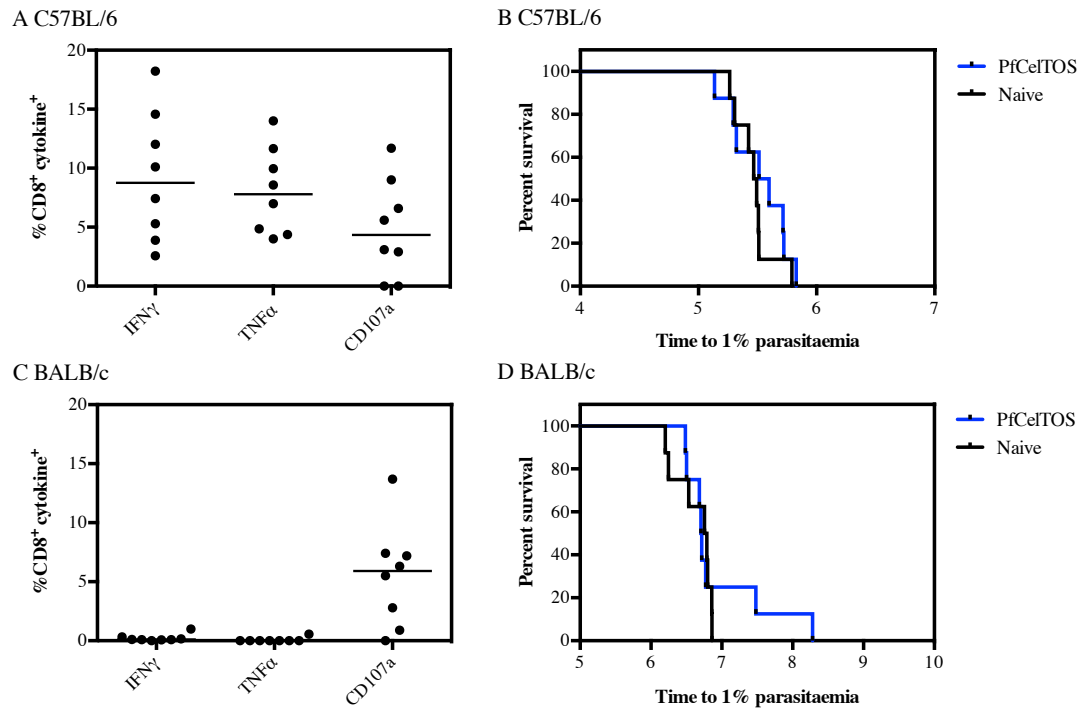


Figure 4.8. Heterologous challenge with *P. berghei* sporozoites in C57BL/6 and BALB/c mice vaccinated with ChAd63-MVA PfCelTOS.

(A) C57BL/6 mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfCelTOS followed eight weeks later by 1×10^6 pfu MVA-PfCelTOS. Blood was collected six days post-MVA boost to assess cellular immunogenicity by ICS, after stimulation for six hours with a pool of overlapping peptides covering the entire PfCelTOS sequence. Results are expressed as the percentage of CD8⁺ T cells expressing the cytokines IFN γ , TNF α or the degranulation marker CD107a. Both median and individual data points are shown. (B) Mice were subsequently challenged i.v. with 1000 *P. berghei* sporozoites two days later (eight days post-MVA boost), along with eight naïve control mice. Mice were monitored daily from four days post-challenge by thin film blood smears and the percent parasitaemia calculated. Following three consecutive positive films, mice were culled. The data collected was used to calculate the time to 1% parasitaemia, using linear regression, and the results are presented in a survival graph. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves; no significant difference was found (p=0.32). (C) BALB/c mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfCelTOS followed eight weeks later by 1×10^7 pfu MVA-PfCelTOS. Blood was collected six days post-MVA boost and ICS was performed as per A. (D) The PfCelTOS vaccinated BALB/c mice, and eight naïve controls, were subsequently challenged with 1000 *P. berghei* sporozoites i.v. as per B. No significant differences were found (p=0.21). The experiment was repeated and the same result was found (data not shown).

Protective efficacy against heterologous *P. berghei* challenge was assessed for PFI0580c vaccination in BALB/c mice, again following the standard eight-week interval prime-boost regimen. Cellular immunogenicity was assessed in the blood six days post-MVA boost by ICS (Figure 4.9 A), and was very low (less than 0.5% for each cytokine from CD8⁺ T cells). Antibodies were not measured in this experiment but previous data indicated that PFI0580c vaccination in BALB/c mice resulted in high levels of antigen-specific antibodies, greater than vaccination with any other antigen. However, no protection was seen upon challenge with 1000 *P. berghei* sporozoites eight days post-MVA boost (Figure 4.9 B). This experiment was repeated and the same result was found.

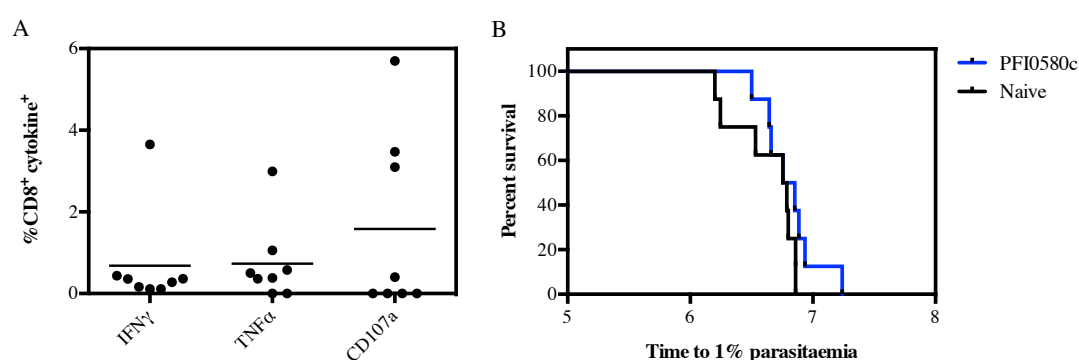


Figure 4.9. Heterologous challenge with *P. berghei* sporozoites in BALB/c mice vaccinated with ChAd63-MVA PFI0580c.

(A) BALB/c mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PFI0580c followed eight weeks later by 1×10^7 pfu MVA-PFI0580c. Blood was collected six days post-MVA boost to assess cellular immunogenicity by ICS, after stimulation for six hours with a pool of overlapping peptides covering the entire PFI0580c sequence. Results are expressed as the percentage of CD8⁺ T cells expressing the cytokines IFN γ , TNF α or the degranulation marker CD107a. Both median and individual data points are shown. (B) The same mice were subsequently challenged i.v. with 1000 *P. berghei* sporozoites two days later (eight days post-MVA boost), along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves; no significant difference was found (p=0.19). The experiment was repeated and the same result was found (data not shown).

Protective efficacy against heterologous *P. berghei* challenge was assessed for PfUIS3 vaccination in BALB/c mice. ICS analysis demonstrated a moderate immune response, with medians of 1.9% IFN γ ⁺, 3% TNF α ⁺ and 4.8% CD107a⁺ from CD8⁺ T cells (Figure 4.10 A). Data is not shown for CD4⁺ T cells or IL-2⁺ due to low-level responses, and again antibodies were not measured in this experiment but previous data showed positive antigen-specific antibody responses to PfUIS3 in BALB/c mice, with relatively high levels compared to those induced by the other antigens (Figure 4.7). Upon i.v. challenge with 1000 *P. berghei* sporozoites eight days post-MVA boost, there was a significant delay in the time taken for parasitaemia in the blood to reach 1% in vaccinated mice compared to controls (p=0.0048, Log-rank (Mantel-Cox) Test) (Figure 4.10 B). No mice were sterilely protected.

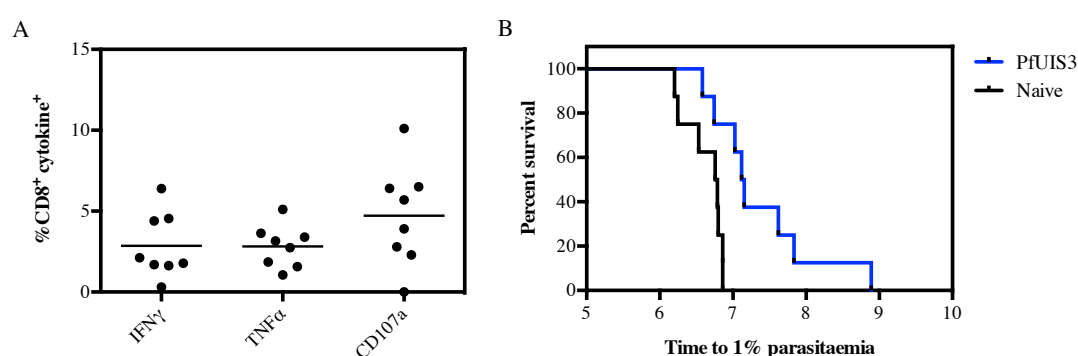


Figure 4.10. Heterologous challenge with *P. berghei* sporozoites in BALB/c mice vaccinated with ChAd63-MVA PfUIS3.

(A) BALB/c mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 1×10^7 pfu MVA-PfUIS3. Blood was collected six days post-MVA boost to assess cellular immunogenicity by ICS, after stimulation for six hours with a pool of overlapping peptides covering the entire PfUIS3 sequence. Results are expressed as the percentage of CD8⁺ T cells expressing the cytokines IFN γ , TNF α or the degranulation marker CD107a. Both median and individual data points are shown. (B) The same mice were subsequently challenged i.v. with 1000 *P. berghei* sporozoites two days later (eight days post-MVA boost), along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, p=0.0048.

4.2.5 Assessment of protective efficacy of the eight *P. falciparum* candidate vaccines using transgenic *P. berghei* sporozoites

Since *P. falciparum* does not infect mice, an alternative challenge model was required to assess the efficacy of the new viral vectored vaccines. As only three antigens contained *P. berghei* homologs, transgenic *P. berghei* parasites were developed that expressed the relevant *P. falciparum* antigen (in addition to the *P. berghei* copy of that antigen, if it existed), referred to as PbPf[antigen]. This work was undertaken in collaboration with the Leiden University Medical Centre; DPhil student Ahmed Salman, Dr. Shahid Khan and Dr. Chris Janse cloned and transfected the transgenic parasites. Each *P. falciparum* antigen was expressed under control of the *P. berghei* UIS4 promoter using the additional strategy (insertion at the 230p locus). To allow *in vivo* assessment of liver-stage infection, each transgenic parasite also contained the luciferase gene.

As fitness assessments of these transgenic parasites had not been undertaken, a standard challenge dose of 1000 sporozoites per mouse injected i.v. was used for all experiments. Experiments were performed in BALB/c mice to allow comparison between antigens (as not all vaccines were immunogenic in C57BL/6 mice). Furthermore, C57BL/6 mice succumb more quickly to *P. berghei* infection than BALB/c mice [127, 452]; using BALB/c mice therefore allowed greater discrimination of small differences in protective efficacy between candidate antigens. Mice were vaccinated in the standard eight-week interval prime-boost regimen, with blood collected six days post-MVA boost to check immunogenicity before proceeding with the challenge. These data are not shown but were comparable to those seen in the immunogenicity studies (Figure 4.2). For each transgenic parasite

line, vaccinated mice were challenged eight days post-MVA boost together with eight unvaccinated controls. The prime-boost regimen was varied slightly for PFI0580c, PFE1590w and PfLSAP2; due to failed sporozoite production mice were given a second MVA boost four weeks after the original boost, and challenged eight days after the second boost.

Each transgenic parasite line resulted in different blood parasitaemia kinetics, and hence each vaccination-challenge experiment is presented on a separate survival graph (Figure 4.11). No protection was conferred by vaccination with PfLSAP1, PFE1590w, PfCeITOS and PfLSA3, with PfCeITOS vaccination resulting in a significantly shorter time to 1% parasitaemia than seen in the control mice ($p=0.0291$, Log-rank (Mantel-Cox) Test), suggesting a negative effect of the vaccination. The transgenic parasite PbPfLSA3 did not efficiently infect all eight naïve controls; three mice showed no signs of parasites in the blood at fourteen days post-challenge.

Figure 4.11. Protective efficacy after ChAd63-MVA vaccination with the *P. falciparum* candidate antigens and challenge with transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen (overleaf).

BALB/c mice ($n=8$) were vaccinated i.m. with 1×10^8 ifu ChAd63-[antigen] followed eight weeks later by 1×10^7 pfu MVA-[antigen]. Blood was collected six days post-MVA boost to check cellular immunogenicity by ICS (data not shown), and two days later the mice were subsequently challenged i.v. with 1000 transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen. An exception was for the antigens PFI0580c, PFE1590w and PfLSAP2, where a second MVA boost was given four weeks after the first, and mice were challenged eight days after the second boost. Eight naïve mice were also challenged for each transgenic parasite line. Mice were monitored daily to enable calculation of the time to 0.5 or 1% parasitaemia. Mice that were slide negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves: (A) PfLSAP1 no significant difference ($p=0.15$), (B) PFE1590w no significant difference ($p=0.29$), (C) PfCeITOS $p=0.0291$, (D) PfUIS3 $p=0.0001$, (E) PfLSAP2 $p<0.0001$, (F) PFI0580c $p=0.0072$, (G) PfLSA1 $p<0.0001$ and (H) PfLSA3 no significant difference ($p=0.45$).

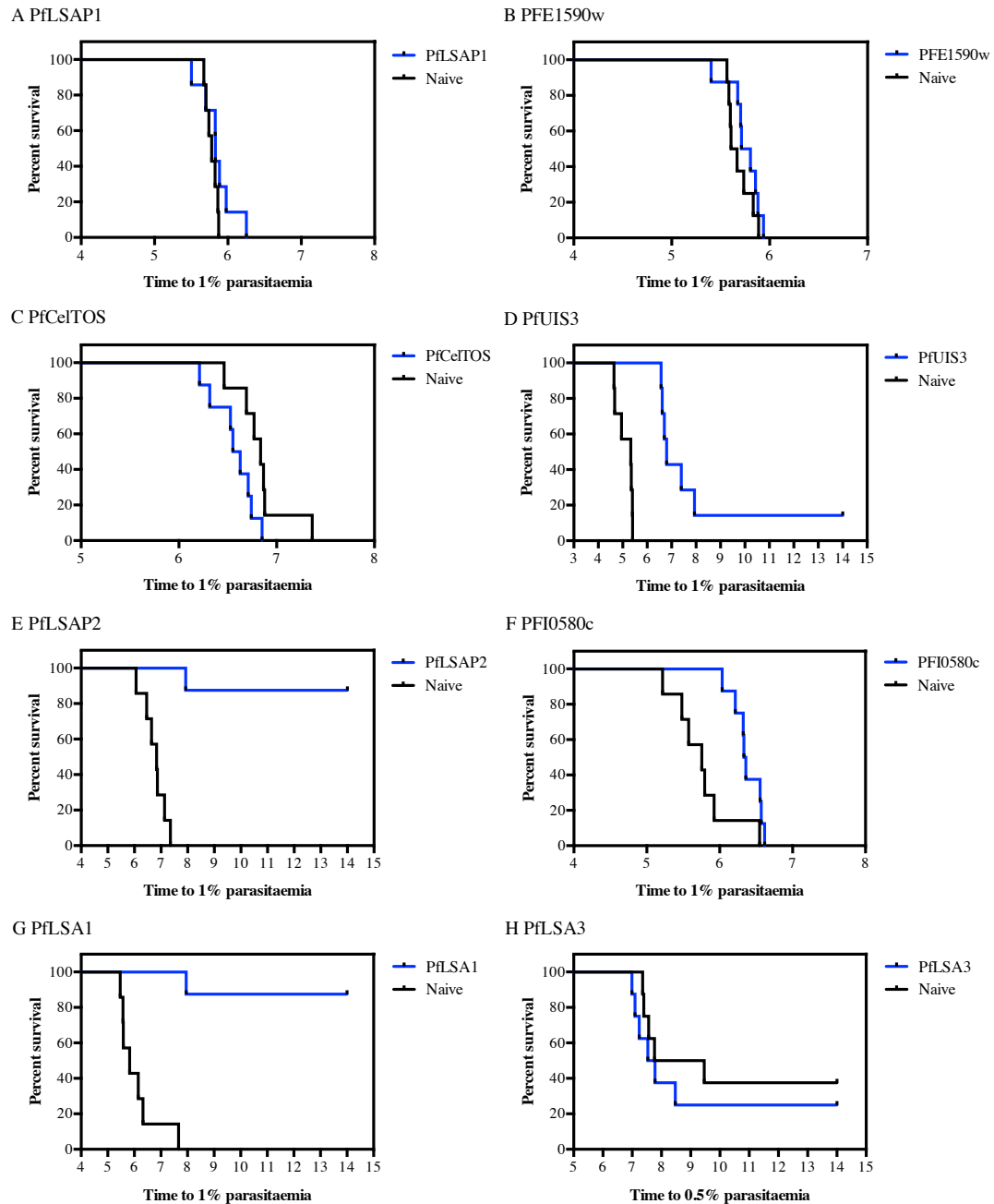


Figure 4.11. Protective efficacy after ChAd63-MVA vaccination with the *P. falciparum* candidate antigens and challenge with transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen (legend on previous page).

Four vaccination regimens resulted in a significant level of protective efficacy when comparing vaccinated mice with controls by the Log-rank (Mantel-Cox) Test: PFI0580c ($p=0.0072$), PfUIS3 ($p=0.0001$), PflSAP2 and PflSA1 (both $p<0.0001$), as a result of sterile protection or a delay in time to 1% parasitaemia (Figure 4.11, previous page). Mice were classified as sterilely protected when there was no evidence of parasites in the blood up to and including the experiment end-point, fourteen days post-challenge. PflSA1 and PflSAP2 conferred sterile protection in seven out of eight vaccinated mice (87.5%) (Table 4.1), whilst only one PfUIS3 vaccinated mouse was sterilely protected (12.5%). The identical vaccination-challenge experiments were also performed for the antigens CSP and TRAP (Figure 4.12), with CSP resulting in 25% sterile protection and TRAP resulting in no sterile protection (Table 4.1). The CSP and TRAP (denoted TRIP) vaccines had previously been generated by other members of the laboratory using the 3D7 sequence and human codon optimization as per the novel candidates tested, as described in Chapter 2.

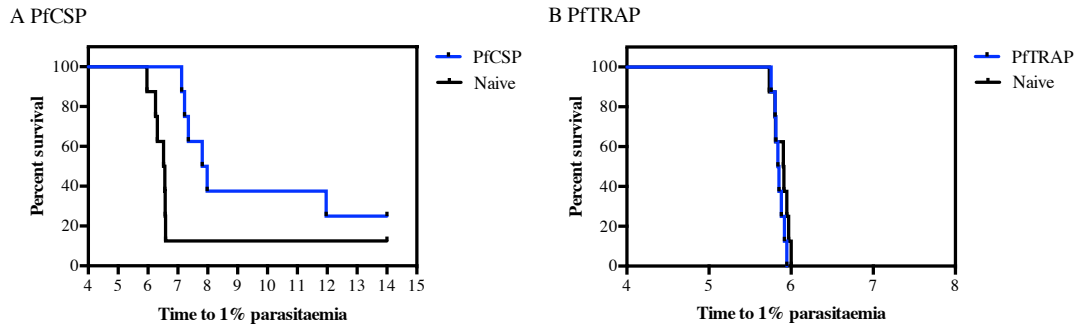


Figure 4.12. Protective efficacy after ChAd63-MVA vaccination with *P. falciparum* CSP or TRAP and challenge with transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen.

Balb/c mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63 followed eight weeks later by 1×10^7 pfu MVA, with either (A) PfCSP or (B) PfTRAP as the antigen. Vaccinated mice were challenged i.v. eight days post boost with 1000 transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen, along with eight naïve controls. Mice were monitored as per Figure 4.11. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves: (A) PfCSP $p=0.0266$ and (B) PfTRAP no significant difference ($p=0.2489$).

Table 4.1. Sterile protection from transgenic *P. berghei* sporozoite challenge after vaccination with the eight *P. falciparum* candidate antigens: comparison with PfCSP or PfTRAP vaccination.

Analysis of sterile protection in mice challenged in Figure 4.11 and Figure 4.12. Mice remaining slide negative until fourteen days post-challenge were considered sterilely protected. The eight new candidate antigens are listed in increasing size order. * Challenge of naïve mice with transgenic PbPfCSP resulted in only seven out of eight mice becoming infected with malaria. ** Challenge of naïve mice with transgenic PbPfLSA3 resulted in only five out of eight mice becoming infected with malaria.

Vaccine	Sterile Protection (%)
PfCSP	25*
PfTRAP	0
PfLSAP1	0
PfE1590w	0
PfCelTOS	0
PfUIS3	12.5
PfLSAP2	87.5
PfI0580c	0
PfLSA1	87.5
PfLSA3	25**

In order to compare the delay in time to 1% parasitaemia (tt1%) across vaccines and transgenic parasite strains, the median delay was calculated by the following formula: (tt1% of vaccinee) - (average tt1% of controls). This then accounts for the various fitness levels of the transgenic parasites (the differing blood parasitaemia kinetics). For PflSA3, the delay in time to 0.5% parasitaemia was calculated. Mice that were sterilely protected, or not infected, were not included in this analysis. Vaccination with PfCSP or PfUIS3 resulted in a significant delay in time to 1% parasitaemia ($p=0.004$, Log-rank (Mantel-Cox) Test), as did PFI0580c ($p=0.0072$, Log-rank (Mantel-Cox) Test), whilst no delay was observed after vaccination with PfTRAP, PflSAP1, PFE1590w, PfCelTOS and PflSA3 (Figure 4.13). Vaccines are listed in increasing size order on the x-axis, after the control vaccines CSP and TRAP. Only one PflSA1 or PflSAP2 vaccinated mouse became parasitaemic, and Figure 4.13 indicates that those mice did have a greater median delay than the naïve controls or mice vaccinated with antigens that resulted in no protective efficacy.

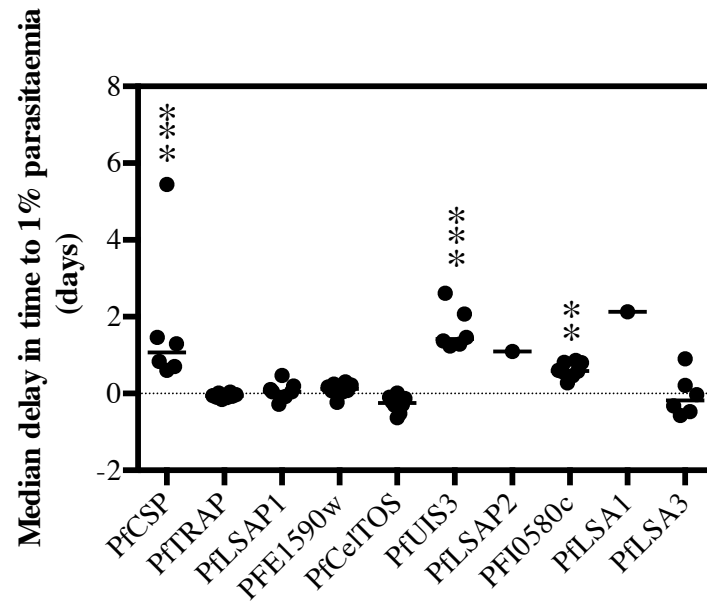


Figure 4.13. Median delay in time to 1% parasitaemia following challenge with transgenic *P. berghei* expressing the cognate *P. falciparum* antigen in mice vaccinated with ChAd63-MVA Pf[antigen].

The median delay in the time to 1% parasitaemia was calculated from the results presented in Figure 4.11 and Figure 4.12, using the formula: (tt1% of vaccinee) – (average tt1% of controls). Both median and individual data points are shown. Statistical significance was assessed using the Log-rank (Mantel-Cox) Test on the survival curves after sterilely protected or non-infected mice were excluded, ** $p=0.01-0.001$ *** $p<0.001$.

A summary of immunogenicity and efficacy for each candidate antigen is provided (Table 4.2). The level of cellular (ELISpot and ICS) or humoral (LIPS) immunogenicity of the candidate antigens did not necessarily predict a delay in parasitaemia or sterile protection. PfLSAP1 vaccination resulted in low levels of cellular immunogenicity (+) and no humoral immunogenicity (-), and therefore the absence of any protective efficacy was not surprising. However, PFE1590w and PfCelTOS both induced moderate levels (++) of cellular immunogenicity, yet no protection was seen. PfLSA3 resulted in high levels of cellular immunogenicity (++++) and moderate levels of humoral immunogenicity (++) and still no protection was observed. Of the antigens that did provide protection, PfUIS3 and PFI0580c exhibited moderate to high levels of both cellular and humoral immunogenicity and resulted in a delay in time to 1% parasitaemia, yet the immune responses to these antigens were comparable to the levels induced by both PfLSA1 and PfLSAP2 vaccination, which resulted in sterile protection.

In each transgenic challenge experiment mice were assessed for liver-stage parasite burden at 44 hours post-challenge by *in vivo* imaging (as the parasites expressed luciferase). For all vaccines that provided protection as determined by blood parasitaemia, protection was also evident by *in vivo* imaging of luciferase; these data are presented for the antigens that provided the highest levels of protection (PfUIS3, PfLSA1 and PfLSAP2) in Chapter 5.

Table 4.2. Summary of the immunogenicity and protective efficacy of the eight candidate *P. falciparum* vaccines in BALB/c mice.

Cellular immunogenicity is based on both *ex vivo* IFN γ ELISpot responses and ICS and antigens are ranked against each other. Humoral immunogenicity is based on antibody measurements via the LIPS assay. Protective efficacy is given after challenge with transgenic *P. berghei* sporozoites expressing the cognate *P. falciparum* antigen. Delay refers to a significant delay in the time to 1% parasitaemia compared to naïve control mice. PfLSA3 was classed as having 0% sterile protection because more naïve mice than vaccinated mice were not infected with malaria.

Vaccine	Cellular Immunogenicity	Humoral Immunogenicity	Efficacy
PfLSAP1	+	-	0
PFE1590w	++	++	0
PfCelTOS	++	+	0
PfUIS3	+++	++	12.5%, Delay
PfLSAP2	++	+++	87.5%
PFI0580c	++	+++	Delay
PfLSA1	+++	++	87.5%
PfLSA3	+++	++	0

4.3 Discussion

The two most advanced malaria vaccines, RTS,S and ChAd63-MVA ME-TRAP, probably do not currently provide high enough levels of protective efficacy for deployment in endemic areas. One approach to increase the effectiveness of a pre-erythrocytic malaria vaccine is to use the viral vectored prime-boost vaccine platform, known to induce very high T cell responses, to express alternative antigenic targets than CSP or TRAP. To this end, eight new liver-stage malaria vaccines were developed using the viral vectors ChAd63 and MVA separately expressing eight candidate antigens, as discussed in Chapter 3. The experiments described in this chapter aimed to continue this work by implementing a comparative screen of these vaccines in mice, through both immunogenicity and efficacy studies.

This chapter confirmed that all the viral vectored vaccines were expressing their target antigens, and induced high levels of IFN γ in a prime-boost regimen as measured by spleen ELISpot. The responses induced were of greater magnitude than immunization with the target antigens in different vaccine platforms, confirming that viral vectors are excellent inducers of cellular immunogenicity. Approximately twice the response was observed after ChAd63-MVA vaccination than previously observed by vaccination with PfCelTOS protein [51], PfLSA1 protein [434] or PfLSA3 DNA [415]. The only regimen with comparable immunogenicity was a recent paper using PfLSA1 and PfCelTOS DNA vaccination (3x30 μ g) with *in vivo* electroporation [253]. Surprisingly, the boosting effect of the MVA only returned the IFN γ level measured by ELISpot to that seen after the prime, for most antigens. However, a clear difference was seen by ICS, as no detectable responses were measured post-prime but were measurable post-boost. As this study encompassed the greatest number of pre-

erythrocytic antigens tested so far in the ChAd63-MVA prime-boost regimen, the ELISpot results may reflect variability or different kinetics leading to the peak time-point in the spleen being missed. Overall, PfUIS3, PflSA1, PfCelTOS, PFI0580c and PflSA3 vaccination, dependent on mouse strain, induced the greatest IFN γ responses.

The IFN γ response induced was predominantly from CD8 $^{+}$ T cells, with comparatively lower CD4 $^{+}$ responses, confirming that viral vectors are excellent at inducing CD8 $^{+}$ T cells [160, 256]. Most cells also produced TNF α and expressed CD107a, suggesting the cells were capable of cytotoxic activity [445]. As the spleen is a secondary lymphoid organ and immune cells travel through the blood to perform their effector functions, initial vaccine induced responses were assessed in these organs. For vaccines targeting liver-stage malaria, the effector immune cells must home to the liver in order to kill the intrahepatic parasites, therefore it is of interest to determine whether T cells induced by these viral vectored vaccines home to the liver [54, 296]. Additionally, since CD8 $^{+}$ T cells induced by the candidate vaccines produced multiple cytokines, it will also be important to determine whether polyfunctional cells contribute to a protective immune response, despite conflicting reports in the literature as to whether monofunctional or polyfunctional T cells are more protective [160, 218, 219, 296]. For the vaccines that induced the highest levels of protective immunity, PfUIS3, PflSA1 and PflSAP2, both the polyfunctionality and the ability to home to the liver was assessed in experiments described in Chapter 5.

All vaccines except PflSAP1 induced detectable antibody responses post-boost. The highest relative responses were to PFI0580c and PflSAP2, both antigens that are either expressed at the sporozoite or blood-stage in addition to the liver-stage. Such a finding confirms previous results that the viral vectored platform can induce high antibody titres in addition to cellular responses (reviewed in [453]). Furthermore, this series of experiments demonstrated the power of the LIPS assay in the absence of purified proteins; this assay was used successfully for all eight antigens, expanding previous work by Burbelo and colleagues [379, 380]. The difficulty with this assay is that the measurements are only semi-quantitative, making it impossible to compare the antibody levels detected with those reported in previous studies. For antigens that may protect against malaria through antibodies, quantitative measurements such as ELISA could be performed in the future to allow comparison, if protein could be expressed. Furthermore, to ensure the antibodies measured are functional, the IFAT should be performed for each against *P. falciparum* sporozoites. Preliminary assessment of binding to the native sequence by IFAT was performed, however no immunofluorescence was detected (except to the control CSP monoclonal antibody); as this was unexpected given results presented in previous studies [51] [407], this finding needs to be confirmed before conclusions can be confidently made.

Overall, cellular immune responses were greater in C57BL/6 mice than BALB/c mice, whilst humoral responses were comparable in both strains. This may be due to the greater innate capacity of the C57BL/6 strain to produce IFN γ compared to BALB/c [447-449], however this was not explored in this thesis. Interestingly, despite the overall higher cellular immunogenicity observed in C57BL/6 mice, not all antigens were capable of inducing a response (PflSAP1 and PflSA1), probably

reflecting the limited MHC repertoire of inbred mice. To overcome this limitation, multiple strains of mice can be used, or outbred mice. Outbred mice will exhibit greater variability, so are not ideal for initial screening studies, but are more representative of an outbred human population. For each of the candidate vaccines assessed in this thesis studies have been limited to C57BL/6 and BALB/c mice. Further testing could be undertaken in alternative strains; this has been done for the vaccines exhibiting the highest levels of protective efficacy, to ensure the protection was not just observed in H-2^d mice (see Chapter 5). However, this could also be pursued for the other candidates, regardless of whether they provided protection in BALB/c mice.

Surprisingly, ChAd63-MVA PfCelTOS vaccination did not induce protection against heterologous *P. berghei* wild-type challenge in BALB/c or C57BL/6 mice, nor homologous protection against transgenic PbPfCelTOS sporozoites. This was unexpected given adjuvanted PfCelTOS protein has previously been reported to induce cross-species protection in both BALB/c and outbred mice (60% sterile) [51]. The protection reported was likely dependent on antibodies given the cellular response measured by IFN γ ELISpot was only a median of 100 SFU per million splenocytes, much lower than observed in the current study. However, a recent study by the same group utilizing bacteria as a vector demonstrated 60% homologous protection with PbCelTOS and 55% heterologous protection with PfCelTOS against *P. berghei* challenge [454]. This seems surprising given there was a negligible production of antibodies and less than 60 SFU per million splenocytes measured by IFN γ ELISpot. Furthermore, results from our laboratory identified no protection from ChAd63-MVA PbCelTOS vaccination against homologous *P. berghei* challenge

(Karolis Bauza, DPhil Thesis). One critical difference between the studies of Bergmann-Leitner and our laboratory was the route of sporozoite injection, with Bergmann-Leitner challenging by sub-cutaneous injection versus intravenous injection in this study. Intravenous injection is a more stringent challenge model [455], so perhaps efficacy would be observed in the ChAd63-MVA regimen if sub-cutaneous challenge was used. However, initial results from the PfCelTOS clinical trial undertaken by the same group indicated there was no protective efficacy (WRAIR, presented at ASTMH 2012), further suggesting PfCelTOS is not the most promising candidate antigen.

ChAd63-MVA PfUIS3 vaccination was able to induce protection against both homologous and heterologous challenge, seen as a delay in time to blood-stage parasitaemia. Whilst cross-species protection has been demonstrated in radiation and genetically attenuated sporozoite models [210, 456, 457], this is one of very few reports showing such efficacy utilizing pre-erythrocytic sub-unit vaccines [51, 415]. As PfUIS3 is expressed at both the sporozoite and liver-stages, and induced both cellular and humoral responses, the mechanism mediating protection needs to be elucidated and was further explored in the experiments described in Chapter 5. The most significant and exciting finding in this chapter was that both PflSA1 and PflSAP2 induced 87.5% sterile protection (seven out of eight mice). This was greater than the protective efficacy induced by ChAd63-MVA PfTRAP or PfCSP, and provides an excellent proof of concept that better target antigens do exist. PflSAP2 was only recently identified as a liver-stage antigen [416], and these results mark the first studies of PflSAP2 as a vaccine candidate. PflSA1 was identified as a promising target in 1992 when an association was found with PflSA1, HLA-B53 and

resistance to severe malaria in Africa [411]. As there are no murine malaria homologs pre-clinical studies have been limited, yet PflSA1 has consistently been associated with protection in studies of naturally acquired immunity and irradiated sporozoite immunization [154-157, 458], and therefore clinical studies with this candidate should be considered.

Interestingly, vaccination with PfCSP provided a higher level of protection than with PfTRAP; this was not necessarily a surprising result, given a head to head comparison found PbCSP was more protective than PbTRAP [163], and in humans protective efficacy following ME-TRAP vaccination requires extremely high levels of T cells [160]. However, a recent clinical trial, Vac45 undertaken in Oxford, showed slightly higher efficacy for ME-TRAP compared to CSP when these antigens were used in ChAd63-MVA vectors (Sheehy *et al.*, in preparation). At least a low level of protection might have been expected with PfTRAP and this finding highlights how little we truly understand about translating results from murine studies to the clinic. One possible explanation is that TRAP is far better expressed on MHC molecules of hepatocytes from *P. falciparum* than *P. berghei* transgenic parasites. Of the other vaccines tested, PFI0580c also provided a delay in the time to blood-stage parasitaemia, albeit reduced compared to the delay induced by PfUIS3 or PfCSP. A combination vaccine of the *P. yoelii* version of PFI0580c and PyUIS3 has shown efficacy in outbred mice [407] and hence the current finding suggests that both antigens warrant further investigation. Neither PflSAP1, PFE1590w nor PflSA3 provided protection against transgenic challenge. This was the first time PflSAP1 and PFE1590w have been assessed as targets for a malaria vaccine, and hence there was no prior evidence on how these antigens might perform. PflSA3, however, has

previously induced protection in mice [415], chimpanzees [412] and monkeys [413], yet the absence of reported data from a clinical trial due for completion in 2008 suggests a lack of efficacy in humans (ClinicalTrials.gov identifier NCT00509158).

Excitingly, both PflSAP2 and PflSA1 conferred excellent protection despite inducing only moderate cellular immune responses compared to the levels of CSP-induced immunity required to provide protection in murine models (Pb9 epitope, 20-30% antigen-specific CD8⁺ cells) [285, 459], suggesting that such immunogenicity and efficacy could be attainable in a clinical setting. Interestingly, the magnitude of the immune response to the various candidate antigens did not predict which vaccines would be protective, as PflSA3 vaccination induced strong immunogenicity and yet no efficacy was seen. This may reflect differential abundance of peptides from the various antigens expressed by MHC molecules on the infected hepatocyte surface. Both PflSA1 and PflSAP2 likely reach the PVM and hence potentially the cytoplasm of the hepatocyte [416, 460], likely allowing them to be processed and presented on the cell surface. These findings indicate that the antigenic target is of high importance, as hypothesized, and suggest that both PflSA1 and PflSAP2 are potentially better targets than CSP or TRAP for a pre-erythrocytic vaccine.

In summary, this chapter evaluated the immunogenicity and protective efficacy of the eight candidate vaccines. All vaccines were immunogenic when administered in the standard eight-week interval prime-boost regimen, producing predominantly CD8⁺ cells secreting IFN γ and TNF α and expressing CD107a, with most vaccines also inducing detectable levels of antibodies. PflUIS3, PflSA1, PflSA3, PflI0580c and PflCelTOS induced the highest cellular responses, whilst PflSAP2 and PflI0580c

induced the highest antibody responses. PfLSA1, PfLSAP2 and PfUIS3 were capable of inducing greater protective efficacy than demonstrated for PfCSP or PfTRAP, providing excellent proof of concept that better target antigens do exist, as has recently been shown for blood-stage vaccines [353]. At this stage, the mechanism of protection of these vaccines is unknown, but could be dependent on both cellular and antibody responses. This chapter also highlighted the promise of transgenic parasites as a new pre-clinical tool, enabling assessment of antigens with no murine homologs, and supports the further use of antigen screens in developing an efficacious, deployable malaria vaccine. However, a limitation does exist: the antigens are expressed under the UIS4 promoter, which changes the expression profile of the antigens and may alter the expression level compared to the native promoter, although this needs to be investigated. For this reason, where possible the vaccines should also be assessed in a system where the target antigen is under control of its native promoter. Such an assay was developed as part of this thesis and the results are presented in Chapter 7. Both strategies combined should reveal promising target antigens to be taken forward into clinical trials.

**Chapter 5: Assessment of PfUIS3, PfLSA1 and
PfLSAP2 as candidates for a liver-stage malaria
vaccine**

5 Assessment of PfUIS3, PflSA1 and PflSAP2 as candidates for a liver-stage malaria vaccine

5.1 Introduction

A malaria vaccine is urgently needed given that current single-component vaccines lack sufficient efficacy for deployment in the field; it is becoming increasingly more accepted that such a vaccine will contain multiple antigens. The work described in this thesis aimed to design, develop and assess new viral vectored sub-unit malaria vaccines based on eight candidate liver-stage antigens. The results presented in Chapter 3 described the selection, design and development of these candidates into viral vectored vaccines. The results presented in Chapter 4 described the comparative assessment of the candidates, through immunogenicity studies in multiple strains of mice and efficacy against transgenic sporozoites in BALB/c mice. The vaccines encoding PfUIS3, PflSA1 and PflSAP2 induced the greatest level of protection, equal to or greater than protection seen with PfTRAP and PfCSP, the two most advanced clinical vaccine antigens.

UIS3 was first identified by transcriptomic profiling of *Plasmodium berghei* and *P. yoelii* sporozoites isolated from mosquito salivary glands, and hence named ‘upregulated in infective sporozoites’ [461, 462]. UIS3 is located in the PVM [463] and is essential for liver-stage growth and development, as demonstrated by UIS3 deficient parasites [52]. The protein has a suggested role in the importation of fatty acids to the PVM, based on evidence showing PbUIS3 directly binds the liver-fatty acid binding protein (L-FABP) in hepatocytes, and downregulation of L-FABP severely impairs parasite growth [463]. Whilst binding of PfUIS3 with L-FABP has

also been demonstrated [464], this was recently questioned by nuclear magnetic resonance interaction studies [465]. UIS3 has primarily been studied in a GAP model, as both Pb and PyUIS3 GAPs provide sterile protection against subsequent sporozoite challenge [52, 211]. Furthermore, PfUIS3 gene expression is downregulated in irradiated sporozoites compared to non-irradiated sporozoites, suggesting the antigen is also involved in this method of attenuation [420]. PyUIS3 was recently studied in a prime-boost sub-unit vaccination regimen (DNA-MVA), and provided 43% sterile protection in outbred mice when administered in combination with the *P. yoelii* homolog of PFI0580c [407].

PfLSAP2 has only recently been identified by transcriptomic profiling of proteins upregulated in sporozoites co-cultured with hepatocytes, compared to salivary gland sporozoites alone [416]. Further assessment of PfLSAP2 in this paper showed the protein was expressed in late liver-stage parasites (from days two to seven post-infection) with expression increasing throughout liver-stage development, as well as moderate expression observed on erythrocytic parasites, but no expression on sporozoites. The staining profile suggested PfLSAP2 was present in the PVM at the liver-stage, which could suggest an important role in the parasite-hepatocyte interaction [466]. Overall, expression was largely localized to the liver-stage, and therefore the protein was named 'liver-stage associated protein'. No murine orthologs were identified. Since this gene was first annotated, no further studies have been published. However, this gene has previously, and independently, been annotated as a blood-stage protein [421].

PfLSA1, however, has been extensively studied as it was one of the first liver-stage proteins identified [410] and one of the only known liver-stage specific proteins. LSA1 is well conserved amongst *P. falciparum* isolates [467, 468], and is essential for late liver-stage development [469]. The likely function of PfLSA1 is in the transition from the liver-stage to the blood-stage, as it is expressed abundantly in the PV as flocculent material surrounding merozoites. Cellular responses to PfLSA1 have consistently been associated with protection in studies of naturally acquired immunity and in volunteers vaccinated with irradiated sporozoites [154-157, 458, 470, 471]. A particularly strong association was found when HLA-B53-restricted cytotoxic T lymphocytes recognized a conserved epitope of PfLSA1, providing a molecular basis for the association of HLA-B53 with resistance to severe malaria in Africa [411]. A clinical trial of recombinant protein PfLSA1 administered with either of the adjuvants AS01 or AS02 (GSK) provided no protection against sporozoite challenge [232]. However, this vaccination regimen induced a strong antibody response and a high proportion of antigen-specific CD4⁺ T cells, but no induction of antigen-specific CD8⁺ T cells. Another clinical trial of a polyprotein construct expressing six antigens including PfLSA1, delivered in a FP9-MVA prime-boost regimen, also demonstrated no efficacy and minimal immunogenicity [472]. The authors suggested this disappointing result was either due to the large size of the construct (over 3000aa) and hence inadequate expression from the vectors, or due to competition between antigens. As PfLSA1 contains no murine homologs, pre-clinical research efforts assessing this antigen as a candidate for a sub-unit vaccine have been hampered.

In the results described in Chapter 4, protection against sporozoite challenge was only assessed in BALB/c mice. The drawback of using inbred mice is that protection may

be MHC-restricted, and hence may not translate into efficacy in humans. Ideally, vaccines identified as protective in inbred strains should also be tested in outbred mice, where there is high genetic diversity and broad MHC expression, similar to human populations. Furthermore, immunogenicity of these candidate vaccines was only assessed in the blood and spleen, yet primed immune cells are most likely required to home to the liver to exert their effect, and the liver is also an immunological organ capable of presenting antigen to naïve T cells [120]. In mice immunized with irradiated sporozoites, which arrest in the liver, research has shown that the number of CD8⁺ cells in the liver secreting IFN γ increases after vaccination [473, 474], and similarly, that a decrease in IFN γ secreting liver-resident CD8⁺ T cells coincides with a decrease in protection [475]. Additionally, previous studies have demonstrated that T cells induced by viral vectors expressing ME-TRAP home to the liver prior to sporozoite challenge [54, 296]; this was particularly evident after an adenoviral prime-MVA boost regimen. Presumably, the presence of antigen-specific cells in the liver prior to sporozoite challenge increases the speed in which an effective immune response can be mounted. It was therefore of relevant interest to assess the ability of T cells induced by PfUIS3, PflSA1 or PflSAP2 vaccination to home to the liver.

The work described in this chapter aimed to further assess the protective efficacy induced by ChAd63-MVA PfUIS3, PflSA1 and PflSAP2 vaccination. The first aim was to confirm protection in BALB/c mice and elucidate the mechanism of protection. The second aim was to assess efficacy in two further strains of mice, C57BL/6 (H-2^b) and CD-1 outbred mice, to determine whether the protection was limited to H-2^d mice. The third aim was to further assess the immune response

induced by these vaccines, by identifying the immunodominant epitopes in BALB/c and C57BL/6 mice and determining whether an antigen-specific response was detectable in the liver prior to challenge. A model was also available to assess the presence of HLA-A2-restricted epitopes within these antigens: transgenic mice expressing HLA-A2 [369]. HLA-A2 is a common MHC type in the general human population [476], and hence finding an HLA-A2-restricted epitope would suggest there is a high probability these vaccines would be immunogenic in humans. A probable clinical regimen using these antigens would be a multi-component malaria vaccine; therefore, the final aim of this chapter was the preliminary assessment of antigen interference or competition if these vaccines were used in combination with each other, or with the leading viral vectored vaccine ME-TRAP.

5.2 Results

5.2.1 Further assessment of ChAd63-MVA PfUIS3 as a candidate vaccine

5.2.1.1 Confirmation of protection in BALB/c mice

To confirm PfUIS3 vaccination results in protection in BALB/c mice, two further independent challenges were performed using transgenic PbPfUIS3 sporozoites. In both repeat challenges, a significant difference was confirmed between vaccinated and naïve control mice ($p=0.0001$ and $p<0.0001$, respectively, Log-rank (Mantel-Cox) Test) (Figure 5.1 A and B). The protection largely presented as a delay in time to 1% parasitaemia, with a median of 7.32 days in vaccinated mice compared to 5.47 days in control mice in the first experiment ($p=0.0011$, Log-rank (Mantel-Cox) Test), and 6.99 compared to 5.13 days in the second ($p=0.0001$, Log-rank (Mantel-Cox) Test). Two out of seven mice were also sterilely protected in the first experiment (26%), and one out of eight in the second (12.5%). Mice were considered sterilely

protected if they were slide-negative at fourteen days post-challenge. As there was no significant difference between experiments in the survival of naïve control mice, the results from the three experiments were combined (Figure 5.1 C). Overall, four out of 22 mice (18%) were sterilely protected with the rest exhibiting a delay in the time to 1% parasitaemia ($p < 0.0001$, Log-rank (Mantel-Cox) Test). Analysis after removing the sterilely protected mice indicated a median time to 1% parasitaemia of 7.06 days in vaccinated mice compared to 5.32 days in naïve control mice ($p < 0.0001$, Log-rank (Mantel-Cox) Test). The median $CD8^+ IFN\gamma^+$ response pre-challenge was 5.9% (Figure 5.1 D).

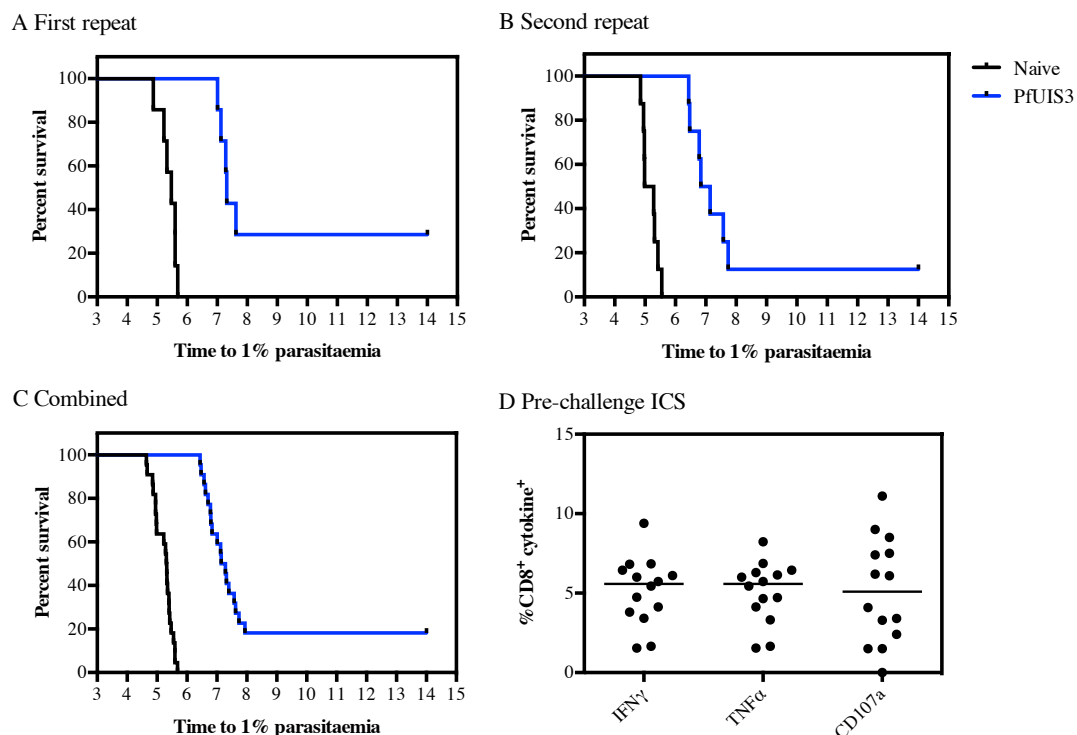


Figure 5.1. Confirmation of protection in BALB/c mice induced by PfUIS3 vaccination.

BALB/c mice (n=7-8 per group) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 1×10^7 pfu MVA-PfUIS3. Mice were challenged i.v. with 1000 transgenic PbPfUIS3 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily from four days post-challenge by thin film blood smears and the percent parasitaemia was calculated. Following three consecutive positive films, mice were culled. The data collected was used to calculate the time to 1% parasitaemia, using linear regression, and the results are presented in a survival graph. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves. Two independent experiments were conducted as shown in (A) $p=0.0001$ and (B) $p<0.0001$. (C) Results from the original and two subsequent repeat experiments were combined, Log-rank (Mantel-Cox) Test $p<0.0001$. (D) Seven days post-boost (three days pre-challenge) blood was taken to check cellular immunogenicity by ICS, after stimulation for six hours with an overlapping peptide pool to PfUIS3. Results are shown from the first two challenge experiments. Both median and individual data points are shown.

As control of all *P. falciparum* antigens in the separate transgenic parasites generated was through the *P. berghei* UIS4 promoter, expression should be specific to the sporozoite and liver-stages [208]. Therefore, protection should be due to an effect of the vaccine at the pre-erythrocytic stage. The transgenic parasites used in the challenges also expressed luciferase, enabling this theory to be confirmed by *in vivo* imaging at 44 hours post-challenge [383, 477]. For PfUIS3 in BALB/c mice, a significant difference in the level of luciferase expression from the liver was seen between vaccinated and naïve mice, with a median of 0.2×10^6 (considered baseline) versus 2.85×10^6 total flux per second ($p=0.0021$, Mann Whitney test) (Figure 5.2 A). In addition, the time to 1% parasitaemia of all mice (vaccinated and naïve) correlated with the luciferase expression measured by *in vivo* imaging (Spearman $r=-0.9416$, $p<0.0001$) (Figure 5.2 B), and consequently *in vivo* imaging was only performed for the original BALB/c challenge experiment.

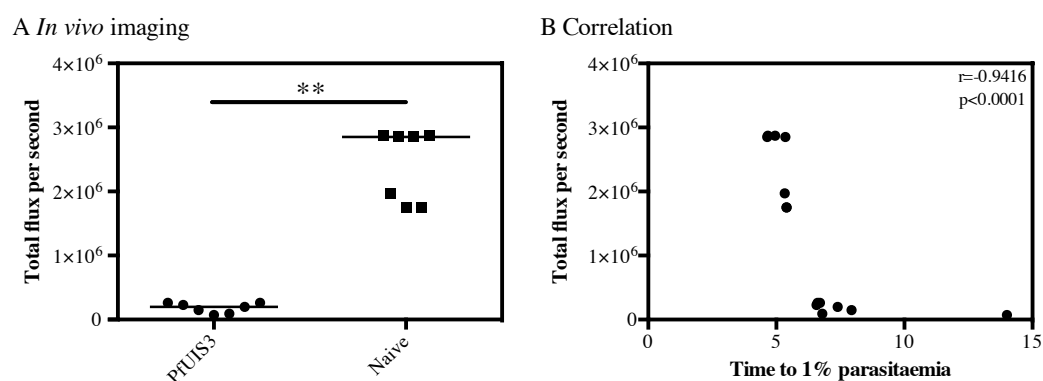


Figure 5.2. The time to 1% parasitaemia correlates with the bioluminescence of transgenic PbPfUIS3 liver-stage parasites.

At 44 hours post-PbPfUIS3 sporozoite challenge in the original experiment, mice were imaged *in vivo* using the IVIS 200 for luciferase expression. (A) Results are presented as the total flux measured per second. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, ** $p=0.0021$. (B) Correlation between time to 1% parasitaemia and IVIS imaging data from both vaccinated and naïve control mice ($n=14$). Spearman $r=-0.9416$, $p<0.0001$.

5.2.1.2 Protection in BALB/c mice is dependent upon CD8⁺ T cells

To assess the mechanism of protection, two methods were employed: *in vivo* depletion of either CD4⁺ or CD8⁺ T cells in mice vaccinated with ChAd63-MVA PfUIS3, or the adoptive transfer of CD4⁺ or CD8⁺ enriched splenocytes from ChAd63-MVA PfUIS3 vaccinated mice into naïve mice, followed by PbPfUIS3 sporozoite challenge. CD4⁺ or CD8⁺ T cells were depleted by injection of monoclonal antibodies (mAb) into vaccinated mice: 100µg injected intraperitoneal on three consecutive days depleted 100% of either cell population (assessed in the blood four days post-challenge, data not shown). No differences were found in the survival of control vaccinated mice and mice injected with a purified IgG control (Figure 5.3). In the absence of CD8⁺ T cells no significant difference in survival compared to naïve controls was observed, whilst a significant difference with IgG control mice was seen ($p < 0.0001$, Log-rank (Mantel-Cox) Test). The median time to 1% parasitaemia was reduced from 7.37 days in IgG control mice to 5.57 days in CD8⁺ depleted mice ($p < 0.0001$, Log-rank (Mantel-Cox) Test). CD4⁺ depletion also significantly reduced efficacy compared to control vaccinated mice and IgG control mice ($p = 0.0007$ and $p = 0.0044$, respectively, Log-rank (Mantel-Cox) Test); however, this regimen still provided some degree of protection compared to naïve mice (median of 6.45 days compared to 5.47 days in naïve controls, $p < 0.0001$, Log-rank (Mantel-Cox) Test).

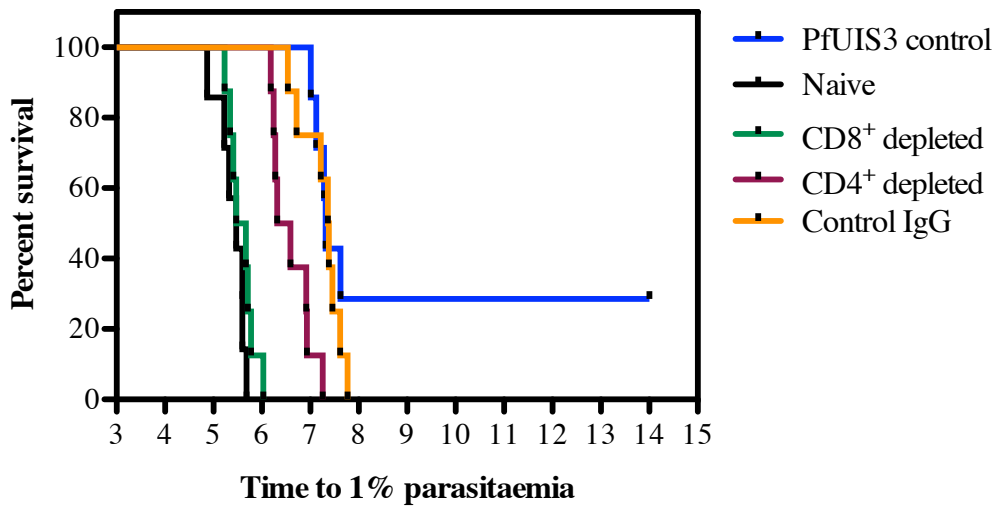


Figure 5.3. Depletion of CD8⁺ T cells abolishes the protection induced by ChAd63-MVA PfUIS3 vaccination in BALB/c mice.

Mice (n=4 groups of 8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 5×10^6 pfu MVA-PfUIS3. Mice were bled seven days post-MVA boost and cellular immunogenicity assessed by ICS, after stimulation for six hours with a pool of overlapping peptides to PfUIS3. No significant difference was found for any cytokine between the four groups (data not shown). Mice were then injected i.p. with 100 μ g of mAb to either CD4⁺ (GK1.5), CD8⁺ (8.43) or an IgG mAb control at days eight, nine and ten post-boost. At day ten, all mice were challenged i.v. with 1000 PbPfUIS3 sporozoites, including seven naïve controls. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves; CD8⁺ depleted versus PfUIS3 control vaccinated $p=0.0001$, CD8⁺ depleted versus control IgG $p<0.0001$, CD4⁺ depleted versus naïve $p<0.0001$, CD4⁺ depleted versus PfUIS3 control vaccinated $p=0.0007$, CD4⁺ depleted versus IgG control $p=0.0044$.

To adoptively transfer antigen-specific T cells, mice were vaccinated with either PfUIS3 or a vector control (PfCelTOS). Splenocytes harvested from these mice were sorted into CD4⁺ and CD8⁺ subsets by magnetic selection prior to transfer into naïve mice. The purity was checked by surface staining: the CD4⁺ sorted cells were 90% pure and the CD8⁺ cells 80% pure (data not shown). IgG purified from sera of these same mice was also transferred into naïve mice, prior to challenge. Adoptive transfer of 7.5×10^7 CD8⁺ T cells from PfUIS3 vaccinated mice provided a minor, but significant, delay in the time to 1% parasitaemia compared to both naïve mice (5.6 days versus 5.13 days, $p=0.0106$, Log-rank (Mantel-Cox) Test) and CD8⁺ splenocytes from vector control immunized mice (5.6 versus 5.42 days, $p=0.0121$, Log-rank (Mantel-Cox) Test) (Figure 5.4). However, there was also a significant difference in the survival of mice receiving PfUIS3 CD8⁺ T cells and control PfUIS3 vaccinated mice ($p=0.0003$, Log-rank (Mantel-Cox) Test). No protection was provided by the adoptive transfer of CD4⁺ T cells or purified IgG from PfUIS3 mice as compared to cells or sera from vector control immunized mice.

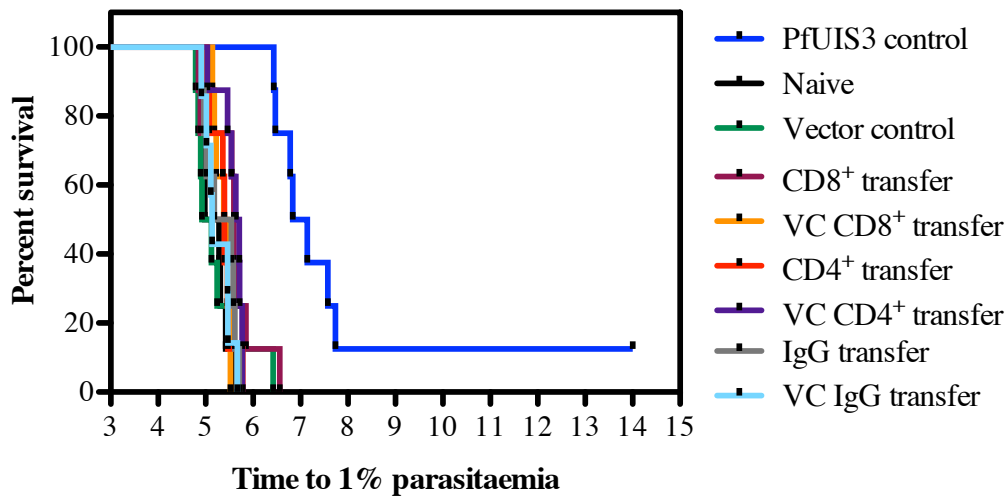


Figure 5.4. Adoptive transfer of splenocytes from ChAd63-MVA PfUIS3 vaccinated mice to naïve mice does not provide the same level of protection from sporozoite challenge as direct vaccination.

BALB/c mice (n=33-34 per group) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 5×10^6 pfu MVA-PfUIS3, or with a vector control (VC) in the same regimen (PfCelTOS). Five days post-boost one mouse was sacrificed and spleen ICS performed to estimate the percentage of antigen-specific cells. Based on this calculation, a vaccinated mouse would have approximately 75 000 antigen-specific CD4⁺ and 243 750 antigen-specific CD8⁺ splenocytes. Accommodating a ten-fold loss upon injection, at least 7.5×10^7 sorted CD4⁺ and 3.75×10^7 sorted CD8⁺ splenocytes were required for adoptive transfer per mouse. Seven days post-boost mice were culled (leaving n=7-8 per group as positive controls), cardiac bled for serum and spleens harvested for T cells. Splenocytes were sorted into CD4⁺ and CD8⁺ cells by magnetic beads, and 7.5×10^7 cells injected per mouse i.v. IgG was purified from the pooled serum, and 173 µg injected i.v. two days later (day of challenge) into eight mice for PfUIS3 and seven mice for the VC. On the day of IgG transfer, and two days post-splenocyte transfer, mice were challenged i.v. with 1000 PbPfUIS3 sporozoites, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves; CD8⁺ PfUIS3 transfer versus CD8⁺ VC transfer p=0.0121, CD8⁺ PfUIS3 transfer versus naïve p=0.0106, CD8⁺ PfUIS3 transfer versus PfUIS3 control p=0.0003.

As depletion of CD8⁺ T cells *in vivo* abolished protection, the efficacy seen in the first two PfUIS3 BALB/c challenge experiments was assessed for correlations with various CD8⁺ T cell subsets. No correlation was found with CD8⁺ IFN γ ⁺, TNF α ⁺, IL-2⁺, CD107a⁺ or antibodies measured pre-challenge (Spearman r, data not shown). Boolean gating was used to assess whether each CD8⁺ T cell was producing one or multiple cytokines. After ChAd63-PfUIS3 prime approximately one-third of antigen-specific CD8⁺ T cells were producing both IFN γ and TNF α , whilst two-thirds were single cytokine producing cells (predominantly IFN γ but also TNF α) (Figure 5.5). After MVA-PfUIS3 boost, approximately 50% of CD8⁺ PfUIS3-specific cells in the blood and 75% in the spleen were double-cytokine producing (IFN γ and TNF α). The same pattern was also found in C57BL/6 mice (data not shown). Given such a large number of cells were polyfunctional, correlations with each permutation were then performed for the first two challenges (against time to 1% parasitaemia); no correlations were identified (Spearman r, data not shown).

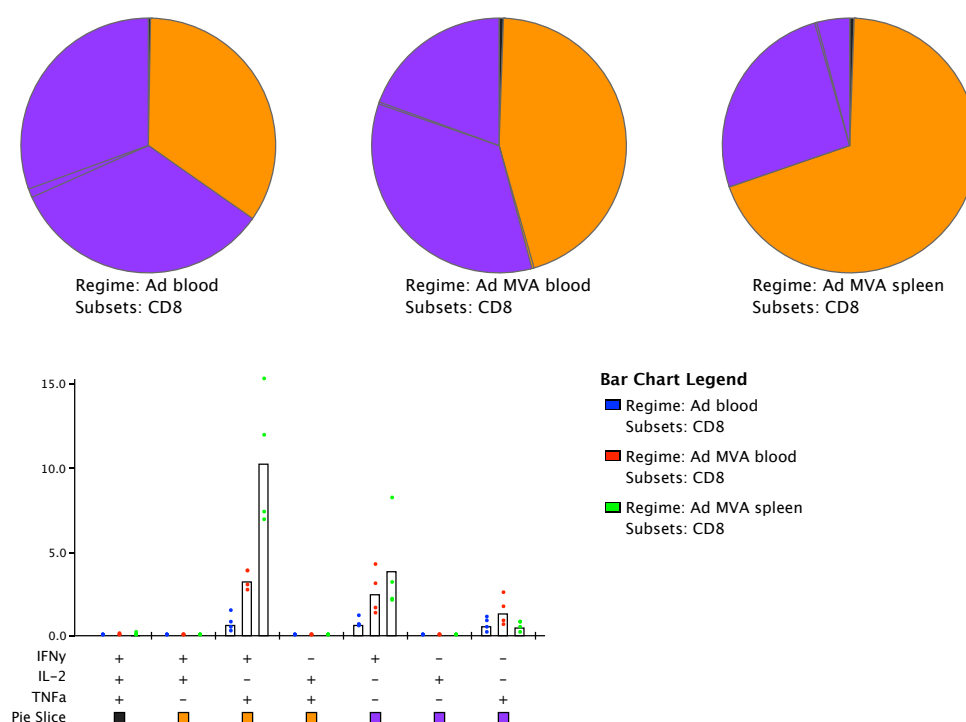


Figure 5.5. Polyfunctionality of CD8⁺ T cells induced by ChAd63-MVA PfUIS3 vaccination in BALB/c mice.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 1×10^7 pfu MVA-PfUIS3, as part of the immunogenicity experiments described in Chapter 4. Two weeks post-ChAd63 prime and one-week post-MVA boost blood was taken and cellular immunogenicity assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PfUIS3. Two weeks post-MVA boost mice were sacrificed, spleens harvested and cellular immunogenicity again assessed by ICS. The proportion of cells at each time-point expressing one, two or three cytokines is shown. The bar chart indicates which cytokines were produced, whilst the slices of the pie chart indicate the proportion of cells producing one (purple), two (orange) or three (black) cytokines. The pie chart showing “Ad MVA Blood” is representative of the proportions seen pre-challenge.

5.2.1.3 ChAd63-MVA PfUIS3 vaccination also provides protection against sporozoite challenge in C57BL/6 mice

To determine whether protection against sporozoite challenge was specific to BALB/c mice (i.e. limited to H-2^d), efficacy was also assessed in C57BL/6 mice (H-2^b) and CD-1 mice (an outbred strain). Two challenge experiments were performed in C57BL/6 mice with PbPfUIS3 after ChAd63-MVA PfUIS3 vaccination; both induced a significant difference in survival compared to naïve control mice (both experiments $p < 0.0001$, Log-rank (Mantel-Cox) Test). As the survival of the naïve controls differed significantly between the two experiments they could not be combined. Representative results from the second experiment are shown (Figure 5.6 B). In the first experiment two mice were sterilely protected (25%) and there was a significant delay in the time to 1% parasitaemia for the remaining mice (7.24 versus 5.45 days in naïve controls, $p = 0.0003$, Log-rank (Mantel-Cox) Test); in the second, three were sterilely protected (37.5%) and again there was a significant delay in the time to 1% parasitaemia for the remaining mice (7.58 versus 4.69 days in naïve controls, $p = 0.0008$, Log-rank (Mantel-Cox) Test). A high level of antigen-specific CD8⁺ T cells were measured prior to challenge, with a median of 4.8% CD8⁺ IFN γ ⁺ (Figure 5.6 A); correlations were performed for all immunogenicity measures (including polyfunctional CD8⁺ T cells and antibody levels) and a significant negative correlation was identified between the time to 1% parasitaemia and CD8⁺ IL-2 secreting cells (Spearman $r = -0.756$, $p = 0.0368$) (Figure 5.6 C). This correlation was not identified in the first C57BL/6 challenge experiment.

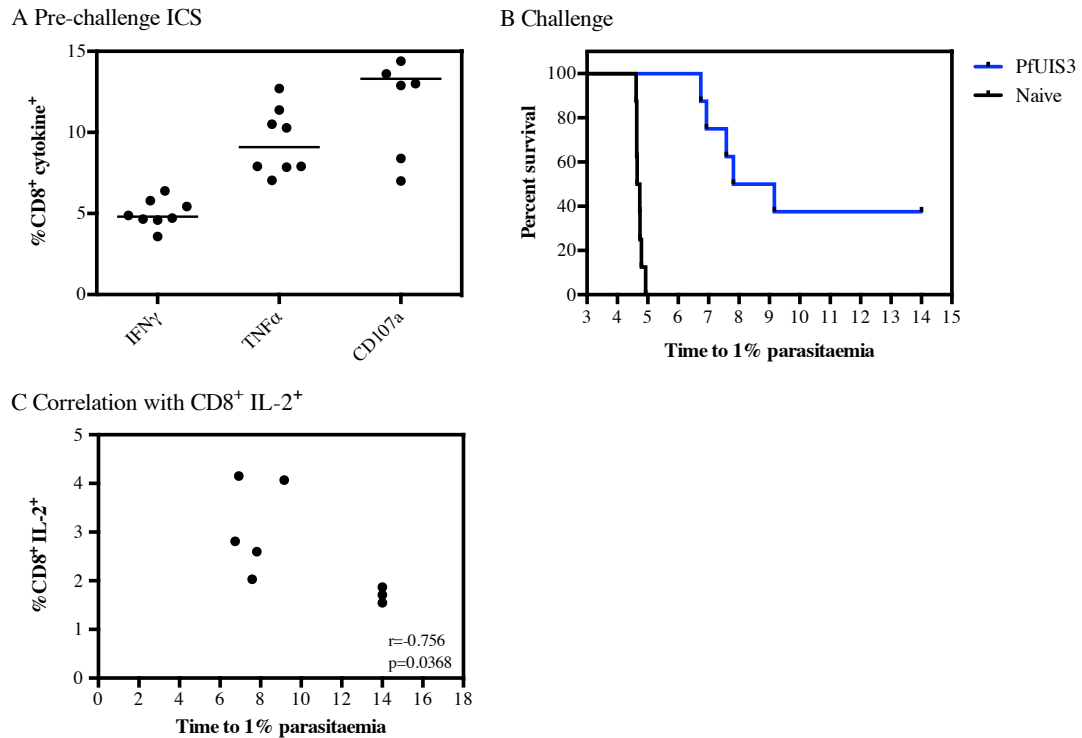


Figure 5.6. ChAd63-MVA PfUIS3 vaccination induces protection against sporozoite challenge in C57BL/6 mice.

C57BL/6 mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 6×10^6 pfu MVA-PfUIS3. Blood was taken seven days post-boost to assess both humoral and cellular immunogenicity. (A) Cellular immunogenicity was assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PfUIS3. Both median and individual data points are shown. (B) Mice were challenged i.v. with 1000 transgenic PbPfUIS3 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, $p < 0.0001$. (C) Correlations were assessed between the time to 1% parasitaemia and both cellular and humoral immunogenicity. The only correlation identified was with CD8⁺ IL-2⁺ cells, Spearman $r = -0.756$ $p = 0.0368$.

One challenge experiment was performed in the outbred laboratory strain CD-1. Whilst an immune response was induced (median CD8⁺ IFN γ ⁺ of 0.9%) (Figure 5.7 A), no significant difference in efficacy was observed between vaccinated and naïve control mice (median time to 1% parasitaemia of 6.77 days in vaccinated mice compared to 5.67 days in control mice) (Figure 5.7 B), despite an initial trend. As increased variability is expected in outbred mice, future experiments should include greater sample sizes. Despite the absence of any protective efficacy, a significant positive correlation was identified between the time to 1% parasitaemia of vaccinated mice and the percentage of both CD8⁺ cells producing IFN γ (Spearman $r=0.7306$, $p=0.0368$) (Figure 5.7 C) or TNF α (Spearman $r=0.7857$, $p=0.0279$) (Figure 5.7 D). An antigen-specific antibody response was also induced in these mice, with a fold-change of 1.05 from naïve, however the levels did not correlate with the time to 1% parasitaemia (Spearman r , data not shown).

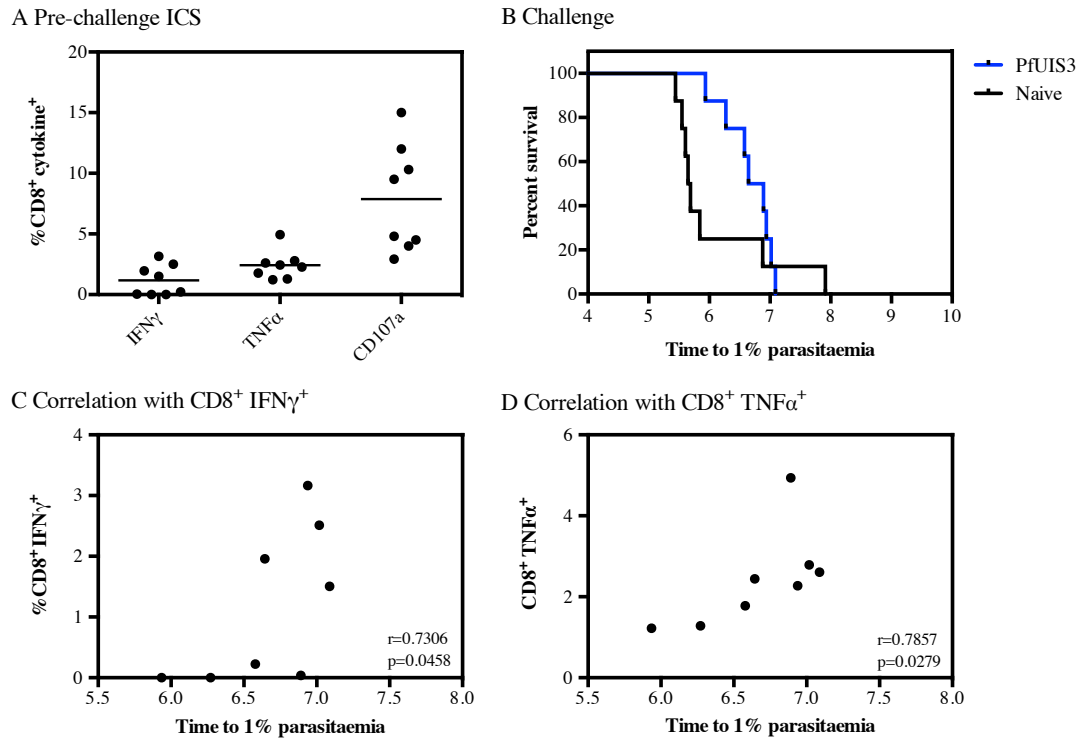


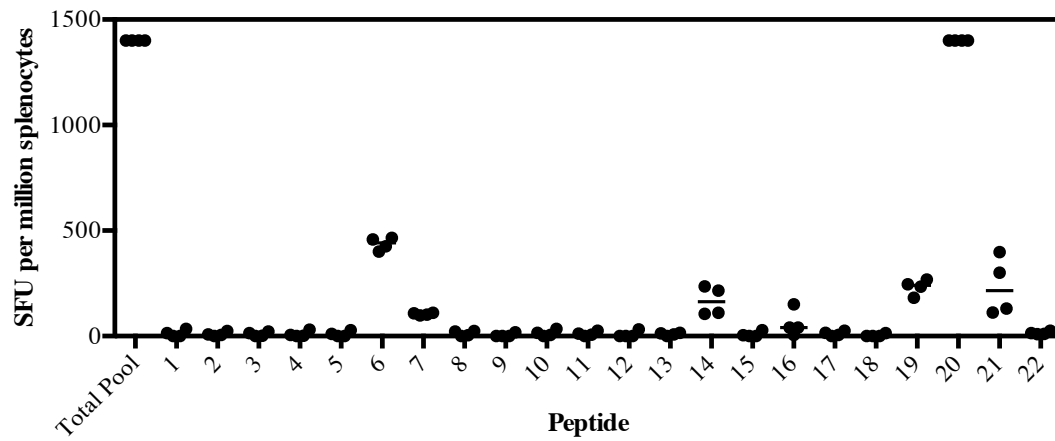
Figure 5.7. ChAd63-MVA PfUIS3 vaccination does not induce protection against sporozoite challenge in CD-1 outbred mice.

CD-1 mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 1×10^7 pfu MVA-PfUIS3. Blood was taken seven days post-boost to assess both humoral and cellular immunogenicity. (A) Cellular immunogenicity was assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PfUIS3. Both median and individual data points are shown. (B) Mice were challenged i.v. with 1000 transgenic PbPfUIS3 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, no difference was found ($p=0.25$). (C+D) Correlations were assessed between the time to 1% parasitaemia and both cellular and humoral immunogenicity. Correlations were identified with both CD8⁺ IFN γ ⁺ cells, Spearman $r=-0.756$ $p=0.0368$, and CD8⁺ TNF α ⁺ cells, Spearman $r=0.7857$ $p=0.0279$.

