Development and characterisation of anti-DBLβ surface-labelling and cytoadhesion-inhibitory mouse monoclonal and polyclonal antibodies

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By

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Table of contents

LIST OF FIGURES ................................................................. 8
LIST OF TABLES ..................................................................... 11
LIST OF ABBREVIATIONS ...................................................... 14
ABSTRACT ........................................................................... 16

CHAPTER 1: INTRODUCTION ................................................ 18
1.1 The burden of malaria disease ........................................... 18
1.2 The life cycle of P. falciparum ............................................ 19
1.3 Pathophysiology of P. falciparum malaria disease ................. 22
  1.3.1 Severe malaria anaemia .............................................. 22
  1.3.2 Pathogenesis of malaria sequestration ......................... 23
  1.3.3 Other pathological consequences ............................... 24
1.4 Sequestration of P. falciparum IEs and major adhesive phenotypes .... 25
  1.4.1 Cerebral malaria ..................................................... 29
  1.4.2 Pregnancy associated malaria .................................... 30
  1.4.3 Rosetting .............................................................. 32
  1.4.4 Auto-agglutination and platelet-mediated clumping .......... 33
1.5 Variant surface antigens on the surface of IEs ....................... 34
  1.5.1 PfEMP1 ................................................................ 35
    1.5.1.1 Structure of PfEMP1 molecules ............................ 35
    1.5.1.2 var genes ......................................................... 37
    1.5.1.3 PfEMP1 Adhesive properties ............................... 38
    1.5.1.4 Antigenic variation .............................................. 40
  1.5.2 RIFIN ................................................................. 41
  1.5.3 STEVOR .............................................................. 43
  1.5.4 SURFIN ............................................................ 44
  1.5.5 Modified band 3 ...................................................... 44
  1.5.6 PfMC-2TM .......................................................... 45
1.6 Human receptors for P. falciparum IE adhesion .................... 46
  1.6.1 ICAM-1 ................................................................ 47
    1.6.1.1 Association between ICAM-1 binding and severe malaria disease .... 49
  1.6.2 CD36 .................................................................... 50
    1.6.2.1 CD36 adhesion properties and association with disease severity .... 51
  1.6.3 EPCR ................................................................. 52
  1.6.4 CSA ................................................................. 54
  1.6.5 PECAM-1 (CD31) .................................................... 55
AIMS OF STUDY ..........................................................................................................................78

CHAPTER 2: INTRODUCTION ..................................................................................................82

2. MATERIALS AND METHODS .............................................................................................84
  2.1 Recombinant Duffy binding-like β (rDBLβ) domains ......................................................84
  2.2 SDS-PAGE and Western blotting for characterising recombinant DBL domains ..............84
     2.2.1 Gel electrophoresis ..................................................................................................84
     2.2.2 Coomassie blue staining .........................................................................................85
     2.2.3 Immunoblotting of recombinant DBLβ domains with anti-polyhistidine antibodies .................................................................86
     2.2.4 Immunoblotting of recombinant DBLβ domains with mouse antisera ....................87
  2.3 Mouse immunisation .........................................................................................................87
  2.4 Characterisation of mouse antisera ..................................................................................88
     2.4.1 Indirect Enzyme Linked Immunosorbent Assay (ELISA) ........................................88
        2.4.1.1 Screening mouse antisera by preliminary ELISA ...........................................88
        2.4.1.2 Checkerboard titration ELISA ........................................................................90
     2.4.2 Analysis of mouse antisera by flow cytometry .........................................................90
        2.4.2.1 Labelling of IEs by antisera ............................................................................90
        2.4.2.2 Data analysis ..................................................................................................91
  2.5 Production and characterisation of mouse monoclonal and polyclonal antibodies ..........92
     2.5.1 Hybridoma cell production ......................................................................................92
        2.5.1.1 SP2/0 myeloma cell line culture ......................................................................92
        2.5.1.2 Extraction and preparation of spleen cells .....................................................93
        2.5.1.3 Fusion of splenocytes with SP2/0 myeloma cells .......................................93
     2.5.2 Indirect ELISA for screening hybridoma clones ......................................................95
2.5.3 Cloning of hybridoma clones by limiting dilution ................................................. 95
2.5.4 Freezing and recovery of Hybridoma clones .......................................................... 96
2.6 Affinity purification of monoclonal and polyclonal antibodies .................................. 97
2.7 Screening eluted fractions by flow cytometry ............................................................ 98
2.8 ELISA for investigating the efficiency of affinity purification ...................................... 98
2.9 Dot blot for estimating the concentration of IgM in eluted fractions ....................... 99
2.10 Double-antibody sandwich ELISA for the quantitation of murine IgM ................. 100
2.11 Parasite culture ........................................................................................................ 101
  2.11.1 P. falciparum isolates ......................................................................................... 101
  2.11.2 Parasite culture .................................................................................................. 101
  2.11.3 Determination of parasitaemia ......................................................................... 101
  2.11.4 Trophozoite enrichment - plasmagel floatation ................................................ 102
  2.11.5 Selection of IgG-IEs on ICAM-1 selection ....................................................... 102
  2.11.6 Selection of A4-IEs on BC6 ............................................................................ 103
  2.11.7 Cryopreservation of parasites ........................................................................... 103
  2.11.8 Reconstitution of frozen parasites ..................................................................... 103

2. RESULTS .................................................................................................................. 105
  2.1 Analysis of recombinant DBLβ domains by SDS-PAGE and Western blotting .............. 105
  2.2 Immunisation of mice with recombinant DBLβ domains ........................................ 108
  2.3 Functional characteristics of mouse antisera .......................................................... 108
    2.3.1 Analysis of mouse antisera by ELISA ............................................................. 108
      2.3.1.1 Indirect ELISA for investigating the reactivity of mouse antisera against recombinant domains .............................................................. 108
      2.3.1.2 Checkerboard ELISA ............................................................................. 112
    2.3.2 Analysis of mouse antisera by Western blotting ............................................... 115
    2.3.3 Analysis of mouse antisera by flow cytometry ............................................... 117
  2.4 Development and characterisation of mouse monoclonal and polyclonal anti-DBLβ antibodies ........................................................................................................ 119
  2.5 Affinity purification and characterisation of purified monoclonal and polyclonal antibodies .............................................................................................................. 123
  2.6 Measurement of IgM concentration in purified mAbs and pAbs ............................. 128
  2.7 Discussion .............................................................................................................. 132
  2.8 Future work ........................................................................................................... 139

CHAPTER 3: INTRODUCTION ...................................................................................... 141

3. MATERIALS AND METHODS .................................................................................... 142
3.1 Measurement of mouse IgM labelling to the surface P. falciparum-IEs by flow cytometry ................................................................. 142
3.2 Competition assays ........................................................................ 143
  3.2.1 Competition between mouse IgMs and BC6 for surface binding to A4-IEs ... 143
  3.2.2 Competition between IgM mAbs and pAbs and HIS for surface binding to A4-IEs 144
  3.2.3 Competition between IgM mAbs and pAbs and rat anti-DBL16 antisera for surface binding to ItG-IEs ................................................................. 145
3.3 Binding of antibodies to trypsin-treated IEs ........................................ 147
3.4 Binding of trypsin-treated ItG-IEs to ICAM-1 receptors .................... 148
3.5 Localisation of IgM surface labelling using IFA ............................. 148
3.6 IgM-mediated IE lysis .................................................................... 149
  3.6.1 Quantitation of lysed IEs by flow cytometry ............................... 149
  3.6.2 Measurement of lysed IEs by microscopy .................................. 150
3.7 Statistical analysis ........................................................................ 150
3. RESULTS .......................................................................................... 151
  3.1 Labelling of mouse mAbs and pAbs to the surface of P. falciparum IEs ..... 151
    3.1.1 Measurement of IgM binding to the surface of ICAM-1 binding P. falciparum-IEs ................................................................. 157
    3.1.2 Measurement of IgM binding to the surface of non-ICAM-1 binding P. falciparum IEs ................................................................. 159
    3.1.3 Measurement of IgM binding to the surface of non-IEs ................. 159
    3.1.4 Correlation of IgM surface labelling among P. falciparum isolates .... 165
    3.1.5 Correlation between IgM reactivity to recombinant domains and IE surface labelling ................................................................. 167
  3.2 Competition assays ........................................................................ 168
    3.2.1 The effect of pre-incubation of A4-IEs with mAbs and pAbs on BC6 surface binding ................................................................. 168
    3.2.2 The effect of pre-incubation of A4-IEs with mAbs and pAbs on HIS surface binding ................................................................. 170
    3.2.3 The effect of pre-incubation of ItG-IEs with mAbs and pAbs on surface labelling of rat anti-DBL16 antisera ................................................................. 173
  3.3 Mouse IgM binding to trypsin-treated IEs ......................................... 175
  3.4 Localisation of IgM surface labelling using liquid-phase immunofluorescence assay (L-IFA) ................................................................. 180
  3.5 Mouse IgM-mediated IE lysis .......................................................... 183
    3.5.1 Quantitation of lysed IEs by flow cytometry ............................... 183
    3.5.2 Estimating the parasitaemia after IgM incubation ....................... 186
    3.5.3 Correlation between IE surface labelling and lysis ....................... 188
3.6.3  Effect of IgM dilution on IE lysis ................................................................. 190
3.7  Discussion ........................................................................................................ 192
3.8  Limitations and Future work............................................................................. 205

CHAPTER 4: INTRODUCTION ............................................................................ 207

4.  MATERIALS AND METHODS ....................................................................... 209
  4.1  Purified proteins used for static and flow adhesion assays ......................... 209
  4.2  Monoclonal antibodies .................................................................................. 209
  4.3  Cytoadhesion assays under static conditions ............................................... 209
        4.3.1  IE adhesion to immobilised protein receptors ..................................... 209
        4.3.2  Blockade of IE adhesion under static conditions ......................... 211
  4.4  Cytoadhesion assays under physiological flow conditions ...................... 212
        4.4.1  IE adhesion to immobilised receptors .............................................. 212
              4.4.1.1  Adhesion to ICAM-1 under flow conditions ....................... 212
              4.4.1.2  Blockade of IE adhesion to ICAM-1 under flow conditions .... 213
        4.4.2  IE adhesion to HUVEC ................................................................. 214
              4.4.2.1  HUVEC culture ................................................................. 214
              4.4.2.2  Analysis of receptor expression on HUVEC by flow cytometry .... 215
              4.4.2.3  IE adhesion to HUVEC under flow conditions .................. 215
              4.4.2.4  Blockade of IE adhesion to HUVEC under flow conditions .... 216
  4.5  IE adhesion reversal to immobilised protein receptors under static conditions .......................................................... 217
  4.6  Statistical analysis .......................................................................................... 217

4.  RESULTS ........................................................................................................... 218
  4.1  Adhesion blocking of ItG-IEs to ICAM-1 and CD36 receptors under static conditions .................................................................................................................. 218
        4.1.1  Blocking ItG-IE binding to ICAM-1 by mAbs and pAbs ................. 218
        4.1.2  Blocking ItG-IEs binding to CD36 by mAbs and pAbs .................. 221
  4.2  Adhesion blocking of C24-IEs to CD36 receptors under static conditions .................................................................................................................. 223
  4.3  Adhesion blocking of ItG-IEs to ICAM-1 receptors under physiological flow conditions .................................................................................................................. 225
  4.4  Adhesion blocking of A4-IEs to HUVEC under physiological flow conditions .................................................................................................................. 227
  4.5  Adhesion blocking of ItG-IEs to ICAM-1 receptors after incubation with different concentrations of mAb G6 ................................................................. 230
  4.6  Adhesion reversal of ItG-IEs to ICAM-1 receptors under static conditions .................................................................................................................. 232
CHAPTER 5: INTRODUCTION

5. MATERIALS AND METHODS

5.1 Serum samples

5.2 Indirect ELISA for screening semi-immune sera against recombinant DBL domains

5.3 Analysis of semi-immune sera by flow cytometry

5.4 Adhesion-blocking of IEs to ICAM-1 or CD36 receptors under static conditions

5.5 Adhesion-blocking of IEs to ICAM-1 receptors under physiological flow conditions

5.6 Adhesion-blocking of IEs to HUVEC under physiological flow conditions

5.7 Agglutination assay

5.8 Statistical analysis

5.9 Ethics approval

5. RESULTS

5.1 Indirect ELISA for investigating the reactivity of semi-immune sera against recombinant DBL domains

5.2 Measurement of IgG binding to the surface of P. falciparum-IEs

5.3 Correlation of IgG surface labelling between P. falciparum parasite isolates

5.4 Adhesion blocking studies with semi-immune sera

5.4.1 Blockade of Ig-IE adhesion to ICAM-1 receptors under static conditions

5.4.2 Blockade of C24-IE adhesion to CD36 receptors under static conditions

5.4.3 Blockade of Ig-IE adhesion to ICAM-1 receptors under physiological flow conditions

5.4.4 Blockade of A4-IE adhesion to HUVEC under physiological flow conditions

5.5 Correlation between surface labelling and adhesion blocking

5.6 Agglutination assay

5.7 Correlation between adhesion blocking and agglutination

5.8 Discussion

5.9 Limitations and future work

CHAPTER 6: GENERAL CONCLUSIONS

ACKNOWLEDGEMENTS
List of figures

Figure 1.2. Parasite-mediated modifications to the IE. ......................................................... 27
Figure 1.3. Cytoadhesion phenotypes of P. falciparum IEs. ..................................................... 28
Figure 1.4. Structural and adhesive properties of PfEMP1 molecules. ...................................... 40
Figure 1.5. Parasite-derived modifications to P. falciparum-IEs. ............................................... 46
Figure 1.6. The crystal structure of the N-terminal domain of human ICAM-1 ......................... 48
Figure 1.7. Effects of P. falciparum-IE adhesion to EPCR ..................................................... 54
Figure 1.8. Possible functional consequences associated with IgM-PfEMP1 binding ............... 68
Figure 2.1. SDS-PAGE and Western blotting for the identification of four rDBL domain proteins expressed in E. coli. ..................................................................................... 106
Figure 2.2. SDS-PAGE and Western Blotting analysis for the identification of four rDBL domain proteins ......................................................................................................................... 107
Figure 2.3. Indirect ELISA for investigating the IgG-class reactivity of antisera obtained from mice immunised with recombinant domains .............................................................................. 109
Figure 2.4. Indirect ELISA for investigating the IgM-class reactivity of antisera obtained from mice immunised with recombinant domains .............................................................................. 110
Figure 2.5. The reactivity of serial dilutions of anti-DBL31 antisera against different concentrations of recombinant DBL domain proteins .......................................................................... 113
Figure 2.6. Western Blotting analysis of mouse polyclonal antisera against immunising DBL proteins ................................................................................................................................. 116
Figure 2.7. Reactivity of anti-DBL13 mouse antisera with the surface of erythrocytes infected by different P. falciparum isolates ................................................................................................. 118
Figure 2.8. Investigating the presence of reactive IgG antibodies in hybridoma culture supernatants (n= 17) against rDBL proteins .............................................................................................. 120
Figure 2.9. Investigating the presence of reactive IgM antibodies in hybridoma culture supernatants (n= 17) against rDBL proteins .............................................................................................. 121
Figure 2.10. Reactivity of all eluted fractions of pAb B12 with the surface of erythrocytes infected by ItG P. falciparum isolate .................................................................................................................. 124
Figure 2.11. Indirect ELISA for investigating the efficiency of affinity purification of mAbs and pAbs ........................................................................................................................................... 127
Figure 2.12. Dot Blot assay for estimating the concentration of IgM antibodies in purified mAbs and pAbs ................................................................................................................................. 129
Figure 2.13. Slope of a standard dilution curve generated from gradual concentrations of commercially provided mouse IgM control .................................................................................. 130
Figure 3.1. FACS histograms of gated erythrocytes infected by ItG isolate and labelled by mAbs E7 and B4 at neat concentrations or at 1:2, 1:4 or 1:8 dilutions ................................................................................. 153
Figure 3.2. FACS histograms of gated erythrocytes infected by ItG isolate and labelled by mAbs and pAbs, ........................................................................................................................................... 158
Figure 3.3. The reactivity of mouse mAbs and pAbs with parasite-derived proteins on the surface of C24-IEs as measured by flow cytometry. ................................................................. 160
Figure 3.4. MAbs and pAbs surface reactivity with 3D7 P. falciparum-IEs as measured by flow cytometry. ................................................................. 161
Figure 3.5. Flow cytometry analysis of the effect of pre-incubating A4-IEs with mouse mAbs and pAbs on the subsequent binding of mAb BC6 (20 µg/ml) and vice versa.................. 169
Figure 3.6. The reactivity of HIS against the surface of A4-IEs as measured by flow cytometry ........................................................................................................... 171
Figure 3.7. Flow cytometry histograms of the effect of pre-incubating A4-IEs with mouse IgM pools on the subsequent binding of HIS (30 mg/ml) and vice versa ...................... 172 .............................................................................................................................. 174
Figure 3.8. Flow cytometry histograms of the effect of pre-incubating of ItG-IEs with mouse IgM pools on the subsequent labelling of rat anti-DBL16 antisera at 1:10 dilution and vice versa. ........................................................................................................... 174
Figure 3.9. Adhesion of trypsin-treated ItG-IEs to immobilised ICAM-1 protein. ................. 176
Table 3.10. Flow cytometry histograms of mAb BC6 labelling to trypsin-treated A4-IEs. ...... 177
Figure 3.11. Flow cytometry histograms of mAb G6, pAb B5, pool 1 of mouse IgMs and HIS labelling to trypsin-treated ItG-IEs ........................................................................... 179
Figure 3.12. L-IFA staining of the surface of live A4-IEs with mAb BC6 (20 µg/ml). .......... 181
Figure 3.14. IgM-mediated IE lysis caused by binding of mouse mAbs/pAbs to C24-IEs...... 184
Figure 3.15. Estimating the percentage of IE lysis after incubation with mouse IgMs over time ........................................................................................................... 187
Figure 3.16. Output of Spearman's rho correlation between surface labelling and IE lysis for 3D7 parasite strain ........................................................................................................... 189
Figure 3.17. Association between IgM concentration and percentage of ItG-IEs lysis by mAbs B4 and G6 as indicated by flow cytometry. ................................................................. 191
Figure 4.1. Coating of ICAM-1 and CD36 receptors onto bacteriological petri dishes for static adhesion assays. ........................................................................................................... 211
Figure 4.2. Vena8 Fluoro+TM biochips .............................................................................. 213
Figure 4.1. Adhesion inhibition of ItG-IEs to purified ICAM-1 receptors under static conditions. ........................................................................................................... 220
Figure 4.2. Adhesion inhibition of ItG-IEs to purified CD36 receptors under static conditions. 222
Figure 4.3. Adhesion inhibition of C24-IEs to CD36 receptors under static conditions. ....... 224
Figure 4.4. Effect of antibody blockade on ICAM-1-mediated ItG-IEs adhesion at an inflow shear stress of 0.05 Pa. ........................................................................................................... 226
Figure 4.5. Representative flow cytometry analysis of the expression of ICAM-1 and CD31 surface receptors on monolayers of HUVEC with or without activation .................. 228
Figure 4.6. Effect of antibody blockade on A4-IEs adhesion to TNF-α stimulated HUVEC under flow conditions at a wall shear stress of 0.05 Pa................................................................. 229

Figure 4.7. Adhesion blocking of ItG-IEs to ICAM-1 receptors under static conditions........ 231

Figure 4.8. Reversal of ItG-IEs binding to purified ICAM-1 receptors with mouse mAbs under static conditions.......................................................... 233

Figure 5.1. The reactivity of serum samples collected from eight female adults living in Kilifi, Kenya against recombinant DBL domains.................................................. 254

Figure 5.2. FACS analysis of ItG-IE surface reactivity with semi-immune sera from female adults living in Kilifi, Kenya............................................................. 257

Figure 5.3. FACS analysis of 3D7-IE surface reactivity with semi-immune sera collected from female adults living in Kilifi, Kenya.................................................. 258

Figure 5.4. Adhesion blocking of ItG-IEs to purified ICAM-1 receptors under static conditions. ........................................................................................................... 262

Figure 5.5. C24-IE adhesion blocking to CD36 receptors with semi-immune sera under static conditions. ........................................................................................................... 264

Figure 5.6. Blockade of ItG-IE adhesion to ICAM-1 at an inflow shear stress of 0.05 Pa........ 266

Figure 5.7. Blockade of A4-IE adhesion by semi-immune sera to TNF-α-stimulated HUVEC under flow conditions at a wall shear stress of 0.05 Pa.................................................. 268

Figure 5.8. A4-IE agglutination mediated by semi-immune adult sera.............................. 273

Figure S1. Indirect ELISA for investigating the efficiency of affinity purification of mAbs and pAbs against different coating antigens..................................................... 315
List of tables

Table 1.1. Vaccine studies based on immunisation of animals with PfEMP1 domains involved in CD36 binding and rosetting .......................................................................................................................... 61
Table 1.2. Vaccine studies based on immunisation of animals with VAR2CSA domains involved in PM ...................................................................................................................................... 64
Table 1.3. Development of mAbs and pAbs based on immunisation of animals with single or full-length PfEMP1 domains .................................................................................................................................. 71
Table 2.1. Calculated and observed molecular weights of recombinant DBLβ domains ................. 107
Table 2.2. Investigating the presence of reactive IgG antibodies in mouse polyclonal antisera against recombinant DBLβ domains .................................................................................................................. 111
Table 2.3. Investigating the presence of reactive IgM antibodies in mouse polyclonal antisera against recombinant DBLβ domains .................................................................................................................. 111
Table 2.4. Summary of the reactivity of serial dilutions of mouse antisera against different concentrations of recombinant DBL domain proteins .............................................................................. 114
Table 2.5. Summary of FACS data for the reactivity of DBLβ domain-specific antisera with erythrocytes infected by different *P. falciparum* isolates ................................................................................................................................. 119
Table 2.6. IgM reactivity of monoclonal and polyclonal hybridoma clones against recombinant *P. falciparum* isolates used in mouse immunisations .......................................................................................................................... 122
Table 2.7. Summary of reactive hybridoma clones raised against variant rDBL proteins .......... 122
Table 2.8. The reactivity of all eluted fractions of mAbs and pAbs resulted from the affinity purification to the surface of ItG-IEs ........................................................................................................................................ 125
Table 2.9. Summary of the final IgM concentration of purified mAbs and as measured by double-antibody sandwich ELISA ......................................................................................................................................... 131
Table 3.1. Summary of primary and secondary antibody incubations for all competition assays ........................................................................................................................................ 146
Table 3.1. Labelling capacity of mouse mAbs and pAbs to the surface of erythrocytes infected with ItG *P. falciparum* isolate .............................................................................................................................................. 152
Table 3.2: The origin and properties of nine distinct *P. falciparum* isolates used in the study ... 155
Table 3.3. The reactivity of monoclonal and polyclonal anti-DBLβ antibodies to the surface of erythrocytes infected by phenotypically and genotypically distinct *P. falciparum* isolates ....... 156
Table 3.4. Spearman's rho correlation of the surface labelling profiles of thirteen mAbs/pAbs to erythrocytes infected by eight *P. falciparum* parasite isolates ........................................................................................................................... 166
Table 3.5. Correlation between surface labelling of 13 mAbs and pAbs to erythrocytes infected by eight *P. falciparum* isolates and reactivity to rDBL31 ................................................................................................................................. 167
Table 3.6. Labelling of mAb BC6 to trypsin-treated A4-IEs as measured by flow cytometry .... 177
Table 3.7. Labelling of mAb G6, pAb B5, pool 1 of mouse IgMs and HIS to ItG-IEs treated with 10 µg/ml, 100 µg/ml or 1000 µg/ml of TPCK-treated trypsin ............................................................................................................ 180
Table 3.8. Summary of IgM-mediated IE lysis caused by binding of thirteen mAbs and pAbs to the surface of erythrocytes infected by eight phenotypically and genotypically distinct *P. falciparum* isolates .......................................................... 185
Table 3.9. Estimating the percentage of IE lysis with/without incubation with mouse IgM antibodies (C2 or B5) at 5, 15, 30 and 60 minutes of incubation .................................................. 188
Table 3.10. Correlation between surface labelling and IE lysis for eight *P. falciparum* isolates 190
Table 4.1. Output of Spearman's rho correlation between surface labelling, IE lysis and adhesion blocking for ItG, C24 and A4 parasite isolates .......................................................... 235
Table 5.1. Characteristics of semi-immune serum samples used in the present study .......... 247
Table 5.2. The reactivity of semi-immune serum samples collected from female adults living in Kilifi, Kenya with the surface of erythrocytes infected by phenotypically and genotypically distinct *P. falciparum* isolates ........................................................................................................ 259
Table 5.3. Spearman's rho correlation of surface labelling profiles of eight semi-immune serum samples obtained from Kenyan adult females to erythrocytes infected by five *P. falciparum* parasite isolates ...................................................... 260
Table 5.4. Output of Spearman's rho correlation between surface labelling and adhesion blocking for ItG, C24 and A4 isolates .................................................................................................... 270
Table 5.5. Agglutination score and frequency for A4-IEs after incubation with eight semi-immune Kenyan sera or UK control serum ............................................................... 272
Table 5.6. Agglutination score and frequency for ItG-IEs after incubation with eight semi-immune Kenyan sera or UK control serum ............................................................................. 272
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Alum</td>
<td>Aluminium hydroxide gel</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>CIDR</td>
<td>Cysteine Rich Interdomain Region</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>CM-CRL</td>
<td>Culture medium control</td>
</tr>
<tr>
<td>CMNS</td>
<td>Foetal bovine serum-free complete medium</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CS</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin Sulfate A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy binding-like</td>
</tr>
<tr>
<td>DC</td>
<td>Domain cassette</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent assay</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
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<tr>
<td>HBEC</td>
<td>Human Brain Endothelial Cells</td>
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<tr>
<td>HDMEC</td>
<td>Human Dermal Microvascular Endothelial Cells</td>
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<td>HIS</td>
<td>Malawian hyper immune serum</td>
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<td>ICAM-1</td>
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<td>Infected erythrocyte</td>
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<td>Polyclonal antibody</td>
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<td>PAM</td>
<td>Pregnancy associated malaria</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS/1%BSA</td>
<td>PBS with 1% bovine serum albumin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet/endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>Plasmodium falciparum</td>
</tr>
<tr>
<td>PiEMP1</td>
<td>Plasmodium falciparum erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PIMC-2TM</td>
<td>Plasmodium falciparum Maurer's cleft 2 transmembrane</td>
</tr>
<tr>
<td>PM</td>
<td>Placental malaria</td>
</tr>
<tr>
<td>PBST</td>
<td>1× PBS containing 0.1% Tween® 20 detergent</td>
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<tr>
<td>PBSTM</td>
<td>1× PBS containing 0.1% Tween® 20 detergent and 5% non-fat milk powder</td>
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<tr>
<td>PV</td>
<td>Parasitophorous Vacuole</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RIFIN</td>
<td>Repetitive interspersed family</td>
</tr>
<tr>
<td>STEVOR</td>
<td>Sub-telomeric variable open reading frame</td>
</tr>
<tr>
<td>SURFIN</td>
<td>Surface-associated interspersed gene family</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TPCK</td>
<td>Tosylamido-2-phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>UCM</td>
<td>Uncomplicated malaria</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant surface antigen</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Abstract

*Plasmodium falciparum* is responsible for most malaria-related morbidity and mortality, mostly affecting young children, non-immune adults and pregnant women. A characteristic feature of the pathogenesis of infection caused by *P. falciparum* is the cytoadherence of infected erythrocytes to the endothelial cells lining the microvessels of host organs. This phenomenon, termed "sequestration", mainly results from the adhesive interactions between *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) proteins on the surface of infected erythrocytes and various host endothelial receptors such intercellular adhesion molecule 1 (ICAM-1), which is hypothesised to have a role in cerebral malaria. PfEMP1 molecules consist of several Duffy binding-like (DBL) and cysteine rich interdomain region (CIDR) domains that have different cytoadhesive functions. The second class of DBL domains, DBLβ, has been associated with adhesion to ICAM-1 receptors.

In the present study, we selected four recombinant PfEMP1ICAM-1-DBLβ domains for mouse immunisations. Thirteen monoclonal (mAbs) and polyclonal antibodies (pAbs) were raised to three recombinant domains (DBL13, DBL31 and DBL41). All mouse mAbs and pAbs comprised IgM antibodies that recognised homologous and heterologous DBLβ domains. Most mAbs and pAbs labelled the surface of erythrocytes infected by *P. falciparum* isolates, with an IgM labelling capacity ranging from 10.1% to 67.6% of total IEs. Mouse antibodies showed similar patterns of reactivity with ICAM-1-binding and non-binding isolates, and reacted with a parasite isolate from a different genome (3D7). Surprisingly, we detected a remarkable reduction in IE population after incubation with mouse mAbs and pAbs, and this was mainly observed with antibodies that strongly labelled the surface of IEs. We demonstrated that this haemolysis was resulted from an immunological interaction between mouse IgMs and a parasite-derived component on the surface of live IEs.