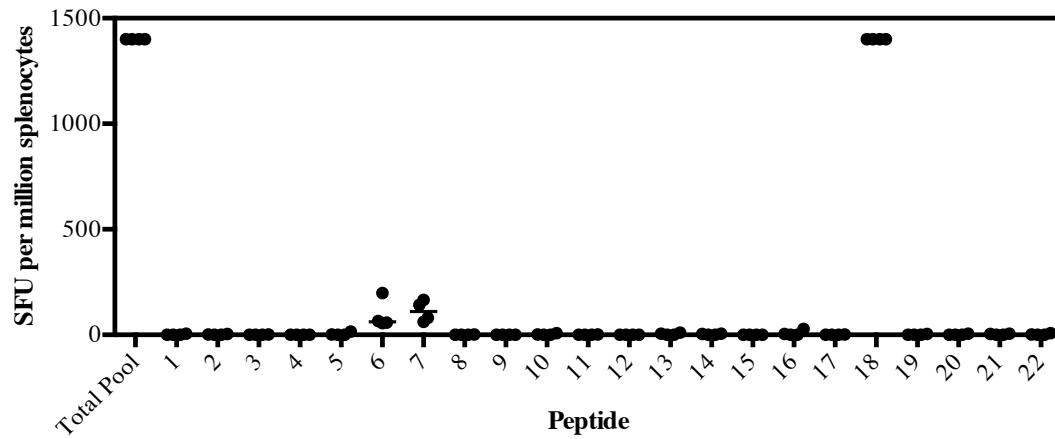
5.2.1.4 Further assessment of immunogenicity induced by ChAd63-MVA PfUIS3 vaccination

As protective efficacy was demonstrated in multiple strains of mice, it was of interest to identify the epitopes associated with protection, and to determine whether any HLA-A2-restricted epitopes could be detected. Epitope mapping was conducted in BALB/c and C57BL/6 mice, as well as with transgenic mice that express HLA-A2 (HHDs), by spleen IFN γ ELISpot to individual peptides (Figure 5.8). Two peptides were identified that could be conserved, as positive responses were seen in each strain of mice, representing amino acids 51 to 80 (labelled as number 6 and 7). The immunodominant epitope in BALB/c was peptide 20, aa191-200, along with five sub-dominant epitopes (including peptides 6 and 7). The immunodominant epitope in C57BL/6 mice was peptide 18, aa171 to 190, and in HHD mice it was peptide 21, aa201 to 220. Full peptide sequences are provided in Appendix Two, for all antigens.

A BALB/c (H-2^d)



B C57BL/6 (H-2^b)



C HHD (HLA-A2)

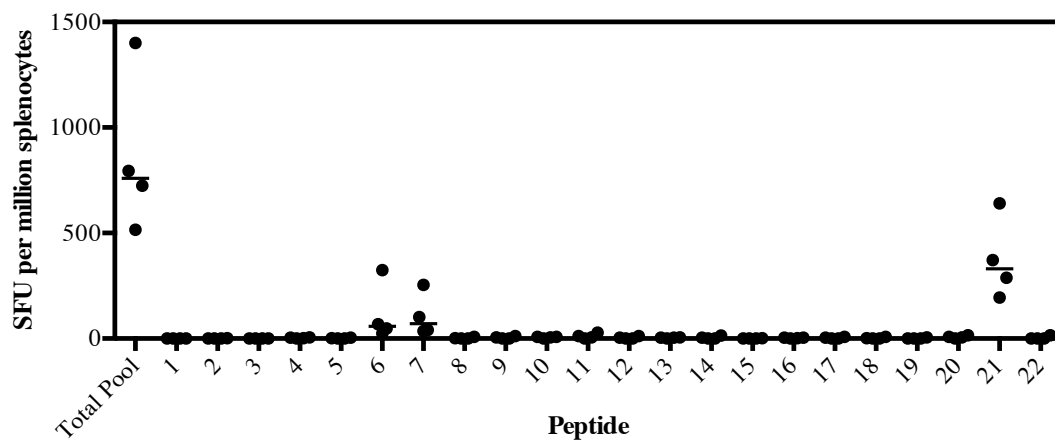


Figure 5.8. Epitope mapping of PfUIS3 in BALB/c, C57BL/6 and HHD mice.

(A) BALB/c, (B) C57BL/6 or (C) HHD mice (n=4 per strain) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfUIS3 followed eight weeks later by 1×10^6 pfu MVA-PfUIS3. Two weeks post-MVA boost, mice were sacrificed and splenocytes isolated to perform an *ex vivo* IFN γ ELISpot. Splenocytes were stimulated with either an overlapping peptide pool to PfUIS3 or individual peptides (20aa each, overlapping by ten). Both median and individual data points are shown.

As minimal correlations were identified between the time to 1% parasitaemia in vaccinated mice and cellular or humoral immunogenicity in the blood, immune responses in other organs may be more indicative of protection. Whilst it is not possible to assay immunogenicity in other organs prior to challenge, it was of interest to know if PfUIS3-specific cells were detectable in the liver of vaccinated mice, given it is the target organ. Livers were perfused with PBS *in situ* to remove cells from the circulating blood, then a single cell suspension prepared and hepatocytes removed via a percoll gradient. PfUIS3-specific cells were observed in the livers of both BALB/c and C57BL/6 mice at a comparable level, or greater, to the frequency of PfUIS3-specific cells observed in the spleen (median CD8⁺ IFN γ ⁺ of 10.97% and 18.01% in the liver, respectively) (Figure 5.9). The only significantly greater level was observed in C57BL/6 mice for CD107a expression ($p < 0.01$, two-way ANOVA). A detectable but low response was identified in livers of HHD mice (median CD8⁺ IFN γ ⁺ of 1.42%). In the same experiment, the presence of effector memory (CD62I⁻ CD127⁺) and central memory (CD62I⁺ CD127⁺) cells was assessed [54, 478]; of the CD8⁺ cells producing IFN γ , 77-99% were of the effector memory subset (Figure 5.10), similar to previously reported studies [54, 479]. For both BALB/c and C57BL/6, a significantly greater proportion of antigen-specific CD8⁺ cells were of the effector memory subset in the liver compared to the spleen ($p < 0.0001$ and $p < 0.01$, respectively, two-way ANOVA) (Figure 5.10 D).

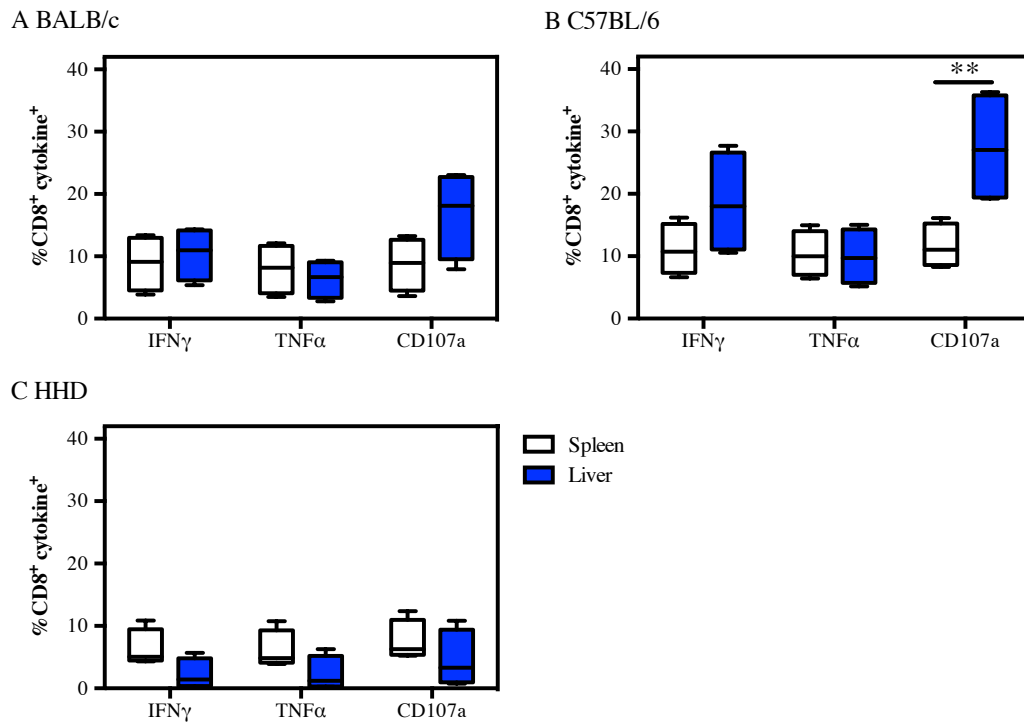


Figure 5.9. PfUIS3-specific cells were observed in both the liver and spleen of mice after ChAd63-MVA vaccination.

In the experiment described in Figure 5.8 above, when mice were sacrificed two weeks post-boost livers were also harvested, following perfusion *in situ*. Single cell suspensions of liver and spleen mononuclear cells were isolated and stimulated for six hours with an overlapping peptide pool to PfUIS3. The percentage of CD8⁺ cytokine⁺ cells is shown for (A) BALB/c, (B) C57BL/6 and (C) HHD mice. Box plots indicate the median response with whiskers representing the minimum and maximum responses. Statistical difference was assessed using a two-way ANOVA with Bonferroni post-test; the only difference was for C57BL/6 mice where the CD8⁺ CD107a⁺ response observed in the liver was greater than in the spleen, ** p<0.01.

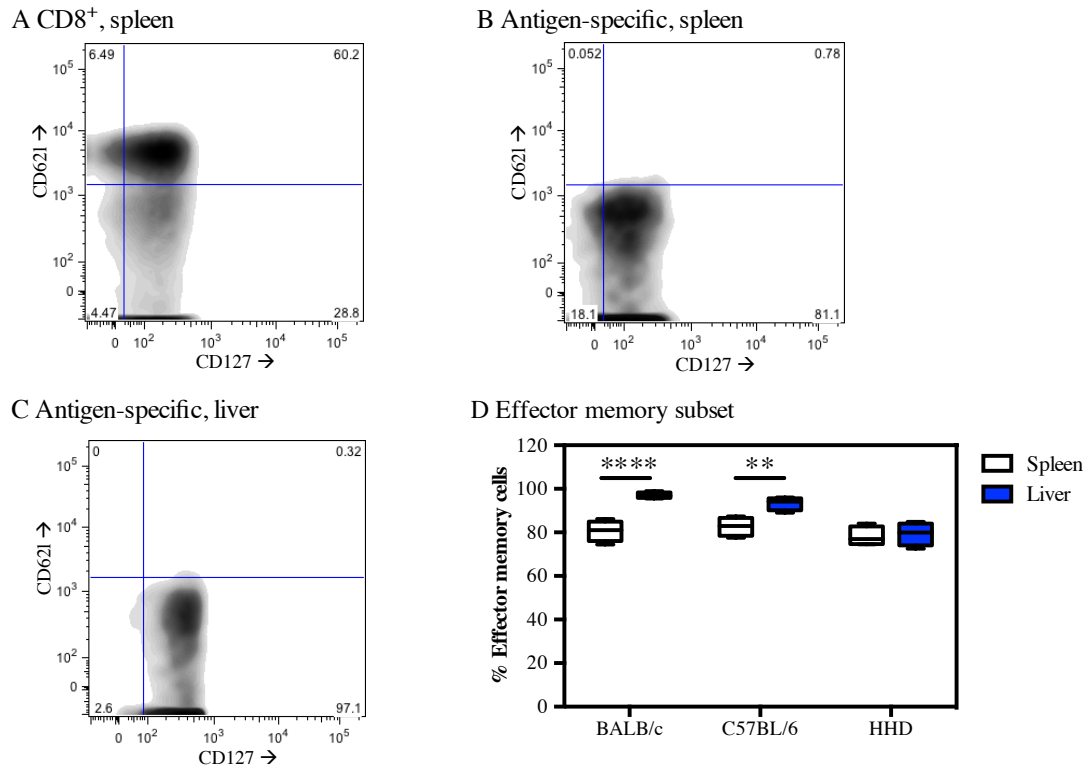


Figure 5.10. PfUIS3 vaccination induces predominantly antigen-specific cells of the effector memory subset.

In the experiment described in Figure 5.9, liver and spleen mononuclear cells were also stained with the markers CD62l and CD127 to calculate the percentage of effector memory (CD62l⁻ CD127⁺) and central memory (CD62l⁺ CD127⁺) subsets. The gating strategy used to identify the populations is shown in panel (A); gating was first performed on CD8⁺ cells, where a clear central memory population can be seen (CD62l⁺ CD127⁺). Antigen-specific cells were then selected based on secretion of IFN γ . Panel (B) demonstrates a representative population in the spleen and (C) demonstrates a representative population in the liver. Panel (D) compares the effector memory population in the spleen and liver for each strain of mice assessed. Statistical difference was assessed using a two-way ANOVA with Bonferroni post-test, **p<0.01 ****p<0.0001.

5.2.2 Further assessment of ChAd63-MVA PfLSA1 as a candidate vaccine

5.2.2.1 Confirmation of sterile protection in BALB/c mice

To confirm PfLSA1 vaccination results in sterile protection in BALB/c mice, an independent repeat challenge was performed using transgenic PbPfLSA1 sporozoites. A significant difference was confirmed between vaccinated and naïve control mice ($p < 0.0001$, Log-rank (Mantel-Cox) Test) (Figure 5.11 A), with six out of eight mice sterilely protected (75%). As there was no significant difference between the repeat and original experiment in the survival of naïve control mice, the results from the two experiments were combined (Figure 5.11 B), resulting in thirteen out of sixteen mice (81.25%) sterilely protected from challenge after PfLSA1 vaccination ($p < 0.0001$, Log-rank (Mantel-Cox) Test). The median $CD8^+ IFN\gamma^+$ response pre-challenge was 1.8% (Figure 5.11 C).

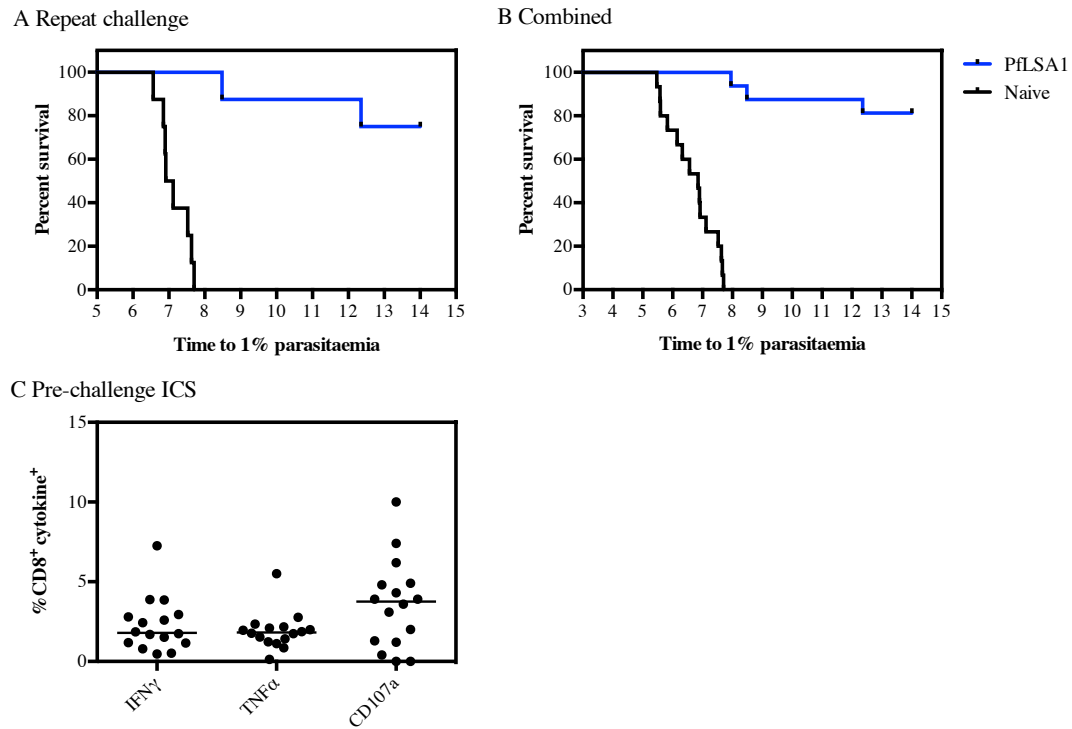


Figure 5.11. Confirmation of pre-erythrocytic protection in BALB/c mice induced by PflSA1 vaccination.

(A) BALB/c mice ($n=8$) were vaccinated i.m. with 1×10^8 ifu ChAd63-PflSA1 followed eight weeks later by 1×10^7 pfu MVA-PflSA1. Mice were challenged i.v. with 1000 transgenic PbPflSA1 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, $p < 0.0001$. (B) Results from the repeat and the original experiment were combined (vaccinated $n=16$, naïve $n=15$), Log-rank (Mantel-Cox) Test $p < 0.0001$. (C) Seven days post-boost (three days pre-challenge) blood was taken to check cellular immunogenicity by ICS, after stimulation for six hours with an overlapping peptide pool to PflSA1. Both median and individual data points are shown for the two experiments combined.

To confirm protection occurred at the pre-erythrocytic stage (see section 5.2.1.1), *in vivo* imaging was performed 44 hours post-challenge to measure bioluminescence. A significant difference in the level of luciferase expression from the liver was seen between vaccinated and naïve mice, with a median of 1.48×10^5 (considered baseline) versus 9.63×10^5 total flux per second ($p=0.0022$, Mann Whitney test) (Figure 5.12 A). In addition, the time to 1% parasitaemia of all mice (vaccinated and naïve) correlated with the luciferase expression measured by *in vivo* imaging (Spearman $r=-0.8884$, $p<0.0001$) (Figure 5.12 B) and consequently *in vivo* imaging was only performed for the original BALB/c challenge experiment.

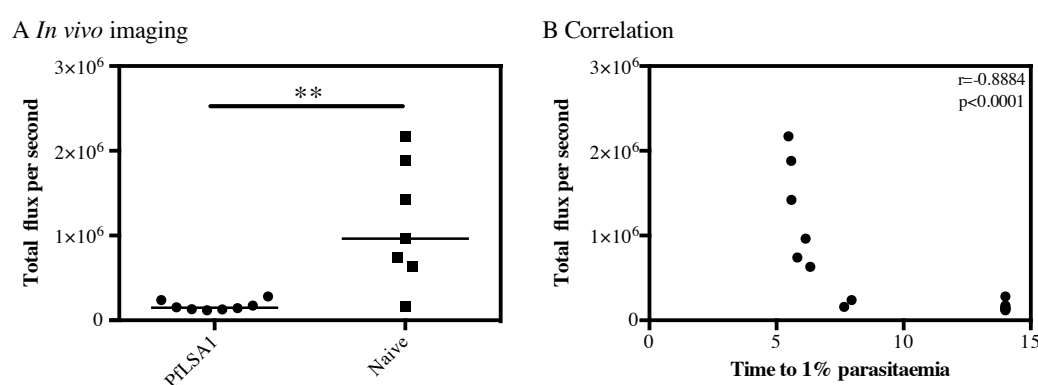


Figure 5.12. The time to 1% parasitaemia correlates with the bioluminescence of transgenic PbPFLSA1 liver-stage parasites.

At 44 hours post-PbPFLSA1 sporozoite challenge in the original experiment, mice were imaged *in vivo* using the IVIS 200 for luciferase expression. (A) Results are presented as the total flux measured per second. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, ** $p=0.0022$. (B) Correlation between time to 1% parasitaemia and IVIS imaging data from both vaccinated and naïve control mice ($n=15$). Spearman $r=-0.8884$, $p<0.0001$.

5.2.2.2 Protection in BALB/c mice is dependent upon CD8⁺ T cells

As thirteen out of sixteen mice were sterilely protected, and hence given the arbitrary value of ‘14’ in the time to 1% parasitaemia analysis, correlations with immune subsets were statistically challenging. Stratifying the mice into ‘delayed’ and ‘sterile protection’ also provided statistical difficulty, given only three mice were delayed. Performing such analysis identified no correlations and no significant differences between mice with a delay in the time to 1% parasitaemia or those sterilely protected when any immune subsets were assessed (see Figure 5.13 for a representative graph). PflSA1 vaccination also induced polyfunctional antigen-specific CD8⁺ T cells, with approximately 50-75% producing both IFN γ and TNF α post-boost in the blood and spleen (Figure 5.14). Assessing all permutations of polyfunctionality found no immune subsets that differed significantly between delayed and protected mice.

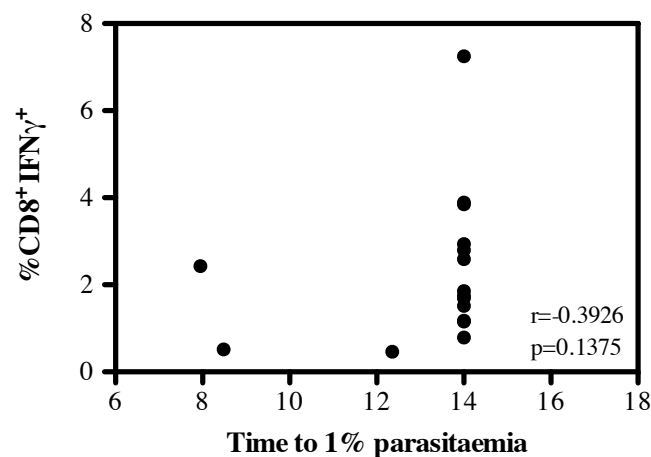


Figure 5.13. The percentage of PflSA1-specific CD8⁺ T cells producing IFN γ in the blood does not correlate with the time to 1% parasitaemia after sporozoite challenge.

In the experiment described in Figure 5.11 above, a correlation analysis was performed between the time to 1% parasitaemia with the percentage of CD8⁺ T cells producing IFN γ . Data was combined from both PflSA1 efficacy challenges performed in BALB/c mice (n=16). No significant correlation was identified, Spearman $r=-0.3926$, $p=0.1375$. This graph can be considered representative for all other immune subsets assessed.

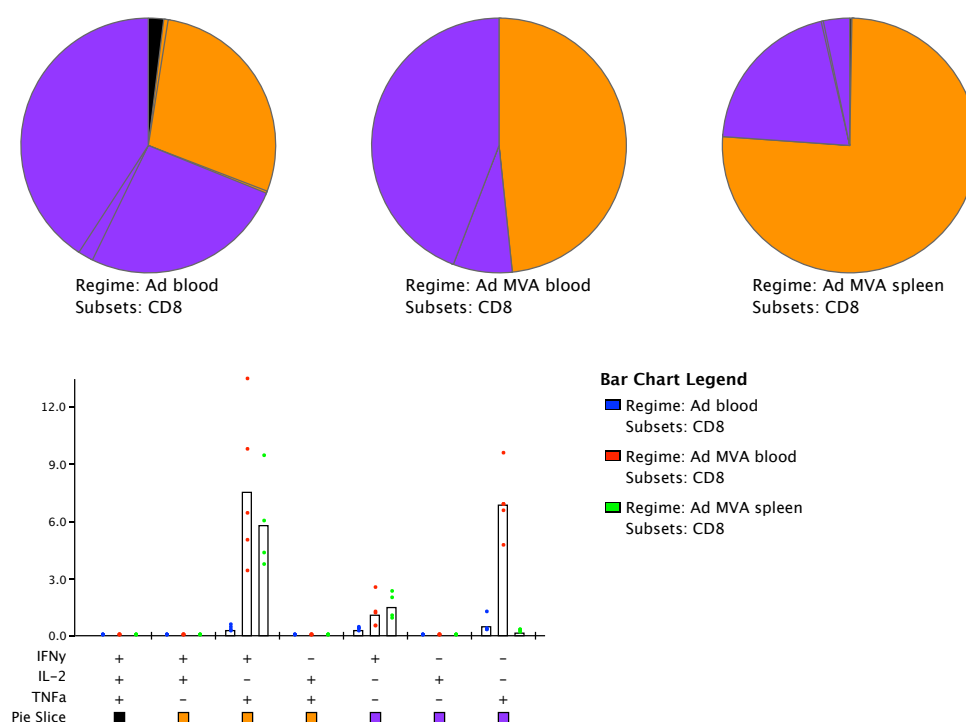


Figure 5.14. Polyfunctionality of CD8⁺ T cells induced by ChAd63-MVA PflSA1 vaccination in BALB/c mice.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-PflSA1 followed eight weeks later by 1×10^7 pfu MVA-PflSA1, as part of the immunogenicity experiments described in Chapter 4. Two weeks post-ChAd63 prime and one-week post-MVA boost blood was taken and cellular immunogenicity assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PflSA1. Two weeks post-MVA boost mice were sacrificed, spleens harvested and cellular immunogenicity again assessed by ICS. The proportion of cells at each time-point expressing one, two or three cytokines is shown. The bar chart indicates which cytokines were produced, whilst the slices of the pie chart indicate the proportion of cells producing one (purple), two (orange) or three (black) cytokines. The pie chart showing “Ad MVA Blood” is representative of the proportions seen pre-challenge.

To overcome this statistical limitation, the dependence on CD8⁺ or CD4⁺ T cells was assessed by *in vivo* depletions of each of these subsets prior to transgenic sporozoite challenge. CD4⁺ or CD8⁺ T cells were depleted by injection of monoclonal antibodies into vaccinated mice: 100µg injected intraperitoneal on three consecutive days depleted 100% of either cell population (assessed in the blood four days post-challenge, data not shown). No differences were found in the survival of PfLSA1 control vaccinated mice and mice depleted with a purified IgG control (Figure 5.15). CD8⁺ depletion reduced the protection induced by PfLSA1 vaccination, as shown by no significant difference in survival compared to naïve mice and a significant difference compared to PfLSA1 vaccinated control mice (p=0.0027, Log-rank (Mantel-Cox) Test). However, one mouse was sterilely protected and there was no significant difference between CD8⁺ depletion and IgG control mice (p=0.0658, Log-rank (Mantel-Cox) Test), despite a clear trend. CD4⁺ depletion also reduced the protection induced by PfLSA1 vaccination, as shown by a significant difference in survival compared to PfLSA1 control vaccinated mice (p=0.026, Log-rank (Mantel-Cox) Test), however this difference was not upheld against IgG control mice (p=0.3425, Log-rank (Mantel-Cox) Test). In addition, these mice still induced a significant level of protection compared to naïve mice (p=0.0003, Log-rank (Mantel-Cox) Test).

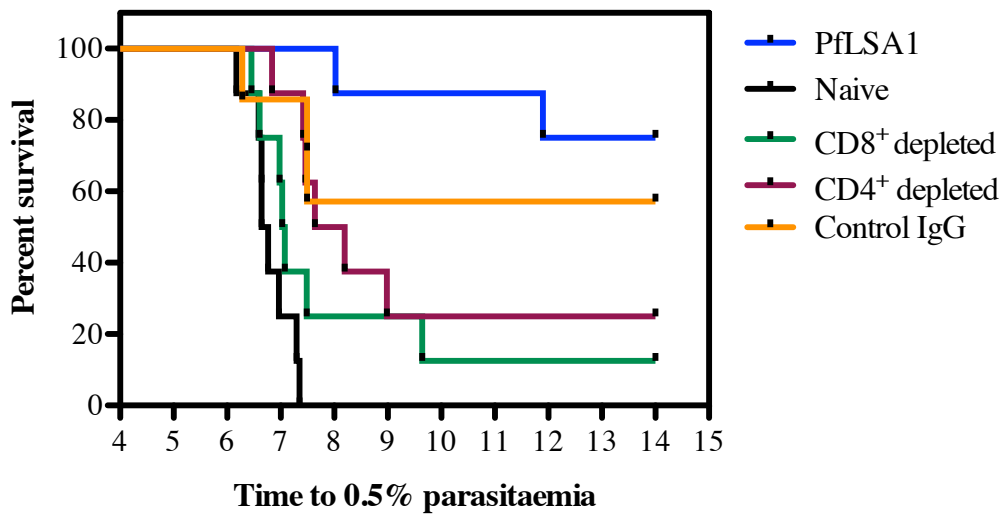


Figure 5.15. Depletion of CD8⁺ T cells abolishes the protection induced by ChAd63-MVA PfLSA1 vaccination in BALB/c mice.

Mice (n=4 groups of 7-8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfLSA1 followed eight weeks later by 1×10^7 pfu MVA-PfLSA1. Mice were bled seven days post-MVA boost and cellular immunogenicity assessed by ICS, after stimulation for six hours with a pool of overlapping peptides to PfLSA1. No significant difference was found for any cytokine between the four groups (data not shown). Mice were injected i.p. with 100 μ g of mAb to either CD4⁺ (GK1.5), CD8⁺ (8.43) or an IgG mAb control at days eight, nine and ten post-boost. At day ten, all mice were challenged i.v. with 1000 PbPfLSA1 sporozoites, including eight naïve control mice. Mice were monitored daily to enable calculation of the time to 0.5% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves; CD8⁺ depleted versus PfLSA1 control vaccinated $p=0.0027$, CD8⁺ depleted versus IgG control $p=0.0658$, CD4⁺ depleted versus naïve $p=0.0003$, CD4⁺ depleted versus PfLSA1 control vaccinated $p=0.026$, CD4⁺ depleted versus IgG control $p=0.3425$.

5.2.2.3 ChAd63-MVA PfLSA1 vaccination also provides protection against sporozoite challenge in CD-1 outbred mice

To determine whether protection against transgenic sporozoite challenge was specific to BALB/c mice (i.e. limited to H-2^d), efficacy was also assessed in C57BL/6 mice (H-2^b) and CD-1 outbred mice. Since no cellular or humoral immune response was observed after ChAd63-MVA PfLSA1 vaccination of C57BL/6 mice, it was not surprising that PbPfLSA1 sporozoite challenge resulted in no protection in this strain (Figure 5.16). Whilst the difference in time to patency between vaccinated and naïve mice was minor, it was significant (median of 5.44 days in vaccinated mice compared to 5.62 days in naïve control mice, $p=0.0375$, Log-rank (Mantel-Cox) Test).

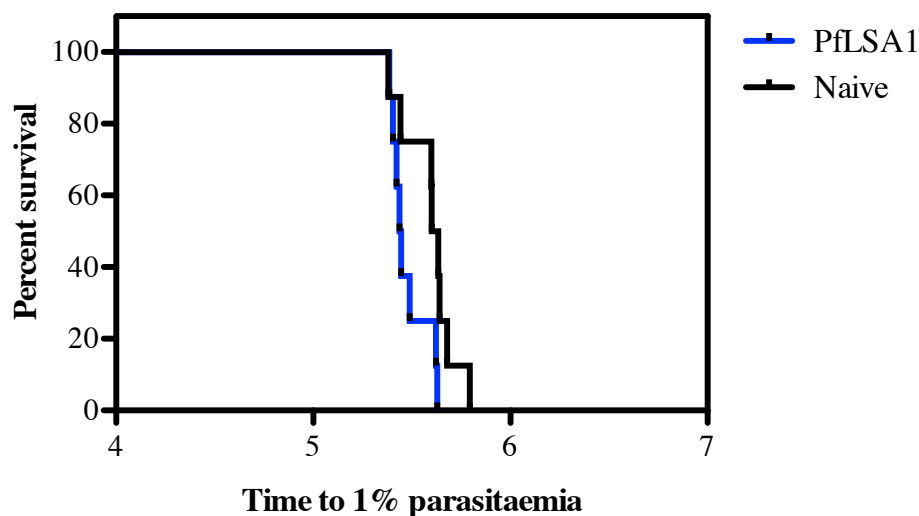


Figure 5.16. ChAd63-MVA PfLSA1 vaccination does not induce protection against sporozoite challenge in C57BL/6 mice.

C57BL/6 mice ($n=8$) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfLSA1 followed eight weeks later by 1×10^7 pfu MVA-PfLSA1. Mice were challenged i.v. with 1000 transgenic PbPfLSA1 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, $p=0.0375$.

PfLSA1 vaccination was able to induce an immune response in CD-1 outbred mice (Figure 5.17 A), with a median CD8⁺ IFN γ ⁺ response of 1.13%, TNF α ⁺ of 1.2% and CD107a⁺ of 5.5%. An antigen-specific antibody response was also induced, with a fold-change of 1.39 from naïve (data not shown). Upon challenge with transgenic PbPfLSA1 sporozoites, seven out of eight mice were sterilely protected (87.5%, $p < 0.0001$, Log-rank (Mantel-Cox) Test) (Figure 5.17 B). As for PfLSA1 efficacy in BALB/c mice, it was difficult to assess correlates of protection given the majority of mice did not develop malaria. In this case, as only one mouse was not sterilely protected, it was not possible to perform analysis of significant differences between delayed and sterile protection.

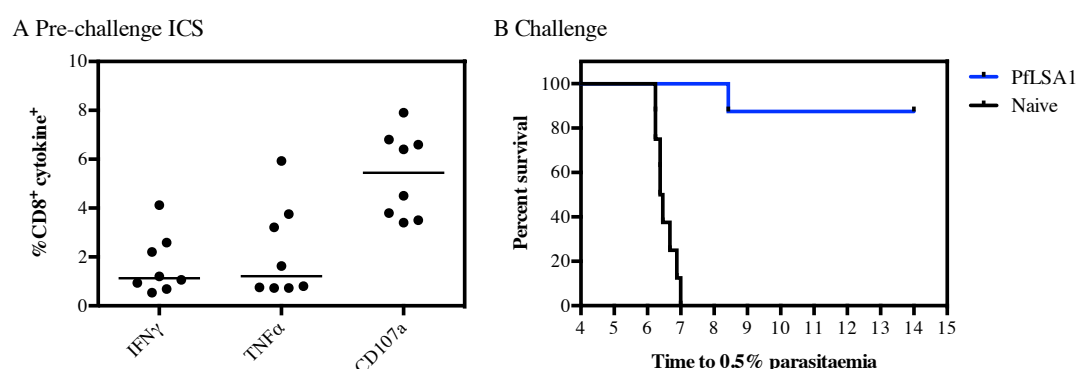


Figure 5.17. ChAd63-MVA PfLSA1 vaccination induces protection against sporozoite challenge in CD-1 outbred mice.

CD-1 mice ($n=8$) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfLSA1 followed eight weeks later by 1×10^7 pfu MVA-PfLSA1. Blood was taken seven days post-boost to assess both humoral and cellular immunogenicity. (A) Cellular immunogenicity was assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PfLSA1. Both median and individual data points are shown. (B) Mice were challenged i.v. with 1000 transgenic PbPfLSA1 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 0.5% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, $p < 0.0001$.

5.2.2.4 Further assessment of immunogenicity induced by ChAd63-MVA PfLSA1 vaccination

As significant protective efficacy was identified in BALB/c mice, it was of interest to know which epitopes were associated with protective responses and whether it was possible to detect an HLA-A2-restricted immune response. Epitope mapping was conducted in BALB/c and HHD (HLA-A2 transgenic) mice by spleen IFN γ ELISpot to individual peptides covering the entire PfLSA1 sequence. Immunodominant responses in BALB/c mice were identified to peptides 20 (aa918 to 937) and 40 (aa1118 to 1137), with three further subdominant responses (Figure 5.18). No HLA-A2-restricted epitopes were identified in HHD mice (data not shown).

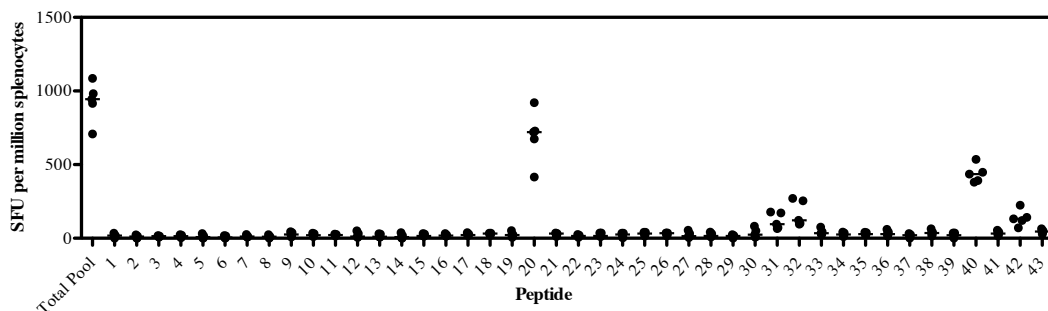


Figure 5.18. Epitope mapping of PfLSA1 in BALB/c mice.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfLSA1 followed eight weeks later by 1×10^7 pfu MVA-PfLSA1. Two weeks post-MVA boost, mice were sacrificed and splenocytes isolated to perform an *ex vivo* IFN γ ELISpot. Splenocytes were stimulated either with an overlapping peptide pool to PfLSA1 or individual peptides (20aa each, overlapping by ten) covering the entire sequence. Both median and individual data points are shown.

Immunogenicity was assessed in liver mononuclear cells of PflSA1 vaccinated BALB/c mice as for PfUIS3 vaccinated mice (see section 5.2.1.4). A low, but detectable, level of PflSA1-specific cells were observed (Figure 5.19), with medians of 0.35% CD8⁺ IFN γ ⁺, 0.38% TNF α ⁺ and 0.94% CD107a⁺. This was significantly lower than levels seen in splenocytes from the same mice (p<0.0001, two-way ANOVA). The values were considered too low to reliably assess the expression of memory cell markers.

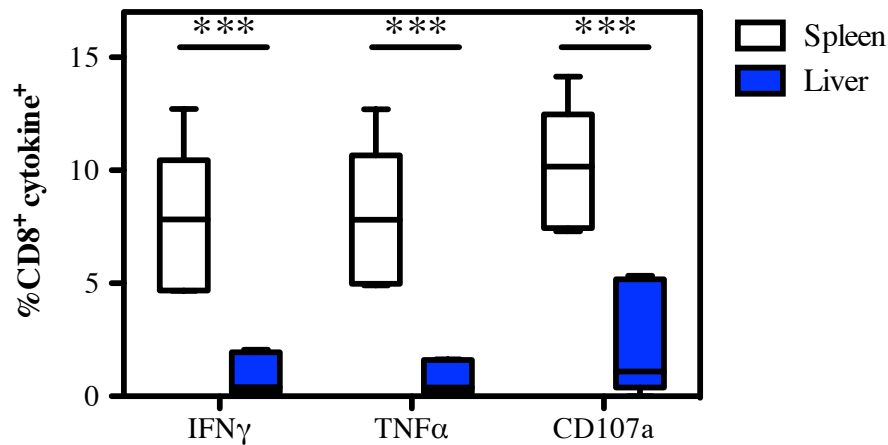


Figure 5.19. ChAd63-MVA PflSA1 vaccination in BALB/c mice induces a low magnitude antigen-specific cellular response in the liver.

In the experiment described in Figure 5.18 above, when mice were sacrificed two weeks post-boost livers were also harvested, following perfusion *in situ*. Single cell suspensions of spleen and liver mononuclear cells were stimulated for six hours with an overlapping peptide pool to PflSA1, and the percentage of CD8⁺ cytokine⁺ cells are shown. Box plots indicate the median response with whiskers representing the minimum and maximum responses. Statistical difference between the response detected in the spleen and liver was assessed by two-way ANOVA with Bonferroni post-test, *** p<0.001, overall p<0.0001.

5.2.3 Further assessment of ChAd63-MVA PfLSAP2 as a candidate vaccine

5.2.3.1 Confirmation of sterile protection in BALB/c mice

To confirm PfLSAP2 vaccination results in sterile protection in BALB/c mice, an independent repeat challenge was performed with transgenic PbPfLSAP2 sporozoites. A significant difference was confirmed between vaccinated and naïve control mice ($p=0.0002$, Log-rank (Mantel-Cox) Test) (Figure 5.20 A), with five out of eight mice sterilely protected (62.5%). As there was no significant difference in the survival of naïve control mice between the repeat and original experiment, the results from the two experiments were combined (Figure 5.20 B). Overall, twelve out of sixteen mice were sterilely protected (75%, $p<0.0001$, Log-rank (Mantel-Cox) Test). The median $CD8^+ IFN\gamma^+$ response pre-challenge was 0.43% (Figure 5.20 C).

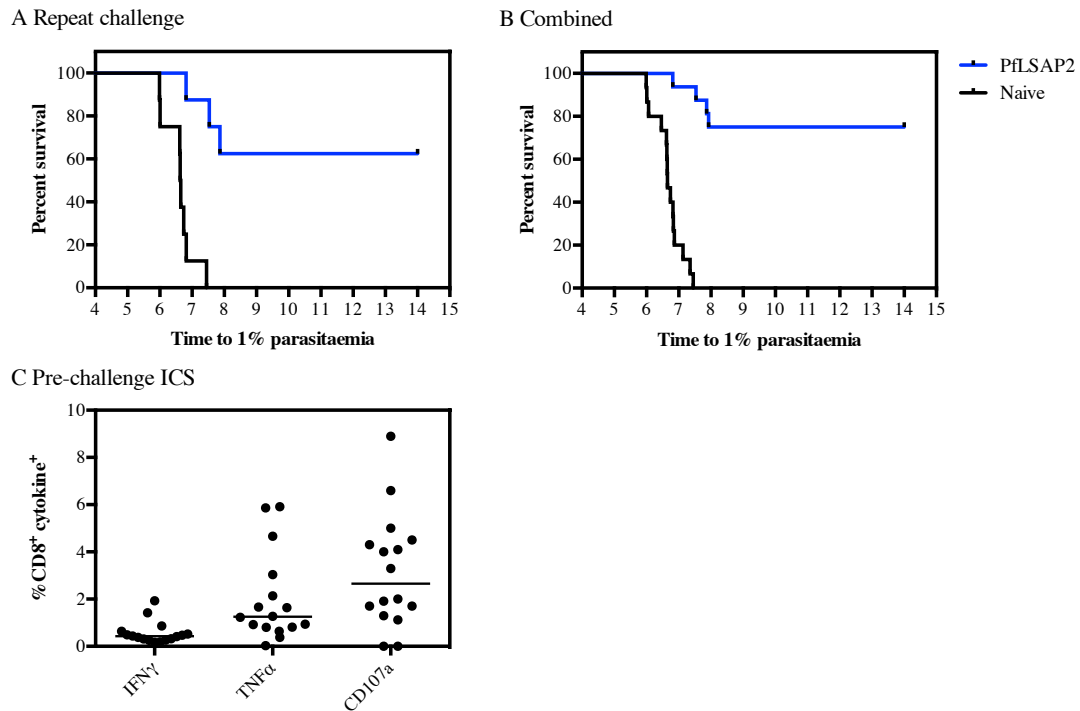


Figure 5.20. Confirmation of protection in BALB/c mice induced by PfLSAP2 vaccination.

(A) BALB/c mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PfLSAP2 followed eight weeks later by 1×10^7 pfu MVA-PfLSAP2. Mice were challenged i.v. with 1000 transgenic PbPfLSAP2 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. Mice that were slide-negative at fourteen days post-challenge were considered sterilely protected. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, $p=0.0002$. (B) Results from the repeat and the original experiment were combined (vaccinated n=16, naïve n=15), Log-rank (Mantel-Cox) Test $p<0.0001$. (C) Seven days post-boost (three days pre-challenge) blood was taken to check cellular immunogenicity by ICS, after stimulation for six hours with an overlapping peptide pool to PfLSAP2. Both median and individual data points are shown for the two experiments combined.

Whilst PflSAP2 is expressed at both the liver and blood-stage [416, 421], in the transgenic parasite the gene is under control of the *P. berghei* UIS4 promoter and hence expression is restricted to the sporozoite and liver-stages [208]. To confirm protection occurred at the pre-erythrocytic stage (see section 5.2.1.1), *in vivo* imaging was performed 44 hours post-challenge to measure bioluminescence. Whilst all vaccinated mice expressed a baseline level of luciferase (considered negative), there was no significant difference compared to naïve mice (Figure 5.21 A). This was likely because the naïve mice became parasitaemic later than seen for other parasite lines (median 6.8 days compared to median 5.4 days for PbPfUIS3 and 5.8 days for PbPflSA1), suggesting 44 hours post-challenge may have been too early to accurately measure the liver burden by *in vivo* imaging. Only a weak correlation was identified between the *in vivo* imaging data and the time to 1% parasitaemia (Spearman $r=-0.527$, $p=0.0435$) (Figure 5.21 B), supporting this hypothesis. Nevertheless, whilst it is likely an effect did occur at the pre-erythrocytic stage, as no luciferase expression was detected, it could not be confirmed with statistical significance in this experiment.

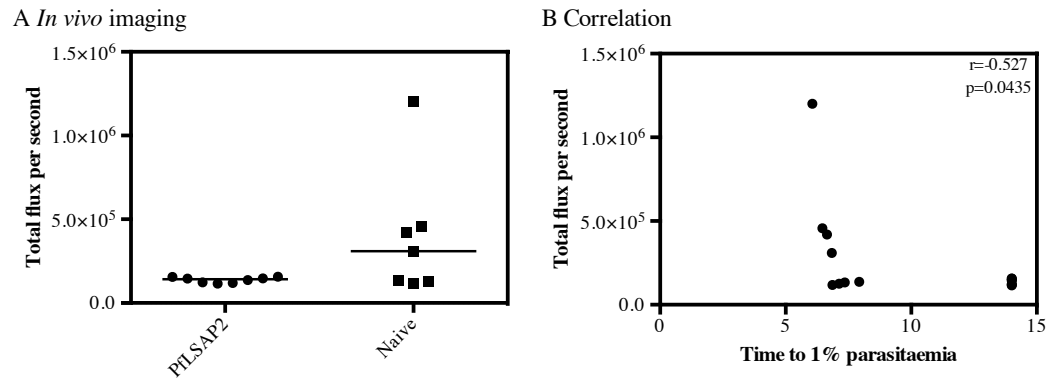


Figure 5.21. The time to 1% parasitaemia correlates weakly with the bioluminescence of transgenic PbPFLSAP2 liver-stage parasites.

At 44 hours post-PbPFLSAP2 sporozoite challenge in the original experiment, mice were imaged *in vivo* using the IVIS 200 for luciferase expression. (A) Results are presented as the total flux measured per second. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, no difference was found ($p=0.2319$). (B) Correlation between time to 1% parasitaemia and IVIS imaging data from both vaccinated and naïve control mice ($n=15$). Spearman $r=-0.527$, $p=0.0435$.

Pre-challenge, PflSAP2 vaccination in BALB/c mice resulted in both a moderate cellular immune response (median CD8⁺ IFN γ ⁺ of 0.43%) (Figure 5.20 C) and a detectable antibody response (fold-change of 1.33 from naïve) (data not shown, see immunogenicity studies in Chapter 4). No correlates of protection could be identified for cellular or humoral immunogenicity, nor was a significant difference seen when grouping vaccinated mice into ‘delayed’ or ‘sterile protection’ (data not shown). As for PflSA1, statistical analysis was difficult, given the low numbers of mice that were not protected. Polyfunctionality was also assessed (Figure 5.22), and unlike PfUIS3 or PflSA1 vaccination, most CD8⁺ T cells were single cytokine producers (IFN γ or TNF α). All permutations of polyfunctionality were analysed for differences between mice sterilely protected and those delayed, but no differences were found (data not shown). As PflSAP2 vaccination induced a cellular immune response in C57BL/6 mice (median 892 SFU per million splenocytes, see Chapter 4) but no antibody response, protective efficacy was then assessed in this strain to determine whether protection was likely mediated through cellular or humoral immunity.

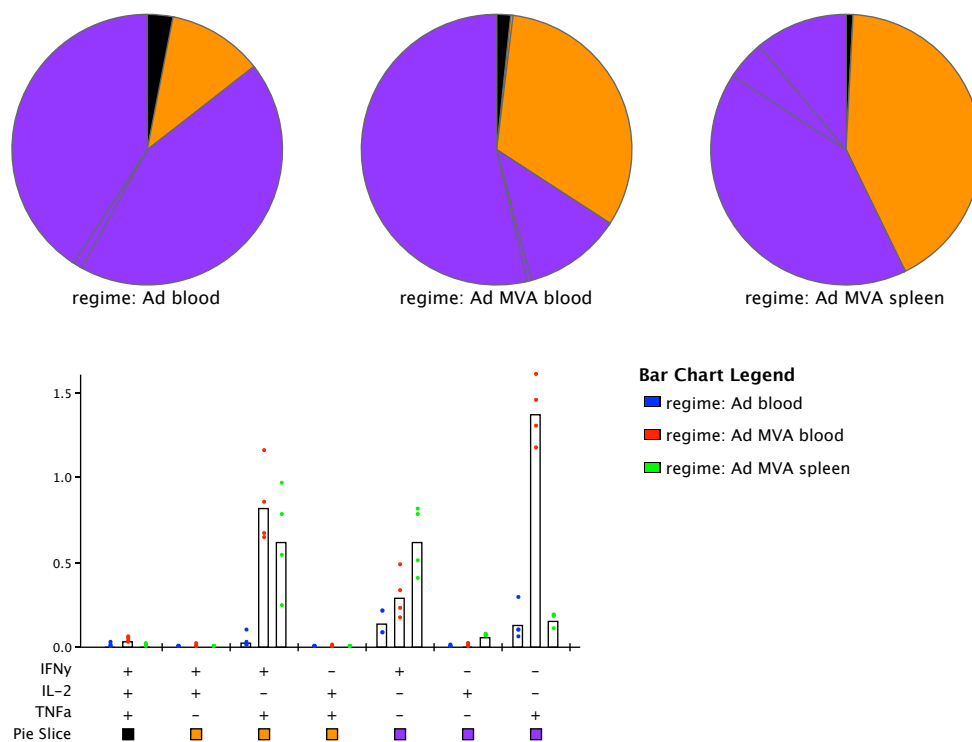


Figure 5.22. Polyfunctionality of CD8⁺ T cells induced by ChAd63-MVA PflSAP2 vaccination in BALB/c mice.

BALB/c mice (n=4) were vaccinated i.m. with 1×10^8 ifu ChAd63-PflSAP2 followed eight weeks later by 1×10^7 pfu MVA-PflSAP2, as part of the immunogenicity experiments described in Chapter 4. Two weeks post-ChAd63 prime and one-week post-MVA boost blood was taken and cellular immunogenicity assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PflSAP2. Two weeks post-MVA boost mice were sacrificed, spleens harvested and cellular immunogenicity again assessed by ICS. The proportion of cells at each time-point expressing one, two or three cytokines is shown. The bar chart indicates which cytokines were produced, whilst the slices of the pie chart indicate the proportion of cells producing one (purple), two (orange) or three (black) cytokines. The pie chart showing “Ad MVA Blood” is representative of the proportions seen pre-challenge.

5.2.3.2 ChAd63-MVA PflSAP2 vaccination does not induce protection against sporozoite challenge in C57BL/6 mice

C57BL/6 mice were vaccinated with PflSAP2 in the standard prime-boost regimen and efficacy tested by transgenic PbPflSAP2 sporozoite challenge. Despite a moderate cellular immune response (median 3.4% of CD8⁺ T cells producing IFN γ , 3.6% TNF α and 3.3% CD107a) (Figure 5.23 A), vaccinated mice were not protected from sporozoite challenge (Figure 5.23 B). The cellular immune response was comparable, or greater, to PflSAP2 vaccination in BALB/c mice, except that a greater proportion of antigen-specific CD8⁺ T cells were double cytokine producers (approximately 75% post-boost in the spleen, data not shown). No antigen-specific antibodies were detected in these mice seven days post-MVA boost (pre-challenge, data not shown).

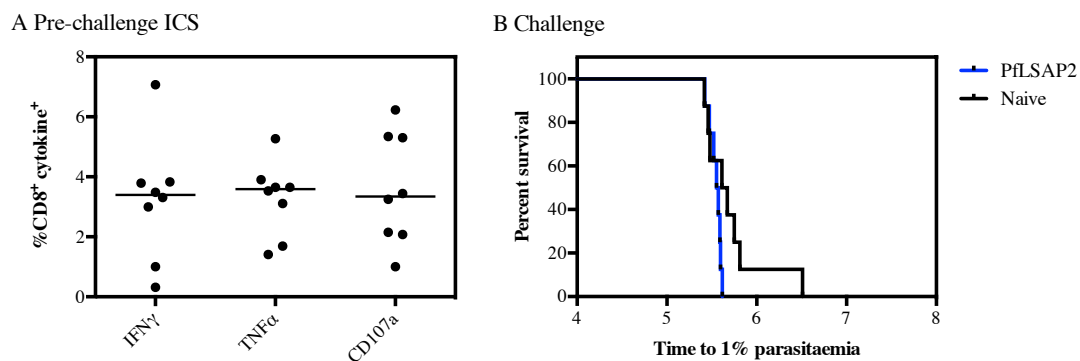


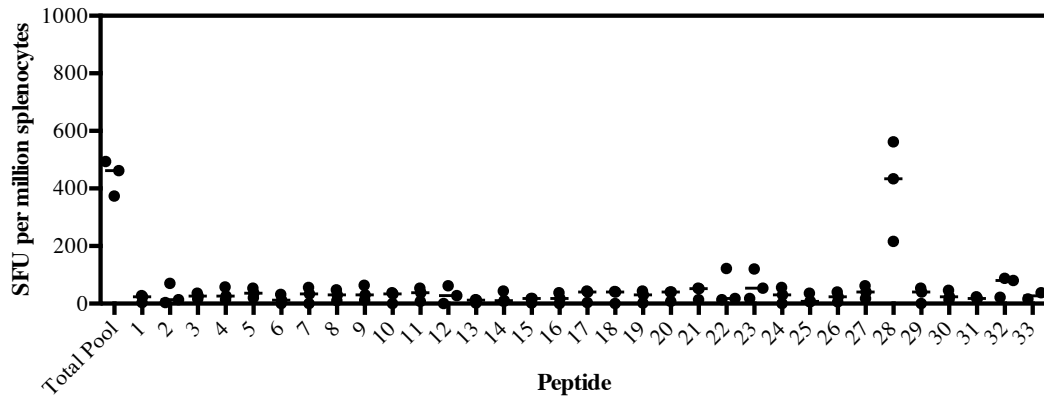
Figure 5.23. ChAd63-MVA PflSAP2 vaccination does not induce protection against sporozoite challenge in C57BL/6 mice.

C57BL/6 mice (n=8) were vaccinated i.m. with 1×10^8 ifu ChAd63-PflSAP2 followed eight weeks later by 1×10^7 pfu MVA-PflSAP2. Blood was taken seven days post-boost to assess both humoral and cellular immunogenicity. (A) Cellular immunogenicity was assessed by ICS, after stimulation for six hours with an overlapping peptide pool to PflSAP2. Both median and individual data points are shown. (B) Mice were challenged i.v. with 1000 transgenic PbPflSAP2 sporozoites ten days post-MVA boost, along with eight naïve control mice. Mice were monitored daily to enable calculation of the time to 1% parasitaemia. The Log-rank (Mantel-Cox) Test was used to assess differences between the survival curves, no difference was found (p=0.0781).

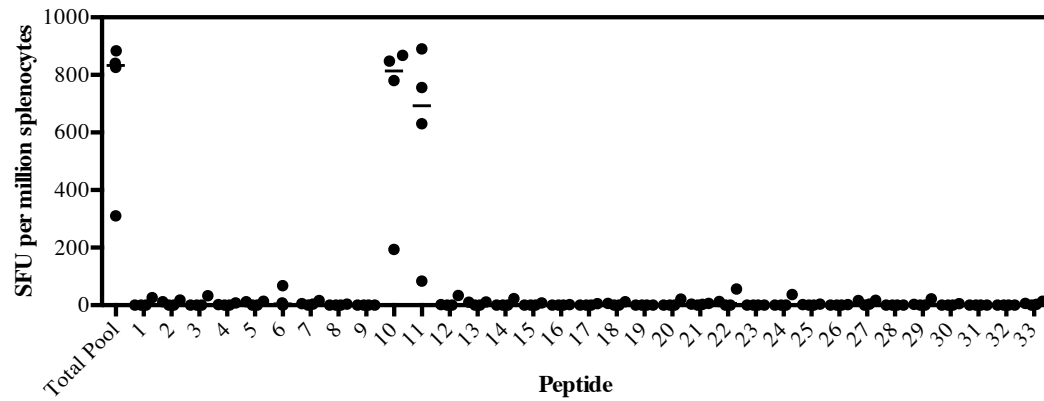
5.2.3.3 Further assessment of immunogenicity induced by ChAd63-MVA PflSAP2 vaccination

As protective efficacy was only demonstrated in BALB/c mice, it was of interest to identify the epitopes associated with protection, and to determine whether any HLA-A2-restricted epitopes could be detected. Epitope mapping was conducted in BALB/c, C57BL/6 and HHD (HLA-A2 transgenic) mice by spleen IFN γ ELISpot to individual peptides (Figure 5.24). One immunodominant epitope was identified in BALB/c mice (thereby presumably the protective epitope, if protection is cell-mediated) in peptide 28, aa241 to 260. Two immunodominant epitopes were identified in C57BL/6 mice, peptides 10 and 11, covering aa61 to 90. An immune response was also induced in HHD mice, with one epitope mapped to peptide 7, aa31 to 50.

A BALB/c (H-2^d)



B C57BL/6 (H-2^b)



C HHD (HLA-A2)

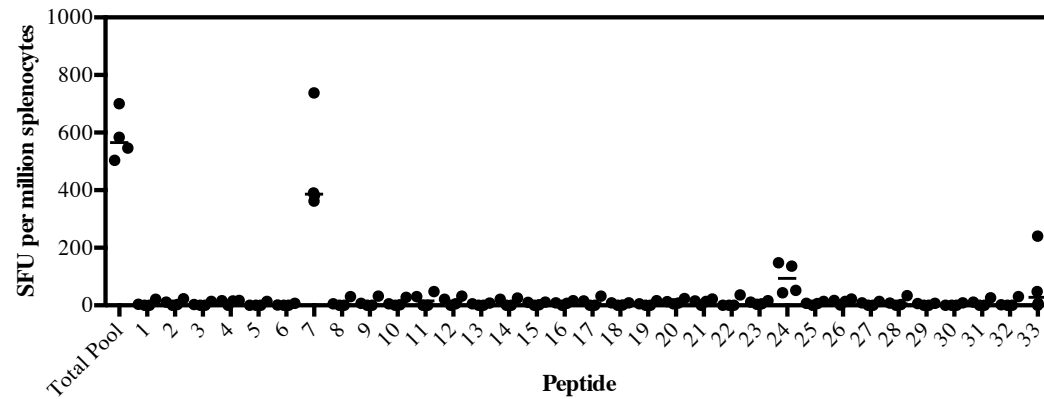


Figure 5.24. Epitope mapping of PflSAP2 in BALB/c, C57BL/6 and HHD mice.

(A) BALB/c (n=3), (B) C57BL/6 (n=4) or (C) HHD (n=4) mice were vaccinated i.m. with 1×10^8 ifu ChAd63-PflSAP2 followed eight weeks later by 1×10^7 pfu MVA-PflSAP2. Two weeks post-MVA boost, mice were sacrificed and splenocytes were isolated to perform an *ex vivo* IFN γ ELISpot. Splenocytes were stimulated either with an overlapping peptide pool to PflSAP2 or individual peptides (20aa each, overlapping by ten) covering the entire sequence. Both median and individual data points are shown.

Immunogenicity was assessed in liver mononuclear cells of PflSAP2 vaccinated mice, as for PfUIS3 vaccinated mice (see section 5.2.1.4). A comparable, or greater, antigen-specific response was detected in the liver of BALB/c and C57BL/6 mice as compared to the spleen (Figure 5.25). The responses in BALB/c and C57BL/6 mice were also comparable to each other, with a median liver CD8⁺ IFN γ ⁺ of 2.1% and 2.2%, TNF α ⁺ of 1.9% and 2.1% and CD107a⁺ of 3.4% and 2.8%, respectively. A low but detectable response was observed in liver mononuclear cells of HHD mice, with a median CD8⁺ IFN γ ⁺ response of 0.8%, TNF α ⁺ 0.6% and CD107a⁺ 0.5%. Of the CD8⁺ cells producing IFN γ , approximately 60% were expressing markers characteristic of effector memory cells (data not shown, see gating strategy in Figure 5.10). There were no significant differences between the three strains nor the liver or spleen, with an overall median of 61.4% (range 38.9-71.5%) of antigen-specific CD8⁺ T cells expressing markers of the effector memory phenotype.

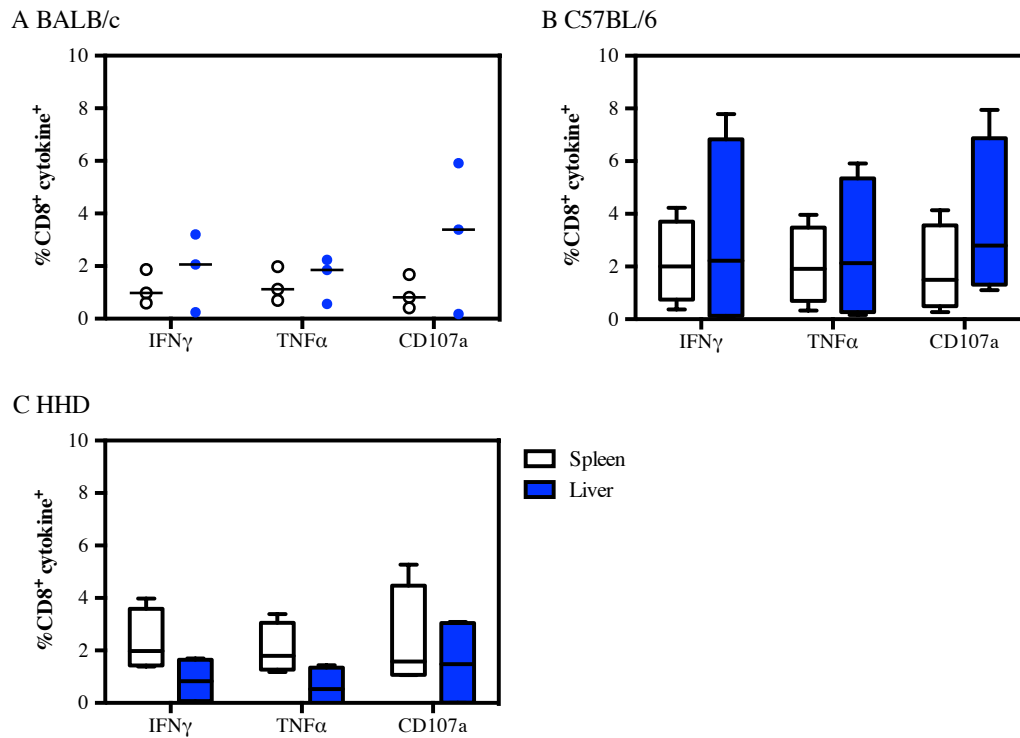


Figure 5.25. ChAd63-MVA PflSAP2 vaccination induces an antigen-specific cellular response in the liver.

In the experiment described in Figure 5.24 above, when mice were sacrificed two weeks post-boost livers were also harvested, following perfusion *in situ*. Single cell suspensions of spleen and liver mononuclear cells were isolated and stimulated for six hours with an overlapping peptide pool to PflSAP2. The percentage of CD8⁺ cytokine⁺ cells is shown for (A) BALB/c, (B) C57BL/6 and (C) HHD mice. Box plots indicate the median response with whiskers representing the minimum and maximum responses. As only three mice were assayed for BALB/c, individual data points are shown. Statistical difference between the spleen and liver responses was assessed using a two-way ANOVA with Bonferroni post-test, no differences were observed.

5.2.4 Comparison of the protective efficacy induced by PfUIS3, PflSA1 and PflSAP2 vaccination and assessment of competition between vaccines

In Chapter 4, PfUIS3, PflSA1 and PflSAP2 were all identified as promising candidate antigens for a pre-erythrocytic malaria vaccine due to the efficacy provided in BALB/c mice. PfUIS3 and PflSA1 subsequently provided protection in another strain of mice, either C57BL/6 or CD-1, but not both (Table 5.1). PflSAP2 vaccination did not provide protection in C57BL/6 mice, but efficacy is still to be assessed in CD-1 outbred mice. From the results described so far in this chapter, PflSA1 was identified as the leading candidate due to protection in outbred mice, given these mice are more representative of an outbred human population. These candidate antigens would likely be used as part of a multi-component malaria vaccine, either in combination with the current leading viral vectored vaccine ME-TRAP, or in combination with each other.

Table 5.1. Comparison of protective efficacy induced by PfUIS3, PflSA1 and PflSAP2 in three different strains of mice.

Mechanism refers to studies in BALB/c mice only.

Antigen	BALB/c	C57BL/6	CD-1	Mechanism
PfUIS3	18% sterile protection, significant delay	37.5% sterile protection, significant delay	No efficacy	CD8 ⁺ T cells critical
PflSA1	81.25% sterile protection	No efficacy	87.5% sterile protection	CD8 ⁺ T cells critical
PflSAP2	75% sterile protection	No efficacy	Not assessed	Unknown

To assess whether combining each of the vaccines with ME-TRAP would affect the immunogenicity of each individual vaccine, C57BL/6 mice were vaccinated with ME-TRAP in combination with either PfUIS3 or PflSAP2. The effect of PfUIS3 and PflSAP2 combination vaccination was also assessed. C57BL/6 mice were chosen as the ME string contains the strong *P. berghei* Pb9 H-2^d-restricted epitope from CSP [393], and hence immunogenicity measured in BALB/c mice would reflect the effect of competition on *P. berghei* CSP rather than *P. falciparum* TRAP. Vaccinating with two vaccines did not significantly reduce or increase the immunogenicity of either vaccine, compared to administration of either vaccine alone (Figure 5.26).

As PflSA1 does not induce an immune response in C57BL/6 mice, it could not be assessed in the experiment outlined above. Instead, to circumvent the Pb9 epitope, the vaccine TRIP was used in BALB/c mice. TRIP is codon optimized *P. falciparum* 3D7 TRAP, without the ME string (ME-TRAP uses the T9/96 TRAP sequence). Vaccinating with both TRIP and PflSA1 together did not significantly reduce or increase the immunogenicity of either vaccine compared to administration of either vaccine alone (Figure 5.27).

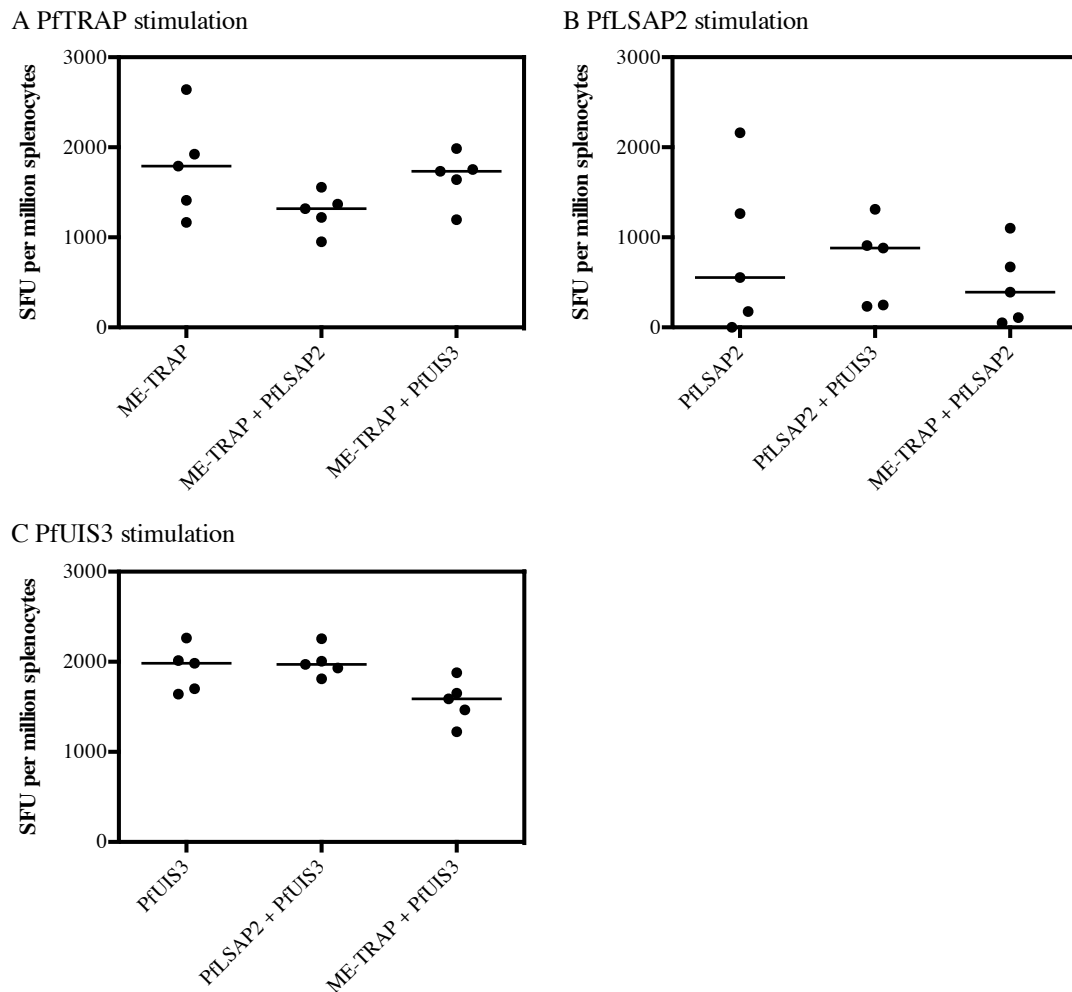


Figure 5.26. Vaccination with combinations of PfUIS3 and PflSAP2 with ME-TRAP, or with each other, does not result in reduced cellular immunogenicity in C57BL/6 mice compared to each vaccine given alone.

C57BL/6 mice (n=5 per group) were vaccinated i.m. with 1×10^8 ifu ChAd63 followed eight weeks later by 1×10^7 pfu MVA, with antigens indicated on the x-axis. When two vaccines were given, mice were vaccinated with a full dose of each vaccine administered in separate legs. Two weeks post-MVA boost, mice were sacrificed and splenocytes were isolated to perform an *ex vivo* IFN γ ELISpot. Splenocytes were stimulated with an overlapping peptide pool to (A) PfTRAP (T9/96), (B) PflSAP2 or (C) PfUIS3. Both median and individual data points are shown. The Kruskal-Wallis test with Dunn's Multiple Comparison post-test was used to assess statistical difference between groups. No differences were found.

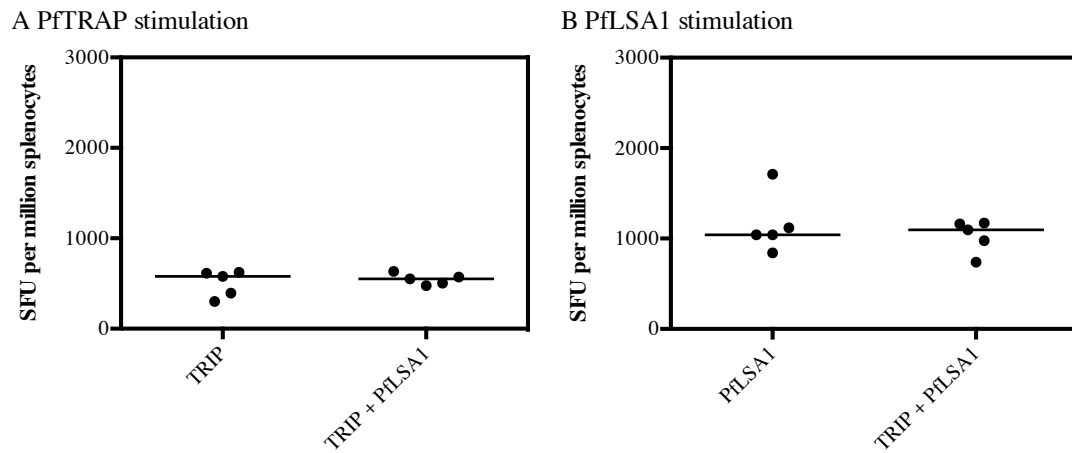


Figure 5.27. Vaccination with both PflSA1 and TRIP does not result in reduced cellular immunogenicity in BALB/c mice compared to vaccination with either alone.

BALB/c mice (n=5 per group) were vaccinated i.m. with 1×10^8 ifu ChAd63 followed eight weeks later by 1×10^7 pfu MVA, with antigens indicated on the x-axis. When two vaccines were given, mice were vaccinated with a full dose of each vaccine administered in separate legs. Two weeks post-MVA boost, mice were sacrificed and splenocytes were isolated to perform an *ex vivo* IFN γ ELISpot. Splenocytes were stimulated with an overlapping peptide pool to (A) PfTRAP (3D7) or (B) PflSA1. Both median and individual data points are shown. The Mann Whitney test was used to assess statistical difference between groups. No differences were found.

5.3 Discussion

The experiments described in this chapter sought to further explore the immunogenicity and protective efficacy of the candidate vaccines PfUIS3, PfLSA1 and PfLSAP2 and to determine whether the protection was limited to H-2^d mice. For each vaccine, the protection originally seen was confirmed in BALB/c mice, validating the choice of these antigens for further analysis.

Protective efficacy elicited by PfUIS3 was not limited to H-2^d mice, as equivalent protection was demonstrated in C57BL/6 mice, manifesting as a delay in the time to 1% parasitaemia rather than a high level of sterile immunity (as seen for BALB/c mice). Limbach *et al.*, have previously shown that PyUIS3 can induce 43% sterile protection in CD-1 outbred mice when used in a DNA prime-MVA boost vaccination regimen in combination with the *P. yoelii* homolog of PFI0580c [407]. In the present study, PfUIS3 was not able to induce protection against transgenic sporozoite challenge in CD-1 mice, though some evidence of a trend towards a delay in parasitaemia was observed. This suggests that when administered alone, PfUIS3 may not be capable of inducing protection in an outbred population but nevertheless it may be beneficial in a multi-component vaccine. Excitingly, two conserved consecutive peptides (aa51 to 80) were identified that induced a positive response on all three backgrounds tested, H-2^d, H-2^b and HLA-A2. This is the first known report of identification of immunodominant epitopes in PfUIS3. This finding warrants further investigation to determine whether this region contains a single epitope, and whether this epitope is, or epitopes are, protective.

A striking level of sterile protection was induced by PfLSA1 vaccination in CD-1 outbred mice, 87.5%, currently the highest level of protection induced by a sub-unit liver-stage vaccine in outbred mice compared to reported studies [51, 407, 480]. Whilst no protection was seen in C57BL/6 mice, this was not surprising as no H-2^b-restricted epitopes were identified for PfLSA1, whilst H-2^d-restricted epitopes were observed, confirming previous reports [434, 481]. Given protection was seen in outbred mice, it is not concerning that protection was only observed in one of the two inbred strains. Whilst no epitopes were identified in HLA-A2 transgenic mice, many studies have previously shown that human populations can respond to PfLSA1 peptides; for example, Fidock *et al.*, reported that 73% of the population in a low transmission region of Madagascar could secrete IFN γ in response to PfLSA1 [467].

PfLSAP2 vaccination failed to induce protection in C57BL/6 mice, which was unexpected given the high level of sterile protection in BALB/c mice and the similar magnitude of the cellular immune response induced between both strains. Two possible reasons should be considered: first, the protection induced in BALB/c mice was antibody rather than cell-mediated. BALB/c mice induced a detectable level of antibodies against PfLSAP2, whilst C57BL/6 mice did not. Whilst native expression of PfLSAP2 does not occur on the sporozoite [416], under control of the UIS4 promoter it should occur [208], and hence protection could be due to inhibition of sporozoite invasion by induction of antigen-specific antibodies. The second reason to explain the difference in efficacy is that standard IFN γ ELISpot and ICS analysis does not reveal the functional or qualitative differences in cellular immunogenicity.

Further assessment indicated that the majority of PflSAP2-specific CD8⁺ T cells in BALB/c mice were monofunctional, producing only IFN γ , whilst in C57BL/6 mice they were largely double cytokine producers (IFN γ and TNF α). The contributions of monofunctional or polyfunctional T cells to immunity against malaria is not clear; polyfunctional cells have been associated with induction of protective immunity after vaccination with sporozoites under chloroquine cover [218, 219], whilst monofunctional CD8⁺ T cells secreting IFN γ have correlated with protective immunity in studies using viral vectors expressing ME-TRAP [160, 296]. Furthermore, whilst correlates of protection were not identified in the recent irradiated sporozoite vaccination study, the CD8⁺ T cells induced in protected volunteers only secreted IFN γ [50]. Together, this suggests that different correlates of protection will likely exist between different vaccination strategies. As both PfUIS3 and PflSA1 CD8⁺ cells were largely polyfunctional in this study, and protective, it seems unlikely the difference in polyfunctionality induced by PflSAP2 vaccination is responsible for the difference in protection between the two strains of mice.

Evidence suggests that protection against malaria is dependent on [482, 483], or at least associated with [473, 475], liver-resident effector memory CD8⁺ T cells secreting IFN γ . As measurement of liver-resident T cells is not possible in human trials, assessment of correlates of protection has largely been performed on T cells in the periphery, similarly in mice. Previous studies have shown that mice vaccinated with viral vectors expressing ME-TRAP have high percentages of antigen-specific CD8⁺ T cells in the liver prior to sporozoite challenge [54, 296]. Upon challenge, this enables a fast and efficient immune response to be mounted, which is critical given the relatively short time the parasite spends in the liver. In the present study, after

vaccination with PflSAP2 or PfUIS3 an antigen-specific CD8⁺ IFN γ ⁺ response was observed in the liver in BALB/c and C57BL/6 mice. Concerning PflSAP2, the response was of equal magnitude between the two strains, so a difference in magnitude is unlikely to account for the differences seen in protective efficacy. The explanation for absence of protection in C57BL/6 mice by PflSAP2 vaccination remains to be elucidated; on the evidence presented, it is likely that either PflSAP2 protection is associated with an H-2^d-restricted epitope (as both strains had different immunodominant epitopes identified), or protection is antibody-mediated. Interestingly, PflSA1 vaccination failed to induce a significant response in the liver compared to the spleen, in BALB/c mice. Given PflSA1 vaccination resulted in sterile protection in BALB/c mice mediated by CD8⁺ cells, it must be assumed that antigen-specific cells homed to the liver after sporozoite challenge, however, this remains to be experimentally confirmed. In addition, the presence of potential liver homing molecules, such as CD44 [484] or CD161 [485, 486], could be assessed.

The protection induced by both PfUIS3 and PflSA1 vaccination in BALB/c mice was dependent upon the presence of CD8⁺ T cells, as shown by the T cell depletion studies. Combined with the *in vivo* imaging data that indicated protection was occurring at the pre-erythrocytic stage, this strongly suggests that protection was mediated by the action of CD8⁺ T cells at the liver-stage of infection. This provides further evidence that CD8⁺ T cells are important effector cell types at the liver-stage, and demonstrates that liver-stage vaccine design should aim to induce not only CD4⁺ T cells, but also CD8⁺ T cells. Furthermore, it supports the suggestion that the reason the clinical trial of PflSA1 recombinant protein vaccination failed to induce protection was the absence of detectable CD8⁺ T cells [232]. Surprisingly, the

adoptive transfer of splenocytes from PfUIS3 vaccinated mice did not provide the same level of protection against sporozoite challenge as PfUIS3 vaccination alone. This could be due to inefficient trafficking to the liver, however previous studies suggest this is unlikely [482, 487, 488]. Importantly, the number of PfUIS3-specific cells was not determined post-transfer, and hence it is not known in this experiment whether enough cells were transferred. A follow-on experiment could be performed with increasing numbers of T cells transferred followed by assessment of protection against challenge.

Unfortunately, although CD8⁺ T cells were identified as an indispensable effector cell type, a correlate of protection could not be identified for PflSA1 (BALB/c or outbred), PfUIS3 (BALB/c) or PflSAP2 (BALB/c). Associations with cytokine secretion and expression of CD107a were performed, as well as all permutations of single, double and triple cytokine secreting cells from the polyfunctionality analysis. In the case of PflSA1 and PflSAP2, the skewed distribution of the results with very high levels of efficacy rendered analysis of protective correlates impossible. Whilst advanced statistical modeling could be tried [286], further experiments are required to increase the size of the data set; for example, with lower vaccine doses to reduce efficacy levels. As PfUIS3 resulted in a greater spread of data, given most mice experienced a delay in the time to 1% parasitaemia rather than sterile protection, the statistical analysis was better powered to test correlations. Surprisingly, no correlate was identified in the largest sample set, BALB/c mice, but correlates were found in C57BL/6 and CD-1 efficacy experiments. In C57BL/6 mice, the time to 1% parasitaemia negatively correlated with the production of IL-2 from CD8⁺ cells. This was a surprising result, given IL-2 has suggested roles in maintaining T cell memory

(reviewed in [489]) and has been associated with protection in vaccination studies [219, 443, 444]. However, the result was not replicated in an independent challenge of PfUIS3 vaccinated C57BL/6 mice. PfUIS3 vaccination in CD-1 mice did not provide protection compared to naïve controls, however, a significant correlation was found between the time to 1% parasitaemia and CD8⁺ T cells producing IFN γ or TNF α . This suggests that if the magnitude of the response had been higher, a greater level of efficacy may have been seen. Again, this was only a small experiment, but warrants further study with an increased sample size.

There is now general consensus that an effective malaria vaccine will contain multiple antigens, either through inclusion of multiple antigens in a sub-unit vaccine, or through whole parasite vaccination [118, 490]. However, evidence suggests that combining sub-unit vaccines can create competition between antigens and reduce overall immunogenicity [253, 491-494]. The competition seen is complex and interference seems to be antigen dependent, as other studies have combined vaccines and reported no competition [495-499]. Research has also suggested that delivery of the vaccines at separate sites can reduce antigen interference [491, 494], but again conflicting results have been reported [492]. In the present study, combinations of PfUIS3, PflSAP2 and PflSA1 with ME-TRAP or TRIP (*P. falciparum* TRAP without the ME string), or in combination with each other, delivered at separate sites resulted in no decrease in cellular immunogenicity when compared to the vaccines administered alone. As both PfTRAP and the candidate antigens are all expressed at the liver-stage, it would be of interest to assess the effect of antigen competition when combining the candidates with a sporozoite or blood-stage antigen vaccine, for example RTS,S or PfRh5. Excitingly, the combination of PflSA1 and RTS,S as

protein-in-adjuvant vaccines has previously demonstrated no antigenic competition [498], which is encouraging given ChAd63-MVA PflSA1 has emerged as one of the most promising candidate vaccines in this thesis. The next stage of pre-clinical testing will be the generation of double transgenic parasites, in order to assess the effect of antigenic competition on efficacy, rather than immunogenicity.

In summary, the results described in this chapter provided further support for PfUIS3, PflSAP2 and PflSA1 as candidate vaccines. PfUIS3 vaccination was able to induce similar levels of efficacy in two inbred strains of mice, most likely through the action of CD8⁺ T cells on liver-stage parasites. There was a trend towards protection in outbred mice, which may be improved if the percentage of antigen-specific cells was increased. PfUIS3 is located in the PVM, providing support of the view that this protein could be exported into the hepatocyte cytoplasm and presented on the cell surface. These are the first results showing the promise of PfUIS3 alone, not just in combination. Whilst PflSAP2 induced a high degree of sterile protection in BALB/c mice, this is likely either H-2^d-restricted or antibody-mediated. These results represent the first assessment of PflSAP2 as a vaccine candidate, and warrant further investigation. PflSA1 was identified as the strongest candidate, with almost complete sterile protection in outbred mice. PflSA1 is indispensable for liver-stage infection, has consistently been associated with protection in naturally acquired immunity and these results strongly suggest it is presented on the hepatocyte cell-surface as a target for CD8⁺ T cells. These results also highlight the value of transgenic parasites, as both PflSA1 and PflSAP2 contain no murine homologs and hence efficacy has not previously been possible to assess in mice. As discussed in Chapter 4, it will be important to independently replicate these findings in a system where the target is

under control of its native promoter, given the promoter controls stage-specific expression [82]; such a system is assessed in the results described in Chapter 7.

Chapter 6: Cellular immune responses to
***Plasmodium falciparum* antigens in human**
volunteers

6 Cellular immune responses to *Plasmodium falciparum* antigens in human volunteers

6.1 Introduction

As introduced previously, the liver-stage is known to be the target of CD8⁺ T cells. Research has not consistently demonstrated whether these CD8⁺ T cells are providing protection directly through cytotoxicity, or indirectly through the induction of cytokines. However, evidence does suggest that the secretion of IFN γ can be important in the inhibition of liver-stage malaria parasites [202, 203, 394, 439, 440], and IFN γ levels have also been associated with protection in naturally acquired immunity [471, 500, 501]. For this reason, the *ex vivo* IFN γ ELISpot has been used as the assay of choice to measure cellular immunogenicity in vaccine trials and in natural infection. The results described in this chapter aimed to assess cellular immune responses to *P. falciparum* in human volunteers, by (1) identifying the epitopes within the vaccine candidate antigen TRAP and (2) assessing responses to multiple malaria antigens in volunteers challenged with *P. falciparum*, to identify potential alternative vaccine candidates.

In a recent Phase II CHMI trial undertaken at the Jenner Institute, denoted Mal34, vaccination with ChAd63-MVA ME-TRAP showed efficacy in 58% (8/14) of volunteers when challenged with *P. falciparum* malaria [160]. Efficacy was defined as either sterile protection or a significant delay in time to patent parasitaemia compared to unvaccinated controls (greater than two days). In previous trials using the same insert, ME-TRAP, in either a DNA-MVA or FP9-MVA regimen, 24% (9/38) of volunteers showed efficacy (sterile or delay to patent parasitaemia) [245,

247, 259]. Whilst the magnitude of cellular immunogenicity induced by ChAd63-MVA ME-TRAP is five to ten fold greater than that induced by the other vectors, this regimen also induced a greater breadth of response. Post-boost, volunteers receiving ChAd63-MVA ME-TRAP responded to a higher number of peptides from within the T9/96 TRAP sequence compared to the other regimens [160, 256]. These results suggest that both the magnitude and the breadth of the CD8⁺ IFN γ ⁺ response correlates with and may contribute to protective efficacy, and hence it is of interest to identify the protective epitopes.

In recent years the emphasis upon characterization of the immune response to infectious diseases, or induced through immunization, has increased. Broadly, it is thought that knowing the protective epitopes induced by immunization, and whether the majority of the population has the capacity to respond to such epitopes, is extremely important [502]. The scientific community has traditionally accepted that CD8⁺ epitopes are restricted by HLA Class I alleles, and hence only a minority of epitopes should be recognized across multiple individuals given the extraordinary genetic diversity within the MHC (reviewed in [503]). However, more recently the number of promiscuous epitopes (those that multiple HLA alleles can bind) identified has increased, both within HLA supertypes [409, 504-508] and between HLA supertypes [509-513]. Why such a great amount of genetic diversity exists within the MHC if multiple HLA alleles have the potential to bind the same epitopes is unclear, but it does suggest that vaccination against infectious pathogens can be successful if promiscuous or universal epitopes are targeted.

For routine analysis of immunogenicity in the clinical trials assessing ChAd63-MVA ME-TRAP, 57 20mer peptides overlapping by ten amino acids (aa) were generated (covering the entire *P. falciparum* T9/96 TRAP sequence) and grouped into six pools of seven to ten peptides each [259], for use in the IFN γ ELISpot assay. Assessment of responses to the six pools gave some indication of the breadth [256], however preliminary assessment of the responsive individual peptides only began in the Mal 34 CHMI trial [160]. Volunteers were assessed for responses to the six pools on the day before challenge (C-1), and where a response of greater than 150 SFC per million PBMCs was detected the corresponding C+7 sample was analysed for a response to the individual peptides within the relevant pool. Seven peptides were strongly recognized by multiple volunteers (Figure 6.1), with volunteers responding to 7.5 peptides on average, with one to two dominant responses. Limited immunogenicity was seen beyond aa310 (peptide 30).

The results described in this chapter aimed to build upon this work; to define the epitopes within *P. falciparum* T9/96 TRAP in volunteers vaccinated with ChAd63-MVA ME-TRAP and to begin to assess whether they were HLA-restricted or promiscuous T cell epitopes. This was achieved by initially mapping minimal epitopes in a sterilely protected volunteer from Mal34, in which fresh PBMCs were readily available, and then confirmation of these epitopes and analysis of HLA restrictions was performed using frozen PBMCs from a previous Phase I vaccination trial, denoted Vac33. Subsequently, another CHMI trial was undertaken at the Jenner Institute, denoted Vac45, which permitted further mapping on fresh PBMCs from selected volunteers.

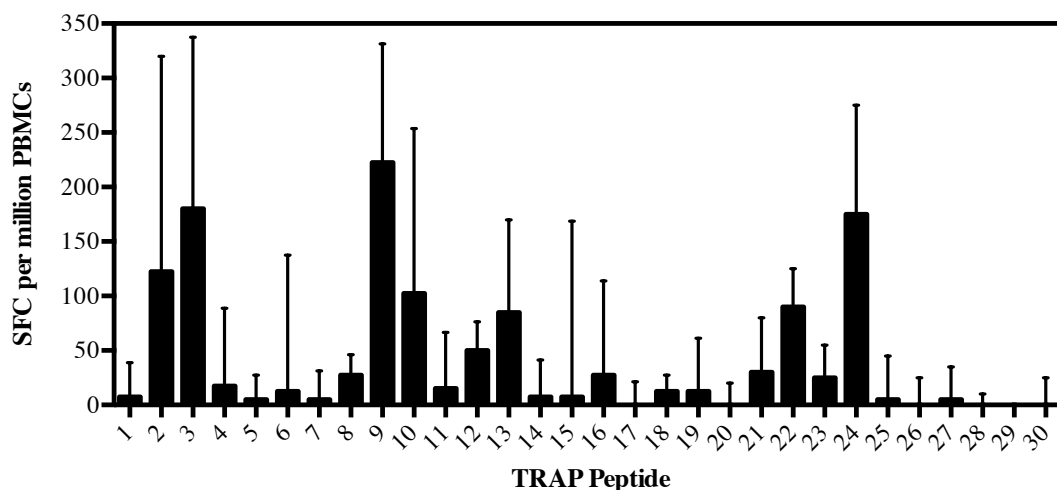


Figure 6.1. Epitope mapping of *P. falciparum* T9/96 TRAP peptides 1-30 (aa1-310) in eighteen volunteers vaccinated with ChAd63-MVA ME-TRAP.

Eighteen volunteers were vaccinated with ChAd63-MVA ME-TRAP and subsequently challenged with *P. falciparum* malaria. Seven days post-challenge fresh PBMCs were isolated and the *ex vivo* IFN γ ELISpot assay was performed using individual 20mer peptides covering the first three pools of *P. falciparum* T9/96 TRAP. Not all peptides were tested for each volunteer; peptides were assessed based on a positive response to the relevant pool of greater than 150 SFC per million PBMCs the day before challenge, n=10-14 vaccinees for each peptide. Samples were tested in single wells and the median response of all volunteers is shown, error bars represent the interquartile range; the background response was subtracted and the results are expressed as SFC per million PBMCs. ELISpots were performed by Dr. Katie Ewer.

The second aim of this chapter was to assess cellular immunogenicity to a range of *P. falciparum* antigens in previously naive volunteers following the first episode of *P. falciparum* malaria infection. Over 5000 different proteins are expressed throughout the parasite's life-cycle [224], yet only a fraction of these have been assessed as vaccine candidates. Numerous studies have assessed targets of cellular immunity in natural infections or volunteers immunized with irradiated sporozoites, yet they have only focused on a narrow subset of well-known *P. falciparum* antigens such as TRAP, CSP, LSA1, LSA3, Exp1 and CelTOS [154-156, 158, 390, 403, 409, 411, 412, 458, 467, 470, 514-523]. Cellular immunogenicity to some of these antigens has

correlated with protection (LSA1 and TRAP, and to a lesser extent CelTOS) [154-158, 390, 411, 458, 470, 471], highlighting the usefulness of assessing other antigens in the hope of identifying new targets for vaccine development. In particular, it was of interest to determine whether an immune response was mounted to any of the candidate antigens analysed as viral vectored vaccines in the previous chapters.

A unique opportunity to assess such responses arose in Oxford: Vac49 was a clinical trial designed to assess the infectivity of cryopreserved sporozoites injected by needle and syringe, to create an alternative and potentially more quantitative challenge method than the traditional mosquito bite model [524]. In total, eighteen previously malaria naïve volunteers were injected with three different doses of cryopreserved sporozoites. Cellular immune responses to sixteen different *P. falciparum* antigens were assessed at various time-points after challenge, using the *ex vivo* IFN γ ELISpot. Memory responses were also measured using the cultured IFN γ ELISpot assay. The antigens assessed included the nine candidate pre-erythrocytic antigens of this thesis (outlined in Chapter 3), the two most successful antigens used in current vaccines, CSP and TRAP, as well five other antigens previously tested as vaccine candidates: the blood-stage antigens MSP1, MSP2 and AMA1 [194, 257, 258, 525, 526], the sexual stage antigen Pfs16 [472, 527] and the pre-erythrocytic antigen STARP [472, 528].

6.2 Results

6.2.1 Epitope mapping of *P. falciparum* TRAP in volunteer 012

In Mal34, volunteer 012 (HLA-A3/23, B65/7, Cw8/7, DR1/4 and 53, DQ7/6) was vaccinated with ChAd63-ME-TRAP followed eight weeks later by MVA-ME-TRAP,

and was subsequently sterilely protected from challenge with *P. falciparum* two weeks post-MVA. The volunteer was re-challenged approximately eight months later (and again, sterilely protected), and the following experiments were performed approximately nine months after the second challenge. Epitope mapping of *P. falciparum* T9/96 TRAP peptides 1-30 (aa1-310) was performed using PBMCs isolated from fresh blood where possible. Sequences for all peptides are provided in Appendix Two, whilst those for the immunodominant epitopes identified are also provided in Chapter 2 and listed below.

Dominant cellular responses, measured by *ex vivo* IFN γ ELISpot, were identified to six peptides (Figure 6.2). These 20mer peptides were then mapped with overlapping 9mers to identify potential CD8⁺ epitopes (Figure 6.3). Strong responses to these 9mers were identified within peptides 2 (aa11-30) and 24 (aa231-250), sub-dominant responses to 9mers were seen within peptides 9 (aa81-100), 12 (aa111-130) and 19 (aa181-200), whilst no 9mer responses were seen to peptide 3 (aa21-40).

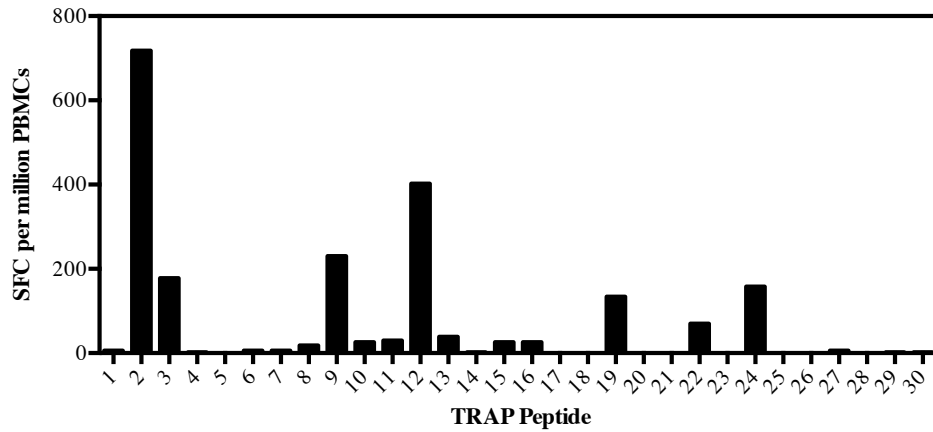


Figure 6.2. Epitope mapping of *P. falciparum* T9/96 TRAP peptides 1-30 (aa1-310) in volunteer 012.

Fresh PBMCs were isolated from volunteer 012 approximately nine months after re-challenge (seventeen months after final MVA vaccination) and the *ex vivo* IFN γ ELISpot assay was performed using individual peptides covering the first three pools of *P. falciparum* T9/96 TRAP. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs.

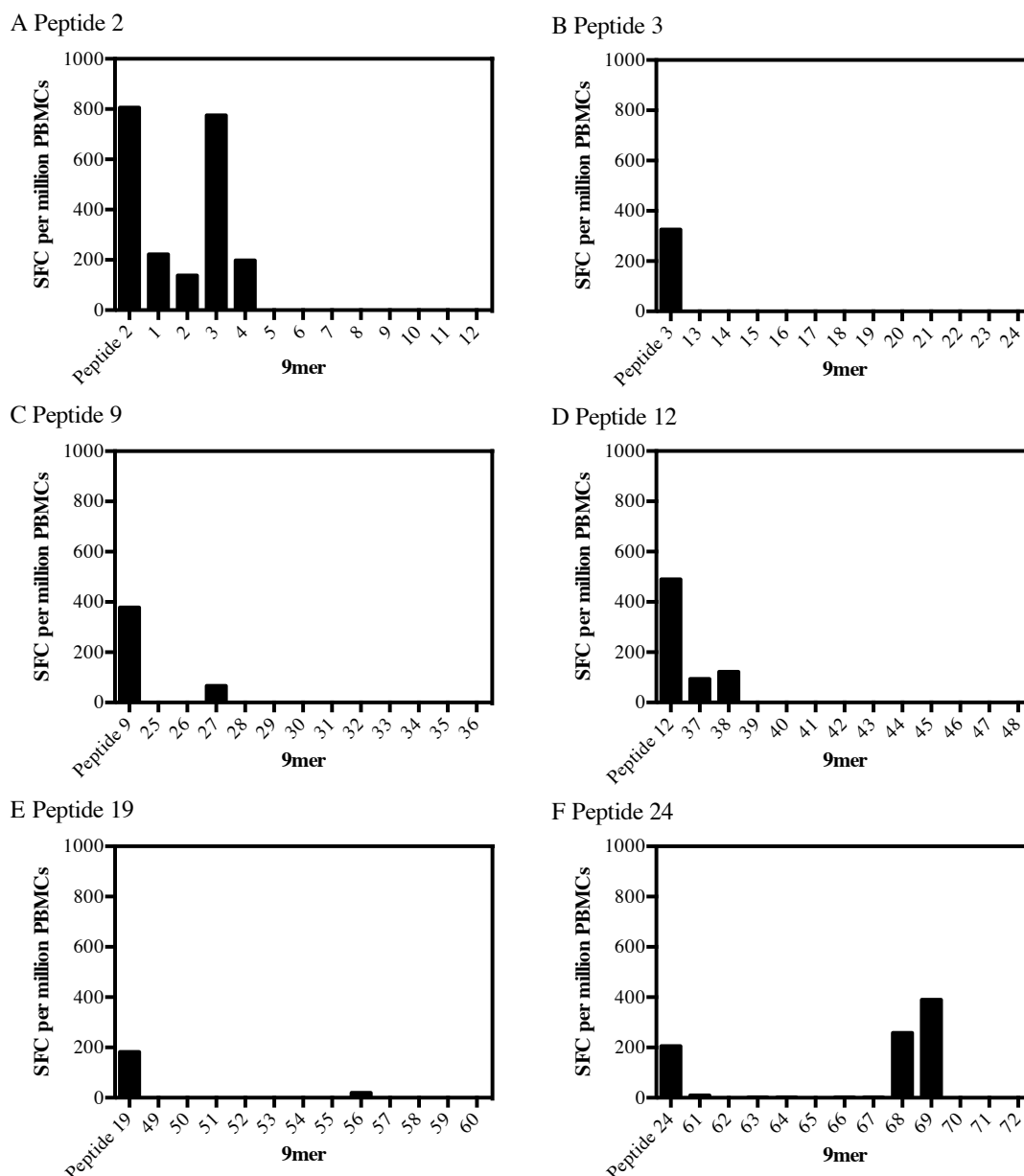


Figure 6.3. Minimal epitope mapping of the six dominant peptides identified for volunteer 012.

Overlapping 9mer peptides were generated to the 20mer epitopes identified in Figure 6.2. Fresh PBMCs were isolated from volunteer 012 approximately nine months after re-challenge (seventeen months after final MVA vaccination) and the *ex vivo* IFN γ ELISpot assay was performed using individual 9mer peptides covering the previously identified 20mer epitopes of *P. falciparum* T9/96 TRAP: (A) peptide 2, (B) peptide 3, (C) peptide 9, (D) peptide 12, (E) peptide 19 and (F) peptide 24. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The results were confirmed in two further independent experiments (data not shown).

Classification of CD8⁺ or CD4⁺ dependent responses was performed by depletion of either subset prior to the *ex vivo* IFN γ ELISpot (Figure 6.4). Essentially all were CD4⁺ epitopes, except for peptide 2 (CD8⁺) and peptides 9 and 24, which were dependent on both CD4⁺ and CD8⁺ cells (Table 6.1). Alternatively, these peptides could contain multiple epitopes.

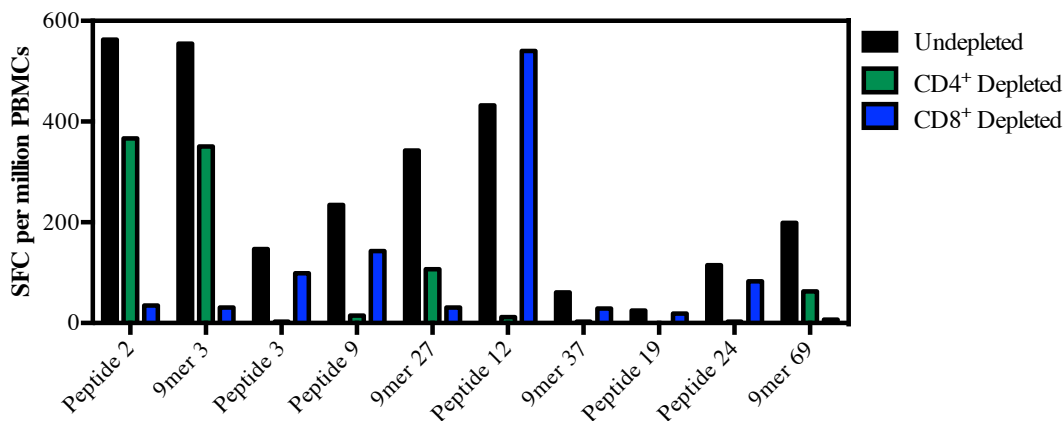


Figure 6.4. Classification of CD4⁺ or CD8⁺ dependent responses for the epitopes identified in volunteer 012.

Fresh PBMCs were isolated from volunteer 012 approximately nine months after re-challenge (seventeen months after final MVA vaccination) and separated into CD4⁺ or CD8⁺ positive fractions by magnetic sorting. The *ex vivo* IFN γ ELISpot assay was performed using individual 20mer and 9mer peptides of *P. falciparum* T9/96 TRAP (constituting previously identified epitopes), using undepleted and depleted PBMCs. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The results were confirmed in a second experiment using undepleted and depleted PBMCs frozen at the time of this experiment (data not shown).

Table 6.1. Summary of minimal epitope mapping in volunteer 012.

Dominant responses reflect 9mers that generated a response similar in magnitude to the peptide it originated from, whereas sub-dominant responses were substantially lower in magnitude than the original response to the parent peptide.

Peptide	9mer	Dominance	CD8⁺ or CD4⁺
2 (aa11-30)	VFLIFFDLF	Dominant	CD8 ⁺
3 (aa21-40)	N/A	N/A	CD4 ⁺
9 (aa81-100)	DNAIHLNVN	Sub-dominant	CD4 ⁺ /CD8 ⁺
12 (aa111-130)	KEKALIIIR/EKALIIIRS	Sub-dominant	CD4 ⁺
19 (aa181-200)	FGIGQGINV	Sub-dominant	CD4 ⁺
24 (aa231-250)	VEKTASCGV/EKTASCGVW	Dominant	CD4 ⁺ /CD8 ⁺

6.2.2 Confirmation of identified epitopes in further volunteers

To confirm the *P. falciparum* T9/96 TRAP epitopes identified in volunteer 012, volunteers from the trials denoted Vac33 and Mal34 were also epitope mapped with peptides 1-30 (aa1-310), dependent on availability of frozen PBMCs (various time-points post-boost were used). Ten volunteers were screened and greater than ten potential epitopes were identified (Figure 6.5 A), including confirmation of five peptides identified in volunteer 012, but not peptide 19 (aa181-200). The dominant epitopes for each volunteer were assessed; this analysis was also performed for the eighteen volunteers originally mapped with fresh PBMCs in Mal34 by Dr. Katie Ewer (Figure 6.1) and seven volunteers subsequently mapped on fresh PBMCs in Vac45 (Figure 6.9), both at C+7. Dominant epitopes were defined as the peptides that could account for the response measured to the minor pool. Eight peptides were classed as dominant in more than two volunteers; peptides 2, 3, 6, 9, 13, 15, 16 and 24 (Figure 6.5 B). Dominant responses in at least one or two volunteers were also seen for nine other peptides, indicating there were at least seventeen potential epitopes within peptides 1-30 (aa1-310). Each volunteer had one to three dominant peptides, and responded on average to 11.7 peptides (range 2-32) with greater than 10 SFC per million PBMCs.

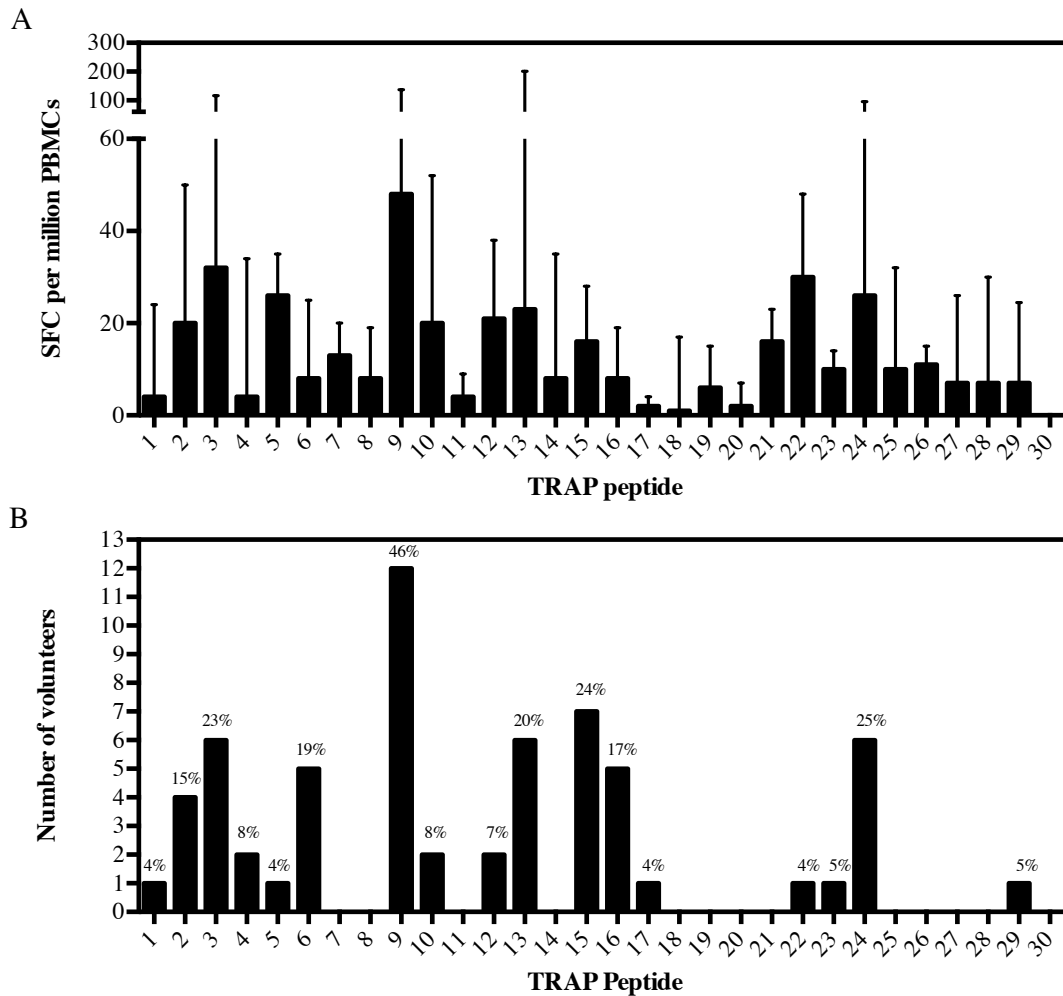


Figure 6.5. Confirmation of epitopes within *P. falciparum* T9/96 TRAP peptides 1-30 (aa1-310).

(A) Frozen PBMCs from ten volunteers at various time-points post-MVA boost (D56, D63, D84, C+7 or C+90) were thawed and the *ex vivo* IFN γ ELISpot assay performed using individual peptides covering the first three pools of *P. falciparum* T9/96 TRAP. Not all peptides were tested for each volunteer: peptides were used based on fresh *ex vivo* IFN γ ELISpot responses to the relevant pool of over 150 SFC per million PBMCs. For peptides 1-20 at least eight volunteers were assayed, for peptides 21-25 at least five volunteers were assayed and for peptides 26-30 four volunteers were assayed. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. Bars represent the median response, error bars indicate the interquartile range. (B) The dominant responses from the ten volunteers were collated and are expressed as the number of volunteers with that peptide as their dominant response, with the percentage of volunteers out of those tested indicated above the bar. Data previously obtained from fresh PBMCs at C+7 in Mal34 was also included (n=18), as was data subsequently obtained from fresh PBMCs at C+7 in Vac45 (n=7). On average, volunteers had two strong responses considered dominant epitopes.

6.2.3 Identification and re-assessment of minimal epitopes in *P. falciparum*

T9/96 TRAP

To re-assess the minimal epitopes identified in peptides 2, 3, 9, 12 and 24 in volunteer 012, 9mer mapping was subsequently performed on frozen PBMCs from previous trials and on fresh PBMCs from the clinical trial Vac45 (Figure 6.6). These epitopes were further narrowed by mapping with 6 or 8mer peptides (Figure 6.7). Minimal epitope mapping was also performed for four of the newly identified peptides, 6, 13, 15 and 16, by 9mer mapping on frozen PBMCs where available from previous trials (Figure 6.8).

The peptide 2 9mer was mapped in four volunteers, and was one amino acid different from the 9mer identified in volunteer 012 (Figure 6.6). Minimal epitope mapping confirmed the epitope was an 8mer common to the two 9mers (Figure 6.7). Peptides 3 and 9 were confirmed to have no dominant 9mers, as shown by mapping in at least eight further volunteers (data not shown). However, there was some evidence for a sub-dominant T cell 9mer within peptide 3 in one volunteer, and in peptide 9 for the majority of volunteers. Peptide 12 was confirmed to have a sub-dominant 9mer response, however in volunteer 659 a second dominant 9mer response was also evident (Figure 6.6). The strong CD8⁺ epitope identified within peptide 24 in volunteer 012 was not replicated in three subsequent volunteers tested (data not shown); these volunteers likely only respond to the CD4⁺ epitope, which is greater than nine amino acids. Minimal epitope mapping in volunteer 012 did indicate that the CD8⁺ epitope was nine amino acids in length (Figure 6.7), however this is not in agreement with the initial 9mer mapping results, where two consecutive responsive 9mers suggested a minimal 8mer epitope. The magnitude of the response to peptide

24 was greatly reduced compared to what was initially measured (see Figure 6.2), potentially accounting for the lack of response to the 8mer.

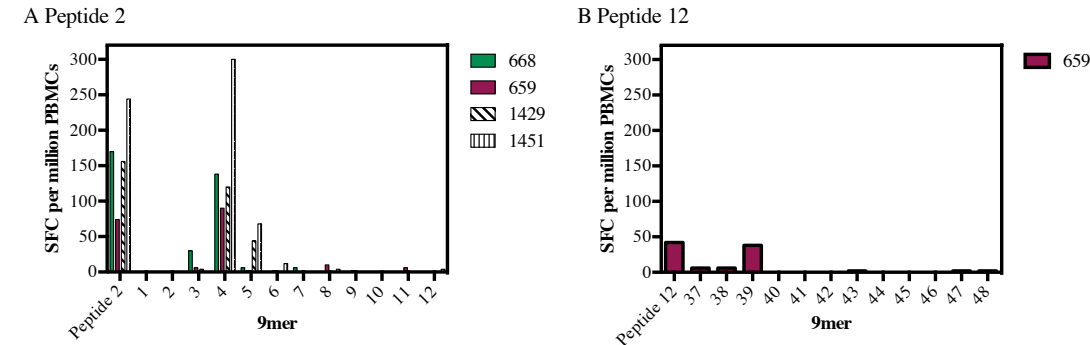


Figure 6.6. Confirmation of epitopes within the *P. falciparum* T9/96 TRAP peptides 2 and 12.

PBMCs (frozen and fresh) from Vac33 and Vac45 at various time-points post-MVA boost (D63, D84 or C+7), dependent on availability, were used to confirm the responsive 9mers in (A) peptide 2 and (B) peptide 12. The *ex vivo* IFN γ ELISpot assay was performed using the 20mer epitopes and appropriate 9mers. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The different volunteers used are colour coded; Vac33 (frozen cells) are in colour, Vac45 (fresh PBMCs) are in black and white.

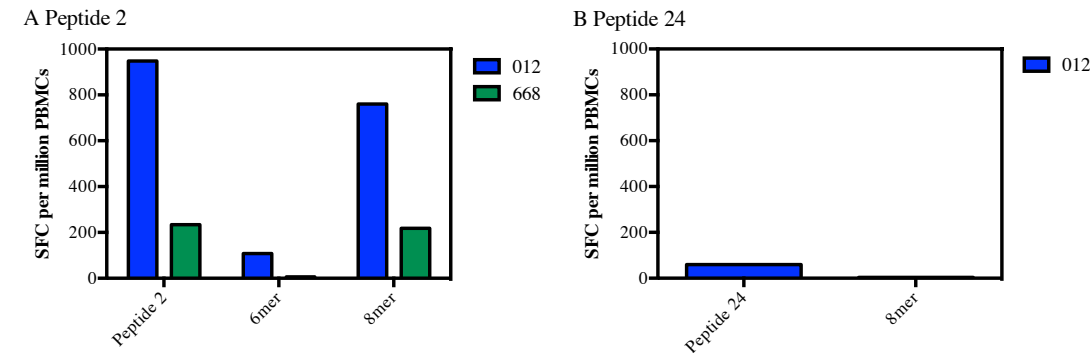


Figure 6.7. Minimal epitope mapping for the *P. falciparum* T9/96 TRAP peptides 2 and 24.

Frozen PBMCs from volunteer 668 (Vac33, D84) and fresh PBMCs from volunteer 012 (Mal34) were used to minimally map (A) peptide 2 and (B) peptide 24. The *ex vivo* IFN γ ELISpot assay was performed using the previously identified 20mer epitopes, and minimal 6 or 8mer peptides generated for those epitopes. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The different volunteers used are colour coded.

9mer mapping for peptides 6, 13, 15 and 16 was undertaken in a limited number of volunteers where frozen PBMCs were available from both Mal34 and Vac33 (Figure 6.8). Initial results suggested that peptide 6 contained at least two different dominant epitopes as shown by mapping in two volunteers. Peptide 13 was also mapped in two volunteers and the results indicated there was one dominant 9mer at the end of the peptide. Peptide 15 was only successfully mapped in one volunteer, and data suggested it was most likely a dominant 7mer epitope as shown by strong responses to three consecutive 9mers. Peptide 16 was mapped in only two responsive volunteers, and the epitope was shown to be one dominant 9mer, however not enough PBMCs were available to test the two 9mers at the end of the peptide (covering the sequence TDGIPDSIQD). A summary of the dominant minimal epitopes mapped for each peptide is provided (Table 6.2).

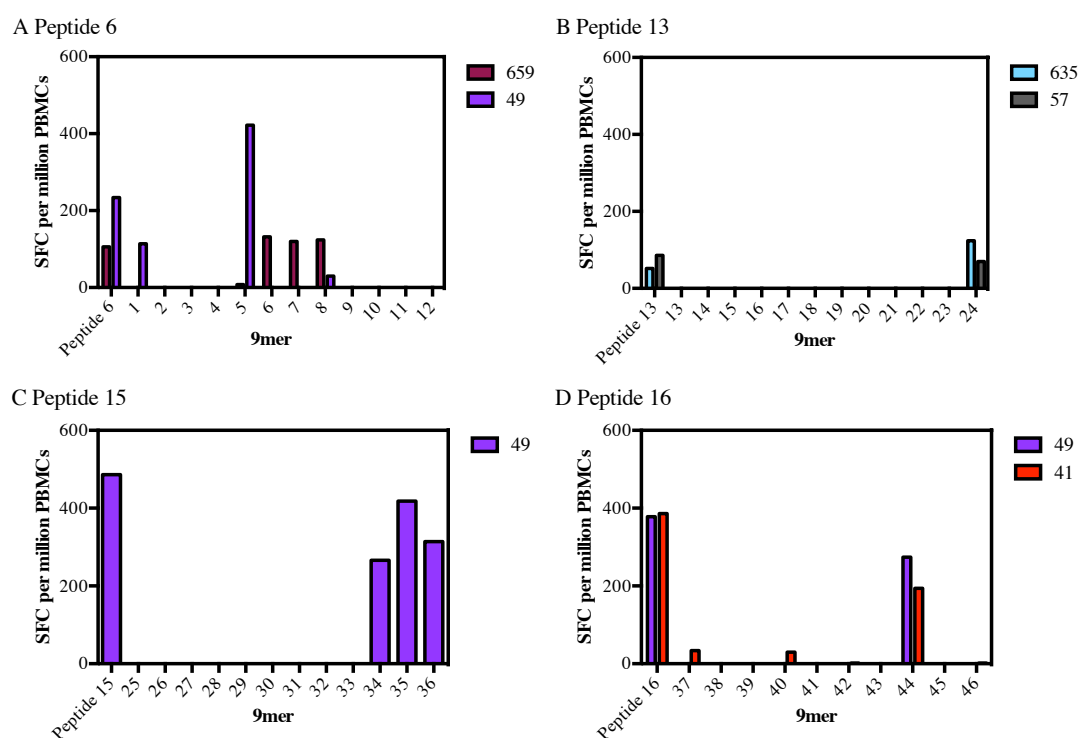


Figure 6.8. Identification of epitopes within the *P. falciparum* T9/96 TRAP peptides 6, 13, 15 and 16.

Frozen PBMCs from five volunteers at various time-points post-MVA boost (D56, D63, D84, C+7 or C+90), dependent on availability, were thawed and the *ex vivo* IFN γ ELISpot assay was performed using the previously identified 20mer epitopes, and overlapping 9mers generated for those epitopes: (A) peptide 6, (B) peptide 13, (C) peptide 15 and (D) peptide 16. Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The different volunteers used are colour coded.

Table 6.2. Summary of the dominant minimal epitopes identified.

Summary of the dominant minimal epitopes identified using fresh and frozen PBMCs from volunteers vaccinated with ChAd63-MVA ME-TRAP. Not all epitopes have been confirmed in multiple volunteers.

Peptide	Minimal epitope
2 (aa11-30)	FLIFFDLF
3 (aa21-40)	FLVNGRDVQNNIVDEIKYSE
6 (aa51-70)	CSGSIRRHN/SIRRHNW
9 (aa81-100)	LNDNAIHLYVNVFSNNAKEI
12 (aa111-130)	KALIIIRSL
13 (aa121-140)	NLTDALLQV
15 (aa141-160)	NANQLVV
16 (aa151-170)	VILTDGIPD
24 (aa231-250)	VEKTASCGV/EKTASCGVW

6.2.4 HLA restriction of identified epitopes

The identified epitopes were assessed for any evident associations with HLA type (Table 6.3). For peptides 2, 3, 9, 12, 13, 15 and 24 there were no obvious HLA restrictions, yet not all volunteers tested were responsive to those peptides. For peptide 6, both responsive volunteers were HLA-DR17, yet each volunteer responded to a different epitope. For peptide 16, the two responsive volunteers were HLA-B53 and HLA-Cw4 positive, whilst none of the non-responsive volunteers expressed these HLA types.

Epitope mapping of *P. falciparum* T9/96 TRAP was subsequently performed on fresh PBMCs from HLA-A3 volunteers in Vac45 at C+7, the follow-on challenge study to Mal34. The HLA-A3 supertype was chosen given the high frequency within the European population [529]. Individual peptides were tested based on a response of over 150 SFC per million PBMCs to the appropriate pool at C-1. The identified epitopes were compared to non-HLA-A3 responses from Mal34, which were also conducted on fresh samples. A number of previously identified epitopes were again confirmed in HLA-A3 volunteers, including peptides 2, 3, 6, 9, 15, 16 and 24 (Figure 6.9). No dominant epitopes were identified in peptides 31-50 (aa301-495, data not shown), confirming the responses were primarily to peptides 1-30 (pools 1-3, aa1-310). No epitopes were HLA-A3-restricted, however responses to peptides 6, 15 and 16 were significantly greater in magnitude and more frequent in HLA-A3 volunteers (peptide 6 $p < 0.05$, peptide 15 and 16 $p < 0.0001$, two-way ANOVA).

Table 6.3. Identified peptides, their minimal epitopes and potential HLA restrictions.

Ten volunteers were initially tested for their *ex vivo* IFN γ ELISpot responses to multiple peptides within *P. falciparum* T9/96 TRAP. During the process of minimal epitope identification three more volunteers from Mal34 were used, as they had known responses to specific 20mer peptides, bringing the total number of volunteers assessed to thirteen. This table presents the identified 20mer peptides, the minimal epitope length and MHC restriction and the number of volunteers who responded to the peptide out of those tested. Only dominant responses were considered positive. Associations with HLA restriction were then made, by comparing the HLA type within responsive volunteers to non-responsive volunteers. SD=sub-dominant.

Peptide	Epitope Length	Responded/Tested	HLA restriction
2 (aa11-30)	8mer (CD8)	5/10	No
3 (aa21-40)	>9mer (CD4)	6/10	No
6 (aa51-70)	9mer	2/11	DR17
	7mer		
9 (aa81-100)	>9mer (CD4)	8/10	No
	SD 9mer (CD8)		
12 (aa111-130)	>9mer (CD4)	2/11	No
	9mer		
13 (aa121-140)	9mer	5/13	No
15 (aa141-160)	7mer	2/13	No
16 (aa151-170)	9mer	2/12	B35 and Cw4
24 (aa231-250)	>9mer (CD4)	6/8	No
	8 or 9mer (CD8)		

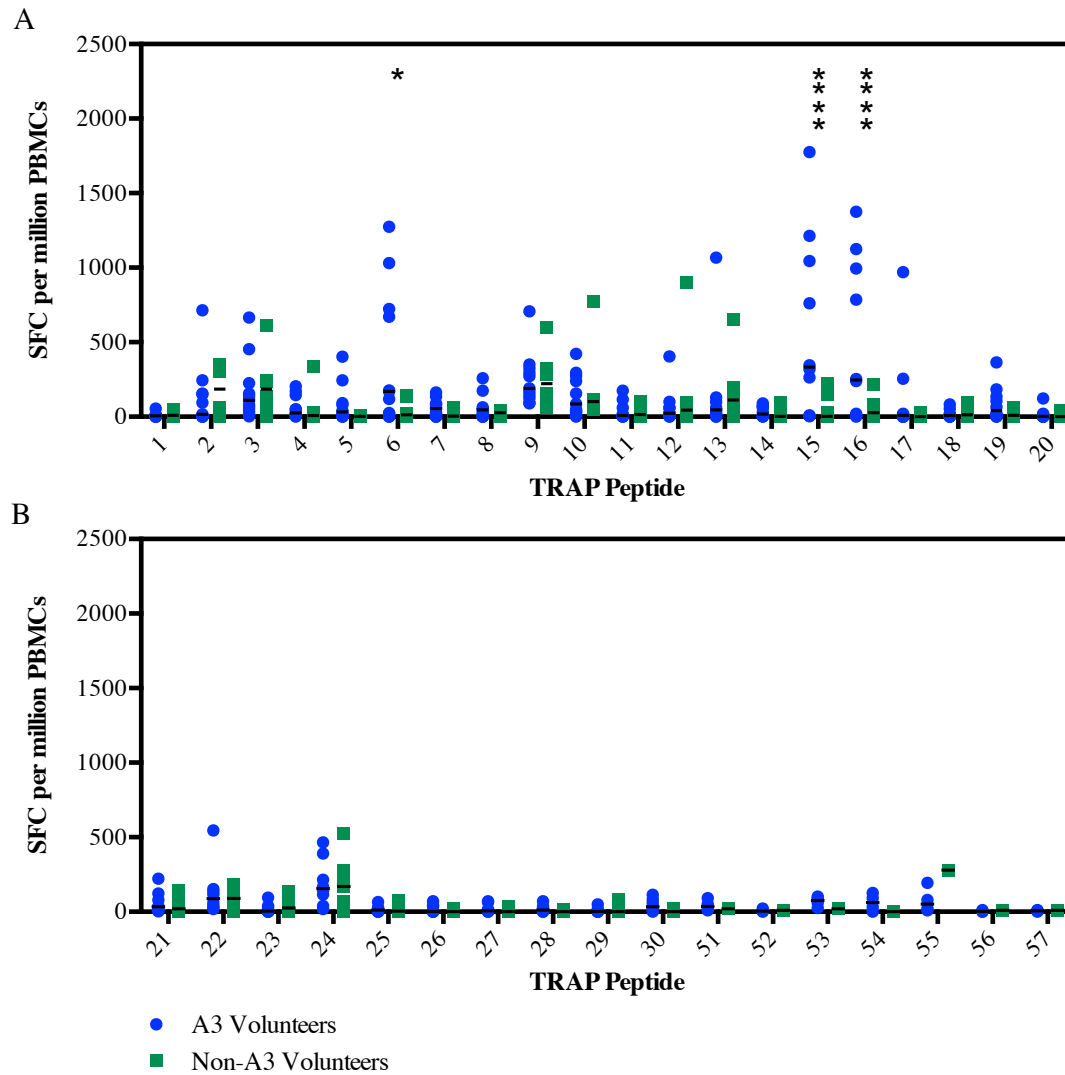


Figure 6.9. *P. falciparum* T9/96 TRAP epitope mapping in HLA-A3 volunteers vaccinated with ChAd63-MVA ME-TRAP, compared to previously obtained data from non-HLA-A3 vaccinated volunteers.

Fresh PBMCs were isolated from volunteers at C+7 in Mal34 and Vac45 and the *ex vivo* IFN γ ELISpot assay was performed using individual peptides to *P. falciparum* T9/96 TRAP. (A) Responses to peptides 1-20 (n=6-11 per group) and (B) responses to peptides 21-30 (n=8-9 per group) and 51-57 (n=1-4 per group). Samples were tested in single wells; the background response was subtracted and the results are expressed as SFC per million PBMCs. The median and individual data points are shown. Significant differences between the HLA-A3 and non-HLA A3 volunteers was assessed using a two-way ANOVA with Bonferroni post-test, * p<0.05 **** p<0.0001.

6.2.5 Assessment of cellular responses to multiple *P. falciparum* antigens in volunteers experimentally challenged with *P. falciparum* malaria

Cellular immune responses, measured by the *ex vivo* IFN γ ELISpot assay, were assessed for sixteen *P. falciparum* 3D7 antigens in volunteers challenged with three different doses of *P. falciparum* sporozoites delivered by injection (Figure 6.10). Group 1 received 2500 sporozoites i.d., group 2 received 2500 sporozoites i.m. and group 3 received the higher dose of 25 000 sporozoites i.m. (n=6 per group). Peptide sequences for all antigens are provided in Appendix Two. As responses were low (the majority less than 60 SFC per million PBMCs), a stringent positive cut off value was set: a background level of 2x media only +5 SFC was first subtracted, followed by subtraction of the volunteer's response at C-1 (before they had been infected with malaria). Responses in group 2 appeared slightly lower than the other groups; group 2 included three volunteers who were never diagnosed with malaria (group 1 included one). However, analysis of the summed responses to all peptides tested indicated there were no significant differences between groups or between uninfected and infected volunteers (Figure 6.11 A and B). Assessment of uninfected verses infected volunteers in more detail at C+90 revealed a greater proportion of the uninfected responses were to pre-erythrocytic antigens, whilst a greater proportion of the infected responses were to blood-stage antigens (PfMSP1, PfMSP2 and PfAMA1) (Figure 6.11 C). However, this effect was non-significant and at earlier time-points the uninfected volunteers did respond to blood-stage antigens (Figure 6.11 D). From these analyses it was not possible to determine whether the 'uninfected' volunteers overcame the liver-stage infection and hence were blood-smear negative, or whether they were never infected and the cellular immunity measured reflects cross-reactivity or molecular mimicry. Therefore, for future analysis the data from all groups was

combined and the four volunteers who were not diagnosed with malaria were included.

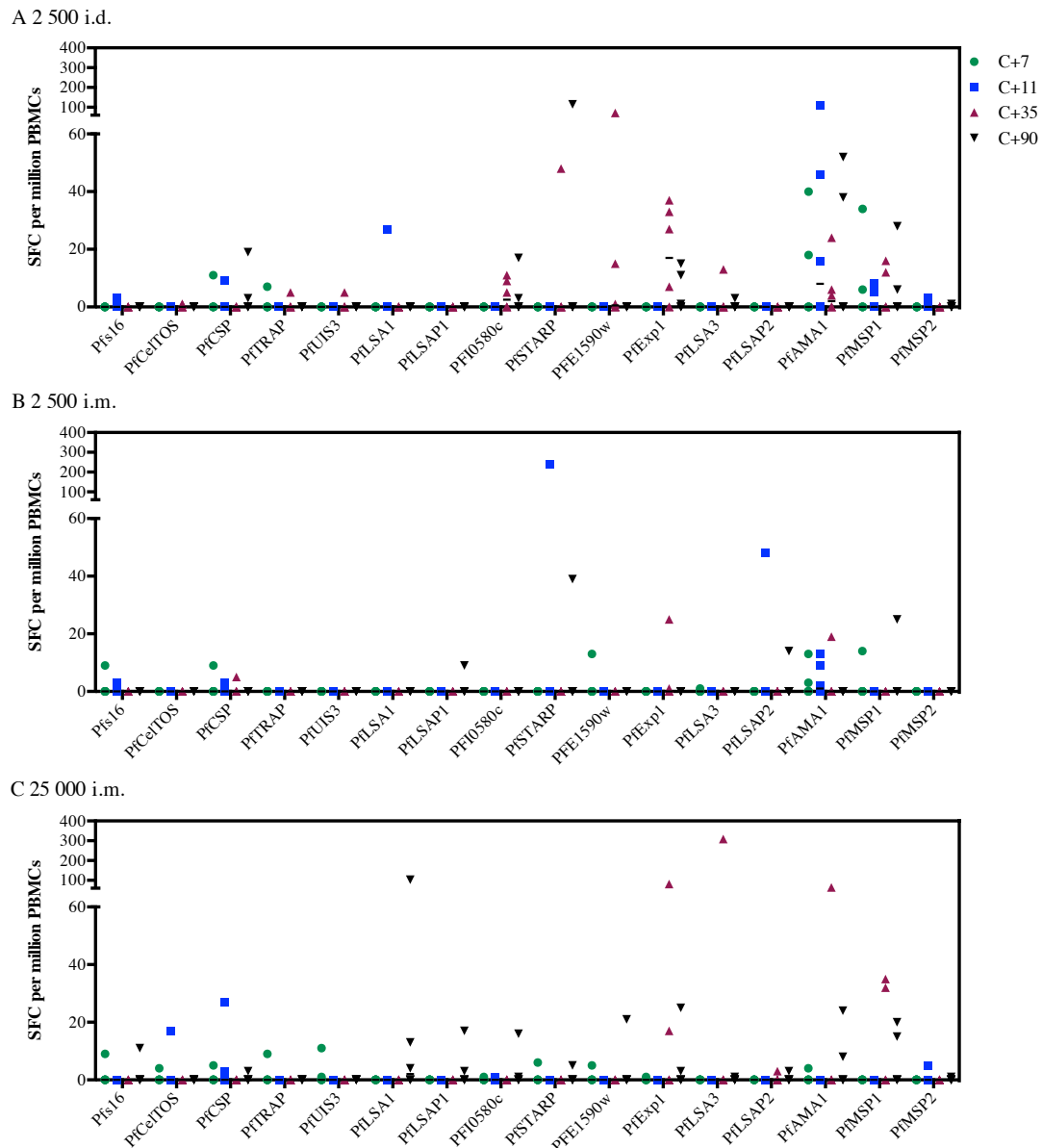


Figure 6.10. Cellular immune responses to sixteen malaria antigens in volunteers challenged with *P. falciparum* by injection.

Eighteen volunteers were challenged with *P. falciparum* sporozoites by injection, and assessed at various time-points post-challenge for responses to sixteen malaria antigens by *ex vivo* IFN γ ELISpot using a single pool of overlapping peptides (concentration 1 μ g/ml final) for each antigen (range 10-130 peptides). The responses are shown for the three different groups: (A) Group 1, 2500 sporozoites i.d., (B) Group 2, 2500 sporozoites i.m. and (C) Group 3, 25 000 sporozoites i.m. The background response was calculated as 2x media only +5 SFC; this value was calculated for each volunteer at each time-point and subtracted from the positive response. The results are expressed as SFC per million PBMCs after background subtraction and subtraction of the response at C-1. Samples were tested in duplicate. The median and individual data points are shown. Antigens are ordered from those expressed at the pre-erythrocytic stage to those at the blood-stage.

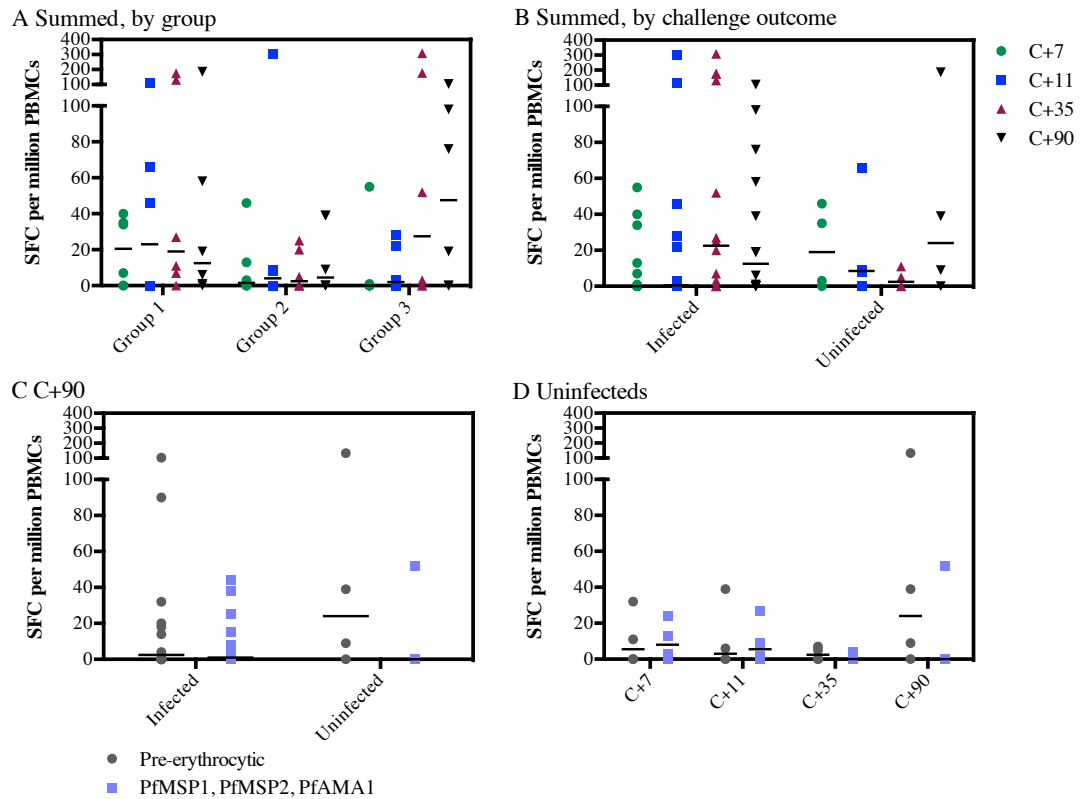


Figure 6.11. Summed responses to sixteen malaria antigens in volunteers challenged with *P. falciparum* by injection.

The individual responses from Figure 6.10 were summed for each volunteer, after background and C-1 subtraction. The summed responses are shown in (A) by group and in (B) by challenge outcome. Four volunteers were classed as ‘uninfected’ as they were PCR negative, blood-film negative and symptom free until the end-point 21 days post-challenge. (C) The individual responses were summed for pre-erythrocytic and blood-stage specific antigens (PfMSP1, PfMSP2 and PfAMA1) separately at C+90 and compared between uninfected and infected volunteers. (D) The summed pre-erythrocytic and blood-stage specific antigens for uninfected volunteers only by time-point. The median and individual data points are shown. No significant differences between groups or challenge status were found using repeated measures two-way ANOVA with Bonferroni post-test.

6.2.6 Assessment of summed responses to multiple *P. falciparum* antigens over time

As there were no significant differences in the summed response between groups, the cellular IFN γ data from all volunteers from different groups was summarized on a single graph to perform further analysis (Figure 6.12 A). The summed responses of the antigens were used to assess differences in the time-points assayed; whilst there was a trend for increased magnitude at C+90 there was no significant difference between the four time-points (Figure 6.12 B). The summed cellular response at any time-point did not correlate with the day of diagnosis (Figure 6.12 C), even when the uninfected volunteers were removed (data not shown).

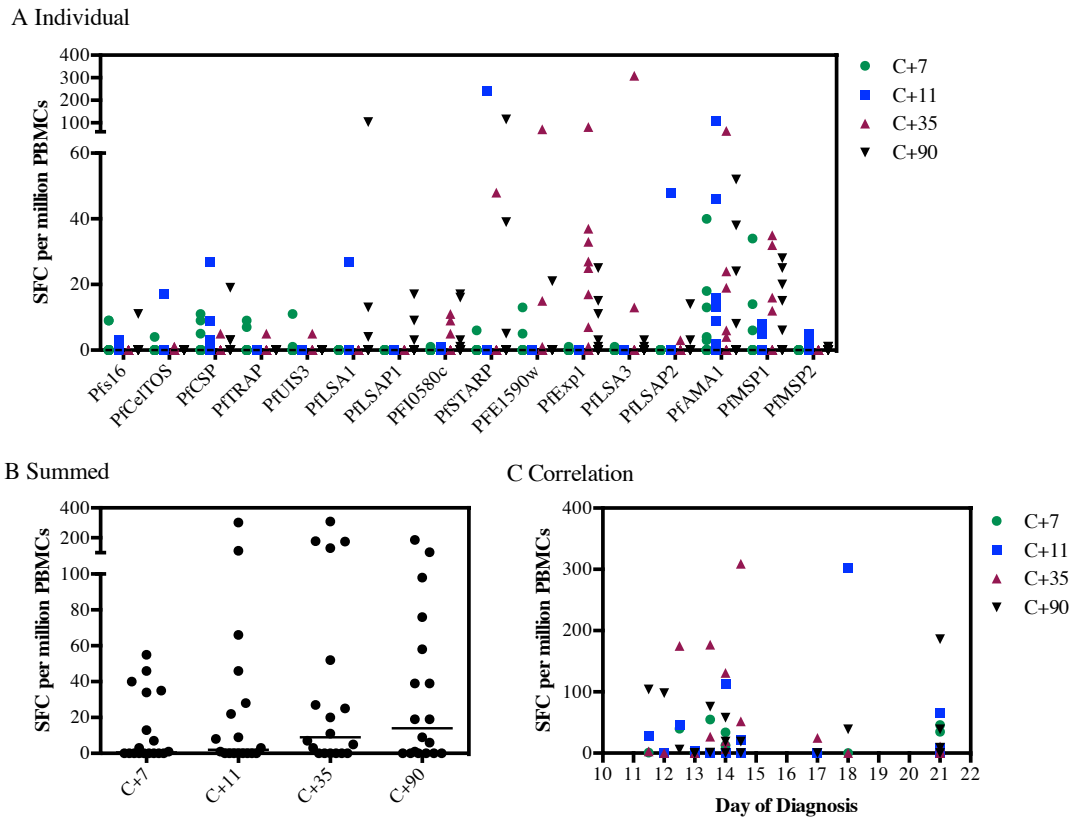


Figure 6.12. Combined cellular responses from volunteers challenged with three different doses of sporozoites indicates there are no significant differences between time-points post-challenge and no correlation between the magnitude of the response and the day of diagnosis with malaria.

Data from all three groups in Figure 6.10 was combined and responses are shown after background and C-1 subtraction for (A) individual antigens and (B) for summed responses to all antigens. There was no significant difference in the summed response over time using Friedman's test with Dunn's Multiple Comparison post-test. (C) There was no correlation of the summed responses to all antigens at any time-point and the day the volunteer was diagnosed with malaria. Spearman's correlation, C+7 $r=0.069$ $p=0.79$, C+11 $r=0.18$ $p=0.48$, C+35 $r=-0.075$ $p=0.77$, C+90 $r=-0.072$ $p=0.78$.

6.2.7 Responses to the *P. falciparum* pre-erythrocytic antigen Exp1

Of the sixteen antigens assayed, a clear hierarchy of immunodominance could be determined (Figure 6.12 A). Five out of eighteen volunteers responded to PfAMA1 at multiple time-points, whilst five of eighteen also responded to PfMSP1 at C+90. At C+35, eight out of eighteen volunteers responded to PfExp1. PfExp1 was therefore the most frequently recognized antigen; whilst limited IFN γ cellular responses were seen at C+7 and C+11, these increased significantly to a median of 26 SFC per million PBMCs for the eight responsive volunteers by C+35 and 11 SFC per million PBMCs for the five responsive volunteers at C+90 ($p=0.0008$, Friedman's test) (Figure 6.13 A). However, of the five positive volunteers at C+90, two were not positive at C+35. No correlation was found between the immune response to PfExp1 at C+35 or C+90 and the day of diagnosis (Figure 6.13 B). The same analysis was performed for PfMSP1 and PfAMA1 and again no significant correlations were found (data not shown).

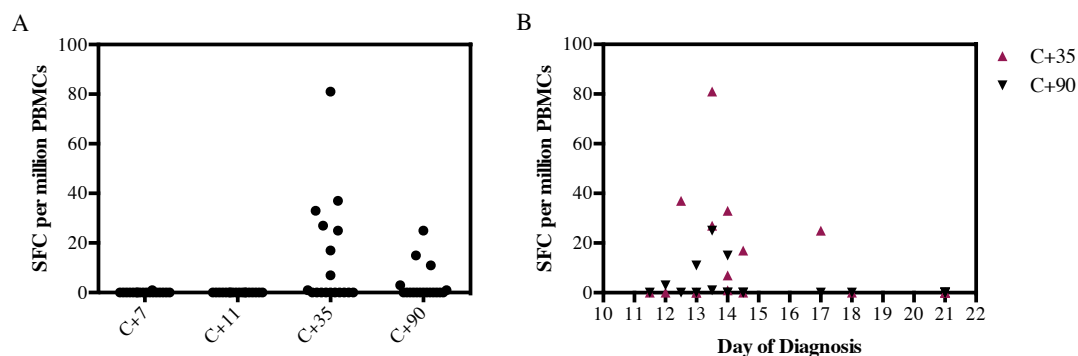


Figure 6.13. Cellular immune responses to the pre-erythrocytic antigen PfExp1 in volunteers challenged with *P. falciparum* sporozoites by injection.

Eighteen volunteers were challenged with *P. falciparum* sporozoites by injection, and assessed at various time-points post-challenge for responses to the *P. falciparum* pre-erythrocytic antigen Exp1 by *ex vivo* IFN γ ELISpot using a single pool of overlapping peptides covering the entire antigen. Samples were tested in duplicate. Data from all three groups was combined and responses are shown after background and C-1 subtraction. (A) There was a significant difference in the median response to PfExp1 over time using Friedman's test with Dunn's Multiple Comparison post-test, overall $p=0.0008$. (B) There was no correlation between the PfExp1 response at C+35 or C+90 with the day the volunteers were diagnosed with malaria. Spearman's correlation, C+35 $r=-0.245$ $p=0.33$ and C+90 $r=-0.432$ $p=0.07$.

6.2.8 Assessment of cellular memory responses to multiple *P. falciparum* antigens in volunteers experimentally challenged with *P. falciparum* malaria

Frozen PBMCs from the C+35 time-point were assessed, where available, for T cell central memory frequency to six pre-erythrocytic antigens by cultured IFN γ ELISpot. The memory responses detected were in general of higher magnitude than those measured by fresh *ex vivo* IFN γ ELISpot (Figure 6.14 A), however the background and variability in responses were also greater. For this reason, two cut-offs for positivity are shown, using data from four volunteers assayed at C-1 using two different antigenic stimulations: 2x the average of C-1 +5 SFC (2xAvC-1+5), and the average of C-1 +2x the standard deviation (AvC-1+2xSD). When the most stringent positive cut-off was applied, only responses to PfUIS3 and PfExp1 reached positivity. The correlation of cellular memory immune responses with the day of diagnosis was then tested; as the data set was not complete for every antigen for every volunteer, the summed response could not be used. Instead, the memory responses to PfUIS3 and PfTRAP were assessed; no correlations were found (Figure 6.14 B).

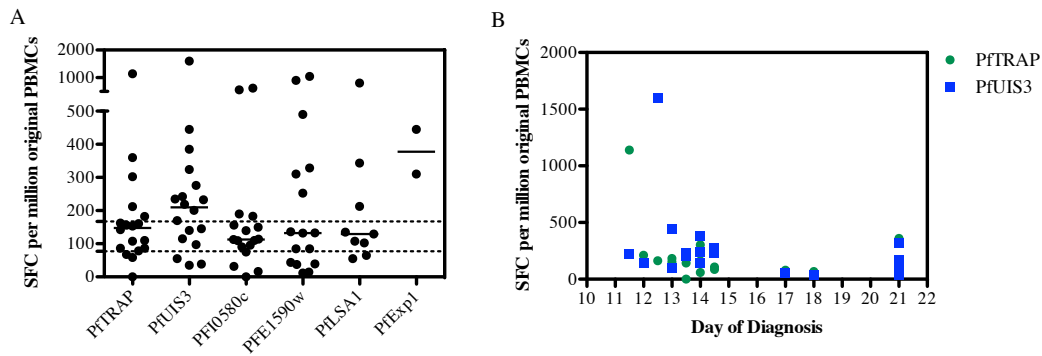


Figure 6.14. Cellular memory responses to six pre-erythrocytic antigens in volunteers challenged with *P. falciparum* sporozoites by injection.

Frozen PBMCs from eighteen volunteers injected with *P. falciparum* sporozoites from C+35 were stimulated for ten days with a pool of overlapping peptides to one of six pre-erythrocytic antigens (PfTRAP, PfUIS3, PFI0580c, PFE1590w, PfLSA1 or PFEExp1) and recombinant human IL-2. The stimulated cells were then used in a standard IFN γ ELISpot assay with a single pool of overlapping peptides to the relevant antigen. The background response of media only wells was subtracted from the positive response. Samples were tested in quadruplicate. (A) Responses to antigens are expressed as SFC per million original PBMCs. Median and individual data points are shown with two positive cut-offs, $2 \times \text{AvC} - 1 + 5$ (=77 SFC per million original PBMCs) or $\text{AvC} - 1 + 2 \times \text{SD}$ (=167 SFC per million original PBMCs). (B) The cultured IFN γ ELISpot response at C+35 for PfTRAP or PfUIS3 did not correlate with the day the volunteers were diagnosed with malaria. Spearman's correlation, PfTRAP $r = -0.358$ $p = 0.14$ and PfUIS3 $r = -0.334$ $p = 0.18$.

6.3 Discussion

Cellular immunity is an important, even indispensable, component of a protective response in murine malaria models using *P. berghei* and *P. yoelii*. However, the cellular immunogenicity directed at various malaria antigens in field studies or volunteers immunized with irradiated sporozoites has rarely correlated with protection, suggesting that immunodominance to one or a few antigens does not exist and that a successful vaccine will need to contain multiple targets [390]. Clearly more research needs to be undertaken in this area and the results described in this chapter sought to (1) analyse the cellular epitopes within the promising pre-erythrocytic target TRAP, to begin to assess the breadth of the response and HLA restrictions, and (2) to assess immunogenicity to multiple antigenic targets in volunteers exposed to *P. falciparum* malaria in a laboratory setting.

Epitope mapping of *P. falciparum* T9/96 TRAP in volunteers vaccinated with ChAd63-MVA ME-TRAP was initially begun by Dr. Katie Ewer using samples from the CHMI trial denoted Mal34. Combined with the mapping performed in this thesis using additional samples from Mal34, Vac33 and Vac45, 35 volunteers were assessed and nineteen potential dominant epitopes identified. As minimal epitope mapping was only performed for nine peptides, it is likely more epitopes do exist. On average, each volunteer had one to three dominant responses, out of a total of 11.7 responsive peptides (range 2-32). The only CD8⁺ epitope confirmed was an 8mer within peptide 2, FLIFFDLF (aa14-21), in the protected volunteer 012, which warrants further investigation to determine whether there is an association with protection from malaria. For use in future experiments, a tetramer could be made to this epitope to allow easy evaluation of responses to this epitope in both vaccinated and naturally

exposed populations. In general, responses were predominantly directed towards the N-terminus of the TRAP sequence (within peptides 1-30, aa1-310). This is strikingly different to what has previously been shown in field studies of malaria-exposed individuals within Africa, where the responses were spread along the entire TRAP sequence [516, 517, 530]. Variation between individuals was also greater in the field and the number of epitopes recognized per volunteer was lower; half of the individuals tested responded to only one peptide, and the maximum number responded to by an individual was five [517]. The breadth of response in TRAP vaccinated volunteers was much greater with individuals responding to an average of 11.7 peptides, as mentioned.

Whilst this might not be unexpected, such a difference between vaccine induced immunogenicity and the natural immune response has not been previously reported (as TRAP is one of the only antigens that has been fully mapped in the field). For CSP, immunodominant responses have been mapped to the polymorphic region in naturally acquired immunity and both naïve and semi-immune adults vaccinated with RTS,S [531-533]. For TRAP, it would be of interest to map the immune response in volunteers vaccinated with ChAd63-MVA ME-TRAP in malaria endemic countries, and compare how it differs or reflects the responses investigated in this chapter and with the work of Flanagan *et al* [516, 517]. The differences seen may be a reflection of differential antigen presentation between liver-stage infection, where multiple antigenic peptides could be expressed on infected hepatocytes, and vaccination with ME-TRAP where the entire sequence of TRAP is available for presentation but only limited epitopes of other antigens. Furthermore, the finding may be a reflection of naturally acquired immunity where multiple infections from different strains result in

a more varied response than seen in vaccination. Finally, the magnitude is much greater in vaccinated volunteers than seen in naturally acquired immunity, and this may allow sub-dominant responses to be more readily detected, resulting in a greater breadth of immune response within each volunteer.

A qualitative assessment of associations between immunodominant epitopes and HLA type revealed a large degree of promiscuity, with multiple HLA types able to bind and present the same epitopes. HLA promiscuity has previously been described for TRAP [530, 534]. Furthermore, non-HLA-A2 volunteers were able to recognize epitopes previously denoted as HLA-A2-restricted (corresponding to within peptide 6 (LLMDCSGSI) and peptide 13 (LLSTNLPYGRTNLTALLQV) [514, 522]). The only potentially HLA-restricted epitope identified by the qualitative analysis was VILTDGIPD (within peptide 16), where both volunteers shared the haplotype HLA-B53 and Cw4; mapping in additional volunteers is needed to confirm this finding. The quantitative assessment of potential HLA-A3-restricted epitopes using fresh PBMCs was more powerful, given the high frequency of this HLA type; two peptides, 15 (aa141-160) and 16 (aa151-170), were found to be significantly associated with HLA-A3 volunteers ($p < 0.0001$), whilst not actually restricted to this HLA type (although the 9mers were not mapped). Given the recent increase in the number of promiscuous epitopes identified [509-513], looking for HLA associations is a more informative and practical method than studying a limited number of individuals for HLA restrictions. In future vaccine trials of ChAd63-MVA ME-TRAP epitope mapping should be included as an immunogenicity measure, to expand the data set begun in this chapter. Given frozen PBMCs are usually of limited

availability, and exhibit reduced *ex vivo* IFN γ ELISpot responses, where possible mapping should be performed on fresh PBMCs.

The results described in the second half of this chapter assessed cellular immunogenicity in volunteers challenged with *P. falciparum* malaria, delivered by needle and syringe. Responses to all sixteen antigens tested were low, however this was expected and correlated well with the magnitude measured in previous field or CHMI studies to similar antigens [206, 390]. No differences were identified when responses were stratified between volunteers receiving different routes or doses of sporozoites, between infected and uninfected volunteers, nor between the different time-points assayed. However, differences were observed between the response rates to each of the antigens; the greatest response rate amongst volunteers was seen to the pre-erythrocytic antigen PfExp1 at C+35, where eight out of eighteen volunteers (44%) gave a positive *ex vivo* IFN γ ELISpot response. The response to PfExp1 increased significantly from C+7 and C+11 to C+35 and C+90, presumably after exposure to the parasite had increased, likely because PfExp1 is also expressed during the blood-stage [405, 406]. Whilst the immune response to PfExp1 did not correlate with the day of diagnosis, it does suggest that PfExp1 warrants further attention as a candidate antigen for a malaria vaccine. As discussed in Chapter 3, PfExp1 was one of the initial candidates of this thesis, however given difficulty with its expression in the ChAd63 vector it was excluded from further study. These results suggest other avenues of vaccination should be explored to test whether this antigen has potential as a vaccine target, for example through plasmid DNA immunization with *in vivo* EP. Interestingly, the best performing candidates identified in the murine studies outlined in the previous chapters of this thesis, PFLSA1, PFLSAP2 and PFUIS3, were not

frequently recognized amongst these volunteers. Whilst their recognition in this population would have provided extra support for these antigens, the fact they were not frequently recognized was not surprising given all responses were minimal. Furthermore, as there is currently no 100% effective sub-unit malaria vaccine based on one or a few antigens, we do not yet know what immune profile such an antigen should have in non-immunized, experimentally challenged individuals.

The cultured IFN γ ELISpot was used to assess responses to a subset of six pre-erythrocytic antigens. This assay measures a different cellular sub-population to the *ex vivo* IFN γ ELISpot [280, 535, 536], thought to be central memory cells [537, 538]. By culturing the cells, the responses are amplified and hence this results in more sensitivity than standard *ex vivo* IFN γ ELISpot when responses are low [159, 537]. Memory responses were detected to both PfUIS3 and PfExp1; this is the first time responses to PfUIS3 have been assessed in malaria-exposed individuals and hence marks the first time such responses have been detected. These results do suggest that an antigen-specific memory response can be mounted after one exposure to *P. falciparum* malaria, and is in support of previous findings in deliberate infection of naïve-adults [479]. Research has also shown that memory responses are mounted in naturally acquired immunity, and furthermore the memory response to TRAP correlated with protection from clinical malaria [158]. Importantly, Todryk *et al.*, identified memory responses of similar magnitude in children of zero to five years to those over twenty, supporting the notion that memory responses can be formed after one or limited malaria episodes [158].