Antibodies raised to DBL41 were the most effective in all assays. Of these, three antibodies (pAb B5, mAb B4, mAb G6) and an anti-DBL31 mAb (E7) significantly blocked IE adhesion to purified proteins (ICAM-1 and CD36) under static and flow conditions. These antibodies also blocked parasite
adhesion to HUVEC under conditions of blood flow. In a separate work, we characterised the immune response of eight semi-immune serum samples obtained from female adults living in Kilifi County, Kenya. Our results indicated that semi-immune sera specifically recognised five recombinant DBLβICAM-1 domains and a VAR2CSA DBL domain, and recognised the surface of erythrocytes infected by diverse parasite isolates with variable levels of reactivity. Some sera, particularly JA225 and JA235, significantly inhibited IE adhesion to ICAM-1 under both static and flow conditions. To our knowledge, this is the first study to examine the use of PfEMP1ICAM-1-DBLβ domains for the development of mouse mAbs and pAbs that recognise homologous and heterologous parasite isolates and block IE adhesion. However, further work is required to identify the surface ligand(s) involved in interaction with mouse IgM and to investigate the mechanisms of IgM-mediated IE lysis.
Chapter 1: Introduction

1.1 The burden of malaria disease

The protozoan parasite *Plasmodium falciparum* is the causative agent of the most virulent forms of malaria disease affecting humans worldwide. Although *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* also cause human infections, *P. falciparum* is responsible for most malaria-related morbidity and mortality, mostly affecting young children, non-immune adults and pregnant women and is the focus of this study (Beeson and Brown, 2002, Weatherall et al., 2002). According to the latest reports, there was an estimated 198 million malaria illness episodes and 584 thousands deaths that occurred globally in 2013, with about 1.2 billion people at high risk of developing malaria infection. The majority of deaths occurred in Africa where an estimated 90% of the total global deaths take place and mainly in children under 5 years of life who represent 78% of all mortalities (Wold malaria report, WHO, 2014). *P. falciparum* malaria is the leading cause of most deaths in children aged < 5 years among all mortalities caused by pathogenic infectious agents, which account for 64% of all child mortalities globally and 81% in Africa (Elliott and Beeson, 2008). The increasing availability of commercial vaccines for most of the other pathogenic causes of child deaths may lead to an increase in the relative contribution of *P. falciparum* to child mortalities, which is considered as the main pathogen for which a vaccine is not yet provided (Elliott and Beeson, 2008). There is an ongoing need for the development of effective vaccines that help to reduce the burden of malaria mortality, especially in light of increasing drug resistance and decreasing efficiency of vector control programs (Chan et al., 2012).
1.2 The life cycle of P. falciparum

*P. falciparum* has a complex life cycle that involves both a mosquito vector and a human host (Rowe et al., 2009). The cycle starts when a parasite-infected female anopheles mosquito injects *P. falciparum* parasites in the form of sporozoites into subcutaneous tissues and less commonly directly into the blood circulation of the human host during a blood meal (Ponnudurai et al., 1991, Menard et al., 2013). The sporozoites migrate through the bloodstream and invade liver cells (hepatocytes). However, some authors prefer the view that sporozoites inoculated into the skin travel to the liver via the lymphatic system rather than by blood capillaries to reach hepatocytes (Ponnudurai et al., 1991, Vaughan et al., 1999). However, the exact proportions of sporozoites that follow each route (blood or lymphatic) is probably influenced by several factors such as the vessel density at the inoculation site and the parasite species (Menard et al., 2013). The main receptor on sporozoites involved in invasion of hepatocytes is the circumsporozoite protein (CS), which binds to heparan sulphate proteoglycans (HSPG) on hepatocytes upon invasion (Frevert et al., 1993). It has been shown that sporozoites traverse several hepatocytes before finally the invasion on one hepatocyte takes place followed by parasite development (Mota et al., 2001).

Inside hepatocytes, an ~8- to 12-day incubation period allows the asexual multiplication of parasite before it finally generates tens of thousands of daughter cells called merozoites, which are released into the bloodstream where they rapidly invade multiple red blood cells [RBCs] (Beeson and Brown, 2002, Weatherall et al., 2002). The merozoites invade RBCs in a cascade of processes including parasite attachment, formation of junctions and entrance. The mechanism involved in this invasion is complex and includes proteins on the surface of merozoites (Mphande et al., 2008). Inside the infected erythrocyte (IE), *P. falciparum* parasites grow, multiply and divide in a cellular compartment known as the parasitophorous vacuole [PV] (Menard et al., 2013). Merozoites develop within IEs into ring stage, trophozoite and schizont stage parasites. Each *P. falciparum* asexual intra-erythrocytic cycle takes 48 hours and is completed by the production of new merozoites into the bloodstream, which re-infect other non-IEs (Rowe et al., 2009). The symptoms of malaria disease
(fever, anaemia, impaired consciousness, chills, etc.) only occur during the asexual cycle of parasite development and multiplication within RBCs and most existing drugs target this stage of parasite life cycle (Wirth, 2002, Weatherall et al., 2002). In addition, the intra-erythrocytic stage of parasite development develops chronic infections that can last for over a year within the infected host (Smith and Craig, 2005).

During the asexual cycle, a small proportion of ring-stage parasites develop into sexual parasite stages known as male and female gametocytes, which may be taken up by the female mosquito during blood meal. The gametocytes are fused in the mosquito gut to form a zygote, which further develops into ookinete. The latter then crosses the wall of the mosquito gut and forms an oocyst filled with sporozoites, which bursts resulting in the release of sporozoites that migrate to the mosquito salivary glands, ready to be injected into another human host (Wirth, 2002). Thus, the life cycle of *P. falciparum* includes three invading stages steps: the ookinete crossing the gut wall in the mosquito, the sporozoites infecting the mosquito salivary glands and the host liver cells, and the merozoites invading the host RBCs (Figure 1.1).
Figure 1.1. The life cycle of *P. falciparum*. The cycle is complex involving mammal and mosquito hosts. The asexual multiplication occurs in the human host, whereas the sexual reproduction takes place within the mosquito. Details about the life cycle are found in the text. The figure was obtained from (Menard et al., 2013).
1.3 **Pathophysiology of *P. falciparum* malaria disease**

The pathogenesis of malaria stems from different parasite and host factors that simultaneously contribute to the severity and consequences of the disease. Most *P. falciparum* infection episodes in endemic regions result in mild symptoms such as headache, fever, lethargy and malaise which often followed by full recovery even without intake of anti-malaria drugs (Beeson and Brown, 2002). Adults in these endemic areas encounter asymptomatic malaria infections which quickly cleared by the host immune response targeting the parasitized RBCs (Storm and Craig, 2014). However, some episodes develop into severe and life-threatening disease, which characterised by several clinical features including severe anaemia, impaired consciousness and respiratory distress (Marsh et al., 1995). There is an increasing recognition that severe malaria is a multi-system disease affecting many tissues and organs, although most marked complications may appear to occur in a single organ such as the brain (Miller et al., 2002).

1.3.1 **Severe malaria anaemia**

The pathogenesis of severe malaria anaemia is mainly due to two groups of contributing factors: increased lysis of infected and non-infected erythrocytes (immune mediated lysis, phagocytosis) and decreased production of RBCs [dyserythropoiesis, effect of inflammatory cytokines and parasite factors] (Ghosh and Ghosh, 2007). Erythrocytes are destroyed with the completion of the parasite erythrocytic asexual cycle inside the red cells. Phagocytosis of parasitized red cells by macrophages following opsonisation by antibodies and/or complement components also contributes to increased destruction of RBCs. Other factors involved in the reduction of non-IEs include the overall increased number of active macrophages, together with the enhanced signals for the detection and clearance of non-IEs during malaria infection leading to hypersplenic state characterised by removal of infected and non-infected RBCs (Weatherall et al., 2002, Ghosh and Ghosh, 2007). Phagocytosis and complement mediated lysis are the primary mechanisms of non-specific immune mediated destruction of RBCs in malaria disease (Autino et al., 2012). Chronic
malaria anaemia is more common in endemic areas and with other contributory factors can lead to severe anaemia (Ghosh and Ghosh, 2007).

1.3.2 Pathogenesis of malaria sequestration

A characteristic feature of the pathogenesis of infection caused by *P. falciparum* is the cytoadherence of IEs to the endothelial lining of small blood vessels [described in detail in the following section] (Miller et al., 2002). This phenomenon, termed "sequestration", results from the adhesive interactions between parasite-derived proteins inserted on the surface of *P. falciparum*-IEs and various host receptors expressed on endothelial cells (ECs), non-infected erythrocytes and placenta (Gardner et al., 1996, Turner et al., 1994). Only mature forms of the parasite sequester from the circulation to vascular endothelium lining various organs (e.g. brain, lung, heart, liver and kidney), the placenta and subcutaneous adipose tissues (Miller et al., 2002, Smith et al., 2013). As a result, only younger developmental forms (i.e. ring stage) of *P. falciparum* parasites are found in the peripheral circulation (Udeinya et al., 1983, Smith et al., 1995). Although only mature parasites sequester *in vivo*, cytoadhesion to some EC lines has been observed *in vitro* in erythrocytes infected by *P. vivax* (Autino et al., 2012). A recent study demonstrated that non-viable parasites killed by different antimalarial drugs retain the ability to cytoadhere for at least 24 hours after fatal dosages of treatment, indicating that sequestration is not exclusive to viable parasites (Hughes et al., 2010).

Sequestration and accumulation of parasitized erythrocytes in host organs and tissues are thought to be the pathogenic basis of severe complications of malaria disease (Autino et al., 2012). Manifestations following parasite sequestration including extensive obstruction of microcirculatory blood flow leading to impaired tissue perfusion (Dondorp et al., 2008), endothelial dysfunction at the site of sequestration by inducing pro-inflammatory cytokines and coagulation pathways (Turner et al., 1994, Moxon et al., 2009), release of parasite toxins, stimulation of the host immune response, resulting in vital organ dysfunction and death even with intense chemotherapy (Ockenhouse et al., 1991, Autino et al., 2012).
Sequestration of IEs is associated with organ-specific malaria syndromes from IE binding to brain (cerebral malaria [CM]) and placenta (placental malaria [PM]). The several ECs lining these organs and syncytiotrophoblasts in the placenta express various host receptors at different expression levels (Miller et al., 2002). Some of these host receptors have been suggested to be associated with organ-specific malaria syndromes such intercellular adhesion molecule 1 (ICAM-1 or CD54) in CM (Turner et al., 1994, Newbold et al., 1999, Ochola et al., 2011) and chondroitin sulphate A (CSA) in PM (Fried and Duffy, 1996, Beeson et al., 2000). Pathogenesis of CM, PM and other adhesive phenotypes such as rosetting (adherence of IEs to non-IEs) and auto-agglutination (adherence of IEs to other IEs) will be discussed in detail later in this chapter.

1.3.3 Other pathological consequences

Metabolic acidosis, respiratory distress, hypoglycaemia and general induction of pro-inflammatory cytokines are other key pathological manifestations of severe malaria (Miller et al., 2002). Respiratory distress could be a consequence of multiple underlying complications acting individually or in combination (Marsh et al., 1995). It may result from fluid retention following malaria infection, and was detected in patients with normal or negative fluid balance and with metabolic acidosis (Beeson and Brown, 2002). Occurrence of respiratory distress in non-immune adults with severe malaria can be life threatening, often leading to pulmonary oedema and adult respiratory distress syndrome (Autino et al., 2012, Marsh et al., 1995). A study conducted on hospitalised Kenyan children admitted with malaria symptoms found that children with respiratory distress or impaired consciousness were at great risk for death (Marsh et al., 1995).

The release of merozoites along with large amounts of malaria toxins and parasite products in the blood following rupture of schizonts leads to stimulation of the innate immune response and release of inflammatory mediators (Autino et al., 2012). Antibodies, cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-12 (IL-12) and mediators such nitric oxide (NO) have been suggested to protect against the blood-stage parasites (Miller et al., 2002, Rovira-Vallbona et al., 2012). However, an imbalance between the release
of pro- or anti-inflammatory cytokines or mediators may contribute to the severity of malaria disease (Rovira-Vallbona et al., 2012, Autino et al., 2012). Higher concentrations of TNF-α were observed in plasma samples collected from severe malaria than non-malaria patients and have been associated with CM (Kwiatkowski et al., 1990). In addition, in vitro studies have demonstrated that stimulation of EC lines with proinflammatory cytokines such as TNF-α caused an up-regulation of ICAM-1 expression (Dobbie et al., 1999, Gray et al., 2003), a receptor that was found at significantly higher levels in the brain vessels of patients who died from CM (Turner et al., 1994).

1.4 Sequestration of *P. falciparum* IEs and major adhesive phenotypes

During the 48-hour parasite maturation inside IEs, the parasite causes several structural and morphological alterations to the host cell. As a result, the IE becomes more rigid, more metabolically active and able to sequester from the peripheral circulation and adhere to the endothelium lining vasculature of different organs (Hughes et al., 2010). A major parasite-derived modification to the host erythrocyte that enables the parasite to sequester is the development of several thousands of electron-dense protrusions called "knobs" (~100 nm in diameter) on the infected cell surface [Figure 1.2] (Kilejian, 1979, Pologe et al., 1987). These knobs only appear on the IE surface with the development of the parasite to trophozoite and schizont stages, but not on cells infected by ring stage parasites (Kilejian, 1979, Udeinya et al., 1983). Different proteins synthesised by the parasite are associated with the formation and expression of knobs on the host cell membrane. Among those, knob-associated histidine rich protein (KAHRP) is the major structural component located at the cytoplasmic side of the erythrocyte membrane (Kilejian, 1979, Pologe et al., 1987, Horrocks et al., 2005). Other parasite-derived proteins such as *P. falciparum* erythrocyte membrane protein 3 ( PfEMP3) and mature infected erythrocyte surface protein (MESA; also called PfEMP2) are also related to establishment of knobs (Biggs et al., 1989, Chan et al., 2014). Knob structures act as sites for anchoring the putative cytoadherence molecule PfEMP1 on the external surface of IEs (Leech...
et al., 1984, Horrocks et al., 2005). PfEMP1 protein family have long been considered as the major parasite-derived protein exported to the IE surface and mediate IE cytoadhesion (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995).

Both in vitro and in vivo studies demonstrated that knobs play a significant role in parasite cytoadhesion. Electron microscopy investigations of post-mortem samples have provided morphologic evidence that the adhesion sites of IEs to ECs to be the knobs, and laboratory studies have linked the loss of knobs from lab-adapted isolates with the loss of ability to mediate cytoadhesion (MacPherson et al., 1985, Horrocks et al., 2005). However, a previous study has shown that parasites were capable of maintaining the formation of knobs on the IE surface under in vitro conditions while losing the capacity to facilitate cytoadhesion, and demonstrated that the loss of binding was not due the loss of knobs (Udeinya et al., 1983). Another study provided evidence for the presence of a knobless parasite isolate capable of facilitating a strong adhesion to C32 melanoma cells (Biggs et al., 1989). This isolate was incapable of expressing KAHRP protein due to deletion of kahrp gene. Although the attachment of melanoma cells to the parasite through knobs was observed, adhesion to areas on the IE surface where knobs were absent was also observed (Biggs et al., 1989). However, a recent study investigated the cytoadhesion properties of knobby and knobless parasite isolates and demonstrated that both static and flow adhesion were significantly decreased in all knobless isolates, and this correlated with the remarkable reduction (~50%) of PfEMP1 expression in isolates with knobs on the surface compared to knobless parasites (Horrocks et al., 2005). Therefore, it has been suggested that knobs could be essential but not sufficient for parasitized cell adhesion to vascular endothelial receptors (Pologe et al., 1987, Biggs et al., 1989). These findings indicate that the expression of PfEMP1 protein, but not the morphologic knobs, is a key essential determinant of parasite adhesion. However, the recent findings that RIFIN (Goel et al., 2015) and STEVOR (Niang et al., 2014) mediate rosetting highlights the importance of investigating the role of other parasite-derived surface antigens in parasite cytoadhesion.
In addition to its role in the parasite virulence, sequestration is believed to play a significant role in the survival of *P. falciparum* parasites within the infected host (Udeinya, 1990, Winograd et al., 2004). First, the parasite sequesters in areas where oxygen tension is low (venules, venous capillaries), an environment that favours the parasite growth (Udeinya et al., 1983, Biggs et al., 1989) as shown in *in vitro* studies (Trager and Jensen, 1976). Parasitized erythrocytes sequester in the endothelial lining of microvessels, where the shear stress is low enough to facilitate the interaction between the parasite ligands and endothelial receptors and allows for the establishment of chronic disease (Winograd et al., 2004, Chakravorty and Craig, 2005). Second, the sequestered mass of mature parasites are protected from host immune response in the peripheral blood circulation and from detection and destruction by the spleen (Udeinya et al., 1983, Biggs et al., 1989, Pologe et al., 1987), as non-adherent mature parasites are destroyed rapidly in the spleen (Miller et al., 2002).

In general, while the number of parasites circulating in the peripheral circulation (parasitaemia) is associated with the clinical severity of malaria infection (MacPherson et al., 1985), patients with lower or undetectable parasitaemias may suffer from severe and life-threatening disease complications due to sequestration and accumulation of mature parasites in the vital organs such as the brain (Ockenhouse et al., 1991, Weatherall et al., 2002). On the other hand, some patients may present with a higher rates of peripheral circulatory parasitaemias but only develop moderate symptoms (Ockenhouse et al., 1991).
Therefore, peripheral parasite count cannot be a sufficient indicator for diagnosing severe infections *per se* (Weatherall et al., 2002) and must be supported by other diagnostic tests for determining the severity of the disease. For the example, levels of plasma concentrations of *P. falciparum* histidine rich protein 2 (PFHRP2) released by the *P. falciparum* into the plasma can be used to measure the total parasite biomass in *P. falciparum* malaria infection and can be used as a parameter reflecting disease severity and outcome (Dondorp et al., 2005).

Three major IE cytoadhesion traits have been recognised (Figure 1.3) and described in detail in the following section; these include i) IE cytoadhesion to receptors in the brain (CM) and placenta (PM), ii) adhesion of IEs to non-IEs (rosetting), and (iii) adherence of parasitized erythrocytes to other IEs (IE clumping).

![Figure 1.3. Cytoadhesion phenotypes of *P. falciparum* IEs. (a): graphic representation of different adhesion traits of IEs to receptors on host cells such as ECs (sequestration), non-IEs (rosetting) and to other IEs in the presence of platelets (platelet-mediated clumping). (b): *In vitro* adhesion of IEs to cultured brain ECs, observed under the light microscope after Giemsa staining. (c): Rosetting of IEs observed *in vitro* under the light microscopy of Giemsa-stained *P. falciparum* culture preparations. (d): Platelet-mediated IE clumps formed after incubation with platelets and visualised under the light microscope after Giemsa staining. The figure was acquired from (Rowe et al., 2009).](image)
1.4.1 Cerebral malaria

CM is one of the most severe and life-threatening complications of malaria disease and remains a major public health concern in Africa and South East Asia (Martins and Daniel-Ribeiro, 2013). About 1% of all malaria episodes caused by *P. falciparum* develop into CM; with 90% of the cases occur in children in Sub-Saharan Africa (Storm and Craig, 2014). Child mortality rate due to CM remains very high (10-20%) despite the sufficient antimalarial treatment (Martins and Daniel-Ribeiro, 2013). Furthermore, the infection causes long-term neurological and cognitive defects such as psychiatric issues, memory impairment and learning difficulties in about 10-20% in children who survived an episode of CM (Desruisseaux et al., 2010).

The primary event for the development of CM is not entirely understood in spite of the large amount of existing data about the disease (Martins and Daniel-Ribeiro, 2013). However, sequestration of IEs to the brain post-capillary venules, inflammatory responses and endothelial dysfunction may account for the majority of mechanisms that govern CM (Berendt et al., 1994, Desruisseaux et al., 2010). Sequestration of the IEs results in the mechanical obstruction of the capillary blood flow (cerebral vessels, capillaries and small venules) leading to reduced perfusion, hypoxia and coma (Berendt et al., 1994). Immediate post-mortem examination of individuals who died from CM clearly revealed an increased number of sequestered trophozoites and schizonts in the brain microvessels relative to patients who died from other malaria-related (non-CM) complications (MacPherson et al., 1985). The release of parasite toxins in the blood and imbalanced inflammatory response leads to brain endothelial dysfunction, breakdown of blood brain barrier (BBB), cerebral oedema and coma (MacPherson et al., 1985, Martins and Daniel-Ribeiro, 2013). Together with the sequestration and inflammation, acute liver failure was recently suggested to be necessary for the development of CM (Martins and Daniel-Ribeiro, 2013).

Patients with CM typically have *P. falciparum* parasitaemia and unarousable deep coma often with convulsions. CM coma is confirmed after excluding other causes of coma such as meningitis, and can be distinguished from impaired consciousness in which patients develop Balantyre coma score of four or less
(Berendt et al., 1994, Marsh et al., 1995). However, any grade of impaired consciousness may indicate the parasite entrance to the brain (Beeson and Brown, 2002).

Sequestration of IEs to the brain vasculature results from specific interaction between PfEMP1 on the surface of IEs with adhesion receptors present on the endothelial surface of cerebral microvasculature such as ICAM-1 (Dobbie et al., 1999, Martins and Daniel-Ribeiro, 2013). However, the involvement of ICAM-1 and other adhesion receptors such as CD36 in the pathogenesis of CM will be discussed later in this chapter.

1.4.2 Pregnancy associated malaria

PM or pregnancy-associated malaria (PAM) is a significant cause of morbidity and mortality for both the mother and the child in Sub-Saharan Africa and caused by parasites that preferentially sequester in the intervillous space of the placenta in pregnant women (Oleinikov et al., 2008). This adhesion phenotype is relatively common with *P. falciparum*, less common with *P. vivax* (Autino et al., 2012) and occurs when mature asexual stages of the parasite express specific variant surface antigens [VSAs] (Barfod et al., 2006) that selectively adhere to CSA expressed on the syncytiotrophoblasts lining the placenta (Fried and Duffy, 1996), and may also bind to other receptors such as HA and immunoglobulins (Igs) (Beeson et al., 2004, Beeson et al., 2000). The major clinical manifestations of the infection are maternal anaemia which may lead to death of the mother, low birth weight and stillbirth, premature delivery and increased mortality of the newborn (Beeson and Brown, 2002, Miller et al., 2002).

A specific PfEMP1, VAR2CSA, is the most important VSA associated with PM (Salanti et al., 2004). VAR2CSA is a large PfEMP1 protein (~350 kDa) encoded by var2csa gene and comprised of six distinct Duffy binding-like (DBL) domains including those involved in CSA binding such as DBL2x and DBL3x (Avril et al., 2006). Var2csa is relatively conserved in sequence and existed a single-copy gene in most parasite genomes (Salanti et al., 2003). Transcription of var2csa gene is markedly upregulated in placental parasites and parasite lines selected for adhesion to CSA *in vitro* (Salanti et al., 2003), and disruption of the
var2csa gene of \textit{P. falciparum} results in the failure to retain CSA-adhesion phenotype (Viebig et al., 2005) suggesting a significant role of \textit{var2csa} in mediating placental binding. Parasites isolated from placentas of infected women are phenotypically distinct from other parasite isolates as they preferentially bind to placental receptors such as CSA, but fail to bind CD36, a common host receptor for parasite adhesion to the microvasculature (Fried and Duffy, 1996, Miller et al., 2002). This suggests that a distinct sub-population of parasites have been selected to sequester not to the endothelium but to the placenta, a site of low immune response, where they can establish maternal malaria (Miller et al., 2002). The reason for the lack of ability to bind CD36 is due to the absence of the CD36-binding CIDRα2-6 domains in \textit{var2csa}-encoded PfEMP1 proteins (Salanti et al., 2003; Smith et al., 2013).

While adult individuals living in malaria endemic areas develop immunity against malaria, pregnant women become highly susceptible to the disease and the risk is highest during their first pregnancy (primigravidae) when mothers lack sufficient immunity against PM (Fried et al., 1998, Viebig et al., 2005). However, women in these areas acquire protective immunity against PM as a function of parity; susceptibility to the infection rapidly reduced with increasing parity and age, and the mechanism of protection seems to be development of adhesion-inhibitory antibodies that block IE adhesion to the placenta (Fried et al., 1998, Beeson et al., 2004). Multigravidae generally have higher amounts of Igs that target placental parasites and block adhesion to CSA than primigravidae or men, reflecting a higher exposure to malaria infection (Beeson et al., 2004, Fried et al., 1998). Higher levels of VAR2CSA-specific IgG are correlated with acquired protection against the clinical manifestations of PM such as low birth weight (Salanti et al., 2004), suggesting that it could be possible to develop a vaccine targeting VAR2CSA to protect pregnant women in PM endemic areas. Furthermore, adhesion-inhibitory sera from African women were found to block CSA adhesion of parasites from other regions in the world (Fried et al., 1998), which propose that the immunogenic targets for PM might be conserved and CSA-binding is a common feature globally.
1.4.3 Rosetting

Rosetting, the adhesion of two or more non-IEs to a mature parasitized erythrocyte (David et al., 1988), is a property of parasite cytoadherence that is thought to contribute to significant microvascular obstruction (Kaul et al., 1991, Carlson and Wahlgren, 1992). Most studies that investigated the association between rosetting and disease outcome suggested that rosetting parasites occur more frequently in patients with severe malaria disease (Newbold et al., 1999), suggesting a significant role of this cytoadherence phenotype in the pathogenesis of severe malaria. Parasites that cause rosetting can be categorised into two types: parasites that mediate the binding of non-immune natural IgM antibodies in the plasma or serum to the IE surface and those that do not do so (Ghumra et al., 2012). Binding to non-immune IgM was originally proposed to act as 'bridges' between the IE and surrounding RBCs (Scholander et al., 1996), but the recent findings that IgM binding site is mapped close to the C-terminus of PfEMP1 makes this suggestion uncertain (Stevenson et al., 2015). Rather, it appears that the function of Fc-dependent IgM in rosetting is strengthening the interaction of multiple domains of PfEMP1 head structure with their receptors on the erythrocyte surface (Stevenson et al., 2015). Although Barfod et al., 2011 indicated that non-specific IgM binding has a role in escaping immunity in the VAR2CSA-type PfEMP1 by masking critical epitopes on the IE surface, inhibiting the subsequent binding of specific antibodies and protecting the parasite from phagocytosis; the recent findings by Stevenson et al., 2015 argued against this and found that IgM binding did not inhibit specific IgG labelling or masked opsonised IEs from phagocytosis in a rosette-mediating PfEMP1.

P. falciparum, P. vivax and P. ovale are all have the capacity to form rosettes, but only P. falciparum-mediated rosettes are correlated with severe malaria especially in African children (Autino et al., 2012). In an early Kenyan study, rosette formation in 154 fresh parasites isolated from Kenyan children with different malaria syndromes was studied and the results showed that the ability to form rosettes was correlated with severe malaria disease (Rowe et al., 1995). Another field study found a strong association between rosette formation and CM in parasites isolated from Gambian children with CM or UCM (Treutiger et al., 1992). Furthermore, a study conducted on 209 Malian children showed that rosetting was significantly higher in P. falciparum parasites isolated from severe malaria patients compared with non-severe and UCM control cases (Doumbo et al., 2009).
Several studies have demonstrated that the key parasite ligands that mediate rosette formation are PfEMP1 molecules on the surface of IEs (Chen et al., 1998a, Rowe et al., 1997), although a very recent work has shown that RIFINs can also mediate rosetting (Goel et al., 2015). The functional erythrocyte binding region on PfEMP1 was identified and found to be the N-terminal DBLα domain (Rowe et al., 1997, Russell et al., 2005), which has been associated with binding to host molecules on the surface of non-IEs such as complement receptor 1 [CR1] (Rowe et al., 1997), heparan sulphate [HS] (Chen et al., 1998a, Chen et al., 2000) and antigens of blood groups A and B (Carlson and Wahlgren, 1992, Rowe et al., 2007) for rosette formation. Previous work has demonstrated that the ABO blood grouping influences the size of rosettes formed but not the general frequency; rosettes with cells from blood group-O patients are generally smaller and tend to be easily disrupted compared to the larger rosettes formed with cells from group A or B individuals, with group-A rosettes likely to be the largest amongst all blood groups (Rowe et al., 2007, Carlson and Wahlgren, 1992). Therefore, blood group-O may be a protective factor against severe disease while non-O blood group individuals may be at a greater risk for developing life-threatening malaria due to the mechanism of increased rosette formation (Carlson and Wahlgren, 1992, Rowe et al., 2007).

1.4.4 Auto-agglutination and platelet-mediated clumping

The cytoadhesion of mature IEs with each other to form clumbs is an adhesion phenotype that was firstly termed auto-agglutination [Figure 1.4] (Roberts et al., 1992). This cytoadhesion phenotype was more frequently observed in children with severe malaria than those with mild disease, suggesting its role in the contribution to severe disease presumably through local disturbance of microcirculatory blood flow and/or other significant physiological functions (Roberts et al., 2000). IE auto-agglutination is distinct from rosetting, since auto-agglutinating parasite clones do not form rosettes and rosetting parasites do not auto-agglutinate IEs (Roberts et al., 1992). It was demonstrated that auto-agglutination of *P. falciparum*-IEs is mediated by platelets and clumping requires the surface expression of platelet glycoprotein CD36 (Pain et al., 2001). Platelet-mediated clumping is an adhesive phenotype expressed by some CD36-binding isolates and is common in field isolates such as those in Kenya. This cytoadherent phenotype is associated with severe disease probably through local obstruction of blood flow (Pain et al., 2001).
1.5 Variant surface antigens on the surface of IEs

Infection of erythrocytes by *P. falciparum* parasites leads to multiple morphological and structural changes that affect cellular membrane rigidity, permeability and host cell surface antigens. These changes benefit the parasite development within the host cell and contribute to the acquisition of nutrients and waste removal, chronicity of infection and establishment of adhesive phenotypes (Smith and Craig, 2005, Sanyal et al., 2012). Although parasite-derived sub-cellular changes are significant for parasite maturation and pathogenesis, the major focus of the following section will be VSAs on the surface of IEs.

During the intra-erythrocytic stage of development, several parasite-derived antigens are synthesised and exported to the surface of IEs (Leech et al., 1984). These VSAs are extremely polymorphic, highly immunogenic in nature and induce an immune response that targets IEs for providing protective immunity against malaria (Bull et al., 1998, Marsh et al., 1989). VSAs are implicated in the cytoadhesion of *P. falciparum*-IEs in the microvessels of various organs, a key pathological feature of severe malaria disease (Berendt et al., 1994). Although the phenomenon of parasite sequestration in humans is only observed extensively with *P. falciparum* infections, all malaria species seem to have the capacity to alter their host surface by expressing VSAs on the IE membrane (Smith et al., 1995).

To date, several VSAs have been identified including PfEMP1 (Leech et al., 1984), repetitive interspersed family (RIFIN) proteins (Cheng et al., 1998, Fernandez et al., 1999, Kyes et al., 1999), sub-telomeric variable open reading frame (STEVOR) proteins (Cheng et al., 1998, Niang et al., 2009), surface-associated interspersed gene family (SURFIN) proteins (Winter et al., 2005) and presumably other proteins such as *P. falciparum* Maurer's cleft 2 transmembrane (PfMC-2TM) proteins (Sam-Yellowe et al., 2004). In addition to parasite-derived surface antigens, altered host proteins such as modified band 3 have also been implicated as immune targets and suggested as surface ligands for cytoadhesion (Winograd et al., 2004).
1.5.1 PfEMP1

The most extensively characterised VSA is PfEMP1, a highly polymorphic protein that has a significant role in the parasite virulence, survival and transmission (Smith, 2014). PfEMP1 proteins are encoded by members of the var multigene family, expressed on the surface of IEs and are associated with cytoadherence of parasitized erythrocytes to various host receptors on ECs, infected and non-IEs, and thus contributing to the pathogenesis of severe malaria disease (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995). PfEMP1 was first recognised by immunoprecipitation with immune sera collected from Aotus monkeys as a highly polymorphic, high molecular weight (MW) protein, sensitive to protease digestion on intact IEs and was suggested as a constituent of the parasite-derived knobs (Leech et al., 1984). PfEMP1 molecules are synthesised and transported to the surface of IEs at ~18 hours post-invasion during the intra-erythrocytic asexual stage of parasite development (Kyes et al., 2000). These proteins are exported to the IE surface with the N-terminal binding region localised at the external surface of the cell and the C-terminal on the cytoplasmic side (Su et al., 1995, Rask et al., 2010).