In summary, this chapter assessed the immune response of human volunteers to various *P. falciparum* antigens following vaccination or exposure to sporozoites. Epitope mapping of TRAP in volunteers vaccinated with ChAd63-MVA ME-TRAP resulted in a different profile of epitopes compared to responses previously identified in volunteers naturally exposed to malaria. At least nineteen epitopes exist within *P. falciparum* T9/96 TRAP; two peptides were identified with a significant association with HLA-A3 individuals, as well as one CD8⁺-restricted epitope in a protected volunteer. Future clinical trials assessing the ChAd63-MVA ME-TRAP vaccination regimen should map individual epitopes on fresh PBMCs, to increase the data set to allow a more complete definition of the number of epitopes, protective epitopes and HLA associations. Identification of commonly recognized immunodominant epitopes would allow a reduction of the TRAP sequence to be included in a multi-component vaccine, if size was a restricting consideration. Assessment of immunogenicity to multiple malaria antigens in volunteers challenged with *P. falciparum* resulted in responses of similar magnitude to those seen in previous studies. A number of antigens were tested for immunogenicity for the first time in humans. PfExp1 was the most frequently recognized antigen, highlighting its potential as a candidate antigen for a malaria vaccine.

**Chapter 7: Development of an *in vitro* T cell
killing assay for the liver-stage of malaria**

7 Development of an *in vitro* T cell killing assay for the liver-stage of malaria

7.1 Introduction

One reason why there is currently no licensed malaria vaccine is the lack of known correlates of protection for use as surrogate measures of efficacy rather than large, expensive and time-consuming field efficacy trials. For blood-stage malaria, the best assay to pre-clinically assess the functionality of vaccine-induced antibodies is the growth inhibition assay (GIA). Whilst a true association between *in vitro* and *in vivo* results has not been proven (reviewed in [539]), it does provide a useful assay for screening and down-selecting candidate vaccines pre-clinically. The GIA was essentially made possible by the development of continuous culture systems of erythrocytic parasites; liver-stage parasites have proved much more difficult to culture and therefore no equivalent assay currently exists for assessment of cellular immunity against the liver-stage.

Rodent *Plasmodium* sporozoites were first successfully cultured in 1979 in embryonic rat fibroblasts [540, 541], followed quickly by culture in the human embryonic lung cell line WI38 [542]. Culture in the liver parenchyma, the natural tissue host, from rodent species was first demonstrated in primary hepatocytes from *Thamnomys gazellae* by *P. yoelii* [543] and laboratory rats by both *P. yoelii* [544] and *P. berghei* [545]. *P. berghei* sporozoites were then found to complete a full developmental cycle in the human hepatoma cell line HepG2 [546], with greater rates of infection than seen in other cell types. It took longer for successful infections to be demonstrated in primary murine hepatocytes [547], because of the increased

difficulty in culturing these cells; in fact, *P. falciparum* was successfully cultured in primary human hepatocytes [548] before rodent species were cultured in murine hepatocytes. The laboratory of Professor Dominique Mazier remains the expert in culturing *P. falciparum in vitro*, yet infection of primary murine hepatocytes remains challenging despite the increased availability of specialized culture reagents and protocol optimization [375]. There are now additional cell lines which support *in vitro* parasite infection: Hepa1-6 and Bnl-1Me A.7R.1 (ATCC TIB-75) are both murine hepatoma cell lines that support *P. berghei* and *P. yoelii* development [549]; a number of human hepatoma cell lines, including Huh7, also support murine species [550]; whilst HC-04 and HHS-102 are both human hepatoma cell lines that support the full cycle of *P. falciparum* development [551, 552], albeit with very low infectivity.

Unfortunately, liver-stage parasites only undergo one round of replication in one hepatocyte, so unlike blood-stage cultures they cannot be maintained continuously. Furthermore, *Plasmodium* species exhibit very low efficiency of invasion and maturation in *in vitro* cell culture systems. Dependent on the species and host cell combination, infectivity can range from approximately 0.1% when using *P. falciparum* or rodent species in primary hepatocytes [547, 548], to 10-20% when using *P. berghei* in HepG2 [546, 553]. For this reason an extremely high number of sporozoites are required for each assay well (usually 20 000 to 50 000), placing a further impediment to study the liver-stage of malaria in culture: institutions are not only required to have the expertise in isolating primary hepatocytes or infecting cell lines, but are also required to have functional insectaries (at containment level 3 for *P. falciparum*) and the ability to produce high yields of sporozoites. Despite these

difficulties, *in vitro* cultures of rodent and human *Plasmodium* parasites have been used relatively frequently over the last 20 years for studying liver-stage biology [439, 554-557], for drug-screening assays [558-561] and to measure the inhibition of antibodies on sporozoite invasion and development [562-567].

Assays to measure the inhibition of sporozoite invasion by antibodies are known as ISI, and those that measure the inhibition of liver-stage development as ILSDA. The methodology of each is similar: sporozoites are pre-incubated with test serum (containing antibodies) then added to the cultured hepatocytes and incubated. For the ISI, cells are fixed and stained approximately three hours post-infection, whilst for the ILSDA parasites are left to develop in the hepatocytes for two to six days depending on the species, with infection measured by fluorescent microscopy. The limitations of these assays include the significant inhibition from controls (naïve sera), reported as up to 30-50% [568], in addition to the loss of cells during fixation and staining. A similar assay to the ILSDA should be possible for measurement of cell-mediated inhibition, however one of the main difficulties is MHC matching between the hepatocytes and the effector T cells.

Efforts were made by two groups in the early 1990s to develop such an *in vitro* T cell killing assay [55, 165-167], and have been continued more recently by a third group [168]. The methodology was similar between all groups: hepatocytes were cultured overnight prior to infection with rodent *Plasmodium* species. This was followed by the addition of cells from vaccinated mice at various concentrations and at various times post-infection depending on the laboratory. Cultures were left for 24-48 hours followed by fixation and staining as for the ISI/ILSDA. Hoffman *et al.*, used total

splenocytes from mice vaccinated with irradiated sporozoites [165], while Weiss *et al.*, used splenocytes from mice vaccinated with irradiated sporozoites that had been pre-cultured with PyCSP peptide and IL-2, to determine whether T cells directed against the PyCSP peptide could specifically inhibit liver-stage parasites [55]. Renia *et al.*, utilized both cells from lymph nodes taken from mice vaccinated with a PyCSP synthetic peptide [167] and a CD4⁺ T cell clone generated from the same vaccination regimen [166]. Trimnell *et al.*, more recently assessed the inhibitory activity of CD8⁺ enriched cells from both the spleens and livers of mice immunized with *P. yoelii* genetically attenuated parasites (PyUIS4-/-) [168]. All studies were able to detect inhibition of liver-stage parasites by fixation, staining and manual counting. Like the ISI/ILSDA, a high level of inhibition was detected in some cases by cells from naïve mice [167].

Recent technological advances make it worth re-visiting such *in vitro* T cell killing assays. First, rodent malaria parasites expressing the markers GFP [72, 381, 569-571], mCherry [572] or luciferase [71, 383, 573] have been developed, removing the need for fixation and staining, and potentially the need for counting by microscopy. Recent studies have measured infection of HepG2 or Huh7 hepatoma cells infected with *P. berghei* GFP by flow cytometry [569, 574], providing a less subjective method that is more suitable for medium-throughput studies, and could be used in an *in vitro* T cell killing assay. Secondly, the development of vaccination regimens inducing high levels of CD8⁺T cells provides a new opportunity to test such an assay. Furthermore, an *in vitro* T cell killing assay has apparently never been attempted using human hepatocytes and *P. falciparum*. The combined expertise of developing CD8⁺ inducing vaccines at Oxford and the culture and infection of human

hepatocytes with *P. falciparum* at Prof. Mazier's lab in Paris created the perfect opportunity to develop such an assay.

The series of experiments described in this chapter were aimed to first re-visit the *in vitro* T cell killing assay conducted with a murine hepatoma cell line, utilizing fluorescent *P. berghei* parasites and alternate methods of measuring infectivity. The antigen PbTRAP was chosen as a model given the high number of antigen-specific CD8⁺ T cells that can be induced in mice by viral vectored vaccination (Karolis Bauza, DPhil Thesis). A secondary aim was to use such an *in vitro* T cell killing assay to explore the mechanism of inhibition induced by pre-erythrocytic vaccines. The final aim was to transfer the *in vitro* model from mice to humans, creating a novel *in vitro* T cell killing assay against *P. falciparum* infected human hepatocytes. Combining such an assay with data collected using *in vivo* challenges with transgenic parasites in mice should reliably identify strong candidate antigens for pre-erythrocytic vaccines.

7.2 Results

7.2.1 Development of the infrastructure for an *in vitro* T cell killing assay

To develop an *in vitro* T cell killing assay for the liver-stage of malaria, the best source of liver cells, the colour of fluorescence and the method of detection of infection were first investigated. Three hepatoma cell lines were obtained: two human, HC-04 (MR4, BEI Resources Depository, NIAID, NIH) and HepG2 (European Collection of Cell Cultures (ECACC), Public Health England), and one mouse, Hepa1-6 (ECACC). The HC-04 and HepG2 cell lines were confirmed to be HLA-A2 positive by HLA typing on extracted DNA (HLA typing performed by the

John Radcliffe Hospital, full typing: HLA-A2/24, B-51/35, Cw-16/4, DR-13/6 and 51/52, DQ-7/6). The Hepa1-6 cell line was derived from a C57L mouse (H-2^b, as per C57BL/6), and the H-2 type confirmed by both PCR and flow cytometry (data not shown). A protocol for obtaining and culturing primary murine hepatocytes was also assessed, however this was never sufficiently optimized to ensure consistent numbers or quality of primary hepatocytes for use in the killing assay. A 96-well plate format was chosen to reduce the required number of hepatoma cells, sporozoites and T cells, with the addition of 5×10^4 hepatoma cells per well resulting in the optimal density after overnight incubation (data not shown).

After establishing the cell lines, methods for determining the infectivity of the cells were assessed. Although wild-type and fluorescent *P. berghei* parasites were readily available in our group (both GFP and mCherry), use of fluorescent parasites was considered to be a more direct and reliable measure of visualization than staining of parasites. To determine whether to use GFP or mCherry parasites, and to analyse the utility of measurements not reliant on manual counting of parasites, a simple assay of infecting the hepatoma cell lines with fluorescent viruses was performed. Hepatoma cells were infected with serial dilutions of AdHu5-GFP or AdHu5-mCherry, and both were visualized by fluorescent microscopy (Table 7.1). The 96-well plates were then measured for fluorescence using the BMG FLUOStar Optima microplate reader. This machine could sufficiently detect the serial dilutions of AdHu5-mCherry, but not of AdHu5-GFP, potentially as the media contains a pH indicator that can increase background fluorescence in the GFP channel. Alternatively, the cells were harvested from the plates and analysed on a flow cytometer; this method allowed clear detection of AdHu5-GFP but not of AdHu5-mCherry (Figure 7.1), due to the dichromatic filter

combination of the flow-cytometer. Given the production of *P. berghei* GFP parasites was better established in our laboratory, it was decided to use this parasite with measurement by flow cytometry.

Table 7.1. Assessment of the best method for measurement of green (GFP) or red (mCherry) fluorescence.

To assess the best method to measure fluorescence, Hepa1-6 or HepG2 cells were infected with serial dilutions of the virus AdHu5 – GFP or AdHu5 – mCherry. Infectivity was assessed by fluorescent microscopy, for which both GFP and mCherry could be detected. To avoid manual counting and the associated reader error, two alternative options were assessed: the BMG FLUOStar Optima, a microplate reader capable of measuring fluorescence over time, and standard flow cytometry. This table represents the results of these experiments.

Fluorescence	Microscopy	BMG FLUOStar	Flow Cytometry
GFP	Yes	Not sufficient	Yes
mCherry	Yes	Yes	Not sufficient

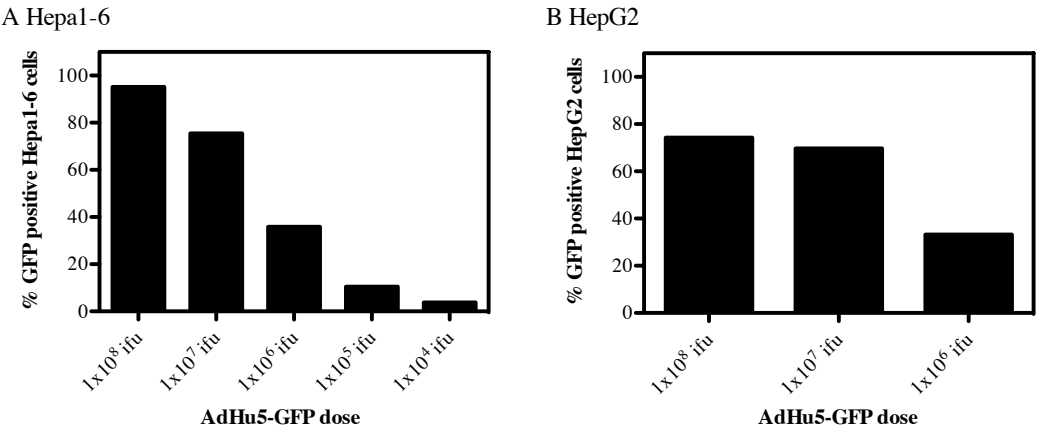


Figure 7.1. Infection of the hepatoma cell lines Hepa1-6 and HepG2 with serial dilutions of AdHu5-GFP.

5x10⁴ (A) Hepa1-6 or (B) HepG2 cells were seeded in a 96-well plate. The following day the cells were infected with serial dilutions of AdHu5-GFP. 48 hours after infection, the cells were removed and run on the flow cytometer; the percentage of GFP positive cells was calculated. Single assay wells were performed. Experiments were also performed using the AdHu5-mCherry virus, however red fluorescence was not detected by the filter combination present on the flow cytometer.

As the infectivity of hepatoma cell lines by *Plasmodium* species is very low, whether the flow cytometry method was sensitive enough to detect changes in infectivity of overall low values was then assessed. The cell lines were infected with the lowest concentration of virus, 1×10^4 ifu, and approximately three hours post-infection splenocytes were added from mice vaccinated with AdHu5-GFP. Despite low infectivity (median 0.59% in Hepa1-6 and 0.295% in HepG2) a statistical difference could be observed following the addition of antigen-specific splenocytes for both Hepa1-6 and HepG2 ($p=0.009$ and $p=0.0023$, respectively, Mann Whitney test) (Figure 7.2).

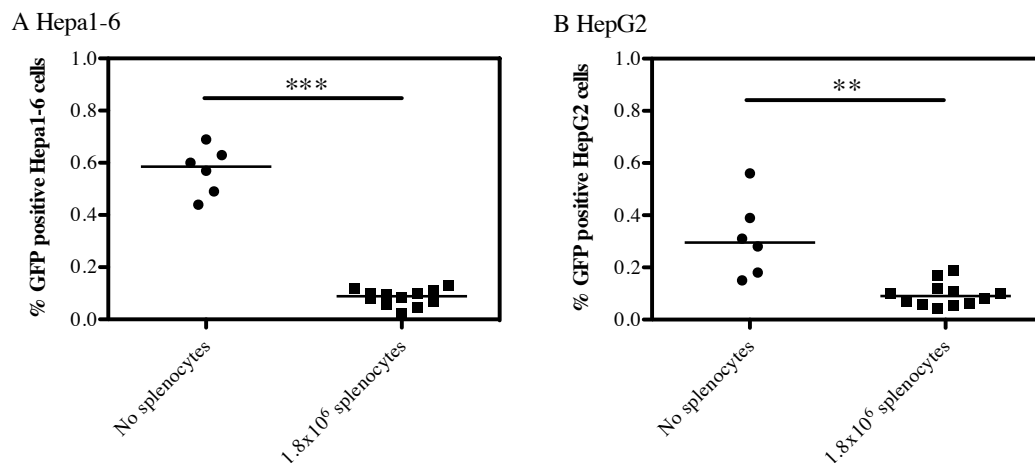


Figure 7.2. Effect on AdHu5-GFP infected Hepa1-6 and HepG2 cells by addition of GFP-specific splenocytes.

HHD mice ($n=2$) were vaccinated i.m. with 5×10^8 ifu AdHu5-GFP. One month later, spleens were harvested and splenocytes isolated. 5×10^4 (A) Hepa1-6 or (B) HepG2 cells were seeded per well of a 96-well plate. 24 hours later the hepatoma cells were infected with 1×10^4 ifu AdHu5-GFP. Approximately three hours post-infection, 1.8×10^6 splenocytes from the AdHu5-GFP vaccinated mice were added to the virus infected hepatoma cells ($n=6$ wells without splenocytes, $n=12$ wells with splenocytes). 48 hours after addition of splenocytes the percentage of virus infected hepatoma cells was calculated by flow cytometry. Both medians and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, ** $p=0.01-0.001$ *** $p<0.001$.

7.2.2 Infection of the hepatoma cell lines Hepa1-6 and HepG2 with *P. berghei* GFP

After establishment of the hepatoma cell lines and proof of concept studies using AdHu5-GFP, whether *P. berghei* GFP sporozoites could infect the cell lines and be detected by microscopy and flow cytometry was then assessed. 24 hours after infection, green liver-stage parasites within Hepa1-6 (Figure 7.3) and HepG2 cells (data not shown) were visible by microscopy, demonstrating the ability of *P. berghei* GFP to infect both hepatoma cell lines.

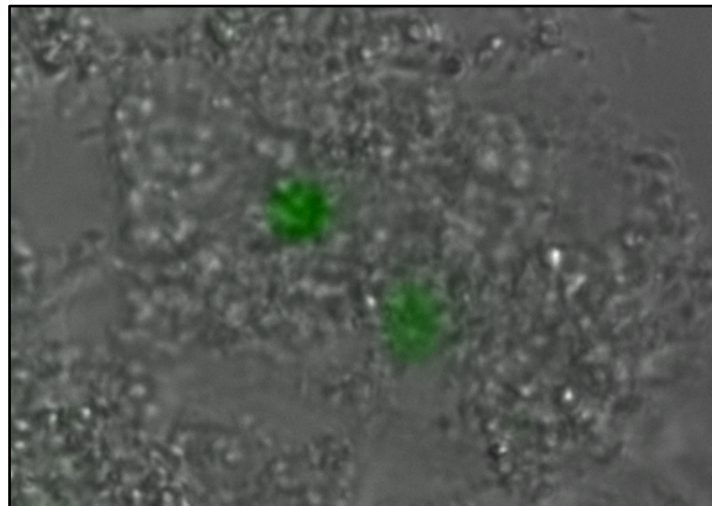


Figure 7.3. *P. berghei* GFP infection in the hepatoma cell line Hepa1-6.

5×10^4 Hepa1-6 cells were seeded per well of a 96-well plate. 24 hours post-infection, media was removed and 40 000 *P. berghei* GFP sporozoites were added per well. Plates were centrifuged at 500xg for five minutes then incubated for a further 24 hours. Image was taken on a Leica DMI3000 B microscope at 40x and is a composite image of the bright field and fluorescent photos.

GFP⁺ hepatoma cells were also detectable by flow cytometry, using the gating strategy outlined in Figure 7.4. To enable detection of the hepatoma cells as a pure population, cells were first labelled with a fluorescent membrane dye, Vibrant® DiD, prior to seeding. A viability marker (DAPI) was also added to each sample prior to running on the flow cytometer.

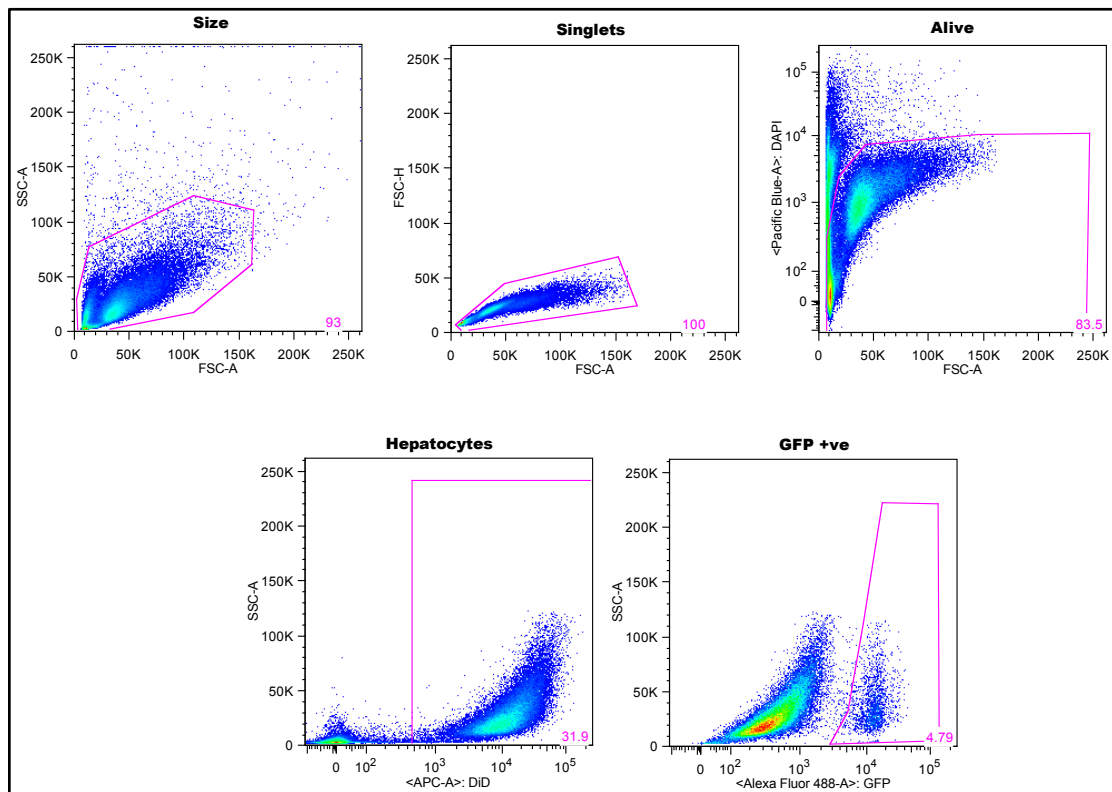


Figure 7.4. An example of the gating strategy used to calculate the percentage of *P. berghei* GFP infected hepatoma cells.

After acquisition on a LSRII flow cytometer, analysis was performed in FlowJo. Cells were first gated by size, followed by single cells. The viable cells were then selected based on the absence of DAPI staining. Hepatoma cells were labelled with the Vybrant® DiD membrane dye prior to seeding, thus enabling identification with DiD fluorescence detected in the same dichromatic filter as APC. Finally, the hepatoma cells that were GFP positive were selected. Uninfected hepatoma cells were run as a control to guide gating of the population of GFP⁺ cells.

To ensure a reliable and detectable number of cells were infected in each experiment, 40 000 sporozoites per well of Hepa1-6 cells and 10 000 sporozoites per well of HepG2 were chosen for all subsequent studies (Figure 7.5).

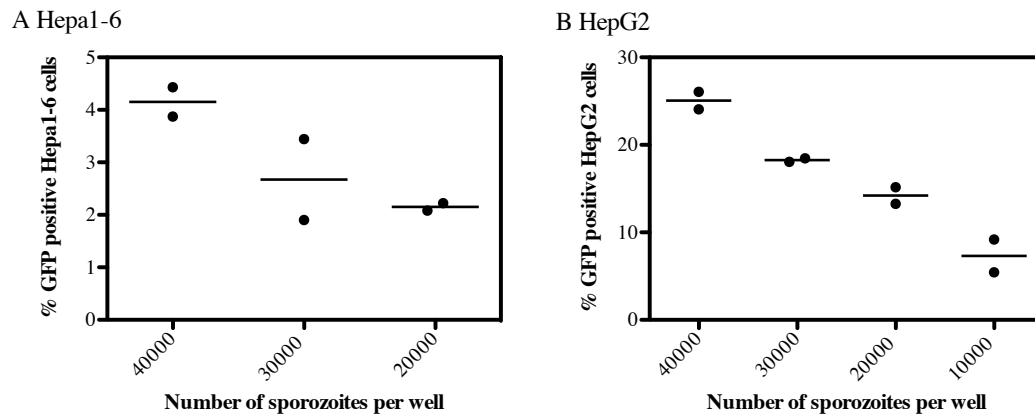


Figure 7.5. Infection of Hepa1-6 and HepG2 cells with dilutions of *P. berghei* GFP sporozoites.

5×10^4 (A) Hepa1-6 or (B) HepG2 cells were seeded in a 96-well plate, after labelling with the membrane dye Vybrant® DiD. After 24 hours incubation, various numbers of *P. berghei* GFP sporozoites were added per well in duplicate, followed by centrifugation of the infected cells. 24 hours post-infection, cells were harvested and run on a flow cytometer. Results are expressed as percentage of GFP positive viable hepatoma cells.

Centrifugation of the 96-well plates immediately after addition of sporozoites to the hepatoma cells was shown to significantly increase the infectivity from a median of 0.56% to 2.76% ($p=0.0001$, Mann Whitney test) (Figure 7.6), and therefore all subsequent experiments included the centrifugation step. To ensure GFP expression was only detected from viable sporozoites, sporozoites were either heat-killed prior to infection or killed by the liver-stage acting drug, primaquine [553]. GFP⁺ cells were not observed in wells containing non-viable sporozoites (Figure 7.6), confirming that all GFP⁺ cells were hepatoma cells infected with a live parasite.

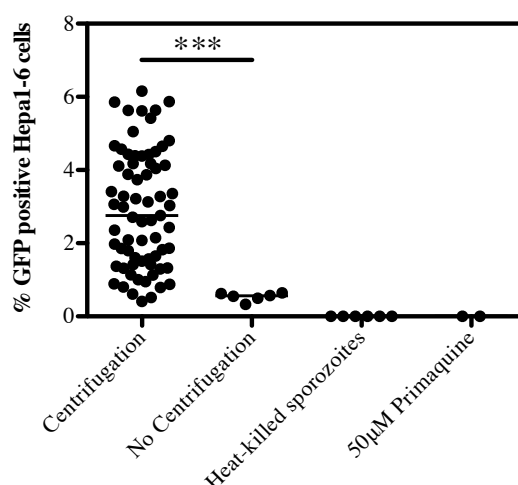


Figure 7.6. Infectivity of Hepa1-6 cells with 40 000 *P. berghei* sporozoites with or without centrifugation.

5×10^4 Hepa1-6 cells were seeded in a 96-well plate, after labelling with the membrane dye Vybrant® DiD. After 24 hours incubation, 40 000 *P. berghei* GFP sporozoites were added per well, either followed by centrifugation at 500xg for five minutes ($n=69$) or without centrifugation ($n=6$). To confirm that non-viable sporozoites no longer expressed GFP, sporozoites were either heat-killed for twenty minutes at 95°C prior to infection ($n=6$) or killed by the addition of 50µM primaquine three hours post-infection ($n=2$). 24 hours post-infection cells were harvested and run on a flow cytometer. Results are expressed as the percentage of GFP positive viable Hepa1-6 cells. The effect of centrifugation on infectivity was assessed using the Mann Whitney test, *** $p=0.0001$. Data was collected over multiple experiments.

7.2.3 The effect of cytokines and naïve T cells on *P. berghei* GFP infected Hepa1-6 cells

Once a protocol had been established to ensure reliable infection and measurement of hepatoma cell lines, the assay was further developed to determine the effect of adding either cytokines or naïve T cells as measured by percent inhibition, based on the percentage infectivity: (1-test well/average of control wells) x100. Control wells contained only sporozoites and Hepa1-6 cells. Addition of either recombinant IFN γ or TNF α three hours post-infection resulted in inhibition of liver-stage parasite development (Figure 7.7), with a trend towards a dose effect, albeit not statistically significant. For both cytokines, the maximum inhibition seen was approximately 40%.

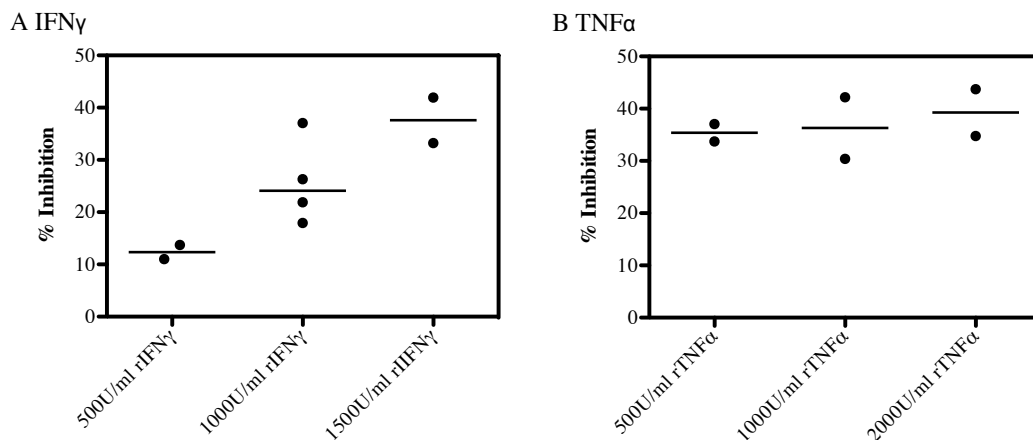


Figure 7.7. Effect of IFN γ and TNF α on *P. berghei* GFP infected Hepa1-6 cells.

After labelling with the membrane dye Vybrant® DiD, 5×10^4 Hepa1-6 cells were seeded in a 96-well plate. After 24 hours incubation, 40 000 *P. berghei* GFP sporozoites were added per well, followed three hours later by dilutions of recombinant mouse (A) IFN γ or (B) TNF α (n=2-4 per group). 24 hours post-infection cells were harvested and run on a flow cytometer; the percentage of GFP positive viable Hepa1-6 cells was calculated. Results are expressed as the % inhibition compared to wells containing no cytokines: 1- (test well/average of control wells) x100. Both median and individual data points are shown. Statistical difference was assessed using the Kruskal-Wallis test with Dunn's Multiple Comparison post-test; no differences were found (IFN γ p=0.077 and TNF α p=0.8).

To assess whether the addition of naïve T cells had any effect on *P. berghei* GFP infected Hepa1-6 cells, an increasing number of splenocytes from naïve (unvaccinated) mice were added to wells three hours after addition of *P. berghei* GFP sporozoites. Addition of splenocytes from naïve mice resulted in a high level of background inhibition, from just below 50% when 1×10^6 to 2×10^6 cells were added to 100% when over 1×10^7 cells were added (Figure 7.8). Whilst there were only two replicates per test, the results indicated that no more than 2×10^6 total splenocytes per well should be added. As the background inhibition was so high, it was decided that CD8⁺ cells should be enriched before addition to the assay and that test wells should be compared to wells containing splenocytes from vector control vaccinated mice, rather than sporozoite only wells.

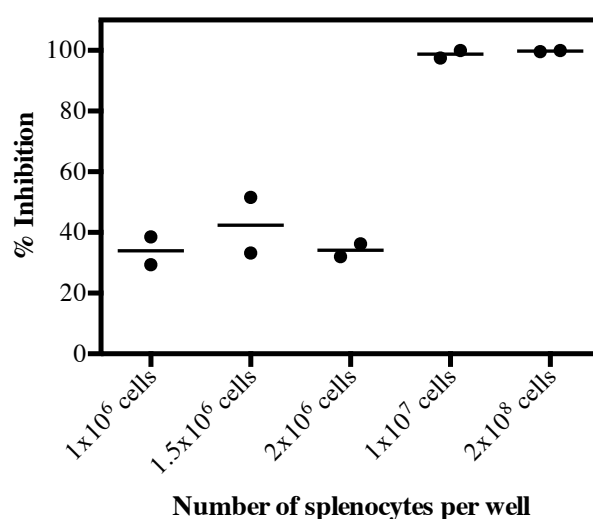


Figure 7.8. Effect of splenocytes from naïve mice on *P. berghei* GFP infected Hepa1-6 cells.

5×10^4 Vybrant® DiD labelled Hepa1-6 cells were seeded in a 96-well plate, with 40 000 *P. berghei* GFP sporozoites added per well 24 hours later, followed three hours later by dilutions of splenocytes from naïve mice (n=2 per group). 24 hours post-infection cells were harvested and run on a flow cytometer; the percentage of GFP positive viable Hepa1-6 cells was calculated. Results are expressed as the % inhibition compared to wells containing no T cells. Both median and individual data points are shown. Statistical difference was assessed using the Kruskal-Wallis test with Dunn's Multiple Comparison post-test; no differences were found (p=0.14).

CD4⁺ T cells could also act directly as effector cells in liver-stage infection if sporozoite infected hepatocytes have the ability to upregulate MHC Class II. In *P. berghei* GFP infected Hepa1-6 cells, no upregulation of MHC Class II was found compared to uninfected Hepa1-6 cells (Figure 7.9). Analysis was performed on all Hepa1-6 cells, not only those infected, due to the limited number of events if only infected cells were assessed.

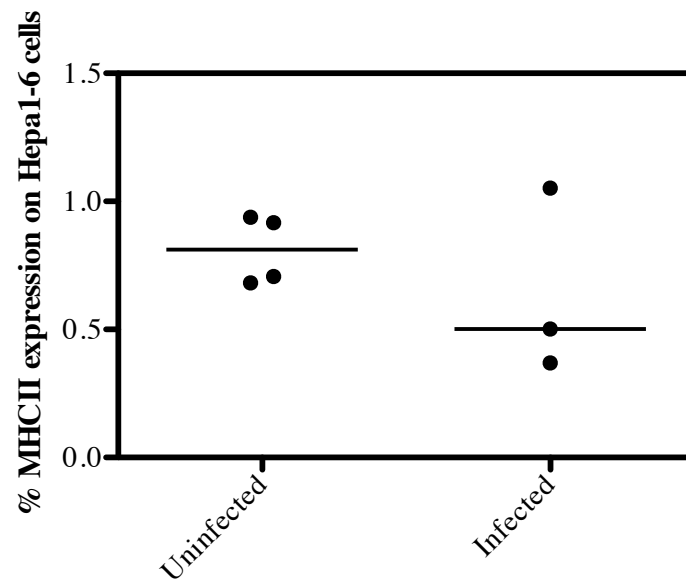


Figure 7.9. Effect of *P. berghei* GFP infection of Hepa1-6 cells on MHC Class II expression.

5x10⁴ Vybrant® DiD labelled Hepa1-6 cells were seeded in a 96-well plate, followed 24 hours later by addition of 40 000 *P. berghei* GFP sporozoites (n=4 uninfected and 3 infected wells). Another 24 hours later cells were harvested, stained with 1/50 anti-MHC II-PE and run on a flow cytometer. Results are expressed as the percentage of MHC Class II positive Hepa1-6 cells. Both median and individual data points are shown. The Mann Whitney test was used to assess statistical significance; no differences were found (p=0.63).

7.2.4 Assessment of inhibition of *P. berghei* GFP infected Hepa1-6 cells by *P. berghei* TRAP-specific splenocytes

Following establishment of the optimal conditions for the *in vitro* T cell killing assay, the system was then used to assess the capacity of PbTRAP vaccinated cells to kill *P. berghei* GFP infected Hepa1-6 cells. Mice were vaccinated in the standard ChAd63-MVA prime-boost regimen and splenocytes harvested the day after seeding of the Vybrant® DiD labelled Hepa1-6 cells. Vaccines were kindly provided by Dr. Karolis Bauza. Three hours after sporozoite addition enriched CD8⁺ splenocytes from vaccinated mice were added; after 24 hours incubation Hepa1-6 cells were harvested and run on a flow cytometer (Figure 7.10).

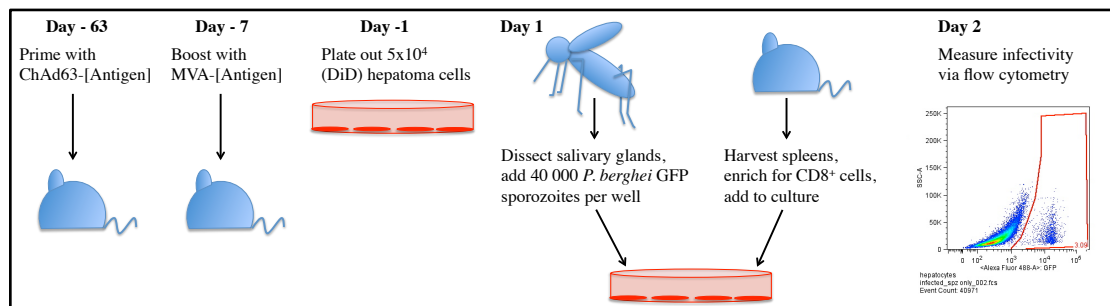


Figure 7.10 A schematic representation of the murine *in vitro* T cell killing assay.

C57BL/6 mice were vaccinated with the standard ChAd63-MVA prime-boost regimen using vaccines expressing PbTRAP. Six days post-MVA boost, Hepa1-6 cells were labelled with the membrane dye Vybrant® DiD and seeded into a 96-well flat bottom plate. The following day salivary glands were dissected from *P. berghei* GFP infected mosquitoes and sporozoites isolated. 40 000 sporozoites were then added per well to the Hepa1-6 cell cultures, followed by centrifugation of plates at 500xg for five minutes. Subsequently, the vaccinated mice were sacrificed, spleens harvested and enriched for CD8⁺ cells via negative depletion using magnetic beads (MACS). The CD8⁺ enriched splenocytes were added to the sporozoite infected Hepa1-6 cell cultures approximately three hours post-infection. Splenocytes were also harvested from control-vaccinated mice and treated in the same way, to determine the effect of non-specific killing. Plates were then incubated for approximately 24 hours prior to harvesting and running cells on the flow cytometer to calculate the percentage infected, based on GFP expression.

Prior to CD8⁺ enrichment, a subset of cells was taken for restimulation and ICS to allow calculation of the antigen-specific effector to target cell ratio. PbTRAP was chosen as the model antigen given the extremely high numbers of antigen-specific cells generated in C57BL/6 mice following prime-boost vaccination (Karolis Bauza, DPhil Thesis). To ensure MHC matching, the Hepa1-6 cell line, which expresses H-2^b, was used in experiments with splenocytes from C57BL/6 mice (H-2^b). Across all experiments performed using PbTRAP for the *in vitro* T cell killing assay, the median CD8⁺ IFN γ ⁺ response was 20.22%, CD8⁺ TNF α ⁺ 15.43% and CD8⁺ CD107a⁺ 20.10% (Figure 7.11). PbTRAP peptide sequences are provided in Appendix Two.

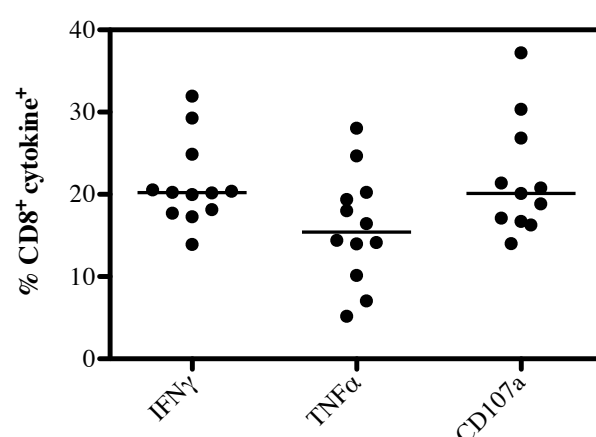


Figure 7.11. Cellular immunogenicity induced by ChAd63-MVA PbTRAP vaccination in C57BL/6 mice.

Cellular immunogenicity from mice used in the *in vitro* T cell killing assays. Each data point represents two sacrificed mice, with splenocytes pooled together prior to the assay or ICS. Twelve sets of mice were culled, resulting in thirteen *in vitro* assays being performed (one set of mice provided for two experiments). C57BL/6 mice were vaccinated i.m. with 1×10^8 ifu ChAd63-PbTRAP followed eight weeks later by 1×10^6 pfu MVA-PbTRAP. Mice were sacrificed one-week post-boost and cellular immunogenicity assessed in the spleen by ICS, after stimulation for six hours with a pool of overlapping peptides covering the entire PbTRAP sequence (60 peptides in total). Results are expressed as the percentage of CD8⁺ cells expressing the listed cytokines. Both individual data points and medians are shown.

Thirteen *in vitro* T cell killing assays were performed in total and inhibition of parasite infected Hepa1-6 cells was achieved (Figure 7.12). The yield of sporozoites obtained from infected mosquitoes (1000 – 10 000 per mosquito) was a limiting factor in performing these experiments. In each experiment no more than 2×10^6 total splenocytes were added, and in each case these splenocytes had been enriched for CD8⁺ cells by negative selection (approximately 70% pure, described in Chapter 2). Each experiment resulted in a different effector-to-target (E:T) ratio which appeared to correlate with the percentage inhibition achieved by the T cells (Figure 7.12 A). Four experiments did not fit the pattern observed, highlighted in blue. From observations while performing the assays, the infectivity of control wells containing only sporozoites and Hepa1-6 cells was compared between the ‘fit’ experiments and the ‘outliers’. A significant difference was found in the level of infectivity, with the percentage of *P. berghei* GFP infected cells at a median of 3.51% for the fit experiments and only 1.39% for the outlier experiments ($p < 0.0001$, Mann Whitney test) (Figure 7.12 B). If the outlier experiments were excluded, a significant correlation was found between the percentage inhibition and the E:T ratio (Spearman $r = 0.8167$, $p = 0.0108$) (Figure 7.12 C). There was also a non-significant trend towards a correlation between the percentage inhibition and the E:T ratio when analyzing only the four outlier experiments (Spearman $r = 0.8$, $p = 0.33$) (Figure 7.12 D).

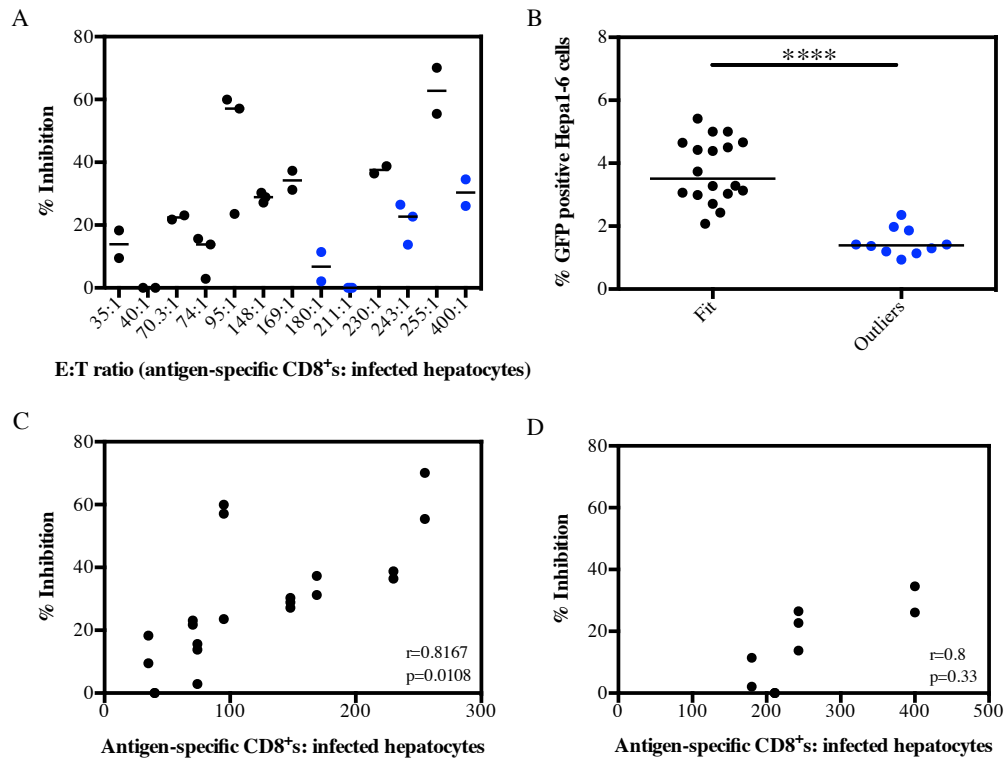


Figure 7.12. CD8⁺ enriched splenocytes from PbTRAP vaccinated mice inhibit *P. berghei* GFP infected Hepa1-6 cells in a dose dependent manner.

5x10⁴ Vybrant® DiD labelled Hepa1-6 cells were seeded in a 96-well plate, followed 24 hours later by addition of 40 000 *P. berghei* GFP sporozoites and a further three hours later by addition of CD8⁺ enriched splenocytes from PbTRAP vaccinated mice. 24 hours after addition of splenocytes, cells were harvested and run on a flow cytometer; the percentage of GFP positive viable Hepa1-6 cells was calculated. (A) Results are expressed as the % inhibition compared to control wells: 1-(test well/average of control wells) x100. Control wells contained the same number of total splenocytes from mice vaccinated with ChAd63-MVA luciferase. A total of thirteen independent experiments were performed with different effector (antigen-specific CD8⁺) to target (infected hepatocytes) ratios. Both the median and individual data points are shown. The number of effector cells was back calculated based on the total number of splenocytes added, the percentage of those that were CD8⁺ and the percentage of the CD8⁺ cells that were antigen-specific (based on the ICS results). Target cells were based on control wells containing only sporozoites. An E:T ratio dependent pattern emerged; those experiments that fit this pattern are coded black, those that appear different are coded blue. (B) The infectivity of wells containing only Hepa1-6 cells and sporozoites; ‘fit’ refers to the experiments from graph A represented by black dots, ‘outliers’ refers to the experiments from graph A represented by blue dots. Both median and individual data points are shown. Statistical difference was assessed using the Mann Whitney test, **** p<0.0001. (C) Correlation of the E:T ratio with the percentage inhibition was calculated, excluding the outliers (n=9 experiments). Spearman r=0.8167, p=0.0108. (D) Correlation of the E:T ratio with the percentage inhibition was calculated for the experiments identified as outliers (n=4 experiments). Spearman r=0.8, p=0.33.

In an effort to elucidate the mechanism of T cell-mediated inhibition, *in vitro* T cell killing experiments were performed to assess the effect of blocking cytotoxic, IFN γ or TNF α -mediated killing (Figure 7.13). To inhibit the action of perforin-mediated cytotoxicity, PbTRAP CD8⁺ enriched splenocytes were pre-incubated with 10nM concanamycin A (conA), an inhibitor of perforin-based cytotoxicity by increasing the pH of lytic granules [575]. The percentage inhibition of parasite infected Hepa1-6 cells was not statistically different between PbTRAP cells and those treated with 10nM conA, when the results of three independent experiments were combined (Figure 7.13 A). One experiment was performed assessing the effect of inhibiting TNF α -mediated killing by addition of an anti-TNF α blocking antibody; no differences were observed (Figure 7.13 B). One experiment was performed assessing the effect of IFN γ -mediated killing by addition of an anti-IFN γ blocking antibody. Whilst the sample size was too small to assess statistical significance, there was a trend towards reduced inhibition when the action of IFN γ was blocked (Figure 7.13 C). Since only a small number of hepatoma cells were infected and thus presenting antigens to CD8⁺ T cells, it is plausible that the limited inhibition observed may be due to inefficient antigen presentation. PbTRAP peptides were therefore added to wells to increase antigen presentation, however, no differences between PbTRAP cells with peptide and those without were observed (Figure 7.13 D), suggesting that sufficient antigen presentation was already occurring.

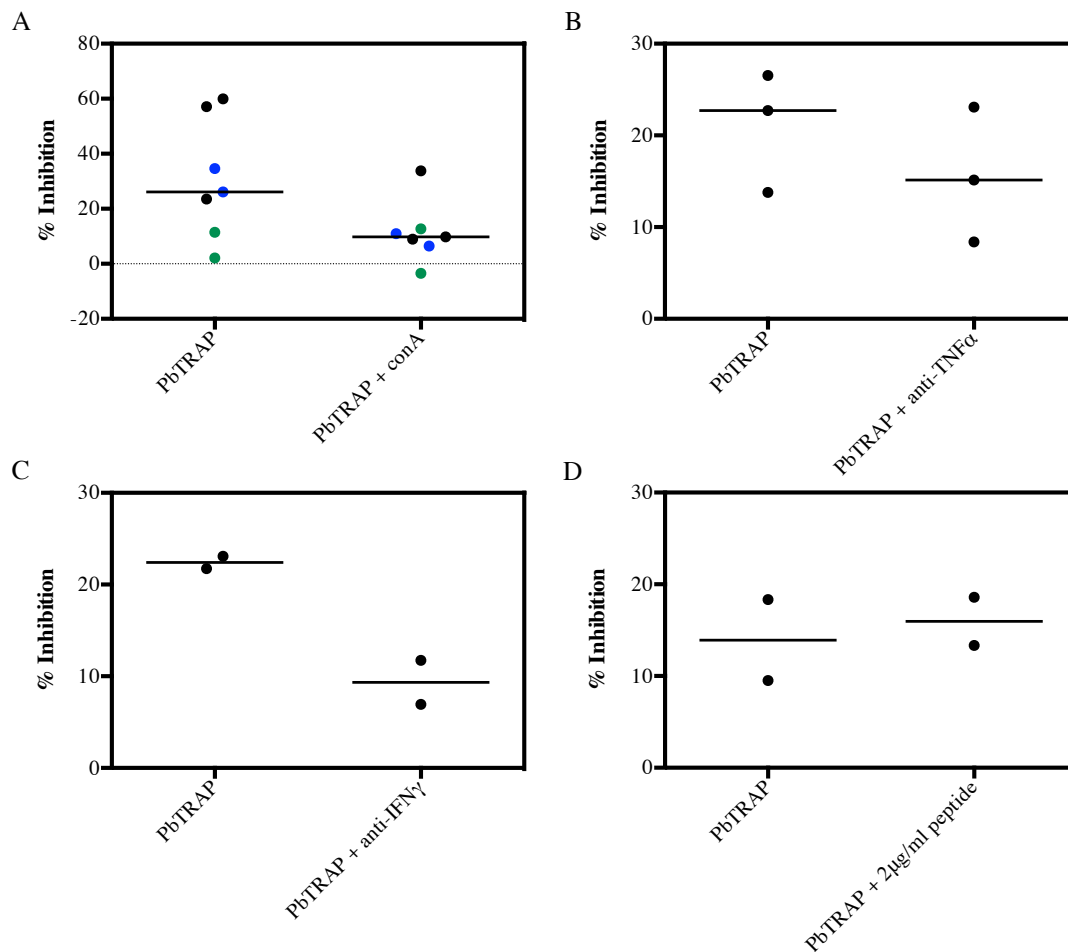


Figure 7.13. Assessment of the mechanism of inhibition observed by PbTRAP-specific CD8⁺ enriched splenocytes on *P. berghei* GFP infected Hepa1-6 cells.

The *in vitro* T cell killing assay was performed as previously described, with the following additions: (A) CD8⁺ enriched splenocytes were pre-incubated with 10nM conA for twenty minutes prior to addition into the infected Hepa1-6 cells. Three independent experiments were performed; results from the same experiment are colour-coded. No statistical difference was found when the three experiments were combined (n=6 per group), as assessed using the Mann Whitney test (p=0.07). (B) CD8⁺ enriched splenocytes were resuspended in 250ng/ml anti-TNFα prior to addition into the infected Hepa1-6 cells (n=3 per group). One experiment was performed. (C) CD8⁺ enriched splenocytes were resuspended in 100μg/ml anti-IFNγ prior to addition into the infected Hepa1-6 cells (n=2 per group). One experiment was performed. (D) CD8⁺ enriched splenocytes were resuspended in 2μg/ml of a PbTRAP pool of overlapping peptides prior to addition into the infected Hepa1-6 cells (n=2 per group). One experiment was performed. Results for each experiment are expressed as the percentage inhibition as compared to splenocytes isolated from mice vaccinated with ChAd63-MVA luciferase; splenocytes were treated in the exact manner as per the PbTRAP-specific cells. Both medians and individual data points are shown.

7.2.5 Development of a human *in vitro* T cell killing assay using *P. falciparum*, primary human hepatocytes and T cells from volunteers vaccinated with ME-TRAP

As the *in vitro* T cell killing assay had been successfully developed using the model antigen PbTRAP in a murine system, the second aim was to transfer this assay into a human system using *P. falciparum* infected human hepatocytes and T cells from volunteers vaccinated with ME-TRAP. PBMCs from ME-TRAP vaccinated volunteers were readily available in Oxford from the Mal34 CHMI trial discussed in Chapter 6. The HC-04 cell line had been obtained and passaged as a source of human hepatocytes, however, during this thesis MR4 revealed that the line they had no longer supported full development of *P. falciparum*, making this option unavailable. Therefore, the first attempt of this assay in Oxford was performed using frozen primary human hepatocytes purchased from ZenBio Incorporated and *P. falciparum* infected mosquitoes purchased from WRAIR. HLA-A3 and HLA-A2 hepatocytes were chosen as they represent common HLA types within the European population [476] and were common among ME-TRAP vaccinees. The hepatocytes were successfully thawed, plated and cultured, however, upon addition of *P. falciparum* sporozoites dissected from salivary glands of infected mosquitoes, fungal contamination quickly developed. It was clear that further methods of decontamination of mosquitoes were required when using *P. falciparum* compared to *P. berghei*, possibly because the mosquitoes were raised in another facility, or because primary hepatocytes are more sensitive to contamination. Prof. Mazier's group in Paris have established themselves as experts in culturing primary human hepatocytes and infections of these cells with *P. falciparum* [548]; for this reason, this section of this DPhil was undertaken in collaboration with Prof. Mazier's laboratory

in Paris, with work performed jointly between Rhea Longley and post-doctoral fellow Dr. Soulard. Research technicians of Prof. Mazier's laboratory isolated the primary hepatocytes.

7.2.5.1 Screening of ME-TRAP vaccinated volunteers in Oxford

To perform the *in vitro* assay experiments in Paris, frozen PBMCs were required to be the source of antigen-specific T cells. Based on the results achieved in Chapter 6, re-stimulation *in vitro* with *P. falciparum* TRAP peptides, as for the cultured IFN γ ELISpot assay, was considered the best method to generate high numbers of TRAP-specific cells. HLA-A2 and HLA-A3 frozen PBMCs from two trials assessing ME-TRAP (Mal34 and Vac33) were screened for the ability of the cells to induce good memory responses as measured by the cultured IFN γ ELISpot assay (Table 7.2). Volunteers from Mal34 had been challenged with *P. falciparum* 3D7 malaria via mosquito bite; whether or not the volunteer was protected from this challenge is also detailed (Table 7.2). To perform the initial experiments in Paris, a HLA-A3 and a HLA-A2 volunteer were chosen based on their cultured IFN γ ELISpot response. Volunteer 09 (HLA-A3) had a strong response of approximately 1000 SFC per million PBMCs and was sterilely protected from challenge with malaria, thereby acting as a positive control. Volunteer 19 (HLA-A2) was also chosen as despite no protection from challenge with malaria, an extremely good cultured IFN γ ELISpot response was recorded (median 2800 SFC per million PBMCs). It was expected that this volunteer might have the ability to inhibit a percentage of the liver-stage parasites, whilst not being able to clear the infection entirely.

Table 7.2. Screening of 22 volunteers vaccinated with ME-TRAP for high cultured IFN γ ELISpot responses.

Volunteers were vaccinated with ChAd63-ME-TRAP and the majority boosted with MVA-ME-TRAP as described in Chapter 2. Frozen PBMCs from various time-points (at least nine weeks after the first vaccination) were thawed and stimulated for ten days with a pool of overlapping peptides to *P. falciparum* T9/96 TRAP and recombinant human IL-2. The stimulated cells were then used in an IFN γ ELISpot assay with a pool of overlapping peptides to *P. falciparum* T9/96 TRAP, as well as stimulation with six separate pools of seven to ten peptides. The background response of media only wells was subtracted from the positive response. Samples were tested in quadruplicate. The cultured IFN γ ELISpot response is given in SFC per original million PBMCs, with the highest median response recorded to either the total pool or one of the six minor pools. In the clinical trial Mal34, volunteers were challenged with *P. falciparum* (3D7) after vaccination with ME-TRAP; whether they were protected or not from this challenge is also indicated in this table.

Trial	Volunteer Number	HLA type	Cultured ELISpot	Challenge
Mal34	7	A32 A3	1000	Delay
Mal34	9	A29 A3	1000	Sterile protection
Mal34	12	A23 A3	500	Sterile protection
Mal34	13	A29 A2	1000	No efficacy
Mal34	15	A2	Less than 200	No efficacy
Mal34	17	A2 A3	Less than 200	No efficacy
Mal34	19	A24 A2	2800	No efficacy
Mal34	20	A2 A3	Less than 200	Delay
Mal34	21	A11 A2	400	No efficacy
Mal34	22	A2	500	No efficacy
Mal34	26	A24 A2	700	No efficacy
Mal34	32	A11 A2	500	No efficacy
Mal34	36	A2	500	No efficacy
Mal34	39	A31 A2	800	No efficacy
Mal34	41	A31 A2	350	No efficacy
Mal34	45	A24 A2	250	No efficacy
Mal34	49	A24 A3	600	Sterile protection
Mal34	52	A31 A3	Less than 200	No efficacy
Mal34	64	A2 A3	400	No efficacy
Vac33	659	A1 A2	250	N/A
Vac33	668	A2 A24	1300	N/A
Vac33	670	A1 A2	1000	N/A

7.2.5.2 First experiment in Prof. Mazier's laboratory

Fresh primary human HLA-A3 hepatocytes were obtained and cultured at 8×10^4 cells per well in a 96-well plate. Five days later (once HLA typing was confirmed), frozen PBMCs from volunteer 09 (HLA-A3) were thawed and stimulated for ten days with a pool of overlapping peptides to *P. falciparum* T9/96 TRAP and human IL-2. PBMCs were used from C+7 and C+35 time-points and antigen-specific responses were confirmed by the IFN γ ELISpot assay (Figure 7.14 A in section 7.2.5.3 below). After the ten days of stimulation, 4×10^4 *P. falciparum* sporozoites were added per well to the cultured human hepatocytes, followed approximately six hours later by addition of PBMCs. *P. falciparum* NF54 infected mosquitoes were purchased from Radboud University, Nijmegen, the Netherlands, and aseptically dissected as described in Chapter 2. Prior to infection, the primary human hepatocytes were in poor condition with many dead cells visible. From experience, Dr. Soulard estimated that approximately 6×10^4 hepatocytes remained viable and total PBMCs were added based on this number of hepatocytes (infected or uninfected). Four ratios were tested, 1:1 (6×10^4 total PBMCs per well), 6.25:1 (3.75×10^5), 12.5:1 (7.5×10^5) and 25:1 (1.5×10^6). A fraction of stimulated PBMCs were also enriched for CD8 $^+$ cells using a negative depletion kit from Miltenyi Biotec. CD8 $^+$ cells were added at a ratio of approximately 8:1 (5×10^5 cells). Infected cultures with added PBMCs were maintained for only two days, given the fragile state of the hepatocytes. Cultures were then fixed and stained with anti-HSP70 immune serum, followed by the secondary antibody goat anti-mouse Alexa Fluor $^{\text{®}}$ 488. *P. falciparum* liver-stage cultures are normally maintained for five to six days, allowing the parasites time to fully develop. As the cultures were stopped much earlier than this the liver-stage parasites visualized were very small (4-5 μ m in diameter). A large number were identified as defective or abortive sporozoites, as

seen by their shape. Due to the greater difficulty in counting such parasites, plates were counted once by Rhea Longley and a second time by Dr. Soulard for verification (with the average count used).

PBMCs from volunteer 09, who received ChAd63-MVA ME-TRAP in the standard regimen, were able to inhibit *P. falciparum* infected human hepatocytes (Figure 7.14 B, in section 7.2.5.3 below). At a ratio of 25 total PBMCs to 1 hepatocyte, a median of 1.5 liver-stage parasites were seen compared to a median of 14 in the control wells ($p < 0.05$, Kruskal-Wallis test). The isolated CD8⁺ T cells at a ratio of 8:1 should have been similar to total PBMCs at a ratio of 16:1 (approximately 50% of total PBMCs were CD8⁺, as determined by flow cytometry staining). However, a ratio of 12.5:1 total PBMCs induced a greater level of inhibition than the CD8⁺s (Figure 7.14 B), potentially indicating a level of non-specific inhibition by total PBMCs. The variation seen in the control wells was quite high, with a range of 10 to 20.5 parasites seen.

7.2.5.3 Second experiment in Prof. Mazier's laboratory

To assess inhibition from PBMCs from volunteer 19 (HLA-A2), frozen primary human hepatocytes had to be used as no fresh HLA-A2 liver samples were received during the collaboration period. Volunteer 19 only received the ChAd63-ME-TRAP vaccine, with no MVA boost. First, the PBMCs from C-1, C+7 and C+35 time-points were thawed and cultured for ten days, as described for volunteer 09, with antigen-specific responses confirmed by the IFN γ ELISpot assay (Figure 7.14 A). At day 8, frozen HLA-A2 hepatocytes were thawed and cultured in a T25 flask, followed the next day by trypsinization and seeding in a 96-well plate. As for the HLA-A3 hepatocytes, these hepatocytes again appeared unhealthy, with many dead cells seen prior to infection. At day 10, the hepatocytes were infected with 4×10^4 *P. falciparum*

sporozoites per well and approximately six hours later the stimulated PBMCs were added at two ratios. Again, the number of cells added was based on an assumed viability of 6×10^4 hepatocytes per well. CD8⁺T cells were also isolated and added at a ratio of 8:1. Cultures were fixed and stained at two days post-infection, and counted as for the first experiment by Dr. Soulard. Whilst this volunteer was not sterilely protected from CHMI, in this *in vitro* assay the T cells were capable of inhibiting *P. falciparum* infected human hepatocytes (Figure 7.14 C). The wells containing CD8⁺ T cells induced the highest level of inhibition, with a median of eight liver-stage parasites identified compared to 30 in the control wells ($p < 0.05$, Kruskal-Wallis test).

The inhibition induced by each volunteer was then compared. The percent inhibition was calculated based on the control wells (sporozoites and liver cells). Whilst no difference between the percentage inhibition induced by the two volunteers was observed when CD8⁺ T cells or total PBMCs at a ratio of 12.5:1 were added, there was a significant difference at a ratio of 6.25:1 ($p < 0.05$, two-way ANOVA) (Figure 7.14 D). Overall, there was no significant difference between the two volunteers tested. The limiting factor in performing this series of experiments was not the number of sporozoites (high yields were achieved, 30-60 000 per mosquito), but the number of viable hepatocytes.

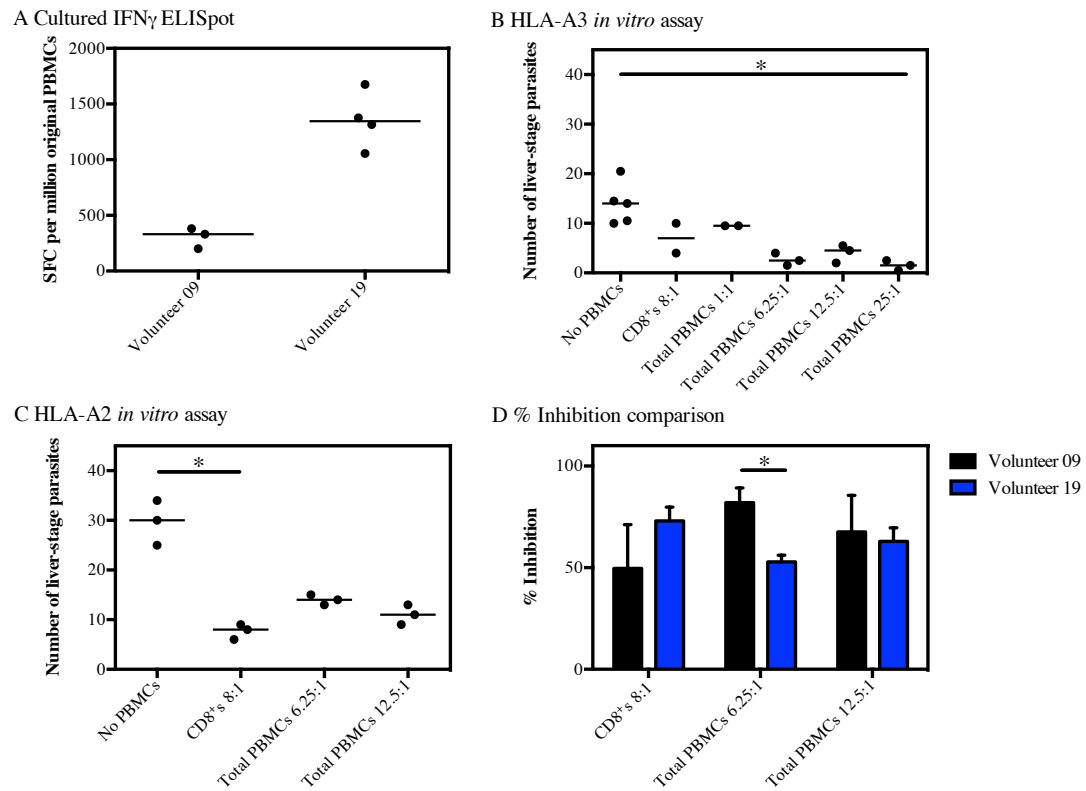


Figure 7.14. Inhibition of *P. falciparum* infected primary human hepatocytes by the addition of PBMCs from volunteers vaccinated with ME-TRAP (legend overleaf).

Figure 7.14. Inhibition of *P. falciparum* infected primary human hepatocytes by the addition of PBMCs from volunteers vaccinated with ME-TRAP (previous page).

Vials of frozen PBMCs from the time-points C-1, C+7 and C+35 were thawed and stimulated for ten days with a pool of overlapping peptides to *P. falciparum* T9/96 TRAP and recombinant human IL-2, for volunteers 09 (HLA-A3) and 19 (HLA-A2) from Mal34. (A) A fraction of the stimulated cells was then used in a standard IFN γ ELISpot assay with a pool of overlapping peptides to *P. falciparum* T9/96 TRAP. The background response of media only wells was subtracted from the positive response. Samples were tested in quadruplicate. The results are expressed as SFC per million original PBMCs, with both medians and individual data points shown. (B) 8×10^4 fresh primary human HLA-A3 (heterozygous) hepatocytes per well were cultured for fifteen days in a 96-well plate, after which 4×10^4 *P. falciparum* sporozoites were added per well. Approximately six hours post-infection, total PBMCs from volunteer 09 were added at various ratios based on a hepatocyte number of 6×10^4 per well (assuming many hepatocytes were not viable). A portion of the stimulated PBMCs was also sorted for CD8 $^+$ cells, which were added to the assay at a ratio of 8:1. Ratios indicate the number of total cells added to the number of hepatocytes. Two days later, cultures were fixed in methanol then stained with anti-HSP70, followed by the secondary antibody Alexa Fluor $^{\text{®}}$ 488. The number of liver-stage parasites was counted using a fluorescent microscope at 63x magnification. The median and individual data points are shown. Statistical difference was assessed using the Kruskal-Wallis test with Dunn's Multiple Comparison post-test, which indicated there was a significant difference between groups due to the difference between PBMCs at a 25:1 ratio and the control wells, * $p < 0.05$. (C) Previously isolated primary human HLA-A2 (homozygous) hepatocytes were thawed and plated at 8×10^4 cells per well in a 96-well plate. After resting overnight, 4×10^4 *P. falciparum* sporozoites were added per well. Approximately six hours later, total PBMCs from volunteer 19 were added at two ratios, along with CD8 $^+$ isolated cells at a ratio of 8:1. Two days later, cultures were fixed, stained and counted as per panel B. Statistical difference was assessed using the Kruskal-Wallis test with Dunn's Multiple Comparison post-test, * $p < 0.05$. (D) The percentage inhibition was calculated for each experiment as compared to control wells containing only sporozoites and liver cells. Results are expressed as the median with error bars representing the interquartile range. Statistical difference was tested using a two-way ANOVA with Bonferroni post-test, * $p < 0.05$.

7.3 Discussion

Currently, there is no standardized *in vitro* assay to test the effectiveness of *P. falciparum* vaccine candidates in the pre-clinical phase of development. Whilst transgenic parasites offer an exciting new model to test such vaccines, they are not without their limitations. For example, the potential overexpression of the insert antigen on the parasite could potentially create a better target than exists in the natural setting. By combining such a technology with an *in vitro* assay to measure the action of T cells on the liver-stage of malaria (where targets are under control of their native promoter), strong target antigens should be identified that could be taken into clinical trials with confidence. This chapter sought to develop such an *in vitro* T cell killing assay, building on work of a murine assay from the early 1990s and translating this into a human model.

Based on a liver-stage T cell assay developed in the 1990s [55, 165-167], and initial experiments in this chapter utilizing fluorescent viruses and parasites, an improved murine *in vitro* T cell killing assay was developed. Through the use of *P. berghei* GFP expressing sporozoites, the need for manual counting was avoided. This was a shortfall of the original assay, as not only is manual counting subjective and time-consuming, the fixing and staining procedure results in the loss of many cells. Furthermore, staining does not distinguish between aborted and live sporozoites, whilst this chapter confirmed that non-viable parasites no longer express GFP. A clear gating strategy was developed, utilizing a fluorescent membrane marker to select hepatoma cells, and the levels of infectivity of two hepatoma cell lines were comparable to previously published results [546, 553, 569, 576].

In previous publications some methods explicitly mention that cultures should be centrifuged after addition of sporozoites to increase the infectivity, however others do not and there is no publication that demonstrates the improvement. From the experiments presented in this chapter, it was clear that centrifugation significantly increased the infectivity of the sporozoites in Hepa1-6 cells. Such a method, known as “spinoculation”, has also been used to increase infectivity of other infectious pathogens [577, 578], so it is perhaps not surprising that an improvement was seen. Whilst the mechanism underlying the increased infectivity was not assessed, previous studies suggested it was the deposition of the infectious particle at the cell surface, not pelleting, that increased the infection rate. This provides a clear advantage in this system given infectivity of liver cells is low, particularly for primary hepatocytes. Furthermore, it was demonstrated in this chapter that the addition of naïve splenocytes to infected cultures induced a significant level of background inhibition. This had previously been mentioned as an aside [167], but has now been shown in a compelling fashion, and highlights the need for appropriate negative controls to be used in this assay, i.e. cells from unvaccinated or mock vaccinated animals, to determine the effect of non-specific killing.

To assess the effect of vaccine induced antigen-specific cells in the improved *in vitro* T cell killing assay, PbTRAP was employed as a model antigen. PbTRAP was chosen for a number of reasons: first, it is the *P. berghei* homolog of the candidate antigen in the leading vaccine ChAd63-MVA ME-TRAP, for which there was a correlation with CD8⁺ T cells and protection against CHMI challenge [160]. Second, a comparable murine vaccine ChAd63-MVA PbTRAP was developed by DPhil student Karolis Bauza that can induce extremely high levels of T cells in C57BL/6 mice and

significant efficacy manifest as a substantial delay in the time to patency. Third, a murine hepatoma cell line, Hepa1-6, was available that is of the same H-2 type as C57BL/6 mice, H-2^b. This provided all the components needed to assess the *in vitro* assay. The results in this chapter demonstrated that PbTRAP-specific CD8⁺ enriched splenocytes were capable of inducing inhibition of liver-stage parasites, in an E:T ratio-dependent manner. C57BL/6 PbTRAP vaccinated mice are not sterilely protected, merely delayed, when challenged with *P. berghei* (Karolis Bauza, DPhil Thesis); in accordance, 100% inhibition was never seen in the *in vitro* assay, perhaps providing an immunological reason as to why sterile protection was not achievable *in vivo*. The maximum inhibition seen was 62.75% at an E:T ratio of approximately 255 PbTRAP-specific CD8⁺ cells to one infected hepatocyte. This is lower than previously seen when assessing cells from radiation or genetically attenuated sporozoite vaccination, but not surprising given that those vaccination regimens result in complete sterile protection [55, 165, 168]. Importantly, these results demonstrate that PbTRAP peptides are processed within hepatocytes and expressed on the cell surface in association with MHC Class I. The only other antigen this has been demonstrated for is PbCSP, in the earlier studies mentioned [55, 166, 167].

There was an interesting trend towards a need for a greater ratio of antigen-specific T cells to infected hepatocytes when fewer hepatocytes were infected. This suggests that a greater number of T cells are required when the infectivity level is low; such a theory could be tested *in vivo* to determine whether it is an artifact of a 2D system or a true biological phenomenon. If proven correct, it would suggest that combining a vaccine targeting inhibition of sporozoite invasion (such as RTS,S) with a liver-stage vaccine (such as ME-TRAP) could have a detrimental rather than a synergistic or

additive effect. However, pre-clinical combination experiments of PbCSP and PbTRAP have indicated a synergistic, not detrimental, effect (Karolis Bauza, DPhil Thesis), likely indicating it is an effect of the *in vitro* system. Unfortunately, the mechanism of inhibition could not be conclusively determined in this series of experiments. However, there was a trend towards dependency on IFN γ , likely in combination with cytotoxicity. The limitation in this series of experiments was the sporozoite yield per mosquito; many rounds of sporozoite production did not provide enough parasites to perform the assay, and of those that did only limited numbers of experimental wells could be included. Efforts will now be focused on increasing the sporozoite yield after consultation with other institutions indicated that their laboratories regularly produce up to 50 000 sporozoites per mosquito (INSERM, Paris and Leiden University, Nijmegen, personal communications), compared with 1000 to 10 000 at Oxford.

The experiments described in this chapter also demonstrated that both IFN γ and TNF α were able to inhibit liver-stage parasites, in agreement with previous studies [555, 579]. However, the inhibition measured (approximately 40%) was lower than previously reported. Interestingly, previous work indicated that the effect of recombinant TNF α was greater in primary hepatocytes than in Hepa1-6 cultured cells [442], suggesting it may be easier to inhibit liver-stage parasites in primary hepatocytes rather than cell lines. Given all of the cellular assays from the 1990s were also performed in primary hepatocytes, another step moving forward will be to repeat the PbTRAP *in vitro* assay using C57BL/6 primary hepatocytes. Isolating and culturing primary hepatocytes was not successfully established throughout the course of this thesis, but with the knowledge gained from experiments performed in Prof.

Mazier's laboratory this procedure will be assessed again. Of interest, no expression of MHC Class II was identified on sporozoite-infected hepatoma cells. Whilst hepatocytes do not normally express MHC Class II, it has previously been reported that its expression can be upregulated in the liver in disease states or in the presence of IFN γ [580, 581]. This finding is of importance to the scientific community to aid in rational vaccine design, and has not previously been assessed, and therefore requires confirmation in an experiment with increased sample sizes and primary hepatocytes. The finding does, however, suggest that cell-mediated immunity observed at the liver-stage is likely to be through CD8⁺ T cells, and supports the notion of enriching for this cellular subset prior to addition into an *in vitro* T cell killing assay. However, the major limitation in this experiment was that all Hepa1-6 cells were assessed for MHC Class II expression, not only the infected cells. This was due to the limited number of events that could be assessed, given the low percentage infectivity of this cell line. Whilst MHC Class II expression may be increased in general in infected wells, due to the initial traversal of the parasite through multiple hepatocytes before initiating infection, the experiment needs to be repeated with greater numbers in order to assess both factors.

Whilst this chapter successfully improved an *in vitro* T cell killing assay first developed in the 1990s, to test the potential clinical application of new vaccines a human system is preferable. Mouse experiments are limited by the small MHC repertoire they possess, particularly given most vaccines induce a single immunodominant epitope in mice, which is not reflective of vaccination in humans. There is also much speculation as to whether pre-clinical mouse models do or do not represent what will be seen in humans. However, establishing a comparable assay in

human hepatocytes is a much more complex task: MHC matching becomes a greater issue than for inbred mice; *P. falciparum* infection of human hepatocytes *in vitro* is much lower than for *P. berghei*; fewer sources of human hepatocytes are available; and *P. falciparum* sporozoites are more difficult to obtain than rodent parasites. Nevertheless, preliminary experiments were successfully undertaken in collaboration with Prof. Mazier's group in Paris, the first time such an assay has ever been performed successfully, and killing of liver-stage parasites was demonstrated.

Performing the assays in Paris relied on the use of frozen PBMCs; such cells from volunteers vaccinated with ME-TRAP in clinical trials undertaken in Oxford were screened for high cultured IFN γ ELISpot responses. Two volunteers were chosen to begin optimization of the *in vitro* assay: volunteer 09, HLA-A3, who was sterilely protected from challenge with *P. falciparum* in Mal34 and gave a cultured IFN γ ELISpot response of approximately 1000 SFC per million PBMCs; and volunteer 19, HLA-A2, who was not sterilely protected upon challenge but generated an exceptionally high cultured IFN γ ELISpot response of 2800 SFC per million PBMCs. It was hypothesized that volunteer 09 would act as a positive control, given they were protected from malaria challenge, whilst volunteer 19 should not induce 100% inhibition of liver-stage parasites given they were not protected, despite their high cellular immunogenicity. However, it was demonstrated that cultured PBMCs from both volunteers were able to induce comparable inhibition of *P. falciparum* infected primary human hepatocytes, perhaps an unexpected finding. Although of course it would be rare to see a clear correlation between only two samples, and furthermore the experimental designs were quite different considering one utilized fresh hepatocytes whilst the other used frozen hepatocytes.