1.5.1.1 Structure of PfEMP1 molecules

Despite the extensive diversity in sequence, size and domain organization of PfEMP1 proteins, they have a common structure and can be grouped according to sequence similarity (Kraemer et al., 2007). PfEMP1 proteins are 150 – 350 kDa in size and each protein contains single intracellular (ID) and transmembrane (TM) domains, and ~2 to 7 tandemly arranged extracellular domains (30-45 kDa per domain) connected with disulphide bonds. From the extracellular N-terminal to the cytoplasmic C-terminal, the modular PfEMP1 consists of an N-terminal segment (NTS), several DBL and CIDR domains, TM region and a highly conserved acidic terminal segment [ATS] (Smith et al., 2000b, Rask et al., 2010). The ATS segment and TM region are thought to anchor the PfEMP1 molecule at the knob structure on the surface of IEs (Su et al., 1995). Due to the variability of the PfEMP1 domain architecture, DBL domains are categorised first by position in the protein and second by type.
(Smith et al., 2000b). For instance, the first N-terminal DBL domain for most PfEMP1 proteins is DBLα type, and thus it is named "DBL1α" which is always followed by a CIDR1 domain. The N-terminal DBL-CIDR tandem is the most conserved extracellular region and known as the semi-conserved head structure (Su et al., 1995, Smith, 2014).

Rask et al. (2010) re-classified PfEMP1 domains based on analyses of 399 distinct PfEMP1 sequences from seven parasite genomes (four Asian, two African and one South American isolate). Their analyses have confirmed the already existed grouping of DBL domains (by Smith et al., 2000b, Gardner et al., 2002 and Lavstsen et al., 2003) into six major classes based on sequence similarity: DBLα, β, γ, δ, ε and ζ. In addition, five relatively smaller different classes were also identified including four N-terminal DBL domains of VAR2CSA and DBLα of VAR3. DBLα was further divided into 3 groups (DBLα0, DBLα1 and DBLα2) and sequences of these groups have been grouped into several subclasses (e.g. DBLα1.3 of VAR3). The CIDR domain has been divided into 5 major classes: CIDRα, β, γ, δ and pam, of which CIDRδ was firstly identified (Rask et al., 2010). The PfEMP1 C2 domain always follows a DBLβ domain and never followed other DBL classes (Smith et al., 2000a). The tandem arrangement DBLβC2 was always found as a combination; with C2 being an essential part of the same DBLβ domain and thus the term (C2) was omitted from all DBLβ domains (Rask et al., 2010, Gullingsrud et al., 2013).

Subsequent work on the classification of PfEMP1 structure has revealed that PfEMP1 proteins are characterised by 23 conserved motifs called domain cassettes (DCs), defined as two or more tandemly arranged domains belonging to a specific sub-class (i.e. combination of DBL and CIDR sub-domains) and existing in three or more of the seven sequenced parasite genomes (Rask et al., 2010). Of particular significance, DC8 and DC13 have been associated with specific clinical consequences. Transcription levels of the conserved DC8 and DC13 have been demonstrated to be elevated in isolates causing severe malaria complications in children than those causing uncomplicated malaria (UCM) (Lavstsen et al., 2012, Almelli et al., 2014). In addition, *P. falciparum* lines panned for binding to human brain microvascular endothelial cells (HBEC) expressed higher levels of DC8 genes (Claessens et al., 2012, Avril et al., 2012),
although DC8-expressing parasites also bound significantly to primary ECs from the heart, dermis and lung (Avril et al., 2012). Despite the potential role of ICAM-1 in CM pathogenesis, IEs selected on expression of DC8 and DC13 by panning on HBECs do not bind to ICAM-1 or only adhere at significantly lower levels (Avril et al., 2012, Claessens et al., 2012). A recent study has identified another DC (termed DC4) present in group A var genes from six genetically diverse P. falciparum isolates. Parasites containing the three domains of DC4 adhered preferentially to ICAM-1 receptors (Bengtsson et al., 2013). These data suggest that specific PfEMP1 DCs may have a critical role in the development of malaria-related pathological outcome.

1.5.1.2 Var genes

Sequencing of P. falciparum parasite genomes has advanced our understanding about the structural and functional properties of var genes. Each parasite genome contains ~60 copies of var genes encoding the multidomain PfEMP1 proteins (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995, Gardner et al., 2002, Kraemer et al., 2007, Lavstsen et al., 2003). The diversity of this gene family is extremely high within and between parasite genomes, and even clonal cultures are enormously heterogeneous phenotypically (Gardner et al., 1996). In most cases, the parasite expresses only one PfEMP1 copy on the surface of IEs at a time in a mutually exclusive fashion (Chen et al., 1998b). Although one var gene is expressed in each parasite, this can switch at a rate of up to 2% per parasite generation (Roberts et al., 1992). However, Horrocks et al. (2004) showed that switching rates vary dramatically between different variants, and each var gene has its own switching rate (Horrocks et al., 2004). Switches in expression of var genes favour the parasite survival through evading the host immune response and allows the parasite to sequester in the microvessels of the host organs (Kraemer et al., 2007).

Despite the high diversity of var gene repertoires, most of these genes are categorised into three major groups (A, B and C) and two intermediate groups (B/A and B/C) on the basis of chromosomal location (central or telomeric), conserved upstream promoter sequence (UpsA, UpsB and UpsC) and gene
orientation (Gardner et al., 2002, Lavstsen et al., 2003). Groups A and B are located in the sub-telomeric regions in the chromosome, whereas group C is present in the central chromosomal regions (Gardner et al., 2002). Comparisons of parasite genomes have revealed the presence of three additional relatively conserved genes at the sequence level: var1csa, var2csa and Type 3 var genes, which are found in almost all isolates with > 75% sequence identity among multiple domains (Kraemer et al., 2007). Var gene groupings have clinical and functional significance and can be linked to certain parasite pathologies. For instance, var2csa encodes a PfEMP1 protein that mediates the adhesion to CSA receptors and therefore has a crucial role in the pathogenesis of PM (Salanti et al., 2004, Salanti et al., 2003). In addition, expression of group A and to some extent group B var genes has been associated with severe malaria, as shown in several published studies (e.g. Kaestli et al. 2006, Jensen et al., 2004, Rottmann et al., 2006, Kyriacou et al., 2006, Falk et al., 2009).

1.5.1.3 PfEMP1 Adhesive properties

The multiple domains of the extracellular region of PfEMP1 proteins have different binding specificities and can predominantly adhere to various host receptors (Smith et al., 2000a, Rowe et al., 1997, Baruch et al., 1997, Chen et al., 2000, Turner et al., 2013). The effectiveness of a given PfEMP1 to bind to host cells mainly depends on the adhesion characteristics of that PfEMP1 variant, availability of host ligands with appropriate binding properties and the host antibody repertoire to PfEMP1 proteins (Lavstsen et al., 2003).

The PfEMP1 adhesive domains for a variety of host receptors have been identified and mapped to different DBL and CIDR domains (Smith et al., 2000a). Although several receptors have been identified as ligands for IE binding (described in detail later in this chapter), CD36 and ICAM-1 are the best understood adhesion candidates. The most common binding specificity of CIDR domain in the semi-conserved head structure is adhesion to human CD36 receptor (Baruch et al., 1997, Chen et al., 2000). This binding property (i.e. binding to CD36) is a common adhesive phenotype for numerous PfEMP1 variants and mainly limited to groups B and C PfEMP1 proteins (Rask et al.,
Subsequent sequence comparisons has led to the sub-grouping of PfEMP1 domains (e.g. CIDRα1.4) (Rask et al., 2010). Whereas CIDRα2-6 sequence types are associated with CD36 binding, CIDRα1 domains (subtypes 1.1, 1.4, 1.5 and 1.7) encode binding to endothelial protein C receptors [EPCR] (Turner et al., 2013). The DBLα1-CIDRβ/γ/δ head structure is associated with rosetting, and CIDRβ/γ/δ domains in this tandem do not bind to CD36 or EPCR indicating that different PfEMP1 head structures domains mediate distinct binding properties (Rask et al., 2010, Smith, 2014).

DBL domains are essential in two main processes associated with malaria pathogenesis: invasion of erythrocytes and mediating IE adhesion to various host receptors (Howell et al., 2008). It has been shown that different DBL domains mediate cytoadhesion to particular host receptors depending on the primary sequence and class of these domains. For example, DBLα domain has been found to bind to host receptors such as CR1 (Rowe et al., 1997), HS molecules (Chen et al., 1998a, Chen et al., 2000) and antigens of blood groups A and B (Carlson and Wahlgren, 1992, Chen et al., 2000) located on the surface of non-IEs for rosette formation. The second class of DBL domains, DBLβ, has been associated with adhesion to ICAM-1 receptors (Smith et al., 2000a, Oleinikov et al., 2009, Howell et al., 2008, Brown et al., 2013). In an analysis of the entire DBLβ domains in the IT4 P. falciparum parasite genome (n=25), six domains bound to ICAM-1 receptors (Howell et al., 2008). It has been demonstrated that PfEMP1-ICAM-1 interaction is mediated fully by single DBLβ domain that binds to the N-terminal region of ICAM-1 to form a 1:1 complex (Brown et al., 2013). Phylogenetic criteria has led to the classification of PfEMP1 domains into small (four extracellular domains) and large (5-9 extracellular domains) PfEMP1 molecules [Figure 1.5] (Smith, 2014). DBLβ type is only found in larger PfEMP1 molecules (Smith, 2014) and therefore ICAM-1 binding is mainly restricted to large PfEMP1 proteins.

Although an early study demonstrated that both CIDR1α and DBL2δ domains bind to platelet-endothelial cell adhesion molecule-1 [PECAM-1] (Chen et al., 2000), a recently published work indicated that IE adhesion to PECAM-1 is mediated by DC5-containing PfEMP1 variants (Berger et al., 2013, see section 1.6.5 for more details).
1.5.1.4 Antigenic variation

PfEMP1 molecules are expressed on the surface of IEs during the second half of the intra-erythrocytic cycle of *P. falciparum* parasite development (Smith et al., 1995). Each mature IE is thought to select and express only one *var* gene (out of a repertoire of ~60 genes) at a time, such that a single PfEMP1 reaches the surface of IEs (Chen et al., 1998b). However, switching of *var* gene expression can occur at each new cycle of erythrocytic invasion, in a phenomenon known as "antigenic variation" (Chen et al., 1998b, Roberts et al., 1992). *In vitro* studies showed that *P. falciparum* parasites undergo a clonal switching in antigenic phenotypes at a rate up to 2% per generation in the absence of immune pressure (Roberts et al., 1992). Since several PfEMP1 molecules have distinct receptor-
binding specificities, clonal switching between expressions of diverse \textit{var} gene variants, in a mutually exclusive fashion, allows the parasite to change its antigenic and cytoadhesion properties (Salanti et al., 2010, Smith et al., 1995). This higher rate of antigenic switching provides an additional complication for laboratory researchers studying parasite cytoadhesion \textit{in vitro}, and assays that require longer periods of parasite cultures must involve regular selection on particular adhesion phenotype to maintain the required binding property (Rowe et al., 2009).

As well as changing the adhesion phenotype, switching of \textit{var} gene expression allows the parasite to evade the host immune response and promote chronic infection (Chakravorty and Craig, 2005). The acquired antibody response to parasite-derived surface antigens demonstrates higher levels of strain-specificity (discussed in detail later in this chapter). This is evident from studies showing that the predominant agglutinating antibody response in humans to the IE surface was principally variant-specific, and cross-reactive antibodies to different serotypes were rare (Newbold et al., 1992). Sera collected from children in the Gambia (Marsh and Howard, 1986) and Papua New Guinea (Forsyth et al., 1989) during convalescence reacted predominantly with homologous but not heterologous parasites in agglutination assays. Since the response is principally variant-specific, the mechanism of antigenic variation allows the development of repeated infections by exploiting gaps in the repertoire of strain-specific acquired antibody response (Bull et al., 1998, Gamain et al., 2001). Immune evasion is likely occur by distracting the host adaptive immune response from the significant cross-reactive antigenic determinants and guiding that response to strong, polymorphic epitopes (Gamain et al., 2001). These data indicate that the parasite antigenic variation is a key factor contributing to the pathogenesis of malaria infection.

1.5.2 RIFIN

RIFIN proteins are the largest family of clonally variant antigens expressed on the surface of \textit{P. falciparum}-IEs. RIFINs are low MW proteins (30–45 kDa) encoded by the \textit{rif} (repetitive interspersed family) multigene family which
contains 150-200 genes per parasite genome (Winter et al., 2005, Kyes et al., 1999, Fernandez et al., 1999). They had originally been called "rosettins" and were proposed to mediate rosetting, before it was clearly demonstrated that PfEMP1 is the main rosetting ligand (Kyes et al., 1999). RIFINs co-express with PfEMP1 on the surface of parasitized erythrocytes, although transcriptional analyses demonstrated that rif genes are expressed after var genes, only for a limited time from late ring to early pigmented trophozoite stages of asexual blood cycle [~18-23 hours after RBC invasion] (Kyes et al., 1999, Kyes et al., 2000). This co-expression has prompted assumptions that expression and export of RIFIN and PfEMP1 proteins are connected (Craig and Scherf, 2001). In contrast to var, rif genes transcribe and express multiple gene copies in a single parasite, resulting in the expression of several RIFINs at the surface of IEs (Fernandez et al., 1999).

In contrast to PfEMP1 of which as little as 1 µg/ml of trypsin treatment can cleave the protein, RIFIN proteins are almost unaffected with up to 100 µg/ml trypsin digestion (Fernandez et al., 1999, Kyes et al., 1999). It has been shown that human immune sera agglutinated IEs treated with trypsin at conditions (> 100 µg/ml) such that the highly trypsin-sensitive PfEMP1 proteins are mostly removed but RIFINs are detected, which suggests the existence of immunogenic epitopes in this family of proteins targeted by agglutinating antibodies (Fernandez et al., 1999). Another study revealed that RIFIN recombinant proteins were well recognised by a large panel of human immune sera suggesting the capacity of these proteins to induce a strong immune response in P. falciparum-infected adults in malaria endemic regions (Abdel-Latif et al., 2002). It has been shown that acquisition of specific anti-RIFIN antibodies was associated with protection against P. falciparum malaria (Abdel-Latif et al., 2003). Although their biological significance and role in natural immunity are largely unknown (Rowe et al., 2009, Chan et al., 2012), the higher expression level of these genes by P. falciparum parasites, their surface localisation and sequence diversity suggest a potential role for these proteins in the parasite interaction with host cells (Kyes et al., 1999). Recently, Goel et al. (2015) have demonstrated that RIFINs are expressed on the surface of IEs, bind to RBCs...
(preferentially of blood group A) to form large rosettes and mediate microvascular adhesion of IEs.

1.5.3 STEVOR

STEVOR proteins, encoded by the stevor multigene family (30-40 copies per parasite genome), represent the third largest family of clonally-variant parasite-derived antigens identified in *P. falciparum* (Cheng et al., 1998). Several stevor gene copies were observed in a single parasite, and the peak transcription takes place during late trophozoite and early schizont stages of parasite development (Niang et al., 2009). Unlike PfEMP1 and RIFIN, data presenting evidence that STEVOR proteins are exported to external surface of parasite-infected RBCs are inadequate. Niang et al. (2009) have demonstrated that STEVORs are expressed on the surface of IEs using a combination of live immunofluorescence assay (IFA) and flow cytometry, and that the expression was only located on erythrocytes infected by parasites at the schizont stage.

Similar to RIFINs, the biological function of STEVOR proteins is largely unknown (Chan et al., 2014). Both protein families have been suggested to play a role in host-parasite interaction and antigenic properties (Beeson et al., 2006, Niang et al., 2009, Rowe et al., 2009). A recent study provided evidence that STEVOR is a RBC binding protein that mediate rosetting independently of PfEMP1 via interation with glycophorin C on the erythrocyte surface and STEVOR-mediated rosetting has a protective role for merozoites from invasion-inhibitory antibodies (Niang et al., 2014). Another study showed that increased expression of stevor genes contributes to decreased deformability and increased rigidity of IEs, which may has a role in enhancing PfEMP1-mediated parasite sequestration (Sanyal et al., 2012). In addition, the clonal diversity of STEVOR proteins makes them potential candidates involved in immune evasion alongside with RIFIN and PfEMP1 proteins (Chan et al., 2014).
1.5.4 SURFIN

SURFINs are high molecular weight polymorphic proteins (~280-300 kDa) encoded by a small family of genes termed surf (surface-associated interspersed genes) multigene family, which contains 10 genes per parasite genome (Winter et al., 2005). Different surf genes are differentially transcribed depending on the developmental stage of intra-erythrocytic parasite cycle; some genes (e.g. surf1.3) are transcribed during late trophozoite and schizont stages, while others (e.g. surf4.2) are transcribed from early rings to schizont stages. This variation in transcriptional profiles may suggest a diversity in the functions of proteins transcribed by the corresponding genes (Mphande et al., 2008).

It has been suggested that SURFIN proteins are present on both IEs and merozoites, the two developmental parasite stages that are exposed to the host immune system (Winter et al., 2005). Surface localisation of SURFINs on IEs was confirmed in two parasite isolates (FCR3 and 3D7), with a member termed SURFIN4.2, which was cleaved off the surface by trypsin treatment. In addition, immuno-electron microscopy analyses revealed that this family of proteins are present in the knob structures, suggesting their co-transportation with PfEMP1 at the surface of parasitized erythrocytes (Winter et al., 2005). However, recent data showed that another member of the SURFIN family (SURFIN4.1) is present in the PV, but not in the erythrocyte cytosol, and on the released merozoites. Agglutination and immunofluorescence data suggested that SURFIN4.1 is not exposed on the surface of IEs (Mphande et al., 2008). Although the biological function of SURFIN is not yet known, some SURFIN members have been associated with parasite invasion to the host erythrocytes (Mphande et al., 2008, Winter et al., 2005). In addition, relation of SURFINs to the surfaces of different parasitic developmental stages may suggest a contribution to parasite survival (Winter et al., 2005).

1.5.5 Modified band 3

One of the earliest identified proteins on the surface of IEs is a parasite-mediated modification of an erythrocyte surface protein, band 3 (Winograd and Sherman, 1989). Band 3 is a protein found abundantly on the surface of RBCs and acts as
an anion transporter (Smith and Craig, 2005). Parasite-modified band 3 has been suggested as an immune target (Chan et al., 2012) since it is trypsin-resistant, located on the surface of IEs (Figure 1.6) and was immunoprecipitated by monoclonal antibodies (mAbs) raised to band 3 (Gardner et al., 1996). In addition, this highly abundant protein on the IE surface has been suggested as a ligand for IE adhesion to thrombospondin (TSP) and CD36 (Winograd et al., 2004, Chan et al., 2014). Selective chemical alteration of modified band 3 has led to a substantial decrease in CD36 adhesion, but not TSP, which supports its essential role in CD36 binding (Winograd et al., 2004). In addition, synthetic peptides obtained from modified band 3 have been demonstrated to block adhesion to CD36 receptors, and antibodies raised against two structural portions of the protein were found to inhibit parasite adhesion to melanoma cells expressing CD36 (Beeson and Brown, 2002). However, its association to PfEMP1-mediated parasite cytoadhesion remains unknown (Chan et al., 2014).

1.5.6 PfMC-2TM

*P. falciparum* Maurer's clefts two-transmembrane proteins (PfMC-2TM) are encoded by a small gene family called *pfmc-2tm* (approximately 13 genes) and located at Maurer's clefts of IEs (Sam-Yellowe et al., 2004, Chan et al., 2014). Almost all *pfmc-2tm* genes are transcribed during the trophozoite stage of parasite development and unlike PfEMP1 proteins, 4 to 10 gene products are expressed at a given time (Sam-Yellowe et al., 2004). The biological function of PfMC-2TM proteins remains to be identified, and it has not been confirmed whether these proteins are related to the IE membrane or exported to outer surface of the parasitized erythrocyte (Chan et al., 2014).
Figure 1.5. Parasite-derived modifications to \textit{P. falciparum}-IEs. Invasion of erythrocytes by \textit{P. falciparum} results in dramatic morphologic and structural modifications to the host erythrocytes, which include the expression of knob structures and insertion of VSAs on the surface of IEs. The graph was acquired from (Chan et al., 2014).

1.6 Human receptors for \textit{P. falciparum}-IE adhesion

A diverse array of human cell receptors have been shown to mediate \textit{P. falciparum}-IE cytoadhesion (reviewed in Rowe et al., 2009) including ICAM-1 (Berendt et al., 1989), CD36 (Barnwell et al., 1989, Ockenhouse et al., 1989), TSP (Roberts et al., 1985), EPCR (Turner et al., 2013), PECAM-1/CD31 (Treutiger et al., 1997), CSA (Rogerson et al., 1995), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) (Ockenhouse et al., 1992b), rosetting receptors including CR1 (Rowe et al., 1997), HS (Chen et al., 1998a), blood groups A and B antigens (Carlson and Wahlgren, 1992) and serum proteins IgG/IgM, fibrinogen and others (Scholander et al., 1996).

The majority of \textit{P. falciparum}-IEs bind to CD36 and to some extent to ICAM-1 receptors which are broadly expressed in the microvasculature (Rogerson et al., 1999, Newbold et al., 1997, Ochola et al., 2011). An exception for this universal binding are IEs isolated from the placenta of malaria-infected women. These receptors are either constitutively expressed on the host cells \textit{in vivo} or up-regulated after stimulation by inflammatory cytokines (Cooke et al., 1995). A single clone of \textit{P. falciparum}-IEs has been shown to have a multiple binding
capacities and bind several endothelial receptors (Ockenhouse et al., 1992b). In order to mediate a firm adhesion to the endothelium, *P. falciparum* IEs first tether, roll before becoming firmly attached to the host endothelial receptors (Miller et al., 2002, Autino et al., 2012). This adhesion mechanism is similar to cytoadhesion of leukocytes (Miller et al., 2002). The majority of host receptors are involved in the tethering and rolling but incapable of mediating firm adhesion on their own such as ICAM-1, which may function as a rolling receptor for IEs in cerebral vessels (Cooke et al., 1994, Miller et al., 2002). On the other hand, only particular receptors such as CD36 and CSA provide stationary and stable attachment of parasitized erythrocytes to the endothelium under flow pressure (Miller et al., 2002, Cooke et al., 1994). This cooperation between capturing and stationary receptors is suggested to promote efficient sequestration (Gray et al., 2003; McCormick et al., 1997).

### 1.6.1 ICAM-1

ICAM-1 (CD54) is an 80-114 kDa transmembrane glycoprotein that belongs to the Ig supergene family (van de Stolpe and van der Saag, 1996). The structure of ICAM-1 molecules is composed of five Ig-like extracellular domains (D1-D5) and a short cytoplasmic tail (Brown et al., 2013). It is widely distributed in various human tissues including vascular endothelium and numerous immune cells (Berendt et al., 1992). This receptor is constitutively expressed on ECs at basal levels and can be significantly up-regulated both *in vivo* and *in vitro* in response to several cytokines such as tumour necrosis factor-α (TNF-α), interlukin-1 (IL-1) and interferon-γ [IFN-γ] (van de Stolpe and van der Saag, 1996, Berendt et al., 1992).

ICAM-1 plays an important role in several biological processes such as inflammatory responses, antigen-independent interactions between lymphocytes and target molecules and T-cell mediated immune response. On the vascular endothelium, ICAM-1 has a significant role in the migration of leukocytes from the blood to tissues at the sites of inflammation. Leukocyte interaction with ICAM-1 is mediated via leukocyte function-associated antigen (LFA-1) and macrophage-1 antigen [Mac-1] (van de Stolpe and van der Saag, 1996, Berendt
et al., 1992). ICAM-1 functions as a ligand for other receptors such as CD43, hyaluronan and fibrinogen, and mediates binding to pathogens such as human rhinoviruses (van de Stolpe and van der Saag, 1996) and *P. falciparum* IEs (Berendt et al., 1989). *In vitro*, cytokine- and endotoxin-activated ECs bound to *P. falciparum*-IEs at increased levels via ICAM-1 ligands compared to binding with non-activated ECs (Berendt et al., 1989), indicating that cytokine-mediated ICAM-1 up-regulation increases parasite adhesion. The binding site on ICAM-1 for *P. falciparum* IEs has been identified and mapped to the opposite face of ICAM-1 binding region for LFA-1 (Figure 1.7). *P. falciparum* IEs have been shown to bind a region on ICAM-1 that is distinct from, but overlaps, the binding sites for LFA-1, human major-type rhinovirus and fibrinogen (Ockenhouse et al., 1992a, Berendt et al., 1992). Adhesion studies have demonstrated that ICAM-1 plays a significant role in capturing parasitized erythrocytes from the flowing blood (Gray et al., 2003), and it has been shown that ICAM-1 and CD36 synergise to facilitate firm adhesion to the endothelium (McCormick et al., 1997).

![Figure 1.6](image)  
*Figure 1.6.* The crystal structure of the N-terminal domain of human ICAM-1 illustrating the binding sites for LFA-1 (blue), fibrinogen (red) and *P. falciparum*-IEs (yellow). The binding site for parasitized erythrocytes appears to involve the BED 'side' of the N-terminus of ICAM-1 Ig-like domains. This binding site was determined using parasite isolates with different ICAM-1 binding avidities, and different panels of ICAM-1 mutants (e.g. K29). The binding to human rhinovirus includes residues distributed along the entire Ig domain [graph acquired from (Tse et al., 2004)].
1.6.1.1 Association between ICAM-1 binding and severe malaria disease

Adhesion of parasitized erythrocytes to host receptors on the brain microvasculature is essential for the development of a fatal malaria-related disease, CM (Smith et al., 2000a). The pathophysiological importance of ICAM-1 binding is not completely understood and evidence to support its association with severe disease, particularly CM, is inconclusive (Cooke et al., 1994, Rowe et al., 2009). However, several lines of evidence have implicated ICAM-1 as having a role in the development of CM. First, immunohistochemical studies on postmortem tissues of patients who died from CM found that ICAM-1 expression was significantly up-regulated in the endothelium of cerebral microvasculature, and this higher ICAM-1 expression co-localised with adhered IEs (Turner et al., 1994). Second, a large study conducted in Kenya, using parasite isolates from patients with defined clinical syndromes as well as isolates from healthy but parasitized community controls, showed an increased, although just missed statistical significance, ICAM-1 adhesion in parasites from patients with CM compared to those with asymptomatic malaria (Newbold et al., 1997). Another more recent large study examined the adhesion patterns of 101 patient isolates from different clinical syndromes showed that ICAM-1 adhesion was higher in isolates from CM patients, whereas increased CD36 adhesion was associated with uncomplicated malaria disease (Ochola et al., 2011). In agreement with these findings, Turner et al. (2013) found that adhesion to ICAM-1 was higher in isolates causing severe malaria than mild or UCM. However, it should be mentioned that a large field study conducted on Malawian children found an inverse association between ICAM-1 adhesion and disease severity (Rogerson et al., 1999) and no difference was observed in adhesion of IEs to ICAM-1 between parasites from severe and UCM in another study (Ockenhouse et al., 1991).

Third, a single natural mutation in the ICAM-1 gene designated ICAM-1\textsuperscript{kilifi} occurs at higher frequency within African populations and was associated with severe malaria; such that individuals homozygous for the Kilifi allele have increased susceptibility to CM in East Africa (Fernandez-Reyes et al.,
1997), although a study in West Africa suggested a protective role of ICAM-1\textsuperscript{kilifi} against severe disease (Kun et al., 1999) and \textit{in vitro} assays showed that adhesion of \textit{P. falciparum}-IEs to ICAM-1\textsuperscript{kilifi} was reduced compared to reference type (Craig et al., 2000). However, another study indicated that this polymorphism only protects against non-malarial febrile illness and does not protect against severe malaria in East Africa (Jenkins et al., 2005).

Despite the potential role of ICAM-1 in CM pathogenesis, IEs selected for expression of DC8 and DC13 by panning on HBECs were found not to adhere to ICAM-1 (Avril et al., 2012, Claessens et al., 2012). In addition, all but one ICAM-1 binding DBL domains belong to DBL\textbeta domains from group B or C PfEMP1 variants (Howell et al., 2008, Oleinikov et al., 2009, Janes et al., 2011). Recent reports also indicated that the role of CD36 in CM pathogenesis may be more important as once thought. Parasite isolates from CM patients have been shown to adhere significantly more to CD36 than those from UCM (Almelli et al., 2014). Surprisingly, the study showed no difference in the binding capacities to ICAM-1 receptors, suggesting a key role of CD36, but not ICAM-1, in parasite sequestration during CM (Almelli et al., 2014). However, the reduced level of binding to ICAM-1 compared to CD36 may occur due to suboptimal binding between IEs and ICAM-1 caused by conformational alterations of the protein when attached to a plastic surface. In addition, Almelli et al. (2014) used ICAM-1 coating concentrations of only 10 µg/ml, which seems to be suboptimal for adhesion assays. Taken together, it might be convinceable to suggest that IE cytoadhesion to ICAM-1 contributes to parasite sequestration and development of severe malaria disease, but additional studies are required to further validate this contribution.

**1.6.2 CD36**

CD36 (also known as platelet glycoprotein IV [GPIV] and GP88) is an 88 kDa integral membrane protein found on several cell types including ECs, macrophages, platelets and monocytes. This receptor has several biological functions including platelet adhesion, TSP-dependent interactions, signal transduction, macrophage phagocytosis of senescent neutrophils, immune responses in humans (Greenwalt et
al., 1992) and acting a receptor for malaria cytoadhesion (Barnwell et al., 1989, Ockenhouse et al., 1989). Direct evidence implicating CD36 as a receptor for *P. falciparum* IEs was provided from *in vitro* studies showing that parasitized erythrocytes were able to bind to plastic-adsorbed CD36 receptors, and this adhesion was blocked by anti-CD36 mAbs (Ockenhouse et al., 1989, Barnwell et al., 1989).

The parasite ligand for CD36 adhesion is CIDRα domain of PfEMP1 (Baruch et al., 1997, Chen et al., 2000) encoded by groups B and C var gene subtypes (Rask et al., 2010, Smith, 2014). Purified CD36 receptors have the capacity to bind to most wild and culture-adapted *P. falciparum* isolates, although quantitative levels of binding are variable (Chilongola et al., 2009, Newbold et al., 1997, Rogerson et al., 1999). However, IEs isolated from placentas of infected pregnant women have been shown to bind to placental receptors such as CSA, but fail to bind to CD36 receptors (Fried and Duffy, 1996). Furthermore, it has been shown that a CIDR domain cloned from the expressed var gene of the CSA-binding FCR3 isolate was found not to bind to CD36 receptors (Buffet et al., 1999). This is due to the lack of CIDRα<sub>2,6</sub> domains, the binding sites for CD36, in the var2csa-encoded PfEMP1 domains (Salanti et al., 2003; Smith et al., 2013).

### 1.6.2.1 CD36 adhesion properties and association with disease severity

Similar to ICAM-1, no definitive association between disease severity and CD36 adhesion has been established (Cooke et al., 1994, Beeson and Brown, 2002, Rowe et al., 2009). In a large field study conducted in Africa (Rogerson et al., 1999) and another study in Thailand (Ockenhouse et al., 1991), no significant difference in CD36 adhesion capacity between parasites from severe and UCM has been found, in contrast to findings from a recent study showing that a higher number of CM isolates bound more to CD36 compared to UCM patients (Almelli et al., 2014). On the other hand, findings from two large studies in Africa showed that parasites isolated from individuals with UCM have significantly higher levels of binding to CD36 than parasites from those with severe malaria, which may suggest a role for this receptor in host control of malaria (Newbold et al., 1997, Ochola et al., 2011). In another study, the low levels of CD36 expression in cerebral vessels of people who died from severe malaria showed no change from controls, in contrast to significantly increased
expression of ICAM-1 (Turner et al., 1994). However, the same study showed a highly significant correlation between CD36 expression and sequestered IEs in the brain endothelium of post-mortem tissues. A recent paper has indicated that CD36 deficiency was associated with protection against P. falciparum malaria anaemia in African children, suggesting a role for this receptor in malaria pathogenesis (Chilongola et al., 2009).

Although CD36 expression in the brain endothelium is negligible (Beeson and Brown, 2002, Chilongola et al., 2009), IE binding to these endothelia can occur indirectly as a bridging interaction via platelets which are widely distributed in the brain and express higher levels of CD36 (Almelli et al., 2014). Moreover, it has been shown that auto-agglutination of P. falciparum IEs is mediated by platelets and requires the surface expression of CD36 platelet receptors (Pain et al., 2001). Despite the fact that CD36 is a common receptor for adhesion of most clinical and laboratory isolates, its implication in severe disease requires further elucidation.

1.6.3 EPCR

EPCR is widely expressed on various arteries and veins throughout the body but also expressed at lower levels in particular vascular beds, including the brain (Aird et al., 2014). EPCR is a receptor of the serum factor protein C and acts as cofactor for its physiological conversion to activated protein C, which also requires thrombodulin. On the EC surface, activated protein C plays a significant role in various functions including anticoagulant activities and protein C-mediated cytoprotective pathway by the activation of protease activated receptor 1 [PAR-1] (Figure 1.8). Cytoprotective effects on ECs include activation of anti-inflammatory and anti-apoptotic responses, modifications of gene expression patterns and protection of endothelial barriers (Aird et al., 2014, Smith, 2014).

Two recently published studies by Turner et al. (2013) and Moxon et al. (2013) have provided significant new understandings about the role of EPCR in P. falciparum malaria disease. Turner et al. (2013) have identified EPCR as a new receptor mediating IE adhesion to ECs on the brain endothelium, and IE adhesion to EPCR was significantly higher among patients with severe malaria
than those with mild or UCM. Parasite binding to EPCR was demonstrated to involve the interaction between the receptor with CIDRα1 domains of DC8-containing PfEMP1 molecules (Turner et al., 2013). In addition, they found that pre-incubation of DC8-CIDRα1.1 or DC13-CIDRα1.4 with EPCR interfered with the binding of EPCR with activated protein C, indicating that PfEMP1 DCs and activated protein C compete for the same binding site. The lack of protein C-EPCR binding complex may lead to endothelial barrier disruption and development of pro-inflammatory pathways, which in turn may contribute to severe malaria (Turner et al., 2013).

Moxon et al. (2013) examined autopsies from Malawian children with CM and found an increased cerebral fibrin clots and loss of EPCR at sites of sequestered IEs in the cerebral microvessels. Using ex vivo endothelial phenotype assays of subcutaneous tissues, they demonstrated that both EPCR and its cofactor thrombodulin are absent at the sites of IE sequestration of non-fatal CM. Changes in EPCR and thrombodulin levels correlated with increased levels of soluble EPCR and soluble thrombodulin in the cerebrospinal fluid. It is therefore hypothesised that IE adhesion in cerebral vessels of CM patients may further down-regulate EPCR and/or thrombodulin expression, which constitutively found at lower levels in the brain, and this reduction makes the brain vulnerable to fibrin deposition, micro-haemorrhages and organ-specific pathology (Moxon et al., 2013).
Figure 1.7. Effects of *P. falciparum*-IE adhesion to EPCR. A: normal functional protein C system (described in the text). B: following *P. falciparum* infection, IEs bind to EPCR leading to inhibition of EPCR functions and enhancement of EC activation, which in turn leads to the release of cytokines, soluble EPCR (sEPCR) and soluble thrombodulin (sTM) in the blood. APC: activated protein C. The graph was acquired from (Aird et al., 2014).

1.6.4 CSA

CSA receptor is expressed on the surface of syncytiotrophoblasts lining the intervillous space of the placenta (Fried and Duffy, 1996). *P. falciparum*-IEs have been shown to adhere to CSA (Rogerson et al., 1995), and the latter was uniquely implicated in the development of PM through the binding of VAR2CSA-expressing *P. falciparum* isolates (Salanti et al., 2003, Salanti et al., 2004). Several studies have implicated CSA as a key adhesion receptor for *P. falciparum* IEs in the placenta. For instance, an early study showed that parasites isolated from placentas of infected pregnant women typically adhered to CSA, and adhesion to placental sections occurred in a CSA-dependent fashion (Fried and Duffy, 1996). Although CSA has been detected in the vasculature of the brain, no steady correlation was observed between parasite binding to CSA and development of severe disease in children or non-pregnant adults (Beeson and Brown, 2002).
1.6.5 PECAM-1 (CD31)

PECAM-1/CD31 is a highly glycosylated 130-kDa polypeptide expressed on ECs, monocytes, granulocytes and platelets. It belongs to the Ig supergene family and contains six extracellular Ig-like domains and a long cytoplasmic tail. *P. falciparum*-IEs have been demonstrated to bind to PECAM-1/CD31 on the vascular endothelium, to PECAM-1-transfected cells and directly to recombinant PECAM-1/CD31 receptors immobilised on plastic (Treutiger et al., 1997). Additionally, soluble PECAM-1 and mAbs raised to the amino-terminal segment of PECAM-1 inhibited IE binding. The binding site on PECAM-1/CD31 was suggested to be residing in the first four domains of the protein (Treutiger et al., 1997). On the IE side, binding of PECAM-1/CD31 was mapped to CIDR1α and DBL2δ domains of PfEMP1 molecules (Chen et al., 2000). Although about 50% of field parasite isolates from Kenya were shown to bind to PECAM-1/CD31 receptors in some reports, no significant association with severe disease has been detected (Rowe et al., 2009).

Recently, Berger et al. (2013) demonstrated that parasite isolates expressing DC5 bind to PECAM-1 receptors. DC5 is a four-domain C-terminal cassette found exclusively in group A PfEMP1 proteins (Rask et al., 2010). Antibodies to each of the four domains in DC5 react with the native PfEMP1 on the surface of IEs. Furthermore, anti-DC5 antibodies blocked adhesion of DC5-expressing isolates to transformed human bone marrow ECs that express PECAM-1. Anti-DC5 antibodies are naturally acquired during the first years of life by individuals living in endemic regions, and people who have higher levels of anti-DC5 antibodies are expected to have less malaria febrile episodes, suggesting a role of DC5 as effectors of malaria immunity (Berger et al., 2013).

1.6.6 Thrombospondin

The adhesive glycoprotein TSP is produced by both melanoma and ECs and suggested to be present on the surface of these cells (Roberts et al., 1985). It is released in the circulating blood in response to thrombin-mediated platelet activation (Rowe et al., 2009) and has been associated with cell-to-cell and cell-to-matrix adhesion (Rock et al., 1988). TSP is considered as the first identified
receptor mediating *P. falciparum* adhesion and implicated in parasite sequestration (Roberts et al., 1985). *P. falciparum*-IEs bind to purified TSP adsorbed onto plastic and TSP-secreting melanoma cells under static conditions (Roberts et al., 1985) and to ECs expressing TSP under conditions of blood flow (Rock et al., 1988). The IE ligand for binding to TSP is debatable, with PfEMP1, the membrane phospholipid "phosphatidylinositol" derived from RBCs and modified band 3 suggested as potential binding ligands. No particular PfEMP1 domains have been shown to mediate the binding to TSP (Rowe et al., 2009).

### 1.6.7 VCAM-1 and E-selectin

VCAM-1 (CD106) is a member of the Ig supergene family and consists of six extracellular domains (Rowe et al., 2009, Ockenhouse et al., 1992b). It mediates the adhesion of lymphocytes to the endothelium in a distinct mechanism from LFA-1 and ICAM-1 (Udomsangpetch et al., 1996). E-selectin (CD62E or endothelial leukocyte adhesion molecule-1 [ELAM-1]) is expressed on ECs at tissue sites of inflammation (Rowe et al., 2009). It belongs to a family of molecules termed selectins; a structurally associated protein similar to the mammalian lectins, epidermal growth factor and complement regulatory molecules (Ockenhouse et al., 1992b), and has been demonstrated to augment the rolling of neutrophils (Udomsangpetch et al., 1996).

*P. falciparum* parasites were selected in *vitro* by consecutive panning on purified VCAM-1 and E-selectin proteins and shown to bind to the same proteins in recombinant soluble forms (Ockenhouse et al., 1992b). Parasites isolated from patients with severe malaria bound to TNF-α-activated human vascular endothelial cells (HUVECs) through VCAM-1 and E-selectin receptors (Ockenhouse et al., 1992b). However, a large African field study of nearly 200 isolates demonstrated that only very few patient-derived parasite isolates bound to VCAM-1, whereas almost none of the isolates bound to E-selectin (Newbold et al., 1997). Another field study conducted on Thai patients showed that the percentage of binding of all tested isolates to VCAM-1 and E-selectin was negligible (Udomsangpetch et al., 1996).
1.7 PfEMP1 vaccine studies

Despite the significant role of PfEMP1 as target of natural immunity and as a key virulence candidate in pathogenesis of severe malaria, the high level of diversity of these proteins limits their potential for the development of a malaria vaccine (Bull et al., 1999, Bull et al., 2000, Nielsen et al., 2004). However, several published studies have provided evidence that the development of a PfEMP1-based vaccine could be possible by showing that PfEMP1 recombinant proteins can mount a protective immune response in experimental animals (Tables 1.1 and 1.2). Immunisation of Aotus monkeys with single CDIR1α domains induced a protective immune response against fatal parasite infection with homologous parasite isolate, although the response was not sufficient for protection against heterologous parasite challenge probably due to the variant-specificity of the immune response and extensive variability of PfEMP1 variants (Baruch et al., 2002).

In another study, the researchers have immunised rats with phylogenetically variant recombinant DBLα domains and the induced antibodies cross-reacted with heterologous domains and reduced sequestration of with homologous and heterologous isolates in a novel in vivo model for human P. falciparum-IE sequestration (Ahuja et al., 2006). This and other published studies (Baruch et al., 2003, Gratepanche et al., 2003, Bengtsson et al., 2013, Angeletti et al., 2013, Avril et al., 2011, Nielsen et al., 2009, Salanti et al., 2010, Saveria et al., 2013, Avril et al., 2008, Oleinikov et al., 2008, Obiakor et al., 2013) have demonstrated that induction of cross-reactive animal antibodies recognising heterologous domains and parasite isolates is possible (Tables 1.1 and 1.2). In an attempt to overcome the variant-specific nature of antibodies raised against PfEMP1 variants, some researchers have used various combinations of PfEMP1 domains to increase the cross-reactivity of the immune response (Gratepanche et al., 2003, Baruch et al., 2003, Moll et al., 2007). For instance, immunisation of mice with different combinations with CIDRα domains induced antibodies capable of agglutinating erythrocytes infected by diverse parasite isolates (Gratepanche et al., 2003, Baruch et al., 2003). Epidemiological studies on pregnant women in malaria-endemic areas have demonstrated that a vaccine that blocks IE binding to CSA and placental sequestration would protect these
women against the consequences of PM (Avril et al., 2006). Women in these areas acquire protection as they develop antibodies against VAR2CSA during consecutive pregnancies (Salanti et al., 2004, Barfod et al., 2006), making VAR2CSA a major target for vaccine development. This is supported by published studies showing that immunising animals with the full-length (Avril et al., 2011, Khunrae et al., 2010) or single domains (Nielsen et al., 2009, Salanti et al., 2010, Saveria et al., 2013, Obiakor et al., 2013, Avril et al., 2010) of VAR2CSA induced antibodies in animal antisera that reacted with several CSA-binding parasites and blocked IE adhesion to CSA receptors (Table 1.2).

For the development of a PfEMP1-based vaccine, two main approaches have been used to overcome the major obstacles of antigenic variation. The first approach aims to develop a multivalent vaccine comprising several distinct PfEMP1 variants for inducing an extensive repertoire of antibodies to cover the high level of antigenic diversity in parasite populations. However, identifying the number of different candidates involved in such PfEMP1-based vaccine remains an obstacle. In addition, the number of distinct variants required would be too large based on the sequence analysis data showing the high level of PfEMP1 sequence diversity (Beeson et al., 2013). Another challenge of this approach is the higher rate of gene recombination (Claessens et al., 2014), which allows the parasite to express other antigenically distinct epitopes that are not included in the vaccine.

The second approach aims to direct the immune response to conserved epitopes or regions in PfEMP1 such that an effective vaccine would elicit cross-reactive antibodies with most if not all PfEMP1 variants in various populations (Gamain et al., 2001, Beeson et al., 2013). Defining conserved PfEMP1 epitopes involved in the pathogenesis of severe disease and shared between genotypically distinct parasites is highly challenging. Despite the high diversity of PfEMP1 proteins, some conservations at the structural level must exist to maintain its cytoadhesion function (Gamain et al., 2001, Bull et al., 1999). It was proposed that common "basic types" of PfEMP1 proteins are expressed during malaria infection, and these types seem to be immunogenic and elicit an immunogenic antibody response (Giha et al., 1999). Immune sera from people living in various African countries agglutinated erythrocytes infected by isolates from distant areas,
suggesting the existence of cross-reactive epitopes expressed by several parasite strains (Aguiar et al., 1992). Another study conducted in a village in Sudan indicated that although parasites circulating in the area are genetically diverse and express distinct PfEMP1 proteins, they also exhibit a significant overlap in their PfEMP1 repertoires (Giha et al., 1999). In another study, parasites collected from severe malaria patients were frequently agglutinated by antibodies in heterologous plasma, indicating that a dominant subset of parasite-derived antigens are present on the surface of IEs and recognised by prevalent antibodies (Bull et al., 1999). The ability of serum IgG to recognise "common" epitopes is encouraging since parasites expressing commonly recognised VSA have been associated with severe disease (Bull et al., 1999, Bull et al., 2000). This and the recent identification of conserved DCs in PfEMP1 molecules and their association with severe disease may raise hope that the achievability of a PfEMP1-based vaccine may be possible. However, further work is needed to characterise the tertiary and quaternary structure of PfEMP1 proteins and identify conserved epitopes as well as their potential to elicit a specific protective immune response.
<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Animal</th>
<th>Reference</th>
<th>Antibody response</th>
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<tbody>
<tr>
<td>CIDRα domains</td>
<td><em>Aotus</em> monkeys</td>
<td>(Baruch et al., 2002)</td>
<td>The produced antibodies protected against fatal parasite infection with homologous, but not heterologous isolates.</td>
</tr>
<tr>
<td>DNA plasmids expressing 3 CIDRα domains</td>
<td>Mice</td>
<td>(Baruch et al., 2003)</td>
<td>The produced antibodies were reactive against corresponding PfEMP1s as shown by ELISA, flow cytometry and agglutination of IEs. Some mouse sera showed a limited cross-reactivity with heterologous parasite isolates and recognised heterologous domains.</td>
</tr>
<tr>
<td>DNA immunisation followed by a protein boost</td>
<td>Mice</td>
<td>(Gratepanche et al., 2003)</td>
<td>A broad range of cross-reactivity to heterologous CIDRα domains was observed in mice immunised with the three domains simultaneously.</td>
</tr>
<tr>
<td>10 different recombinant DBLα domains</td>
<td>Rats</td>
<td>(Oguariri et al., 2003)</td>
<td>The produced antibodies recognised conserved PfEMP1 synthetic peptides but failed to label the surface of trophozoite-IEs and were incapable of recognising the full length PfEMP1 from laboratory strains. Antibodies also failed to agglutinate IEs.</td>
</tr>
<tr>
<td>DBL1α, CIDR1α and DBL2δ domains</td>
<td>Mice / rats / rabbits</td>
<td>(Chen et al., 2004)</td>
<td>Immune sera reacted with the surface of live IEs as well as linear recombinant proteins in ELISA and immunoblotting. Anti-DBL1α antisera and sera raised against a mixture of all domains disrupted rosettes and auto-agglutinates. Anti-DBL1α antisera protected against <em>P. falciparum</em> IE sequestration in an <em>in vivo</em> model of severe malaria.</td>
</tr>
<tr>
<td>NTS-DBL1α/x domains of 3D7-PfEMP1</td>
<td>Rats</td>
<td>(Ahuja et al., 2006)</td>
<td>The antisera showed a wide range of cross-reactivity to diverse DBL1α/x domains in ELISA. In addition, the produced antibodies reduced parasite sequestration in an <em>in vivo</em> model.</td>
</tr>
<tr>
<td>DBLα alone or in combination with other DBLα</td>
<td>Rats / monkeys</td>
<td>(Moll et al., 2007)</td>
<td>The produced antibodies reacted with the surface of homologous and heterologous parasites. Antibodies significantly reduced PfEMP1-mediated sequestration of homologous isolates in immunised rats and protected against heterologous parasite sequestration after direct and indirect immunisation of rats. Antibodies significantly blocked IE adhesion in immunised monkeys.</td>
</tr>
<tr>
<td>Domain Type</td>
<td>Species</td>
<td>Reference</td>
<td>Description</td>
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<tr>
<td>NTS-DBL1α of varO parasites</td>
<td>Mice</td>
<td>(Vigan-Womas et al., 2008)</td>
<td>Anti-NTS-DBL1α antisera produced high antibody titres in ELISA against recombinant NTS-DBL1α domains, recognised the surface of varO-IEs and disrupted varO-IE rosettes.</td>
</tr>
<tr>
<td>Three NTS-DBL1α domains</td>
<td>Mice</td>
<td>(Vigan-Womas et al., 2011)</td>
<td>Mouse antibodies produced by each recombinant NTS-DBL1α domain cross-reacted with their allelic forms by ELISA but showed a strict variant-specific IE surface reactivity and rosette-disruption.</td>
</tr>
<tr>
<td>DBLα, CIDRα and DBLβ domains from DC4-containing PfEMP1s</td>
<td>Rats</td>
<td>(Bengtsson et al., 2013)</td>
<td>The produced sera reacted with the surface of erythrocytes infected by heterologous <em>P. falciparum</em> isolates expressing DC4-containing PfEMP1 variants.</td>
</tr>
<tr>
<td>DBLα1.5 and DBLα1.8 domains from group A PfEMP1 variants</td>
<td>Rabbits</td>
<td>(Ghumra et al., 2012)</td>
<td>The produced antibodies labelled the surface of IEs, inhibited rosetting and induced phagocytosis against homologous isolates. Furthermore, the antibodies cross-reacted with heterologous rosetting isolates, including clinical parasites from Africa.</td>
</tr>
<tr>
<td>Peptide sequences of subdomain 2 of DBL1α</td>
<td>Rats / rabbits</td>
<td>(Blomqvist et al., 2013)</td>
<td>Antisera recognised the recombinant peptides as shown in ELISA and labelled the surface of live IEs. 50% of tested isolates (laboratory or clinical) were surface reactive as indicated by flow cytometry and IFA.</td>
</tr>
<tr>
<td>Three NTS-DBLα domains</td>
<td>Rats / goats</td>
<td>(Angeletti et al., 2013)</td>
<td>All recombinant proteins induced high antibody titres and exhibited substantial levels of cross-reactivity with heterologous NTS-DBLα proteins as shown by ELISA and flow cytometry. All antisera labelled the surface of erythrocytes infected by homologous PfEMP1 variants and showed some levels of cross-reactivity with heterologous isolates.</td>
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Table 1.1. Vaccine studies based on immunisation of animals with PfEMP1 domains involved in CD36 binding and rosetting.
<table>
<thead>
<tr>
<th><strong>Immunogen</strong></th>
<th><strong>Animal</strong></th>
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<th><strong>Antibody response</strong></th>
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<tbody>
<tr>
<td>Full-length VAR2CSA / 3 domain constructs (DBL4-6)</td>
<td>Mice / rabbits</td>
<td>(Avril et al., 2011)</td>
<td>Antibodies in animals immunised by either immunogen recognised several VAR2CSA single domains in ELISA. Both immunogens elicited cross-reactive antibodies recognising diverse CSA-binding <em>P. falciparum</em> isolates. Only rabbit antibodies raised to the full-length VAR2CSA inhibited homologous CSA-binding parasite adhesion.</td>
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<tr>
<td>DNA plasmid of NTS-DBL2X of VAR2CSA</td>
<td>Mice / rabbits</td>
<td>(Bigey et al., 2011)</td>
<td>The produced antibodies significantly inhibited IE adhesion to CSA similar to antibodies raised against the full-length VAR2CSA.</td>
</tr>
<tr>
<td>VAR2CSA single domains, sub-domains and multi-domains</td>
<td>Rats</td>
<td>(Nielsen et al., 2009)</td>
<td>Antibodies raised against VAR2CSA DBL4 domains were surface reactive and efficiently blocked CSA adhesion of several genetically-distinct parasite isolates. Overall, multi-domains elicited higher levels of surface reactivity than single domains.</td>
</tr>
<tr>
<td>All VAR2CSA domains from 3D7 and HB3 parasites</td>
<td>Rats</td>
<td>(Salanti et al., 2010)</td>
<td>All 3D7 recombinant proteins except DBL6 elicited cross-reactive IgG antibodies that labelled VAR2CSA on the surface of IEs. Only antibodies raised against DBL3-HB3 T1 and DBL1-3D7 almost completely inhibited IE adhesion to CSA.</td>
</tr>
<tr>
<td>Individual domains of 3D7 VAR2CSA variant</td>
<td>Rats / mice</td>
<td>(Saveria et al., 2013)</td>
<td>Antibodies raised against DBL4ε, DBL5ε, DBL6ε and tandems of DBL4-DBL5 and DBL5-DBL6 all bind to placental parasites and laboratory isolates selected for CSA binding. Antisera to DBL5ε and DBL6ε blocked placental parasite adhesion to placental tissues and inhibited binding of several field isolates to CSA.</td>
</tr>
<tr>
<td>DBL3 and DBL5 (VAR2CSA-3D7), DBL5 (VAR2CSA-FCR3) and DBL2 (PFL0008-3D7)</td>
<td>Rabbits</td>
<td>(Magistrado et al., 2008)</td>
<td>Antibodies raised against recombinant domains of VAR2CSA labelled the surface of <em>P. falciparum</em> parasites isolated from infected placentas.</td>
</tr>
<tr>
<td>All VAR2CSA DBL</td>
<td>Rabbits</td>
<td>(Andersen et al., 2008)</td>
<td>The produced antibodies reacted with the surface of erythrocytes infected by CSA-binding isolates (3D7 and</td>
</tr>
<tr>
<td>Domains and ID2 domain</td>
<td>Species</td>
<td>Year</td>
<td>Study Details</td>
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<tr>
<td>DBL3X and DBL6ε domains of VAR2CSA</td>
<td>Mice</td>
<td>2008</td>
<td>(Fernandez et al., 2008) Antisera raised against DBL6ε-VAR2CSA-FCR3 domain specifically recognised the surface of CSA-binding <em>P. falciparum</em> isolates and partially blocked IE adhesion to CSA receptors.</td>
</tr>
<tr>
<td>DBL1, DBL3, DBL4, DBL5 and DBL6 of VAR2CSA-3D7</td>
<td>Rabbits / mice</td>
<td>(Oleinikov et al., 2008) Anti-DBL5 antisera cross-reacted with the surface of CSA-binding <em>P. falciparum</em> laboratory isolates (3D7 and FCR3) and a clinical PM isolate. Anti-DBL6 antisera only reacted with 3D7-IEs.</td>
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<tr>
<td>All six domains of VAR2CSA-3D7</td>
<td>Rabbits / mice</td>
<td>(Barfod et al., 2006) Antisera raised in animals immunised with each of the domains reacted exclusively with the immunising domain as shown in ELISA. Some of the antisera reacted with the surface of IEs.</td>
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<tr>
<td>Full-length VAR2CSA from FCR3 isolate</td>
<td>Rats</td>
<td>(Khunrae et al., 2010) The produced antibodies completely inhibited recombinant VAR2CSA binding, as well as IE adhesion to chondroitin sulphate proteoglycans (CSPG). Of particular significance, anti-DBL4ε inhibited FCR3-IE adhesion to CSA by 90%.</td>
<td></td>
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<tr>
<td>DBL2X, DBL3X, DBL2X-S3, DBL3X-S3 of FCR3 line</td>
<td>Rats / rabbits</td>
<td>(Obiakor et al., 2013) Purified IgG from antisera raised against all domains recognised the surface of erythrocytes infected by homologous and some heterologous isolates. Anti-DBL3X-S3 inhibited IE binding to CSA receptors.</td>
<td></td>
</tr>
<tr>
<td>Individual VAR2CSA DBL domains</td>
<td>Rabbits</td>
<td>(Avril et al., 2008) Rabbit antisera reacted with homologous FCR3 isolates. Antisera to several DBL domains cross-reacted with different <em>P. falciparum</em> isolates.</td>
<td></td>
</tr>
<tr>
<td>Full-length VAR2CSA from FCR3 isolate, CSA-binding VAR2CSA fragments</td>
<td>Rats</td>
<td>(Clausen et al., 2012) Antibodies raised to CSA-binding fragments were efficient at blocking IE binding to CSA. Some sera partially cross-inhibited placental isolates.</td>
<td></td>
</tr>
<tr>
<td>VAR2CSA-DBL1 and DBL5 domains</td>
<td>Rabbits</td>
<td>(Avril et al., 2010) Immunisation with DBL5 domains induced a broadly cross-reactive response that recognised almost all tested lab-adapted CSA-binding isolates and clinical parasites from pregnant women from distinct regions. On the other hand, immunisation with DBL1 elicited antibodies with a relatively less reactivity with tested parasites.</td>
<td></td>
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<tr>
<td>Domain Description</td>
<td>Animal Species</td>
<td>Reference</td>
<td>Summary</td>
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<tr>
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<tr>
<td>DBL5ε, DBL6ε and DBL5ε-DBL6ε</td>
<td>Mice / goats</td>
<td>(Fernandez et al., 2010)</td>
<td>All recombinant domains induced cross-reactive antibodies that blocked 40 – 96% of IE adhesion to CSA. Antibodies raised to DBL5ε from 3 distinct parasite genotypes exhibited strain-specific IE blocking capacity.</td>
</tr>
<tr>
<td>CSA-binding DBL3γ of VAR1CSA</td>
<td>Mice</td>
<td>(Bir et al., 2006)</td>
<td>The produced antibodies cross-reacted with diverse CSA-binding <em>P. falciparum</em> isolates and blocked their adhesion to placental cryosections under flow conditions. Antibodies to DBL3γ domains cross-reacted with DBL3X of VAR2CSA.</td>
</tr>
<tr>
<td>Full-length VAR2CSA from 3D7 isolate</td>
<td>Rabbits</td>
<td>(Srivastava et al., 2011)</td>
<td>Rabbit antibodies against the full-length VAR2CSA inhibited the interaction between DBL1X-6ε and decorin by 72%, and these antibodies predominantly target DBL3X and to some extent DBL5ε single domains.</td>
</tr>
<tr>
<td>Several single and multiple VAR2CSA domains</td>
<td>Rats</td>
<td>(Dahlback et al., 2011)</td>
<td>Purified IgG antibodies from immunised rats reacted with VAR2CSA of homologous FCR3-IEs. The multidomains induced higher flow cytometry response than single domains. Antibodies against NTS-DBL4ε and NTS-DBL3X efficiently blocked FCR3-IE adhesion to CSPG. Antibodies to the full-length VAR2CSA almost completely abolished IE binding.</td>
</tr>
<tr>
<td>5 single or double domain constructs of VAR2CSA</td>
<td>Mice</td>
<td>(Bordbar et al., 2012)</td>
<td>All domains but DBL2X and Id1 induced a high-titre antibody response as measured by ELISA. Sera from mice immunised by NTS-DBL1X-Id1 showed a partial blocking capacity, whereas antibodies in antisera raised to Id1-DBL2X completely ablated IE binding to CSA. The latter antisera also labelled the surface of erythrocytes infected by field isolates from pregnant women, and blocked adhesion of 8 tested isolates.</td>
</tr>
</tbody>
</table>

Table 1.2. Vaccine studies based on immunisation of animals with VAR2CSA domains involved in PM.
1.8 Antibodies to malaria disease

Immunoglobulins or antibodies are classified into five main classes (IgG, IgA, IgE, IgD and IgM), which in humans is further divided into nine subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgE, IgD and IgM), all secreted from B cells. Each antibody is visualised as a Y shape and consists of four polypeptides: two pairs of heavy (µ, γ1, γ2, γ3, γ4, α1, α2, ε and δ) and light (κ and λ) chains both comprising variable domain and constant domains (Pleass and Holder, 2005, Greenfield, 2014). The heavy and light chains are joined together with covalent and non-covalent bonds. The variable domains comprise three hyper-variable or complementarity determining regions (CDR) which recognise the antigen. The main function of antibodies is binding to antigens, and in malaria disease they have several functions such as neutralisation of malaria toxins and inhibition of parasite invasion. Passive administration of human Igs from sera of hyperimmune adults has been shown to be effective in controlling malaria disease, indicating that Igs have an essential role in the protection against malaria (Cohen et al., 1961). However, direct binding to pathogens can be insufficient and antibody-mediated activation of other effector mechanisms is often required for the identification and destruction of invading pathogens. Activation is achieved through the Fc region of antibodies, which facilitates the effector mechanism via binding to Fc receptors (FcR) and/or complement (Pleass and Holder, 2005, Greenfield, 2014). There are different FcRs for all classes of antibodies (reviewed in Pleass, 2009), which are widely expressed on various types of cells of the immune system. Examples of FcRs include receptors for IgG (FcγRI, FcγRII, FcγRIII), IgE (FceRI), IgA (FcaRI, Fca/µR) and IgM (Fca/µR) (Pleass, 2009). FcRs can activate or block different immune effector mechanisms including phagocytosis, antioxidant secretion, antibody-dependent cellular toxicity and production of cytokines and other inflammatory mediators (Pleass and Holder, 2005, Greenfield, 2014).