The limitation of both experiments was the poor condition of the hepatocytes, with many dead cells visible prior to infection and a poor cell monolayer. This resulted in extremely low numbers of liver-stage parasites per well compared to what is normally seen (300 – 1000, Dr. Soulard, personal communication) and a high level of variability. In addition, cultures had to be fixed at two days post-infection rather than five to six days, to ensure a result was obtained before the cells completely broke down. For this reason, the liver-stage parasites were very small and more difficult to identify. Hence, the inhibition seen needs to be considered carefully given it could be impacted by the variability and the unpredictable nature of the experiment due to the poor state of the hepatocytes, and it could reflect non-specific killing given appropriate controls were not included (e.g. from NK cells [582, 583]). Generally, fresh primary human hepatocyte cultures can survive for up to three weeks in good condition, so it was unfortunate that this was not the case in the first experiment. Cryopreserving primary human hepatocytes and subsequent thawing has been carried out in the past [385, 584], but has not been a necessity in Prof. Mazier's laboratory over recent years due to a constant supply of fresh liver samples. Given the more complicated nature of cellular assays than drug or antibody assays, there is a greater reliance on frozen hepatocytes in order to plan the timing of such experiments and to HLA type the samples. This protocol will therefore be optimized before further attempts at the *in vitro* assay are made. This assay could be trialed using the HC-04 cell line [551], however this limits the assay to HLA-A2 positive volunteers.

Once the culture of frozen primary human hepatocytes is optimized, future work will include further *in vitro* experiments undertaken on more ME-TRAP vaccinated volunteers, with additional controls including pre-vaccination PBMCs and PBMCs

stimulated with irrelevant antigens rather than PfTRAP (for example, influenza peptides) to determine the level of non-specific inhibition. A secondary aim is to produce a T cell clone from a volunteer providing 100% inhibition to act as a positive control that could be included in each experiment. To generate a T cell clone (or line) the protective epitopes need to be known; the epitope mapping described in Chapter 6 could be utilized for this aim. It is hoped that once sufficient data are obtained, a correlation can be performed between the percentage inhibition achieved *in vitro* and protection *in vivo*. Whilst it is likely that other factors contribute to the protection seen *in vivo*, such an *in vitro* assay would still prove useful for assessment of the mechanism of protection. Further improvements could include the use of recently developed *P. falciparum* parasites expressing GFP [585, 586], to enable visualization without staining (including visualization in real-time) and the potential use of flow cytometry as per the murine *in vitro* assay; the use of cryopreserved sporozoites to reduce variability in infection from different batches [524]; the potential use of a recently described 3D liver architecture to more closely mimic the natural setting [561]; and even combining the assay with new *in vitro* imaging techniques [168].

In summary, this chapter aimed to develop an *in vitro* T cell killing assay to test the effectiveness of vaccine induced T cells to inhibit liver-stage parasites. A new and improved assay was developed in a murine system based on GFP expressing parasites. The improvements made have generated a more standardized assay that is not influenced by the subjectivity of the operator, and has clarified the methodology concerning centrifugation and background inhibition. Furthermore, it is the first time the *in vitro* T cell killing assay has been performed using a hepatoma cell line, making the assay more accessible to laboratories that do not have the facilities and

experience necessary to culture primary cells. These improvements can also be applied to ISI and ILSDA assays. The results in this chapter also demonstrated the first known attempt at such an *in vitro* assay using primary human hepatocytes, *P. falciparum* and antigen-specific T cells from vaccinated human volunteers. Whilst only preliminary experiments were performed, these results pave the way for the development of an exceptionally useful and exciting assay. It is envisaged that this assay could be used for screening new *P. falciparum* vaccine candidates by immunising HLA transgenic mice as a source of MHC matched T cells (e.g. HHD mice [369]) or through *in vitro* vaccination such as the MIMIC® technology (<http://www.vaxdesign.com/mimic-technology>). Perhaps when the results of such an assay are combined with *in vivo* challenges using transgenic parasites, or potentially the recently described humanized mice [587, 588], definitive answers on the likely protective efficacy of *P. falciparum* vaccine candidates could be achieved pre-clinically.

Chapter 8: Discussion

8 Discussion

8.1 Overview

A malaria vaccine of high efficacy suitable for deployment in malaria endemic countries has yet to be developed. The most advanced vaccine RTS,S, and the most advanced T cell inducing vaccine ChAd63-MVA ME-TRAP, express the pre-erythrocytic antigens CSP and TRAP, respectively. CSP and TRAP have important functional roles in sporozoite motility, attachment to hepatocytes and liver-stage development [73, 307-309, 337-340]. Yet these vaccines based on CSP and TRAP have not been able to replicate the high levels of protective efficacy induced by irradiated sporozoites delivered by mosquito bite [45-49], or more recently, by intravenous injection [50]. However, a sub-unit vaccine has a more amenable manufacturing process, a more standard delivery route (intramuscular immunization) and more favourable storage conditions (without the requirement for LN₂), leading to the conclusion that a sub-unit vaccine is more feasible for field deployment and more likely to lead to licensure. Hence, the focus of both our laboratory and that of GSK (manufacturer of RTS,S) is to increase the immunogenicity and protective efficacy of these sub-unit vaccines. One option is to administer them together, thereby combining the antibody-mediated inhibition of sporozoite invasion induced by RTS,S with the CD8⁺-mediated killing of infected hepatocytes induced by ChAd63-MVA ME-TRAP. This has been demonstrated in a pre-clinical model using a ChAd63 prime-protein boost regimen expressing PbCSP and ChAd63-MVA expressing PbTRAP (Karolis Bauza, DPhil Thesis). Sterile protection increased from approximately 0-40% when either vaccine was used alone, to 100% when administered together. A

clinical trial to assess the efficacy of RTS,S delivered in combination with ChAd63-MVA ME-TRAP has just begun at the Jenner Institute.

However, another feasible option remains; given neither TRAP nor CSP alone can account for the protection induced by irradiated sporozoites, it is highly likely that a broad immune response to multiple target antigens is required. Two studies have provided solid evidence that other antigens are implicated in a protective immune response: first, mice tolerized to CSP epitopes are still able to induce protection in the irradiated sporozoite model [389] and second, the immune responses generated to known antigens in the irradiated sporozoite model do not amount to the level of protection induced, and in fact several unknown antigens were more predictive of protection [390]. An extension of this work demonstrated that the combined immunogenicity of a panel of nineteen antigens could account for protection [589]. Together, this suggests that other pre-erythrocytic candidate antigens exist that could be included in a multi-component vaccine, which is not surprising given over 5000 proteins are expressed throughout the parasite's life-cycle [224]. Furthermore, including multiple antigenic targets expressed at the liver-stage in one vaccine might overcome limitations based on genetically restricted responses to certain epitopes [590].

The work in this thesis provides further support for this notion. Through a comparative screen of eight novel viral vectored vaccines, two antigens, PflSA1 and PflSAP2, were identified that provided greater protection in a pre-clinical murine model than either CSP or TRAP. Another, PflUIS3, was shown to provide protective efficacy equivalent to CSP. As discussed in Chapter 4, this is excellent proof of

concept that other protective antigens exist, and that they are not necessarily the most immunogenic. The risk of using a murine model is that the findings may not translate into protective efficacy in humans. To account for this, outbred mice with diverse MHC repertoires similar to humans can be used, and as demonstrated in Chapter 7 continued development of the *in vitro* T cell killing assay should allow pre-clinical assessment in a human model. Further assessment of each of the candidates will continue pre-clinically, however there is a strong case for taking PFLSA1 straight into clinical trials, as discussed below.

8.2 Conclusions and future directions

8.2.1 Comparative assessment of pre-erythrocytic vaccines and identification of novel candidates

Eight novel viral vectored vaccines were comparatively assessed by immunogenicity and efficacy; of these, PfUIS3, PFLSA1 and PFLSAP2 provided the highest level of efficacy in BALB/c mice and hence were chosen for further assessment. Protective efficacy induced by PfUIS3 and PFLSA1 vaccination was not found to be H-2^d dependent, yet this may be the case for PFLSAP2 (Table 8.1) and therefore the five non-protective vaccines should also be assessed in further mouse strains. This highlights the problem of using a murine system for assessment of *P. falciparum* antigens: we do not know how reflective the results are of what would be seen in humans [591], given the differences in MHC and immunodominance, and hence confidence in such experiments involves extensive assessment in multiple strains of mice. However, as a proof of concept study, multiple vaccines were found that induced greater levels of efficacy than CSP or TRAP, and hence further candidate

antigens warrant assessment in the same system. First, however, candidates need to be down-selected from the 1000 or so proteins expressed at the liver-stage [398, 592].

Table 8.1. Summary of immunogenicity and efficacy of the protective vaccines identified in this thesis.

PfLSA1, PfLSAP2, PfUIS3 and PFI0580c all induced protective efficacy compared to control mice when challenged with the appropriate transgenic parasite. The pre-challenge immunogenicity and efficacy is outlined below. Cellular immunogenicity is reported as the percentage of CD8⁺ IFN γ ⁺ cells in the blood, whilst humoral immunogenicity refers to the antibody level measured by LIPS and is expressed as the fold change from naive. All values given are medians. * Indicates that the value was not assessed pre-challenge, and hence the representative median from the immunogenicity experiments is given. Efficacy refers to sterile protection, given as a percentage, or a significant delay in the time to 1% parasitaemia compared to control mice, denoted “delay”. Data is also shown for PfCSP and PfTRAP for comparison where available: the BALB/c results were presented in Chapter 4, Rhea Longley performed the PfTRAP experiment in CD-1 mice and Dr. Alex Spencer performed the PfTRAP experiment in C57BL/6 mice. PfCSP has not yet been assessed in C57BL/6 or CD-1 mice.

	BALB/c			C57BL/6			CD-1		
Vaccine	Cellular	Humoral	Protection	Cellular	Humoral	Protection	Cellular	Humoral	Protection
PfLSA1	1.80%	1.26*	81.25%	0.21%	0	None	1.13%	1.39	87.50%
PfLSAP2	0.43%	1.33	75%	3.40%	0	None	N/A	N/A	N/A
PfUIS3	5.59%	1.34*	18%, delay	4.80%	1.16	37.5%, delay	0.87%	1.05	None
PFI0580c	0	1.57*	Delay	N/A	N/A	N/A	N/A	N/A	N/A
PfCSP	12.22%	N/A	25%, delay	N/A	N/A	N/A	N/A	N/A	N/A
PfTRAP	0.61%	N/A	None	3.51%	N/A	None	9.48%	N/A	None

This series of experiments has shown that whilst vaccines are required to be immunogenic, the magnitude alone does not predict protective efficacy. The aim of using viral vectors to express these antigens was to induce predominantly CD8⁺ T cells as this subset has been strongly associated with protection in the irradiated sporozoite model [164, 202-204, 395]. Protection induced by both PfUIS3 and PflSA1 was shown to be largely dependent on CD8⁺ T cells, supporting the basis of this rationale. In order to provide protection, these antigens needed to be processed and presented on the surface of the infected hepatocyte in association with MHC Class I. However, liver-stage development occurs within a parasitophorous vacuole, which physically separates the parasite from the hepatocyte cytoplasm. For a parasite antigen to be processed and presented it first needs to interact with the host cell cytoplasm, which is most likely to occur due to an important role in liver-stage development. It could be assumed that proteins expressed in the PVM would be processed, given the PVM is the parasite-hepatocyte interface [466], however, this is still likely dependent on the actual function of each protein. In essence, not all parasite proteins will be expressed as peptides on the cell surface, and this is the likely reason that not all immunogenic candidates conferred protection against transgenic sporozoite challenge. However, this is assuming that the *P. falciparum* antigen behaves the same in a transgenic *P. berghei* parasite as it does natively. Therefore, to down-select the 1000 or so proteins likely expressed at the liver-stage, those that are presented as peptides in complex with MHC Class I on the hepatocyte surface should be prioritized.

Rational vaccine design requires an in-depth understanding of the biological role of candidate antigens and antigen presentation. Unfortunately, for most targets such

studies are lacking, largely because such small numbers of hepatocytes ever become infected with *Plasmodium*. In fact, the only studies that have identified CD8⁺T cell killing through peptide-MHC Class I complexes on infected hepatocytes have been the *in vitro* T cell killing assays of radiation or genetically attenuated sporozoites and studies of CSP [55, 165-168], and now TRAP. Down-selection has been achieved by assessing *P. falciparum* sporozoite proteome data [593] with bioinformatic predictions of the MHC binding capacity, HLA supertype considerations and immunogenicity in volunteers vaccinated with irradiated sporozoites [390]. However, this strategy failed to identify PflSA1 given it is only expressed at the liver-stage, and the liver-stage specific proteome could not be assessed due to the small number of liver-stage parasites that could be obtained. This suggests that other liver-stage proteins with important roles in liver-stage development could have been missed, particularly since antigens with late liver-stage expression likely contribute to the protection seen in the irradiated sporozoite model [594], and late liver-stage antigens have made the most successful GAPs [210]. PflSAP2 was identified by transcriptomic profiling of *P. falciparum* sporozoites co-cultured with hepatocytes [416], supporting this notion. However, the authors were required to dissect 1200 mosquitoes in order to have a high enough number of infected cells to assess. They only incubated sporozoites and hepatocytes together for one hour, so whilst this study did reveal PflSAP2, it is possible that other late liver-stage antigens were still missed. Interestingly, a recent publication provided evidence that the MHC Class I complex was almost completely downregulated in late liver-stage development [595], suggesting appropriate antigenic targets will also need to be expressed earlier on.

Three methods of isolating *P. falciparum* infected hepatocytes are immediately available: (1) infection of primary human hepatocytes in culture, as for PflSAP2 identification, (2) infection of non-human primates and removal of the liver or (3) infection of humanized mice and removal of the liver. Method (1) is difficult given the low infectivity of primary hepatocytes by *P. falciparum* (approximately 0.1%), however, could be plausible if a large-scale approach was taken, as previously discussed. Method (2) is undesirable given the limited availability and cost of non-human primates; furthermore, they are generally not culled but treated and re-used after malaria infection. Method (3) has become recently available: a number of laboratories now routinely infect various models of humanized mice with *P. falciparum*, after repopulation of the liver with human hepatocytes [587, 588, 596]. This method should enable the collection of large enough numbers of infected hepatocytes to enable proteomic studies, or elution of the peptides bound to MHC Class I.

Elution of peptides bound to MHC Class I from infected hepatocytes would allow identification of antigens that can be recognized by the human immune system. Such a strategy [597] has previously been used for diseases such as influenza [598], hepatitis C virus [599], cancer [600] and mycobacterium tuberculosis [601]. This is a new research avenue beginning at the Jenner Institute to select potential target antigens for a pre-erythrocytic malaria vaccine. Down-selection from the 1000 or so liver-stage proteins in this manner should leave a reasonable number of targets that can be assessed in the transgenic sporozoite challenge model, or in the *in vitro* T cell killing assay.

8.2.2 T cell-mediated killing of liver-stage parasites

This thesis describes the development of an *in vitro* T cell killing assay, both in murine and human models. The murine assay demonstrated huge improvements on the studies from the 1990s, through the use of fluorescent parasites, flow cytometry and clarification of the methodology. This assay will also be useful to assess mechanisms of protection. Development of the human assay has begun, and whilst large improvements still need to be made, the future of this assay has potential. First, whilst murine models are useful and have traditionally been used to screen vaccines pre-clinically, it would be preferable to screen in a human system. As discussed in Chapter 7, this is largely because of the limited MHC repertoire of mice and, in turn, vaccination tends to induce only one immunodominant epitope. This is extremely different to vaccination-induced immunity in humans, where multiple epitopes exist and are often different between individuals. The *in vitro* assay could change the way we screen *P. falciparum* vaccines, and is potentially more likely to generate reliable candidates by reflecting the human response more accurately.

Secondly, apart from screening vaccines, these studies also inform us about the biology of the antigen. For example, T cell-mediated inhibition of liver-stage parasites directly demonstrates that the antigen is presented on the hepatocyte surface, and would be a desirable target *in vivo*. From an immunological point of view, it can allow the mechanism to be elucidated, which in turn helps inform rationale vaccine design (e.g. do we want to induce cytotoxic CD8⁺ T cells or cells secreting IFN γ , or both?). For a liver-stage specific vaccine, such as PfLSA1, where protection will be solely due to inhibition of liver-stage parasites, this assay could also act as a surrogate for protection. Then, instead of CHMI trials, only immunogenicity and safety studies

would need to be performed in humans, and efficacy could be determined in the assay (with appropriate HLA matching). Furthermore, this assay could be used to define protective epitopes. For example, the epitopes within PfTRAP (identified in Chapter 6) could be screened for their ability to inhibit liver-stage parasites in the *in vitro* assay, by the generation of epitope specific T cell clones or lines.

Another interesting model that has recently been developed is the generation of humanized mice that can be infected with *P. falciparum*, as mentioned previously. As this method develops, it would be of interest to determine whether such a model is better than an *in vitro* T cell killing assay. Two different models currently exist, SCID Alb-uPA [587, 596] and FRG NOD [588], both based on immunocompromised mice in which the liver can be repopulated with human hepatocytes (up to 80-90%). The advantages over an *in vitro* model are that larger liver-stage parasites develop, up to 80µm compared to 15-40µm in culture [548, 551, 588]. The larger size is more similar to results from studies of human infections [602, 603]. Furthermore, often liver-stage artifacts or abortive forms of parasites are detected in cultures (as seen in the results described in this thesis), yet this has not been identified in humanized mice. However, the disadvantage is that antigen-specific T cells would need to be adoptively transferred into these mice; this has yet to be assessed, and so the number required is unknown. Given the system is an entire mouse rather than a culture well more cells might be required to assess protection. Furthermore, at present these humanized mouse models are challenging to work with, given some require injections to control non-adaptive immune defenses in order to maintain the phenotype [587], and they all need to be specially housed (increasing the expense). However, as these models develop it will be important to consider the use of each in assessing T cell-

mediated inhibition, and determining which better reflects the *in vivo* response in humans.

8.2.3 Naturally acquired immunity versus vaccine-induced immunity

Chapter 6 described the assessment of immunodominant epitopes in naïve-adults vaccinated with ChAd63-MVA ME-TRAP and demonstrated that the profile of epitopes was remarkably different to the response in natural infections. From these studies, it has been difficult to identify the underlying reason, of which there are many potential causes. The different epitopes identified between exposed and vaccinated volunteers may reflect variation and underlying genetic differences in the two populations, or the effect of pre-existing immunity on epitope immunodominance. Volunteers in clinical trials are only vaccinated against one strain, whilst naturally acquired immunity would most likely have developed from exposure to multiple strains of parasites, and there could also be differences in antigen processing between a viral vectored vaccine and intrahepatic parasites. As suggested in Chapter 6, future work should aim to address these differences, primarily by building up the database on known epitopes in both populations, but also by assessing immunodominant epitopes induced in vaccinated adults from endemic areas. This would be interesting not only for identifying protective epitopes, but also to determine whether such epitopes fall in the conserved or variable regions of TRAP, which may in turn provide information on the potential of vaccine escape.

As naturally acquired immunity is non-sterilizing, and largely due to antibodies, liver-stage vaccines are not aiming to replicate but improve upon this form of immunity. However, an understanding of naturally acquired immunity is still essential to identify what types of responses are protective, so that a vaccine can

amplify this immunity. Assessment of immune responses in volunteers naturally or experimentally infected with malaria can also be useful for this purpose. Experimental malaria infection is more controlled, and experiments can be performed in laboratories where all the equipment and reagents are available. So whilst not a true reflection of naturally acquired immunity, such studies can still be informative. In Chapter 6, the highest response rate in experimentally infected volunteers was to the antigen PfExp1. Whilst this does not necessarily suggest PfExp1 will be a component of the next malaria vaccine, it does demonstrate that the sequence of PfExp1 is immunogenic in humans. At the present time when so little is known about most new candidate antigens, demonstration of immunogenicity in humans is an interesting finding, particularly given the *P. yoelii* homolog of Exp1 (PyHep17) has been able to induce high levels of sterile protection in mice dependent upon cellular immune responses [405, 480, 590]. As PfExp1 was unable to be developed in the ChAd63 vector, this antigen could be assessed in a DNA regimen with *in vivo* EP, given the recent encouraging results using this platform [253], with or without a MVA boost. As DNA constructs are made during the initial cloning for viral vectors, it would be a straightforward experiment to compare PfExp1 with PflSA1 or PflSAP2 in this regimen.

8.2.4 Identifying correlates of protection

The identification of correlates of protection has become a major focus in malaria vaccine research, as such a correlate could be used as a surrogate for protection. However, in the murine studies described in this thesis it was statistically difficult to identify correlates, using either Spearman's correlation or by stratifying the data. This is also a difficulty in clinical trials where often only small proportions of volunteers are protected or delayed. In this scenario, the *in vitro* T cell killing assay may become

a valuable tool in identifying potential mechanisms of protection, or identifying where the vaccine has failed. However, a further problem in clinical trials is that there are limited numbers of PBMCs available for use, but increasingly more assays to test them. Priority should be given to the *ex vivo* IFN γ ELISpot and multi-parameter ICS on fresh PBMCs, to confirm the vaccine was immunogenic. Whilst ideally the *in vitro* assay would also be performed on fresh PBMCs, frozen PBMCs can be used if they are not available. Furthermore, this still allows for frozen PBMCs to also be used for transcriptomic profiling. This approach only requires a small number of cells, and in turn gives a wealth of information on the genes and pathways involved in the immune response [604, 605]. Hopefully combining four different methodologies in a systems biology approach would allow a greater understanding of all the immune components at play.

8.2.5 PflSA1 as a vaccine candidate

Through challenges in mice with transgenic parasites, PflSA1 was demonstrated to be a strong candidate antigen. Whilst future experiments can continue to assess the protective capacity of immune responses generated against PflSA1 pre-clinically, there is evidence to suggest this vaccine should be taken straight into a clinical trial. First, PflSA1 provided 87.5% sterile protection in outbred mice, a level of protection that has not previously been reached for any vaccine. Outbred mice are more comparable to an outbred human population, and the protection cannot be due to one immunodominant epitope. Second, the basis of the mechanism of protection is known. CD8⁺ T cells were shown to be indispensable, with CD4⁺ T cells also contributing. The level of CD8⁺ T cells was of a moderate magnitude that should be achievable in humans, unlike the very high levels of CD8⁺s required to Pb9 [606], and it has been demonstrated that the viral vectored platform provides the highest

level of CD8⁺ T cells compared to other vaccination regimens [160, 256]. Furthermore, there has been consistent evidence in field studies that cytokine responses against PfLSA1 are associated with protection [154-157, 411, 471]. Given PfLSA1 is only expressed at the liver-stage, it is likely these cytokines have been induced by CD8⁺ T cells recognizing PfLSA1 peptide presented by hepatocytes. It also demonstrates that human populations can induce an LSA1-specific immune response.

Whilst the two known clinical trials of PfLSA1, as a recombinant protein-in-adjuvant vaccine [232] or as part of a large polyprotein construct delivered by viral vectors [472], were not protective, there are logical reasons as to why these efficacy trials failed. In the first study, the immune response induced was predominantly LSA1-specific antibodies and CD4⁺ T cells, and the results described in this thesis demonstrated that CD8⁺ T cells were indispensable for protection. When PfLSA1 was delivered as part of a polyprotein construct, the sequence was very large: the construct contained five other antigens and was over 3000aa. The authors demonstrated little immunogenicity to each component of the vaccine, and suggested that the size was too large for appropriate expression from the viral vectors, or that there was antigenic competition. Given these trials were not conducted using ideal vaccine platforms to induce CD8⁺ T cells, ChAd63-MVA PfLSA1 should be assessed as a vaccine candidate in clinical trials.

8.3 Final remarks

In conclusion, this thesis approached the development of a novel, protective liver-stage vaccine in two ways: through an antigen screen of currently annotated liver-stage proteins, and by the development of a novel *in vitro* assay. PfLSA1 was

identified as a strong candidate antigen that should be tested in clinical trials, delivered in the ChAd63-MVA regimen. As more liver-stage antigens are down-selected through the processes discussed above, they could be assessed in the transgenic parasite challenge system and the *in vitro* assay. In addition, the *in vitro* T cell killing assay may be able to elucidate mechanisms of protection of existing vaccines and could be used to confirm that target antigens are presented on the hepatocyte cell surface in association with MHC Class I. Such an assay should help guide vaccine research efforts in the future.

References

References

1. World Malaria Report, 2012, World Health Organization.
2. Murray, C.J., et al., *Global malaria mortality between 1980 and 2010: a systematic analysis*. Lancet, 2012. **379**(9814): p. 413-31.
3. White, N.J., et al., *New global estimates of malaria deaths*. Lancet, 2012. **380**(9841): p. 559-60.
4. Battle, K.E., et al., *The global public health significance of Plasmodium vivax*. Adv Parasitol, 2012. **80**: p. 1-111.
5. Cox-Singh, J., et al., *Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening*. Clin Infect Dis, 2008. **46**(2): p. 165-71.
6. Goh, X.T., et al., *Increased detection of Plasmodium knowlesi in Sandakan division, Sabah as revealed by PlasmoNex*. Malar J, 2013. **12**: p. 264.
7. Maguire, J.D. and J.K. Baird, *The 'non-falciparum' malarias: the roles of epidemiology, parasite biology, clinical syndromes, complications and diagnostic rigour in guiding therapeutic strategies*. Ann Trop Med Parasitol, 2010. **104**(4): p. 283-301.
8. William, T., et al., *Increasing incidence of Plasmodium knowlesi malaria following control of P. falciparum and P. vivax Malaria in Sabah, Malaysia*. PLoS Negl Trop Dis, 2013. **7**(1): p. e2026.
9. Sachs, J. and P. Malaney, *The economic and social burden of malaria*. Nature, 2002. **415**(6872): p. 680-5.
10. Sicuri, E., et al., *The economic costs of malaria in children in three sub-Saharan countries: Ghana, Tanzania and Kenya*. Malar J, 2013. **12**(1): p. 307.
11. Teklehaimanot, A., G.C. McCord, and J.D. Sachs, *Scaling up malaria control in Africa: an economic and epidemiological assessment*. Am J Trop Med Hyg, 2007. **77**(6 Suppl): p. 138-44.
12. Tusting, L.S., et al., *Socioeconomic development as an intervention against malaria: a systematic review and meta-analysis*. Lancet, 2013. **382**(9896): p. 963-72.
13. Jones, C.O. and H.A. Williams, *The social burden of malaria: what are we measuring?* Am J Trop Med Hyg, 2004. **71**(2 Suppl): p. 156-61.
14. Lengeler, C., *Insecticide-treated bed nets and curtains for preventing malaria*. Cochrane Database Syst Rev, 2004(2): p. CD000363.

15. Greenwood, B., *Anti-malarial drugs and the prevention of malaria in the population of malaria endemic areas*. Malar J, 2010. **9 Suppl 3**: p. S2.
16. John, G.K., et al., *Primaquine radical cure of Plasmodium vivax: a critical review of the literature*. Malar J, 2012. **11**: p. 280.
17. Kremsner, P.G. and S. Krishna, *Antimalarial combinations*. Lancet, 2004. **364**(9430): p. 285-94.
18. Mita, T. and K. Tanabe, *Evolution of Plasmodium falciparum drug resistance: implications for the development and containment of artemisinin resistance*. Jpn J Infect Dis, 2012. **65**(6): p. 465-75.
19. Plowe, C.V., *The evolution of drug-resistant malaria*. Trans R Soc Trop Med Hyg, 2009. **103 Suppl 1**: p. S11-4.
20. Howes, R.E., et al., *G6PD deficiency: global distribution, genetic variants and primaquine therapy*. Adv Parasitol, 2013. **81**: p. 133-201.
21. Karema, C., et al., *Trends in malaria cases, hospital admissions and deaths following scale-up of anti-malarial interventions, 2000-2010, Rwanda*. Malar J, 2012. **11**: p. 236.
22. Okiro, E.A., et al., *The decline in paediatric malaria admissions on the coast of Kenya*. Malar J, 2007. **6**: p. 151.
23. Okiro, E.A., et al., *Childhood malaria admission rates to four hospitals in Malawi between 2000 and 2010*. PLoS One, 2013. **8**(4): p. e62214.
24. Dondorp, A.M., et al., *Artemisinin resistance in Plasmodium falciparum malaria*. N Engl J Med, 2009. **361**(5): p. 455-67.
25. Miotto, O., et al., *Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia*. Nat Genet, 2013. **45**(6): p. 648-55.
26. Na-Bangchang, K., et al., *Identification of resistance of Plasmodium falciparum to artesunate-mefloquine combination in an area along the Thai-Myanmar border: integration of clinico-parasitological response, systemic drug exposure, and in vitro parasite sensitivity*. Malar J, 2013. **12**: p. 263.
27. Noedl, H., et al., *Evidence of artemisinin-resistant malaria in western Cambodia*. N Engl J Med, 2008. **359**(24): p. 2619-20.
28. Phyto, A.P., et al., *Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study*. Lancet, 2012. **379**(9830): p. 1960-6.
29. Payne, D., *Spread of chloroquine resistance in Plasmodium falciparum*. Parasitol Today, 1987. **3**(8): p. 241-6.

30. Roper, C., et al., *Intercontinental spread of pyrimethamine-resistant malaria*. Science, 2004. **305**(5687): p. 1124.
31. Hargreaves, K., et al., *Anopheles funestus resistant to pyrethroid insecticides in South Africa*. Med Vet Entomol, 2000. **14**(2): p. 181-9.
32. Ranson, H., et al., *Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?* Trends Parasitol, 2011. **27**(2): p. 91-8.
33. Plowe, C.V., P. Alonso, and S.L. Hoffman, *The potential role of vaccines in the elimination of falciparum malaria and the eventual eradication of malaria*. J Infect Dis, 2009. **200**(11): p. 1646-9.
34. *A research agenda for malaria eradication: vaccines*. PLoS Med, 2011. **8**(1): p. e1000398.
35. Greenwood, B. and G. Targett, *Do we still need a malaria vaccine?* Parasite Immunol, 2009. **31**(9): p. 582-6.
36. Penny, M.A., et al., *What should vaccine developers ask? Simulation of the effectiveness of malaria vaccines*. PLoS One, 2008. **3**(9): p. e3193.
37. Strassburg, M.A., *The global eradication of smallpox*. Am J Infect Control, 1982. **10**(2): p. 53-9.
38. Ehrenfeld, E., J. Modlin, and K. Chumakov, *Future of polio vaccines*. Expert Rev Vaccines, 2009. **8**(7): p. 899-905.
39. Moss, W.J. and D.E. Griffin, *Global measles elimination*. Nat Rev Microbiol, 2006. **4**(12): p. 900-8.
40. Adegbola, R.A., et al., *Elimination of Haemophilus influenzae type b (Hib) disease from The Gambia after the introduction of routine immunisation with a Hib conjugate vaccine: a prospective study*. Lancet, 2005. **366**(9480): p. 144-50.
41. Takala, S.L. and C.V. Plowe, *Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'*. Parasite Immunol, 2009. **31**(9): p. 560-73.
42. Knox, D.P. and D.L. Redmond, *Parasite vaccines - recent progress and problems associated with their development*. Parasitology, 2006. **133** Suppl: p. S1-8.
43. Sharman, P.A., et al., *Chasing the golden egg: vaccination against poultry coccidiosis*. Parasite Immunol, 2010. **32**(8): p. 590-8.
44. Suarez, C.E. and S. Noh, *Emerging perspectives in the research of bovine babesiosis and anaplasmosis*. Vet Parasitol, 2011. **180**(1-2): p. 109-25.

45. Clyde, D.F., *Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites*. Am J Trop Med Hyg, 1975. **24**(3): p. 397-401.
46. Clyde, D.F., et al., *Immunization of man against sporozite-induced falciparum malaria*. Am J Med Sci, 1973. **266**(3): p. 169-77.
47. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites*. J Infect Dis, 2002. **185**(8): p. 1155-64.
48. Rieckmann, K.H., et al., *Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria*. Bull World Health Organ, 1979. **57 Suppl 1**: p. 261-5.
49. Rieckmann, K.H., et al., *Letter: Sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparum*. Trans R Soc Trop Med Hyg, 1974. **68**(3): p. 258-9.
50. Seder, R.A., et al., *Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine*. Science, 2013.
51. Bergmann-Leitner, E.S., et al., *Immunization with pre-erythrocytic antigen CelTOS from Plasmodium falciparum elicits cross-species protection against heterologous challenge with Plasmodium berghei*. PLoS One, 2010. **5**(8): p. e12294.
52. Mueller, A.K., et al., *Genetically modified Plasmodium parasites as a protective experimental malaria vaccine*. Nature, 2005. **433**(7022): p. 164-7.
53. Nussenzweig, R.S., et al., *Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei*. Nature, 1967. **216**(5111): p. 160-2.
54. Reyes-Sandoval, A., et al., *CD8+ T effector memory cells protect against liver-stage malaria*. J Immunol, 2011. **187**(3): p. 1347-57.
55. Weiss, W.R., et al., *Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes*. J Exp Med, 1990. **171**(3): p. 763-73.
56. Wirth, D.F., *Biological revelations*. Nature, 2002. **419**(6906): p. 495-6.
57. Vanderberg, J.P., *Plasmodium berghei: quantitation of sporozoites injected by mosquitoes feeding on a rodent host*. Exp Parasitol, 1977. **42**(1): p. 169-81.
58. Rosenberg, R., et al., *An estimation of the number of malaria sporozoites ejected by a feeding mosquito*. Trans R Soc Trop Med Hyg, 1990. **84**(2): p. 209-12.

59. Beier, J.C., et al., *Quantitation of Plasmodium falciparum sporozoites transmitted in vitro by experimentally infected Anopheles gambiae and Anopheles stephensi*. Am J Trop Med Hyg, 1991. **44**(5): p. 564-70.
60. Sidjanski, S. and J.P. Vanderberg, *Delayed migration of Plasmodium sporozoites from the mosquito bite site to the blood*. Am J Trop Med Hyg, 1997. **57**(4): p. 426-9.
61. Yamauchi, L.M., et al., *Plasmodium sporozoites trickle out of the injection site*. Cell Microbiol, 2007. **9**(5): p. 1215-22.
62. Amino, R., et al., *Quantitative imaging of Plasmodium transmission from mosquito to mammal*. Nat Med, 2006. **12**(2): p. 220-4.
63. Chakravarty, S., et al., *CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes*. Nat Med, 2007. **13**(9): p. 1035-41.
64. Tavares, J., et al., *Role of host cell traversal by the malaria sporozoite during liver infection*. J Exp Med, 2013. **210**(5): p. 905-15.
65. Mota, M.M., et al., *Migration of Plasmodium sporozoites through cells before infection*. Science, 2001. **291**(5501): p. 141-4.
66. Sturm, A., et al., *Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids*. Science, 2006. **313**(5791): p. 1287-90.
67. Reilly, H.B., et al., *Quantitative dissection of clone-specific growth rates in cultured malaria parasites*. Int J Parasitol, 2007. **37**(14): p. 1599-607.
68. Hafalla, J.C., O. Silvie, and K. Matuschewski, *Cell biology and immunology of malaria*. Immunol Rev, 2011. **240**(1): p. 297-316.
69. Janse, C.J., et al., *High efficiency transfection of Plasmodium berghei facilitates novel selection procedures*. Mol Biochem Parasitol, 2006. **145**(1): p. 60-70.
70. van Dijk, M.R., C.J. Janse, and A.P. Waters, *Expression of a Plasmodium gene introduced into subtelomeric regions of Plasmodium berghei chromosomes*. Science, 1996. **271**(5249): p. 662-5.
71. de Koning-Ward, T.F., et al., *Analysis of stage specificity of promoters in Plasmodium berghei using luciferase as a reporter*. Mol Biochem Parasitol, 1999. **100**(1): p. 141-6.
72. de Koning-Ward, T.F., et al., *Stable expression of green fluorescent protein in blood and mosquito stages of Plasmodium berghei*. Mol Biochem Parasitol, 1998. **97**(1-2): p. 247-52.

73. Tewari, R., et al., *Function of region I and II adhesive motifs of Plasmodium falciparum circumsporozoite protein in sporozoite motility and infectivity*. J Biol Chem, 2002. **277**(49): p. 47613-8.
74. Cao, Y., D. Zhang, and W. Pan, *Construction of transgenic Plasmodium berghei as a model for evaluation of blood-stage vaccine candidate of Plasmodium falciparum chimeric protein 2.9*. PLoS One, 2009. **4**(9): p. e6894.
75. Espinosa, D.A., et al., *Development of a Chimeric Plasmodium berghei Strain Expressing the Repeat Region of the P. vivax Circumsporozoite Protein for In Vivo Evaluation of Vaccine Efficacy*. Infect Immun, 2013. **81**(8): p. 2882-7.
76. Mlambo, G., J. Maciel, and N. Kumar, *Murine model for assessment of Plasmodium falciparum transmission-blocking vaccine using transgenic Plasmodium berghei parasites expressing the target antigen Pfs25*. Infect Immun, 2008. **76**(5): p. 2018-24.
77. Persson, C., et al., *Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid Plasmodium falciparum circumsporozoite protein*. J Immunol, 2002. **169**(12): p. 6681-5.
78. Porter, M.D., et al., *Transgenic parasites stably expressing full-length Plasmodium falciparum circumsporozoite protein as a model for vaccine down-selection in mice using sterile protection as an endpoint*. Clin Vaccine Immunol, 2013. **20**(6): p. 803-10.
79. Ramjanee, S., et al., *The use of transgenic Plasmodium berghei expressing the Plasmodium vivax antigen P25 to determine the transmission-blocking activity of sera from malaria vaccine trials*. Vaccine, 2007. **25**(5): p. 886-94.
80. Wengelnik, K., et al., *The A-domain and the thrombospondin-related motif of Plasmodium falciparum TRAP are implicated in the invasion process of mosquito salivary glands*. EMBO J, 1999. **18**(19): p. 5195-204.
81. Jacobs-Lorena, V.Y., et al., *A dispensable Plasmodium locus for stable transgene expression*. Mol Biochem Parasitol, 2010. **171**(1): p. 40-4.
82. Kocken, C.H., et al., *Precise timing of expression of a Plasmodium falciparum-derived transgene in Plasmodium berghei is a critical determinant of subsequent subcellular localization*. J Biol Chem, 1998. **273**(24): p. 15119-24.
83. Scott, J.A., et al., *Relation between falciparum malaria and bacteraemia in Kenyan children: a population-based, case-control study and a longitudinal study*. Lancet, 2011. **378**(9799): p. 1316-23.
84. Leech, J.H., et al., *Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes*. J Exp Med, 1984. **159**(6): p. 1567-75.

85. Newbold, C.I., et al., *PfEMP1, polymorphism and pathogenesis*. Ann Trop Med Parasitol, 1997. **91**(5): p. 551-7.
86. Rowe, J.A., et al., *P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1*. Nature, 1997. **388**(6639): p. 292-5.
87. Doumbo, O.K., et al., *High levels of Plasmodium falciparum rosetting in all clinical forms of severe malaria in African children*. Am J Trop Med Hyg, 2009. **81**(6): p. 987-93.
88. Pongponratn, E., et al., *An ultrastructural study of the brain in fatal Plasmodium falciparum malaria*. Am J Trop Med Hyg, 2003. **69**(4): p. 345-59.
89. White, N.J., et al., *Malaria*. Lancet, 2013.
90. Turner, G.D., et al., *An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration*. Am J Pathol, 1994. **145**(5): p. 1057-69.
91. Newbold, C., et al., *Receptor-specific adhesion and clinical disease in Plasmodium falciparum*. Am J Trop Med Hyg, 1997. **57**(4): p. 389-98.
92. Mota, M.M., et al., *Plasmodium chabaudi-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way*. Infect Immun, 2000. **68**(7): p. 4135-44.
93. McCormick, C.J., et al., *Intercellular adhesion molecule-1 and CD36 synergize to mediate adherence of Plasmodium falciparum-infected erythrocytes to cultured human microvascular endothelial cells*. J Clin Invest, 1997. **100**(10): p. 2521-9.
94. Ho, M. and N.J. White, *Molecular mechanisms of cytoadherence in malaria*. Am J Physiol, 1999. **276**(6 Pt 1): p. C1231-42.
95. Yadava, A., et al., *Trafficking of Plasmodium chabaudi adami-infected erythrocytes within the mouse spleen*. Proc Natl Acad Sci U S A, 1996. **93**(10): p. 4595-9.
96. Engwerda, C.R., L. Beattie, and F.H. Amante, *The importance of the spleen in malaria*. Trends Parasitol, 2005. **21**(2): p. 75-80.
97. Kumar, S., et al., *Interdependence of CD4+ T cells and malarial spleen in immunity to Plasmodium vinckei vinckei. Relevance to vaccine development*. J Immunol, 1989. **143**(6): p. 2017-23.

98. Cunnington, A.J., E.M. Riley, and M. Walther, *Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes*. Trends Parasitol, 2013. **29**(12): p. 585-92.
99. Looareesuwan, S., et al., *Dynamic alteration in splenic function during acute falciparum malaria*. N Engl J Med, 1987. **317**(11): p. 675-9.
100. Buffet, P.A., et al., *The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology*. Blood, 2011. **117**(2): p. 381-92.
101. Lamikanra, A.A., et al., *Malarial anemia: of mice and men*. Blood, 2007. **110**(1): p. 18-28.
102. Awandare, G.A., et al., *Increased levels of inflammatory mediators in children with severe Plasmodium falciparum malaria with respiratory distress*. J Infect Dis, 2006. **194**(10): p. 1438-46.
103. Ayimba, E., et al., *Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria*. Clin Exp Immunol, 2011. **166**(2): p. 218-26.
104. Felli, N., et al., *Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis*. J Immunol, 2005. **175**(3): p. 1464-72.
105. Martiney, J.A., et al., *Macrophage migration inhibitory factor release by macrophages after ingestion of Plasmodium chabaudi-infected erythrocytes: possible role in the pathogenesis of malarial anemia*. Infect Immun, 2000. **68**(4): p. 2259-67.
106. Owusu-Agyei, S., et al., *Incidence of symptomatic and asymptomatic Plasmodium falciparum infection following curative therapy in adult residents of northern Ghana*. Am J Trop Med Hyg, 2001. **65**(3): p. 197-203.
107. Baird, J.K., et al., *Age-dependent susceptibility to severe disease with primary exposure to Plasmodium falciparum*. J Infect Dis, 1998. **178**(2): p. 592-5.
108. Smith, T., et al., *Premunition in Plasmodium falciparum infection: insights from the epidemiology of multiple infections*. Trans R Soc Trop Med Hyg, 1999. **93 Suppl 1**: p. 59-64.
109. Gupta, S., et al., *Immunity to non-cerebral severe malaria is acquired after one or two infections*. Nat Med, 1999. **5**(3): p. 340-3.
110. Okell, L.C., et al., *Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis*. J Infect Dis, 2009. **200**(10): p. 1509-17.
111. Medzhitov, R., D.S. Schneider, and M.P. Soares, *Disease tolerance as a defense strategy*. Science, 2012. **335**(6071): p. 936-41.

112. Menendez, C., et al., *Development of vaccines to prevent malaria in pregnant women: WHO MALVAC meeting report*. Expert Rev Vaccines, 2011. **10**(9): p. 1271-80.
113. Finney, O.C., E.M. Riley, and M. Walther, *Regulatory T cells in malaria--friend or foe?* Trends Immunol, 2010. **31**(2): p. 63-70.
114. Hansen, D.S. and L. Schofield, *Natural regulatory T cells in malaria: host or parasite allies?* PLoS Pathog, 2010. **6**(4): p. e1000771.
115. Riley, E.M., et al., *Regulating immunity to malaria*. Parasite Immunol, 2006. **28**(1-2): p. 35-49.
116. Liehl, P. and M.M. Mota, *Innate recognition of malarial parasites by mammalian hosts*. Int J Parasitol, 2012. **42**(6): p. 557-66.
117. Offeddu, V., et al., *Naturally acquired immune responses against Plasmodium falciparum sporozoites and liver infection*. Int J Parasitol, 2012. **42**(6): p. 535-48.
118. Riley, E.M. and V.A. Stewart, *Immune mechanisms in malaria: new insights in vaccine development*. Nat Med, 2013. **19**(2): p. 168-78.
119. Crispe, I.N., et al., *Cellular and molecular mechanisms of liver tolerance*. Immunol Rev, 2006. **213**: p. 101-18.
120. Jenne, C.N. and P. Kubes, *Immune surveillance by the liver*. Nat Immunol, 2013. **14**(10): p. 996-1006.
121. Medzhitov, R., *Recognition of microorganisms and activation of the immune response*. Nature, 2007. **449**(7164): p. 819-26.
122. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition*. Int Immunol, 2009. **21**(4): p. 317-37.
123. Epiphonio, S., et al., *Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection*. Cell Host Microbe, 2008. **3**(5): p. 331-8.
124. Khan, Z.M. and J.P. Vanderberg, *Role of host cellular response in differential susceptibility of nonimmunized BALB/c mice to Plasmodium berghei and Plasmodium yoelii sporozoites*. Infect Immun, 1991. **59**(8): p. 2529-34.
125. van de Sand, C., et al., *The liver stage of Plasmodium berghei inhibits host cell apoptosis*. Mol Microbiol, 2005. **58**(3): p. 731-42.
126. Khan, Z.M., C. Ng, and J.P. Vanderberg, *Early hepatic stages of Plasmodium berghei: release of circumsporozoite protein and host cellular inflammatory response*. Infect Immun, 1992. **60**(1): p. 264-70.

127. Scheller, L.F., R.A. Wirtz, and A.F. Azad, *Susceptibility of different strains of mice to hepatic infection with Plasmodium berghei*. Infect Immun, 1994. **62**(11): p. 4844-7.
128. Usynin, I., C. Klotz, and U. Frevert, *Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells*. Cell Microbiol, 2007. **9**(11): p. 2610-28.
129. Singh, A.P., et al., *Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite*. Cell, 2007. **131**(3): p. 492-504.
130. Leiriao, P., et al., *HGF/MET signalling protects Plasmodium-infected host cells from apoptosis*. Cell Microbiol, 2005. **7**(4): p. 603-9.
131. Rennenberg, A., et al., *Exoerythrocytic Plasmodium parasites secrete a cysteine protease inhibitor involved in sporozoite invasion and capable of blocking cell death of host hepatocytes*. PLoS Pathog, 2010. **6**(3): p. e1000825.
132. Ockenhouse, C.F., et al., *Common and divergent immune response signaling pathways discovered in peripheral blood mononuclear cell gene expression patterns in presymptomatic and clinically apparent malaria*. Infect Immun, 2006. **74**(10): p. 5561-73.
133. Walther, M., et al., *Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage Plasmodium falciparum correlate with parasitological and clinical outcomes*. J Immunol, 2006. **177**(8): p. 5736-45.
134. Gazzinelli, R.T. and E.Y. Denkers, *Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism*. Nat Rev Immunol, 2006. **6**(12): p. 895-906.
135. Krishnegowda, G., et al., *Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity*. J Biol Chem, 2005. **280**(9): p. 8606-16.
136. Coban, C., et al., *Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin*. J Exp Med, 2005. **201**(1): p. 19-25.
137. Zhu, J., G. Krishnegowda, and D.C. Gowda, *Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: the requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-kappaB pathways for the expression of proinflammatory cytokines and nitric oxide*. J Biol Chem, 2005. **280**(9): p. 8617-27.

138. Parroche, P., et al., *Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9*. Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1919-24.
139. Schofield, L., et al., *Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria*. Nature, 2002. **418**(6899): p. 785-9.
140. Coban, C., et al., *Effect of CpG oligodeoxynucleotides on the immunogenicity of Pfs25, a Plasmodium falciparum transmission-blocking vaccine antigen*. Infect Immun, 2004. **72**(1): p. 584-8.
141. Kumar, S., et al., *CpG oligodeoxynucleotide and Montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine*. Infect Immun, 2004. **72**(2): p. 949-57.
142. Pinzon-Charry, A., et al., *Low doses of killed parasite in CpG elicit vigorous CD4+ T cell responses against blood-stage malaria in mice*. J Clin Invest, 2010. **120**(8): p. 2967-78.
143. Coban, C., et al., *The malarial metabolite hemozoin and its potential use as a vaccine adjuvant*. Allergol Int, 2010. **59**(2): p. 115-24.
144. Jensen, P.E., *Recent advances in antigen processing and presentation*. Nat Immunol, 2007. **8**(10): p. 1041-8.
145. Bevan, M.J., *Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming*. J Immunol, 1976. **117**(6): p. 2233-8.
146. Amigorena, S. and A. Savina, *Intracellular mechanisms of antigen cross presentation in dendritic cells*. Curr Opin Immunol, 2010. **22**(1): p. 109-17.
147. Crotzer, V.L. and J.S. Blum, *Autophagy and adaptive immunity*. Immunology, 2010. **131**(1): p. 9-17.
148. Vyas, J.M., A.G. Van der Veen, and H.L. Ploegh, *The known unknowns of antigen processing and presentation*. Nat Rev Immunol, 2008. **8**(8): p. 607-18.
149. Janeway CA Jr, T.P., Walport M, et al. , *Immunobiology: The Immune System in Health and Disease*. 5th Edition ed. 2001, New York: Garland Science.
150. Klein, I. and I.N. Crispe, *Complete differentiation of CD8+ T cells activated locally within the transplanted liver*. J Exp Med, 2006. **203**(2): p. 437-47.
151. Winau, F., et al., *Ito cells are liver-resident antigen-presenting cells for activating T cell responses*. Immunity, 2007. **26**(1): p. 117-29.
152. Portugal, S., et al., *Host-mediated regulation of superinfection in malaria*. Nat Med, 2011. **17**(6): p. 732-7.

153. Vaughan, A.M. and S.H. Kappe, *Malaria vaccine development: persistent challenges*. Curr Opin Immunol, 2012. **24**(3): p. 324-31.
154. John, C.C., et al., *Cytokine responses to Plasmodium falciparum liver-stage antigen 1 vary in rainy and dry seasons in highland Kenya*. Infect Immun, 2000. **68**(9): p. 5198-204.
155. Kurtis, J.D., et al., *Interleukin-10 responses to liver-stage antigen 1 predict human resistance to Plasmodium falciparum*. Infect Immun, 1999. **67**(7): p. 3424-9.
156. Luty, A.J., et al., *Parasite antigen-specific interleukin-10 and antibody responses predict accelerated parasite clearance in Plasmodium falciparum malaria*. Eur Cytokine Netw, 1998. **9**(4): p. 639-46.
157. Migot-Nabias, F., et al., *Immune response to Plasmodium falciparum liver stage antigen-1: geographical variations within Central Africa and their relationship with protection from clinical malaria*. Trans R Soc Trop Med Hyg, 2000. **94**(5): p. 557-62.
158. Todryk, S.M., et al., *Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans*. PLoS One, 2008. **3**(4): p. e2027.
159. Reece, W.H., et al., *A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease*. Nat Med, 2004. **10**(4): p. 406-10.
160. Ewer, K.J., *Protective CD8+ T cell Immunity to Human Malaria Induced by Chimpanzee Adenovirus-MVA Immunisation*. Nature Communications, 2013. **In Press**.
161. Rodrigues, E.G., et al., *Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria*. J Immunol, 1997. **158**(3): p. 1268-74.
162. Rodrigues, M., R.S. Nussenzweig, and F. Zavala, *The relative contribution of antibodies, CD4+ and CD8+ T cells to sporozoite-induced protection against malaria*. Immunology, 1993. **80**(1): p. 1-5.
163. Schneider, J., et al., *Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara*. Nat Med, 1998. **4**(4): p. 397-402.
164. Weiss, W.R., et al., *CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites*. Proc Natl Acad Sci U S A, 1988. **85**(2): p. 573-6.

165. Hoffman, S.L., et al., *Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes*. Science, 1989. **244**(4908): p. 1078-81.
166. Renia, L., et al., *Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against Plasmodium yoelii liver stages*. J Immunol, 1993. **150**(4): p. 1471-8.
167. Renia, L., et al., *In vitro activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide*. Proc Natl Acad Sci U S A, 1991. **88**(18): p. 7963-7.
168. Trimnell, A., et al., *Genetically attenuated parasite vaccines induce contact-dependent CD8+ T cell killing of Plasmodium yoelii liver stage-infected hepatocytes*. J Immunol, 2009. **183**(9): p. 5870-8.
169. Warren, A., et al., *T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells*. Hepatology, 2006. **44**(5): p. 1182-90.
170. Balam, S., et al., *CSP--a model for in vivo presentation of Plasmodium berghei sporozoite antigens by hepatocytes*. PLoS One, 2012. **7**(12): p. e51875.
171. Leiriao, P., M.M. Mota, and A. Rodriguez, *Apoptotic Plasmodium-infected hepatocytes provide antigens to liver dendritic cells*. J Infect Dis, 2005. **191**(10): p. 1576-81.
172. Parker, G.A. and C.A. Picut, *Liver immunobiology*. Toxicol Pathol, 2005. **33**(1): p. 52-62.
173. Carvalho, L.H., C.J. Fontes, and A.U. Krettli, *Cellular responses to Plasmodium falciparum major surface antigens and their relationship to human activities associated with malaria transmission*. Am J Trop Med Hyg, 1999. **60**(4): p. 674-9.
174. Elloso, M.M., et al., *Inhibition of Plasmodium falciparum in vitro by human gamma delta T cells*. J Immunol, 1994. **153**(3): p. 1187-94.
175. Pombo, D.J., et al., *Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum*. Lancet, 2002. **360**(9333): p. 610-7.
176. Riley, E.M., et al., *Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of Plasmodium falciparum are associated with reduced malaria morbidity*. Parasite Immunol, 1992. **14**(3): p. 321-37.

177. John, C.C., et al., *Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection*. Am J Trop Med Hyg, 2005. **73**(1): p. 222-8.
178. John, C.C., et al., *Antibodies to pre-erythrocytic Plasmodium falciparum antigens and risk of clinical malaria in Kenyan children*. J Infect Dis, 2008. **197**(4): p. 519-26.
179. White, M.T., et al., *The relationship between RTS,S vaccine-induced antibodies, CD4(+) T cell responses and protection against Plasmodium falciparum infection*. PLoS One, 2013. **8**(4): p. e61395.
180. Cohen, S., G.I. Mc, and S. Carrington, *Gamma-globulin and acquired immunity to human malaria*. Nature, 1961. **192**: p. 733-7.
181. Sabchareon, A., et al., *Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria*. Am J Trop Med Hyg, 1991. **45**(3): p. 297-308.
182. Astagneau, P., et al., *Antibodies to the 4-mer repeat of the ring-infected erythrocyte surface antigen (Pf155/RESA) protect against Plasmodium falciparum malaria*. Int J Epidemiol, 1994. **23**(1): p. 169-75.
183. Astagneau, P., et al., *Antibodies to a Plasmodium falciparum blood-stage antigen as a tool for predicting the protection levels of two malaria-exposed populations*. Am J Trop Med Hyg, 1995. **53**(1): p. 23-8.
184. Astagneau, P., et al., *Antibodies to ring-infected erythrocyte surface antigen (Pf155/RESA) protect against P. falciparum parasitemia in highly exposed multigravidas women in Malawi*. Acta Trop, 1994. **57**(4): p. 317-25.
185. Bull, P.C., et al., *Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria*. Nat Med, 1998. **4**(3): p. 358-60.
186. Chizzolini, C., et al., *Natural antibodies against three distinct and defined antigens of Plasmodium falciparum in residents of a mesoendemic area in Gabon*. Am J Trop Med Hyg, 1988. **39**(2): p. 150-6.
187. Crompton, P.D., et al., *A prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray*. Proc Natl Acad Sci U S A, 2010. **107**(15): p. 6958-63.
188. Marsh, K. and S. Kinyanjui, *Immune effector mechanisms in malaria*. Parasite Immunol, 2006. **28**(1-2): p. 51-60.
189. Polley, S.D., et al., *Human antibodies to recombinant protein constructs of Plasmodium falciparum Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria*. Vaccine, 2004. **23**(5): p. 718-28.

190. Wipasa, J., et al., *Long-lived antibody and B Cell memory responses to the human malaria parasites, Plasmodium falciparum and Plasmodium vivax*. PLoS Pathog, 2010. **6**(2): p. e1000770.
191. Fowkes, F.J., et al., *The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis*. PLoS Med, 2010. **7**(1): p. e1000218.
192. Beeson, J.G., F.H. Osier, and C.R. Engwerda, *Recent insights into humoral and cellular immune responses against malaria*. Trends Parasitol, 2008. **24**(12): p. 578-84.
193. Genton, B., et al., *A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase I-2b trial in Papua New Guinea*. J Infect Dis, 2002. **185**(6): p. 820-7.
194. Sheehy, S.H., et al., *ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans*. Mol Ther, 2012. **20**(12): p. 2355-68.
195. Spring, M.D., et al., *Phase I/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A*. PLoS One, 2009. **4**(4): p. e5254.
196. *Complete List of Vaccines Licensed for Immunization and Distribution in the US*. 2013 3/09 [cited 2013 1/09]; Available from: <http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM093833>.
197. McAleer, W.J., et al., *Human hepatitis B vaccine from recombinant yeast*. Nature, 1984. **307**(5947): p. 178-80.
198. Donegan, S., R. Bellamy, and C.L. Gamble, *Vaccines for preventing anthrax*. Cochrane Database Syst Rev, 2009(2): p. CD006403.
199. Wheeler, C.M., et al., *Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial*. Lancet Oncol, 2012. **13**(1): p. 100-10.
200. Russell, P.F. and B.N. Mohan, *THE IMMUNIZATION OF FOWLS AGAINST MOSQUITO-BORNE PLASMODIUM GALLINACEUM BY INJECTIONS OF SERUM AND OF INACTIVATED HOMOLOGOUS SPOROZOITES*. J Exp Med, 1942. **76**(5): p. 477-95.
201. Mellouk, S., et al., *Protection against malaria induced by irradiated sporozoites*. Lancet, 1990. **335**(8691): p. 721.

202. Schofield, L., et al., *Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites*. Nature, 1987. **330**(6149): p. 664-6.
203. Seguin, M.C., et al., *Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated Plasmodium berghei infected mosquitoes: involvement of interferon gamma and CD8+ T cells*. J Exp Med, 1994. **180**(1): p. 353-8.
204. Weiss, W.R. and C.G. Jiang, *Protective CD8+ T lymphocytes in primates immunized with malaria sporozoites*. PLoS One, 2012. **7**(2): p. e31247.
205. Hoffman, S.L., et al., *Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria*. Hum Vaccin, 2010. **6**(1): p. 97-106.
206. Epstein, J.E., et al., *Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity*. Science, 2011. **334**(6055): p. 475-80.
207. Douradinha, B., et al., *Immunization with genetically attenuated P52-deficient Plasmodium berghei sporozoites induces a long-lasting effector memory CD8+ T cell response in the liver*. J Immune Based Ther Vaccines, 2011. **9**(1): p. 6.
208. Mueller, A.K., et al., *Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface*. Proc Natl Acad Sci U S A, 2005. **102**(8): p. 3022-7.
209. van Dijk, M.R., et al., *Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells*. Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12194-9.
210. Butler, N.S., et al., *Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites*. Cell Host Microbe, 2011. **9**(6): p. 451-62.
211. Tarun, A.S., et al., *Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells*. J Infect Dis, 2007. **196**(4): p. 608-16.
212. Annoura, T., et al., *Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates*. Vaccine, 2012. **30**(16): p. 2662-70.
213. Khan, S.M., et al., *Genetic engineering of attenuated malaria parasites for vaccination*. Curr Opin Biotechnol, 2012. **23**(6): p. 908-16.

214. VanBuskirk, K.M., et al., *Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design*. Proc Natl Acad Sci U S A, 2009. **106**(31): p. 13004-9.
215. Purcell, L.A., et al., *Chemically attenuated Plasmodium sporozoites induce specific immune responses, sterile immunity and cross-protection against heterologous challenge*. Vaccine, 2008. **26**(38): p. 4880-4.
216. Purcell, L.A., et al., *Chemical attenuation of Plasmodium berghei sporozoites induces sterile immunity in mice*. Infect Immun, 2008. **76**(3): p. 1193-9.
217. Yayon, A., et al., *Stage-dependent effects of chloroquine on Plasmodium falciparum in vitro*. J Protozool, 1983. **30**(4): p. 642-7.
218. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation*. N Engl J Med, 2009. **361**(5): p. 468-77.
219. Roestenberg, M., et al., *Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study*. Lancet, 2011. **377**(9779): p. 1770-6.
220. Belnoue, E., et al., *Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment*. J Immunol, 2004. **172**(4): p. 2487-95.
221. Bijker, E.M., et al., *Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity*. Proc Natl Acad Sci U S A, 2013. **110**(19): p. 7862-7.
222. McCarthy, J.S. and M.F. Good, *Whole parasite blood stage malaria vaccines: a convergence of evidence*. Hum Vaccin, 2010. **6**(1): p. 114-23.
223. Hill, A.V., *Vaccines against malaria*. Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1579): p. 2806-14.
224. Gardner, M.J., et al., *Genome sequence of the human malaria parasite Plasmodium falciparum*. Nature, 2002. **419**(6906): p. 498-511.
225. Gwadz, R.W., et al., *Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of Plasmodium knowlesi and characterization of surface antigens of these parasites*. Bull World Health Organ, 1979. **57 Suppl 1**: p. 165-73.
226. Nardin, E.H., et al., *Circumsporozoite proteins of human malaria parasites Plasmodium falciparum and Plasmodium vivax*. J Exp Med, 1982. **156**(1): p. 20-30.
227. Schwartz, L., et al., *A review of malaria vaccine clinical projects based on the WHO rainbow table*. Malar J, 2012. **11**: p. 11.

228. Duffy, P.E., et al., *Pre-erythrocytic malaria vaccines: identifying the targets*. Expert Rev Vaccines, 2012. **11**(10): p. 1261-80.
229. Angov, E., et al., *Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host*. PLoS One, 2008. **3**(5): p. e2189.
230. Kester, K.E., et al., *Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naïve adults: safety, efficacy, and immunologic associates of protection*. J Infect Dis, 2009. **200**(3): p. 337-46.
231. Olotu, A.I., G. Fegan, and P. Bejon, *Further analysis of correlates of protection from a phase 2a trial of the falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naïve adults*. J Infect Dis, 2010. **201**(6): p. 970-1.
232. Cummings, J.F., et al., *Recombinant Liver Stage Antigen-1 (LSA-1) formulated with AS01 or AS02 is safe, elicits high titer antibody and induces IFN-gamma/IL-2 CD4+ T cells but does not protect against experimental Plasmodium falciparum infection*. Vaccine, 2010. **28**(31): p. 5135-44.
233. Zhu, Q., et al., *Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines*. Proc Natl Acad Sci U S A, 2008. **105**(42): p. 16260-5.
234. Schwarz, K., et al., *Role of Toll-like receptors in costimulating cytotoxic T cell responses*. Eur J Immunol, 2003. **33**(6): p. 1465-70.
235. Sjolander, S., et al., *Intranasal immunisation with influenza-ISCOM induces strong mucosal as well as systemic antibody and cytotoxic T-lymphocyte responses*. Vaccine, 2001. **19**(28-29): p. 4072-80.
236. McCluskie, M.J., et al., *CpG ODN and ISCOMATRIX adjuvant: a synergistic adjuvant combination inducing strong T-Cell IFN-gamma responses*. Biomed Res Int, 2013. **2013**: p. 636847.
237. Lauterbach, H., et al., *Genetic Adjuvantation of Recombinant MVA with CD40L Potentiates CD8 T Cell Mediated Immunity*. Front Immunol, 2013. **4**: p. 251.
238. Rowe, H.M., et al., *Immunization with a lentiviral vector stimulates both CD4 and CD8 T cell responses to an ovalbumin transgene*. Mol Ther, 2006. **13**(2): p. 310-9.
239. Hoffman, S.L., M. Sedegah, and R.C. Hedstrom, *Protection against malaria by immunization with a Plasmodium yoelii circumsporozoite protein nucleic acid vaccine*. Vaccine, 1994. **12**(16): p. 1529-33.

240. Sedegah, M., et al., *Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein*. Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9866-70.
241. Doolan, D.L. and S.L. Hoffman, *DNA-based vaccines against malaria: status and promise of the Multi-Stage Malaria DNA Vaccine Operation*. Int J Parasitol, 2001. **31**(8): p. 753-62.
242. Epstein, J.E., et al., *Safety, tolerability, and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes*. Hum Gene Ther, 2002. **13**(13): p. 1551-60.
243. Wang, R., et al., *Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine*. Science, 1998. **282**(5388): p. 476-80.
244. Wang, R., et al., *Induction of CD4(+) T cell-dependent CD8(+) type I responses in humans by a malaria DNA vaccine*. Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10817-22.
245. Dunachie, S.J., et al., *A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naïve adults against Plasmodium falciparum sporozoite challenge*. Infect Immun, 2006. **74**(10): p. 5933-42.
246. Gilbert, S.C., et al., *Ty virus-like particles, DNA vaccines and Modified Vaccinia Virus Ankara; comparisons and combinations*. Biol Chem, 1999. **380**(3): p. 299-303.
247. McConkey, S.J., et al., *Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans*. Nat Med, 2003. **9**(6): p. 729-35.
248. Rogers, W.O., et al., *Protection of rhesus macaques against lethal Plasmodium knowlesi malaria by a heterologous DNA priming and poxvirus boosting immunization regimen*. Infect Immun, 2002. **70**(8): p. 4329-35.
249. Sedegah, M., et al., *Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine*. Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7648-53.
250. Degano, P., et al., *Gene gun intradermal DNA immunization followed by boosting with modified vaccinia virus Ankara: enhanced CD8+ T cell immunogenicity and protective efficacy in the influenza and malaria models*. Vaccine, 1999. **18**(7-8): p. 623-32.

251. Richie, T.L., et al., *Clinical trial in healthy malaria-naïve adults to evaluate the safety, tolerability, immunogenicity and efficacy of MuStDO5, a five-gene, sporozoite/hepatic stage Plasmodium falciparum DNA vaccine combined with escalating dose human GM-CSF DNA*. Hum Vaccin Immunother, 2012. **8**(11): p. 1564-84.
252. Watts, A.M. and R.C. Kennedy, *DNA vaccination strategies against infectious diseases*. Int J Parasitol, 1999. **29**(8): p. 1149-63.
253. Ferraro, B., et al., *Inducing humoral and cellular responses to multiple sporozoite and liver stage malaria antigens using pDNA*. Infect Immun, 2013.
254. Dobaño, C., et al., *Enhancement of antibody and cellular immune responses to malaria DNA vaccines by in vivo electroporation*. Vaccine, 2007. **25**(36): p. 6635-45.
255. LeBlanc, R., et al., *Markedly enhanced immunogenicity of a Pfs25 DNA-based malaria transmission-blocking vaccine by in vivo electroporation*. Vaccine, 2008. **26**(2): p. 185-92.
256. O'Hara, G.A., et al., *Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector*. J Infect Dis, 2012. **205**(5): p. 772-81.
257. Sheehy, S.H., et al., *Phase Ia clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors*. PLoS One, 2012. **7**(2): p. e31208.
258. Sheehy, S.H., et al., *Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors*. Mol Ther, 2011. **19**(12): p. 2269-76.
259. Webster, D.P., et al., *Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara*. Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4836-41.
260. Moorthy, V.S., et al., *Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage P. falciparum malaria in non-immune volunteers*. Vaccine, 2003. **21**(17-18): p. 1995-2002.
261. Dudareva, M., et al., *Prevalence of serum neutralizing antibodies against chimpanzee adenovirus 63 and human adenovirus 5 in Kenyan children, in the context of vaccine vector efficacy*. Vaccine, 2009. **27**(27): p. 3501-4.
262. Nwanegbo, E., et al., *Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States*. Clin Diagn Lab Immunol, 2004. **11**(2): p. 351-7.
263. Moorthy, V.S., et al., *Phase I evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month reboosting vaccination, for malaria vaccination in Gambian men*. J Infect Dis, 2004. **189**(12): p. 2213-9.

264. Pincha, M., B.S. Sundarasetty, and R. Stripecke, *Lentiviral vectors for immunization: an inflammatory field*. Expert Rev Vaccines, 2010. **9**(3): p. 309-21.
265. Tangy, F. and H.Y. Naim, *Live attenuated measles vaccine as a potential multivalent pediatric vaccination vector*. Viral Immunol, 2005. **18**(2): p. 317-26.
266. Monath, T.P., et al., *Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen*. J Infect Dis, 2003. **188**(8): p. 1213-30.
267. Hansen, S.G., et al., *Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine*. Nature, 2011. **473**(7348): p. 523-7.
268. Mayr, A., et al., *[The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]*. Zentralbl Bakteriell B, 1978. **167**(5-6): p. 375-90.
269. Meisinger-Henschel, C., et al., *Genomic sequence of chorioallantois vaccinia virus Ankara, the ancestor of modified vaccinia virus Ankara*. J Gen Virol, 2007. **88**(Pt 12): p. 3249-59.
270. Im, E.J. and T. Hanke, *MVA as a vector for vaccines against HIV-1*. Expert Rev Vaccines, 2004. **3**(4 Suppl): p. S89-97.
271. Mwau, M., et al., *A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans*. J Gen Virol, 2004. **85**(Pt 4): p. 911-9.
272. Fournillier, A., et al., *An accelerated vaccine schedule with a poly-antigenic hepatitis C virus MVA-based candidate vaccine induces potent, long lasting and in vivo cross-reactive T cell responses*. Vaccine, 2007. **25**(42): p. 7339-53.
273. Harrop, R., et al., *Cross-trial analysis of immunologic and clinical data resulting from phase I and II trials of MVA-5T4 (TroVax) in colorectal, renal, and prostate cancer patients*. J Immunother, 2010. **33**(9): p. 999-1005.
274. McShane, H., et al., *Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials*. Tuberculosis (Edinb), 2005. **85**(1-2): p. 47-52.
275. Tameris, M.D., et al., *Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial*. Lancet, 2013. **381**(9871): p. 1021-8.

276. Anderson, R.J., et al., *Enhanced CD8+ T cell immune responses and protection elicited against Plasmodium berghei malaria by prime boost immunization regimens using a novel attenuated fowlpox virus*. J Immunol, 2004. **172**(5): p. 3094-100.
277. Tartaglia, J., et al., *NYVAC: a highly attenuated strain of vaccinia virus*. Virology, 1992. **188**(1): p. 217-32.
278. Ockenhouse, C.F., et al., *Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria*. J Infect Dis, 1998. **177**(6): p. 1664-73.
279. Taylor, J. and E. Paoletti, *Fowlpox virus as a vector in non-avian species*. Vaccine, 1988. **6**(6): p. 466-8.
280. Vuola, J.M., et al., *Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers*. J Immunol, 2005. **174**(1): p. 449-55.
281. Bejon, P., et al., *A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya*. PLoS Clin Trials, 2006. **1**(6): p. e29.
282. Bejon, P., et al., *Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya*. PLoS One, 2007. **2**(8): p. e707.
283. Ophorst, O.J., et al., *Immunogenicity and protection of a recombinant human adenovirus serotype 35-based malaria vaccine against Plasmodium yoelii in mice*. Infect Immun, 2006. **74**(1): p. 313-20.
284. Rodriguez, A., et al., *Impact of recombinant adenovirus serotype 35 priming versus boosting of a Plasmodium falciparum protein: characterization of T- and B-cell responses to liver-stage antigen 1*. Infect Immun, 2008. **76**(4): p. 1709-18.
285. Reyes-Sandoval, A., et al., *Single-dose immunogenicity and protective efficacy of simian adenoviral vectors against Plasmodium berghei*. Eur J Immunol, 2008. **38**(3): p. 732-41.
286. Chuang, I., et al., *DNA prime/Adenovirus boost malaria vaccine encoding P. falciparum CSP and AMA1 induces sterile protection associated with cell-mediated immunity*. PLoS One, 2013. **8**(2): p. e55571.
287. Sedegah, M., et al., *Adenovirus 5-vectored P. falciparum vaccine expressing CSP and AMA1. Part A: safety and immunogenicity in seronegative adults*. PLoS One, 2011. **6**(10): p. e24586.

288. Tamminga, C., et al., *Adenovirus-5-vectored P. falciparum vaccine expressing CSP and AMA1. Part B: safety, immunogenicity and protective efficacy of the CSP component*. PLoS One, 2011. **6**(10): p. e25868.
289. Casimiro, D.R., et al., *Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene*. J Virol, 2003. **77**(11): p. 6305-13.
290. Casimiro, D.R., et al., *Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type 5 vectors expressing a human immunodeficiency virus type 1 gag gene*. J Virol, 2003. **77**(13): p. 7663-8.
291. Miao, J., et al., *Immune responses in mice induced by prime-boost schemes of the Plasmodium falciparum apical membrane antigen 1 (PfAMA1)-based DNA, protein and recombinant modified vaccinia Ankara vaccines*. Vaccine, 2006. **24**(37-39): p. 6187-98.
292. Capone, S., et al., *Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates*. Vaccine, 2010. **29**(2): p. 256-65.
293. Draper, S.J., et al., *Recombinant viral vaccines expressing merozoite surface protein-1 induce antibody- and T cell-mediated multistage protection against malaria*. Cell Host Microbe, 2009. **5**(1): p. 95-105.
294. Draper, S.J., et al., *Effective induction of high-titer antibodies by viral vector vaccines*. Nat Med, 2008. **14**(8): p. 819-21.
295. Goodman, A.L., et al., *New candidate vaccines against blood-stage Plasmodium falciparum malaria: prime-boost immunization regimens incorporating human and simian adenoviral vectors and poxviral vectors expressing an optimized antigen based on merozoite surface protein 1*. Infect Immun, 2010. **78**(11): p. 4601-12.
296. Reyes-Sandoval, A., et al., *Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses*. Infect Immun, 2010. **78**(1): p. 145-53.
297. Bruna-Romero, O., et al., *Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen*. Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11491-6.
298. Li, S., et al., *Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria*. Proc Natl Acad Sci U S A, 1993. **90**(11): p. 5214-8.

299. de Cassan, S.C., et al., *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*. J Immunol, 2011. **187**(5): p. 2602-16.
300. Douglas, A.D., et al., *Tailoring subunit vaccine immunogenicity: maximizing antibody and T cell responses by using combinations of adenovirus, poxvirus and protein-adjuvant vaccines against Plasmodium falciparum MSP1*. Vaccine, 2010. **28**(44): p. 7167-78.
301. Draper, S.J., et al., *Enhancing blood-stage malaria subunit vaccine immunogenicity in rhesus macaques by combining adenovirus, poxvirus, and protein-in-adjuvant vaccines*. J Immunol, 2010. **185**(12): p. 7583-95.
302. Stewart, V.A., et al., *Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to Plasmodium falciparum CS compared to that with either malaria vaccine alone*. Infect Immun, 2007. **75**(5): p. 2283-90.
303. Cohen, J., et al., *From the circumsporozoite protein to the RTS, S/AS candidate vaccine*. Hum Vaccin, 2010. **6**(1): p. 90-6.
304. Nussenzweig, V. and R.S. Nussenzweig, *Circumsporozoite proteins of malaria parasites*. Cell, 1985. **42**(2): p. 401-3.
305. Nussenzweig, V. and R.S. Nussenzweig, *Circumsporozoite proteins of malaria parasites*. Bull Mem Acad R Med Belg, 1989. **144**(11): p. 493-504.
306. Yoshida, N., et al., *Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite*. Science, 1980. **207**(4426): p. 71-3.
307. Wang, Q., H. Fujioka, and V. Nussenzweig, *Exit of Plasmodium sporozoites from oocysts is an active process that involves the circumsporozoite protein*. PLoS Pathog, 2005. **1**(1): p. e9.
308. Rathore, D., et al., *Binding and invasion of liver cells by Plasmodium falciparum sporozoites. Essential involvement of the amino terminus of circumsporozoite protein*. J Biol Chem, 2002. **277**(9): p. 7092-8.
309. Frevert, U., et al., *Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes*. J Exp Med, 1993. **177**(5): p. 1287-98.
310. Pradel, G., S. Garapaty, and U. Frevert, *Proteoglycans mediate malaria sporozoite targeting to the liver*. Mol Microbiol, 2002. **45**(3): p. 637-51.
311. Zavala, F., et al., *Rationale for development of a synthetic vaccine against Plasmodium falciparum malaria*. Science, 1985. **228**(4706): p. 1436-40.