1.8.1 IgM antibodies

IgM is the only antibody class expressed by all vertebrates and the first antibody to be produced during an immune response. It is extremely effective at
neutralising pathogens and activating the classical complement cascade (Czajkowsky et al., 2010, Couper et al., 2005). While the heavy chains of most Igs contain three constant domains, the heavy chains of IgM contain a fourth C\textmu domain (i.e. C\textmu4). The extra two (C\textmu2) domains are located in the hinge regions and provide extra flexibility of the antigen-binding Fab domains (Czajkowsky and Shao, 2009). There are two classes of IgM: immune (or antigen-specific) IgM which is secreted in response to foreign pathogens, and non-immune (or natural) IgM which has a low affinity and broad reactivity, and secreted without previous exposure to any specific antigen (Czajkowsky et al., 2010). The latter class is produced by B-1 B cells and has a major role to control the dissemination of viruses and bacteria as part of the host first line defence (Czajkowsky et al., 2010, Ghumra et al., 2008).

Parasite-specific IgM has been shown to play a significant role in limiting the parasite replication in a murine model (Couper et al., 2005). However, its role in human malaria remains largely unknown (Ghumra et al., 2008). Similarly, the role of non-immune IgM in malaria is not entirely clear, although it has been demonstrated that non-immune IgM binds to the surface of *P. falciparum*-IEs through Fc, and this was demonstrated to correlate with rosetting and severe malaria in field isolates and laboratory clones (Rowe et al., 2002). An early study showed that *P. falciparum* parasites were unable to form rosettes when cultured in serum-free medium, and rosetting was restored after the addition of IgM from normal donors, indicating the importance of natural IgM in rosetting (Clough et al., 1998). Furthermore, CSA-binding *P. falciparum*-IEs (implicated in PM) are also able to bind non-immune IgM, and VAR2CSA variants involved in CSA-adhesion contain IgM-binding domains (Rasti et al., 2006). However, binding to non-immune IgM is only restricted to *P. falciparum* isolates with particular virulence-associated binding phenotypes (i.e. rosetting and CSA-binding), and isolates with other adhesion phenotypes such as ICAM-1 or CD36 binding do not seem to bind non-immune IgM (Rowe et al., 2002).
1.8.2 Consequences of PfEMP1-IgM binding

It is not completely understood why *P. falciparum* parasites have evolved to bind IgM molecules through Fc regions. Several potential functional consequences of IgM-PfEMP1 interaction that favour the parasite development have already been described (reviewed in Czajkowsky et al., 2010) including inhibition of the binding of specific antibodies by masking critical PfEMP1 domains, interference with the binding of IgM with FcµR on B cells and T cells, interference with immune functions associated with binding of Fcα/µR expressed on follicular dendritic cells (FDCs), enhancement of IE sequestration in host microvessels or placenta, or interference with immunological signalling pathways and pathogen clearance mechanisms [summarised in Figure 1.9] (Czajkowsky et al., 2010). A recent study has demonstrated that *P. falciparum* parasites evade the host specific immune response by masking critical epitopes on PfEMP1 by binding non-specific IgM (Barfod et al., 2011). Non-specific IgM binding to VAR2CSA-expressing *P. falciparum* isolates interfered with subsequent binding of specific mAbs (IgG isotype) to the critical domains DBL3X and DBL5ε, protected the parasite from phagocytosis and did not interfere with their ability to bind CSA receptors (Barfod et al., 2011). In the case of rosetting phenotype, IgM binding was suggested to act as bridge between IEs and non-IEs to stabilise rosettes (Somner et al., 2000).

Complement activation is one of the earliest markers of immune response to malaria (Roestenberg et al., 2007). The polymeric structure of IgM predominantly benefits these molecules to efficiently agglutinate and neutralise pathogens or pathogen-infected cells. This sole structure makes IgM extremely efficient at activating the classical cascade, as a single IgM molecule is capable of activating the complement system leading to the lysis of a single RBC (Czajkowsky et al., 2010). Upon binding to the surface epitope on IEs, IgM may undergo a conformational change favouring the binding of C1q (Czajkowsky and Shao, 2009) which facilitates downstream components terminating in the assembly of membrane attack complexes (MAC) that lyse the cell. However, binding of IgM by PfEMP1 could interfere with complement activation by locking out C1q-binding sites on IgM or forcing IgM to be oriented with its functional flat site facing the IE surface, thus hiding C1q-binding sites from C1q (Czajkowsky et al., 2010).
Figure 1.8. Possible functional consequences associated with IgM-PfEMP1 binding. A: activation of B cells through interactions with BCR (and CR1/2). A: facilitation immunological synapse formation through FcµRs found on T cells. B: activation of B cells through interaction with BCR. C: direct interaction between BCR on B cells and PfEMP1 variants. D: activation of B cells and FDCs through interaction with Fcα/µRs. E: inhibition of potential antigens on the surface of IEs through masking mechanism resulted from IgM binding to PfEMP1. F: activation of ECs through interaction with the endothelial receptor CD300LG. G: activation of the classical pathway through the binding of the potent activator, C1q. The graph was obtained from (Czajkowsky et al., 2010).

1.8.3 IgM-PfEMP1 binding sites

The parasite ligands that mediate IE binding to IgM have been identified and shown to be the PfEMP1 molecules on the surface of IEs. A number of different PfEMP1 domains from IgM-binding isolates have been demonstrated to bind IgM; these include DBL4β, DBL5ε, DBL6ε, DBL7ε and DBL2-X domains of different parasite strains (Ghumra et al., 2008, Semblat et al., 2006, Rasti et al., 2006). Using interaction studies with domain swapped antibodies, IgM mutants and anti-IgM mAbs, the region of polymeric IgM molecule that mediates PfEMP1 binding was identified as Cµ4 (Ghumra et al., 2008). This demonstrates that PfEMP1 is an Fc-binding protein of *P. falciparum* isolates that have the specificity to binding IgM. This IgM binding region was found to be shared by diverse IgM-binding *P. falciparum* isolates of different adhesion phenotypes, suggesting that different parasite isolates expressing diverse PfEMP1 variants all bind to the same region on human IgM molecules (Ghumra et al., 2008).
1.9 Development of monoclonal antibodies against PfEMP1 recombinant proteins

Strategies aiming to block PfEMP1 domains involved in IE cytoadhesion have been hypothesised to decrease the severity of malaria disease by decreasing or suppressing the capacity of parasite-infected erythrocytes to sequester to the host microvessels (Chattopadhyay et al., 2004). The development of hybrid cells (hybridomas) secreting antibodies with monoclonal specificity to defined *P. falciparum* antigens is an early approach that has long been shown to be successful. The use of purified malaria antigens can be used for immunising animals and studying the immune response (Perrin et al., 1980). MAbs directed against diverse adhesive domains of PfEMP1 may have a therapeutic value in reducing severe disease. Antibody-mediated adhesion-inhibition and/or reversal methods have been demonstrated not only reduce capacity of IEs to cytoadhere, but also to play a significant role in the removal of IEs from the peripheral circulation by the host spleen and reduce the harmful inflammatory responses in vital tissues (Rowe et al., 2009, Salanti et al., 2010). In addition to their adhesion-blocking role, these antibodies appear to opsonise IEs and induce phagocytosis (Ghumra et al., 2008, Ghumra et al., 2011). However, attempts to develop mAbs targeting the conformational VSAs have mostly been unsuccessful, and in spite of intensive efforts in several laboratories, only a small number of mAbs have been successfully developed (Table 1.3). This shortage of success may be due to the large number of immunodominant VSAs that have the capacity to elicit a strong immune response in animals, making the induction specific mAbs directed to minor or relatively less immunogenic antigens or conformational epitopes very difficult (Lekana Douki et al., 2002).

The majority of mAb studies in anti-adhesive therapies have focussed on the relatively-conserved domains: CIDR1-α, DBL-1α and VAR2CSA domains involved in IE adhesion to CD36, rosetting-related receptors and CSA, respectively (Table 1.3). In one of these studies, mouse mAbs obtained by immunisation with CIDRα domains have demonstrated substantial levels of surface reactivity with erythrocytes infected by genetically diverse *P. falciparum* isolates. One of these mAbs reacted with 90% of tested isolates, and only failed
to react with a non-CD36 binding isolate (Gamain et al., 2001). VAR2CSA variant is a vaccine candidate for protection against PM (Chan et al., 2014). Mouse mAbs generated against the full-length (Avril et al., 2006) or single domains (Costa et al., 2003) of VAR2CSA blocked IE adhesion to CSA receptors, suggesting that production of antibodies with in vivo therapeutic functions based on VAR2CSA domains may be feasible. Although these domains are not completely conserved, it was hoped that their variation is more relatively limited unlike the other highly diverse functional candidates such as DBLβ domains. Despite the potential role of ICAM-1 receptors in the pathogenesis of CM, no studies have been published - to our knowledge - for the development of mAbs targeting the ICAM-1-binding parasite ligand DBLβ domains. Only some work has been carried out to block the parasite adhesion at the ICAM-1 side using inhibitors to the IE-binding determinants on ICAM-1 (e.g. Tse et al., 2004).

The presence of numerous VSAs on the surface of IEs makes the significance of PfEMP1 relative to other variant antigens difficult to quantify. Most immunoassays that use recombinant PfEMP1 domains for antibody production cannot highlight the relative importance of antibodies targeting PfEMP1 proteins independently of antibody responses directed to other VSAs (Beeson et al., 2013, Marsh and Kinyanjui, 2006). Nevertheless, a recent novel approach using genetically modified P. falciparum lines with suppressed PfEMP1 expression has shed some light on the significance of PfEMP1 and other VSAs as targets for protective antibody response among serum samples collected from individuals living in malaria endemic areas in Eastern Africa (Chan et al., 2012). The study showed that binding of IgG to the surface of erythrocytes infected by the transgenic line was greatly reduced in comparison with the parental line expressing PfEMP1, suggesting that acquired antibody response to the surface of parasitized erythrocytes is mainly directed towards PfEMP1, with other VSAs playing minor roles as immune targets (Chan et al., 2012). The study also revealed that PfMEP1-specific antibodies were correlated with protective immunity against symptomatic malaria and mediated opsonic phagocytosis of IEs.
<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Animal</th>
<th>Reference</th>
<th>Antibody response (mAbs/pAbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length VAR2CSA of FCR3&lt;sub&gt;CSA&lt;/sub&gt; isolate</td>
<td>Mice</td>
<td>(Avril et al., 2006)</td>
<td>Mouse mAbs blocked 0-60% of <em>P. falciparum</em> adhesion to CSA and immunoprecipitated PfEMP1&lt;sub&gt;CSA&lt;/sub&gt; (VAR2CSA). These mAbs also specifically selected CSA-adhering IEs and mediated the purification of VAR2CSA from IE.</td>
</tr>
<tr>
<td>6 extracellular domains of rosetting PfEMP1-R29 isolate</td>
<td>Rabbits</td>
<td>(Ghumra et al., 2011)</td>
<td>Antibodies to all PfEMP1 domains reacted with the surface of IEs, and antibodies to all domains except DBL2 inhibited rosetting, opsonise IEs and induced phagocytosis. Anti-NTS-DBL1α antibodies were most effective in all assays.</td>
</tr>
<tr>
<td>Intact IE&lt;sub&gt;CSA&lt;/sub&gt; or CHO cells expressing rDBL-γ3&lt;sub&gt;CSA&lt;/sub&gt; from FCR3 isolate</td>
<td>Mice</td>
<td>(Lekana Douki et al., 2002)</td>
<td>The produced mAbs, of which ~70% were IgMs, recognised the surface of IE&lt;sub&gt;CSA&lt;/sub&gt; and several of these mAbs cross-reacted with <em>P. falciparum</em> isolates from diverse West African areas.</td>
</tr>
<tr>
<td>CIDR1 from PfEMP1 that is functionally conserved for CD36 adhesion</td>
<td>Mice</td>
<td>(Gamain et al., 2001)</td>
<td>Two mAbs targeting different regions of CIDR1 domains recognised several <em>P. falciparum</em> isolates expressing distinct PfEMP1 variants. The mAbs cross-reacted with CIDR1 domains of various CD36-binding parasites. One of these mAbs reacted with 90% of tested isolates.</td>
</tr>
<tr>
<td>rDBL-γ3&lt;sub&gt;CSA&lt;/sub&gt; from FCR3 and 3D7 isolates</td>
<td>Mice</td>
<td>(Costa et al., 2003)</td>
<td>The produced antibodies recognised homologous and heterologous rDBL-γ3&lt;sub&gt;CSA&lt;/sub&gt; domains. Only one mAb inhibited and reversed IE&lt;sub&gt;CSA&lt;/sub&gt; adhesion to ECs <em>in vitro</em>.</td>
</tr>
</tbody>
</table>

Table 1.3. Development of mAbs and pAbs based on immunisation of animals with single or full-length PfEMP1 domains.
1.10 Acquired immunity against *P. falciparum* infection

1.10.1 Early development of acquired immunity

Malaria is a significant cause of morbidity, but not every malaria infection develops into severe life-threatening disease (Autino et al., 2012). The outcome of the disease and development into severe pathology depend on a combination of multi-factors that include both the host and the infecting parasite. Such factors that affect the progression to clinical infection include the age and immune status of the host, and endemicity of the parasite (Miller et al., 2002). The first exposure to *P. falciparum* malaria in individuals without previous experience usually leads to febrile illness; but in rare occasions it develops into severe disease or even death. In endemic areas where malaria transmission is very high and stable, repeated exposure to malaria episodes results in the gradual acquisition of a clinical immunity, which limits the risk of severe malaria disease (Gupta et al., 1999). Protective immune responses acquired this way are primarily mediated by IgG antibodies (Cohen et al., 1961, Piper et al., 1999, Yone et al., 2005). The risk of severe and life-threatening disease in these malaria-endemic areas is usually highest during the first few years of life, after which the frequency and severity of disease rapidly decrease over several years despite continuous exposure, as individuals acquire natural protective immunity (Bull et al., 1998, Gupta et al., 1999).

Although the diversity of the parasite population is huge, epidemiological data suggests that only one or two malaria infections are required for developing substantial immune response against non-CM severe disease that require hospital admission (Gupta et al., 1999). From approximately 6 months of age, young children may encounter severe malaria episodes and death. From the age of 5 years, the frequency of symptomatic malaria disease starts to decline and malaria-related mortality becomes very rare, though children may continue to harbour higher rates of parasitaemia. From adolescence onwards, severe malaria episodes do not occur except under exceptional conditions (Marsh, 1992). Sterile protective immunity is very rare or never achieved as adults remain vulnerable to asymptomatic disease and harbour low parasitaemia (Marsh and Kinyanjui, 2006).
The mechanisms underlying the slow development of clinical immunity to malaria are not completely understood, but at least it is partly due to the requirement for the host either to recognise most parasites circulating in the area or to mount an extensive cross-reactive antibody response to various isolates (Newbold et al., 1992). On the parasite side, slow progression of immunity is attributable to the immune evasion mechanisms: antigenic variation (substantial inter- and intra-clonal diversity of PfEMP1), presence of cross-reactive antigenic determinants, and limited immunogenicity of several surface antigens (Barfod et al., 2011, Autino et al., 2012). Although antigenic variation in vivo facilitates chronic disease, exposure of the host immune system to distinct parasite variant antigens resulted from a higher rate of antigenic switching may allow the host to rapidly recognise future heterologous infections (Roberts et al., 1992). This may explain why a protective immune response is acquired after few malaria episodes (Gupta et al., 1999) despite the extensive number of parasite antigenic types.

Parasites causing severe malaria are hypothesised to express PfEMP1 variants that are superior in their capacity to cytoadhere due to their greater binding affinities to EC receptors. Such parasites are believed to be responsible for earlier infections in younger children when protective immunity against these parasites has not yet been developed, and first immune responses are thought to be directed to these virulent variants (Bull et al., 2000, Lavstsen et al., 2003). This may explain why younger children in areas with high transmission rates develop severe and life-threatening disease, whereas adults continue to harbour parasites that cause mild or non-complicating malaria diseases (Berger et al., 2013).

During infection, the parasite is under two contrasting selection pressures; the first preferring expression of antigenic variants with significant cytoadherence capacity to host cells (functional selection) and the second preferring expression of new antigenic determinants that are not identified by previous antibodies (immune selection). In young children, functional selection may be more significant than immune selection, taken into account that the previous immunity in such individuals is mainly poor. In older individuals, the immune selection
may be more important to escape pre-existing antibody responses (Bull et al., 2000).

1.10.2 Antibodies to VSAs confer protection

Several studies have provided evidence that VSAs on *P. falciparum*-IEs are important targets of naturally acquired immunity, and antibodies directed against these highly immunogenic antigens are associated with protection against homologous parasite isolates (Giha et al., 2000, Bull et al., 1998). In some situations, development of antibodies to heterologous parasites also occurs and this has been postulated as an evidence for the presence of cross-reactive antibody responses against antigens on the surface of IEs (Chattopadhyay et al., 2003). However, it is not completely clear whether such cross-reactive heterologous responses or responses directed against less immunogenic conserved antigens on the IE surface have a significant role in protection against clinical disease (Mackintosh et al., 2008).

The major target for anti-VSA antibodies is believed to be the highly diverse PfEMP1 (Newbold et al., 1992, Baruch et al., 1995, Beeson et al., 2006), and antibodies directed against this protein are thought to confer protection against malaria disease (Baruch et al., 2002, Giha et al., 1999, Bull et al., 1998, Bull et al., 1999, Bull et al., 2000, Gupta et al., 1999). A study was conducted on plasma samples collected before and after malaria season in a Sudanese village showed that both susceptible (individuals who had malaria) and protected (individuals who did not have malaria) persons acquired antibodies to VSAs during the malaria season, and pre-existing anti-PfEMP1 antibodies can reduce the risk of clinical malaria caused by parasites expressing homologous variant antigens (Giha et al., 2000). On the other hand, failure to induce antibodies recognising the surface of *P. falciparum*-IEs in another study was associated with increased susceptibility to clinical malaria (Mackintosh et al., 2008).

The mechanisms by which antibodies to VSAs confer protection are not entirely clear. Three main hypotheses have been proposed; (1) anti-VSA antibodies, particularly those directed to PfEMP1, may provide protection by interfering with one or more of the cytoadhesive properties mediated by different domains.
of PfEMP1 proteins (Marsh et al., 1989, Fried et al., 1998, Marsh and Howard, 1986). (2) Antibodies to VSAs may also provide protection by opsonising parasitized erythrocytes for subsequent phagocytosis, an essential mechanism of parasite elimination (Celada et al., 1982, Marsh and Kinyanjui, 2006). Several studies on animal models have demonstrated that existence of antibodies opsonising IEs is associated with protection. For instance, immunisation of rabbits with extracellular PfEMP1 domains elicited antibodies that opsonised IEs and induced phagocytosis (Ghumra et al., 2011). A recent study also showed that PfEMP1 is the main target for natural immunity and individuals with higher levels of anti-PfEMP1 antibodies have enhanced opsonic phagocytosis and acquired protection against malaria (Chan et al., 2012). (3) Antibodies to VSAs could promote other anti-parasite effector mechanisms such as complement-mediated lysis and antibody-dependent cytotoxicity (Marsh et al., 1989).

1.10.3 Acquired immune response is mainly variant-specific

During the development of clinical immunity, especially during the first few years of life, production of strain-specific antibodies to PfEMP1 domains is essential for preventing infection with previously challenged isolates (Newbold et al., 1992, Marsh and Howard, 1986, Bull et al., 1998, Iqbal et al., 1993). This type of immune response is important during and after infections with isolates causing severe disease (Miller et al., 2002). Several published studies that measured the agglutination responses to *P. falciparum* isolates have demonstrated that children acquired strain-specific anti-VSA antibodies rapidly following malaria infection (Bull et al., 1998, Bull et al., 1999). This suggests that immune response acquired following an infection comprises antibodies targeting erythrocytes infected by the homologous isolate, and supports the essential role of strain-specific antibodies in protection against malaria infection and severe disease (Bull et al., 1998, Newbold et al., 1992).

Despite the fact that the dominant immune response following an infection is variant-specific (Newbold et al., 1992), evidence for the existence of at least some degree of cross-reactive response to distinct parasite isolates has been observed in some studies (Marsh and Howard, 1986, Newbold et al., 1992, Giha
et al., 1999, Ofori et al., 2002). Antibodies in convalescent sera collected from adults agglutinated distinct *P. falciparum* isolates (Chattopadhyay et al., 2003), and immune sera obtained from people living in diverse African countries agglutinated parasitized erythrocytes from different geographical areas, suggesting the presence of cross-reactive epitopes expressed by different parasite isolates (Aguiar et al., 1992).

Marsh and Howard (1986) investigated parasites isolated from ten Gambian children with acute and convalescent sera from each child, and showed that most acute sera did not react with homologous isolates whereas each convalescent serum was highly reactive with homologous but not heterologous isolates. This study suggested that infection by *P. falciparum* results in a great diversity of antigenic phenotypes expressed on the surface of IEs, which was evident from the variant-specific nature of antibody response. Moreover, semi-immune sera from Gambian adults agglutinated erythrocytes infected by the ten children, suggesting that by adulthood most individuals acquire a broader cross-reactive response that protects against heterologous isolates (Marsh and Howard, 1986). However, it should be noted that the exposure to cross-reactive, conserved determinants on the surface of IEs was the key factor for induction of a broadly reactive response in adult semi-immune sera in this study but not the breadth of response to recognise distinct important targets on the surface of the parasitized erythrocyte. It was suggested that the parasite evades strong immune responses directed to conserved sites on PfEMP1, such as sites involved in cytoadhesion, by "drawing the attention" of the host humoral immune response to extremely diverse and at the same time more immunogenic epitopes on the protein (Staalso et al., 1998). If the immune response to conserved regions is significant for protection and essential for the parasite pathogenesis, such conserved regions might useful for vaccine development.

Bull et al. (1999, 2000) have provided evidence for the presence of rare and prevalent parasite isolates, and suggested that isolates causing severe disease express a unique subset of VSAs associated with severe disease in younger children with relatively lower immunity. This was evident from the findings that parasites isolated from children with severe malaria had a significantly higher agglutination frequency than those isolated from patients with mild malaria.
infection (Bull et al., 2000). Consistent with these findings, Nielsen et al. (2002) showed that parasites isolated from children with severe malaria were broadly and more frequently recognised by VSA-specific antibodies in plasma samples of local healthy individuals than parasites isolated from children with non-severe disease (Nielsen et al., 2002). Thus, P. falciparum isolates that cause clinical disease to semi-immune young children appear to express VSAs that are not identified by previously existed strain-specific antibodies, which supports the "hole in the antibody repertoire" hypothesis (Nielsen et al., 2002, Ofori et al., 2002, Bull et al., 1998). Malaria exposure may increase the levels of specific antibodies that recognise VSAs expressed by parasite isolates causing current infections (Bull et al., 1998, Giha et al., 1999), and acquisition of VSA-specific antibodies after repeated exposure appears to gradually limit VSA variants causing severe disease and help to close the "holes" in antibody repertoire (Nielsen et al., 2002).
Aims of study

The main aim of this thesis was to develop monoclonal and polyclonal antibodies in mice against recombinant PfEMP1\textsuperscript{ICAM-1-DBLβ} domains and characterise the immune response of these antibodies. One of the existing approaches to overcome the polymorphism of PfEMP1 in the development of therapeutic interventions is to target PfEMP1 subdomains involved in parasite binding (Gratepanche et al., 2003). Recombinant proteins corresponding to different PfEMP1 domains have been synthesised and used for the induction of specific immune responses in standard immunoassays. A benefit of this approach is the ability to direct a specific immune response to anticipated regions or epitopes for extending the protective response whilst excluding undesirable responses. Another advantage is the production of a response with an extensive broad-range of antigen coverage maintained under correct protein expression and production (Richards and Beeson, 2009). Individual DBLβ domains were used rather than the full-length PfEMP1 protein to map the minimal receptor-adhesion parts for ICAM-1 binding parasites and to understand the functional requirements for receptor adhesion. The specific aims of the different chapters were as follows:

Chapter 2

- Study the immune response of serum samples obtained from mice immunised with recombinant PfEMP1\textsuperscript{ICAM-1-DBLβ} domains expressed as hexahistidine-tagged proteins in \textit{Escherichia coli} (\textit{E. coli}). This was crucial since seroreactivity is an important indication for the efficiency of immunisation. In addition, it reflects the specificity of immune response towards immunising antigens.

- Develop mouse monoclonal and polyclonal antibodies against these DBL domains, and characterise the reactivity of these antibodies against corresponding homologous and heterologous domains. MAbs and pAbs directed against various PfEMP1 domains have been suggested as valuable tools for recognising critical functional sites on PfEMP1 involved in parasite adhesion and determining the cross-reactivity with parasite strains from
irrelevant regions (Lekana Douki et al., 2002). Development of reactive mAbs and pAbs secreted by immortal hybridoma lines is required for the production of therapeutic interventions. The limited amount of immune sera makes them only available for restricted preliminary screening assays.

**Chapter 3**

- Investigate the capacity of mAbs and pAbs to recognise VSAs on the surface of erythrocytes infected by ICAM-1-binding and non-binding *P. falciparum* isolates, and evaluate their cross-reactivity with parasite isolates from different genomes. VSAs are implicated in the cytoadhesion of *P. falciparum*-IEs in the microvessels of various organs, a key pathological feature of severe malaria disease (Berendt et al., 1994). Several studies have provided evidence that these VSAs are important targets of naturally acquired immunity, and antibodies directed against these highly immunogenic antigens are associated with protection against homologous parasite isolates (Giha et al., 2000, Bull et al., 1998).
  - Identify the surface epitope(s) labelled by mouse mAbs and pAbs.
  - Localise the surface labelling of mouse IgM antibodies using immunofluorescence assays.

**Chapter 4**

- Evaluate the capacity of mouse mAbs and pAbs to block the adhesion of IEs to immobilised receptors (ICAM-1 or CD36) or primary HUVEC under static or physiological flow conditions. MAbs directed against varied adhesive domains of PfEMP1 may have a therapeutic value in reducing the severity of malaria disease. Antibody-mediated adhesion-blocking have been demonstrated not only decrease the capacity of IEs to sequester, but also to play an important role in the elimination of IEs from the circulation by the host spleen and reduce the damaging inflammatory responses in vital organs (Rowe et al., 2009, Salanti et al., 2010). In addition to their adhesion-inhibition role, these antibodies seem to opsonise IEs and induce phagocytosis (Ghumra et al., 2008, Ghumra et al., 2011).
Chapter 5

- Characterise the functional antibody response of eight serum samples obtained from adult female donors living in Junju sub-location, Kilifi County, Kenya against a panel of DBL domains.
- Investigate the reactivity of semi-immune sera against the surface of live erythrocytes infected by diverse human lab-adapted P. falciparum isolates.
- Assess the ability of semi-immune sera to inhibit the adhesion of IEs to immobilised receptors (ICAM-1 or CD36) or HUVEC under static or physiological flow conditions.
- Investigate the presence of IgG antibodies that have the capacity to agglutinate IEs.
Chapter 2

Production and characterisation of monoclonal and polyclonal antibodies raised to PfEMP1$^{\text{ICAM-1-DBL}\beta}$ domains
Chapter 2: Introduction

During the intra-erythrocytic stage of *P. falciparum* development, several parasite-derived antigens are synthesised and exported to the surface of IEs (Leech et al., 1984). These VSAs are extremely polymorphic, highly immunogenic in nature and induce an immune response that targets IEs for providing protective immunity against malaria (Bull et al., 1998, Marsh et al., 1989). The most extensively characterised VSA is PfEMP1, a highly polymorphic protein associated with cytoadherence of parasitized erythrocytes to various host receptors (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995). The modular PfEMP1 consists of several DBL and CIDR domains that mediate parasite cytoadhesion (Rask et al., 2010). The second class of DBL domains, DBLβ, has been associated with adhesion to ICAM-1 receptors (Smith et al., 2000a, Oleinikov et al., 2009, Howell et al., 2008, Brown et al., 2013). A diverse array of human cell receptors have been shown to mediate *P. falciparum*-IE cytoadhesion (reviewed in Rowe et al., 2009) including ICAM-1 (Berendt et al., 1989), which implicated in the pathogenesis of CM.

One of the current approaches to overcome the diversity of PfEMP1 domains in the development of vaccines and therapeutic interventions is targeting PfEMP1 subdomains involved in parasite cytoadhesion (Gratepanche et al., 2003). Recombinant purified proteins for variable PfEMP1 domains have been synthesised and used for the induction of specific antibody responses in standard immunoassays. An advantage of this approach is the capacity to direct a specific immune response to a desired region or epitope for extending the protective response whilst excluding unwanted responses. Another advantage is the production of a response with broad-range of antigen coverage maintained under correct protein expression and production (Richards and Beeson, 2009). However, due to the large size of PfEMP1, expression of the full-length recombinant proteins is challenging (Beeson et al., 2013), and therefore only single domains or combinations of two or more domains have been mainly designed and used for the induction of antibody response. Even the expression of several single domains of PfEMP1 is difficult because of their conformational nature and/or large size. Some of these domains require particular adjuvants and
there is much doubt about choosing the accurate adjuvant-protein combination to induce a response with the right fine specificity and constant duration (Richards and Beeson, 2009). Recognition of functional domains that have the potential to contribute in protection against severe disease, obtainment of the correct folding and presentation of recombinant domains, and the likelihood that these domains would induce a specific immune response are further challenges for the production of specific antibody response against PfEMP1 domains (Richards and Beeson, 2009, Beeson et al., 2013).