312. Brown, A.E., et al., *Safety, immunogenicity and limited efficacy study of a recombinant Plasmodium falciparum circumsporozoite vaccine in Thai soldiers*. Vaccine, 1994. **12**(2): p. 102-8.
313. Sherwood, J.A., et al., *Plasmodium falciparum circumsporozoite vaccine immunogenicity and efficacy trial with natural challenge quantitation in an area of endemic human malaria of Kenya*. Vaccine, 1996. **14**(8): p. 817-27.
314. Gordon, D.M., et al., *Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine*. J Infect Dis, 1995. **171**(6): p. 1576-85.
315. Garcon, N., P. Chomez, and M. Van Mechelen, *GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives*. Expert Rev Vaccines, 2007. **6**(5): p. 723-39.
316. Ulrich, J.T. and K.R. Myers, *Monophosphoryl lipid A as an adjuvant. Past experiences and new directions*. Pharm Biotechnol, 1995. **6**: p. 495-524.
317. Kensil, C.R., *Saponins as vaccine adjuvants*. Crit Rev Ther Drug Carrier Syst, 1996. **13**(1-2): p. 1-55.
318. Stoute, J.A., et al., *A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group*. N Engl J Med, 1997. **336**(2): p. 86-91.
319. Kester, K.E., et al., *Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria*. J Infect Dis, 2001. **183**(4): p. 640-7.
320. Bojang, K.A., et al., *Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial*. Lancet, 2001. **358**(9297): p. 1927-34.
321. Alonso, P.L., et al., *Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial*. Lancet, 2005. **366**(9502): p. 2012-8.
322. Alonso, P.L., et al., *Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial*. Lancet, 2004. **364**(9443): p. 1411-20.
323. Sacarlal, J., et al., *Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children*. J Infect Dis, 2009. **200**(3): p. 329-36.
324. Abdulla, S., et al., *Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants*. N Engl J Med, 2008. **359**(24): p. 2533-44.

325. Aponte, J.J., et al., *Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial*. Lancet, 2007. **370**(9598): p. 1543-51.
326. Polhemus, M.E., et al., *Evaluation of RTS,S/AS02A and RTS,S/AS01B in adults in a high malaria transmission area*. PLoS One, 2009. **4**(7): p. e6465.
327. Asante, K.P., et al., *Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial*. Lancet Infect Dis, 2011. **11**(10): p. 741-9.
328. Bejon, P., et al., *Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age*. N Engl J Med, 2008. **359**(24): p. 2521-32.
329. Olotu, A., et al., *Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial*. Lancet Infect Dis, 2011. **11**(2): p. 102-9.
330. Lell, B., et al., *A randomized trial assessing the safety and immunogenicity of AS01 and AS02 adjuvanted RTS,S malaria vaccine candidates in children in Gabon*. PLoS One, 2009. **4**(10): p. e7611.
331. Owusu-Agyei, S., et al., *Randomized controlled trial of RTS,S/AS02D and RTS,S/AS01E malaria candidate vaccines given according to different schedules in Ghanaian children*. PLoS One, 2009. **4**(10): p. e7302.
332. Agnandji, S.T., et al., *First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children*. N Engl J Med, 2011. **365**(20): p. 1863-75.
333. Agnandji, S.T., et al., *A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants*. N Engl J Med, 2012. **367**(24): p. 2284-95.
334. Olotu, A., et al., *Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure*. N Engl J Med, 2013. **368**(12): p. 1111-20.
335. Robson, K.J., et al., *A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite*. Nature, 1988. **335**(6185): p. 79-82.
336. Rogers, W.O., et al., *Characterization of the gene encoding sporozoite surface protein 2, a protective Plasmodium yoelii sporozoite antigen*. Mol Biochem Parasitol, 1992. **53**(1-2): p. 45-51.
337. Ghosh, A.K., et al., *Malaria parasite invasion of the mosquito salivary gland requires interaction between the Plasmodium TRAP and the Anopheles saglin proteins*. PLoS Pathog, 2009. **5**(1): p. e1000265.

338. Kappe, S., et al., *Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites*. J Cell Biol, 1999. **147**(5): p. 937-44.
339. Muller, H.M., E. Scarselli, and A. Crisanti, *Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum in parasite-host cell interactions*. Parasitologia, 1993. **35 Suppl**: p. 69-72.
340. Sultan, A.A., et al., *TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites*. Cell, 1997. **90**(3): p. 511-22.
341. Rogers, W.O., et al., *Characterization of Plasmodium falciparum sporozoite surface protein 2*. Proc Natl Acad Sci U S A, 1992. **89**(19): p. 9176-80.
342. Khusmith, S., M. Sedegah, and S.L. Hoffman, *Complete protection against Plasmodium yoelii by adoptive transfer of a CD8+ cytotoxic T-cell clone recognizing sporozoite surface protein 2*. Infect Immun, 1994. **62**(7): p. 2979-83.
343. Gilbert, S.C., et al., *A protein particle vaccine containing multiple malaria epitopes*. Nat Biotechnol, 1997. **15**(12): p. 1280-4.
344. Takala, S.L., et al., *Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development*. Sci Transl Med, 2009. **1**(2): p. 2ra5.
345. Thera, M.A., et al., *A field trial to assess a blood-stage malaria vaccine*. N Engl J Med, 2011. **365**(11): p. 1004-13.
346. Belard, S., et al., *A randomized controlled phase Ib trial of the malaria vaccine candidate GMZ2 in African children*. PLoS One, 2011. **6**(7): p. e22525.
347. Lusingu, J.P., et al., *Satisfactory safety and immunogenicity of MSP3 malaria vaccine candidate in Tanzanian children aged 12-24 months*. Malar J, 2009. **8**: p. 163.
348. Nebie, I., et al., *Humoral and cell-mediated immunity to MSP3 peptides in adults immunized with MSP3 in malaria endemic area, Burkina Faso*. Parasite Immunol, 2009. **31**(8): p. 474-80.
349. Sirima, S.B., et al., *Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children*. PLoS One, 2009. **4**(10): p. e7549.
350. Crosnier, C., et al., *Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum*. Nature, 2011. **480**(7378): p. 534-7.
351. Baum, J., et al., *Reticulocyte-binding protein homologue 5 - an essential adhesin involved in invasion of human erythrocytes by Plasmodium falciparum*. Int J Parasitol, 2009. **39**(3): p. 371-80.

352. Cowman, A.F. and B.S. Crabb, *Invasion of red blood cells by malaria parasites*. Cell, 2006. **124**(4): p. 755-66.
353. Douglas, A.D., et al., *The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody*. Nat Commun, 2011. **2**: p. 601.
354. Patel, S.D., et al., *Plasmodium falciparum merozoite surface antigen, PfRH5, elicits detectable levels of invasion-inhibiting antibodies in humans*. J Infect Dis, 2013. **208**(10): p. 1679-87.
355. Tran, T.M., et al., *Naturally Acquired Antibodies Specific for Plasmodium falciparum Reticulocyte-Binding Protein Homologue 5 Inhibit Parasite Growth and Predict Protection From Malaria*. J Infect Dis, 2013.
356. Kang, J.M., et al., *Limited sequence polymorphisms of four transmission-blocking vaccine candidate antigens in Plasmodium vivax Korean isolates*. Malar J, 2013. **12**: p. 144.
357. Carter, R. and D.H. Chen, *Malaria transmission blocked by immunisation with gametes of the malaria parasite*. Nature, 1976. **263**(5572): p. 57-60.
358. Malkin, E.M., et al., *Phase I vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria*. Vaccine, 2005. **23**(24): p. 3131-8.
359. Wu, Y., et al., *Phase I trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51*. PLoS One, 2008. **3**(7): p. e2636.
360. Dobaño, C. and J.J. Campo, *Understanding protective immune mechanisms induced by malaria vaccines in the context of clinical trials*. Hum Vaccin, 2009. **5**(8): p. 562-5.
361. Thakur, A., L.E. Pedersen, and G. Jungersen, *Immune markers and correlates of protection for vaccine induced immune responses*. Vaccine, 2012. **30**(33): p. 4907-20.
362. Newell, E.W., et al., *Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes*. Immunity, 2012. **36**(1): p. 142-52.
363. Bendtsen, J.D., et al., *Improved prediction of signal peptides: SignalP 3.0*. J Mol Biol, 2004. **340**(4): p. 783-95.
364. Ny, T., F. Elgh, and B. Lund, *The structure of the human tissue-type plasminogen activator gene: correlation of intron and exon structures to functional and structural domains*. Proc Natl Acad Sci U S A, 1984. **81**(17): p. 5355-9.

365. Kozak, M., *At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells*. J Mol Biol, 1987. **196**(4): p. 947-50.
366. Yuen, L. and B. Moss, *Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes*. Proc Natl Acad Sci U S A, 1987. **84**(18): p. 6417-21.
367. Cottingham, M.G., et al., *Preventing spontaneous genetic rearrangements in the transgene cassettes of adenovirus vectors*. Biotechnol Bioeng, 2012. **109**(3): p. 719-28.
368. Berthoud, T.K., et al., *Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1*. Clin Infect Dis, 2011. **52**(1): p. 1-7.
369. Pascolo, S., et al., *HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice*. J Exp Med, 1997. **185**(12): p. 2043-51.
370. Rozen, S. and H. Skaletsky, *Primer3 on the WWW for general users and for biologist programmers*. Methods Mol Biol, 2000. **132**: p. 365-86.
371. Bednarek, M.A., et al., *The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2*. J Immunol, 1991. **147**(12): p. 4047-53.
372. Gotch, F., et al., *Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2*. Nature, 1987. **326**(6116): p. 881-2.
373. Hillen, W. and C. Berens, *Mechanisms underlying expression of Tn10 encoded tetracycline resistance*. Annu Rev Microbiol, 1994. **48**: p. 345-69.
374. Stanton, R.J., et al., *Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function*. Biotechniques, 2008. **45**(6): p. 659-62, 664-8.
375. Li, W.C., K.L. Ralphs, and D. Tosh, *Isolation and culture of adult mouse hepatocytes*. Methods Mol Biol, 2010. **633**: p. 185-96.
376. Kim, Y., et al., *Immune epitope database analysis resource*. Nucleic Acids Res, 2012. **40**(Web Server issue): p. W525-30.
377. Rammensee, H., et al., *SYFPEITHI: database for MHC ligands and peptide motifs*. Immunogenetics, 1999. **50**(3-4): p. 213-9.
378. Roederer, M., J.L. Nozzi, and M.C. Nason, *SPICE: exploration and analysis of post-cytometric complex multivariate datasets*. Cytometry A, 2011. **79**(2): p. 167-74.

379. Burbelo, P.D., R. Goldman, and T.L. Mattson, *A simplified immunoprecipitation method for quantitatively measuring antibody responses in clinical sera samples by using mammalian-produced Renilla luciferase-antigen fusion proteins*. BMC Biotechnol, 2005. **5**: p. 22.
380. Burbelo, P.D., et al., *Antibody profiling by Luciferase Immunoprecipitation Systems (LIPS)*. J Vis Exp, 2009(32).
381. Franke-Fayard, B., et al., *A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle*. Mol Biochem Parasitol, 2004. **137**(1): p. 23-33.
382. Lin, J.W., et al., *A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent malaria parasites*. PLoS One, 2011. **6**(12): p. e29289.
383. Ploemen, I.H., et al., *Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging*. PLoS One, 2009. **4**(11): p. e7881.
384. Guguen-Guillouzo, C., et al., *High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver*. Cell Biol Int Rep, 1982. **6**(6): p. 625-8.
385. Silvie, O., et al., *A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites*. J Biol Chem, 2004. **279**(10): p. 9490-6.
386. Renia, L., et al., *A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells*. Eur J Immunol, 1990. **20**(7): p. 1445-9.
387. Barbosa, A., et al., *Plasmodium falciparum-specific cellular immune responses after immunization with the RTS,S/AS02D candidate malaria vaccine in infants living in an area of high endemicity in Mozambique*. Infect Immun, 2009. **77**(10): p. 4502-9.
388. Gruner, A.C., et al., *Sterile protection against malaria is independent of immune responses to the circumsporozoite protein*. PLoS One, 2007. **2**(12): p. e1371.
389. Kumar, K.A., et al., *The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites*. Nature, 2006. **444**(7121): p. 937-40.
390. Doolan, D.L., et al., *Identification of Plasmodium falciparum antigens by antigenic analysis of genomic and proteomic data*. Proc Natl Acad Sci U S A, 2003. **100**(17): p. 9952-7.
391. Doolan, D.L., *Plasmodium immunomics*. Int J Parasitol, 2011. **41**(1): p. 3-20.

392. Rodrigues, M.M., et al., *CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria*. Int Immunol, 1991. **3**(6): p. 579-85.
393. Romero, P., et al., *Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria*. Nature, 1989. **341**(6240): p. 323-6.
394. Weiss, W.R., et al., *A T cell clone directed at the circumsporozoite protein which protects mice against both Plasmodium yoelii and Plasmodium berghei*. J Immunol, 1992. **149**(6): p. 2103-9.
395. Doolan, D.L. and S.L. Hoffman, *IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8+ T cells in the Plasmodium yoelii model*. J Immunol, 1999. **163**(2): p. 884-92.
396. Mendes, E.A., et al., *Recombinant vaccines against T. gondii: comparison between homologous and heterologous vaccination protocols using two viral vectors expressing SAG1*. PLoS One, 2013. **8**(5): p. e63201.
397. Ratto-Kim, S., et al., *Heterologous prime-boost regimens using rAd35 and rMVA vectors elicit stronger cellular immune responses to HIV proteins than homologous regimens*. PLoS One, 2012. **7**(9): p. e45840.
398. Tarun, A.S., et al., *A combined transcriptome and proteome survey of malaria parasite liver stages*. Proc Natl Acad Sci U S A, 2008. **105**(1): p. 305-10.
399. Ishino, T., et al., *LISP1 is important for the egress of Plasmodium berghei parasites from liver cells*. Cell Microbiol, 2009. **11**(9): p. 1329-39.
400. Bergmann-Leitner, E.S., et al., *Cellular and humoral immune effector mechanisms required for sterile protection against sporozoite challenge induced with the novel malaria vaccine candidate CelTOS*. Vaccine, 2011. **29**(35): p. 5940-9.
401. Kariu, T., et al., *CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts*. Mol Microbiol, 2006. **59**(5): p. 1369-79.
402. Vignali, M., et al., *Interaction of an atypical Plasmodium falciparum ETRAMP with human apolipoproteins*. Malar J, 2008. **7**: p. 211.
403. Aidoo, M., et al., *Cytotoxic T-lymphocyte epitopes for HLA-B53 and other HLA types in the malaria vaccine candidate liver-stage antigen 3*. Infect Immun, 2000. **68**(1): p. 227-32.
404. Caspers, P., et al., *A Plasmodium falciparum malaria vaccine candidate which contains epitopes from the circumsporozoite protein and a blood stage antigen, 5.1*. Mol Biochem Parasitol, 1991. **47**(2): p. 143-50.

405. Doolan, D.L., et al., *Identification and characterization of the protective hepatocyte erythrocyte protein 17 kDa gene of Plasmodium yoelii, homolog of Plasmodium falciparum exported protein 1*. J Biol Chem, 1996. **271**(30): p. 17861-8.
406. Simmons, D., et al., *A malaria protein exported into a new compartment within the host erythrocyte*. EMBO J, 1987. **6**(2): p. 485-91.
407. Limbach, K., et al., *Identification of two new protective pre-erythrocytic malaria vaccine antigen candidates*. Malar J, 2011. **10**: p. 65.
408. Pei, Y., et al., *Plasmodium yoelii inhibitor of cysteine proteases is exported to exomembrane structures and interacts with yoelipain-2 during asexual blood-stage development*. Cell Microbiol, 2013.
409. Doolan, D.L., et al., *Degenerate cytotoxic T cell epitopes from P. falciparum restricted by multiple HLA-A and HLA-B supertype alleles*. Immunity, 1997. **7**(1): p. 97-112.
410. Guerin-Marchand, C., et al., *A liver-stage-specific antigen of Plasmodium falciparum characterized by gene cloning*. Nature, 1987. **329**(6135): p. 164-7.
411. Hill, A.V., et al., *Molecular analysis of the association of HLA-B53 and resistance to severe malaria*. Nature, 1992. **360**(6403): p. 434-9.
412. Daubersies, P., et al., *Protection against Plasmodium falciparum malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3*. Nat Med, 2000. **6**(11): p. 1258-63.
413. Perlaza, B.L., et al., *Protection against Plasmodium falciparum challenge induced in Aotus monkeys by liver-stage antigen-3-derived long synthetic peptides*. Eur J Immunol, 2008. **38**(9): p. 2610-5.
414. Prieur, E. and P. Druilhe, *The malaria candidate vaccine liver stage antigen-3 is highly conserved in Plasmodium falciparum isolates from diverse geographical areas*. Malar J, 2009. **8**: p. 247.
415. Sauzet, J.P., et al., *DNA immunization by Plasmodium falciparum liver-stage antigen 3 induces protection against Plasmodium yoelii sporozoite challenge*. Infect Immun, 2001. **69**(2): p. 1202-6.
416. Siau, A., et al., *Temperature shift and host cell contact up-regulate sporozoite expression of Plasmodium falciparum genes involved in hepatocyte infection*. PLoS Pathog, 2008. **4**(8): p. e1000121.
417. Aly, A.S., et al., *SAPI is a critical post-transcriptional regulator of infectivity in malaria parasite sporozoite stages*. Mol Microbiol, 2011. **79**(4): p. 929-39.

418. Aly, A.S., et al., *Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection*. Mol Microbiol, 2008. **69**(1): p. 152-63.
419. Zhao, J., et al., *Immunogenicity, protective efficacy and safety of a recombinant DNA vaccine encoding truncated Plasmodium yoelii sporozoite asparagine-rich protein 1 (PySAP1)*. Hum Vaccin Immunother, 2013. **9**(5).
420. Hoffman, B.U. and R. Chattopadhyay, *Plasmodium falciparum: effect of radiation on levels of gene transcripts in sporozoites*. Exp Parasitol, 2008. **118**(2): p. 247-52.
421. Sargeant, T.J., et al., *Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites*. Genome Biol, 2006. **7**(2): p. R12.
422. Mackellar, D.C., et al., *Plasmodium falciparum PF10_0164 (ETRAP10.3) is an essential parasitophorous vacuole and exported protein in blood stages*. Eukaryot Cell, 2010. **9**(5): p. 784-94.
423. Bett, A.J., et al., *An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3*. Proc Natl Acad Sci U S A, 1994. **91**(19): p. 8802-6.
424. Becker, S.I., et al., *Protection of mice against Plasmodium yoelii sporozoite challenge with P. yoelii merozoite surface protein 1 DNA vaccines*. Infect Immun, 1998. **66**(7): p. 3457-61.
425. Li, Z., et al., *Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences*. Infect Immun, 1999. **67**(9): p. 4780-6.
426. Luo, M., et al., *Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice*. J Virol Methods, 2008. **154**(1-2): p. 121-7.
427. Narum, D.L., et al., *Codon optimization of gene fragments encoding Plasmodium falciparum merozoite proteins enhances DNA vaccine protein expression and immunogenicity in mice*. Infect Immun, 2001. **69**(12): p. 7250-3.
428. Yadava, A. and C.F. Ockenhouse, *Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems*. Infect Immun, 2003. **71**(9): p. 4961-9.
429. Dobaño, C., et al., *Plasmodium: mammalian codon optimization of malaria plasmid DNA vaccines enhances antibody responses but not T cell responses nor protective immunity*. Exp Parasitol, 2009. **122**(2): p. 112-23.

430. Nielsen, M., et al., *Reliable prediction of T-cell epitopes using neural networks with novel sequence representations*. Protein Sci, 2003. **12**(5): p. 1007-17.
431. Lundegaard, C., et al., *NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11*. Nucleic Acids Res, 2008. **36**(Web Server issue): p. W509-12.
432. Wang, P., et al., *A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach*. PLoS Comput Biol, 2008. **4**(4): p. e1000048.
433. Wang, P., et al., *Peptide binding predictions for HLA DR, DP and DQ molecules*. BMC Bioinformatics, 2010. **11**: p. 568.
434. Brando, C., et al., *Murine immune responses to liver-stage antigen 1 protein FMP011, a malaria vaccine candidate, delivered with adjuvant AS01B or AS02A*. Infect Immun, 2007. **75**(2): p. 838-45.
435. Yewdell, J.W., L.C. Anton, and J.R. Bennink, *Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules?* J Immunol, 1996. **157**(5): p. 1823-6.
436. Bergmann-Leitner, E.S., et al., *Computational and experimental validation of B and T-cell epitopes of the in vivo immune response to a novel malarial antigen*. PLoS One, 2013. **8**(8): p. e71610.
437. Yu, K., et al., *Methods for prediction of peptide binding to MHC molecules: a comparative study*. Mol Med, 2002. **8**(3): p. 137-48.
438. Yang, X. and X. Yu, *An introduction to epitope prediction methods and software*. Rev Med Virol, 2009. **19**(2): p. 77-96.
439. Ferreira, A., et al., *Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon*. Science, 1986. **232**(4752): p. 881-4.
440. Schofield, L., et al., *Interferon-gamma inhibits the intrahepatocytic development of malaria parasites in vitro*. J Immunol, 1987. **139**(6): p. 2020-5.
441. Seder, R.A., P.A. Darrah, and M. Roederer, *T-cell quality in memory and protection: implications for vaccine design*. Nat Rev Immunol, 2008. **8**(4): p. 247-58.
442. Depinay, N., et al., *Inhibitory effect of TNF-alpha on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations*. PLoS One, 2011. **6**(3): p. e17464.

443. Horowitz, A., et al., *Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine*. J Immunol, 2012. **188**(10): p. 5054-62.
444. Lumsden, J.M., et al., *Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells*. PLoS One, 2011. **6**(7): p. e20775.
445. Betts, M.R., et al., *Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation*. J Immunol Methods, 2003. **281**(1-2): p. 65-78.
446. van Dijk, M.R., et al., *Three members of the 6-cys protein family of Plasmodium play a role in gamete fertility*. PLoS Pathog, 2010. **6**(4): p. e1000853.
447. Gessner, A., H. Blum, and M. Rollinghoff, *Differential regulation of IL-9-expression after infection with Leishmania major in susceptible and resistant mice*. Immunobiology, 1993. **189**(5): p. 419-35.
448. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm*. J Immunol, 2000. **164**(12): p. 6166-73.
449. Schulte, S., G.K. Sukhova, and P. Libby, *Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis*. Am J Pathol, 2008. **172**(6): p. 1500-8.
450. Watanabe, H., et al., *Innate immune response in Th1- and Th2-dominant mouse strains*. Shock, 2004. **22**(5): p. 460-6.
451. Biswas, S., et al., *Recombinant viral-vectored vaccines expressing Plasmodium chabaudi AS apical membrane antigen 1: mechanisms of vaccine-induced blood-stage protection*. J Immunol, 2012. **188**(10): p. 5041-53.
452. Jaffe, R.I., G.H. Lowell, and D.M. Gordon, *Differences in susceptibility among mouse strains to infection with Plasmodium berghei (ANKA clone) sporozoites and its relationship to protection by gamma-irradiated sporozoites*. Am J Trop Med Hyg, 1990. **42**(4): p. 309-13.
453. Draper, S.J., M.G. Cottingham, and S.C. Gilbert, *Utilizing poxviral vectored vaccines for antibody induction-Progress and prospects*. Vaccine, 2013. **31**(39): p. 4223-30.
454. Bergmann-Leitner, E.S., et al., *Self-adjuvanting bacterial vectors expressing pre-erythrocytic antigens induce sterile protection against malaria*. Front Immunol, 2013. **4**: p. 176.

455. Leitner, W.W., E.S. Bergmann-Leitner, and E. Angov, *Comparison of Plasmodium berghei challenge models for the evaluation of pre-erythrocytic malaria vaccines and their effect on perceived vaccine efficacy*. Malar J, 2010. **9**: p. 145.
456. Douradinha, B., et al., *Genetically attenuated P36p-deficient Plasmodium berghei sporozoites confer long-lasting and partial cross-species protection*. Int J Parasitol, 2007. **37**(13): p. 1511-9.
457. Mauduit, M., et al., *A role for immune responses against non-CS components in the cross-species protection induced by immunization with irradiated malaria sporozoites*. PLoS One, 2009. **4**(11): p. e7717.
458. Krzych, U., et al., *T lymphocytes from volunteers immunized with irradiated Plasmodium falciparum sporozoites recognize liver and blood stage malaria antigens*. J Immunol, 1995. **155**(8): p. 4072-7.
459. Reyes-Sandoval, A., et al., *Mixed vector immunization with recombinant adenovirus and MVA can improve vaccine efficacy while decreasing antivector immunity*. Mol Ther, 2012. **20**(8): p. 1633-47.
460. Hollingdale, M.R., et al., *Non-CS pre-erythrocytic protective antigens*. Immunol Lett, 1990. **25**(1-3): p. 71-6.
461. Kaiser, K., et al., *Differential transcriptome profiling identifies Plasmodium genes encoding pre-erythrocytic stage-specific proteins*. Mol Microbiol, 2004. **51**(5): p. 1221-32.
462. Matuschewski, K., et al., *Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage*. J Biol Chem, 2002. **277**(44): p. 41948-53.
463. Mikolajczak, S.A., et al., *L-FABP is a critical host factor for successful malaria liver stage development*. Int J Parasitol, 2007. **37**(5): p. 483-9.
464. Sharma, A., et al., *Crystal structure of soluble domain of malaria sporozoite protein UIS3 in complex with lipid*. J Biol Chem, 2008. **283**(35): p. 24077-88.
465. Favretto, F., et al., *Evidence from NMR interaction studies challenges the hypothesis of direct lipid transfer from L-FABP to malaria sporozoite protein UIS3*. Protein Sci, 2013. **22**(2): p. 133-8.
466. Spielmann, T., et al., *Molecular make-up of the Plasmodium parasitophorous vacuolar membrane*. Int J Med Microbiol, 2012. **302**(4-5): p. 179-86.
467. Fidock, D.A., et al., *Plasmodium falciparum liver stage antigen-1 is well conserved and contains potent B and T cell determinants*. J Immunol, 1994. **153**(1): p. 190-204.

468. Yang, C., et al., *Sequence variations in the non-repetitive regions of the liver stage-specific antigen-1 (LSA-1) of Plasmodium falciparum from field isolates*. Mol Biochem Parasitol, 1995. **71**(2): p. 291-4.
469. Mikolajczak, S.A., et al., *Disruption of the Plasmodium falciparum liver-stage antigen-1 locus causes a differentiation defect in late liver-stage parasites*. Cell Microbiol, 2011. **13**(8): p. 1250-60.
470. Connelly, M., et al., *T-cell immunity to peptide epitopes of liver-stage antigen 1 in an area of Papua New Guinea in which malaria is holoendemic*. Infect Immun, 1997. **65**(12): p. 5082-7.
471. Luty, A.J., et al., *Interferon-gamma responses are associated with resistance to reinfection with Plasmodium falciparum in young African children*. J Infect Dis, 1999. **179**(4): p. 980-8.
472. Porter, D.W., et al., *A human Phase I/IIa malaria challenge trial of a polyprotein malaria vaccine*. Vaccine, 2011. **29**(43): p. 7514-22.
473. Berenzon, D., et al., *Protracted protection to Plasmodium berghei malaria is linked to functionally and phenotypically heterogeneous liver memory CD8+ T cells*. J Immunol, 2003. **171**(4): p. 2024-34.
474. Krzych, U., et al., *The role of intrahepatic lymphocytes in mediating protective immunity induced by attenuated Plasmodium berghei sporozoites*. Immunol Rev, 2000. **174**: p. 123-34.
475. Nganou-Makamdop, K., et al., *Long term protection after immunization with P. berghei sporozoites correlates with sustained IFNgamma responses of hepatic CD8+ memory T cells*. PLoS One, 2012. **7**(5): p. e36508.
476. Sidney, J., et al., *Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs*. Immunol Today, 1996. **17**(6): p. 261-6.
477. Miller, J.L., et al., *Quantitative bioluminescent imaging of pre-erythrocytic malaria parasite infection using luciferase-expressing Plasmodium yoelii*. PLoS One, 2013. **8**(4): p. e60820.
478. Krzych, U., et al., *Memory CD8 T cells specific for plasmodia liver-stage antigens maintain protracted protection against malaria*. Front Immunol, 2012. **3**: p. 370.
479. Teirlinck, A.C., et al., *Longevity and composition of cellular immune responses following experimental Plasmodium falciparum malaria infection in humans*. PLoS Pathog, 2011. **7**(12): p. e1002389.
480. Charoenvit, Y., et al., *CD4(+) T-cell- and gamma interferon-dependent protection against murine malaria by immunization with linear synthetic peptides from a Plasmodium yoelii 17-kilodalton hepatocyte erythrocyte protein*. Infect Immun, 1999. **67**(11): p. 5604-14.

481. Prieur, E., et al., *A Plasmodium falciparum candidate vaccine based on a six-antigen polyprotein encoded by recombinant poxviruses*. Proc Natl Acad Sci U S A, 2004. **101**(1): p. 290-5.
482. Guebre-Xabier, M., R. Schwenk, and U. Krzych, *Memory phenotype CD8(+) T cells persist in livers of mice protected against malaria by immunization with attenuated Plasmodium berghei sporozoites*. Eur J Immunol, 1999. **29**(12): p. 3978-86.
483. Morrot, A., et al., *IL-4 receptor expression on CD8+ T cells is required for the development of protective memory responses against liver stages of malaria parasites*. J Exp Med, 2005. **202**(4): p. 551-60.
484. Frevert, U. and E. Nardin, *Cellular effector mechanisms against Plasmodium liver stages*. Cell Microbiol, 2008. **10**(10): p. 1956-67.
485. Billerbeck, E., et al., *Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties*. Proc Natl Acad Sci U S A, 2010. **107**(7): p. 3006-11.
486. Fergusson, J.R., V.M. Fleming, and P. Klenerman, *CD161-expressing human T cells*. Front Immunol, 2011. **2**: p. 36.
487. Klonowski, K.D., et al., *Dynamics of blood-borne CD8 memory T cell migration in vivo*. Immunity, 2004. **20**(5): p. 551-62.
488. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets*. Nat Immunol, 2003. **4**(3): p. 225-34.
489. Schluns, K.S. and L. Lefrancois, *Cytokine control of memory T-cell development and survival*. Nat Rev Immunol, 2003. **3**(4): p. 269-79.
490. Good, M.F. and D.L. Doolan, *Malaria vaccine design: immunological considerations*. Immunity, 2010. **33**(4): p. 555-66.
491. Forbes, E.K., et al., *Combining liver- and blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell interference*. J Immunol, 2011. **187**(7): p. 3738-50.
492. Pichyangkul, S., et al., *Evaluation of the safety and immunogenicity of Plasmodium falciparum apical membrane antigen 1, merozoite surface protein 1 or RTS,S vaccines with adjuvant system AS02A administered alone or concurrently in rhesus monkeys*. Vaccine, 2009. **28**(2): p. 452-62.
493. Sedegah, M., et al., *Effect on antibody and T-cell responses of mixing five GMP-produced DNA plasmids and administration with plasmid expressing GM-CSF*. Genes Immun, 2004. **5**(7): p. 553-61.
494. Sedegah, M., et al., *Reduced immunogenicity of DNA vaccine plasmids in mixtures*. Gene Ther, 2004. **11**(5): p. 448-56.

495. Grifantini, R., et al., *Multi-plasmid DNA vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid*. Eur J Immunol, 1998. **28**(4): p. 1225-32.
496. Jiang, G., et al., *Induction of multi-antigen multi-stage immune responses against Plasmodium falciparum in rhesus monkeys, in the absence of antigen interference, with heterologous DNA prime/poxvirus boost immunization*. Malar J, 2007. **6**: p. 135.
497. Jones, T.R., et al., *Absence of antigenic competition in Aotus monkeys immunized with Plasmodium falciparum DNA vaccines delivered as a mixture*. Vaccine, 2002. **20**(11-12): p. 1675-80.
498. Pichyangkul, S., et al., *Preclinical evaluation of the safety and immunogenicity of a vaccine consisting of Plasmodium falciparum liver-stage antigen 1 with adjuvant AS01B administered alone or concurrently with the RTS,S/AS01B vaccine in rhesus primates*. Infect Immun, 2008. **76**(1): p. 229-38.
499. Wang, R., et al., *Simultaneous induction of multiple antigen-specific cytotoxic T lymphocytes in nonhuman primates by immunization with a mixture of four Plasmodium falciparum DNA plasmids*. Infect Immun, 1998. **66**(9): p. 4193-202.
500. D'Ombrain, M.C., et al., *Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children*. Clin Infect Dis, 2008. **47**(11): p. 1380-7.
501. Dodoo, D., et al., *Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to Plasmodium falciparum malaria*. J Infect Dis, 2002. **185**(7): p. 971-9.
502. Li Pira, G., et al., *High throughput T epitope mapping and vaccine development*. J Biomed Biotechnol, 2010. **2010**: p. 325720.
503. Sette, A. and B. Peters, *Immune epitope mapping in the post-genomic era: lessons for vaccine development*. Curr Opin Immunol, 2007. **19**(1): p. 106-10.
504. Bertoni, R., et al., *Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis*. J Clin Invest, 1997. **100**(3): p. 503-13.
505. Burrows, S.R., et al., *Promiscuous CTL recognition of viral epitopes on multiple human leukocyte antigens: biological validation of the proposed HLA A24 supertype*. J Immunol, 2003. **171**(3): p. 1407-12.
506. Chang, K.M., et al., *Identification of HLA-A3 and -B7-restricted CTL response to hepatitis C virus in patients with acute and chronic hepatitis C*. J Immunol, 1999. **162**(2): p. 1156-64.

507. Lyke, K.E., et al., *HLA-A2 supertype-restricted cell-mediated immunity by peripheral blood mononuclear cells derived from Malian children with severe or uncomplicated Plasmodium falciparum malaria and healthy controls*. Infect Immun, 2005. **73**(9): p. 5799-808.
508. Threlkeld, S.C., et al., *Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development*. J Immunol, 1997. **159**(4): p. 1648-57.
509. Barber, L.D., et al., *Overlap in the repertoires of peptides bound in vivo by a group of related class I HLA-B allotypes*. Curr Biol, 1995. **5**(2): p. 179-90.
510. Frahm, N., et al., *Extensive HLA class I allele promiscuity among viral CTL epitopes*. Eur J Immunol, 2007. **37**(9): p. 2419-33.
511. Masemola, A.M., et al., *Novel and promiscuous CTL epitopes in conserved regions of Gag targeted by individuals with early subtype C HIV type 1 infection from southern Africa*. J Immunol, 2004. **173**(7): p. 4607-17.
512. Nakagawa, M., et al., *HLA class I binding promiscuity of the CD8 T-cell epitopes of human papillomavirus type 16 E6 protein*. J Virol, 2007. **81**(3): p. 1412-23.
513. Ueno, T., H. Tomiyama, and M. Takiguchi, *Single T cell receptor-mediated recognition of an identical HIV-derived peptide presented by multiple HLA class I molecules*. J Immunol, 2002. **169**(9): p. 4961-9.
514. Aidoo, M., et al., *Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria*. Lancet, 1995. **345**(8956): p. 1003-7.
515. Dodoo, D., et al., *Measuring naturally acquired immune responses to candidate malaria vaccine antigens in Ghanaian adults*. Malar J, 2011. **10**: p. 168.
516. Flanagan, K.L., et al., *Ex vivo interferon-gamma immune response to thrombospondin-related adhesive protein in coastal Kenyans: longevity and risk of Plasmodium falciparum infection*. Am J Trop Med Hyg, 2003. **68**(4): p. 421-30.
517. Flanagan, K.L., et al., *Broadly distributed T cell reactivity, with no immunodominant loci, to the pre-erythrocytic antigen thrombospondin-related adhesive protein of Plasmodium falciparum in West Africans*. Eur J Immunol, 1999. **29**(6): p. 1943-54.
518. Malik, A., et al., *Human cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein*. Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3300-4.

519. Perlaza, B.L., et al., *Long synthetic peptides encompassing the Plasmodium falciparum LSA3 are the target of human B and T cells and are potent inducers of B helper, T helper and cytolytic T cell responses in mice*. Eur J Immunol, 2001. **31**(7): p. 2200-9.
520. Sedegah, M., et al., *Naturally acquired CD8+ cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein*. J Immunol, 1992. **149**(3): p. 966-71.
521. Toure-Balde, A., et al., *Evidence for multiple B- and T-cell epitopes in Plasmodium falciparum liver-stage antigen 3*. Infect Immun, 2009. **77**(3): p. 1189-96.
522. Wizel, B., et al., *HLA-A2-restricted cytotoxic T lymphocyte responses to multiple Plasmodium falciparum sporozoite surface protein 2 epitopes in sporozoite-immunized volunteers*. J Immunol, 1995. **155**(2): p. 766-75.
523. Wizel, B., et al., *Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the Plasmodium falciparum sporozoite surface protein 2*. J Exp Med, 1995. **182**(5): p. 1435-45.
524. Sheehy, S.H., et al., *Optimising Controlled Human Malaria Infection Studies Using Cryopreserved Parasites Administered by Needle and Syringe*. PLoS One, 2013. **8**(6): p. e65960.
525. Flueck, C., et al., *Evaluation of two long synthetic merozoite surface protein 2 peptides as malaria vaccine candidates*. Vaccine, 2009. **27**(20): p. 2653-61.
526. McCarthy, J.S., et al., *A phase 1 trial of MSP2-C1, a blood-stage malaria vaccine containing 2 isoforms of MSP2 formulated with Montanide(R) ISA 720*. PLoS One, 2011. **6**(9): p. e24413.
527. Moelans, II, et al., *Induction of Plasmodium falciparum sporozoite-neutralizing antibodies upon vaccination with recombinant Pfs16 vaccinia virus and/or recombinant Pfs16 protein produced in yeast*. Mol Biochem Parasitol, 1995. **72**(1-2): p. 179-92.
528. Fidock, D.A., et al., *Cloning and characterization of a novel Plasmodium falciparum sporozoite surface antigen, STARP*. Mol Biochem Parasitol, 1994. **64**(2): p. 219-32.
529. Sidney, J., et al., *Definition of an HLA-A3-like supermotif demonstrates the overlapping peptide-binding repertoires of common HLA molecules*. Hum Immunol, 1996. **45**(2): p. 79-93.
530. Flanagan, K.L., et al., *Cellular reactivity to the p. Falciparum protein trap in adult kenyans: novel epitopes, complex cytokine patterns, and the impact of natural antigenic variation*. Am J Trop Med Hyg, 2006. **74**(3): p. 367-75.

531. Good, M.F., et al., *Human T-cell recognition of the circumsporozoite protein of Plasmodium falciparum: immunodominant T-cell domains map to the polymorphic regions of the molecule*. Proc Natl Acad Sci U S A, 1988. **85**(4): p. 1199-203.
532. Lalvani, A., et al., *Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine*. J Infect Dis, 1999. **180**(5): p. 1656-64.
533. Pinder, M., et al., *Cellular immunity induced by the recombinant Plasmodium falciparum malaria vaccine, RTS,S/AS02, in semi-immune adults in The Gambia*. Clin Exp Immunol, 2004. **135**(2): p. 286-93.
534. Doolan, D.L., et al., *HLA-DR-promiscuous T cell epitopes from Plasmodium falciparum pre-erythrocytic-stage antigens restricted by multiple HLA class II alleles*. J Immunol, 2000. **165**(2): p. 1123-37.
535. Calarota, S.A., et al., *HIV-I-specific T cell precursors with high proliferative capacity correlate with low viremia and high CD4 counts in untreated individuals*. J Immunol, 2008. **180**(9): p. 5907-15.
536. Flanagan, K.L., et al., *Unique T cell effector functions elicited by Plasmodium falciparum epitopes in malaria-exposed Africans tested by three T cell assays*. J Immunol, 2001. **167**(8): p. 4729-37.
537. Keating, S.M., et al., *Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria*. J Immunol, 2005. **175**(9): p. 5675-80.
538. Todryk, S.M., et al., *The relationship between human effector and memory T cells measured by ex vivo and cultured ELISPOT following recent and distal priming*. Immunology, 2009. **128**(1): p. 83-91.
539. Duncan, C.J., A.V. Hill, and R.D. Ellis, *Can growth inhibition assays (GIA) predict blood-stage malaria vaccine efficacy?* Hum Vaccin Immunother, 2012. **8**(6): p. 706-14.
540. Sinden, R.E. and J. Smith, *Culture of the liver stages (exoerythrocytic schizonts) of rodent malaria parasites from sporozoites in vitro*. Trans R Soc Trop Med Hyg, 1980. **74**(1): p. 134-6.
541. Strome, C.P., P.L. De Santis, and R.L. Beaudoin, *The cultivation of the exoerythrocytic stages of Plasmodium berghei from sporozoites*. In Vitro, 1979. **15**(7): p. 531-6.
542. Hollingdale, M.R., et al., *In vitro cultivation of the exoerythrocytic stage of Plasmodium berghei from sporozoites*. Science, 1981. **213**(4511): p. 1021-2.

543. Mazier, D., et al., [*In vitro* infestation of adult *thamnomys* hepatocytes with *Plasmodium yoelii* sporozoites; schizogony and release of infecting merozoites]. C R Seances Acad Sci III, 1982. **294**(19): p. 963-5.
544. Lambiotte, M., et al., [*Development of schizonts in cultured hepatocytes of adult rats after in vitro infection with Plasmodium yoelii* sporozoites]. C R Seances Acad Sci III, 1981. **293**(8): p. 431-3.
545. Pirson, P., *Culture of the exoerythrocytic liver stages of Plasmodium berghei sporozoites in rat hepatocytes*. Trans R Soc Trop Med Hyg, 1982. **76**(3): p. 422.
546. Hollingdale, M.R., P. Leland, and A.L. Schwartz, *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. Am J Trop Med Hyg, 1983. **32**(4): p. 682-4.
547. Long, G.W., et al., *Cultivation of the exoerythrocytic stage of Plasmodium berghei in primary cultures of mouse hepatocytes and continuous mouse cell lines*. In Vitro Cell Dev Biol, 1989. **25**(9): p. 857-62.
548. Mazier, D., et al., *Complete development of hepatic stages of Plasmodium falciparum in vitro*. Science, 1985. **227**(4685): p. 440-2.
549. Mota, M.M. and A. Rodriguez, *Plasmodium yoelii*: efficient in vitro invasion and complete development of sporozoites in mouse hepatic cell lines. Exp Parasitol, 2000. **96**(4): p. 257-9.
550. Calvo-Calle, J.M., et al., *In vitro* development of infectious liver stages of *P. yoelii* and *P. berghei* malaria in human cell lines. Exp Parasitol, 1994. **79**(3): p. 362-73.
551. Sattabongkot, J., et al., *Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites Plasmodium falciparum and P. vivax*. Am J Trop Med Hyg, 2006. **74**(5): p. 708-15.
552. Karnasuta, C., et al., *Complete development of the liver stage of Plasmodium falciparum in a human hepatoma cell line*. Am J Trop Med Hyg, 1995. **53**(6): p. 607-11.
553. Sinden, R.E., et al., *The development and routine application of high-density exoerythrocytic-stage cultures of Plasmodium berghei*. Bull World Health Organ, 1990. **68 Suppl**: p. 115-25.
554. Gruner, A.C., et al., *Insights into the P. y. yoelii hepatic stage transcriptome reveal complex transcriptional patterns*. Mol Biochem Parasitol, 2005. **142**(2): p. 184-92.
555. Nussler, A., et al., *TNF inhibits malaria hepatic stages in vitro via synthesis of IL-6*. Int Immunol, 1991. **3**(4): p. 317-21.

556. Pied, S., et al., *IL-6 induced by IL-1 inhibits malaria pre-erythrocytic stages but its secretion is down-regulated by the parasite*. J Immunol, 1992. **148**(1): p. 197-201.
557. Silvie, O., et al., *Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the Plasmodium species*. Cell Microbiol, 2006. **8**(7): p. 1134-46.
558. Carraz, M., et al., *Isolation and antimalarial activity of new morphinan alkaloids on Plasmodium yoelii liver stage*. Bioorg Med Chem, 2008. **16**(11): p. 6186-92.
559. Chattopadhyay, R., et al., *Establishment of an in vitro assay for assessing the effects of drugs on the liver stages of Plasmodium vivax malaria*. PLoS One, 2010. **5**(12): p. e14275.
560. Lazaro, J.E., et al., *Antimalarial activity of crambescidin 800 and synthetic analogues against liver and blood stage of Plasmodium sp.* J Antibiot (Tokyo), 2006. **59**(9): p. 583-90.
561. March, S., et al., *A Microscale Human Liver Platform that Supports the Hepatic Stages of Plasmodium falciparum and vivax*. Cell Host Microbe, 2013. **14**(1): p. 104-15.
562. Brahimi, K., et al., *Human antibodies against Plasmodium falciparum liver-stage antigen 3 cross-react with Plasmodium yoelii preerythrocytic-stage epitopes and inhibit sporozoite invasion in vitro and in vivo*. Infect Immun, 2001. **69**(6): p. 3845-52.
563. Hollingdale, M.R., et al., *Activity of human volunteer sera to candidate Plasmodium falciparum circumsporozoite protein vaccines in the inhibition of sporozoite invasion assay of human hepatoma cells and hepatocytes*. Trans R Soc Trop Med Hyg, 1990. **84**(3): p. 325-9.
564. Hollingdale, M.R., et al., *Plasmodium falciparum: elicitation by peptides and recombinant circumsporozoite proteins of circulating mouse antibodies inhibiting sporozoite invasion of hepatoma cells*. Exp Parasitol, 1987. **63**(3): p. 345-51.
565. Hollingdale, M.R., et al., *Inhibition of entry of Plasmodium falciparum and P. vivax sporozoites into cultured cells; an in vitro assay of protective antibodies*. J Immunol, 1984. **132**(2): p. 909-13.
566. Mazier, D., et al., *Effect of antibodies to recombinant and synthetic peptides on P. falciparum sporozoites in vitro*. Science, 1986. **231**(4734): p. 156-9.
567. Mellouk, S., et al., *Evaluation of an in vitro assay aimed at measuring protective antibodies against sporozoites*. Bull World Health Organ, 1990. **68 Suppl**: p. 52-9.

568. House, B.L., et al., *Functional immunoassays using an in-vitro malaria liver-stage infection model: where do we go from here?* Trends Parasitol, 2009. **25**(11): p. 525-33.
569. Natarajan, R., et al., *Fluorescent Plasmodium berghei sporozoites and pre-erythrocytic stages: a new tool to study mosquito and mammalian host interactions with malaria parasites.* Cell Microbiol, 2001. **3**(6): p. 371-9.
570. Ono, T., T. Tadakuma, and A. Rodriguez, *Plasmodium yoelii yoelii 17XNL constitutively expressing GFP throughout the life cycle.* Exp Parasitol, 2007. **115**(3): p. 310-3.
571. Tarun, A.S., et al., *Quantitative isolation and in vivo imaging of malaria parasite liver stages.* Int J Parasitol, 2006. **36**(12): p. 1283-93.
572. Graewe, S., et al., *Going live: a comparative analysis of the suitability of the RFP derivatives RedStar, mCherry and tdTomato for intravital and in vitro live imaging of Plasmodium parasites.* Biotechnol J, 2009. **4**(6): p. 895-902.
573. Mwakingwe, A., et al., *Noninvasive real-time monitoring of liver-stage development of bioluminescent Plasmodium parasites.* J Infect Dis, 2009. **200**(9): p. 1470-8.
574. Prudencio, M., et al., *Dissecting in vitro host cell infection by Plasmodium sporozoites using flow cytometry.* Cell Microbiol, 2008. **10**(1): p. 218-24.
575. Kataoka, T., et al., *Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity.* J Immunol, 1996. **156**(10): p. 3678-86.
576. Silvie, O., et al., *Alternative invasion pathways for Plasmodium berghei sporozoites.* Int J Parasitol, 2007. **37**(2): p. 173-82.
577. O'Doherty, U., W.J. Swiggard, and M.H. Malim, *Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding.* J Virol, 2000. **74**(21): p. 10074-80.
578. Scanlan, P.M., et al., *Spinoculation of heparan sulfate deficient cells enhances HSV-1 entry, but does not abolish the need for essential glycoproteins in viral fusion.* J Virol Methods, 2005. **128**(1-2): p. 104-12.
579. Mellouk, S., et al., *IFN-gamma inhibits development of Plasmodium berghei exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism.* J Immunol, 1991. **146**(11): p. 3971-6.
580. Franco, A., et al., *Expression of class I and class II major histocompatibility complex antigens on human hepatocytes.* Hepatology, 1988. **8**(3): p. 449-54.

581. Speeti, M., et al., *Upregulation of major histocompatibility complex class II antigens in hepatocytes in Doberman hepatitis*. Vet Immunol Immunopathol, 2003. **96**(1-2): p. 1-12.
582. Norris, S., et al., *Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes*. J Hepatol, 1998. **28**(1): p. 84-90.
583. Norris, S., et al., *Natural T cells in the human liver: cytotoxic lymphocytes with dual T cell and natural killer cell phenotype and function are phenotypically heterogenous and include Valpha24-JalphaQ and gammadelta T cell receptor bearing cells*. Hum Immunol, 1999. **60**(1): p. 20-31.
584. Meis, J.F., et al., *Infection of cryopreserved adult human hepatocytes with Plasmodium falciparum sporozoites*. Cell Biol Int Rep, 1985. **9**(11): p. 976.
585. Talman, A.M., A.M. Blagborough, and R.E. Sinden, *A Plasmodium falciparum strain expressing GFP throughout the parasite's life-cycle*. PLoS One, 2010. **5**(2): p. e9156.
586. Vaughan, A.M., et al., *A transgenic Plasmodium falciparum NF54 strain that expresses GFP-luciferase throughout the parasite life cycle*. Mol Biochem Parasitol, 2012. **186**(2): p. 143-7.
587. Morosan, S., et al., *Liver-stage development of Plasmodium falciparum, in a humanized mouse model*. J Infect Dis, 2006. **193**(7): p. 996-1004.
588. Vaughan, A.M., et al., *Complete Plasmodium falciparum liver-stage development in liver-chimeric mice*. J Clin Invest, 2012. **122**(10): p. 3618-28.
589. Trieu, A., et al., *Sterile protective immunity to malaria is associated with a panel of novel P. falciparum antigens*. Mol Cell Proteomics, 2011. **10**(9): p. M111 007948.
590. Doolan, D.L., et al., *Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity*. J Exp Med, 1996. **183**(4): p. 1739-46.
591. Druilhe, P. and J.W. Barnwell, *Pre-erythrocytic stage malaria vaccines: time for a change in path*. Curr Opin Microbiol, 2007. **10**(4): p. 371-8.
592. Sacci, J.B., Jr., et al., *Transcriptional analysis of in vivo Plasmodium yoelii liver stage gene expression*. Mol Biochem Parasitol, 2005. **142**(2): p. 177-83.
593. Florens, L., et al., *A proteomic view of the Plasmodium falciparum life cycle*. Nature, 2002. **419**(6906): p. 520-6.
594. Silvie, O., et al., *Effects of irradiation on Plasmodium falciparum sporozoite hepatic development: implications for the design of pre-erythrocytic malaria vaccines*. Parasite Immunol, 2002. **24**(4): p. 221-3.

595. Ma, J., et al., *Dynamics of the Major Histocompatibility Complex Class I Processing and Presentation Pathway in the Course of Malaria Parasite Development in Human Hepatocytes: Implications for Vaccine Development*. PLoS ONE, 2013. **8**(9): p. e75321.
596. Sacci, J.B., Jr., et al., *Plasmodium falciparum* infection and exoerythrocytic development in mice with chimeric human livers. Int J Parasitol, 2006. **36**(3): p. 353-60.
597. Hunt, D.F., et al., *Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry*. Science, 1992. **255**(5049): p. 1261-3.
598. Testa, J.S., et al., *MHC class I-presented T cell epitopes identified by immunoproteomics analysis are targets for a cross reactive influenza-specific T cell response*. PLoS One, 2012. **7**(11): p. e48484.
599. Wolk, B., et al., *Identification of naturally processed hepatitis C virus-derived major histocompatibility complex class I ligands*. PLoS One, 2012. **7**(1): p. e29286.
600. Ramakrishna, V., et al., *Naturally occurring peptides associated with HLA-A2 in ovarian cancer cell lines identified by mass spectrometry are targets of HLA-A2-restricted cytotoxic T cells*. Int Immunol, 2003. **15**(6): p. 751-63.
601. Flyer, D.C., et al., *Identification by mass spectrometry of CD8(+)-T-cell Mycobacterium tuberculosis epitopes within the Rv0341 gene product*. Infect Immun, 2002. **70**(6): p. 2926-32.
602. Jeffery, G.M., et al., *Exo-erythrocytic stages of Plasmodium falciparum*. Am J Trop Med Hyg, 1952. **1**(6): p. 917-25.
603. Shortt, H.E., et al., *The pre-erythrocytic stage of Plasmodium falciparum*. Trans R Soc Trop Med Hyg, 1951. **44**(4): p. 405-19.
604. Matsumiya, M., et al., *Roles for Treg expansion and HMGB1 signaling through the TLR1-2-6 axis in determining the magnitude of the antigen-specific immune response to MVA85A*. PLoS One, 2013. **8**(7): p. e67922.
605. Querec, T.D., et al., *Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans*. Nat Immunol, 2009. **10**(1): p. 116-25.
606. Schmidt, N.W., et al., *Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria*. Proc Natl Acad Sci U S A, 2008. **105**(37): p. 14017-22.

Appendices

Table of Contents

Table of Contents	369
Appendix One: Jenner Protocols	370
J098: DNA Ligation	371
J160: Gateway LR Clonase reaction using a shuttle vector and destination vector	376
J004: Transfection of HEK293 or 293TREx cells with adenoviral DNA.....	379
J090: Alkaline phosphatase treatment of DNA	384
J005: Adenovirus purification using discontinuous and isopycnic CsCl gradients	386
J006: Plaque forming unit (pfu) assay	393
J245: ID and Purity PCR for recombinant virus.	396
J013: CEF culture	402
J176: Recombination of either MVA or FP9 with shuttle vector	406
J131: 96-well plate MoFlo sort of CEF infected with recombinant poxviruses ...	410
J018: Purification of recombinant Poxvirus by plaque picking in 6-well plates ..	416
J019: Bulking up and purifying poxviruses	421
J079: ID and Purity PCR for recombinant MVA	428
J111: MVA Immunostaining titration using CEF in 6-well plates	431
J135: IgG purification for <i>Plasmodium falciparum</i> Growth Inhibition Assay	438
Appendix Two: Peptide Sequences	445
List of Tables.....	446
Appendix Three: PflSA1 and PflSA3 sequence modifications	476
PflSA1 Sequence modifications	477
PflSA3 Sequence modifications	478

Appendix One: Jenner Protocols



J098: DNA Ligation

1 Version Number: 07

2 Other documents

Refer to MSDS for the relevant safety information on the individual reagents:

\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH

assessments\Manufacturers material safety data sheets

J084 DNA band excision from agarose and DNA extraction

J089 Quantification of DNA using nanodrop

J065 Agarose Gel Electrophoresis and Gel Imaging

3 Definitions

DNA deoxyribonucleic acid

TAE Tris-acetate EDTA

SAP Shrimp-alkaline phosphatase

CIP Calf intestinal phosphatase

4 Objective

This protocol describes methods using both NEB T4 DNA ligase and Invitrogen T4 DNA ligase. Methods for the NEB enzyme are shown in black text; [those for Invitrogen are shown in blue text](#).

The insertion of DNA into a vector is achieved by prior digestion of both species with restriction enzymes. Ideally, identical or compatible enzymes are used to create cohesive ends, which will anneal specifically. Alternatively, blunt-cutting enzymes are used or the overhangs filled-in to allow ligation of non-complementary DNA ends.

Preparation of DNA for ligation.

Restriction enzyme digest (and filling in of ends where necessary) should be followed with purification of the digested DNA from other DNA species and/or the enzymic mix and the removal of 5' phosphate groups from one of the fragments.

Notes:

Generally the 5' phosphate groups are removed from the vector backbone as this prevents potential self-ligation of a molecule containing antibiotic resistance.

Self-ligation of the plasmid can occur where a single enzyme has been used, or if one of the enzymes in a double-digest has not cut to completion.

DNA Ligase

T4 DNA ligase is unstable on ice and should be stored at -20°C until immediately before it is required. It should then be placed in a labtop cooler while out of the freezer.

The buffer contains ATP and therefore is stored as single-use aliquots to avoid freeze/thawing. The buffer should be vortexed before use to resuspend any precipitated matter and any residual buffer discarded.

Vector:insert ratios.

A vector: insert ratio of 1:1 to 1:3 works well in most cases. DNA can be quantified by running on an agarose gel with molecular weight standards of known concentration.

Alternatively, running a known volume on a gel will allow the concentration of the two products to be assessed in relation to each other. To calculate the ratio, the mass of the fragments must be known, for equivalent fluorescence when stained with SYBRsafe, under UV light, a 900bp fragment will contain 3x lower moles of DNA than a 300bp fragment.

In practice, volume ratios of 1:1, 1:3 and 1:10 vector: insert are used. Obviously, if one of the fragments is a lot fainter when run on a gel this should be compensated for accordingly.

Control reactions

- **Uncut DNA** added to a reaction without digested plasmid or insert is used to verify that the bacteria used for transformation at the end of the reaction are viable.
- **No ligase** added will show if there is any uncut plasmid DNA present in the reaction once bacterial cells are transformed with the ligation-reaction products.
- **No insert** demonstrates the presence of plasmid DNA that is capable of self-ligation in the presence of ligase once bacterial cells are transformed with the ligation reaction products.

5 Reagents

Sterile distilled water (RNase & DNase Free)	Sigma	W4502
400 U / µl T4 DNA ligase + 10 x reaction buffer*	NEB	M0202
5U/µl T4 DNA ligase + 5 x buffer*	Invitrogen	15224-041

*the units are not equivalent.

6 Equipment

Gilson Pipettes – P1000, P200, P20, P10
Pipette tips – P1000, P200, P20, P10

7 Method

1) Reagent mix when using NEB DNA ligase:

Prepare ligation reactions, omitting no. 4, according to the following table (assuming plasmid and insert DNA are of similar intensity when stained with SYBRGold and viewed under UV light). If the concentration of the insert DNA is low, include reaction no. 4:

Reagent	1	2	3	4	5	6
distilled water	16µl	15µl	13µl	6µl	16µl	14µl
10x ligation buffer	2µl	2µl	2µl	2µl	2µl	2µl
Plasmid DNA	1µl uncut	1µl	1µl	1µl	1µl	1µl
Insert DNA	-	1µl	3µl	10µl	-	3µl
T4 DNA ligase	1µl	1µl	1µl	1µl	1µl	-

2) Reagent mix when using Invitrogen DNA ligase to ligate cohesive ends:

Reagent	1	2	3	4
distilled water	Up to 20µl	Up to 20µl	Up to 20µl	Up to 20µl
5 x ligation buffer	4µl	4µl	4µl	4µl
Plasmid DNA	30 fmol uncut	30 fmol	30 fmol	30 fmol
Insert DNA	-	90 fmol	-	90 fmol
T4 DNA ligase (1U final)	0.2µl	0.2µl	0.2µl	-

Incubate at RT for 1 hour (or overnight at 14°C)

3) Reagent mix when using Invitrogen DNA ligase to ligate blunt ends:

Reagent	1	2	3	4
distilled water	Up to 20µl	Up to 20µl	Up to 20µl	Up to 20µl
5 x ligation buffer	4µl	4µl	4µl	4µl
Plasmid DNA	60 fmol uncut	60 fmol	60 fmol	60 fmol
Insert DNA	-	180 fmol	-	180 fmol
T4 DNA ligase (1U final)	0.2µl	0.2µl	0.2µl	-

Incubate overnight at 14°C.

Refer to manufacturer's manuals for alternative incubation times.

Dilute the reactions 1:5 with water (>10ng of DNA is toxic to cells).

Transform bacterial cells with 3µl of the reaction as per J062.

Notes:

- Once complete, ligation reactions can be stored at 4°C until transformation. **DO NOT FREEZE REACTIONS.**
- Glycerol content of the reactions should not exceed 10%, therefore T4 DNA ligase (50% glycerol) should not exceed 20% of the total volume.

Calculation for amount of DNA

For blunt ligation,

60 fmol vector DNA

180 fmol insert DNA

Vector DNA

1000 bp	60 fmol = 20 ng
2000 bp	60 fmol = 40 ng
3000 bp	60 fmol = 60 ng
4000 bp	60 fmol = 80 ng
5000 bp	60 fmol = 100 ng
6000 bp	60 fmol = 120 ng
7000 bp	60 fmol = 140 ng
8000 bp	60 fmol = 160 ng

Insert DNA

100 bp	180 fmol = 6 ng
200 bp	180 fmol = 12 ng
300 bp	180 fmol = 18 ng
400 bp	180 fmol = 24 ng
500 bp	180 fmol = 30 ng
600 bp	180 fmol = 36 ng
700 bp	180 fmol = 42 ng
800 bp	180 fmol = 48 ng
900 bp	180 fmol = 54 ng
1000 bp	180 fmol = 60 ng

For cohesive ends:

30 fmol vector DNA

90 fmol insert DNA

1000 bp	30 fmol = 10 ng
2000 bp	30 fmol = 20 ng
3000 bp	30 fmol = 30 ng
4000 bp	30 fmol = 40 ng
5000 bp	30 fmol = 50 ng
6000 bp	30 fmol = 60 ng
7000 bp	30 fmol = 70 ng
8000 bp	30 fmol = 80 ng

100 bp	90 fmol = 3 ng
200 bp	90 fmol = 6 ng
300 bp	90 fmol = 9 ng
400 bp	90 fmol = 12 ng
500 bp	90 fmol = 15 ng
600 bp	90 fmol = 18 ng
700 bp	90 fmol = 21 ng
800 bp	90 fmol = 24 ng
900 bp	90 fmol = 27 ng
1000 bp	90 fmol = 30 ng

RE digest and gel extraction yields approximately 60% expected recovery at worst, 90% at best.

Therefore, if the plasmid is 8kb and your insert is 1kb, the insert forms 12.5% of the total DNA.

The maximum yield for 1µg is 125ng. For a blunt ligation you require 60ng for a 1:3 molar ratio as described above. This would be achieved with a 50% recovery.

Remember to digest extra DNA to be able to prepare control ligation reactions (+ ligase, - ligase etc).

8 Revision History

Version Number	Why it Changed	Who Changed it	Date changed
1.0		Matt Dicks	
2.0		A.Turner	
3.0	Changed to include overnight incubation recommendation.	A.Turner	
4.0	To include reaction volumes for Invitrogen enzyme	Ali Turner	
5	Change of Invitrogen ligase method	Ali Turner	17 th Nov 2010
6	Addition of example amounts of DNA for correct concentration in ligation reactions	Ali Turner	6 th December 2010
7	Changed Invitrogen table to read that water is used to make total volume up to 20 µl	Ali Turner	13 th Jan 2011



J160: Gateway LR Clonase reaction using a shuttle vector and destination vector

1 Version Number 2.0

This protocol uses GMO. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH. If you are not currently registered with the Occupational Health Service for work with GMO, or need to check your registration, please contact the ORCRB

2 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:

\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH assessments\Manufacturers material safety data sheets

J160	Appendix I Manual pAd/CMV/V5-DEST and pAd/PL-DEST Gateway Vectors Manual- Invitrogen
J004	Transfection of 293 cells with Adenoviral DNA
J065	Agarose Gel Electrophoresis and Gel Imaging
J062	Transformation and culture of E-coli
J115	PCR screening of transformed bacterial colonies
J103	Pouring LB agar plates containing antibiotic
C032	Antibiotics for use in molecular biology
C008	Agarose gel electrophoresis
R002	Adenoviruses GMO
R004	Appendix antigens

3 Objective

Gateway® is a universal cloning technology that takes advantage of the site specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. To express your gene of interest in mammalian cells using the Gateway® Technology, simply (overleaf):

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone (**Note:** If you are using pAd/PL-DEST™, your insert will need to include a promoter of choice, the gene or sequence of interest, and a polyadenylation signal) then

2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (e.g. pAd/PLDEST™ or pAd/CMV/V5-DEST™).

4 Equipment

Purified plasmid DNA of entry clone (150ng/μl)

pAd/PL-DEST™ vector (150ng/μl)

Gateway® LR Clonase™ II Enzyme Mix Invitrogen cat no. 11791-020
(£150 per 20 reactions)

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

2μg/μl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)

pENTR™-gus positive control (supplied with the LR Clonase™ II enzyme mix)

Competent *E. coli* host and growth media for expression

S.O.C. Medium

LB agar plates containing 100μg/ml ampicillin to select for expression clones

LB agar plates containing 30μg/ml chloramphenicol to counter select

5 Method

- 1) Clone the gene of interest into a Gateway® entry vector of choice to create an entry clone.
- 2) Add all the components shown below except for the LR clonase enzyme.

Component	Sample	Positive Control	Negative Control
Entry vector (50-150ng/rxn)	1-7μl	/	1-7μl
pENTRgus (50ng/μl)	/	2μl	/
Destination vector (150ng/μl)	1μl	1μl	1μl
TE buffer pH 8.0	to 8μl	5μl	to 8μl
LR Clonase™ II enzyme mix	2μl	2μl	/

- 3) Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
- 4) Vortex the LR Clonase enzyme mix briefly twice (2 seconds each time).
- 5) To each sample above, add 2μl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down. **Return LR Clonase enzyme mix to -20°C immediately after use.**
- 6) Incubate reactions at 25°C (RT) for 1 hour (longer incubation (up to overnight) is recommended for plasmids >10kb to increase the number of colonies containing the desired expression clone).

- 7) Add 1µl of the Proteinase K solution to each reaction and incubate for 10 minutes at 37°C.
- 8) Transform 2-3µl of the LR recombination reaction into a suitable *E. coli* host. Excessive manipulations can shear the DNA, do not vortex or pipette up and down.
- 9) Select for expression clones (True expression clones will be ampicillin-resistant and chloramphenicol-sensitive). Note: the plasmids containing the backbone genomes alone are Amp^R, upon LR clonase they become *cmp* sensitive.
- 10) Carry out a plasmid prep on an overnight culture (type depends on the yield of DNA required).

Confirming the expression clone

The *ccdB* gene mutates at a low frequency, resulting in a low number of false positives. True expression clones will be ampicillin/carbenicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be ampicillin/carbenicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30µg/ml chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Linearization by digestion with PacI

Digest the expression clone with Pac I to expose the viral inverted terminal repeats (ITRs). See Jenner Laboratory protocol: Transfection of 293 wells with Adenoviral DNA in T25 flasks (J004 v09).

6 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First Written	N/A	Aisling Vaughan
02	OH statement added	To comply with safety regulations	S. Morris (24.03.11)
03	Method clarified and explanation about longer incubation times added		Ali Turner (19.06.12)



J004: Transfection of HEK293 or 293TREx cells with adenoviral DNA in T25 flasks

1 Version Number 11

2 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets
R002 Adenovirus
R004 GMO RA appendix

3 Definitions

DMEM	Dulbecco's Modified Eagle's Media
FBS	Foetal Bovine Serum
RNase	Ribonuclease
DNase	Deoxyribonuclease
BSA	Bovine serum albumin

4 Objective

To transfect HEK293 or 293TREx cells with recombinant adenoviral plasmids to generate a new virus stock. It is recommended to perform a control transfection with serotype-matched GFP or mCherry-expressing virus at same time. This will help to separate problems due to poor transfection efficiency from problems related to virus growth/antigen toxicity. It may also be useful to perform a mock transfection with Lipofectamine alone to enable any toxic effects of the transfection reagent to be observed.

5 Reagents

1 x T25 per transfection being performed of HEK293 or 293TREx cells at approximately 90-95% confluency in 5ml antibiotic-free 10% DMEM (for seeding densities see below).

DMEM	Sigma	D6546
OptiMEM	Invitrogen	
200mM Glutamine	Sigma	G7513

100x Penicillin/Streptomycin solution (10 000 Units penicillin-G and 10mg strep per ml)	Sigma	P0781
Foetal Bovine Serum	Sigma	F2442
Lipofectamine™ 2000 transfection reagent	Invitrogen	11668-019
5µg recombinant destination plasmid created using Gateway Technology (linearised by digesting with PacI or PmeI as appropriate- see below)		
Sterile water (RNase/DNase free)	Sigma	
NEB Buffer 1		
10x BSA		
10U/µl PacI or 10U/µl PmeI	NEB	

6 Equipment

15ml Falcon Tubes	
50ml Falcon Tubes	
Heating block	
Class II BioSafety Cabinet	Walker safety cabinets Ltd
Microscope	Leica DMIL
Centrifuge	Beckmann

7 Method

A) Digestion of Adenoviral plasmid

Note: remember that the plasmid will be used to transfect cells in tissue culture. It is therefore essential that it is kept sterile.

The quantities described here are sufficient for transfection of 1 x T25 flask

Reaction mix

6µg pAd DEST plasmid
 20U PacI (if AdHu5) or PmeI (if ChAd63 in buffer 4 with BSA)
 10µl 10x BSA
 10µl NEB Buffer 1 (10x)
 Make up to 100µl total in sterile water

- 1) Incubate at 37°C for at least 2 hours.
- 2) Heat-inactivate the restriction enzyme for 25 minutes at 65°C.
- 3) Remove 15µl (0.9µg) to a separate tube; add 5µl water and 4µl 6x loading dye.
- 4) Run on a 1% agarose gel to check plasmid was linearised by PacI digest. The digest should release a fragment of ~2000bp and the Ad backbone which is about 35kb.
- 5) Use the remaining 85µl for transfection.

B) Transfection Procedure

Day 1 Cell Seeding

Prepare sufficient T25 flasks for the following:

- 1 x T25 for untransfected control
- 1 x T25 to be transfected with mcherry control DNA
- 1 x T25 to be mock-transfected with Lipofectamine alone
- ? x T25 for experimental transfection.

Seed T25 flasks at the following cell densities – two sets of flasks are usually set up such that those reaching 90–95% can be selected (density recommended by Invitrogen).

T-REX seed 2×10^6 cells in 5ml **antibiotic-free** D10 media in one set of flasks

Seed 2.5×10^6 cells in 5ml **antibiotic-free** D10 media in a second set of flasks

Incubate overnight at 37°C, 5% CO₂.

Day 2 Cell transfection

Warm an aliquot of OptiMEM for the preparation of DNA-Lipofectamine complexes to ROOM TEMPERATURE – (not 37°C).

Warm an aliquot of OptiMEM for cell washing/media replacement to 37°C

Warm 10% FCS DMEM without antibiotics to 37°C

Prepare:

1 x sterile 2ml tube containing 85µl restriction enzyme mix + 215µl OptiMEM without serum.

1 x sterile 2ml tube containing 30µl lipofectamine 2000 + 270µl OptiMEM without serum.

- 1) Mix tubes gently by tapping.
- 2) Incubate at room temperature for 5 minutes.
- 3) Mix the contents of the 2 tubes. Add the DNA to the lipofectamine, not vice versa. Pipette gently up and down with a P1000 to mix, and incubate at room temperature for at least 20 minutes (complexes are stable for 6 hours at RT).
- 4) Remove the media from the flasks of cells and wash twice with OptiMEM (no additions)
- 5) Add 1.4ml of OptiMEM without antibiotics, to each flask.
- 6) Add the 600µl of DNA/lipid complexes to the flasks and mix by tilting the flask gently to ensure that the transfection mix covers all the cells.
- 7) Place the cells at 37°C, 5% CO₂ for 4 hours. 293 cells can be left overnight but for T-REX cells the transfection mix **must be removed after 4 hours** to prevent toxicity.
- 8) Replace the media with 5ml of 10% DMEM without antibiotics.
- 9) Place the cells at 37°C, 5% CO₂.

Day 4 Passaging cells to maintain cell viability and promote virus rescue.

- 1) Inspect cells using microscope, they should be confluent and beginning to appear crowded.
- 2) Check for mcherry protein expression to assess transfection efficiency.
- 3) Warm TrypLE Express, PBS and 10% FCS DMEM without antibiotics to 37°C. The cells are fragile at this stage and must be treated very gently.
- 4) Transfer media from the T25 to a fresh T75.
- 5) Wash cells carefully with 5ml PBS (aim fluids along the top of the flask to prevent dislodging the cells).
- 6) Add 2ml TrypLE Express and place flask at 37°C for approximately 2 minutes to detach cells.
- 7) Add 10ml 10% FCS DMEM to the flask and transfer to the T75.
- 8) Place at 37°C, 5% CO₂ and monitor for the appearance of CPE, this should begin 48-72 hours after cell passage

Day 7 – 11 Harvesting of adenovirus-infected cells

293 cells showing a CPE should detach from the flask with gentle tapping. Transfer cells and media to a sterile, 50ml tube. This stage is called the transfection stock (TF).

Freeze/thaw 3 times to release virus from the cells.

Centrifuge (2000rpm for 10 minutes), transfer the supernatant to a fresh tube and store at -20°C.

Day 14 Infection of cells with rescued adenovirus

The aim of the production of a premaster stock of adenovirus is to try and achieve samples that contain a relatively standard amount of virus such that the hyperflasks can be infected at an almost optimal MOI. We do not have capacity to titre all of the viruses we produce at this stage and therefore this is a means of trying to standardize the viral preps.

The amount of virus produced from the transfection/rescue stage will vary greatly. In order to achieve a sample of virus from optimally infected cells, a six-well plate is prepared that is infected with a serial dilution of the cell lysate from the previous step (If the cells round up by the following day, this is not true CPE but is due to the effects of the excess penton capsomere that is produced during virus growth).

- 1) The well that displays uniform CPE after 72 hours is selected and harvested to form the premaster stock for both hyperflask infection and archiving.
- 2) Prepare a 6-well plate with either HEK293 or HEK293TREx cells the day before required.
- 3) On the day of infection, replace media with 2ml fresh D10 (this also serves to remove non-adhered cells to which adenovirus would otherwise attach).
- 4) Add 1ml cell lysate supernatant to the first well and move plate backward, forwards and side to side to evenly distribute the virus.
- 5) Take 100µl of media from the first well, add to the second well and repeat the mixing step.

- 6) Repeat for the remaining 4 wells
- 7) Place plate at 37°C, 5% CO₂ for ~72 hours.
- 8) Harvest the well that shows uniform CPE.
- 9) Harvest cells when they show a CPE, centrifuge (2500rpm for 5 minutes), transfer the supernatant to a fresh tube and store at -20°C until bulk prep. Freeze/thaw 3 times to release virus from the cells.
- 10) Centrifuge (2500rpm for 5 minutes) and transfer the supernatant to a fresh tube. Store at -20°C.
- 11) Infect 1 x HF with 300µl cell lysate depending on the amount of CPE observed.

8 Revision History

Version Number	What Changed	Who Changed it	Date changed
02	Not obvious that need to double the amount of plasmid to be digested if setting up transfections in duplicate	Nicky Green	
02	Only needs 4h	Nicky Green	
02	Cells showing a CPE should bang off easily	Nicky Green	
02	To release virus	Nicky Green	
03	To fill gap in protocols To reflect what is done in practice, media removed and replaced with fresh.	Fionnadh	
04	Changed to include heat inactivation of RE and also to change method to 6-well plates		
05	Minor changes to method as per Matt Dicks	Sandy Douglas	
06	Alternative method using 1 x T25 flask per transfection. Comments regarding TREX cells added	Nicky Green	
07	Using microcentrifuge tubes to preparation of DNA/lipid mix Addition of images in appendix	Ali Turner	
08	Alteration of scale of cultures post virus rescue. Altered to T75 and 1 x HF	Ali Turner	
09	Note about temperature to warm Opti-MEM to	Ali Turner	12 th Jan 2011
10	Image of example CPE added, protocol changed to 6-well plate for premaster	Ali Turner	17 th Jan 2011



J090: Alkaline phosphatase treatment of DNA

1 Version Number: 2

2 Other documents

J084	DNA band excision from agarose and DNA extraction
J056	DNA restriction digest
J086	Qiagen PCR purification kit

3 Definitions

DNA	deoxyribonucleic acid
NEB	New England Biolabs
CIP	Calf intestinal phosphatase
EDTA	ethylenediaminetetraacetic acid
BSA	bovine serum albumin
RNase	ribonuclease
DNase	deoxyribonuclease

4 Objective

Ligation of two DNA fragments often involves a previous digest step with a single enzyme. DNA cut in this manner would be able to self-ligate under appropriate conditions. The ability to self-ligate is also inherent to DNA with incompatible ends that have been filled in to create blunt-ended DNA. Removal of the 5' phosphate groups prevents this but allows ligation with untreated insert DNA.

The total reaction volume may need to be adjusted as the phosphatase enzyme is supplied in 50% glycerol, which should not exceed 10% of the final reaction volume.

This method includes protocols for using either CIP or Antarctic phosphatase. Additional information about which to use is available at the NEB website.

5 Reagents

Samples

Gel-purified DNA previously cut with restriction enzymes.

1.7ml microfuge tube

Pipettes and tips

Calf Intestinal phosphatase (10U/μl) and buffer	NEB	M0290S
Or		
Antarctic Phosphatase	NEB	M0289S
Sterile distilled water (RNAse & DNAse Free)	Sigma	W4502
37°C water bath		

6 Equipment

Pipettes – P1000, P200, P20, P10
 Pipette tips – P1000, P200, P20, P10
 0.5ml microcentrifuge tubes
 Heat block
 Vortex
 Micro-centrifuge

7 Method

Method for Calf Intestinal phosphatase (NEB cat no. M0290)

The following reaction is prepared:

30μl DNA solution (in sterile water)
 4μl 10x NEB buffer 3
 1μl CIP enzyme
 5μl sterile water

The reaction is incubated at 37°C for 1 hour in a water bath.
 The DNA is purified using a spin-column as described in J086

Protocol from NEB for Antarctic Phosphatase (cat no. M0289)

- 1) Add 1/10 volume of 10X Antarctic Phosphatase Reaction Buffer to 1-5μg of DNA cut with any restriction endonuclease in any buffer.
- 2) Add 1μl of Antarctic Phosphatase (5 units) and mix.
- 3) Incubate for 15 minutes at 37°C for 5' extensions or blunt-ends, 60 minutes for 3' extensions.
- 4) Heat inactivate (or as required to inactivate the restriction enzyme) for 5 minutes at 65°C.
- 5) Proceed with ligation.

The DNA can be stored on ice or at -20°C until required.

8 Revision History

Version Number	What Changed	Who Changed it	Date changed
V2	Addition of Antarctic phosphatase method	Ali Turner	04.05.10



J005: Adenovirus purification using discontinuous and isopycnic CsCl gradients

1 Version Number 13

This protocol uses GMO. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work with GMO, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:

\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

R002 Adenovirus

R004 GMO RA appendix

J008 Infecting 293 cells with adenovirus

J011 Passaging 293 cells

J104 Adenovirus Titre Immunoassay

J005 App I Annotated images of CsCl centrifugation steps for troubleshooting.

3 Definitions

Ad Adenovirus

CsCl Caesium chloride

CPE Cytopathic effect

PBS Phosphate buffered saline

HF Hyperflasks

4 Objective

The purification of recombinant adenovirus by two different methods of density centrifugation using Caesium chloride solutions as solvents. The first step involves a form of rate zonal centrifugation whereby the component particles are separated in a

manner based upon mass (larger particles will sediment faster). The second centrifugation step is isopycnic centrifugation whereby the particles are separated according to density.

This method describes purification of recombinant adenovirus from a single hyperflask – scale up or down as necessary.

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

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

Note about CsCl solutions

In January 2011 we had an issue with bands not forming on two separate viruses, one on first banding and one on second banding (lab book VC27 5th Jan 2001; Production records VC261 And VC296). The virus that failed the first banding – one tube was correct and the other had a diffuse band where the empty and complete capsids should have banded. Both tubes were pooled and the viruses banded perfectly during the overnight centrifugation. The second virus banded perfectly during the 2 hour spin but was diffuse after the overnight centrifugation. To solve this issue, 1) the centrifuge was cleaned thoroughly and all of the o-rings and gaskets were greased. 2) Nicky suggested that she has previously seen CsCl settling out during prolonged storage and as a consequence loaded CsCl of incorrect density.

The virus that didn't band on the overnight centrifugation step was re-banded o/n with thoroughly mixed CsCl and worked perfectly.

Therefore – ensure all CsCl solutions are thoroughly mixed both prior to aliquoting and prior to adding to centrifugation tubes.

General virus handling:

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

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

5 Reagents

Hyperflasks containing adenovirus-infected HEK293A or TREx293 cells. Cells should be showing a cytopathic effect CPE (round grapelike clusters), but the majority should still be attached to the flask.

18.2 MΩ water

1M Trizma-HCl pH7.8 Sigma T-2569-100ml

Caesium Chloride Sigma 203025

PBS containing Ca & Mg PAA H15-001

1M MgCl₂ Sigma 63069-100ML

Sucrose* Fluka 84097

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

Solution preparation:

Pre-rinse Duran bottles with 18 MΩ-water. Add solids to a Duran bottle and add some 18.2 MΩ-water, then add liquids. Add a clean magnetic stir bar and place on a stir plate until solids have dissolved. Transfer to a volumetric flask and top up with water. Filter sterilise the solution into 500ml sterile tissue culture bottles.

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

1.25g/ml density CsCl (density = 1.25g/mL = 1.98M) in 10mM Tris pH 7.8.

166.89g CsCl

5ml 1M Tris pH 7.8

Add 18.2 MΩ water to 500ml using volumetric flask

Filter sterilise.

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

233.65g CsCl

5ml 1M Tris pH 7.8

18.2 MΩ water to 500ml using volumetric flask.

Filter sterilise.

Lysis buffer (10mM Tris, 1mM MgCl₂, pH 7.8)

5ml 1M Tris-HCl pH 7.8

0.5ml 1M MgCl₂

Make up to 500ml with 18.2 MΩ water to 500ml using a measuring cylinder

Filter sterilise.

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

Weigh 75g sucrose and transfer to a 1 litre measuring cylinder.

Add 18.2 MΩ water up to ~800ml.

Add 10ml 1M Tris-HCl pH 7.8

Seal top of measuring cylinder with parafilm and invert cylinder repeatedly until sucrose has full dissolved.

Adjust volume to 1000ml.

Filter sterilise and store either at 4°C in the virus lab or on the shelf in molecular biology.

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
IMS	Fisher	M/4400/17
Corning conical tubes, 250ml PP		
Centrifuge tube, plug seal cap	Fisher	CFT-900-021Y
Bottle-top filters	Fisher	TKV-238-050Y
Volumetric flasks		
500ml and 1l sterile Duran bottles		
5ml syringes	Fisher	SZR-150-170T19G
Needles	nu-care products	M1500
Ultraclear centrifuge tubes 14.0ml	Beckman Coulter	344060
Dialysis cassettes 3-12ml & 0.5-3ml	Thermo-Fisher	66453 & 66455
Slide-A-Lyzer Buoys for 3-12ml and 0.5-3ml dialysis cassettes		66432 & 66430
250ml polypropylene bottles		
Dry ice in suitable container		
Beckman ultracentrifuge	Beckman Coulter	Optima L-80 XP
SW40Ti rotor & buckets	Beckman Coulter	
Balance		
Forceps		
Tube holder		

7 Method

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

If preparing two viruses at the same time ensure that they are kept completely separate and that tubes containing different viruses are NEVER open at the same time in the hood. Wipe down all surfaces carefully between handling different viruses and change gloves.

- 1) Mix aliquots of CsCl thoroughly before use.
- 2) Set up the relevant number (3 tubes per hyperflask) of Caesium Chloride gradients in 14ml Beckman Ultraclear tubes.
 - 1) Add 3.5ml 1.25g/ml CsCl solution to the tubes using a pipette.
 - 2) Take up 10ml of 1.35g/ml CsCl solution into a 10ml pipette and use this to underlay the 1.25g/ml CsCl solution with 3.5ml 1.35g/ml CsCl solution. Pipette very slowly and only add 3.5ml

Ensure that the interface between the two solutions is visible.

This is absolutely crucial to this centrifugation method as the interface remains intact during centrifugation and is the limit to which the adenovirus migrates.

- 3) Add the cell lysate supernatant evenly to each tube containing CsCl gradients. Take care not to disturb the interface whilst adding the sample. This can be avoided by adding the sample slowly down the side of the tube. Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 2mm of the top to prevent tube failure.
- 4) Place centrifuge tubes in the buckets for the SW40 Ti rotor and balance them to within 0.1g of each other.
- 5) Centrifuge for 2 hours at **110 000xg** at 4°C. (This is shown as 109 388xg when entered into the display).
NOTE: use all 6 buckets correctly sealed and mounted in the rotor
- 6) Once the centrifuge run is complete, remove rotor from the ultracentrifuge, transfer the buckets to the holder and move to the biological safety cabinet.
- 7) Unscrew the bucket lids and remove the tubes using forceps.
- 8) Place the tube in a clamp stand above an empty beaker (this should be treated with a biocide after use).
- 9) Two virus bands should be visible close to the centre of the tube; the upper band is incomplete virus and the lower band intact virus (see figure 1).
- 10) Decide how many tubes are required for the second centrifugation step:
This is based on experience. The major concern is that placing too much virus in each tube will lead to aggregation of the virus particles.
- 11) Set up the second caesium gradients in 14ml Beckman ultraclear tubes:
Mix aliquots of CsCl thoroughly before use.
Using 10ml serological pipettes, add 6ml of 1.35g/ml CsCl to each tube
- 12) Using a 19G needle and 5ml syringe pierce the tube from the first centrifugation step approximately 5mm below the band by gently twisting and pushing the needle through the wall of the tube. *Be careful not to hold your other hand on the other side of the tube whilst doing this for risk of injury.*
- 13) Once the needle is inside the tube gently pull on the syringe with the bevel pointing upwards to extract the lower band.
- 14) Discard the used centrifuge tube into the beaker.
- 15) Repeat for all tubes. The same needle and syringe can be used, however care should be taken not to apply pressure to the plunger of the syringe whilst piercing the tube.
- 16) Divide the virus supernatant between the tubes set up for the second centrifugation step. Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 2mm of the top to prevent tube failure.
- 17) Place the tubes in the buckets for the SW40 Ti rotor. Balance the tubes to within 0.1g of each other with lysis buffer and centrifuge at **160 000xg for 18-19 hours** (i.e. overnight). (This is shown as 159 005xg when entered into the display).

- 18) Once the centrifuge run has finished, remove rotor from the centrifuge, transfer the buckets to the holder and move to the biological safety cabinet.
- 19) Remove the centrifuge tube using forceps and place in the retort clamp. Two bands may be visible. However, the upper band may not be present if the virus added onto the 2nd gradient was very pure. Remove the lower band using a 19G needle and 5ml syringe. This can be placed safely to one side to be directly loaded into the dialysis cassette.

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

- 20) Discard the used centrifuge tubes into the beaker of Trigene.
- 21) Add the purified virus band to a dialysis cassette using a needle and syringe:
 - 1) Transfer the wetted dialysis cassette to the hood.
 - 2) Insert cassette into the buoy to function as a holder.
 - 3) Place a needle on a 5ml syringe and insert the needle so that just the bevel of the needle is inside the cassette. Insert a small amount of air to the cassette so that it is slightly inflated which makes it easier to add the virus without piercing the membrane.
 - 4) Add virus solution
 - 5) Tilt cassette and remove air such that the cassette is full with no air bubble.
- 22) Place the cassette back into the beaker of 500ml of cold storage buffer, cover with foil and place on a magnetic stir plate. Dialyse for 90 minutes changing the buffer every 30 minutes.
- 23) Remove virus from dialysis cassette, tilting the cassette ensure all of the solution is removed, and aliquot.

Ensure there are at least 10 x 10µl aliquots for QC

Label virus clearly with stock number. Store at -80°C.

Enter in the File Maker Pro Virus database the details and location of the new virus.

8 Revision History

Version Number	What Changed	Who Changed it	Date changed
2	Amount of Benzonase added	Nicky Green	
2	2nd gradient preparation, dilution of band from first gradient and only using heavy CsCl	Nicky Green	
3	Amount of Benzonase added	Nicky Green	
3	Dialysis step added between bandings 2 x 2 step gradients	Nicky Green	
4	Buffer recipes to include ready-made stocks of Tris buffer and MgCl ₂ . Removal of dialysis step between bandings. Second, optimised, isopycnic centrifugation procedure added instead of step gradient.	Ali turner	
5	Modified for new ultracentrifuge	Nicky Green	
6	Slight modifications to format. Addition of note about preparing two viruses at the same time.	Ali Turner	
7	Changed to single HF	Ali	
8	Centrifugation speeds corrected – RCF is entered as RCF (max) from rotor tables	Ali	
9	Note about CsCl settling and relevant change to protocol	Ali	07.01.11
10	Method clarification and images added	Ali	07.03.11
11	OH statement added	S. Morris	7.04.11
12	Removal of part of methods already covered in protocol J004. Replace Microsol with Trigene.	R.Hossack	06.11.12
13	Underlying of gradients clarified. Pictures replaced. Displayed speed added.	R. Hossack	30.05.13
14	Change in centrifuge spin time at step 9.17	Louisa Rose	20.08.13



J006: Plaque forming unit (pfu) assay

1 Version Number 03

This protocol uses GMO. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work with GMO, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other documents

- J011 Passaging 293 cells
- C030 Culture of primary cell and cell lines including freezing and reviving cells
- C024 Use of penicillin in tissue culture by sensitized individuals
- R002 Adenovirus
- R004 GMO RA appendix

3 Objective

To calculate the infectious viral titre by visualizing plaque forming units

4 Reagents

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

L-Glutamine (Sigma, product # 8540)

Penicillin/Streptomycin solution (Sigma # 4333)

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

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

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

5 Method

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

- 1) Warm media (DMEM with no additions) to 37°C in a water bath for 10 minutes.
- 2) Prepare work space in hood by swabbing with microsol.

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

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

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

- 4) Add **1ml** of pure virus to tube 1 containing 1ml of media. Mix well by pipeting or inverting the tube.
- 5) Add **1ml** of diluted virus from tube 1 to tube 2 and mix well by pipeting or inverting the tube. Continue to serially dilute the virus as detailed in the table above. For example the subsequent dilution (-7) will be created by adding 100ml of diluted virus from tube 2 to 900ml of media in tube 3.
- 6) Aspirate media from 6 well plates prepared the previous day. Take care not to touch the cell monolayer with the pipette. This can be avoided by tilting the plate so that the media flows to the corner of the well.
- 7) Add **200ml** of each dilution per well, gently tilt plate so that the media covers the monolayer. Incubate at 37° C for 2 hours rocking the plates every 15 minutes to ensure that the cells do not dry out. Usually a dilution range of -6 to -11 (in duplicate) is sufficient to determine viral titre.
- 8) Warm 2X complete media in a 37 °C water bath for 30 minutes.

- 9) Heat 2% agarose in microwave for 3 minutes or until it boils, allow to cool at 37°C water bath for approximately 20 minutes. Ensure that the agarose is not above 40°C when added as this may cause damage to the cells.
- 10) Prepare work space in hood by swabbing with microsol. Before placing bottles in the hood they should also be wiped with microsol taking particular care around the bottle necks.
- 11) Mix media and agarose together in a 1:1 ratio in a pre-warmed tube, allow 3mls per well
- 12) Aspirate media from 6-well plates and gently add 3ml of complete media/agarose mix to each well using a pre-warmed serological pipette. Allow to solidify in laminar flow hood for approximately 10 minutes before placing in 37°C incubator.
- 13) After 3 days overlay with 2ml of 2xDMEM/2% agarose. An additional overlay is usually required 9 days post infection if the agarose appears yellow.
- 14) 10 days post infection stain living cells using MTT (5mg per ml PBS, add 100ml per ml of overlay) and count the number of plaques. Alternatively if the adenoviral construct contains GFP, plaques can be counted using the inverted microscope.

6 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
02	Volume of media cells infected in	Should be as small volume as possible	Nicky Green
02	Timing of agarose overlay, after 2 hours NOT overnight	Standard protocol	Nicky Green
02	Use of pre-warmed tubes and pipettes	To prevent agarose from setting	Nicky Green
03 (07.04.11)	Safety data added	Safety regulations	S. Morris



J245: ID and Purity PCR for recombinant virus using KAPA Long Range Hot-start Polymerase ReadyMix with dye.

1 Version Number: 01

This protocol uses GMOs. All users **MUST be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.**

If you are not currently registered with the Occupational Health Service for work with GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other Documents

J065 Agarose Gel Electrophoresis and Gel Imaging
J099 Viral DNA extraction from mammalian cells or purified virus using Sigma Genelute kit
J173 Preparation of template DNA using DNA Releasey
R002 Adenovirus
R003 Poxvirus Risk Assessment
\\msnw3_jenner_server\jenner\hill_group\Safety\GMO RAR003
poxvirus.doc
R004 GMO RA appendix

3 Definitions

PCR	Polymerase Chain Reaction
dH ₂ O	Deionised water
RNAse	Ribonuclease
DNAse	Deoxyribonuclease
MVA	Modified vaccinia Ankara
RFP	Red Fluorescent Protein

4 Objective

This procedure seeks to confirm, using specific primers, the presence of various antigens in the relevant insertion site of a virus, and hence the identity of the virus sample. It also describes methods to determine the presence of contaminating viruses using primers that bind the promoter regions and regions downstream of the antigens that are common to many of the viruses in production.

For poxviruses, the conditions are also given to check for the presence of parental virus that was used in the original recombination reaction.

Product Information:

The KAPALongRange HotStart PCR system is a blend of *Taq* DNA polymerase and a modified archaeal (Type B) DNA polymerase possessing proofreading capability. This two-enzyme system is designed specifically to support long range and/ or sensitive PCR. In the HotStart formulation, the enzyme is combined with a proprietary antibody that inactivates the enzyme until the first denaturation step. This eliminates spurious amplification products resulting from non-specific priming events during reaction setup and initiation, and increases overall reaction efficiency.

The KAPALongRange HotStart system polymerizes DNA from a primer annealed to a DNA template in the presence of deoxyribonucleotide triphosphates. Both enzymes possess 5'-3' polymerase activity, but only *Taq* possesses double strand dependent 5'-3' exonuclease activity and only the Type B polymerase possesses 3'-5' exonuclease (proofreading) activity. KAPALongRange HotStart displays higher fidelity than *Taq* polymerase. KAPAHiFi™ DNA Polymerase is recommended for applications that require the highest fidelity.

Taq polymerase lacks proofreading activity and is unable to efficiently extend beyond misincorporated bases. **Mismatched base pairing generates truncated products that accumulate during PCR and contribute to reaction failure if the target is long and/or the template DNA is supplied in low amounts.** In contrast, proofreading high fidelity enzymes are extremely accurate, but do not perform well over longer target distances or with **low template concentration because the 3'-5' exonuclease (proofreading) activity destroys primers** and affects sensitivity.

The addition of a proofreading polymerase to *Taq* allows mismatches incorporated at the 3' end of the growing strand to be repaired. Less primer destruction occurs because the ratio of proofreading activity to polymerase activity in the blend is lower than for a pure proofreading polymerase. The resulting polymerase blend is, therefore, able to support PCR of longer targets over more cycles than either enzyme alone. The fidelity is improved as compared to *Taq*, but is lower than that of pure Type B high fidelity polymerases.

5 Reagents

DNA extracted from virus

10 ng μl^{-1} Positive control plasmid DNA

KAPA LR HS ReadyMix with dye (500 reactions) (~70p per/reaction)	Anachem	KK3602
Sterile distilled water (RNase & DNase Free)	Sigma	W4502
10μM DNA primers (see primers database)	MWG	

e.g.

for parental virus: either two viral backbone primers spanning insert region
or parental insert (for MVA = RFP) + backbone primer
 for specific insert: specific primer + viral backbone primer

6 Equipment

Microfuge PCR machine Tetrad	MJ Research	DNA engine
200μl PCR tubes Pipette tips with hydrophobic filters Gilson pipettes	Appleton Woods	

7 Method

Sample details:

For adenovirus, two master mixes are made up per virus; one containing the antigen-specific primer and the relevant downstream adenovirus backbone primer (Hu5, Ch63 etc). The second primer set contains one binding the BGH polyA tail and the other is a CMV promoter specific primer. Four reaction are prepared per virus sample: sterile water; positive control plasmid; test sample and an irrelevant adenovirus control of the same serotype.

For poxviruses, three master mixes are made up per virus. The ID PCR reaction master mix contains the antigen-specific primer and the poxvirus (MVA or FP) backbone primer. Four samples are run for each virus sample: sterile water, positive control plasmid, the test sample and an irrelevant virus control (MVA-RFP).

The flank to promoter PCR screens for various insertions/deletions, and uses a TKL primer and the p7.5 primer, or a primer specific to other promoters if they have been cloned. Four samples are run for each virus sample: sterile water, positive control plasmid, the test sample and an irrelevant virus control (MVA-RFP).

The Purity PCR master mix contains a specific parental (e.g. RFP, wild type, mcherry) primer and the poxvirus (MVA or FP) backbone primer. Five samples are run for each virus sample: sterile water, positive control plasmid (p459), negative control plasmid (relevant to the test virus sample), the test sample and an irrelevant virus control (MVA-RFP).

Prepare the master mixes using the table below for recipe with the omission of the template DNA that is added after transferring the mix to the bench in the immunology lab. 2µl of template DNA prepared using the Release reagent is required for those samples containing genomic DNA prepared from virus (or infected-cell lysate) while 10ng of plasmid DNA is sufficient.

General Working Practice:

- Work on the VVCF pre-PCR bench.
- Spray the bench with 70% IMS and wipe thoroughly (Ethanol precipitates DNA and must be wiped away after spraying).
- Use filter tips and use good pipetting technique.
- Lock the pipettes once the volume has been set – they are not accurate when unlocked.
- Wear the paper lab coat specific for VVCF PCR.
- Our PCR reactions are extremely sensitive and therefore very susceptible to any low level contaminants.
- Mol Biol Grade water:
 - This is purchased from Sigma and the entire bottle aliquoted into 50ml tubes when it is first opened. This can be poured from the bottle (no need to use pipettes). The aliquots are stored in the bottom drawer beneath the Pre-PCR bench.
 - The water is then aliquoted into 2ml screw-capped tubes, using a 2ml serological pipette. The entire 50ml tube is aliquoted when it is first opened.
 - One 2ml tube of water is used by all staff. When first opened, write the date on the side. Each tube of water is kept for one day and then discarded.
- KAPA LR HS ReadyMix with dye:
 - Always ensure we have 2 unopened, 500 reaction tubes in stock (we must ensure we NEVER run out of this reagent).
 - Upon opening a new tube, aliquot into 500µl samples in 2ml screw-capped tubes. Store at -20°C.
 - All staff to use the same aliquot.
- Primers
 - Primers are designed using Primer3 software using the recombinant genome as template.
 - Standard primers (those annealing to the virus backbones) are ordered at a scale of 0.05µmol and purified by Desalting.
 - Upon first use, dilute to 10µM using a fresh aliquot of molecular biology grade water. Prepare 500µl aliquots in 2 ml screw-capped tubes. Aliquot one of these into 200µl PCR tubes (20µl per tube). Store the rest at -20°C in the primers freezer.
 - Antigen specific primers are ordered at a scale of 0.025 µmol and purified by Desalting. These are diluted to 100µM with a fresh aliquot of water and then 100µl at 10µM prepared and used.

Reaction preparation:

- 1) Prepare the following master mix for the required number of reactions (+ 2 reactions for pipetting error)
- 2) Add water, master mix and the primers (in that order) to a 2ml screw-capped tube.
- 3) Invert the tube 10x to thoroughly mix the contents. Flick to collect the liquid at the base of the tube.
- 4) Transfer 23µl to the required number of PCR tubes.
- 5) Close the lids of the tubes.
- 6) Transfer reaction mixes to the immunology lab.
- 7) Add 2µl templates to the tubes. **ALWAYS HANDLE THE TEST MATERIAL FIRST.** Then add the test plasmid, and finally the positive controls (any small carry over on gloves etc will be amplified by the PCR and produce a false positive result).
- 8) Invert the tubes 10x to thoroughly mix the contents. Flick to collect the liquid at the base of the tubes.
- 9) Place tubes in thermal cycler, use blank tubes at the corners of the block to support the lid if necessary.
- 10) Run the PCR program as detailed below.

25µl Mix for addition of 2µl template DNA (e.g. prepared using DNA Releasey).

	1	2	3	4	5	6	7	8	9	10
Water	8	16	24	32	40	48	56	64	72	80
2x master mix	12.5	25	37.5	50	62.5	75	87.5	100	112.5	125
Primer 1	1.25	2.5	3.75	5	6.25	7.5	8.75	10	11.25	12.5
Primer 2	1.25	2.5	3.75	5	6.25	7.5	8.75	10	11.25	12.5
Total template	2	4	6	8	10	12	14	16	18	20

10µl mix for addition of 1µl template DNA (e.g. prepared using DNA Releasey).

	1	2	3	4	5	6	7	8	9	10
Water	3	6	9	12	15	18	21	24	27	30
2x master mix	5	10	15	20	25	30	35	40	45	50
Primer 1	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Primer 2	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Total template	1	2	3	4	5	6	7	8	9	10

ID/Purity PCR Conditions

Primary Denature	95°C	3 min	} X 25
Denature	95°C	40sec*	
Anneal	60°C	15 sec	
Extension	68°C	1 min/kb	
Final Extension	72°C	3 min	

Flank-Promoter PCR Conditions

Primary Denature	95°C	3 min	} X 25
Denature	95°C	40sec*	
Anneal	60°C	15 sec	
Extension	68°C	3 min**	
Final Extension	72°C	5 min	
End			

*The denaturation step is longer than in the manufacturer's tech data sheet as this compensates for the temperature lag of the tube contents when using a fast-block thermal cycler (which we have).

**The flank PCR has a standard extension time as this needs to be sufficient for the amplification of any contaminating virus DNA.

Start the relevant PCR run.

Completed reactions are analyzed by standard agarose gel electrophoresis.

FOR ALL PCR WHERE THE PRODUCT IS LESS THAN 500bp (E.G. MVA PURITY) – RUN PRODUCTS ON A 2% AGAROSE GEL, USING A SINGLE GEL TANK AND ADDING SOME SYBRSAFE TO THE LOWER TANK RESERVOIR – OCCASIONALLY THERE ARE PRODUCTS THAT ARE A SIMILAR SIZE TO MVA-RED THAT WE NEED TO DIFFERENTIATE.

Run 10µl of the reaction mix using the thin, 14-well combs for optimal resolution.

Always load samples in the following way:

5µl Smartladder
10µl –ve control (water)
(10µl p459 if performing MVA purity PCR)
10µl plasmid control
10µl test sample
10µl irrelevant virus control
5µl Smartladder

Retain the samples in case we need to run a gel with more or less sample. In some cases we may need to run out less sample volume if bands need to be more finely differentiated.

Samples may be stored at -20°C in the molecular biology lab until analysis by electrophoresis.

NOTE:

If unpredicted bands are present when examining the agarose gel, excise the band in a gel slice so that it is available for sequencing if required.

8 Revision History

Version Number	What Changed	Who Changed it	Date changed
1	First written	Ali Turner	10 th April 2012



J013: CEF culture

1 Version Number: 08

2 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

Note: Trypan blue solution is a possible **carcinogen** and should be handled using the appropriate PPE

C030 Culture of primary cells and cell lines, including freezing and reviving
C024 Use of penicillin in tissue culture for sensitized users
J114 Counting cells

3 Definitions

CEF Chicken embryo fibroblasts
FCS Foetal calf serum
DMEM Dulbecco's modified Eagle's media
PPE Personal protective equipment

4 Objective

To receive CEF and culture cells in preparation for poxvirus infection.

5 Reagents

Primary CEF	Institute for Animal Health, Compton,
DMEM	Sigma D6546
100x Pen/Strep solution (10 000 units penicillin-G and 10mg strep per ml)	Sigma P0781
200mM Glutamine	Sigma G7513
FCS	PAA A15-503
TrypLE Express	Gibco I2605
Trypan blue	Sigma T8154
PBS	Sigma D8537

Complete 2% (10%) FCS DMEM:

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

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mar
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
Disposable haemocytometer		
Culture flasks (T150 or T75 or T25)	Corning	
TC plates (6 or 24 or 96 wells)	Nunc	

7 Method

Background information: confluent cell numbers and sub-culturing seeding densities.

T175	Confluent:	~2 x 10 ⁷ CEF / flask	
Seed	5.8 x 10 ⁶ CEF/flask	→	confluent after 3 days
Seed	1.2 x 10 ⁷ CEF/flask	→	confluent after 2 days
Seed	1.7 x 10 ⁷ CEF/flask	→	confluent after 1 day
T150			
Confluent:	~2 x 10 ⁷ CEF / flask		
Seed	5.0 x 10 ⁶ CEF/flask	→	confluent after 3 days
Seed	1.0 x 10 ⁷ CEF/flask	→	confluent after 2 days
Seed	1.5 x 10 ⁷ CEF/flask	→	confluent after 1 day
T75			
Seed	2.5 x 10 ⁶ CEF/flask	→	confluent after 3 days
Seed	5 x 10 ⁶ CEF/flask	→	confluent after 2 days
Seed	1.0 x 10 ⁷ CEF/flask	→	confluent after 1 day
T25			
Seed	2.5 x 10 ⁶ CEF/flask	→	confluent after 1 day
6-well plate			
Confluent	~1 x 10 ⁶ CEF/well		
Seed	1.8 x 10 ⁷ CEF/plate	→	confluent overnight
Seed	6 x 10 ⁶ CEF/plate	→	confluent overnight
Seed	3 x 10 ⁵ CEF/plate	→	confluent after 2 days
24-well plate			
Seed	4.8 x 10 ⁶ CEF/plate	→	confluent overnight

Culturing primary CEF in T175 Culture flasks

Receive vial of CEF from Compton (Wednesday).

- 1) Perform cell count as described in J114
- 2) Add 1.2×10^7 viable cells to T175 flasks in a total of 50ml of 10% FCS DMEM.
- 3) Seed any additional culture vessels required.
- 4) Incubate at 37°C with 5% CO₂ until cells are 90% confluent (~ 2 days).

Subculturing CEF in T175 flasks

- 5) When cells are 90% confluent, aspirate the culture media from the flasks, rinse with 10ml PBS and then add 10ml TrypLE Express.
- 6) Incubate at 37°C until the cells are detached (3 – 5 minutes).
- 7) Add 10ml of 10% FCS DMEM (Total 20ml/flask).
- 8) Count the cells and seed flasks as required.
- 9) Incubate the flasks at 37°C with 5% CO₂ until they are 90% confluent

Check for bacterial growth in antibiotic free media once a month

- 1) On the first Monday of every month seed a T25 flask at 2 days confluency in antibiotic free media.
- 2) Regularly check the flask for bacterial growth until the cells die.

8 Revision History

Version Number	What Changed	Who Changed it	Date Changed
01	First written	Ali Turner	
02	To accommodate counting of P0 CEF from IAH Compton	Ali Turner	
03	Counting chamber image altered and batch number assigning details	Ali Turner	
05	Reference to C030 added	Ali Turner	17.05.10
06	Addition of step 10	Louisa Rose	11.11.11
07	Addition of T175 seeding densities	Louisa Rose	07.01.13
08	Clarification	Ali Turner	30.05.13



J176: Recombination of either MVA of FP9 with shuttle vector using Effectene Transfection reagent

1 Version Number: 09

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

Poxvirus production must ALWAYS be performed on CEF

(not DF-1 cell line that may be used for titration).

NOTE: DF-1 cells are not validated for the generation of vaccines for GMP manufacture. Therefore, they must **ONLY** be used for **virus titration** (NOT plaque picking or any stage of bulk up) during routine production. This will prevent any inadvertent misuse of the cells.

2 Other documents

J013 CEF Culture

J017 Linearisation of plasmids

J015 Cell sorting following infection-transfection and first screen

MSDS refer to MSDS for the relevant safety information on the individual reagents:

\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH assessments\Manufacturers material safety data sheets

Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE

C030 Culturing of primary cells and cell lines including freezing and reviving

C024 Use of penicillin for tissue culture by sensitised individuals

R003 Poxvirus Risk Assessment
\\msnw3_jenner_server\jenner\hill_group\Safety\GMO RA\R003 poxvirus.doc

R004 GM RA appendix

3 Definitions

DF1	Doug Foster strain 1 cells
CEF	Chicken Embryo Fibroblasts
DMEM	Dulbecco's Modified Eagles Medium
FCS	Foetal Calf Serum

4 Objective

To recombine an FP9 or MVA shuttle vector with FP9-mCherry or MVA-RFP parental virus respectively. This protocol should result in the production of recombinant, GFP positive viruses for subsequent MoFlo sorting.

This protocol refers to the use of linearised plasmid DNA. However, if marker-less virus is being prepared using the transient/dominant selection system, the plasmid should NOT be linearised.

Note: CEF are used for MVA recombination. **When recombining FP9, primary (P0) CEF MUST be used.**

5 Equipment/materials

MVA

6-well plates containing ~100% confluent CEF

These are seeded at 1×10^6 CEF/well in 2ml D10 media 24 hours prior to use or 0.5×10^6 cells/well 48 hours prior to use.

FP9

6-well plates containing ~100% confluent **PRIMARY** CEF (i.e. non-passaged).

These are seeded at 3×10^6 cells/well in 2ml D10 media. Due to the primary nature of the cells, they adhere to a greater or lesser extent. However, this seeding density, in our hands, results in sufficient levels of infection and transfection and subsequent recombination.

DMEM	Sigma	D6546
100x Pen/Strep solution (10,000 units penicillin-G and 10mg strep per ml)	Sigma	P0781
200mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605

Complete 2% FCS DMEM:

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

Effectene Transfection Reagent Kit	Qiagen	3014
MVA.RFP/FP9-mcherry		
1.5µg of linearised shuttle vector DNA		
Class II BioSafety Cabinet	Scanlaf	Mars
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
6-well tissue culture plates		
Disposable haemocytometer		
1.5ml microcentrifuge tube (heat sterilised)		
Aspirator	Integra BioSciences	Vacunsafe
Sterile 2ml screw-capped tubes		
Cell lifter	Fisher	FB55160

6 Method

Day 1:

- 1) Seed 1x10⁶ CEF cells (in 2ml D10 media) in a single well of a 6-well plate. One plate is needed for each virus to be made – **multiple recombinations must NOT be performed on the same plate.** When recombining FP9, primary cells must be seeded at 3 x 10⁶ in 2ml in a single well upon arrival from IAH Compton.
- 2) Incubate at 37°C with 5% CO₂ overnight.

Day 2:

- 3) Transfer the 6-well plate to the virus lab incubator.
- 4) Pre-warm D2 media to 37°C.
- 5) Thaw MVA RFP (or FP9-mCherry) - these are stored in a nalgene box in the -80°C freezer in immunology.
- 6) Aspirate media from the 6-well plate
- 7) Infect cells with FP9-mCherry or MVA-RFP:

Previous recombinations have worked well using 5 x 10⁶ total PFU/well (MOI ~ 2.5) with both CEF - therefore, infect with 5 x 10⁶ total PFU, in 1ml D2 media.

More than one recombination is typically being prepared at a time and therefore the media is normally in a 50ml tube. When adding the virus to this tube, do not insert the Gilson pipette into the tube but just “dot” the virus in the upper side of the tube and then mix thoroughly by inverting the tube 10 times.

- 8) Incubate at 37°C 5% CO₂ for 90 minutes.

- 9) Approximately 75 minutes into the above incubation period, begin preparing transfection complexes in a sterile 2ml tube: 1µg linearised DNA is added per well. Give the DNA a brief vortex (1 sec) and then flick tube to collect contents towards the base of the tube. DNA used should be high quality plasmid DNA, purified using a Qiagen purification kit.

DNA	Volume of Enhancer	Volume of Buffer EC
1.5µg	3µl	Make total volume up to 112.5µl

- 10) Incubate the reaction mix at room temperature for 5 minutes and pulse centrifuge for a few seconds to remove all droplets from the lid.
- 11) Add 37.5µl of Effectene Transfection reagent to the DNA-Enhancer mixture and vortex for 10 seconds, flick tube to collect contents towards the base of the tube.
- 12) Incubate at room temperature for 10 minutes to allow transfection complex formation.
- 13) During incubation, aspirate media from the well of CEF to be transfected and replace with 2ml, fresh, warm DMEM + 2% FCS.
- 14) Make final volume of transfection mix up to 0.75ml using warm DMEM + 2% FCS and triturate gently 10 times to ensure the complexes are thoroughly resuspended.
- 15) Add 0.5ml transfection complex mix to each well and ensure equal distribution by rocking the plate backwards, forwards and side to side.
- 16) Incubate 48 hours at 37°C 5% CO₂.

Day 4:

Observe fluorescence – GFP positive cells should be visible. Where no GFP fluorescence is seen in the entire well it will be necessary to repeat the recombination. Harvest cells and media by scraping (using a cell lifter) and transfer to a sterile 2ml screw-capped tube using **a 2ml serological pipette**. Store at -20°C until required.

7 Revision History

Version Number	Why it changed/ What Changed	Who Changed it	Date Changed
1	First written	Chris	30.06.10
2	Volumes adjusted to account for the use of only 1 well rather than 2.	Chris	28.07.10
3	To account for both MVA and Fp9 shuttle vectors	Chris	01.10.10
4	Method clarified	Ali	08.12.10
5	OH statement added	S. Morris	19.04.11
6	Method adapted for use of DF-1 cells for MVA	J. Matthews	30.03.12
7	Method clarification	Ali Turner	24.07.12
8	Method clarification	Ali Turner	8.04.13
9	Note to only use CEF for poxvirus production – not DF-1 cells	Ali Turner	18.04.13



J131: 96-well plate MoFlo sort of CEF infected with recombinant poxviruses

1 Version Number 11

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

Poxvirus production must ALWAYS be performed on CEF
(not DF-1 cell line that may be used for titration).

NOTE: DF-1 cells are not validated for the generation of vaccines for GMP manufacture. Therefore, they must **ONLY** be used for **virus titration** (NOT plaque picking or any stage of bulk up) during routine production. This will prevent any inadvertent misuse of the cells.

2 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

R003 Poxvirus

C030 Culture of primary cells and cell lines including freezing and reviving

C024 Use of penicillin in tissue culture by sensitised individuals

J176 Recombination of Poxvirus with shuttle vector using Effectene Transfection reagent

J018 Purification of recombinant MVA by plaque picking in 6-well plates

J173 Preparation of template DNA using DNA Release

J175 Virus ID and Purity PCR using KAPA2G robust polymerase

3 Definitions

DF-1 Doug Foster strain 1 cells

Organ: embryo

Strain: East Lansing Line (ELL-0)

Cell Type: fibroblasts spontaneously transformed

FBS Foetal bovine serum

MEM Minimum essential media

RFP Red fluorescent protein.

GFP Green fluorescent protein.

DMEM Dulbecco's modified Eagle's media

CEF Chicken embryonic fibroblasts

4 Objective

Purification of fluorescent, recombinant poxviruses can be accelerated by a MoFlo sort whereby infected cells are single-cell sorted into a 96-well plate based on their fluorescence.

This method is superior to that of plaque picking as none of the cells surrounding the plaque are co-transferred to the well. The resultant single cells are lysed by sorting them into wells containing sterile water and then CEF added to the wells to permit virus propagation.

Wells containing appropriate virus are identified by microscopy and the entire well is harvested, screened by PCR and cell lysates from the relevant well titrated following protocol J018.

This method is performed using CEF cells for both MVA and FP9 viruses. A T75 flask of 80% confluent CEF cells is infected with a small volume of cell lysate harvested from the recombination between Poxvirus and shuttle vector.

It is important that the cell culture does not contain a large amount of virus as although the cells are sorted based on fluorescence, if there are virus particles attached to the outside of the cell membrane they will be co-transferred to the wells where they can then replicate.

We usually recover at least 5 wells that have been independently sorted and harvest the "dump well" in addition to these. The lysate from the dump well is considerably enriched for recombinant virus but usually not pure.

5 Reagents

CEF

DMEM Sigma D6546

100x Pen/Strep solution (10 000 units penicillin-G and 10mg strep per ml)

Sigma P0781

200mM Glutamine Sigma G7513

FBS Sigma F2442

TrypLE Express	Gibco	I2605
Trypan blue stain	Sigma	T8154

Complete 2% (10%) FCS DMEM:

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

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Disposable haemocytometer		
-80°C freezer		
Dry ice		
96-well tissue culture plates	Fisher	TKT-520-030T
Gilson pipettes and filter tips		

7 Method

Day 1:

- 1) Seed a 6-well plate with CEF cells at 1×10^6 per well for MVA in 10% FBS DMEM for MoFlo controls (2 wells RFP; 2 wells GFP; 2 wells uninfected for MVA) (2 wells mCherry; 2 wells GFP; 2 wells uninfected for FP9).
- 2) Prepare one T75 flask per virus - Seed at 8×10^6 CEF cells for MVA in 10% FBS DMEM.
- 3) Incubate at 37°C with 5% CO₂ overnight.

Day 2:

- 4) Transfer the 6-well plate and T75 flasks of cells to virus lab.
- 5) Place plate and flasks of cells in incubator (37°C, 5% CO₂) until required.
- 6) Freeze/thaw the cell lysate(s) to be MoFlo sorted 3x and vortex for 15 sec.
- 7) Aliquot media into Falcon tubes:
3ml D2 media into each of 3 x 15ml Falcon tubes (for controls)
15ml D2 media into 50ml tubes for each test sample.
- 8) Control wells are infected at an MOI ~0.001, VVCF have stocks of pre-diluted control viruses to use for this purpose. To prepare controls, add either MVA-RFP and MVA-GFP or FP9- mCherry and FP9-GFP cell lysate to respective 3ml aliquots of D2 media and vortex for up to 5 seconds. Leave one of the 3ml aliquots of D2 to add to uninfected controls. Aspirate media from all wells and add 1ml of each control to each of 2 wells.
- 9) To prepare viruses, thoroughly clean cabinet, taking particular care to clean Gilson pipettes, and change gloves. Add 10µl of test sample cell lysate to 15ml D2 media and vortex (up to 5 sec).

- 10) Aspirate the D10 media from the T75 flask of cells and replace with 15ml of virus/D2 suspension.
- 11) Incubate infected cells for up to 3 days at 37°C with 5% CO₂

Day 4/5

- 12) Observe the plate/flasks using the fluorescence microscope to check for GFP positive recombinant virus. Also use red fluorescence to check RFP/mCherry is expressing.
- 13) Prepare one 96-well plate per virus to be sorted by adding 50µl sterile water to each well.
- 14) Prepare 50ml fresh sterile PBS in 50ml Falcon tubes – 3 tubes for controls and **separate tubes for each test sample.**
- 15) Pre-warm the PBS and TrypLE to 37°C.

Preparation of control cells

- 1) Aspirate the media from the control plate.
- 2) Wash cells once with ~5ml of PBS.
- 3) Add 1ml of TrypLE per well and incubate at 37°C until 80% of the cells are detached – monitor frequently under microscope.
- 4) Add 5ml PBS to each well then take cells up and down gently and transfer to 50ml Falcon tubes.
- 5) Rinse wells with an additional 5ml PBS and transfer to the 50ml Falcon tube.
- 6) Centrifuge at 190xg (900rpm) for 3 minutes at RT.
- 7) Aspirate the supernatant.
- 8) Resuspend the cells gently by tapping the tube.
- 9) Add ~20ml PBS.
- 10) Centrifuge at 190xg (900rpm) for 3 minutes at RT.
- 11) Aspirate the supernatant (leaving approximately 200µl residual volume) and then flick the tube gently in the liquid to get a single cell suspension.
- 12) Place all the samples in a Virus carrying box. (Wipe it with Microsol before use).
- 13) Prepare all controls and take them to Drew to set up the MoFlo.

Preparation of experimental cells

- 1) Aspirate the media from one T75 flask.
- 2) Wash cells once with ~10ml PBS
- 3) Add 5ml of TrypLE and incubate at 37°C until 80% of the cells are detached – monitor frequently under microscope.

- 4) Add 10ml PBS then take cells up and down gently and transfer to 50ml Falcon tubes.
- 5) Rinse cells with an additional 10ml PBS and transfer to the 50ml Falcon tube.
- 6) Centrifuge at 190xg (900rpm) for 3 minutes at RT.
- 7) Aspirate the supernatant.
- 8) Resuspend the cells gently by tapping the tube.
- 9) Add ~20ml PBS.
- 10) Centrifuge at 190xg (900rpm) for 3 minutes at RT.
- 11) Aspirate the supernatant (leaving approximately 200µl residual volume) and then flick the tube gently in the liquid to get a single cell suspension.
- 12) Place the sample in a Virus carrying box. (Wipe it with Microsol before use) and taken to Drew for sorting.
- 13) 96-well plates are returned to the virus room and CEF cells added: 50 000 cells per well in 200µl 2% DMEM. (5×10^6 cells in 20ml per plate).
NOTE –CELLS MAY BE TRYPSINISED AND COUNTED TOGETHER FOR MULTIPLE PLATES. HOWEVER, THEY MUST NOT BE PLACED IN A SINGLE REAGENT RESERVIOR TO SEED MULTIPLE PLATES AS THE MULTICHANNEL PIPETTE WILL DIP INTO THE 96-WELL PLATES CONTAINING DIFFERENT VIRUSES.
 Alternatively, the 96-well plates can be placed at -20°C and the cells added later.
 Place plate in incubator (37°C, 5% CO₂) for 2-4 days until recombinant virus can be observed.
- 14) The entire wells containing recombinant virus (as visualized by fluorescence) are harvested. Where possible, at least 5 wells should be harvested in the first instance + the dump well. In cases where MoFlo sorting has previously failed for a particular virus it may be worth harvesting as many candidate wells as possible for PCR screening to maximize the chance of obtaining correct virus.

Wells to be harvested are scraped with a P1000 tip, transferred into a 2ml Starstedt tube and frozen/thawed x3. 20µl of lysate is taken and added to 30µl DNA RelEasy in a PCR tube and extracted using the 'Tough DNA RelEasy' program (see J173). Samples are screened with reference to J175. The remaining lysate is stored at -20°C in the virus lab freezer for subsequent plaque purification and amplification.

8 Revision History

Version Number	What Changed	Who Changed it	Date Changed
01	First written	Ali Turner	
02	Infecting T150 flasks with virus instead of 6-well plates	Chris Schultz	
03	Testing of dump well virus by PCR screen	Ali Turner	
04	Made applicable to both MVA and Fp9 sorts	Chris Schultz	
05	Method clarified	Ali Turner	15 th December 2010
06	Not recorded		
07	OH statement added	S. morris	19.04.11
08	Note about seeding multiple plates	Ali turner	07.10.11
09	Method adapted for use of DF-1 cells when sorting MVA	J Matthews	30.03.12
10	DF-1 cells for FP9 as well. Method clarified	Ali Turner	19.06.12
11	CEF only – not DF-1 cells + explanatory note	Ali Turner	18.04.13
12	Modified to use PBS only instead of PBS + 2% FBS (step 9.3 onwards)	Jake Matthews	13.05.13



J018: Purification of recombinant Poxvirus by plaque picking in 6-well plates

1 Version Number: 09

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

Poxvirus production must ALWAYS be performed on CEF

(not DF-1 cell line that may be used for titration).

NOTE: DF-1 cells are not validated for the generation of vaccines for GMP manufacture. Therefore, they must **ONLY** be used for **virus titration** (NOT plaque picking or any stage of bulk up) during routine production. This will prevent any inadvertent misuse of the cells.

2 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

Note: Trypan blue solution is a possible **carcinogen** and should be handled using the appropriate PPE

C030	Culturing primary cells and cell lines including freezing and reviving
C024	Use of penicillin for tissue culture by sensitized individuals
R003	Poxvirus Risk Assessment
\\msnw3_jenner_server\jenner\hill_group\Safety\GMO RA\R003 poxvirus.doc	
J013	CEF culture
J019	Bulking up poxviruses

3 Definitions

CEF	Chicken embryo fibroblasts
FCS	Foetal calf serum
MEM	Minimum essential media
RFP	Red fluorescent protein.
GFP	Green fluorescent protein.
DMEM	Dulbecco's modified Eagle's media

4 Objective

To purify recombinant poxvirus from parental virus by plaque picking following infection of CEF (MVA) with a serial dilution of plaque lysate.

After each round of purification ID, Purity and Flank PCR are performed to screen for the presence of the desired antigen and to check for the presence of parental poxvirus (MVA-RFP or FP9-mcherry).

When one round of plaque picking is confirmed as free of parental virus by PCR, a plaque from the next round of plaque picking is used to infect a T175.

This T175 is then also screened by ID, Flank and Purity PCR and is used as a premaster for subsequent MSV Infection if found to be correct and pure.

Ensure cells infected with different viruses are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different vectors.

5 Reagents

CEF	Institute for Animal Health, Compton.
DMEM	Sigma D6546
2xMEM without phenol red	Gibco 21935
100x Pen/Strep solution (10 000 units penicillin-G and 10mg strep per ml)	Sigma P0781
200mM Glutamine	Sigma G7513
FCS	Sigma F2442
TrypLE Express	Gibco I2605
Trypan blue stain	Sigma T8154
CMC high viscosity	BDH# 279294T

Complete 2% (10% for FP9) FCS DMEM:

500ml DMEM	
10 (50ml for FP9) ml FCS	(2% (10% for FP9) final conc)
5ml Pen/strep	(100 U Penicillin, 0.1mg strep ml ⁻¹ final conc)
10ml L-glutamine	(4mM final conc)

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

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope with objective marker attachment	Leica	DMIL
Disposable haemocytometer		
-80°C freezer		
Dry ice		
6-well tissue culture plates	Fisher	TKT-520-030T
Gilson pipettes and filter tips		

7 Method

Infection of CEF.

- 1) Prepare one 6-well plate of CEF per virus plaque to be titrated. Standard procedure is to purify one plaque.
- 2) Incubate at 37°C with 5% CO₂ until ~90% confluent.
- 3) Transfer the 6-well plates to the virus lab.
- 4) Aspirate the media and replace with 1ml fresh, pre-warmed D2.
- 5) Infect one well of the 6-well plate with 20µl of the material to be purified (This will either be lysate that was harvested from the MoFlo sorted 96-well plate or a plaque pick from a previous titration). Tilt the plate side to side and front to back 5 times to ensure mixing (do not move the plate in circles as this forces the virus to the edge of the well).
- 6) Take 100µl from the well that has just been infected and transfer it to the next well using a Gilson with a P200 tip. Tilt the plate as in 5 to mix.
- 7) Repeat step 6 four more times to give the following plate layout:

Well 1 ~2x 10 ⁻² dilution 1ml D2 + 20µl lysate	Well 2 ~2x 10 ⁻³ dilution 1ml D2 + 100µl from well 1	Well 3 ~2x 10 ⁻⁴ dilution 1ml D2 + 100µl from well 2
Well 4 ~2x 10 ⁻⁵ dilution 1ml D2 + 100µl from well 3	Well 5 ~2x 10 ⁻⁶ dilution 1ml D2 + 100µl from well 4	Well 6 ~2x 10 ⁻⁷ dilution 1ml D2 + 100µl from well 5

- 8) Incubate for 90 ± 30 minutes at 37°C with 5% CO₂.
- 9) Mix sufficient D2 media and CMC in a ratio 2/3 D2 : 1/3 CMC for 2ml per well required.
- 10) Aspirate media containing virus.
- 11) Replace with 2ml D2/CMC overlay per well.

- 12) Incubate for 2-4 days.
- 13) Pick plaques as required.

Plaque picking.

- 1) 2ml screw capped tubes containing 500ml 10mM Tris pH 9.0 are stored in the VVCF drawers in the virus room. These are for the plaques to be picked into. Transfer one to the BSC2.
- 2) Using the microscope, identify a well isolated plaque of desired fluorescence/no fluorescence and use the objective cell dotter to mark a ring around the plaque.
- 3) Transfer the plate to the BSC2 and use a p1000 pipette and tip to remove the plaque from the monolayer.
- 4) Check, using the microscope that the correct region has been harvested.
- 5) Place the tubes on dry ice to freeze; thaw in 37°C water bath and vortex very hard.
- 6) Repeat freeze, thaw and vortex twice more for each tube. (Virus may be frozen at -20°C for storage).
- 7) In the BSC2, harvest well 1 (the well that was infected with the most concentrated lysate) by scraping and transfer to a 2ml tube. Freeze-thaw-vortex three times before taking 20µl for DNA RelEasy extraction and freezing the remainder at -20°C.

Note: After performing titration of first round plaques it is not necessary to harvest the most concentrated well for PCR screening because this is equivalent to the material screened after MoFlo harvest. Harvest of the most concentrated well is necessary after titration of later round plaques.

Recombinant virus purity

When a plaque passes ID, Flank and Purity PCR 50ul of a subsequent pick is taken and used to infect a T150 as premaster. For example, if plaque 1 (from MoFlo) passes all PCR, this plaque should be titrated and plaque 1.1 picked for premaster infection. See J019 Bulking Up Poxviruses.

After premaster harvest 20ul of premaster lysate should be PCR screened – if this passes it can then be used to infect multiple T150s to produce the MSV.

8 Revision History

Version Number	What Changed	Who Changed it	Date changed
01	First written	A.Turner	
02	Altered to contain CMC method variation	A.Turner	
03	Method altered	Ali Turner	
04	Agarose method removed	Ali Turner	15 th Dec 2010
05	Safety data added	S. Morris	19.04.11
06	Clarification of which wells should be plated in step 9.2.6.	C.Schultz	06.07.11
07	Method altered. Adapted for use of DF-1 cells for MVA	J Matthews	30.03.12
08	Reduced to picking only 1 plaque/plate	J Matthews	26.07.12
09	Altered to stipulate CEF only and to detail how to plaque pick in the BSC2, not under the microscope	A Turner	11.04.12



J019: Bulking up and purifying poxviruses

1 Version Number: 09

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH. If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

Note: Trypan blue solution is a possible **carcinogen** and should be handled using the appropriate PPE

Risk assessments covering the use of poxviruses within the Jenner Institute are listed below and can be found in the safety folder on the s: drive or hard copies are available in the office area. These must be read and understood prior to commencing work using these viruses. In addition, the appendix to the GMO RA (R004) documents all of the antigens might be encoded by the poxviruses. These should be read and understood as relevant to the work being undertaken.

GMO	RA R003 poxvirus
GMO	RA R004 GM RA app
C052	use of sonicator
C030	Culturing primary cells and cell lines including freezing and reviving
J013	CEF culture
J018	plaque picking
J208	preparation of 36% sucrose
J209	preparation of 10mM Tris for MVA production

3 Definitions

CEF	Chicken embryo fibroblasts
CPE	Cytopathic effects

FCS	Foetal calf serum
MVA	Modified vaccinia Ankara
FP9	a plaque-purified, high passage attenuated, European strain of Fowlpox virus
DMEM	Dulbecco's modified Eagle's media
GMO	Genetically modified organism
RA	Risk assessment

4 Objective

Large-scale preparation of MVA/FP9 requires the infection of CEF, the extraction of virus from the cell lysate and crude purification to remove cell debris from the sample.

This protocol describes the preparation of a premaster stock of virus, infection of CEF with premaster lysate to form a master seed virus prep and purification through a sucrose cushion.

Sucrose cushion purification separates the viral particles from the cell lysate.

The potency, ID, purity and sterility of the virus are then assayed using standard methods.

Ensure cells infected with different viruses are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different vectors.

5 Reagents

CEF		
DMEM	Sigma	D6546
Pen/Strep	Sigma	
Glutamine	Sigma	
PBS (without Ca ²⁺ and Mg ²⁺)	Sigma	D8537
FCS	Sigma	F2442
Trypan blue stain	Sigma	T8154
TrypLE Express	Gibco	I2605
10mM Tris [pH 9]		
From 1M Tris pH 9 stock solution	SIGMA	T2819-100ml
36% w/v sucrose solution (in 10mM Tris pH 9), (filter sterilised)		
It is important that this material is low in endotoxins		
	Fluka	84097

Premaster cell/media lysate

Cells and media from a T150 flask infected with material confirmed free from parental poxvirus by PCR. If this is the first time the virus has been made, the premaster will have been prepared from a plaque pick one round on from a plaque that passed QC PCR (for example plaque 1.1 is usually used if plaque 1 passed QC PCR). If this is a rebulk prep, the premaster is prepared by infection of a CEF T150 with cell lysate from the original premaster.

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
Industrial methylated spirit (IMS)	Fischer	M/4400/17
2ml screw cap tubes.		
Tube, Thinwall, Ultra-Clear™, 14ml, 14x95mm		
<i>These actually have a max capacity of 13ml</i>		
	Beckman Coulter	344060
Corning conical tubes, 250ml PP Centrifuge tube, plug seal cap	Fisher	CFT-900-021Y
Bottle, Polyallomer, assembly with cap and plug	Beckman Coulter	334205
Disposable haemocytometer		
Cup-horn Sonicator	Sonics and Materials	
Sterile transfer pipette (pastette)		
-80°C freezer	New Brunswick Scientific	U57085
25cm cell scrapers		
Storage bottle disposable with plug seal cap sterile	Fisher Scientific	tkv-185-030f
50ml centrifuge tubes		
15ml centrifuge tubes		
Dry ice		
Optima L-80 XP Ultracentrifuge	Beckman Coulter	
Type 19 fixed-angle centrifuge rotor	Beckman Coulter	

7 Method

Preparing and infecting CEF with virus in T150 flasks (Premaster).

- 1) Prepare one T150 flask per virus with DF-1 cells such that they are 90% confluent at time of infection. One T150 is required for each set of 5 x T150 flasks required.
- 2) Incubate (37°C, 5% CO₂) until 90% confluent.
- 3) Transfer T150 flask to the virus lab
- 4) For each flask, pipette 50µl of prepared plaque pick (three times freeze, thaw and vortexed) into 15ml of 2% FCS DMEM in a 15ml falcon tube and vortex thoroughly.
- 5) Aspirate the 10% DMEM and replace with the 2% media containing the virus.
- 6) Incubate (37°C, 5% CO₂) and monitor appearance of CPE (~100% infected cells should be evident after approximately 3 days).
- 7) Scrape off the virus-infected cells from the T150 flask into 50ml falcon tubes. *(Cells and supernatant may be frozen for storage at this stage).*
- 8) Place the 50ml falcon tubes with virus infected CEF on dry ice to freeze, thaw in 37°C water bath and vortex very hard.

- 9) Repeat freeze/thaw once more before taking a 20µl aliquot and adding to 30µl DNA RelEasy in a PCR tube for DNA extraction. Freeze the remaining lysate and store until needed for MSV infection.

Preparing and infecting CEF with virus in T150 flasks (Master seed virus).

- 1) Prepare 5x T150 flasks/virus for a standard prep (adjust if a large prep is to be made).
- 2) Incubate (37°C, 5% CO₂) until ~90% confluent.
- 3) Transfer T150 flasks to virus lab.
- 4) Prepare D2 media containing virus lysate by pipetting 140ml D2 media per 5x T150 flask prep into an empty 250ml conical tube. Take 1ml of premaster lysate and transfer to a 1.5ml O-ring tube and store this at -80°C as a premaster for future preps – this should be labeled with the stock number of the current prep and the date + operator. Then add the remaining ~34ml of the premaster lysate to the 250ml conical tube and mix by vortexing hard for 10 seconds.
- 5) Aspirate the 10% FCS DMEM and replace with 35ml of 2% FCS DMEM/virus.
- 6) Incubate (37°C, 5% CO₂) until all cells are infected and beginning to show signs of CPE (3-5 days).

Harvesting virus.

- 1) Scrape off the virus-infected cells with the cell scraper and transfer everything, using a 25ml pipette, into 250ml conical bottomed tubes.
- 2) Centrifuge the 250ml conical tubes at 670xg for 10 minutes to pellet cell debris.
- 3) Transfer the supernatants to the autoclaved 250ml flat-bottom Ultracentrifuge tubes (Maximum volume ~250ml). Retain cell pellet.

NOTE: These ultracentrifuge tubes must be filled to the bottom of the thread on which the lid screws down, otherwise the tubes can deform during a spin. If the fluid level is not high enough top up with PBS to the required level.

- 4) Centrifuge the tubes (33 000xg, 60 minutes, 4°C.) to pellet the virus particles in Beckman Coulter Optima L-80 XP Ultracentrifuge using the Type 19 fixed-angle centrifuge rotor
- 5) During the 60 minute centrifugation, process the cell debris.
- 6) Add 2ml of 10mM Tris [pH 9] to the pelleted cell debris, resuspend thoroughly and transfer to 15ml Falcon tube.
- 7) Freeze the pellets on dry ice, thaw in 37°C water bath and vortex.
- 8) Repeat freeze/thaw/vortex twice more and place tubes on ice until the 60 minute centrifugation of the supernatants has been completed.
- 9) Following the 60 minute centrifugation of the supernatants (step 4), aspirate the supernatant from the 250ml bottles, leaving a virus-rich pellet.
- 10) Centrifuge the 15ml Falcon tubes from step 8 at 670xg for 5 minutes.
- 11) Carefully remove the supernatant using a pipette and transfer to the 250ml bottle containing the virus-rich pellet, store this material on ice while continuing with the processing of the cell pellet.
- 12) Resuspend the cell pellet in the 15ml tubes by adding 3ml 10mM Tris [pH 9].

- 13) Sonicate for 1 minute, 57 Hz. (*Switch on the sonicator, connect the lower pipe of the tube holder to the cold tap water and drain the upper pipe just in the sink, keep the tube in the holder and push the start button. Leave the lab, until it stops. After finishing disconnect everything and turn the sonicator off*)
- 14) Centrifuge the 15ml tube (670xg, 5 minutes) and pool the supernatant with that obtained previously in the 250ml bottle.
- 15) Discard the cell debris pellets.
- 16) Transfer virus suspension to a 15ml Falcon tube. This material can be stored at -20°C or -80°C.
- 17) Clean the 250ml flat-bottomed tubes

Purification through 36% w/v sucrose cushion.

- 1) If previously frozen: thaw the impure virus in 37°C water bath and vortex.
- 2) Ensure no clumps are present in the virus suspension – centrifuge samples (950xg, 3 minutes, 4°C)
- 3) For each 5 x T150 flask prep, use one ~7.5ml sucrose cushions.
- 4) Prepare the ultracentrifuge buckets by placing in the biological safety cabinet, wiping the bucket lids thoroughly with 10% Trigene solution. Remove the Trigene completely by spraying with 70% IMS and wiping with blue roll.
Note: traces of Trigene in contact with the virus will inactivate the virus. Spray the internal surfaces of buckets of the SW40 rotor with 70% IMS, turn upside-down to drain and then place inside the virus lab biological safety cabinet to allow residual ethanol to evaporate.
- 5) Add 7.5ml of 36% sucrose (in 10mM Tris [pH 9]) to a 13ml thin-walled ultracentrifuge tube.
- 6) Layer the virus solution (max. volume of 5.5ml for this size centrifuge tube) onto the top of the sucrose, slowly and carefully with a sterile transfer pipette without disturbing the sucrose layer.
- 7) If preparing two viruses, seal the first tube, wipe down the surfaces and change gloves before loading the second virus.
- 8) Place the ultracentrifuge tubes inside the buckets and balance using the balance scale.
- 9) Centrifuge at 30 000xg, 4°C for 2 hours using the Optima L-80 XP Ultracentrifuge. During the centrifuge run prepare the top and side labels for the vials of purified virus – tubes should be fully labeled and placed in the virus lab fridge before the run is finished.
- 10) Remove and discard the supernatant carefully using the aspirator – remove the top of the upper layer first to ensure this does not contact the purified pellet at the base of the tube.
- 11) Re-suspend the pellet in 10mM Tris [pH 9] in a total volume of 800µl.
- 12) Transfer the virus to a 2ml screw cap tube.
- 13) Wash the centrifuge tube with ~300µl of 10mM Tris [pH 9] and transfer this to the screw cap tube.
- 14) Sonicate the tube (1 minute, 57 Hz)
- 15) Vortex the tube for 10 seconds.
- 16) Carefully aliquot the virus into 2ml screw-capped vials (overleaf):

First, transfer 6 x 10µl into vials and transfer to the -80°C freezer in immunology. These aliquots are used for viral titration and PCR.

Aliquot 10µl into a single vial and place in the virus lab fridge for sterility testing.

Split the remainder into 200µl aliquots and place these in the -80°C freezer in immunology. Note the number of aliquots for entry into the virus database.

- 17) Immerse the centrifuge buckets and tubes in a container containing 10% Trigene for a maximum of 10 minutes, rinse thoroughly 4 times with tap water and then 15MΩ Elga water.
- 18) The disposable storage bottles with plug seal caps should be placed in autoclave bags (unassembled) and sent for autoclaving. **The screw-on lids should NOT be autoclaved.**
- 19) Centrifuge buckets are allowed to air-dry – do not put lids on the buckets, leave them loose on the holder to allow and residual water to evaporate.

Database maintenance

Virus storage (s:Reagent storage info/virus storage/virus stock database)

Account name: user

Password: virus

Add new record and fill in details.

MVA production chart: (S:\VIRUS PRODUCTION RECORDS)

Notes:

MVA IMV and EEV can also be purified using CsCl gradients (IMV and EEV sediment at 1.27 and 1.23g/ml respectively). However, sucrose cushion is much less expensive.

We perform only a single round of purification through sucrose (a single 36% w/v sucrose centrifugation). This generates a pellet that contains both virus and cell debris.

A further purification step is possible using a sucrose gradient (40%, 36%, 32%, 28% and 24%) that is left for 2.5 hours to diffuse and then overlaid with virus from the first purification step. Centrifugation is performed (19 000xg, 50 minutes, 4°C)

8 Revision History

Version Number	What changed and Why it Changed	Who Changed it	Date changed
01	N/A	Ali Turner	
02	Altered to specify one virus in the hood at any one time. Final resuspension volume changed Premaster stock changed from 1 x T75 to 1 x T150	Ali Turner	
03		Ali Turner	
04	Addition of references to GMO RA Alterations for new ultracentrifuge	Ali Turner	
05	Centrifugation of sample prior to loading on sucrose cushion. Sonication of sample prior to aliquoting	Ali Turner	18 th Oct 2010
06	Reference to risk assessment for sonicator Method clarification	Ali Turner	15 th Dec 2010
07	Safety data added (OH statement)	S. Morris	19.04.11
08	Note added about topping up fluid level in 250ml ultra tubes	Jake Matthews	24.07.12
09	Premaster changed to use DF-1 cells. Standard prep changed to 5x T150, purified over a single sucrose column (Volumes in steps 3.6.6 and 3.6.12 adjusted to allow this). Aliquot details changed.	Jake Matthews	26.07.12



J079: ID and Purity PCR for recombinant MVA

1 Version Number: 02

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH. If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other Documents

R003	Poxviruses GMO RA
J065	Agarose Gel Electrophoresis and Gel Imaging
J014	Infection and transfection of CEF with MVA and shuttle vector
J002	DNA extraction from Adenovirus and MVA

3 Definitions

PCR	polymerase chain reaction
BSA	Bovine serum albumin
dH ₂ O	Distilled water
RE	Restriction enzyme
RNase	Ribonuclease
DNase	Deoxyribonuclease
MVA	Modified vaccinia Ankara

4 Objective

This procedure confirms, using specific primers, the insertion of various antigens into the thymidine kinase locus of MVA, and hence the identity of the MVA virus sample.

Using parental virus-specific primers the presence of contaminating parental virus can be determined in the test samples.

5 Reagents

DNA extracted from MVA as per J002
Positive control plasmid DNA

AmpliTaq Gold with Geneamp (10x PCR buffer II & MgCl ₂)	Applied Biosystems	4311818
5U/μl AmpliTaq DNA polymerase		
10x PCR buffer (100mM Tris-HCl, pH8.3, 500mM KCl)		
25mM MgCl ₂		
8mM equal mix dNTPs		
Sterile distilled water (RNase & DNase Free)	Sigma	W4502
10 μM DNA primers (see primers database)	MWG	
e.g. for MVA.Red primers:	Redvirus 3 and TKL	
for specific insert:	specific primer + TKL	

6 Equipment

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

7 Method

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

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

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

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

1 x PCR mix

4μl	sterile water		
1μl	10x AmpliTaq buffer	final conc	1x
0.6μl	25mM MgCl ₂	final conc.	1.5mM
1μl	10μM forward primer	final conc	1μM
1μl	10μM reverse primer	final conc	1μM
1μl	8mM equal dNTP mix	final conc	200μM each
0.4μl	5U/μl AmpliTaq polymerase	final conc	2U
1μl	DNA sample		

	Final Conc.	Vol. per 1 reaction (μ l)	Vol. per 2 reactions (μ l)	Vol. per 3 reactions (μ l)	Vol. per 4 reactions (μ l)	Vol. per 5 reactions (μ l)	Vol. per 6 reactions (μ l)
Sterile H ₂ O	-	4	8	12	16	20	24
Amplitaq Buffer (10x)	(1x)	1	2	3	4	5	6
25mM MgCl ₂	150 μ M	0.6	1.2	1.8	2.4	3.0	3.6
Forward primer (10 μ M)	1 μ M	1	2	3	4	5	6
Reverse primer (10 μ M)	1 μ M	1	2	3	4	5	6
<i>Equal mix dNTP's (8mM)</i>	200 μ M	1	2	3	4	5	6
AmpliTaQ Polymerase (5U/ μ l)	0.2 Unit/ μ l	0.4	0.8	1.2	1.6	2.0	2.4
Total		9	18	27	36	45	54

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

PCR Conditions

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

Fix the adhesive lid into place on the 96-well plate and place the plate in the PCR machine.

Start the relevant PCR run, using the heated lid option.

Completed reactions are analyzed by standard agarose gel electrophoresis.

Samples may be stored at -20°C until analysis by electrophoresis.

8 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First written		
02	OH statement added	Safety regulations	S. Morris (19.04.11)



J111: MVA Immunostaining titration using CEF in 6-well plates

1 Version Number: 03

This protocol uses GMOs. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH. If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\msnw3_jenner_server\jenner\hill_group\Safety\COSHH
assessments\Manufacturers material safety data sheets

R003 Poxvirus Risk Assessment
\\msnw3_jenner_server\jenner\hill_group\Safety\GMO RA\R003 poxvirus.doc

C009 MVA Immunostaining Titre
C030 Culture of primary cells and cell lines including freezing and reviving
C024 Use of penicillin for tissue culture by sensitized individuals

J013 CEF culture
J019 Bulking up poxviruses

3 Definitions

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

4 Objective

To titrate a preparation of MVA using CEF in 6-well plates. Method is given for screening by immunostaining for markerless MVA virus. Each virus preparation should be tested in quadruplicate: Two different CEF preps are used at different passage number (i.e. seed 6-well plates from fresh CEF and the previous week's delivery).

Two separate serial dilutions are titrated on each cell batch. A single concentration of a reference virus should be titred at the same time. Therefore, plate one 6-well plate of each cell batch for the virus titration and on a separate plates seed two wells with each cell batch for the reference virus.

GFP expressing reference virus can be counted by immunostaining to gauge accuracy of initial GFP plaque counting, but this is not essential.

5 Reagents

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

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

Complete 2% (10%) FCS DMEM:

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

4% FCS 2xMEM:

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

PBS + 3% FCS:

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

CMC:

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

4% Paraformaldehyde in PBS:

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

0.2% Triton X-100 in PBS:

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

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

6 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO ₂ incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
6-well tissue culture plates		
Benchtop Centrifuge	Beckman Coulter	Allegra X-
12R		
Disposable haemocytometer		
1.5ml microcentrifuge tubes (heat sterilised)		
10µl filter tips		
200µl filter tips		
1000µl filter tips		
Vacuum aspirator		
Aspirator pipettes		
5, 10 and 25ml serological pipettes		
Vortex		
500ml waste bottle and lid (Duran) marked for formaldehyde waste		
250ml waste bottle and lid (Duran) marked for DAB waste		

7 Method

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



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

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

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

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

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

- 8) Incubate at 37°C ($\pm 2^\circ\text{C}$) + 5% CO_2 for 1 hour.
- 9) Proceed with either Agarose or CMC overlay:

Agarose overlay:

- 10) During the incubation, take the bottle of 2% LMP agarose (stored in the tissue culture lab) to melt (2-3 minutes on high power in the microwave in the mol. biol. lab). Transfer required amount of agarose to 50ml Falcon tubes in the tissue culture lab biological safety cabinet.

Pass the Falcon tubes through to the virus lab and place in the water bath at 37°C for at least 15 minutes.

- 11) Place the 4% FCS MEM in 37°C water bath in the virus lab.
- 12) Aspirate the culture medium from the wells (do not allow cells to dry out).
- 13) Pour 4% FCS MEM into an equal volume of 2% low melting point agarose in 50ml Falcon tube (*note: add FCS MEM to the agarose to prevent agarose setting in the 50ml Falcon tube as it is poured*).
- 14) Quickly pipette 2ml per well of this mixture (to prevent the agarose solidifying).
- 15) Incubate for 2-4 days and count the plaques under fluorescent microscope.

CMC overlay:

- 16) Mix sufficient D2 media and CMC in a ratio 2/3 D2:1/3 CMC for 2ml per well required.
- 17) Aspirate media containing virus.
- 18) Replace with 2ml D2/CMC overlay per well.
- 19) Incubate for 2-4 days and count the plaques under fluorescent microscope.

Staining:

- 1) Remove the fluid from the wells with the Vacusafe aspirator
- 2) Fix the cells with approximately 1ml per well of paraformaldehyde (4% in PBS) for 5 minutes at room temperature.
- 3) Remove the fixative **with a pipette to the formaldehyde waste bottle.**
- 4) Wash once with 2ml per well of PBS. Again remove waste with a pipette to formaldehyde waste bottle.
- 5) Permeabilise cells with 0.2% Triton X-100 in PBS. Add 1ml per well for 5 minutes at room temperature. Aspirate waste
- 6) Wash cells twice with 2mls per well PBS using Vacusafe aspirator.
- 7) Block with 1ml per well PBS + 3% FCS and incubate for 1 hour at room temperature
- 8) Remove PBS + 3% FCS using Vacusafe aspirator.
- 9) Prepare 1ml per well of primary antibody diluted 1:1000 in PBS + 3% FCS
- 10) Add 1ml of the primary antibody per well and incubate for 1 hour at room temperature.
- 11) Remove antibody solution and wash twice with approximately 2ml of PBS per well. Remove using Vacusafe aspirator
- 12) Prepare the secondary HRP-conjugated antibody by diluting 1:300 in PBS + 3% FCS.
- 13) Add 1ml of secondary antibody solution per well. Incubate for 45 minutes.
- 14) Remove the antibody solution and wash twice with approximately 2ml of PBS per well. Remove using Vacusafe aspirator
- 15) Make up the DAB Liquid Chromagen developing solution: (allow 1ml/well) add one drop of Chromogen liquid to every 1ml of DAB

Liquid buffer solution that is required and mix well. (Always make up this solution freshly)

- 16) Add 1ml of developing solution per well. Plaques will stain a distinct brown colour. Leave for 10 minutes.
- 17) Discard solution into a glass bottle (marked DAB waste) containing one Pre-Sept tablet
- 18) Wash plates with approximately 2mls tap water per well. Discard water into DAB waste bottle. Bottle left over night and contents discarded with plenty of water down sink.
- 19) Allow plates to dry for half hour in air. Ideally leave upside down on a paper towel.

Determination of Titre (pfu/ml):

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

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

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

Database maintenance:

Virus storage (s:Reagent storage info/virus storage/virus stock database)

Account name: user

Password: virus

Add new record and fill titre details.

MVA production chart: (s:Technical group meetings/vector core meetings/MVA production chart).

Add virus titre to relevant entry

8 **Revision History**

Version Number	What Changed	Why it Changed	Who Changed it
01	First written	N/A	S. Elias
02	Reference dilutions and dilution mixing clarified		S. Elias
03	OH statement added	Safety regulations	S. Morris (19.04.11)



J135: IgG purification for *Plasmodium falciparum* Growth Inhibition

Assay

1 Version Number 2.2

This protocol uses human/animal blood. All users **MUST** be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work with human/animal blood, or need to check your registration, please contact the ORCRB NDM Personnel team.

2 Other documents

See also J063-Rabbit IgG purification from sera
NIH MVDU Protocol IM-6018-00
J133 RBC preparation for *P. falciparum* cultures

3 Definitions

OD: or optical density, a measurement of absorbance of a specific wavelength of ultraviolet or visible light by a solution.

4 Objective

Purification of IgG from mouse, rat, rabbit, Aotus, rhesus or human sera for growth inhibition assays or other tests.

This protocol should not be used for purifying IgG from guinea pig sera.

Not to be confused with preparation of human serum for use as part of malaria culture medium- see protocol J134.

5 Materials

Equipment:

10ml Polypropylene columns with accessories, Pierce Cat. No. 29924
Refrigerated Centrifuge capable of spinning 50ml Falcons at 3095G- e.g. Sorvall machines in Mouse or Human TC.
Parafilm
Pipetboy
Disposable serological sterile pipettes, Costar, or equivalent
Disposable conical tubes with screw cap, sterile, 15ml and 50mL, Falcon, or equivalent
Eppendorf 1.5ml tubes
Spectrophotometer – Nanodrop or Jenner Immunology lab Beckman Coulter with Nanovette system
Amicon Ultra-15 Centrifugal filter device, 30 000 MWCO, Millipore, Cat. No. UFC903024.
Millex-Gv Syringe-driven filter unit, 0.22µm, small diameter, Millipore, Cat. No. SLGVOO4SL or SLGVO13SL, or equivalent
1ml or 2ml syringes, sterile, with Luer Lok attachment if possible
19G Syringe needles, sterile, any source

Reagents:

Immunopure Plus Immobilized Protein G Gel, Pierce Cat. No. 22852
(Or Immobilized Protein G Gel, Pierce Cat. No. 20399)
Immunopure IgG Binding buffer, Pierce, Cat. No. 21011
Immunopure IgG Elution buffer, Pierce, Cat. No. 21009
Tris (Tris [hydroxymethyl] aminomethane), any source
Sodium azide
RPMI Medium 1640
Incomplete *P falciparum* medium – see J132
50% haematocrit O+ erythrocytes – see J133

Solutions to prepare:

Neutralization buffer - 1M Tris buffer, pH 9.0

Storage solution - 0.02% sodium azide = 0.01g in 50ml
(May be more accurate to make 2% azide stock solution- 1g sodium azide in 50ml H₂O- mark as toxic and keep).
Prepare 50ml aliquots of 0.02% azide by adding 500µl of 1% azide stock to 50ml H₂O.