In this chapter, we selected four recombinant PfEMP1ICAM-1-DBLβ domains that were expressed as hexahistidine-tagged proteins in E. coli for mouse immunisations. All domains have been shown to bind ICAM-1 receptors (Kraemer et al., 2007, Howell et al., 2008). Individual DBLβ domains were used rather than the full-length PfEMP1 protein to map the minimal receptor-binding regions for ICAM-1 binding isolates and to understand the functional requirements for receptor adhesion. We present evidence that it is possible to elicit mAbs and pAbs in mice by immunisation with single recombinant DBLβ domains from ICAM-1-binding variants. MAbs and pAbs comprising reactive antibodies recognised homologous and heterologous DBLβ domains and labelled the surface of erythrocytes infected by P. falciparum isolates. To our knowledge, this is the first study to examine the use of PfEMP1ICAM-1-DBLβ domains for the development of mAbs and pAbs that recognise homologous and heterologous parasite isolates and block IE adhesion.
2. Materials and Methods

2.1 Recombinant Duffy binding-like β (rDBLβ) domains

Recombinant DBL13, DBL27, DBL31 and DBL41 domains used in this work were obtained from Matthew Higgins, Oxford. These particular domains have been chosen for the present work because they are amongst the limited number of expressed DBLβ domains that mediate ICAM-1 binding (see Howell et al., 2008). Expression and purification of recombinant domains are described in detail elsewhere (Brown et al., 2013). DBLβ domains of corresponding PfEMP1 variants (IT4VAR13, IT4VAR27, IT4VAR31 and IT4VAR41) were cloned into modified pET15b vector, and the His-tagged proteins were expressed in *E. coli* Origami B cells. Cells were lysed and the proteins were purified using nickel-nitrilotriacetic acid-Sepharose (Qiagen) and size-exclusion chromatography.

2.2 SDS-PAGE and Western blotting for characterising recombinant DBL domains

2.2.1 Gel electrophoresis

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out to characterise recombinant PfEMP1ICAM-1-DBLβ proteins (rDBL13, rDBL27, rDBL31 and rDBL41) used for mouse immunisations. Each protein was adjusted to a concentration of 5µg/20µl PBS and diluted in NuPAGE® LDS sample buffer at 1:4 ratio (Novex®, 4×, Cat. No. NP0007) and Nuclease Free Water (NFW). Proteins were fully denatured using DL-dithiothreitol solution (DTT) reducing agent at a ratio of 1:10 relative to total sample volume (Thermo Scientific, R0861). 4-12% polyacrylamide NuPAGE® Novex® Bis-Tris pre-cast gels (Novex®, Life Technologies, NP0322PK2) were washed with distilled water and assembled in electrophoresis chambers following manufacturer’s instructions. The chambers were filled with 1×NuPAGE® MOPS/SDS Running buffer (20×, Novex®, Life Technologies, ,
NP0001) for resolving medium to large sized proteins (14 kDa to 200 kDa). Protein samples were heated on a heating block for ~3 minutes at 90°C and 16 µl of each sample was loaded into wells of acrylamide gel. A protein ladder of known molecular weights (SeeBlue® Plus2 Pre-stained Protein standard, Novex®, Life Technologies, LC5925) was also loaded on the gel to recognise molecular masses of loaded proteins. The chamber was connected to a power supply and an electrical current of 200 volts was run for 45 minutes. Negatively-charged proteins migrated across the gel from the negative cathode to positive anode and separated according to their molecular weights. Following electrophoresis, gels were either stained with Coomassie blue stain (PageBlue® protein stain, (Thermo Scientific®) or prepared for electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes for immunoblotting.

2.2.2 Coomassie blue staining

Staining of the gel was carried out to visualise all proteins in the sample and to evaluate the efficiency of the SDS-PAGE by ensuring that proteins have migrated evenly and uniformly. The gel to be stained was thoroughly washed in distilled water (×3, 5 minutes each) to remove excess SDS and incubated with PageBlue® protein staining solution (Thermo Scientific®, No. 24620) with gentle agitation for 30 minutes. The gel can additionally be microwaved for 30-60 seconds with gentle agitation to accelerate staining with the same equivalent sensitivity. PageBlue® contains Coomassie G-250 dye that sensitively stains proteins on polyacrylamide gels. The gel was then washed overnight to remove excess stain and stained proteins visualised on a light box. Naïve mouse serum (NMS) was included as an indicator for correct reduction of proteins; as reduced NMS shows two bands (55 kDa for the heavy chain and 26 kDa for the light chain).
2.2.3 Immunoblotting of recombinant DBLβ domains with antipolyhistidine antibodies

In Western blotting, electrophoresed proteins are transferred from the gel to a membrane to make proteins accessible for immunological identification by antibodies. This method provides details about the molecular mass of proteins, abundance and heterogeneity. Six pieces of Whatman® filter papers (Sigma-Aldrich®, diam. 30 mm, product no. Z695033) and three pieces of 0.45 µm PVDF membranes (Whatman® Westran®, Sigma-Aldrich®, Z671061) were cut to exact size of polyacrylamide gels (75 × 90 mm). The PVDF membranes were pre-soaked in 100% methanol for 2 minutes and equilibrated in ×1 working solution of NuPAGE® transfer buffer (Novex®, Life Technologies, NP0006-1). Filter papers as well as blotting pads were also soaked in transfer buffer. The electrotransfer unit was prepared by placing two pre-soaked pads on the sandwich cassette, followed by a piece of filter paper and a PVDF membrane. The electrophoresed gel was gently removed and placed over the PVDF membrane followed by a piece of filter paper and two blotting pads above the membrane. All sheets were aligned exactly on top of each other and trapped air bubbles squeezed out to ensure that proteins transferred properly. The sandwich was placed in an electrotransfer chamber pre-filled with transfer buffer and an electrical current of 100 volts was run for 60-65 minutes for transferring proteins from the gel to the PVDF membrane.

After transfer, non-specific binding sites in the membrane were blocked by incubating the membrane in 1× PBS containing 0.1% Tween® 20 detergent (Sigma-Aldrich®, P1379) and 5% non-fat milk powder (PBSTM). After 2 hours of blocking, PVDF membranes were washed three times in 1× PBS containing 0.1% Tween20 (PBST) and probed with 1:200 diluted HRP-conjugated antipolyhistidine monoclonal antibodies (clone HIS-1, Sigma-Aldrich®, A7058) for 120 minutes with gentle shaking at room temperature. The antibody recognises native or denatured proteins tagged with polyhistidine in the sample. It was conjugated with HRP and thus was detected without use of a secondary detecting antibody. Membranes were then washed three times in PBST and bands were resolved by incubating the membranes with developing solution including 1× PBS, 0.02% hydrogen peroxide [H₂O₂] (Sigma, lot number 082
K3250) and 0.005% 3,3-Diaminobenzidine HCL substrate [DAB] (Millipore, NG16 16211). The substrate reaction proceeded for 5-10 minutes before the reaction was blocked by washing membranes with distilled water and the blots were dried and analysed. NMS that lacks the polyhistidine tag was included as a negative control.

### 2.2.4 Immunoblotting of recombinant DBLβ domains with mouse antisera

In this assay, recombinant DBLβ domains were loaded onto 4-12% polyacrylamide NuPAGE® gels and transferred to six separate PVDF membranes following the same procedure described in the previous section (2.2.3). An additional recombinant protein (VAR2CSA-DBL4ε) was included in the blots as a negative control. After transferring proteins, PVDF membranes were blocked in PBSTM. After 2 hours of blocking, PVDF membranes were washed three times in PBST washing buffer and four blots probed with each of the mouse antisera (anti-DBL13, anti-DBL27, anti-DBL31 and anti-DBL41), a blot probed by HRP-conjugated anti-polyhistidine mAbs (clone HIS-1, Sigma-Aldrich®, A7058) and the sixth blot was probed with NMS for 120 minutes with gentle shaking at room temperature. Immune and control sera were used at 1:250 dilution (sera: PBST) and anti-polyhistidine antibodies at 1:250 dilution. The membranes were then washed three times in PBST and binding of primary antibodies in immune or control sera was detected by incubation with goat anti-mouse IgG (Fc-specific)-HRP conjugated antibodies (Sigma-Aldrich®, product No. A2554) at a dilution of 1:500 (antibody: PBST). The membranes were incubated for 120 minutes at room temperature with gentle shaking before they were washed and developed as described in the previous section (2.2.3).

### 2.3 Mouse immunisation

In the present work, two groups of mice were immunised by His-tagged recombinant domains (rDBL13, rDBL27, rDBL31 and rDBL41) and stimulation of the immune response was carried out using two different adjuvants. Immunisation of the first group of animals was carried out at Severn Biotech
Ltd® (Worcestershire, United Kingdom) using Freund's complete/incomplete adjuvant (FCA) for immune stimulation. Briefly, a group of eight Balb/c mice (two per protein) were injected subcutaneously with 100 µg/ml of each of the four recombinant proteins (100 µl) emulsified in an equal volume of FCA. The initial injection was followed by three subsequent subcutaneous injections at one-week intervals with the same amount of antigens emulsified in Freund's incomplete adjuvant for immune boosting. One week after the final booster injection, venous blood was collected from mice and immune sera were tested for the presence of reactive antibodies by ELISA, immunoblotting and flow cytometry. Spleens were gently extracted and preserved in pre-warmed FBS-free complete medium (CMNS) for hybridoma production. Two mice were immunised and boosted with an emulsion of PBS/FCA following the same procedure and their sera were used as negative controls.

The second immunisation was carried out by Professor Richard Pleass (Liverpool School of Tropical Medicine, Liverpool) using Aluminium salts (Alum) (Sigma) as an adjuvant following a slightly different protocol. Eight Balb/c mice (2 per antigen) were inoculated with 50 µg of recombinant proteins emulsified in an equal volume of Alum. The initial immunisation was followed by two intermittent subcutaneous injections with 50 µg of recombinant proteins in Alum at 2-week intervals for stimulating the immune response. Mouse antisera and spleens were collected three days post final injection and used immediately for immortalization. Two mice were immunised with PBS/Alum and used as controls.

2.4 Characterisation of mouse antisera

2.4.1 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

2.4.1.1 Screening mouse antisera by preliminary ELISA

Recognition of immunising DBL domains by antisera from mice stimulated with FCA or Alum adjuvants was assessed by indirect ELSIA. The first assay was carried out to screen mouse antisera for the presence of IgG-class antibodies that recognise homologous and heterologous domains, as described by Hornbeck et
al. (1991) with slight modifications. Briefly, 96-well MaxiSorb microtitre polystyrene plates (Nunc®) were coated with 100 µl of recombinant domains (rDBL13, rDBL27, rDBL31 and rDBL41) dissolved in 0.1 M carbonate bicarbonate buffer at 1 µg/ml and incubated overnight at 4°C. Unbound antigens were removed by washing with PBST (×3) and free residual binding sites were blocked in PBSTM for 2 hours at room temperature. Plates were washed as previous and incubated with mouse immune sera (anti-DBL13, anti-DBL27, anti-DBL31 and anti-DBL41) diluted 1:500 in duplicates for 2 hours at room temperature with gentle agitation. NMS at 1:500 dilution or mouse mAb BC6 IgG antibody (20 µg/ml) were also incubated with recombinant antigens and set as negative controls. After incubation, wells were washed as previous and incubated at room temperature for 1 hour with 100 µl of goat anti-mouse IgG (Fc-specific)-HRP conjugated (Sigma-Aldrich®, product No. A2554) diluted 1:500 in complete medium. Wells were washed again and the reaction was developed by adding 100 µl of substrate 3,3′,5′,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich®, product No. T3405) diluted in phosphate-citrate buffer with Sodium Perborate (Sigma-Aldrich, product No. P4922) according to manufacturer instructions. Plates were incubated for approximately 10 minutes in the dark at room temperature before the reaction was blocked upon colour development by adding 50 µl of 2M H₂SO₄ to the wells. Optical densities (OD) were measured using an ELISA plate reader with a 450-nm reference filter. OD values are shown as the average of duplicate wells with standard deviation (SD) expressed as error bars. The results were considered positive if the mean OD value obtained from immune sera was above the cut-off point set at 3 SDs above the mean background absorbance (OD) of NMS. Capacity of recognition was set as a grade from + to +++++ as shown in the Results section.

The second indirect ELISA was carried out following the same procedure but immune-detection was performed by incubating antisera-antigen complex with goat anti-mouse IgM (µ chain specific)-HRP conjugated (Southern Biotech, Cat. No. 1021-05) for detecting the presence of IgM reactive antibodies in tested immune sera. Developing and blocking were carried out as described above.
2.4.1.2 Checkerboard titration ELISA

Indirect titration ELISAs were carried out to evaluate the antibody titre of mouse polyclonal antisera using serial dilutions of immune sera against different concentrations of recombinant DBL proteins. The same protocol was followed as the previous ELISA (section 2.4.1.1) with some modifications. Briefly, 96-well polystyrene plates were coated with recombinant domains (DBL13, DBL27, DBL31, DBL41 and DBL4ε) at different concentrations (1 µg/ml, 0.5 µg/ml, 0.1 µg/ml and 0.01 µg/ml) and incubated overnight at 4°C. Plates were washed and blocked as described previously, and wells were incubated with polyclonal antisera diluted 1:500, 1:1,000, 1:5,000, 1:10,000, 1:50,000 and 1:100,000 in complete medium and incubated overnight at room temperature with gentle shaking. Plates were then washed and incubated with goat anti-mouse IgG (Fc-specific)-HRP conjugated (Sigma-Aldrich®, product No. A2554). Subsequent washing, developing and blocking were carried out as described in section 2.4.1.1. Incubation of polyclonal antisera with VAR2CSA-DBL4ε was included as a negative control. The results were considered positive if the mean absorbance (OD) value obtained from a particular immune serum was 3 SDs above the mean absorbance value of that antiserum against control DBL4ε domain. Capacity of recognition were set as a grade from + to +++++ as shown in the Results section.

2.4.2 Analysis of mouse antisera by flow cytometry

2.4.2.1 Labelling of IEs by antisera

The ability of mouse polyclonal antisera to recognise the surface of erythrocytes infected by homologous and heterologous *P. falciparum* isolates was assessed by flow cytometry. Briefly, *P. falciparum* parasites (A4, C24, GC503, 2B2, ItG, 2F6 and outgrown A4) were cultured and maintained following standard laboratory conditions. Mature pigmented trophozoites at mid-late stage were enriched from stable parasite cultures at 3-8% parasitaemia (i.e. 3-8 parasites per 100 erythrocytes) and a haematocrit (HCT) of 1-2%, using plasmagel floatation (Pasvol et al., 1978). 60-80% of mid-late trophozoites and schizonts were resolved from the enrichment, and 3 µl of the enriched pellet was added to 1 ml
of PBS with 1% bovine serum albumin (PBS/1%BSA) to prepare IE suspension. Aliquots of the suspension (100 µl, 0.8-1 × 10⁶ cells) were washed in 1.5 ml microfuge tubes and the pellet was re-suspended with mouse immune or control sera at 1:50 dilution and incubated for 60 minutes at 37°C. After incubation, cells were washed and bound IgM or IgG antibodies were detected by incubation with 1:100 dilution of APC-conjugated goat anti-mouse IgM (µ) secondary antibody (Molecular probes®, Cat. No. M31505) or goat anti-mouse IgG (Fc)-APC conjugated (Thermo Scientific, Product No. 31981), respectively, plus 10 µg/ml ethidium bromide for staining nuclei of IEs. Cells were incubated in the dark for 60 minutes at 37°C. After incubation, cells were washed once, re-suspended with 400 µl of Cell Wash and analysed using Becton-Dickinson FACSCalibur flow cytometer (BD LSRII). All washes and antibody dilutions were carried out in PBS/1%BSA. Cells incubated with NMS and stained with ethidium bromide and secondary antibodies or those only incubated with secondary antibodies and ethidium bromide were included as negative controls. Labelling of A4-infected erythrocytes with mAb BC6 (20 µg/ml), which recognises an exposed epitope on the native PfEMP1 on A4-IE surface, was included as a positive control. Labelling of uninfected cells with mouse IgG or IgM antibodies was found at a range of 1-3%, indicating that labelling is specific for IEs. Differences in the binding capacity of polyclonal sera against tested isolates were further confirmed by repeating experiments.

2.4.2.2 Data analysis

Data were acquired using FACSCalibur flow cytometer and 50,000 events were collected and analysed with FlowJo software (X 10.0, San Carlos, CA, USA). Gating on FSC-A vs. SSC-A parameters was carried out to identify RBC populations and to exclude cell debris and dead cells, whereas gating on a SSC-A vs. FITC-A was performed to differentiate infected RBC population (ethidium bromide positive) from uninfected erythrocytes. After gating on IEs, APC-positive cells were gated to show IgM or IgG-labelled IE population. This was firstly carried out with a negative control sample (cells incubated with NMS) to define APC-positive / APC-negative subsets and to exclude background labelling, then the same subset was applied for test antibodies. Thus, specific surface staining for IEs was determined in terms of percentage of APC-positive
IE population labelled by test antibodies relative to control antibody. A second negative control was included for some experiments (cells only incubated with secondary antibody plus ethidium bromide) to confirm the lack of reactivity for control antibody (NMS). In all assays, a labelling of > 10% of IE population was considered positive for surface labelling, whereas ≤ 10% was considered negative. Grading of capacity of surface recognition was set as followed:

+: if labelled IE population was within a range of 10.1-20%
++: if labelled IE population was within a range of 20.1-40%
+++: if labelled IE population was within a range of 40.1-60%
++++: if labelled IE population was > 60%.

2.5 Production and characterisation of mouse monoclonal and polyclonal antibodies

2.5.1 Hybridoma cell production

2.5.1.1 SP2/0 myeloma cell line culture

SP2/0 murine myeloma cell lines were recovered from liquid nitrogen (LiN$_2$) storage and cultured 10-15 days in advance prior to fusion with splenocytes and maintained in a log phase of growth as described elsewhere (Fuller et al., 1988a), with minor modifications. SP2/0 cells were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin and one unit of 8-azaguanine (at 20 µg/ml). Myeloma cells were cultured in a medium containing 8-azaguanine to ensure their aminopterin sensitivity to Hypoxanthine Aminopterin Thymidine (HAT) selection medium following the fusion with splenocytes (Fuller et al., 1988a). Cells were cultured in T75 (75 cm$^2$) ventilated culture flasks at 37°C in CO$_2$ incubator (9% CO$_2$) and split in a 1/4 ratio every 2-3 days to maintain a log-phase growth at the appropriate exponential rate and avoid decline in cell viability. Prior to fusion with spleen cells, SP2/0 cells were checked for viability and maintained at ~10$^6$-10$^7$ cells/ml. In addition, morphology and potential contamination were examined using inverted microscope for obtaining a successful fusion. SP2/0 myeloma cells at appropriate log-phase growth (~10$^6$ cells/ml) were frozen in 10% dimethyl sulphoxide in FBS (10%DMSO/FBS) in LiN$_2$ for further use.
2.5.1.2 Extraction and preparation of spleen cells

Spleens from immunised mice were dissected out aseptically and placed in pre-warmed CMNS within the animal facility. Two spleens obtained from mice immunised with the same antigen were placed in a 100-mm sterile Petri dish containing 10 ml of pre-warmed CMNS for extracting splenocytes inside a sterile hood. Spleens were gently crushed using a sterile syringe plunger and a needle into tiny pieces (1 mm in diameter). The suspension was collected in 50-ml falcon tubes and unwanted debris were allowed to sediment down by gravity at room temperature for 3-6 minutes. The upper 95% of suspension containing harvested spleen cells was cautiously collected and re-suspended in CMNS. Splenocytes were washed three times in CMNS, counted using haemocytometer chamber and prepared for fusion.

2.5.1.3 Fusion of splenocytes with SP2/0 myeloma cells

Freshly extracted spleen cells and SP2/0 myeloma cells were co-pelleted by centrifugation and fused using QED Fuse-It® Hybridoma development kit (QED Bioscience Inc., product No. K50000) following manufacturer's instructions. The fusion protocol was carried out as described elsewhere (Fuller et al., 1988b). SP2/0 myeloma cells from stable cultures were detached by agitation and centrifuged at 1200 rounds per minute (rpm) for 5 minutes. The pellet was washed three times in CMNS to remove serum components and the concentration of cells was adjusted to $1 \times 10^6$ cells/ml. Splenocytes and SP2/0 cells were combined at a ratio of 5:1 (splenocytes to SP2/0 cells) in sterile 50-ml falcon tube.

Cells were thoroughly mixed and co-pelleted at 1000 rpm for 5 minutes before the supernatant was discarded and the cell mixture was left at a drying state. Polyethylene glycol (PEG) fusion reagent was prepared as per manufacturer's instructions and 1 ml was added to the cell mixture drop-wise over a 30-second period with gentle agitation. This was followed by the addition of 1 ml CMNS at a drop-wise manner over 30 seconds to dilute the PEG and further 10 ml of CMNS were dropped over a period of 3 minutes. The former dilutions were carried out with gentle agitation throughout the procedure. Cells were
centrifuged at 1000 rpm for 5 minutes, the supernatant containing traces of PEG was extracted and the pellet was re-suspended at a concentration of $1.5 \times 10^6$ cells/ml in cloning medium (DMEM medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 10% BriClone hybridoma cloning medium (Qed Bioscience Inc., Product No. BRI10000) supplemented with one unit of HAT. As the total number of cells resulted from the fusion was $60 \times 10^6$ cells, this was re-suspended in 40 ml cloning medium to yield a concentration of $1.5 \times 10^6$ cells/ml. 200 µl of the cell suspension was aliquoted into 96-well plates (~200 wells, $3 \times 10^5$ cells/well). Plates were incubated at 37°C in CO2 incubator for at least 3 days. On the fourth day, wells were inspected for colony growth, and 50 µl of fresh cloning medium was added to wells with signs of colony growth (Figure 2.1). Wells were checked for the presence of a milky cloudy aggregation of cells at the base of each well that indicates hybridoma formation. However, cell clumping may also indicate the presence of SP2/0 aggregates; therefore, hybridomas were inspected under inverted microscope and differentiated from SP2/0 aggregates by the size and morphology of cells (Figure 2.1). Wells were inspected at one-day intervals and growing hybridoma clones at 10-50% confluence were assayed with ELISA for the production of antigen-specific antibodies. Hybridoma cell lines were diluted and expanded gradually from 96-well plates to 24-well plates, 6-well plates, 25 cm$^2$ and 75 cm$^2$ culture flasks containing appropriate volumes of cloning medium.

Figure 2.1. Growing hybridoma colonies as inspected under inverted microscope.
2.5.2 Indirect ELISA for screening hybridoma clones

Multiple screening ELISAs were carried out to investigate the presence of reactive IgG or IgM antibodies in culture supernatants of confluent hybridoma cell lines in 96-well plates. Reactivity was checked against recombinant DBL domains following the same ELISA protocol described in section 2.4.1.1 with some modifications. Briefly, 96-well ELISA plates (Nunc) were coated with 100 µl of recombinant domains (rDBL13, rDBL27, rDBL31 and rDBL41) dissolved in 0.1 M carbonate bicarbonate buffer at 1 µg/ml and incubated overnight at 4°C. Washing off unbound antigens and blocking were carried out as described section 2.4.1.1. Plates were washed and incubated with 50 µl from the supernatant of confluent hybridoma cell line in duplicates for 2 hours at room temperature with gentle agitation. After incubation, wells were washed as previous and incubated at room temperature for 1 hour with 100 µl of 1:500 diluted goat anti-mouse IgG (Fc-specific)-HRP conjugated antibody (Sigma-Aldrich©, product No. A2554) for the detection of reactive IgG or with goat anti-mouse IgM (µ chain specific)-HRP conjugated (Southern Biotech, Cat. No. 1021-05) for the detection of reactive IgM antibodies in culture supernatants. Following secondary antibody incubation, assays were completed as described in section 2.4.1.1. Wells coated with recombinant antigens and incubated with complete medium were used as negative controls. Positive hybridomas were then either cloned by limiting dilution or expanded and assayed as polyclonal antibodies.

2.5.3 Cloning of hybridoma clones by limiting dilution

Hybridoma cell lines that showed positive reactivity with ELISA were further cloned by limiting dilution at 0.8 cells/well to produce monoclonal cell lines that secrete monoclonal antibodies, following a protocol described by Fuller et al., (1988c). Hybridoma cell lines to be cloned were transferred to 24-well culture plates containing 0.5 ml cloning medium and cultured to log-phase of growth overnight at 37°C. Each confluent line was counted with a haemocytometer and transferred to 6-well plates at 1:100 dilution in 3 ml in the first well, 80 cells/ml in the second well and 8 cells/ml in the third well. Then, cells at 80 cells/ml and
8 cells/ml were aliquoted in 96-well plates in cloning medium and incubated at 37°C. Wells containing 0.8 cells/well were inspected for growth and the resulted monoclonal colonies were scanned by ELISA for reactivity with recombinant domains as described in section 2.4.2. Positive clones were further cultured and maintained for further study.

2.5.4 Freezing and recovery of Hybridoma clones

Confluent hybridoma cell lines at a log-phase growth at ~10^6 cells/ml were resuspended in 10%DMSO/FBS to a final concentration of 5 × 10^6 cells/cryovial. Vials were rapidly transferred to a freezing chamber containing isopropanol and frozen overnight at -70°C. On the following day, vials were transferred to liquid nitrogen, the method of choice for long-term storage of hybridoma clones that ensures the endurance of antibody production in frozen aliquots (Fuller et al., 1988d). Hybridoma cell lines to be recovered post-freezing were thawed in 37°C water bath, washed in CMNS to remove traces of DMSO, re-suspended in an appropriate volume of cloning medium and incubated at 37°C.

Over-confluent growth of cells in expanded cultures was suspended by the addition sodium azide (0.02% w/v). Culture flasks containing dead cells were firstly centrifuged at 1200 rpm for 5 minutes, the supernatant was collected and filtered through 0.2 µm pore-size filters to ensure the removal of dead cells, and recovered culture supernatant containing reactive antibodies was stored at 4°C. A total of 300 ml of culture supernatant was collected from each hybridoma cell line and preserved for further study. Further ELISAs were carried out at this point to confirm positivity for antigen (section 2.4.1.1) following the same procedure described in section 2.5.2 but with the addition of 200 µl of hybridoma culture supernatants (preserved in 0.02% w/v sodium azide) to wells pre-coated with recombinant domains at 5 µg/ml. Although there was no clear difference in the capacity of antibody recognition to wells coated with 1 µg/ml or 5 µg/ml (data not shown), the latter concentration was used in all subsequent ELISA procedures. Mouse polyclonal antisera diluted 1:50 dilution were assayed as positive controls, whereas mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®, M5909, 200µg/ml) was included as a negative control (20 µg/ml).
2.6 Affinity purification of monoclonal and polyclonal antibodies

A total of thirteen monoclonal and polyclonal hybridoma cell lines that secrete reactive murine IgM antibodies were affinity-purified from stable culture supernatants using manual gravity flow column affinity chromatography. First, the column was packed with 5 ml of goat anti-mouse IgM (µ-chain specific) coupled to agarose beads (Sigma-Aldrich®, product No. A4540) and washed with 5-6 column volumes of Hank's balanced salt solution (HBSS) (Gibco®, Life Technologies, Cat. No. 14175-095). Then, culture supernatants (300 ml) were run through the resin of the column at a low flow rate (~0.5-1 ml/min). The sample was re-circulated 2-3 times to increase the potential of retaining larger amounts of functional antibodies from culture supernatants. The column was washed with HBSS as previously to remove contaminants that bind with low affinity, before the lower end of the column was capped and captured antibodies were eluted from agarose beads by adding 0.1 M glycine pH 2.5 to the suspension with gentle agitation for 2-3 minutes. Seven fractions (~2 ml) of affinity purified antibodies were then collected in 15 ml falcon tubes containing appropriate volume (240-260 µl) of 1 M Tris-HCl pH 9.0 for neutralising the pH of the eluted fraction. The pH of eluted fractions was then checked to confirm pH of 7.0-8.0. The antibody-depleted flow-through fluid was stored at 4°C for further study to confirm the absence of antigen-specific antibodies by ELISA. Purified antibodies were stored in aliquots (100 µl) at -20°C for further study. The column was then washed with 2-3 column volumes of 0.1 M glycine pH 2.5, then neutralised with 7-8 column volumes of HBSS and stored at 4°C. For long-term storage, the column was filled with 20% ethanol.
2.7 Screening eluted fractions by flow cytometry

All eluted fractions were screened by flow cytometry for the presence of antibodies that label the surface of live IEs. This was to identify fraction(s) containing the highest amount of reactive antibodies for further study. Erythrocytes infected by ItG *P. falciparum* parasites (IT4var16) were assayed as described in section 2.4.2.1 with some modifications. Briefly, aliquots of parasite suspension (100 μl, ~μl 0.8-1 × 10^6 cells) were washed and the pellet was re-suspended with 50 μl of each of the eluted fractions and incubated for 60 minutes at 37°C. The pellet of cells was then washed and incubated with 1:100 dilution of APC-conjugated goat anti-mouse IgM (μ chain specific) secondary antibody (Southern Biotech®, Cat. No. 1020-11L, 0.5mg) plus 10 μg/ml ethidium bromide for 60 minutes at 37°C. After incubation, cells were washed and analysed as described in section 2.4.2.2. Cells incubated with 20 μg/ml of mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®, M5909, 200 μg/ml) and stained with ethidium bromide and secondary antibodies were used to exclude background labelling (1-9% of IE population) and included as a negative control. A labelling of > 10% of IE population was considered positive.

2.8 ELISA for investigating the efficiency of affinity purification

The recovery of murine IgM from hybridoma culture supernatants by affinity purification was assessed by ELISA using either cultures supernatants, purified eluted fractions and flow-through medium. A similar procedure was carried out as described in section 2.5.2 with some modifications. Briefly, 96-well ELISA plates (Nunc) were coated with 100 μl of rDBL31 proteins dissolved in 0.1 M carbonate bicarbonate buffer at 5 μg/ml and incubated overnight at 4°C. Washing off unbound antigens and blocking were carried out as described section 2.4.1.1. Plates were washed and incubated with 200 μl of crude culture supernatant, 200 μl of flow-through fluid or 50 μl of purified antibodies in duplicates for 2 hours at room temperature with gentle agitation. Purified
antibody fraction that showed the highest reactivity in flow cytometry was used in this assay. After incubation, wells were washed as previously and incubated at room temperature for 1 hour with 1:500 dilution of goat anti-mouse IgM (µ chain specific)-HRP conjugated [Southern Biotech, Cat. No. 1021-05] (100 µl). Subsequent washing and developing was carried out as described in section 2.4.1.1. Incubation of 100 µl of complete medium or 100 µl of 20 µg/ml mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®, M5909, 200µg/ml) with recombinant proteins were included in the assay as negative controls, whereas anti-DBL31 mouse serum (50 µl, 1:10 dilution) was used as a positive control.

2.9 Dot blot for estimating the concentration of IgM in eluted fractions

IgM concentration of eluted fractions was estimated semi-quantitatively in comparison with mouse IgM control standard spotted down at gradual concentrations onto a nitrocellulose membrane. Briefly, 5 µl of each eluted fraction was spotted manually onto Whatman® Protran® Nitrocellulose membrane (Whatman® GmbH, Germany). Mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®, M5909, 200 µg/ml) was titrated at doubling dilutions from 200 µg/ml down to 0.2 µg/ml in glycine/Trizma-HCL pH 7.0 buffer and spotted onto the membrane. Blots were left to dry at room temperature for 10-20 minutes, before the membrane was blocked with PBSTM at room temperature for 120 minutes with gentle agitation. The membrane was washed in distilled water and incubated with HRP-conjugated goat anti-mouse IgM (Southern Biotech®, Cat. No. 1021-05, Lot E5406-R690B) at a dilution of 1:500 overnight at room temperature. On the following day, the membrane was washed in distilled water and developed in 1xPBS/1%DAB/0.5%H2O2 solution for 10 minutes, before it was thoroughly washed for avoiding excess staining and imaged by Gel Doc™ EZ imager (Bio-Rad, Hercules, CA, USA) and analysed using Image Lab software version 3.0. 5 µl of complete medium or mAb BC6 at 20 µg/ml were spotted onto the membrane and used as negative controls.
2.10 Double-antibody sandwich ELISA for the quantitation of murine IgM

The concentration of IgM antibodies in purified fractions was quantified using double-antibody sandwich ELISA as described elsewhere (Mushens et al., 1993). Briefly, unlabelled µ-chain specific rat anti-mouse IgM (clone 1B4B1, Southern Biotech®, Cat. No. 1140-01, 0.5 mg/ml) was coated onto the plate at 5 µg/ml in 0.1 M carbonate bicarbonate buffer and incubated overnight at 4°C. The plate was washed three times with PBST and blocked in PBSTM for 60 minutes at room temperature. The plate was washed as previously and 100 µl of mAbs/pAbs diluted at 1:100 in complete medium were added to the pre-coated wells. Mouse IgM isotype positive control (clone MOPC 104E, Sigma®, M5909, 200 µg/ml) was titrated from 4000 to 0.12 ng/ml by doubling dilution in complete medium and added to the plate (100 µl in duplicate). The plate was incubated on the shaker for 120 min at room temperature, before it was thoroughly washed and incubated with HRP-conjugated goat anti-mouse IgM (Southern Biotech®, Cat. No. 1021-05) at 1:500 dilution for 60 min at RT. Wells were washed again and the reaction was developed by adding 100 µl of 3,3’5,5’-tetramethylbenzidine dihydrochloride diluted in phosphate-citrate buffer with Sodium Perborate and left for 10 - 15 minutes in the dark at room temperature. Finally, 50 µl of 2 M H2SO4 was added to block the reaction and the optical densities were measured using an ELISA plate reader with a 450-nm reference filter. Incubation of 100 µl of complete medium or 100 µl of 20 µg/ml of mAb BC6 with coated antibody were included in the assay as negative controls. After generating a standard dilution curve for the mean concentration and absorbance values of the mouse IgM standard, the unknown concentration of purified mAbs and pAbs was calculated using the excel function =TREND (all concentration values of standard, [all absorbance values of standard], [absorbance of unknown sample], [constant]).
2.11 Parasite culture

2.11.1 P. falciparum isolates

Characteristics of parasite isolates used in the present study are indicated in the results of Chapter 3 (Table 3.2). All parasites are subject to antigenic switching and thus were maintained for 1-3 weeks after selection to minimise the effect of mixed populations, since most isolates in continuous cultures lose the capacity to bind after a short period (~ 24-43 days), although some continue to bind after longer periods (Udeinya et al., 1983). ItG was selected on ICAM-1 and the expression of the corresponding var gene (var16) was confirmed in our laboratory by PCR. A4 was selected on mAb BC6 (Horrocks et al., 2002) followed by FACS analysis of the expressed PfEMP1 with the corresponding parasite isolate (A4).

2.11.2 Parasite culture

Parasites were cultured under standard conditions described elsewhere (Trager and Jensen, 1976). Blood-stage parasites were maintained in complete culture medium (RPMI 1640 medium supplemented with 37.5 mM HEPES, 7 mM glucose, 6 Mm NaOH, 25 mg/ml of gentamicin sulphate, 2 mM L-glutamine and 10% human serum) at 1-2% HCT in O+ human erythrocytes, pH of 7.2 and in a gas mixture of 96% nitrogen, 3% carbon dioxide and 1% oxygen. Cultures were checked every 48 hours and maintained at 2 – 10% parasitaemia by dilution in washed human RBCs with exchanging culture media and gassing of flasks. RBCs were obtained from the British transfusion services (Liverpool, UK) in bags lacking anti-coagulant and centrifuged at 3000 rpm for 20 minutes through Histopaque (Sigma) to discard plasma and leukocytes. Washed RBCs were resuspended in serum-free culture medium to a final HCT of 50% and stored at 4°C for 7-10 days.

2.11.3 Determination of parasitaemia

Parasitaemia and stage of growth were determined every alternate day by thin blood smears. 300-400 µl of culture was centrifuged in Eppendorf tubes for 30
seconds and the pellet was smeared onto a microscope slide, air-dried and fixed in 100% methanol for 5 seconds. Fixed smears were rinsed in water and stained with 5% Giemsa stain (in phosphate-buffered water, at pH 7.2) for 10-20 minutes. The slides were rinsed in water, air-dried and inspected under the light microscope using 100 × 10 (×1000) magnification under oil immersion objective lens. Five hundred (500) RBCs were counted and the number of parasites (rings or trophozoites) were counted relative to non-IEs.

2.11.4 Trophozoite enrichment - plasmagel floatation

This method is based on the capacity of knobby IEs to float on Plasmion, a physiological saline solution containing 3% gelatine. The culture to be synchronised was transferred to a 50 ml Falcon tube and centrifuged at 1800 rpm for 5 minutes. The pellet was resuspended in 1.5-volume of serum-free complete medium and 2.5-volume of Plasmion. The suspension was mixed, transferred to a 15 ml Falcon tube and allowed to settle for 20 minutes at 37°C. knobby trophozoites in the pink top layer were carefully transferred to a clean 15 ml Falcon tube, washed in serum-free complete medium (by centrifugation at 1800 rpm for 5 minutes) and used in assays or added in complete medium for continuous cultures. The efficiency of enrichment was assessed by counting the parasitaemia in a stained then smear to evaluate the percentage of trophozoites in the enriched pellet. Smears taken after this procedure usually showed ~50-80% of enriched trophozoites. During microscopic examination of enriched trophozoites, spontaneous agglutination was checked and found to be absent at higher trophozoite concentrations.

2.11.5 Selection of ItG-IEs on ICAM-1 protein

ItG-IEs were selected on ICAM-1 to increase the homogeneity of parasites for ICAM-1 binding phenotype. 50 µl of Protein A dynabeads (Invitrogen) were washed three times in 200 µl PBS/1%BSA using a magnet that recovers the beads with every wash, and resuspended in 200 µl of PBS/1%BSA after the last wash. 2.5 µg/ml of purified ICAM-1 protein was mixed with the bead suspension on a rotator for 60 minutes at room temperature. ICAM-1-coated
dynabeads were purified on the magnet and washed three times in 200 µl of PBS/1%BSA. Trophozoites enriched in Plasmion were incubated with ICAM-1-coated dynabeads and incubated on a rotator for 45 minutes at room temperature. Unbound IEs were removed by repeated washing (3×) in PBS/1%BSA. IE-bound beads were resuspended in fresh complete medium and cultured as described above.

2.11.6 Selection of A4-IEs on BC6

Selection of A4 (IT4var14)-IEs was carried out using mAb BC6, which recognises an exposed epitope on PfEMP1 of erythrocytes infected by A4 P. falciparum isolate (Roberts et al., 1992, Horrocks et al., 2002). A similar protocol to that used in ICAM-1 selection was followed except that Protein G dynabeads were used instead of Protein A dynabeads. Following ICAM-1 or BC6 selection, batches of selected parasite stabilates were cryopreserved to be used in subsequent work.

2.11.7 Cryopreservation of parasites

Parasites at 5-8% parasitaemia (ring stage) were centrifuged and the pellet was cryopreserved in glycerolyte freezing medium at a ratio of 5-volume freezing medium to 3-volume pellet. Addition of the cryopreservation medium was carried out in two steps. First, one-volume of the medium was added and the cells were allowed to stand for 5 minutes before the remaining 4-volumes were added slowly. The suspension was gently mixed and transferred to pre-labelled cryovials (500 µl per vial). Vials were kept in a polystyrene rack, wrapped with tissue papers and allowed to freeze slowly at -80°C overnight before they were transferred to liquid nitrogen for long-term storage.

2.11.8 Reconstitution of frozen parasites

Appropriate volumes of 12% NaCL, 1.8% NaCL, and 0.9% NaCL in 0.2% glucose, serum-free complete medium and complete medium were warmed at 37°C before the thawing starts. Frozen parasite stabilates were carefully removed from liquid nitrogen and allowed to thaw at room temperature. As soon as the stabilate was thawed, the 500 µl parasite contents was transferred to a 15
ml Falcon tube, resuspended in 100 µl of 12% NaCL and allowed to stand for 5 minutes at room temperature. Then, 5 ml 1.8% NaCL was slowly added over 5 minutes and the suspension was allowed to stand for 5 minutes. This was followed by the addition of 5 ml 0.9% NaCL over 5 minutes and allowed to stand for 5 minutes as previous. After that, the suspension was centrifuged at 1800 rpm for 5 minutes. The pellet was washed in serum-free complete medium, before washed parasites were resuspended appropriate volume of complete medium (8-10 ml) and cultured as described above.
2. Results

2.1 Analysis of recombinant DBLβ domains by SDS-PAGE and Western blotting

The four recombinant DBLβ domains used in this work were obtained from Matthew Higgins, Oxford (Brown et al., 2013). All domains, namely rDBL13, rDBL27, rDBL31 and rDBL41, were cloned from ICAM-1-binding PfEMP1 variants from *P. falciparum* (IT4 genotype) and expressed in *E. coli* vectors. SDS-PAGE was carried out for the separation and recognition of rDBLβ domains on 4-12% polyacrylamide gradient gels. Gels were stained with PageBlue™ Coomassie brilliant blue protein stain or electrophoretically transferred to PVDF membranes and probed with primary monoclonal anti-polyHistidine HRP-conjugated antibodies. The separation and recognition of reduced and non-reduced recombinant proteins are shown in Figures 2.1 and 2.2, respectively. Cloned DBLβ domains of different lengths comprised ~391-404 residues and estimated to have molecular masses of approximately 44.8-46 kDa. All rDBLβ migrated in the stained gel and visualised on the blots at molecular weights slightly larger than the calculated masses (46-47 kDa), which can be explained by presence of vector-specific amino acids and the histidine tag that increased the overall size of the protein core. Recombinant DBL41 was observed as a relatively faint band at the expected MW range in the stained gel, but the band was stronger and specific in the immunoblot at the same MW. The calculated and observed MWs of each recombinant protein are summarised in Table 2.1.
Figure 2.1. SDS-PAGE and Western blotting for the identification of four rDBL domain proteins expressed in *E. coli*. Purified recombinant proteins were adjusted to a concentration of 5 µg in 20 µl buffer (5 µg of protein was used per well), reduced with NuPAGE® reducing agent and 16 µl of each sample was loaded into wells of 4-12% polyacrylamide SDS-PAGE gels. Proteins were either stained with PageBlue™ protein stain (A) or transferred to PVDF membrane and probed with primary monoclonal anti-polyHistidine HRP-conjugated antibodies at 1:250 dilution (B). Blots were developed in a developing solution (1×PBS, 0.02% H₂O₂ and 0.005% DAB). Naive Mouse serum (NMS) was loaded onto the gel (5 µl / 20 µl) and included as an internal control for the reduction which showed two bands at 26 kDa (light chain) and 52 kDa (heavy chain) in the stained gel, but was not reactive with anti-polyhistidine antibodies since it lacks histidine tags. A protein ladder of known MWs (SeeBlue® Plus2 Pre-stained Protein standard) was loaded on the gel and indicated in kDa. Lanes 13, 27, 31 and 41 represent rDBL13, rDBL27, rDBL31 and rDBL41 domains, respectively.
Figure 2.2. SDS-PAGE and Western Blotting analysis for the identification of four rDBL domain proteins. Purified recombinant proteins were prepared following the same procedure as Figure 2.1, but under non-reducing conditions. Proteins were either stained with PageBlue™ protein stain (A) or transferred to PVDF membrane and probed with primary monoclonal Anti-polyHistidine HRP-conjugated antibodies (B). A protein ladder of known MWs was loaded on the gel and indicated in kDa. Lanes 13, 27, 31 and 41 represent rDBL13, rDBL27, rDBL31 and rDBL41 domains, respectively. NMS: Naive Mouse serum.

<table>
<thead>
<tr>
<th>Recombinant domains</th>
<th>Accession number</th>
<th>Length (amino acid residues)</th>
<th>Calculated MW (kD)</th>
<th>Observed MW (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL13</td>
<td>EF158072.1</td>
<td>391</td>
<td>45.3</td>
<td>~47</td>
</tr>
<tr>
<td>DBL27</td>
<td>EF158081.1</td>
<td>404</td>
<td>45.8</td>
<td>~47</td>
</tr>
<tr>
<td>DBL31</td>
<td>AF193424.1</td>
<td>403</td>
<td>46.0</td>
<td>~47</td>
</tr>
<tr>
<td>DBL41</td>
<td>EF158090.1</td>
<td>393</td>
<td>44.8</td>
<td>~46</td>
</tr>
</tbody>
</table>

Table 2.1. Calculated and observed MWs of recombinant DBLβ domains. All recombinant proteins were observed at slightly larger MWs than calculated weights.
2.2 Immunisation of mice with recombinant DBLβ domains

In the present work, two groups of mice were immunised with recombinant domains and stimulated with different adjuvants. Immunisation of the first group was carried out at Severn Biotech Ltd® (Worcestershire, United Kingdom) using Freund’s complete/incomplete adjuvant (FCA), whereas the second immunisation was performed by Professor Richard Pleass, using Alum (Sigma) as an adjuvant. After mouse immunisation, sera were collected and analysed for reactivity against immunising domains with ELISA and flow cytometry.

2.3 Functional characteristics of mouse antisera

2.3.1 Analysis of mouse antisera by ELISA

Indirect ELISAs were carried out to investigate the antibody reactivity of polyclonal antisera from mice immunised with various recombinant DBL domains using either FCA or Alum for immune stimulation. The assays also aimed to determine the immunological class of reactive antibodies present in mouse antisera. The results showed that serum samples from mice challenged with FCA were devoid of reactive IgG or IgM antibodies that recognise homologous and heterologous immunising domains (data not shown). Therefore, only analysis of Alum-stimulated mice will be discussed henceforth.

2.3.1.1 Indirect ELISA for investigating the reactivity of mouse antisera against recombinant domains

The first ELISA was carried out to screen antisera from mice stimulated with Alum for the presence of IgG class antibodies capable of recognising homologous and heterologous antigens (Figure 2.3, Table 2.2). As expected, all polyclonal sera showed higher reactivity with their corresponding fusion domains compared to the non-immune mouse sera (NMS) negative control, except anti-rDBL41 antisera, which recognised their own fusion proteins very weakly. However, all polyclonal anti-sera showed variable levels of cross-
reactivity against other heterologous domains, but had almost no reactivity with the rDBL41 protein. A pattern of homologous domain-specificity was observed as antisera showed a relatively higher reactivity with their immunising antigens compared to other heterologous proteins. For instance, anti-DBL13 antisera reacted exclusively with homologous domains, indicating that mice injected with rDBL13 antigens elicited specific IgG antibodies that only recognised corresponding immunising antigens.

Figure 2.3. Indirect ELISA for investigating the IgG-class reactivity of antisera obtained from mice immunised with recombinant domains. 100 µl of rDBL13, rDBL27, rDBL31 and rDBL41 domains at 1 µg/ml in 0.1 M carbonate bicarbonate buffer were coated onto ELISA plates. Plates were blocked and washed as described in Materials and Methods, then incubated with 1:500 diluted mouse antisera (anti-DBL13, anti-DBL27, anti-DBL31 and anti-DBL41), PBS-injected mouse serum (NMS) or mouse monoclonal BC6 IgG antibody (20 µg/ml). Reactive IgG antibodies were detected with HRP-conjugated goat anti-mouse IgG antibodies (Sigma) at 1:500 dilution. Recognition was measured in terms of the magnitude of the response demonstrated as optical density (OD) values and shown as the average of duplicate wells with SD values expressed as error bars. Incubation of NMS mouse sera or mAb BC6 with recombinant proteins was included in the assay as negative control.
In the second ELISA, we used a secondary antibody that enables the detection of reactive IgM antibodies in mouse polyclonal antisera (Figure 2.4, Table 2.3). Mouse immune sera exhibited an overall reduced reactivity with homologous and heterologous domains compared to IgG antigen-recognition profile. All antisera cross-reacted with heterologous domains, with anti-DBL13 and anti-DBL27 antisera showing a relatively increased pattern of homologous domain-specificity. NMS control showed a slightly higher reactivity compared to mAb BC6 control, which can be explained by the presence of heterophilic low-affinity IgM antibodies in naïve mouse serum capable of recognising some epitopes on coated proteins.

![Figure 2.4. Indirect ELISA for investigating the IgM-class reactivity of antisera obtained from mice immunised with recombinant domains. 100 µl of rDBL13, rDBL27, rDBL31 and rDBL41 domains at 1 μg/ml in 0.1 M carbonate bicarbonate buffer were coated onto ELISA plates. Plates were blocked and washed as described in Materials and Methods, then incubated with 1:500 diluted mouse antisera (anti-DBL13, anti-DBL27, anti-DBL31 and anti-DBL41), PBS-injected mouse serum (NMS) or mouse monoclonal BC6 IgG antibody (20 μg/ml). Reactive IgM antibodies were detected with HRP-conjugated goat anti-mouse IgM (Southern Biotech) at 1:500 dilution. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars. Incubation of NMS mouse sera or mAb BC6 with recombinant proteins were included in the assay as negative controls.](image-url)
### Table 2.2. Investigating the presence of reactive IgG antibodies in mouse polyclonal antisera against recombinant DBLβ domains as measured by indirect ELISA.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Recombinant DBLβ domains</th>
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<tbody>
<tr>
<td></td>
<td>DBL13</td>
</tr>
<tr>
<td>Anti-DBL13 antisera</td>
<td>++</td>
</tr>
<tr>
<td>Anti-DBL27 antisera</td>
<td>++</td>
</tr>
<tr>
<td>Anti-DBL31 antisera</td>
<td>+++</td>
</tr>
<tr>
<td>Anti-DBL41 antisera</td>
<td>+++</td>
</tr>
<tr>
<td>NMS</td>
<td>-</td>
</tr>
<tr>
<td>BC6</td>
<td>-</td>
</tr>
</tbody>
</table>

The results were considered positive if the mean absorbance value obtained from immune sera was 3 SDs above the mean of NMS absorbance values. Capacity of recognition was as followed: +: ˃ 3 SD values above the mean of NMS absorbance value (0.11) to OD value of 0.31, ++: OD values of 0.32 to OD values of 0.51, +++: OD values of 0.52 to OD values of 0.71, ++++: OD values of 0.72 to OD values of 0.91, +++++: ˃ OD values of 0.91.

### Table 2.3. Investigating the presence of reactive IgM antibodies in mouse polyclonal antisera against recombinant DBLβ domains as measured by indirect ELISA.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Recombinant DBLβ domains</th>
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<tbody>
<tr>
<td></td>
<td>DBL13</td>
</tr>
<tr>
<td>Anti-DBL13 antisera</td>
<td>+</td>
</tr>
<tr>
<td>Anti-DBL27 antisera</td>
<td>-</td>
</tr>
<tr>
<td>Anti-DBL31 antisera</td>
<td>+</td>
</tr>
<tr>
<td>Anti-DBL41 antisera</td>
<td>-</td>
</tr>
<tr>
<td>NMS</td>
<td>-</td>
</tr>
<tr>
<td>BC6</td>
<td>-</td>
</tr>
</tbody>
</table>

The results were considered positive if the mean absorbance value obtained from immune sera was 3 SDs above the mean of NMS absorbance values. Capacity of recognition was as followed: +: ˃ 3 SD values above the mean of NMS absorbance values (0.25) to OD values of 0.45, ++: OD values of 0.46 to OD values of 0.65.
2.3.1.2 Checkerboard ELISA

An indirect ELISA was then carried out to screen mouse polyclonal antisera for the presence of rDBL-specific antibodies using serial dilutions of immune sera against different concentrations of fusion rDBL domains, in order to determine the antibody titre of immune antisera (Table 2.4). Figure 2.5 shows the titration for anti-DBL31 antisera against different concentrations of homologous and heterologous proteins used for mouse immunisation. Only IgG response was measured in this assay, as primary screening ELISA showed a relatively weak IgM response in mouse antisera (Figure 2.4). The antibody titre was defined as the minimum dilution of antisera that shows an OD value above the cut-off point set at three SDs above the mean OD value of DBL4ε control. In general, titres for most antibodies ranged from 1/1000 and 1/100,000, and an adequate antibody response is achieved when antibody titre of test serum was > 1/1000 (Fuller et al., 1992).

Overall, titration curves showed that all antisera efficiently recognised their corresponding rDBL antigens down to 1:100,000 dilution (Table 2.4). An exception was the anti-rDBL41 immune serum, which had not been successful in recognising its corresponding protein but cross-reacted with the heterologous domains with a titre of 1/1000 with DBL13 and DBL31, and 1:5000 with DBL27. Frequencies of recognition were shown to be dependent on the coating concentration of proteins as well as the dilution of antisera, i.e. higher concentrations of coating protein and lower serum dilution showed the maximum reactivity in all assays, and vice versa. All recombinant proteins, particularly rDBL31, were recognised well by mouse antisera except DBL41 which only weekly recognised by anti-DBL13 and anti-DBL27 antisera. In addition, all polyclonal sera showed no reactivity with DBL4ε control protein indicating that the positive response was specific to recombinant DBLβ domains of PfEMP1 from the IT4 parasite genome.
Figure 2.5. The reactivity of serial dilutions of anti-DBL31 antisera against different concentrations of recombinant DBL domain proteins as measured by indirect ELISA. 96-well ELISA plates were coated with rDBL proteins (rDBL13, rDBL27, rDBL31, rDBL41 and rDBL4ε control) dissolved in 0.1 M carbonate bicarbonate buffer at 1, 0.5, 0.1 or 0.01 µg/ml. Plates were blocked and washed as described in Materials and Methods, then incubated with serial dilutions of mouse polyclonal antisera (1:500, 1:1000, 1:5000, 1:10,000, 1:50,000 and 1:100,000). Reactive IgG antibodies were detected with HRP-conjugated goat anti-mouse IgG antibodies (Sigma) at 1:500 dilution. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars. Incubation of NMS mouse sera or mAb BC6 with recombinant proteins was included in the assay as negative controls. Recombinant DBL4ε domain was cloned from VAR2CSA and used as a negative control in the assay.
<table>
<thead>
<tr>
<th>Antisera</th>
<th>Dilution</th>
<th>DBL13 (μg/ml)</th>
<th>DBL27 (μg/ml)</th>
<th>DBL31 (μg/ml)</th>
<th>DBL41 (μg/ml)</th>
<th>DBL4ε (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.01</td>
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<tr>
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<td>++++</td>
<td>+++</td>
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<td>-</td>
<td>++</td>
</tr>
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<td></td>
<td>1:1000</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1:5000</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti-DBL27</td>
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<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
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<td>-</td>
<td>++++</td>
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<tr>
<td></td>
<td>1:5000</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Anti-DBL31</td>
<td>1:500</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>++++</td>
</tr>
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<td></td>
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<td>-</td>
<td>++</td>
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<td>-</td>
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<td>-</td>
<td>+++</td>
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<td></td>
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<td>-</td>
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</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.4. Summary of the reactivity of serial dilutions of mouse antisera against different concentrations of recombinant DBL domain proteins. The results were considered positive if the mean absorbance value obtained from a particular immune serum was 3 SDs above the mean absorbance values of that antisera against control DBL4ε domain. Then, capacity of recognition of + to ++++ was determined with an interval of 0.2 OD units. For instance, 3 SD values above the mean absorbance of anti-DBL13 antisera against control DBL4ε domain was 0.12. Therefore, all absorbance readings > 0.12 were considered positive. Capacity of recognition for anti-DBL13 was as followed: +: > 3 SD values above the mean of control (0.12) to OD values of 0.32, ++: OD values of 0.33 to OD values of 0.52, +++: OD values of 0.53 to OD values of 0.72, ++++: OD values of 0.73 to OD values of 0.92. ++++: > OD values of 0.92. Capacity of recognition for the remaining sera were not stated for brevity.
2.3.2 Analysis of mouse antisera by Western blotting

To further investigate the immunoreactivity of mouse antisera, recombinant DBLβ domains used in mouse immunisation and DBL4ε control domain were resolved on SDS-PAGE gels, transferred to PVDF membrane and probed with polyclonal immune sera, NMS control serum or primary monoclonal anti-polyHistidine antibodies (Figure 2.6). All immune sera, except anti-DBL41 antisera, strongly recognised their corresponding antigens, revealing a major band at the expected MW (45 to 46 kDa). Anti-DBL41 antisera exhibited faint bands at the range DBL domains MW with DBL27 and DBL31 and a very faint band with homologous domain at the same MW (Figure 2.6).

Recombinant DBL13, DBL27 and DBL31 proteins were recognised not only by sera from mice immunised with the homologous proteins, but also by sera obtained from heterologous mouse groups. Recombinant DBL31 was similarly recognised by homologous sera as well as by anti-DBL13 and anti-DBL27 antisera. Interestingly, anti-DBL13 mouse sera recognised DBL31 more specifically than its corresponding domain, which is inconsistent with the previous ELISA (Figure 2.3) that showed a homologous specificity of anti-DBL13 antisera against corresponding domains. On the other hand, recombinant DBL41 only showed a strong band with anti-DBL13 antisera and faint band with homologous sera at the MW range of DBL domains. Beside the recognition of expressed domains, polyclonal sera also frequently recognised multiple bands of variable MWs, particularly a band of ~25 kDa which was recognised by all antisera. These bands are most likely breakdown products of recombinant proteins and bacterial contaminants and aggregates resulting from expression in *E. coli*.

Recombinant DBL13, DBL27 and DBL31 proteins were recognised by anti-polyHistidine antibodies at expected MW sizes of recombinant DBL proteins. On the contrary, rDBL41 only showed a very faint band at the same MW. The control DBL4ε was identified at a MW of 35-37 kDa with anti-polyhistidine antibodies. NMS at the same dilution did not react with recombinant domains. In addition, all antisera almost showed no reactivity with VAR2CSA-DBL4ε control domain.
Figure 2.6. Western Blotting analysis of mouse polyclonal antisera against immunising DBL proteins. Recombinant proteins (rDBL13, rDBL27, rDBL31, rDBL41 and rDBL4ε) at 5 µg/20µl concentration were reduced with DTT, blotted onto PVDV membrane and probed with 1:250 dilution of mouse immune sera (anti-DBL13, anti-DBL27, anti-DBL31 and anti-DBL41), NMS or with HRP-conjugated anti-polyHistidine antibodies at 1:200 dilution. Primary antibody binding in blots A, B, C, D and F was detected by 1:500 diluted HRP-conjugated goat anti-mouse IgG antibodies (Sigma). Recombinant DBL domains probed with anti-DBL13 mouse antisera (A), anti-DBL27 antisera (B), anti-DBL31 antisera (C) and anti-DBL41 anti-sera (D). A positive control blot of all proteins was probed with primary monoclonal anti-polyHistidine HRP-conjugated antibodies (E). An additional blot was probed with NMS and included as a negative control (F). VAR2CSA-DBL4ε recombinant protein was included as an internal negative control (lane 40). The molecular weight standard is indicated in kDa.
2.3.3 Analysis of mouse antisera by flow cytometry

The ability of mouse serum IgG or IgM to recognise the surface of IEs was tested by flow cytometry using erythrocytes infected with homologous (GC503 and 2F6) and heterologous *P. falciparum* strains (Table 2.5). In general, sera from mice immunised with rDBL13 domains were the most effective in labelling erythrocytes infected with homologous (GC503) and heterologous *P. falciparum* isolates (Figure 2.7, only showing labelling profile of anti-DBL13 antisera). On the contrary, there was little or no binding with sera raised to the remaining domains. DBL13-induced antibodies, principally of the IgM isotype, highly recognised erythrocytes infected with homologous (A4, C24, GC503, 2B2 and ItG. Anti-DBL13 antisera also comprised IgG reactive antibodies; however, the capacity of surface recognition for IgG was significantly lower than IgM recognition (Figure 2.7).