100% hematocrit human O+ RBC - To prepare 500µl of 100% of hematocrit human O+RBC place 1000µl of 50% hematocrit O+ RBCs (refer to J133) into an Eppendorf safe-lock 1.5ml tube and centrifuge at 1700xg for 10 minutes at room temperature.

The 100% RBC pellet is the bottom 500µl in the tube.

6 Method

Note: This SOP is for 2ml of Protein G or Protein G Plus gel but may be scaled up or down according to manufacturer's instructions. However, purifications involving other volumes are NOT covered by this SOP.

Column preparation + record keeping

First use:

Pack columns according to manufacturer's instructions or use pre-packed columns. Use 2ml of packed (settled) gel per column for up to 3ml of serum. Label the column. Use the first three letters of the species name followed by three number places as the standard format for the column number (i.e. for the first column used for purifying human serum, identify the column as "HUM001").

Re-use :

Columns may be used for 10 purifications or until binding capacity becomes reduced. Each time the column is used, note the sample ID on the Falcon in which it is stored.

Retirement:

When columns are retired, discard them and transfer the information about which samples it has been used for into the Protein G column Excel sheet. S:\Protocols\Parasitology\Bloodstage\Protein G column records.

Degassing:

Gas bubbles can collect under either foam disc in the column, and within the bead slurry. Bubbles substantially slow down buffer flow rates. Bubble accumulation can be reduced by warming buffers up to room temperature thoroughly before use.

REMOVAL OF BUBBLES FROM BELOW BOTTOM DISC

Fill column with dH₂O, leaving the top disc in place.

Put Parafilm over the top of the column to form a seal. Invert the column

Remove bottom cap. Press with a finger on the Parafilm.

Fluid and bubbles should be displaced out of the tip.

Replace bottom cap before releasing pressure on the Parafilm.

REMOVAL OF BUBBLES FROM BELOW TOP DISC + FROM SLURRY:

Leave bottom cap on column

Half fill the column with dH₂O.

Remove the top disc using forceps and place it in water.

Use a 5 or 10ml pipette to resuspend the beads.

Place the column in a 50ml conical tube, centrifuge at 1000xg for 10 minutes.

Replace the disc to approximately 1mm from the slurry without trapping bubbles underneath the disc.

dH₂O above the top disc can be thrown off

Prepare the serum sample

- 1) Obtain the volumes of sera specified in the following table. Save at least 10 μ l at -20°C for future ELISA.

Species	Minimum volume (mL)	Maximum volume (mL)	Expected IgG Yield per mL serum (mg)
Normal Mouse	2 mL	4 mL	0.3 – 2 mg
Immunized Mouse	1.3 mL	2 mL	0.3 – 2 mg
Immunized Rat	1 mL	2 mL	3 – 6 mg
Normal Rabbit	2 mL	4 mL	2 – 5 mg
Immunized Rabbit	1 mL	2 mL	2 – 5 mg
Aotus	1 mL	2 mL	2 – 7 mg
Rhesus	1 mL	2 mL	2 – 7 mg
Human	1 mL	2 mL	2 – 7 mg

In practice, 3ml samples of rabbit serum seem to work well.
Dilute serum with an equal volume of binding buffer.

IgG purification

- 2) Warm buffers to room temperature.
- 3) Dispense 300 μ l 1 M Tris, pH 9.0 into a 50ml Falcon labeled with each sample's ID.
- 4) Use 10ml binding buffer to equilibrate the column.
- 5) Apply the serum sample – binding buffer mixture to the column.
- 6) Wash the column using 20ml binding buffer.
- 7) Elute the IgG from the column using 10ml elution buffer into the Falcon containing Tris pH 9.
- 8) Rinse the column with 10ml elution buffer (discard flow through).
- 9) Regenerate the column with 10ml binding buffer, or prepare the column for storage by adding at least 5ml of 0.02% sodium azide (section 5.11.2) and running 2.0-3.0mL through it. Cap and store at 4°C until the next preparation.
- 10) At this point, the protocol can be continued and completed the same day, or the IgG samples can be stored at 4°C overnight for buffer exchange and IgG concentration the next day.

Buffer exchange and IgG concentration.

Cool centrifuge to 4°C

Buffer exchange for total fraction volumes less than 15ml.

This does not need to be done in a sterile fashion unless you wish the bottle of medium to remain sterile.

- 1) Pool the fractions into the Amicon centrifugal filter device (Amicon) and add RPMI 1640 into the filter to total 15ml and mix. Centrifuge at 3095xg for 30 to 45 minutes (until ~250 μ l remains above the filter).
- 2) Discard flow-through. Add RPMI1640 to fill device (approximately 15ml), mix and centrifuge at 3095xg for 30 to 45 minutes (until ~250 μ l remain). Discard flow-through. Add incomplete medium- not RPMI 1640 to fill device (approximately 15ml), mix and centrifuge at 3095xg for 30 to 45 minutes (until ~250 μ l remain).

Buffer exchange for total fraction volumes equal to or greater than 15ml

- 1) Pool 15ml of the fraction volume into the Amicon. Do not add medium. Centrifuge at 3095xg for 30 to 45 minutes (until ~250µl remains above the filter).
- 2) Pool the remaining fractions into the Amicon and add RPMI 1640 into the filter to total 15ml and mix. Centrifuge at 3095xg for 30 to 45 minutes (until ~250µl remains above the filter).
- 3) Discard flow-through. Add RPMI1640 to fill device (approximately 15ml), mix and centrifuge at 3095xg for 30 to 45 minutes (until ~250µl remains above the filter).
- 4) Discard flow-through. Add incomplete medium- not RPMI 1640 to fill device (approximately 15ml), mix and centrifuge at 3095xg for 30 to 45 minutes (until ~250µl remain).

Collection and sterilization of concentrated IgG solution

In a microbiological safety cabinet- e.g. in mouse TC.

- 1) Draw up the sample from the Amicon using a 1-2ml syringe and needle (e.g. 19G). Keep the sample in the syringe with the needle attached. Syringes must be clearly labeled if working with more than one sample.
- 2) Using a 200µl Gilson with a clean tip for each sample, apply 150µl incomplete medium to each side of the Amicon filter, rinsing each side of the apparatus five times. Centrifuge the filter for just a few seconds to bring all the liquid to the bottom. Collect the liquid with the same syringe and needle. Keep the sample in the syringe.
- 3) Remove needle from syringe. Push the sample through a small diameter Millex-Gv filter (section 6.20) into a sterile 1.5ml Eppendorf, labeled with the sample ID. Be very careful not to let the filter come off the end of the syringe under pressure. A LuerLok syringe is preferable to prevent this.

Quantification of IgG

- 1) Gently mix the sample.
- 2) Prepare a 1:20 dilution of the purified IgG by adding 5µl of purified IgG to 95µl incomplete medium (8-tube PCR strips are helpful for this).
- 3) Blank spectrophotometer with the same batch of incomplete media used in sections 8.5.2 - 8.5.5. Read OD. Unneeded diluted sample may be discarded.
- 4) If the OD is above 2, additional dilutions may be made and read. Consult supervisor to determine appropriate dilutions.
- 5) Determine antibody concentration in mg/ml
- 6) Add the same batch of incomplete media to the sample to make the desired IgG concentration. Consult a supervisor to determine the desired concentration (usually 20mg/ml) and see section 9.3 and/ or the *IgG purification and concentration record* spreadsheet for calculations.

Diluting IgG solution to appropriate concentration

If the concentration is less than the desired concentration, do not add any media.

If the concentration is greater than the desired concentration,

-find the net weight in mg of the concentrated IgG by weighing the Eppendorf containing the concentrated IgG, having zero'd the balance with an empty Eppendorf. This is a close approximation to the volume in µl of the concentrated IgG

-Use the IgG purification and concentration record sheet to calculate the volume of incomplete medium to be added to the tube.

-The formula used is:

$$([\text{Initial conc}] * \text{initial volume})/[\text{desired conc}] - \text{initial volume}$$

RBC Adsorption

- 1) Prepare 100% hematocrit O+ RBC
- 2) Add 50ul of 100% hematocrit human O+ RBCs per 1ml of original serum sample that was used to prepare the IgG in a sealed sterile tube.
- 3) Mix the samples by gentle inversion, wrap or cover with foil, and place them on a rocker/shaker for one hour at room temperature.
- 4) Centrifuge the samples at 5000xg using the microcentrifuge for 2 minutes to pellet RBCs.
- 5) Recover the adsorbed IgG by pipetting the supernatant into a new, sterile centrifuge tube. Recover as much as possible. If RBCs are carried across into the new tube, this can be re-spun and the supernatant carried across to a fresh tube. Discard the RBC pellet.
- 6) Label sample with sample ID, IgG concentration, and store at -20°C for future use.

Calculations

Determining antibody concentration

The extinction coefficient for IgG is given in the following table, meaning a 1mg/ml of mouse IgG has an OD of 1.38 at 280nm.

Species	Extinction coefficient (OD of 1 mg/mL IgG at 280 nm)
Mouse	1.38
Rat	1.43
Rabbit	1.44
Antus	1.43
Rhesus	1.43
Human	1.43

To calculate antibody concentration from OD: $(\text{OD} \times \text{dilution factor}) / \text{extinction coefficient} = \text{mg/ml}$

For example, if the measured OD of a 1:20 dilution of mouse IgG is 0.610, then the concentration is:

$$0.610 \times 20 / 1.38 = 8.84 \text{mg/ml}$$

7 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
1.0, 14/8/2009	First draft	Initial version	Sandy Douglas
2.0 6/4/10	Rewording to improve explanations.	Original protocol unclear + not adapted to Jenner equipment	Sandy Douglas
2.1	IgG conc adjustment method	Clarification and use of weights to estimate volume of IgG	Sandy Douglas
2.2 (24.03.11)	OH statement added	Comply with safety regulations	S. Morris

Appendix Two: Peptide Sequences

List of Tables

Table 1. <i>P. falciparum</i> 3D7 AMA1 peptide sequences	447
Table 2. <i>P. falciparum</i> 3D7 CelTOS peptide sequences	449
Table 3. <i>P. falciparum</i> 3D7 CSP peptide sequences	450
Table 4. <i>P. falciparum</i> 3D7 Exp1 peptide sequences	452
Table 5. <i>P. falciparum</i> 3D7 LSA1 peptide sequences	453
Table 6. <i>P. falciparum</i> 3D7 LSA3 peptide sequences	454
Table 7. <i>P. falciparum</i> 3D7 LSAP1 peptide sequences	457
Table 8. <i>P. falciparum</i> 3D7 LSAP2 peptide sequences	458
Table 9. <i>P. falciparum</i> 3D7 MSP1 peptide sequences	459
Table 10. <i>P. falciparum</i> 3D7 MSP2 peptide sequences	462
Table 11. <i>P. falciparum</i> 3D7 PFE1590w peptide sequences	464
Table 12. <i>P. falciparum</i> 3D7 PFI0580c peptide sequences	465
Table 13. <i>P. falciparum</i> 3D7 Pfs16 peptide sequences	466
Table 14. <i>P. falciparum</i> 3D7 STARP peptide sequences	467
Table 15. <i>P. falciparum</i> 3D7 TRAP peptide sequences	469
Table 16. <i>P. falciparum</i> 3D7 UIS3 peptide sequences	471
Table 17. <i>P. falciparum</i> T9/96 TRAP peptide sequences	472
Table 18. <i>P. berghei</i> ANKA TRAP peptide sequences	474

Table 1. *P. falciparum* 3D7 AMA1 peptide sequences.

Peptide	Sequence
AMA1-1	QNYWEHPYQNSDVYRPINEH
AMA1-2	SDVYRPINEHREHPKEYEYP
AMA1-3	REHPKEYEYPLHQEHTYQQE
AMA1-4	LHQEHTYQQEDSGEDENTLQ
AMA1-5	DSGEDENTLQHAYPIDHEGA
AMA1-6	HAYPIDHEGAEPAPQEQNLF
AMA1-7	EPAPQEQNLFSSIEIVERSN
AMA1-8	SSIEIVERSNYMGNPWTEYM
AMA1-9	YMGNPWTEYMAKYDIEEVHG
AMA1-10	AKYDIEEVHGS GIRVDLGED
AMA1-11	SGIRVDLGEDAEVAGTQYRL
AMA1-12	AEVAGTQYRLPSGKCPVFGK
AMA1-13	PSGKCPVFGKGIIIENSKTT
AMA1-14	GIIIENSKTTFLTPVATGNQ
AMA1-15	FLTPVATGNQYLKDGGFAFP
AMA1-16	YLKDGGFAFPTEPLMSPMT
AMA1-17	PTEPLMSPMTLDEMRFYKD
AMA1-18	LDEMRFYKDNKYVKNLDEL
AMA1-19	NKYVKNLDELTLCSRHAGNM
AMA1-20	TLCSRHAGNMIPDNDKNSNY
AMA1-21	IPDNDKNSNYKYPVYDDKD
AMA1-22	KYPVYDDKDKKCHILYIAA
AMA1-23	KKCHILYIAAQENNGPRYCN
AMA1-24	QENNGPRYCNKDESKRNSMF
AMA1-25	KDESKRNSMFCFRPAKDISF
AMA1-26	CFRPAKDISFQNLVYLSKNV
AMA1-27	QNLVYLSKNVVDNWEKVCPR
AMA1-28	VDNWEKVCPRKNLQNAKFGL
AMA1-29	KNLQNAKFGLWVDGNCEDIP
AMA1-30	WVDGNCEDIPHVNEFPAIDL
AMA1-31	HVNEFPAIDLFECNKL VFEL
AMA1-32	FECNKL VFELSASDQPKQYE
AMA1-33	SASDQPKQYEQHLTDYEKIK
AMA1-34	QHLTDYEKIKEGFKNKNREM
AMA1-35	EGFKNKNREMIKSAFLPTGA
AMA1-36	IKSAFLPTGAFKADRYKSHG
AMA1-37	FKADRYKSHGKGYNWGNNT
AMA1-38	KGYNWGNNTETQKCEIFNV
AMA1-39	ETQKCEIFNVKPTCLINDKN
AMA1-40	KPTCLINDKNYIATTALSHP
AMA1-41	YIATTALSHPIEVENNFPCS
AMA1-42	IEVENNFPCSLYKDEIMKEI
AMA1-43	LYKDEIMKEIERESKRIKLN
AMA1-44	ERESKRIKLNNDNDEGNKKI

AMA1-45	DNDDEGNKKIIPRIFISDD
AMA1-46	IAPRIFISDDKDSLKCPCDP
AMA1-47	KDSLKCPCDPEMVSQSTCRF
AMA1-48	EMVSQSTCRFFVCKCVERRA
AMA1-49	FVCKCVERRAEVTSNNEVVV
AMA1-50	EVTSNNEVVVKEEYKDEYAD
AMA1-51	KEEYKDEYADIPEHKPTYDK
AMA1-52	IPEHKPTYDKMKGGGPGGG
AMA1-53	QNYWEHPYQKSDVYHPINEH
AMA1-54	SDVYHPINEHREHPKEYEYP
AMA1-68	FLTPVATGNQDLKDGGFAFP
AMA1-69	DLKDGGFAFPPTNPLISPMT
AMA1-70	PTNPLISPMTLNGMRDFYKN
AMA1-71	LNGMRDFYKNNEYVKNLDEL
AMA1-72	NEYVKNLDELTLCSRHAGNM
AMA1-73	TLCSRHAGNMNPDNDKNSNY
AMA1-74	NPDNDKNSNYKYPVYDYND
AMA1-75	KYPVYDYNDKKCHILYIAA
AMA1-77	QENNGPRYCNKDQSKRNSMF
AMA1-78	KDQSKRNSMFCFRPAKDKLF
AMA1-79	CFRPAKDKLFENLVYLSKNV
AMA1-80	ENLVYLSKNVVVDNWEEVCPR
AMA1-81	VDNWEEVCPRKNLENAKFGL
AMA1-82	KNLENAKFGLWVDGNCEDIP
AMA1-83	WVDGNCEDIPHVNEFSANDL
AMA1-84	HVNEFSANDLFECNKLVFEL
AMA1-90	FKADRYKSHGKGYNWGNYNR
AMA1-91	KGYNWGNYNRETQKCEIFNV
AMA1-94	YIATTALSHPIEVEHNFPCS
AMA1-95	IEVEHNFPCSLYKDEIKKEI
AMA1-96	LYKDEIKKEIERESKRIKLN
AMA1-105	IPEHKPTYDNMKIIIASSAA
AMA1-106	MKIIIASSAAVAVLATILMV
AMA1-107	VAVLATILMVYLYKRKGNAE
AMA1-108	YLYKRKGNAEKYDKMDQPQH
AMA1-109	KYDKMDQPQHYGKSTSRNDE
AMA1-110	YGKSTSRNDEMLEDPEASFWG
AMA1-111	MLDPEASFWGEEKRASHTTP
AMA1-112	EEKRASHTTPVLMEKPYY

Table 2. *P. falciparum* 3D7 CelTOS peptide sequences

Peptide	Sequences
CelTOS-1	MNALRRLPVICSFLV
CelTOS-2	RLPVICSFLVFLVFS
CelTOS-3	CSFLVFLVFSNVLCF
CelTOS-4	FLVFSNVLCFRGNNG
CelTOS-5	NVLCFRGNNGHNSSS
CelTOS-6	RGNNGHNSSSSLYNG
CelTOS-7	HNSSSSLYNGSQFIE
CelTOS-8	SLYNGSQFIEQLNNS
CelTOS-9	SQFIEQLNNSFTSAF
CelTOS-10	QLNNSFTSAFLESQS
CelTOS-11	FTSAFLESQSMNKIG
CelTOS-12	LESQSMNKIGDDLAE
CelTOS-13	MNKIGDDLAETISNE
CelTOS-14	DDLAETISNELVSVL
CelTOS-15	TISNELVSVLQKNSP
CelTOS-16	LVSVLQKNSPTFLES
CelTOS-17	QKNSPTFLESSFDIK
CelTOS-18	TFLESSFDIKSEVKK
CelTOS-19	SFDIKSEVKKHAKSM
CelTOS-20	SEVKKHAKSMLKELI
CelTOS-21	HAKSMLKELIKVGLP
CelTOS-22	LKELIKVGLPSFENL
CelTOS-23	KVGLPSFENLVAENV
CelTOS-24	SFENLVAENVKPPKV
CelTOS-25	VAENVKPPKVDPATY
CelTOS-26	KPPKVDPATYGIIVP
CelTOS-27	DPATYGIIVPVLTSL
CelTOS-28	GIIVPVLTSLFNKVE
CelTOS-29	VLTSLFNKVETAVGA
CelTOS-30	FNKVETAVGAKVSDE
CelTOS-31	TAVGAKVSDEIWNYN
CelTOS-32	KVSDEIWNYNSPDVS
CelTOS-33	IWNYNSPDVSESEES
CelTOS-34	SPDVSESEESLSDDF
CelTOS-35	DVSESEESLSDDFFD

Table 3. *P. falciparum* 3D7 CSP peptide sequences

Peptide	Sequences
CSP-1	MRKLAILSVSSFLFV
CSP-2	ILSVSSFLFVEALFQ
CSP-3	SFLFVEALFQEYQCY
CSP-4	EALFQEYQCYGSSSN
CSP-5	EYQCYGSSSNTRVLN
CSP-6	GSSSNTRVLNELNYD
CSP-7	TRVLNELNYDNAGTN
CSP-8	ELNYDNAGTNLYNEL
CSP-9	NAGTNLYNELEMNYY
CSP-10	LYNELEMNYYGKQEN
CSP-11	EMNYYGKQENWYSLK
CSP-12	GKQENWYSLKKNSRS
CSP-13	WYSLKKNSRSLGEND
CSP-14	KNSRSLGENDDGNNE
CSP-15	LGENDDGNNEEDNEKL
CSP-16	DGNNEEDNEKLKPKH
CSP-17	DNEKLKPKHKKLKQ
CSP-18	RKPKHKKLKQPADGN
CSP-19	KKLKQPADGNPDPNA
CSP-20	PADGNPDPNANPNVD
CSP-21	PDPNANPNVDPNANP
CSP-22	NPNVDPNANPNVDPN
CSP-23	PNANPNVDPNANPNV
CSP-24	NVDPNANPNVDPNAN
CSP-25	ANPNVDPNANPNANP
CSP-26	DPNANPNANPNANPN
CSP-27	PNANPNANPNANPNNA
CSP-28	NANPNANPNANPNAN
CSP-29	ANPNANPNANPNANP
CSP-30	NPNANPNANPNANPN
CSP-31	PNANPNANPNANPNNA
CSP-32	NANPNANPNANPNKN
CSP-33	ANPNANPNKNNQGNG
CSP-34	NPNKNNQGNGQGHNM
CSP-35	NQGNGQGHNMPNDPN
CSP-36	QGHNMPNDPNRNVDE
CSP-37	PNDPNRNVDEANANAN
CSP-38	RNVDEANANANSVKN
CSP-39	NANANSVKNNNNEE
CSP-40	SAVKNNNNNEEPSDKH
CSP-41	NNNEEPSDKHIKEYL
CSP-42	PSDKHIKEYLNKIQN
CSP-43	IKEYLNKIQNLSLSTE
CSP-44	NKIQNLSLSTEWSPCS

CSP-45	SLSTEWSPCSVTCGN
CSP-46	WSPCSVTCGNGIQVR
CSP-47	VTCGNGIQVRIKPGS
CSP-48	GIQVRIKPGSANKPK
CSP-49	IKPGSANKPKDELDY
CSP-50	ANKPKDELDYANDIE
CSP-51	DELDYANDIEKKICK
CSP-52	ANDIEKKICKMEKCS
CSP-53	KKICKMEKCSSVFNV
CSP-54	MEKCSSVFNVVNSSI
CSP-55	SVFNVVNSSIG

Table 4. *P. falciparum* 3D7 Exp1 peptide sequences

Peptide	Sequence
EXP1-1	MKILSVFFLVLFFIIFNKES
EXP1-2	LFFIIFNKESLAEKTNKETG
EXP1-3	LAEKTNKETGSGVSSKKKNK
EXP1-4	SGVSSKKKNKKGSGEPLIDV
EXP1-5	KGSGEPLIDVHDLISDMIKK
EXP1-6	HDLISDMIKKEEELVEVNKR
EXP1-7	EEELVEVNKRKSKYKLATSV
EXP1-8	KSKYKLATSVLAGLLGVVST
EXP1-9	LAGLLGVVSTVLLGGVGLVL
EXP1-10	VLLGGVGLVLYNTEKGRHPF
EXP1-11	YNTEKGRHPFKIGSSDPADN
EXP1-12	KIGSSDPADNANPDADSESN
EXP1-13	ANPDADSESNGEPNADPQVT
EXP1-14	GEPNADPQVTAQDVTPEQPQ
EXP1-15	AQDVTPEQPQGDDNNLVSGP
EXP1-16	GDDNNLVSGPEH

Table 5. *P. falciparum* 3D7 LSA1 peptide sequences

Peptide	Sequences
LSA1-1	MKHILYISFYFILVNLLIFH
LSA1-2	FILVNLLIFHINGKIIKNSE
LSA1-3	INGKIIKNSEKDEIIKSNLR
LSA1-4	KDEIIKSNLRSGSSNSRNRI
LSA1-5	SGSSNSRNRINEEKHEKKHV
LSA1-6	NEEKHEKKHVLSHNSYEKTK
LSA1-7	LSHNSYEKTKNNENNKFDDK
LSA1-8	NNENNKFDDKDKELTMSNVK
LSA1-9	DKELTMSNVKNVSQTNFKSL
LSA1-10	NVSQTNFKSLLRNLGVSENI
LSA1-11	LRNLGVSENI FLKENKLNKE
LSA1-12	FLKENKLNKEGKLIIEHIIND
LSA1-13	GKLIIEHIINDDDDDKKKYIKG
LSA1-14	DDDDKKKYIKGQDENRQEDPQ
LSA1-15	QDENRQEDPQRDLEQRKADT
LSA1-16	RDLEQRKADTKKNLERKKEH
LSA1-17	KKNLERKKEHGDVLAEDLYG
LSA1-18	GDVLAEDLYGRLEIPAIELP
LSA1-19	RLEIPAIELPSENERGYIIP
LSA1-20	SENERGYIIPHQSLLPQDNR
LSA1-21	HQSLLPQDNRGNSRDSKEIS
LSA1-22	GNSRDSKEISIIIEKTNRESI
LSA1-23	IIIEKTNRESITTNVEGRRDI
LSA1-24	TTNVEGRRDIHKGHLEEKKD
LSA1-25	HKGHLEEKKDGSIKPEQKED
LSA1-26	GSIKPEQKEDKSADIQNHTL
LSA1-27	KSADIQNHTLETVNISDVND
LSA1-28	ETVNISDVNDFQISKYEDEI
LSA1-29	FQISKYEDEISAEYDDSLID
LSA1-30	SAEYDDSLIDEEEDDEDLDE
LSA1-31	EEEDDEDLDEFKPIVQYDNF
LSA1-32	FKPIVQYDNFQDEENIGIYK
LSA1-33	QDEENIGIYKELEDLIEKNE
LSA1-34	ELEDLIEKNENLDDLDEGIE
LSA1-35	NLDDLDEGIEKSSEELSEEK
LSA1-36	KSSEELSEEKIKKGKKYEKT
LSA1-37	IKKGKKYEKTKDNNFKPNDK
LSA1-38	KDNNFKPNDKSLYDEHIKKY
LSA1-39	SLYDEHIKKYKNDKQVNKEK
LSA1-40	KNDKQVNKEKEKFIKSLFHI
LSA1-41	EKFIKSLFHIFDGDNEILQI
LSA1-42	FDGDNEILQIVDELSEDITK
LSA1-43	VDELSEDITKYFMKL

Table 6. *P. falciparum* 3D7 LSA3 peptide sequences

Peptide	Sequence
LSA3-1	MDAMKRGLCCVLLLCGAVFV
LSA3-2	VLLLCGAVFVSPSQEIHARF
LSA3-3	SPSQEIHARFRRMNSDINKS
LSA3-4	RRMNSDINKSWKKNTYVDKK
LSA3-5	WKKNTYVDKKLNKLFNRSLG
LSA3-6	LNKLFNRSLGESQVNGELAS
LSA3-7	ESQVNGELASEEVKEKILDL
LSA3-8	EEVKEKILDLLEEGNTLTES
LSA3-9	LEEGNTLTESVDDNKNLEEA
LSA3-10	VDDNKNLEEAEDIKENILLS
LSA3-11	EDIKENILLSNIEEPKENII
LSA3-12	NIEEPKENIIDNLLNIGQN
LSA3-13	DNLLNIGQNSEKQESVSEN
LSA3-14	SEKQESVSENVQVSDELFNE
LSA3-15	VQVSDELFNELLNSVDVNGE
LSA3-16	LLNSVDVNGEVKENILEESQ
LSA3-17	VKENILEESQVND DIFNSLV
LSA3-18	VND DIFNSLVKSVQQEQQHN
LSA3-19	KSVQQEQQHNVEEKVEESVE
LSA3-20	VEEKVEESVEENDEESVEEN
LSA3-21	ENDEESVEENVEENVEENDD
LSA3-22	VEENVEENDDGSVASSVEES
LSA3-23	GSVASSVEESIASSVDESID
LSA3-24	IASSVDESIDSSIEENVAPT
LSA3-25	SSIEENVAPTVEEIVAPTVE
LSA3-26	VEEIVAPTVEEIVAPSVVES
LSA3-27	EIVAPSVVESVAPSVVEESVA
LSA3-28	VAPSVVESVAPSVVEESVAEN
LSA3-29	PSVEESVAENVEESVAENVE
LSA3-30	VEESVAENVEEIVAPSVVES
LSA3-31	EIVAPSVVESVAENVEEIVA
LSA3-32	VAENVEEIVAPTVEESVAEN
LSA3-33	PTVEESVAENVATNLSGNLL
LSA3-34	VATNLSGNLLSNLLGGIETE
LSA3-35	SNLLGGIETEEIKDSILNEI
LSA3-36	EIKDSILNEIEEVKENVVTT
LSA3-37	EEVKENVVTTILENVEETTA
LSA3-38	ILENVEETTAESVTTFSNIL
LSA3-39	ESVTTFSNILEEIQENTITN
LSA3-40	EEIQENTITNDTIEEKL EEL
LSA3-41	DTIEEKL EELHENVLSAALE
LSA3-42	HENVLSAALENTQSEEEKKE
LSA3-43	NTQSEEEKKEVIDVIEEVKE
LSA3-44	VIDVIEEVKEEVATTLIETV

LSA3-45	EVATTLIETVEQAEEESAST
LSA3-46	EQAEEESASTITEIFENLEE
LSA3-47	ITEIFENLEENAVESSENA
LSA3-48	NAVESSENAENLEKLNETV
LSA3-49	ENLEKLNETVFNTVLDKVEE
LSA3-50	FNTVLDKVEETVEISGESLE
LSA3-51	TVEISGESLENNEMDKAFFS
LSA3-52	NNEMDKAFFSEIFDNVKGIQ
LSA3-53	EIFDNVKGIQENLLTGMFRS
LSA3-54	ENLLTGMFRSIETSIVIQSE
LSA3-55	IETSIVIQSEEKVDLNENVV
LSA3-56	EKVDLNENVVSSILDNIENM
LSA3-57	SSILDNIENMKEGLLNKLEN
LSA3-58	KEGLLNKLENISSTEGVQET
LSA3-59	ISSTEGVQETVTEHVEQNVY
LSA3-60	VTEHVEQNVYVDVDVPAMKD
LSA3-61	VDVDVPAMKDQFLGILNEAG
LSA3-62	QFLGILNEAGGLKEMFFNLE
LSA3-63	GLKEMFFNLEDVFKSESDVI
LSA3-64	DVFKSESDVITVEEIKDEPV
LSA3-65	TVEEIKDEPVQKEVEKETVS
LSA3-66	QKEVEKETVSIIEEMEEENIV
LSA3-67	IIIEEMEEENIVDVLEEEKEDL
LSA3-68	DVLEEEKEDLTDKMIDAVEE
LSA3-69	TDKMIDAVEESIEISSDSKE
LSA3-70	SIEISSDSKEETESIKDKEK
LSA3-71	ETESIKDKEKGVSLVVEEVQ
LSA3-72	GVSLVVEEVQDNDMDDESVEK
LSA3-73	DNDMDDESVEKVLELKNMEEE
LSA3-74	VLELKNMEEELMKDAVEIND
LSA3-75	LMKDAVEINDITSKLIEETQ
LSA3-76	ITSKLIEETQELNEVEADLI
LSA3-77	ELNEVEADLIKDMEKLKELE
LSA3-78	KDMEKLKELEKALSSEDKEI
LSA3-79	KALSSEDKEIIDAKDDRLEK
LSA3-80	IDAKDDRLEKVIEEEHDITT
LSA3-81	VIEEEHDITTTLDEVVELKD
LSA3-82	TLDEVVELKDVEEDKIEKVS
LSA3-83	VEEDKIEKVSDLKDLEEDIL
LSA3-84	DLKDLEEDILKEVKEIKELE
LSA3-85	KEVKEIKELESEILEDYKEL
LSA3-86	SEILEDYKELKTIETDILEE
LSA3-87	KTIETDILEEKKEIEKDHFE
LSA3-88	KKEIEKDHFEKFEEEAEEIK
LSA3-89	KFEEEAEEIKDLEADILKEV
LSA3-90	DLEADILKEVSSLEVEEEKK

LSA3-91	SSLEVEEEKKLEEVHELKEE
LSA3-92	LEEVHELKEEVEHIISGDAH
LSA3-93	VEHIISGDAHIKGLEEDDLE
LSA3-94	IKGLEEDDLEEVDLKGSL
LSA3-95	EVDDLKGSILDMLKGDMELG
LSA3-96	DMLKGDMELGDMDKESLEDV
LSA3-97	DMDKESLEDVTAKLGERVES
LSA3-98	TAKLGERVESLKDVLSSALG
LSA3-99	LKDVLSSALGMDEEQMKTRK
LSA3-100	MDEEQMKTRKKAQRPKLEEV
LSA3-101	KAQRPKLEEVLLKEEVKEEP
LSA3-102	LLKEEVKEEPKKKITKKKVR
LSA3-103	KKKITKKKVRFDIKDKEPKD
LSA3-104	FDIKDKEPKDEIVEVEMKDE
LSA3-105	EIVEVEMKDEDEDIEEDIEEDI
LSA3-106	DIEEDIEEDIEEDKVEDIDE
LSA3-107	EEDKVEDIDEDIDEDIDEDI
LSA3-108	DIDEDIDEDIGEDKDEVIDL
LSA3-109	GEDKDEVIDLIVQKEKRIEK
LSA3-110	IVQKEKRIEKVKEKKKKLEK
LSA3-111	VKEKKKKLEKKVEEGVSLK
LSA3-112	KVEEGVSLKKHVDEVMKYV
LSA3-113	KHVDEVMKYVQKIDKEVDKE
LSA3-114	QKIDKEVDKEVSKALESKND
LSA3-115	VSKALESKNDVTNVLKQNQE
LSA3-116	VTNVLKQNQEFSKVKNFVK
LSA3-117	FFSKVKNFVKKYKVFAAPFI
LSA3-118	KYKVFAAPFISAVAAFASYV
LSA3-119	SAVAAFASYVVGFFTFSLFS
LSA3-120	VGFFTFSLFSSCVTIASSTY
LSA3-121	SCVTIASSTYLLSKVDKTIN
LSA3-122	LLSKVDKTINKNKERPFYSF
LSA3-123	KNKERPFYSFVFDIFKNLKH
LSA3-124	VFDIFKNLKHYLQQMKEKFS
LSA3-125	YLQQMKEKFSKEKNNNVIEV
LSA3-126	KEKNNNVIEVTNKAEEKGNV
LSA3-127	TNKAEEKGNVQVTNKTEKTT
LSA3-128	QVTNKTEKTTKVDKNNKVPK
LSA3-129	KVDKNNKVPKKRRTQKSKGG
LSA3-130	KRRTQKSKGGGPGGGRS

Table 7. *P. falciparum* 3D7 LSAP1 peptide sequences

Peptide	Sequence
LSAP1-1	MKTIIIVTLFILILNTIIN
LSAP1-2	ILILNTIINPCTCVPNTLP
LSAP1-3	PCTCVPNTLPIEYFDYETLK
LSAP1-4	IEYFDYETLKENVKIRDSNT
LSAP1-5	ENVKIRDSNTKTQLIILSAV
LSAP1-6	KTQLIILSAVCASILLSLSS
LSAP1-7	CASILLSLSSLWGIRIHYNH
LSAP1-8	LWGIRIHYNHEMNKKEINNY
LSAP1-9	EMNKKEINNYELNSDLLKDA
LSAP1-10	ELNSDLLKDADFYMVE

Table 8. *P. falciparum* 3D7 LSAP2 peptide sequences

Peptide	Sequence
LSAP2-1	MKRGLCCVLLLCGAVFVSPS
LSAP2-2	LCGAVFVSPSQEIHARFRRG
LSAP2-3	QEIHARFRRGMWLCKRGLSV
LSAP2-4	MWLCKRGLSVNDTTKCDVPC
LSAP2-5	NDTTKCDVPCKDFYMLFLSN
LSAP2-6	KDFYMLFLSNKKEKIKCGTF
LSAP2-7	KKEKIKCGTFFGYIFLSKFM
LSAP2-8	FGYIFLSKFMKLSISLLLLA
LSAP2-9	KLSISLLLLALIQNILLSNV
LSAP2-10	LIQNILLSNVSLISGSHLYK
LSAP2-11	SLISGSHLYKRNSRKFAEGY
LSAP2-12	RNSRKFAEGYMKGSGSEKNV
LSAP2-13	MKGSGSEKNVYLSNKNKEIN
LSAP2-14	YLSNKNKEINMNQQSDNKM
LSAP2-15	MNQQSDNKMCDCEDDMNQPG
LSAP2-16	DECDDMNQPGDVNKNKDTSN
LSAP2-17	DVNKNKDTSNDQANSSSDSC
LSAP2-18	DQANSSSDCEPLPFGLKPS
LSAP2-19	EPLPFGLKPSDLNRKVTEED
LSAP2-20	DLNRKVTEEDLERMIIELPG
LSAP2-21	LERMIIELPGKLERKDMYLI
LSAP2-22	KLERKDMYLIWHYSHSLLRD
LSAP2-23	WHYSHSLLRDKFNKMKSSLW
LSAP2-24	KFNKMKSSLWSICGKLAHEH
LSAP2-25	SICGKLAHEHKLFPKIKMCK
LSAP2-26	KLPFKIKMKKWWKCCGHVTD
LSAP2-27	WWKCCGHVTDELLIKEHDDY
LSAP2-28	ELLIKEHDDYNSIYNYINNE
LSAP2-29	NSIYNYINNESSSREQFLIF
LSAP2-30	SSSREQFLIFLNMIKHSWTT
LSAP2-31	LNMIKHSWTTFTMETFIKCK
LSAP2-32	FTMETFIKCKISLENNMRNV
LSAP2-33	ISLENNMRNVTN

Table 9. *P. falciparum* 3D7 MSP1 peptide sequences

Peptide	Sequence
MSP1-1	MKIIFFLCSFLFFIINTQCV
MSP1-2	LFFIINTQCVTHESYQELVK
MSP1-3	THESYQELVKKLEALED AVL
MSP1-4	KLEALED AVL TGYSLFQKEK
MSP1-5	TGYSLFQKEKMVLNELFDLT
MSP1-6	MVLNELFDLTNHMLTLC DNI
MSP1-7	NHMLTLC DNIHGFKYLIDGY
MSP1-8	HGFKYLIDGYEEINELLYKL
MSP1-9	EEINELLYKLNFYFDLLRAK
MSP1-10	NFYFDLLRAKLNDVCANDYC
MSP1-11	LNDVCANDYCQIPFNLKIRA
MSP1-12	QIPFNLKIRANELDVLKKLV
MSP1-13	NELDVLKKLVFGYRKPLDNI
MSP1-14	FGYRKPLDNIKDNVGKMEDY
MSP1-15	KDNVGKMEDYIKKNKTTIAN
MSP1-16	IKKNKTTIANINELIEGSKK
MSP1-17	INELIEGSKKTIDQKNADN
MSP1-18	TIDQKNADNEEGKKKLYQA
MSP1-19	EEGKKKLYQAQYDLSIYNKQ
MSP1-20	QYDLSIYNKQLEE AHN LISV
MSP1-21	LEE AHN LISVLEKRIDTLKK
MSP1-22	LEKRIDTLKKNENIKIKEIA
MSP1-23	NENIKIKEIAKTIKFNIDSL
MSP1-24	KTIKFNIDSLFTDPLELEY Y
MSP1-25	FTDPLELEY YLREKNKKMQI
MSP1-26	LREKNKKMQIKKL TLLKEQL
MSP1-27	KKL TLLKEQLESKLNSLNNP
MSP1-28	ESKLNSLNNPHNVLQNFSVF
MSP1-29	HNVLQNFSVFFNKKKEAEIA
MSP1-30	FNKKKEAEIAETENTLENTK
MSP1-31	ETENTLENTKILLKHYKGLV
MSP1-32	ILLKHYKGLVKYYNGESSPL
MSP1-33	KYYNGESSPLKTLSEVSIQT
MSP1-34	KTLSEVSIQTEDNYANLEGQ
MSP1-35	EDNYANLEGQVVTGEAVTPS
MSP1-36	VVTGEAVTPSVIDNILSKIE
MSP1-37	VIDNILSKIENEYEVLYLKP
MSP1-38	NEYEVLYLKPLAGVYRSLKK
MSP1-39	LAGVYRSLKKQLENNVMTFN
MSP1-40	QLENNVMTFNVNVKDILNSR
MSP1-41	VNVKDILNSRFNKRENFKNV
MSP1-42	FNKRENFKNVLES DLIPYKD
MSP1-43	LES DLIPYKDLTSSNYVVKD
MSP1-44	LTSSNYVVKDPYKFLNKEKR

MSP1-45	PYKFLNKEKRDKFLSSYNYI
MSP1-46	DKFLSSYNYIKDSIDTDINF
MSP1-47	KDSIDTDINFANDVLGYKYI
MSP1-48	ANDVLGYKILSEKYKSDLD
MSP1-49	LSEKYKSDLSIKKYINDKQ
MSP1-50	SIKKYINDKQGENEKYLPFL
MSP1-51	GENEKYLPFLNNIETLYKTV
MSP1-52	NNIETLYKTVNDKIDLFVIH
MSP1-53	NDKIDLFVIHLEAKVLNYTY
MSP1-54	LEAKVLNYTYEKSNEVEVKIK
MSP1-55	EKSNEVEVKIKELNYLKTIQD
MSP1-56	ELNYLKTIQDKLADFKKNNN
MSP1-57	KLADFKKNNNFVGIADLSTD
MSP1-58	FVGIADLSTDYNHNNLLTKF
MSP1-59	YNHNNLLTKFLSTGMVFENL
MSP1-60	LSTGMVFENLAKTVLSNLLD
MSP1-61	AKTVLSNLLDGNLQGMLNIA
MSP1-62	GNLQGMLNIAQHQCCKKQIP
MSP1-63	QHQCCKKQIPQNSGCFRHL
MSP1-64	QNSGCFRHLDEREEWKCLLN
MSP1-65	EREEWKCLLNKQEGDKCVE
MSP1-66	YKQEGDKCVENPNPTCNENN
MSP1-67	NPNTCNENNNGGCDADAKCT
MSP1-68	GGCDADAKCTEEDSGSNGKK
MSP1-69	EEDSGSNGKKITCECTKPS
MSP1-70	ITCECTKPSYPLFDGIFCS
MSP1-71	YPLFDGIFCSSSNGGGPGGG
MSP1-72	SSNGGGPGGGDQVVTGEAIS
MSP1-73	DQVVTGEAISVTMDNILSGF
MSP1-74	VTMDNILSGFENEYDVIYK
MSP1-75	ENEYDVIYKPLAGVYRSLK
MSP1-76	PLAGVYRSLKKQIEKNIFT
MSP1-77	KQIEKNIFTFNLNLNDILNS
MSP1-78	NLNLNDILNSRLKKRKYFLD
MSP1-79	RLKKRKYFLDVLES DLMQFK
MSP1-80	VLES DLMQFKHISSNEYIIE
MSP1-81	HISSNEYIIEDSFKLLNSEQ
MSP1-82	DSFKLLNSEQKNTLLKSYKY
MSP1-83	KNTLLKSYKYIKESVENDIK
MSP1-84	IKESVENDIKFAQEGISYYE
MSP1-85	FAQEGISYYEKVLAKYKDDL
MSP1-86	KVLAKYKDDLESIKKVIKEE
MSP1-87	ESIKKVIKEEKEKFPSSPPT
MSP1-88	KEKFPSSPPTTPPSPAKTDE
MSP1-89	TPPSPAKTDEQKKESKFLPF
MSP1-90	QKKESKFLPFLTNIETLYNN

MSP1-91	LTNIETLYNNLVNKIDDYLI
MSP1-92	LVNKIDDYLINLKAKINDCN
MSP1-93	NLKAKINDCNVEKDEAHVKI
MSP1-94	VEKDEAHVKITKLSDLKAID
MSP1-95	TKLSDLKAIDDKIDLFKNPY
MSP1-96	DKIDLFKNPYDFEAIKKLIN
MSP1-97	DFEAIKKLINDDTKKDMLGK
MSP1-98	DDTKKDMLGKLLSTGLVQNF
MSP1-99	LLSTGLVQNFNPNTIISKLIE
MSP1-100	PNTIISKLIEGKFQDMLNIA
MSP1-101	GKFQDMLNIAQHQCCKKQIP
MSP1-102	QHQCCKKQIPENSGCFRHL
MSP1-103	ENSGCFRHLDEREEWKCLLN
MSP1-106	NPNTCNENNGGCDADATCT
MSP1-107	GGCDADATCTEEDSGSSRKK
MSP1-108	EEDSGSSRKKITCECTKPDS
MSP1-110	YPLFDGIFCSSLNLILYSFI

Table 10. *P. falciparum* 3D7 MSP2 peptide sequences

Peptide	Sequences
MSP2-1	MKVIKTLSIINFFIF
MSP2-2	TLSIINFFIFVTFNI
MSP2-3	NFFIFVTFNIKNESK
MSP2-4	VTFNIKNESKYSNTF
MSP2-5	KNESKYSNTFINNAY
MSP2-6	YSNTFINNAYNMSIR
MSP2-7	INNAYNMSIRRSMAE
MSP2-8	NMSIRRSMAESKPST
MSP2-9	RSMAESKPSTGAGGS
MSP2-10	SKPSTGAGGSAGGSA
MSP2-11	GAGGSAGGSAGGSAG
MSP2-12	AGGSAGGSAGGSAGG
MSP2-13	GGGAGGSAGGSAGGS
MSP2-14	GSAGGSAGGSAGSGD
MSP2-15	SAGGSAGSGDGNGAD
MSP2-16	AGSGDGNGADAEGSS
MSP2-17	GNGADAEGSSSTPAT
MSP2-18	AEGSSSTPATTTTTTK
MSP2-19	STPATTTTTTKTTTTT
MSP2-20	TTTTKTTTTTTTTND
MSP2-21	TTTTTTTTNDAEAST
MSP2-22	TTTNDAEASTSTSSE
MSP2-23	AEASTSTSENPNHK
MSP2-24	STSENPNHKNAETN
MSP2-25	NPNHKNAETNPKGKG
MSP2-26	NAETNPKGKGGEVQEP
MSP2-27	PKGKGGEVQEPNQANK
MSP2-28	EVQEPNQANKETQNN
MSP2-29	NQANKETQNNNSNVQQ
MSP2-30	ETQNNNSNVQQDSQTK
MSP2-31	SNVQQDSQTKSNVPP
MSP2-32	DSQTKSNVPPTQDAD
MSP2-33	SNVPPTQDADTKSPT
MSP2-34	TQDADTKSPTAQPEQ
MSP2-35	TKSPTAQPEQAENSA
MSP2-36	AQPEQAENSAPTAEQ
MSP2-37	AENSAPTAEQTESPE
MSP2-38	PTAEQTESPELQSAP
MSP2-39	TESPELQSAPENKGT
MSP2-40	LQSAPENKGTGQHGH
MSP2-41	ENKGTGQHGHHMHGSR
MSP2-42	GQHGHHMHGSRNNHPQ
MSP2-43	MHGSRNNHPQNTSDS
MSP2-44	NNHPQNTSDSQKECT

MSP2-45	NTSDSQKECTDGNKE
MSP2-46	QKECTDGNKENC GAA
MSP2-47	DGNKENC GAATSLN
MSP2-48	NCGAATSLN NSSNI
MSP2-49	TSLLN NSSNIASINK
MSP2-50	NSSNIASINKFV VLI
MSP2-51	ASINKFVVLISATLV
MSP2-52	FVVLISATLVLSFAI
MSP2-53	VLISATLVLSFAIFI

Table 11. *P. falciparum* 3D7 PFE1590w peptide sequences

Peptide	Sequence
PFE1590w-1	MKRGLCCVLLLCGAVFVSPS
PFE1590w-2	LCGAVFVSPSQEIHARFRRG
PFE1590w-3	QEIHARFRRGMRFSKVFSFF
PFE1590w-4	MRFSKVFSFFAFFIALKYFN
PFE1590w-5	AFFIALKYFNRLGDQLDMG
PFE1590w-6	RCLGDQLDMGSVHNNNSVVG
PFE1590w-7	SVHNNNSVVGNSSSHSPSSS
PFE1590w-8	NSSHSPSSSSSSPSSSSSS
PFE1590w-9	SSSPSSSSSSSSSPSASSS
PFE1590w-10	SSSPSASSSSSSSPASSS
PFE1590w-11	SSSSPASSSSSPSSTSDDS
PFE1590w-12	SSPSSTSDDSKNASLDKIDE
PFE1590w-13	KNASLDKIDEELQKKKKNEK
PFE1590w-14	ELQKKKKNEKLLLISSIATG
PFE1590w-15	LLLISSIATGLAVLVGGIIG
PFE1590w-16	LAVLVGGIIGTALYASRKSS
PFE1590w-17	TALYASRKSSKACKNNVDDL
PFE1590w-18	KACKNNVDDLDSVVEEADVT
PFE1590w-19	DSVVEEADVTEESAVETKKD
PFE1590w-20	ESAVETKKDEVKTEEPKKEQ

Table 12. *P. falciparum* 3D7 PFI0580c peptide sequences

Peptide	Sequence
PFI0580c-1	MKRGLCCVLLLCGAVFVSPS
PFI0580c-2	LCGAVFVSPSQEIHARFRRG
PFI0580c-3	QEIHARFRRGMNLLVFFCF
PFI0580c-4	MNLLVFFCFLLSCIVHLSR
PFI0580c-5	LLSCIVHLSRCSDNNSYSFE
PFI0580c-6	CSDNNSYSFEIVNRSTWLN
PFI0580c-7	IVNRSTWLNIAERIFKGNAP
PFI0580c-8	AERIFKGNAPFNFTIIPYNY
PFI0580c-9	FNFTIIPYNYVNNSTEENNN
PFI0580c-10	VNNSTEENNNKDSVLLISK
PFI0580c-11	KDSVLLISKNLKNSSNPVDE
PFI0580c-12	LKNSSNPVDENNHIDSTKK
PFI0580c-13	NNHIDSTKKNTSNNNNNNS
PFI0580c-14	NTSNNNNNNSNIVGIYESQV
PFI0580c-15	NIVGIYESQVHEEKIKEDNT
PFI0580c-16	HEEKIKEDNTRQDNINKKEN
PFI0580c-17	RQDNINKKENEIINNHHQIP
PFI0580c-18	EIINNHHQIPVSNIFSENID
PFI0580c-19	VSNIFSENIDNNKNYIESNY
PFI0580c-20	NNKNYIESNYKSTYNNNP
PFI0580c-21	KSTYNNNP
PFI0580c-22	IHSTDFIGSNNNHTFNFLSR
PFI0580c-23	NNHTFNFLSRYNNSVLNNMQ
PFI0580c-24	YNNSVLNNMQGNTKVP
PFI0580c-25	GNTKVP
PFI0580c-26	ELKARIFSEEENTEVE
PFI0580c-27	ENTEVE
PFI0580c-28	NHTNSLNP
PFI0580c-29	SCDQIIKLGD
PFI0580c-30	IINSVNEKI
PFI0580c-31	SINSTVNNVLCINLDSVNGN
PFI0580c-32	CINLDSVNGNGFVWTL
PFI0580c-33	GFVWTL
PFI0580c-34	KKKPLIDPSNFPTKRVTQSY
PFI0580c-35	FPTKRVTQSYVSPDISVTNP
PFI0580c-36	VSPDISVTNPVPIPKNSNTN
PFI0580c-37	VPIPKNSNTNKDD
PFI0580c-38	KDD
PFI0580c-39	GSQNNTTTNHFPKPREQLVG
PFI0580c-40	FPKPREQLVGGSSMLISKIK
PFI0580c-41	GSSMLISKIKPHKPGKYFIV
PFI0580c-42	PHKPGKYFIVYSYRPFDP
PFI0580c-43	YSYRPFDPTRDTNTRIVEL
PFI0580c-44	RDTNTRIVELNVQ

Table 13. *P. falciparum* 3D7 Pfs16 peptide sequences

Peptide	Sequence
Pfs16-1	MNIRKFIPSLALMLIFFAFA
Pfs16-2	ALMLIFFAFANLVLSDANDK
Pfs16-3	NLVLSDANDKAKKPAGKGGSP
Pfs16-4	AKKPAGKGGSPSTLQTPGSSS
Pfs16-5	STLQTPGSSSGASLHAVGPN
Pfs16-6	GASLHAVGPNLGGLSQGLSG
Pfs16-7	LGGLSQGLSGKDSADKMPLE
Pfs16-8	KDSADKMPLETQLAIEEIKS
Pfs16-9	TQLAIEEIKSLSNMLDKKTT
Pfs16-10	LSNMLDKKTTVNRNLIISTA
Pfs16-11	VNRNLIISTAVTNMIMIKLI
Pfs16-12	VTNMIMIKLIILSGIVGFKV
Pfs16-13	ILSGIVGFKVKKTKNADDDK
Pfs16-14	KKTKNADDDKGDKDKDKDNT
Pfs16-15	GDKDKDKDNTDEGDEGDDS

Table 14. *P. falciparum* 3D7 STARP peptide sequences

Peptide	Sequence
STARP-1	NLKCNFYNNNNLSTYVIKH
STARP-2	NNLSTYVIKHNRFLSEYQSN
STARP-3	NRFLSEYQSNFLGGGYSAAL
STARP-4	FLGGGYSAALKLVNSKKSGT
STARP-5	KLVNSKKSGTNVNTKYNSSEN
STARP-6	NVNTKYNSSENTNTNNNIPES
STARP-7	TNTNNNIPESSTYTNTRLA
STARP-8	SSTYTNTRLAANNSTTTSTT
STARP-9	ANNSTTTSTTKVTDNNKTNI
STARP-10	KVTDNNKTNIKLTGNNSTTI
STARP-11	KLTGNNSTTINTNSTENTSA
STARP-12	NTNSTENTSATKKVTENVIT
STARP-13	TKKVTENVITNQILTGNNNT
STARP-14	NQILTGNNNTTTNTSTTEHN
STARP-15	TTNTSTTEHNNNINTNTNST
STARP-16	NNINTNTNSTENTSATKKVT
STARP-17	ENTSATKKVTENVITNQILT
STARP-18	ENVITNQILTGNNNTTTNTS
STARP-19	GNNNTTTNTSTTEHNNNINT
STARP-20	TTEHNNNINTNTNSTDNSNT
STARP-21	NTNSTDNSNTNTNLTDNTST
STARP-22	NTNLTDNTSTTKKLTDNINT
STARP-23	TKKLTDNINTTQNLTTSTNT
STARP-24	TQNLTTSTNTTTVSTDNNNI
STARP-25	TTVSTDNNNINTKPIDNNNT
STARP-26	NTKPIDNNNTDIKSTDNYNT
STARP-27	DIKSTDNYNTGTKETDNKNT
STARP-28	GTKETDNKNTDIKATDNNNI
STARP-29	DIKATDNNNITTTTDNTNTN
STARP-30	TTTTDNTNTNVISTDNSKTN
STARP-31	VISTDNSKTNVISTDNSKTN
STARP-32	VISTDNSKTNISTDNDNAD
STARP-33	TISTDNDNADTILTDNDNNT
STARP-34	TILTDNDNNTDIILTDNNNT
STARP-35	DIILTDNNNTDTISTDNDNA
STARP-36	DTISTDNDNADTKATDNNNN
STARP-37	DTKATDNNNNNTNTKATDNNN
STARP-38	TNTKATDNNNTKIIISPNNN
STARP-39	TKIIISPNNNTKTTSTDNNN
STARP-40	TKTTSTDNNNNNTNTKATDNN
STARP-41	NTNTKATDNNNTKTISTDNN
STARP-42	NTKTISTDNNNTKTISTDNN
STARP-43	NTKTISTDNNNTKTISTDNN
STARP-44	NTKTISTDNNNTKTISNDNN

STARP-45	NTKTISNDNNNTNTISTDNN
STARP-46	NTNTISTDNNNNNTNQYVFA
STARP-47	NNNTNQYVFANNYNETTSDD
STARP-48	NNYNETTSDDDELNKDSCDYS
STARP-49	ELNKDSCDYSEEKENIKSMI
STARP-50	EEKENIKSMINAYLDKLDLE
STARP-51	NAYLDKLDLETVRKIHSDIS
STARP-52	TVRKIHSDISTCIEKKNNPR
STARP-53	TCIEKKNNPRNQITHLNNLK
STARP-54	NQITHLNNLKNMYNIIKFIV
STARP-55	NMYNIIKFIVVIYIAFNWSE
STARP-56	VIYIAFNWSEVIYKYVGKLI
STARP-57	VIYKYVGKLILAFALYMLIN
STARP-58	LAFALYMLIN

Table 15. *P. falciparum* 3D7 TRAP peptide sequences

Peptide	Sequence
TRAP-1	MNHLGNVKYLVIVFLIFFDL
TRAP-2	VIVFLIFFDLFLVNGRDVQN
TRAP-3	FLVNGRDVQNNIVDEIKYRE
TRAP-4	NIVDEIKYREEVCNDEVLDY
TRAP-5	EVCNDEVLDYLLMDCSGSIR
TRAP-6	LLMDCSGSIRRHNVNHAVP
TRAP-7	RHNWVNHAVPLAMKLIQQLN
TRAP-8	LAMKLIQQLNLNDNAIHLA
TRAP-9	LNDNAIHLAASVFSNNAREI
TRAP-10	SVFSNNAREIIRLHSDASKN
TRAP-11	IRLHSDASKNKEKALIIKS
TRAP-12	KEKALIIKSLLSTNLPYGK
TRAP-13	LLSTNLPYGKTNLTDALLQV
TRAP-14	TNLTALLQVRKHLNDRINR
TRAP-15	RKHLNDRINRENANQLVVIL
TRAP-16	ENANQLVVILTDGIPDSIQD
TRAP-17	TDGIPDSIQDSLKESRKLS
TRAP-18	SLKESRKLSDRGVKIAVFGI
TRAP-19	RGVKIAVFGIGQGINVAFNR
TRAP-20	GQGINVAFNRFLVGCHPSDG
TRAP-21	FLVGCHPSDGKCNLYADSAW
TRAP-22	KCNLYADSAWENVKNVIGPF
TRAP-23	ENVKNVIGPFMKAVCVEVEK
TRAP-24	MKAVCVEVEKTASCGVWDEW
TRAP-25	TASCGVWDEWSPCSVTCGKG
TRAP-26	SPCSVTCGKGTRSRKREILH
TRAP-27	TRSRKREILHEGCTSELQEQ
TRAP-28	EGCTSELQEQCEEERCLPKR
TRAP-29	CEEERCLPKREPLDVPDEPE
TRAP-30	EPLDVPDEPEDDQPRPRGDN
TRAP-31	DDQPRPRGDNFAVEKPNENI
TRAP-32	FAVEKPNENIIDNNPQEPSP
TRAP-33	IDNNPQEPSPNPEEGKGENP
TRAP-34	NPEEGKGENPNGFDLDENPE
TRAP-35	NGFDLDENPENPPNPPNPPN
TRAP-36	NPPNPPNPPNPPNPPNPPNP
TRAP-37	PPNPPNPPNPDIPEQKPNIP
TRAP-38	DIPEQKPNIPEDSEKEVPSD
TRAP-39	EDSEKEVPSDVPKNPEDDRE
TRAP-40	VPKNPEDDREENFDIPKKPE
TRAP-41	ENFDIPKKPENKHDNQNNLP
TRAP-42	NKHDNQNNLPNDKSDRYIPY
TRAP-43	NDKSDRYIPYSPLAPKVLDN
TRAP-44	SPLAPKVLDNERKQSDPQSQ

TRAP-45	ERKQSDPQSQDNNGNRHVPN
TRAP-46	DNNGNRHVPNSEDRETRPHG
TRAP-47	SEDRETRPHGRNNENRSYNR
TRAP-48	RNNENRSYNRKHNNTPKHPE
TRAP-49	KHNNTPKHPEREEHEKPDNN
TRAP-50	REEHEKPDNNKKKAGSDNKY

Table 16. *P. falciparum* 3D7 UIS3 peptide sequences

Peptide	Sequence
UIS3-1	MKVSKLVLFAHIFFIINILC
UIS3-2	HIFFIINILCQYICLNASKV
UIS3-3	QYICLNASKVNKKGKIAEEK
UIS3-4	NKKGKIAEEKKRKNIDK
UIS3-5	KRKNIDKAIEEHNKRKK
UIS3-6	AIEEHNKRKKLIYYSLIASG
UIS3-7	LIYYSLIASGAIASVAAILG
UIS3-8	AIASVAAILGLGYYGYKKS
UIS3-9	LGYYGYKKSREDDLYNKYL
UIS3-10	EDDLYYNKYLEYRNGEYNIK
UIS3-11	EYRNGEYNIKYQDGAIAS
UIS3-12	YQDGAIASSEFYIEPEGIN
UIS3-13	EFYIEPEGINKINLNKPIIE
UIS3-14	KINLNKPIIENKNNVDVSIK
UIS3-15	NKNNVDVSIKRYNNFVDIAR
UIS3-16	RYNNFVDIARLSIQKHFEHL
UIS3-17	LSIQKHFEHLSNDQKDSHVN
UIS3-18	SNDQKDSHVNNMEYMQKFVQ
UIS3-19	NMEYMQKFVQGLQENRNISL
UIS3-20	GLQENRNISLSKYQENKAVM
UIS3-21	SKYQENKAVMDLKYHLQKVY
UIS3-22	DLKYHLQKVYANYLSQEN

Table 17. *P. falciparum* T9/96 TRAP peptide sequences

Peptide	Sequence
TRAP-1	MNHLGNVKYLVIVFLIFFDL
TRAP-2	VIVFLIFFDLFLVNGRDVQN
TRAP-3	FLVNGRDVQNNIVDEIKYSE
TRAP-4	NIVDEIKYSEEVVCNDQVDLY
TRAP-5	EVCNDQVDLYLLMDCSGSIR
TRAP-6	LLMDCSGSIRRHNVNHAVP
TRAP-7	RHNWVNHAVPLAMKLIQQLN
TRAP-8	LAMKLIQQLNLNDNAIHLVY
TRAP-9	LNDNAIHLVNVVFSNNAKEI
TRAP-10	LNDNAIHLVNVVFSNNAKEI
TRAP-11	IRLHSDASKNKEKALIIIRS
TRAP-12	KEKALIIIRSLLSTNLPYGR
TRAP-13	LLSTNLPYGRTNLTDALLQV
TRAP-14	TNLTDALLQVRKHLNDRINR
TRAP-15	RKHLNDRINRENANQLVVIL
TRAP-16	ENANQLVVILTDGIPDSIQD
TRAP-17	TDGIPDSIQDSLKESRKLS
TRAP-18	SLKESRKLSDRGVKIAVFGI
TRAP-19	RGVKIAVFGIGQGINVAFNR
TRAP-20	GQGINVAFNRFLVGCHPSDG
TRAP-21	FLVGCHPSDGKCNLYADSAW
TRAP-22	KCNLYADSAWENVKNVIGPF
TRAP-23	ENVKNVIGPFMKAVCVEVEK
TRAP-24	MKAVCVEVEKTASCGVWDEW
TRAP-25	TASCGVWDEWSPCSVTCGKG
TRAP-26	SPCSVTCGKGTRSRKREILH
TRAP-27	TRSRKREILHEGCTSEIQEQ
TRAP-28	EGCTSEIQEQCEEERCPPKW
TRAP-29	CEEERCPPKWEPLDVPDEPE
TRAP-30	EPLDVPDEPEDDQPRPRGDN
TRAP-31	DDQPRPRGDNSSVQKPEENI
TRAP-32	SSVQKPEENIIDNNPQEPSP
TRAP-33	IDNNPQEPSPNPEEGKDENP
TRAP-34	NPEEGKDENPNGFDLDENPE
TRAP-35	NGFDLDENPENPPNPDIPEQ
TRAP-36	NPPNPDIPEQKPNIPEDSEK
TRAP-37	<i>PPNPPNPPNPDIPEQKPNIP</i>
TRAP-38	DIPEQKPNIPEDSEKEVPSD
TRAP-39	EDSEKEVPSDVPKNPEDDRE
TRAP-40	VPKNPEDDREENFDIPKKPE
TRAP-41	ENFDIPKKPENKHDNQNNLP
TRAP-42	NKHDNQNNLPNDKSDRNIPY
TRAP-43	NDKSDRNIPYSPLPPKVLDN
TRAP-44	SPLPPKVLDNERKQSDPQSQ

TRAP-45	ERKQSDPQSQDNNGNRHVPN
TRAP-46	DNNGNRHVPNSEDRETRPHG
TRAP-47	SEDRETRPHGRNNENRSYNR
TRAP-48	RNNENRSYNRKYNDTPKHPE
TRAP-49	KYNDTPKHPEREEHEKPDNN
TRAP-50	REEHEKPDNNKKKGESDNKY
TRAP-51	KKKGESDNKYKIAGGIAGGL
TRAP-52	KIAGGIAGGLALLACAGLAY
TRAP-53	ALLACAGLAYKFVVPGAATP
TRAP-54	KFVVPGAATPYAGEPAPFDE
TRAP-55	YAGEPAPFDETLGEEDKDLD
TRAP-56	TLGEEDKDLDEPEQFRLPEE
TRAP-57	EPEQFRLPEENEWN

Table 18. *P. berghei* ANKA TRAP peptide sequences

Peptide	Sequence
TRAP-1	MKLLGNSKYFFVVLRLCISV
TRAP-2	FVVLRLCISVFLNGQEILDE
TRAP-3	FLNGQEILDEIKYSEEV
TRAP-4	IKYSEEV
TRAP-5	QIDLHILLDGSIGHSNWI
TRAP-6	SGSIGHSNWISHVIPMLTTL
TRAP-7	SHVIPMLTTLVDNLNISRDE
TRAP-8	VDNLNISRDEINISMTLFT
TRAP-9	INISMTLFTYARELVRLKR
TRAP-10	YARELVRLKRYGSTSKASLR
TRAP-11	YGSTSKASLRFIIAQLQNNY
TRAP-12	FIIAQLQNNYSPHGTTNLT
TRAP-13	SPHGTTNLTALLNVDNLIQ
TRAP-14	ALLNVDNLIQKKMNRPNAIQ
TRAP-15	KKMNRPNAIQLVIILTDGIP
TRAP-16	LVILTDGIPNNLKKSTTVV
TRAP-17	NNLKKSTTVVNQLKKKDVNV
TRAP-18	NQLKKKDVNVAIIGVGAGVN
TRAP-19	AIIGVGAGVNNMFNRILVGC
TRAP-20	NMFNRILVGCGLGPCPYYS
TRAP-21	GKLGPCPYYSYGSWDQAQTM
TRAP-22	YGSWDQAQTMIKPFLSKVCQ
TRAP-23	IKPFLSKVCQEVEKVALCGK
TRAP-24	EVEKVALCGKWEEWSECSTT
TRAP-25	WEEWSECSTTCDNGTKIRKR
TRAP-26	CDNGTKIRKRKVLHPNCAGE
TRAP-27	KVLHPNCAGEMTAPCKVRDC
TRAP-28	MTAPCKVRDCPPKPVAPPVI
TRAP-29	PPKPVAPPVIPIKVPDVPVK
TRAP-30	PIKVPDVPVKPVEPIEPAEP
TRAP-31	PVEPIEPAEP
TRAP-32	AEPAEP
TRAP-33	EPAEP
TRAP-34	PAEP
TRAP-35	AEPAEP
TRAP-36	EPAEP
TRAP-37	PAEP
TRAP-38	AEPAEP
TRAP-39	EPVNP
TRAP-40	PIKPEEP
TRAP-41	AEPLN
TRAP-42	PFIIP
TRAP-43	PIIAP
TRAP-44	KPIIPEE

TRAP-45	LPNNLPESPSDSQVEYPRPN
TRAP-46	DSQVEYPRPNDNGDNSNTI
TRAP-47	DNGDNSNTINSNKNIPNKH
TRAP-48	NSNKNIPNKHVPPTDDNPYK
TRAP-49	VPPTDDNPYKGQEERIPKPH
TRAP-50	GQEERIPKPHRSNDEYIYYN
TRAP-51	RSNDEYIYYNNANNNDKLEP
TRAP-52	NANNNDKLEPEIPSKDYEEN
TRAP-53	EIPSKDYEENKSKKQSKSNN
TRAP-54	KSKKQSKSNNGYKIAGGIIG
TRAP-55	GYKIAGGIIGGLAIIGCIGV
TRAP-56	GLAIIGCIGVGYNFIAGSSA
TRAP-57	GYNFIAGSSAAAMAGEAAPF
TRAP-58	AAMAGEAAPFEDVMADDEKG
TRAP-59	EDVMADDEKGIVENEQFKLP
TRAP-60	DEKGIVENEQFKLPEDNDWN

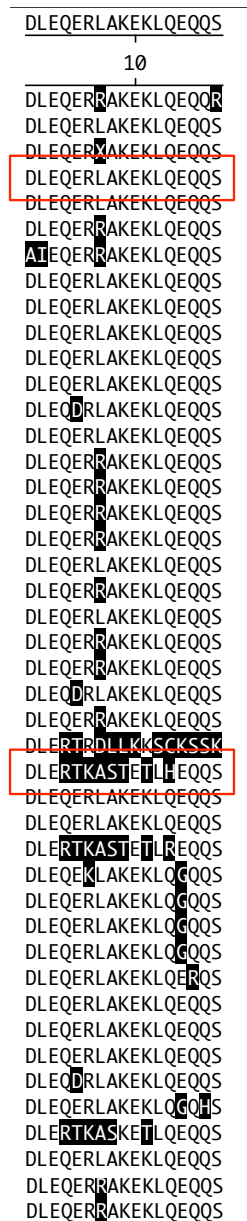
**Appendix Three: PfLSA1 and PfLSA3 sequence
modifications**

PfLSA1 Sequence modifications

The 43 degenerative seventeen amino acid repeats of *P. falciparum* 3D7 LSA1 were aligned in MegAlign using clustalw. The alignment is shown below (Figure 1).

Figure 1. Sequence alignment of *P. falciparum* 3D7 LSA1 repeats.

The seventeen amino acid repeats were aligned using MegAlign and ClustalW. The shaded amino acids represent those different to the majority. The repeats outlined in red were included in the vaccine construct.



PfLSA3 Sequence modifications

The repetitive regions of sequence in *P. falciparum* 3D7 LSA3 were deleted to one copy of each. The alignment of the sequence with repeat regions deleted and the original are shown below.

Figure 2. PfLSA3 sequence: comparison of the modified and original sequences.

The modified sequence (repeats deleted to one copy) was compared with the original sequence using the EMBOSS protein sequence alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The lower sequence is the original sequence, the upper is the modified sequence.

#=====		
EMBOSS_001	1 mtnsnyksnnktyennneqittifnrtnmpikkchmrekinkyfflik	50
EMBOSS_001	1 mtnsnyksnnktyennneqittifnrtnmpikkchmrekinkyfflik	50
EMBOSS_001	51 iltctiliwavyannsdinkswkkntyvdkklnklfnrslgesqvngel	100
EMBOSS_001	51 iltctiliwavyannsdinkswkkntyvdkklnklfnrslgesqvngel	100
EMBOSS_001	101 aseevkekildlleegntltesvddnknleaeedikenillsnieepken	150
EMBOSS_001	101 aseevkekildlleegntltesvddnknleaeedikenillsnieepken	150
EMBOSS_001	151 iidnllnngqnsekqesvsenvqvsdelfnellnsvdngevkenilee	200
EMBOSS_001	151 iidnllnngqnsekqesvsenvqvsdelfnellnsvdngevkenilee	200
EMBOSS_001	201 sqvnddifnslvksvqqeqqhnveekveesveendees-----veen	242
EMBOSS_001	201 sqvnddifnslvksvqqeqqhnveekveesveendeesveenveenveen	250
EMBOSS_001	243 ddesvassveesiassvdesidssieen-----vaptveeivapsv	284
EMBOSS_001	251 ddesvassveesiassvdesidssieenvaptveeivaptveeivapsv	300
EMBOSS_001	285 esvapsveesve-----	296
EMBOSS_001	301 esvapsveesveenveesvaenveesvaenveesvaenveesvaenvees	350
EMBOSS_001	297 --enveesvaenveeivaptveesvaptveeivapsveesvapsveeivv	344
EMBOSS_001	351 vaenveesvaenveeivaptveesvaptveeivapsveesvapsveeivv	400
EMBOSS_001	345 ptveesvaen-----veeivapsveeivaptveesvaptveeivaps	386
EMBOSS_001	401 ptveesvaenveeivapsveeivapsveeivaptveesvaptveeivaps	450
EMBOSS_001	387 veesvapsveeivptveesvaen-----	410
EMBOSS_001	451 veesvapsveeivptveesvaenveesvaenveeivapsveeivapsve	500
EMBOSS_001	411 -----veei	414
EMBOSS_001	501 eivapsveeivapsveeivapsveeivapsveeivapsveeivapsveei	550

EMBOSS_001	415	vapsveevaptveevapsveevaptveesvaenvatnlsdnllsnll :	464
EMBOSS_001	551	vaptveevaptveevapsveevaptveesvaenvatnlsdnllsnll 	600
EMBOSS_001	465	ggieteeikdsilneieevkenvvtilenveettaesvtffsnileei 	514
EMBOSS_001	601	ggieteeikdsilneieevkenvvtilenveettaesvtffsnileei 	650
EMBOSS_001	515	entitndtieekleelhenvlsaalentqseeekkevidvieevkeev 	564
EMBOSS_001	651	entitndtieekleelhenvlsaalentqseeekkevidvieevkeev 	700
EMBOSS_001	565	tlietveqaeesastiteifenleenavesnenvaenleklnetvntv 	614
EMBOSS_001	701	tlietveqaeesastiteifenleenavesnenvaenleklnetvntv 	750
EMBOSS_001	615	ldkveetveisgeslennemdkaffseifdnvkgiqenlltgmfrsiets 	664
EMBOSS_001	751	ldkveetveisgeslennemdkaffseifdnvkgiqenlltgmfrsiets 	800
EMBOSS_001	665	iviqseekvdlennvssildnienmkegllnklenisstegvqetvteh 	714
EMBOSS_001	801	iviqseekvdlennvssildnienmkegllnklenisstegvqetvteh 	850
EMBOSS_001	715	veqnvvydvdpamkdqflgilneagglkemffnledvfkssesdvitvee 	764
EMBOSS_001	851	veqnvvydvdpamkdqflgilneagglkemffnledvfkssesdvitvee 	900
EMBOSS_001	765	ikdepvqkeveketvsiieemeenivdvleekedltdkmidaveesiei 	814
EMBOSS_001	901	ikdepvqkeveketvsiieemeenivdvleekedltdkmidaveesiei 	950
EMBOSS_001	815	ssdskeetesikdkekdvslvveevqndmdesvekvlelknmeeelmkd 	864
EMBOSS_001	951	ssdskeetesikdkekdvslvveevqndmdesvekvlelknmeeelmkd 	1000
EMBOSS_001	865	aveinditsklieetqelneveadlikdmekllekalsedskeiidak 	914
EMBOSS_001	1001	aveinditsklieetqelneveadlikdmekllekalsedskeiidak 	1050
EMBOSS_001	915	ddtlekvieehditttldvvelkdveedkiekvsdkdleedilkev 	964
EMBOSS_001	1051	ddtlekvieehditttldvvelkdveedkiekvsdkdleedilkev 	1100
EMBOSS_001	965	eikeleseiledykelktietdilekkeiekdhfekfeeeaeikdle 	1014
EMBOSS_001	1101	eikeleseiledykelktietdilekkeiekdhfekfeeeaeikdle 	1150
EMBOSS_001	1015	dilkevssleveeekleevhelkeevahiisgdahikgleeddleevdd 	1064
EMBOSS_001	1151	dilkevssleveeekleevhelkeevahiisgdahikgleeddleevdd 	1200
EMBOSS_001	1065	lksildmllkgdmeigmdkesledvtaklgerveslkdvlssalgmdee 	1114
EMBOSS_001	1201	lksildmllkgdmeigmdkesledvtaklgerveslkdvlssalgmdee 	1250
EMBOSS_001	1115	qmkrkkaqrpkleevllkeevkeepkkkitkkkvrfdikdkepkdeive 	1164
EMBOSS_001	1251	qmkrkkaqrpkleevllkeevkeepkkkitkkkvrfdikdkepkdeive 	1300
EMBOSS_001	1165	vemk----dedieedveedieedkv-----edidedigedkdevidl 	1202
EMBOSS_001	1301	vemkdedidedieedveedieedkvedidedidedidedigedkdevidl 	1350

EMBOSS_001	1203	ivqkekriekvkekkllekkveegvsglkkhvdevmkyvqkidkevdk	1252
EMBOSS_001	1351	ivqkekriekvkekkllekkveegvsglkkhvdevmkyvqkidkevdk	1400
EMBOSS_001	1253	vskaleskndvtnvlkqnqdfsvknfvkkykvfaapfisavaafasyv	1302
EMBOSS_001	1401	vskaleskndvtnvlkqnqdfsvknfvkkykvfaapfisavaafasyv	1450
EMBOSS_001	1303	vgfftfslfsscvtiasstylskvdtinknerpfysfvdfknlkh	1352
EMBOSS_001	1451	vgfftfslfsscvtiasstylskvdtinknerpfysfvdfknlkh	1500
EMBOSS_001	1353	ylqqmekfskeknnvievtlnaekkgvqvtntekttkvdknkvpk	1402
EMBOSS_001	1501	ylqqmekfskeknnvievtlnaekkgvqvtntekttkvdknkvpk	1550
EMBOSS_001	1403	ksrtqsk	1410
EMBOSS_001	1551	ksrtqsk	1558

#-----
#-----