Mouse immune sera raised to DBL27 only labelled the surface of 2F6-IEs, a parasite isolate expected to express the homologous var27 gene. On the other hand, anti-DBL31 and -DBL41 mouse antisera only recognised the surface of outgrown A4-IEs, which expected to express a mixture of genes including var31 variants. Parasite isolates that dominantly express var31 or var41 genes were not available for testing, therefore anti-DBL31 and -DBL41 mouse antisera were only tested with heterologous isolates. Labelling of non-IEs was observed mainly at low frequency, demonstrating the efficacy of the immunisation and specificity of reactive antibodies towards parasite-derived proteins on the surface of parasitized erythrocytes. NMS had not recognised the surface of IEs, indicating the absence of heterophilic antibodies that react with the surface of IEs. Taken together, these results indicate that immunised mice elicited antibodies, principally of the IgM isotype, capable of staining the surface of erythrocytes infected with homologous and heterologous DBL domains.
Figure 2.7. Reactivity of anti-DBL13 mouse antisera with the surface of erythrocytes infected by different *P. falciparum* isolates (A4, C24, GC503, 2B2 and ItG) as measured by flow cytometry. Labelling was detected using APC-conjugated goat anti-mouse IgM (µ) secondary antibody (Molecular probes®) for the detection of reactive IgM antibodies or with goat anti-mouse IgG (Fc)-APC conjugated (Thermo Scientific) for the detection of reactive IgG class antibodies. Population of IEs labelled with reactive IgM or IgG antibodies are shown in blue. Cells incubated with NMS at the same dilution and stained with ethidium bromide and secondary antibodies were included as a negative control and shown in red. Labelling of NMS was used to exclude background labelling, which was usually found at a range of 1-3% of IE population compared to erythrocytes incubated with secondary antibody alone (not shown). A labelling of > 10% of IE population was considered positive. Positive control includes the labelling of A4-infected erythrocytes with mAb BC6 (20 µg/ml), which recognises an exposed epitope on PfEMP1 of A4-IE surface. Uninfected cells were shown to be negative with labelling IgG or IgM antibodies (not shown).

<table>
<thead>
<tr>
<th>Anti-DBL13 antisera</th>
<th>PBS-injected mouse sera</th>
<th>BC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>A4</td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>C24</td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
<tr>
<td>GC503</td>
<td><img src="image9" alt="Graph" /></td>
<td><img src="image10" alt="Graph" /></td>
</tr>
<tr>
<td>2B2</td>
<td><img src="image13" alt="Graph" /></td>
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<tr>
<td>ItG</td>
<td><img src="image17" alt="Graph" /></td>
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</tr>
</tbody>
</table>
Table 2.5. Summary of FACS data for the reactivity of DBLβ domain-specific antisera with erythrocytes infected by different *P. falciparum* isolates. - (labelled IE population ≤ 10%), + (labelled IE population 10.1 to 20%), ++ (labelled IE population 20.1 to 40%), +++ (labelled IE population 40.1 to 60%). 1 Outgrown A4 was expected to express mixed var genes including var31. Results correspond to two experiments. ND: no data.

### 2.4 Development and characterisation of mouse monoclonal and polyclonal anti-DBLβ antibodies

We produced mAbs and pAbs by fusing splenocytes extracted from mice immunised with four recombinant DBLβ domains with SP2/0 myeloma cell line. Culture supernatants of the resulting hybridoma lines were screened with ELISA for reactivity against immunising DBL domains (data not shown). The assays also aimed to determine the immunological class of reactive antibodies present in culture supernatants. Some hybridoma clones that showed positive reactivity by ELISA have been cloned by limiting dilution to produce single clones that secrete antibodies with mono-specificity (mAbs), others have been tested in the study without limiting dilution (pAbs).

Following preliminary screening, two ELISAs were carried out to detect IgG or IgM reactive antibodies in culture supernatants from hybridomas (Figures 2.8 and 2.9, respectively). Screening ELISAs demonstrated that out of the initial panel of hybridoma clones (n= 17) that showed confluent growth post-fusion,
thirteen clones were found to contain reactive antibodies against immunising DBL domains (Table 2.6). Interestingly, all positive hybridoma clones only secreted reactive IgM antibodies, whereas none of the clones comprised IgG antibodies that recognise immunising domains. No pattern of homologous domain specificity was observed with hybridoma lines, as all clones exhibited variable levels of IgM reactivity with homologous and heterologous DBL domains. If a hybridoma clone was reactive with a particular recombinant protein, it cross-reacted with the other DBL domains. Table 2.7 summarises all hybridoma clones that reacted by ELISA, their specificity and immunising DBL domains. These mAbs and pAbs (n= 13) were selected for subsequent characterisation in our work.

Figure 2.8. Investigating the presence of reactive IgG antibodies in hybridoma culture supernatants (n= 17) against rDBL proteins. 100 µl of rDBL13, rDBL27, rDBL31 and rDBL41 domains at 5 µg/ml in 0.1 M carbonate bicarbonate buffer were coated onto ELISA plates. Plates were blocked and washed as described in Materials and Methods, then incubated with 200 µl of hybridoma culture supernatants, culture media control (CM-CRL) or anti-DBL27 mouse antiserum at 1:500 dilution. Reactive IgG antibodies were detected with HRP-conjugated goat anti-mouse IgG antibodies (Sigma) at 1:500 dilution. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars. Incubation of CM-CRL with recombinant proteins was included in the assay as negative control, whereas anti-DBL27 mouse antiserum was used as a positive control previously shown to give a positive reactivity with all DBL proteins.
Figure 2.9. Investigating the presence of reactive IgM antibodies in hybridoma culture supernatants (n= 17) against rDBL proteins. 100 μl of rDBL13, rDBL27, rDBL31 and rDBL41 domains at 5 μg/ml in 0.1 M carbonate bicarbonate buffer were coated onto ELISA plates. Plates were blocked and washed as described in Materials and Methods, then incubated with 200 μl of hybridoma culture supernatants, CM-CRL or anti-DBL27 mouse antiserum at 1:500 dilution. Reactive IgM antibodies were detected with 100 μl HRP-conjugated goat anti-mouse IgM (Southern Biotech®) at 1:500 dilution. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars. Incubation of CM-CRL with recombinant proteins was included in the assay as negative control, whereas anti-DBL27 mouse antiserum was used as a positive control previously shown to give a positive reactivity with all DBL proteins.
<table>
<thead>
<tr>
<th>Hybridoma clone</th>
<th>Immunising DBLβ domains (5 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBL13</td>
</tr>
<tr>
<td>E11</td>
<td>++++</td>
</tr>
<tr>
<td>B12</td>
<td>++++</td>
</tr>
<tr>
<td>A6A</td>
<td>++++</td>
</tr>
<tr>
<td>H1A</td>
<td>-</td>
</tr>
<tr>
<td>A5B</td>
<td>-</td>
</tr>
<tr>
<td>A1B</td>
<td>-</td>
</tr>
<tr>
<td>2A9</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>+</td>
</tr>
<tr>
<td>E4</td>
<td>++++</td>
</tr>
<tr>
<td>A4</td>
<td>++++</td>
</tr>
<tr>
<td>E7</td>
<td>++++</td>
</tr>
<tr>
<td>C9</td>
<td>++++</td>
</tr>
<tr>
<td>2E10</td>
<td>++++</td>
</tr>
<tr>
<td>B5</td>
<td>+</td>
</tr>
<tr>
<td>D5</td>
<td>+++</td>
</tr>
<tr>
<td>B4</td>
<td>++</td>
</tr>
<tr>
<td>G6</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 2.6. IgM reactivity of monoclonal and polyclonal hybridoma clones against recombinant DBL domains used in mouse immunisations as measured by ELISA. + (OD value > 0.2 above negative control), ++ (OD value > 0.4 above negative control), +++ (OD value > 0.6 above negative control) and ++++ (OD value > 1.0 above negative control).

<table>
<thead>
<tr>
<th>Hybridoma clone</th>
<th>DBL domain</th>
<th>Antibody isotype</th>
<th>Specificity</th>
<th>Parent clone (if monoclonal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11</td>
<td>DBL13</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
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<td>DBL13</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
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<td>DBL13</td>
<td>IgM</td>
<td>Monoclonal</td>
<td>C1</td>
</tr>
<tr>
<td>C2</td>
<td>DBL31</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
<td>E4</td>
<td>DBL31</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
<td>A4</td>
<td>DBL31</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
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<td>DBL31</td>
<td>IgM</td>
<td>Monoclonal</td>
<td>E4</td>
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</tr>
<tr>
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<td>DBL31</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>DBL41</td>
<td>IgM</td>
<td>Polyclonal</td>
<td>-</td>
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<tr>
<td>D5</td>
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<td>IgM</td>
<td>Monoclonal</td>
<td>B5</td>
</tr>
<tr>
<td>G6</td>
<td>DBL41</td>
<td>IgM</td>
<td>Monoclonal</td>
<td>B5</td>
</tr>
</tbody>
</table>

Table 2.7. Summary of reactive hybridoma clones raised against variant rDBL proteins. Hybridomas were generated from the fusion of myeloma cell lines with immunised mice splenocytes. Some of the antibody-secreting hybridoma clones have been cloned by limiting dilution to single clones (monoclonal specificity); others have been used in the study without limiting dilution (polyclonal specificity).
2.5 Affinity purification and characterisation of purified monoclonal and polyclonal antibodies

A total of thirteen monoclonal and polyclonal hybridoma cell lines were found to secrete reactive IgM antibodies capable of recognising rDBL domains. Antibodies were affinity-purified from stable culture supernatants using manual gravity flow column chromatography. All eluted fractions resulted from the purification were screened by flow cytometry for their ability to recognise the surface of erythrocytes infected by ItG parasites, in order to select fraction(s) with the highest labelling capacity for further work. Table 2.8 only summarises the IgM surface reactivity of all eluted fractions to ItG-IEs, as none of the purified mAbs and pAbs comprised surface-reactive IgG antibodies (data not shown). Antibody fractions with the highest labelling capacity are shown in green and were selected for further analysis. A representative flow cytometry profile of surface labelling by pAb B12 eluted fractions is shown in Figure 2.10, which clearly demonstrated that fraction 1 comprised all reactive antibodies eluted from the purification.
Figure 2.10. Reactivity of all eluted fractions of pAb B12 with the surface of erythrocytes infected by ItG P. falciparum isolate as measured by flow cytometry. 50 µl of each eluted fraction at neat concentrations was incubated with trophozoite-IEs. Labelling was detected using APC-conjugated goat anti-mouse IgM secondary antibody (Southern Biotech®) at 1:100 dilution. Cells incubated with 20 µg/ml of mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®) and stained with ethidium bromide and secondary antibodies were used to exclude background labelling and included as a negative control. Percentages of IEs labelled with reactive IgM antibodies of pAb B12 fractions are shown as APC-A+ subset and were obtained relative to a background of control IgM labelling. A labelling of > 10% of IE population was considered positive.
Table 2.8. The reactivity of all eluted fractions of mAbs and pAbs resulted from the affinity purification to the surface of ItG-IEs as measured by flow cytometry. Antibody fraction(s) selected for further characterisation is/are marked in green. Results of surface reactivity correspond to at least two experiments. 
- (labelled IE population ≤ 10%), + (labelled IE population 10.1 to 20%), ++ (labelled IE population 20.1 to 40%), +++ (labelled IE population 41 to 60%), ++++ (labelled IE population > 60.1%).

<table>
<thead>
<tr>
<th>mAbs/pAbs</th>
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<tbody>
<tr>
<td></td>
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<tr>
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<td>C9</td>
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<td>-</td>
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<td>B4</td>
<td>-</td>
</tr>
<tr>
<td>G6</td>
<td>-</td>
</tr>
</tbody>
</table>
Then, an indirect ELISA assay was carried out to assess the efficiency of the purification by comparing the level of antibody reactivity for culture supernatants prior to purification, purified antibody yield and depleted flow-through fluid resulted from the purification (Figure 2.11). Recombinant DBL31 domain was used as a representative coating antigen in this assay. As expected, frequencies of recognition of the coated protein for all purified mAbs and pAbs were markedly higher than those of pre-purification culture supernatants or flow-through media, except purified pAb B12 which almost failed to recognise the coated antigen. This was surprising since pAb B12 exhibited positive IgM surface labelling with ItG-IEs. This suggests the presence of trivial amounts of surface-reactive IgM antibodies resolved from the purification and capable of labelling conformational epitopes by flow cytometry but failed to recognise the recombinant protein by ELISA. It should be noted that the overall reactivity of culture supernatants in this experiment is very low compared to that observed in Figure 2.9 and Table 2.6 (e.g. B12), and this inconsistency can be due to the use of culture supernatants preserved for longer periods in sodium azide at 4°C in the present experiment, whereas fresh supernatants collected immediately after hybridoma culture were used in Figure 2.9.

Another experiment was carried out to investigate the reactivity of mouse IgM antibodies against VAR2CSA-DBL4ε (Figure S1, Appendix). A representative pAb (A4) was used and the experiment was carried out following the same procedure as in Figure 2.11. The results showed that pAb A4 significantly recognised recombinant DBL31 protein but failed to react with DBL4ε.
Figure 2.11. Indirect ELISA for investigating the efficiency of affinity purification of mAbs and pAbs. The reactivity of purified antibody yield, culture supernatant prior to purification and the resulted flow-through media were tested against recombinant DBL31 proteins (5 μg/ml). 50 μl purified mAbs/pAbs, 200 μl of hybridoma culture supernatants or flow-through fluid resulted from the purification were incubated with coated antigens. Reactive IgM antibodies were detected with 100 μl HRP-conjugated goat anti-mouse IgM (Southern Biotech®) at 1:500 dilution. Incubation of CM-CRL or 20 μg/ml of mouse IgM isotype control (Sigma®) with recombinant proteins were included in the assay as negative controls, whereas anti-DBL31 mouse serum (50 μl, 1:50 dilution) was used as a positive control. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars.
2.6 Measurement of IgM concentration in purified mAbs and pAbs

A semi-quantitative Dot Blot assay was carried out to estimate the amount of IgM in purified mAbs and pAbs (Figure 2.12). IgM concentration in eluted fractions was estimated by comparison to a mouse IgM control standard also spotted onto nitrocellulose membranes. Immunoblotting analysis confirmed the presence of mouse IgM antibodies in purified mAbs and pAbs at a range of 10-80 μg/ml for most fractions. Fraction 1 of pAb B12 showed a very faint dot although it labelled the surface of IEs as shown in Figure 2.10. This can be explained by the presence of finite amounts of highly specific IgM antibodies in the purified yield that could produce a positive signal for surface labelling but failed to be detected in the semi-quantitative Dot Blot.
Figure 2.12. Dot Blot assay for estimating the concentration of IgM antibodies in purified mAbs and pAbs. 5 μl of all eluted fractions was spotted onto Whatman® Protran® Nitrocellulose membrane. Gradual concentrations (200 μg/ml - 0.2 μg/ml) of mouse IgM isotype control (Sigma®) were also blotted onto the membrane as a standard for determining the concentration of purified antibodies. Control IgM dilutions were carried out in neutralised glycine/Trizma-HCL pH 7.0 buffer solution, the same buffer for eluted mouse IgMs. The membrane was blocked and washed as described in Materials and Methods. IgM reactivity was detected with HRP-conjugated goat anti-mouse IgM (Southern Biotech®) at 1:500 dilution. 5 µl of complete medium (CM) or mAb BC6 at 20 μg/ml were spotted and used as negative controls.

Then, IgM antibodies in affinity-purified yields were quantified using the indirect double-antibody sandwich ELISA. In this ELISA, the heavy chain of mouse IgM antibodies was captured by unlabelled heavy chain-specific anti-mouse IgM before the complex was detected with HRP-conjugated goat anti-
mouse IgM antibodies. The unknown IgM concentration was calculated with regard to a standard curve based on known concentration and absorbance values derived from the mouse IgM control (Figure 2.13). We found that quantitated IgM concentration in purified yields varied from 0.1 to 66.3 µg/ml. Table 2.9 summarises the concentration of IgM antibodies in all purified mAbs and pAbs selected for the study. Quantitation of pAb B12 confirms previous ELISA (Figure 2.11) and Dot Blot (Figure 2.12).

Figure 2.13. Slope of a standard dilution curve generated from gradual concentrations of commercially provided mouse IgM control as indicated by double-antibody sandwich ELISA. Briefly, unlabelled μ-chain specific rat anti-mouse IgM (Southern Biotech®) at 5 µg/ml in 0.1 M carbonate bicarbonate buffer was coated onto the plate. The plate was blocked and washed as described previously, before mAbs/pAbs were added at 1:100 dilution. Mouse IgM isotype control (Sigma®) was diluted by doubling dilution into gradual concentrations (4000-0.12 ng/ml) and added to the plate. Reactive IgM antibodies were detected with 100 µl HRP-conjugated goat anti-mouse IgM (Southern Biotech®) at 1:500 dilution. Incubation of CM-CRL or 20 µg/ml of BC6 mAb (IgG class antibodies) with coated antibody were included in the assay as negative controls. The unknown concentration of IgM in purified mAbs and pAbs was obtained with regard to a standard curve of known concentration and absorbance values using the excel function =TREND (all concentration values of standard, [all absorbance values of standard], [absorbance of unknown concentrtion], [constant]).
<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>IgM control (standard)</th>
<th>Purified mAbs and pAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance at 450 nm (OD values)</td>
<td>Antibody Fraction</td>
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<tr>
<td>4000</td>
<td>1.03965</td>
<td>E11 fr4</td>
</tr>
<tr>
<td>2000</td>
<td>0.9702</td>
<td>B12 fr1</td>
</tr>
<tr>
<td>1000</td>
<td>0.96245</td>
<td>A6A fr7</td>
</tr>
<tr>
<td>500</td>
<td>0.8773</td>
<td>C2 fr4</td>
</tr>
<tr>
<td>250</td>
<td>0.76905</td>
<td>E4 fr3</td>
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<tr>
<td>125</td>
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<td>A4 fr5</td>
</tr>
<tr>
<td>62.5</td>
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<td>E7 fr3</td>
</tr>
<tr>
<td>31.25</td>
<td>0.46765</td>
<td>C9 fr5</td>
</tr>
<tr>
<td>15.6</td>
<td>0.3787</td>
<td>2E10 fr6</td>
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<td>7.8</td>
<td>0.2881</td>
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<td>0.1855</td>
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<td>A6A fr6</td>
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<td>C2 fr5</td>
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<td>A4 fr6</td>
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<tr>
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<td>CM</td>
</tr>
<tr>
<td></td>
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<td>BC6</td>
</tr>
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</table>

Table 2.9. Summary of the final IgM concentration of purified mAbs and as measured by double-antibody sandwich ELISA. Pool 1: a pool of pAb E11, pAb B12, mAb A6A and pAb E4 of equal volumes was prepared and quantitated for further investigations. Pool 2: mAb E7, pAb B4, pAb D5 and mAb G6 was prepared for the same purpose. Fr: fraction.
2.7 Discussion

Sequestration of IEs to endothelial cells lining the post-capillary venules of various host organs is a distinctive feature in the virulence of *P. falciparum* infection (Cooke et al., 1995, Pasloske and Howard, 1994). Among host endothelial ligands that mediate cytoadhesion, ICAM-1 was linked to severe forms of malaria diseases, including CM (Newbold et al., 1997, Turner et al., 1994). PfEMP1 is a major parasite-derived protein ligand exported to the surface of IEs and is associated with the adhesion of IEs to host endothelium (Cooke et al., 1995). Early studies have revealed that the binding domain of ICAM-1 receptors on the parasite PfEMP-1 ligand has been mapped and located within two domains; DBLβ2 and C2 (Smith et al., 2000a) which termed DBLβ in this work as C2 domain is basically a part of the main DBLβ domain (Gullingsrud et al., 2013).

MAbs and pAbs directed against various PfEMP1 domains have been suggested as valuable tools for recognising critical functional sites on PfEMP1 involved in parasite adhesion and determining the cross-reactivity with parasite strains from irrelevant regions (Lekana Douki et al., 2002). However, it remains unknown whether DBLβ domains from PfEMP1 of ICAM-1 binding isolates can induce functional and cross-reactive antibodies that are effective in blocking parasite adhesion to ICAM-1. In the present study, we selected four recombinant PfEMP1<sup>ICAM-1</sup>-DBLβ domains that were expressed as hexahistidine-tagged proteins in *E. coli* and shown to bind ICAM-1 proteins (Kraemer et al., 2007, Howell et al., 2008) for mouse immunisations. DBLβ domains were used rather than the full-length PfEMP1 protein to map the minimal receptor-binding regions for ICAM-1 binding isolates and to understand the functional requirements for receptor adhesion. In addition, if we had used the full-length PfEMP1 domains we may not be able to recover any relevant anti-DBLβ mAbs. We present evidence that it is possible to elicit mAbs and pAbs in mice by immunisation with single recombinant DBLβ domains from PfEMP1<sup>ICAM-1</sup> variants of the IT4 genome. MAbs and pAbs comprising reactive antibodies recognised homologous and heterologous DBLβ domains and labelled the surface of erythrocytes infected by *P. falciparum* isolates. To our knowledge, this is the first study to examine the use of PfEMP1<sup>ICAM-1</sup>-DBLβ domains for the
development of mAbs and pAbs that block parasite adhesion to immobilised ICAM-1/CD36 receptors or endothelial cell lines, although the same technique was used for several other domains and interactions.

Stimulation of an efficient humoral immune response in mice is crucial for the production of mAbs against specific antigens (Fuller et al., 1992). In our study, mice were immunised with recombinant domains complexed with either FCA or Alum as adjuvants, to increase the probability of a good response towards the immunising antigens and to compare the efficiency of both boosting adjuvants. Unfortunately, the former adjuvant failed to elicit reactive mouse antibodies and all reactive mAbs and pAbs were generated using Alum adjuvant. The failure of FCA to induce a specific immune response was surprising since this adjuvant has been proven to elicit antibodies with malarial proteins. For instance, Barfod et al. (2006) used both Freund's incomplete/complete adjuvant and aluminium hydroxide gel for mice immunisation with recombinant VAR2CSA domains and found that both adjuvants induced surface-reactive antibodies and no difference was observed in eliciting sera with both adjuvant systems. In addition, Fernandez et al. (2010) provided evidence that immunisation of mice with single DBL domains from VAR2CSA using alum as an adjuvant has induced an antibody response that efficiently blocked parasite adhesion to CSA. Since the same immunogens were used in both immunisations, we suggest that the response was either lost during immunisation or not induced at all, due to failure in antigen delivery to FCA-stimulated mice or suboptimal dose that was insufficient for inducing an immune response. Failure in the induction of antigen-specific immune response with FCA was observed in previous studies. For example, a recent study showed that a previously induced inhibitory response in immunised rat sera towards specific DBL domains was not reproducible in subsequent immunisations (Salanti et al., 2010). The authors suggested that different immunisations with the same antigen did not give the same fine specificity in each immunisation, as shown by variation in the inhibitory response of induced antibodies.

After mouse immunisation, sera were screened for the presence of reactive antibodies that recognise the recombinant immunising domains by ELISA or the surface of live IEs by flow cytometry. Indirect primary and checkerboard
ELISAs showed that mouse antisera raised to all recombinant domains, except DBL41, had higher IgG titres against corresponding immunising antigens. Anti-rDBL41 antiserum failed to recognise its own domain but cross-reacted with heterologous antigens. Moreover, all antisera exhibited variable degrees of cross-reactivity with the other heterologous DBL domains but almost failed to recognise rDBL41 antigen. These results are consistent with Western Blotting data and suggest that rDBL41 used in our work either has not been correctly expressed and purified, or has been degraded during expression or after purification. In general, poor success of a given antigen in eliciting an adequate immune response can be attributed to several factors including unsuitably prepared emulsion when using the adjuvant, site of immunisation, dose of the immunogen and frequency of immunisation (Fuller et al., 1992). The ability of anti-DBL41 antisera to recognise heterologous but not homologous domains as shown by ELISA, together with the cross-reactivity of all immune sera against all domains except DBL41, strongly suggest that the immunogen has been fragmented after mouse immunisation. Alternatively, these data can also suggest that the recombinant protein has been degraded prior to immunisation causing the loss of critical and functional epitopes on the native protein or that the mouse response was targeting a discontinuous section on a conformational epitope in the structure of DBL41.

Protein expression in E. coli is simple, fast, and cheap with expressed proteins comprising up to 50% of the cellular protein composition (Francis and Page, 2010). In addition, E. coli proteins are not glycosylated similar to the native malaria proteins (Oleinikov et al., 2008). These are some of the advantages that made E. coli the expression system of choice for malarial proteins in many labs with more than 30 P. falciparum proteins in the Protein Data Bank having been successfully expressed in E. coli (Mehlin et al., 2006). However, the rapid rate of bacterial protein expression often results in issues with protein folding, including the production of misfolded or unfolded proteins, especially for heterologous proteins that require long periods of time to fold correctly (Francis and Page, 2010). In the case of malarial proteins, soluble expression in E. coli is a challenging task that may lead to lack of expressed proteins or in expression of proteins as insoluble inclusion bodies (Mehlin et al., 2006). These obstacles may
explain the existence of multiple bands of variable MWs in the immunoblots, as bacterial proteins may have been co-purified with the histidine tagged recombinant proteins, which is commonly encountered during expression in *E. coli* (Graslund et al., 2008).

One of the broadly used methods for detecting the presence of reactive antibodies against malaria-derived surface antigens is fluorescence-activated cell sorting (FACS) analysis. Our FACS data indicated that mouse immune sera only labelled the surface of IEs but mainly not non-IEs, confirming that binding was specific for *P. falciparum*-derived VSAs exported to the surface of live IEs. We found that antisera raised to DBL27, DBL31 and DBL41 only comprised IgM antibodies that showed variable reactivities with ItG and outgrown A4 isolates, but almost none of these sera contained reactive IgGs. This is the opposite to that found in ELISA, as mouse antisera comprised more reactive IgG response whereas the IgM response was generally weak (Figure 2.4; Table 2.3). This could be due to the higher avidity of IgM molecules that comprise multiple antigen binding sites binding to VSAs on the surface of IEs compared to only two binding sites for IgG antibodies. The latter has higher affinity for binding to linear epitopes in ELISA and their interaction could not be disrupted by subsequent washing, contrary to the low affinity binding of IgM antibodies.

Among all mouse sera, DBL13-specific antiserum comprised cross-reactive IgM antibodies that labelled the surface of erythrocytes infected by homologous and heterologous isolates. These results indicate that immunised mice elicited antibodies, principally of the IgM isotype, capable of staining the surface of erythrocytes infected with homologous and heterologous parasite isolates. Of particular significance, cross-reactivity with heterologous isolates suggest that induced antibodies recognised surface-exposed epitopes on the surface of IEs that are shared between tested parasite isolates. However, it is unresolved whether these sera were cross-reactive to conserved epitopes on the surface of IEs or alternatively they contain a mixture of antibodies of multiple specificities. Although surface-labelling was only specific for IEs, we could not confirm that the surface protein recognised by antisera was DBLβ or another variant epitope in the native PfEMP1, since several immunogenic VSAs co-express with PfEMP1 on the parasitized cell surface and can act as antibody binding ligands.
In the present study, we identified thirteen hybridoma clones that secrete reactive IgM antibodies against DBL13, DBL31 and DBL41 domains. MAbs and pAbs not only recognised their corresponding antigens, but also efficiently cross-reacted with heterologous domains with a similar capacity of recognition. Interestingly, mouse IgM antibodies effectively reacted with rDBL41 domains, which was weakly recognised by mouse immune sera. Although mouse antisera comprised IgG antibodies that showed a significant reactivity with recombinant domains over IgM-class antibodies, we believe that only the small subpopulation of IgM-secreting cells were selectively expressed and amplified after fusion. Failure to produce anti-DBL27 reactive mAbs and pAbs may indicate that antibody-secreting spleen cells were lost during fusion. This cannot be attributed to failure in antigen delivery during immunisation or protein degradation, since anti-DBL27 antisera showed positive reactivity against recombinant domains and recognised the surface of erythrocytes infected by homologous isolates.

The higher level of cross-reactivity observed in ELISA may propose that all recombinant domains share conserved immunogenic epitopes, despite their lower level of sequence identity (46%), that were easily recognisable and accessible to IgM antibodies. This cross-reactivity could also be explained by the presence of bacterial contaminants that were co-purified with histidine tagged recombinant proteins. These contaminants include multiple histidine residues and molecular chaperones that bind to recombinant proteins during expression (Graslund et al., 2008) and may be more immunogenic than the desired antigen to produce an antibody response at lower specificity (Fuller et al., 1992).

All hybridoma culture supernatants were tested for their ability to recognise the surface of P. falciparum-IEs by FACS and none of the clones demonstrated IgG-specific surface labelling (data not shown). Therefore, only IgM antibodies in hybridoma culture supernatants were affinity-purified using high liquid performance chromatography (HPLC) to obtain a concentrated yield of pure IgMs. However, the reactivity of eluted antibodies yielded from the automated system was very low when measured by flow cytometry (data not shown) and therefore the purification was repeated using manual gravity flow. All IgM antibodies resulting from gravity flow purification, except pAb B12, were highly
efficient at recognising immunising domains and labelling the surface of IEs, and were therefore used in all subsequent in vitro characterisations. The amount of IgM in purified yields was firstly estimated semi-quantitatively by dot blot and the precise IgM concentration was measured with indirect double-antibody sandwich ELISA. This method is simple, sensitive and reliable for quantification of mouse IgM antibodies in purified yields obtained from cell culture supernatants by comparison with a standard curve produced by a purified mAb of the same class (Mushens et al., 1993). NanoDrop UV spectrophotometer was also used for IgM quantitation. However, this method was unreliable due to the frequent discrepancies obtained in repeated quantitation most likely due to the presence of glycine in the elution buffer.

All eluted fractions were tested for surface fluorescence with live IEs and the most reactive fraction was used to confirm the efficiency of the purification by ELISA. Purified IgM antibodies were used in flow cytometry at concentrations ranging from 0.1-66.3 µg/ml, as the signal was markedly reduced at 1:2 dilution and almost lost at further dilutions (discussed in Chapter 3). The lack of IgG reactive antibodies in purified mAbs and pAbs, as indicated by ELISA and flow cytometry, was consistent with flow cytometry data showing higher surface labelling of IgM-class antibodies for mouse antisera.

An interesting finding in our study was that all positive hybridoma clones only secreted reactive IgM antibodies, and none of the clones comprised reactive IgG antibodies despite the higher IgG titre of mouse antisera. This was also encountered in several published studies revealing the production of reactive mouse mAbs of the IgM isotype. For instance, Lekana Douki et al. (2002) elicited mAbs in mice against the DBL-γ3CSA of the FCR3 strain. The produced mAbs, of which 70.8% were IgMs, labelled the surface of IE5CSA and cross-reacted with CSA-binding parasites from different geographic areas. In another study, 250 mAbs were produced by immunising mice with var2CSA (FCR3CSA) and 240 mAbs (96%) were of the IgM isotype that recognised IECSA (Avril et al., 2006).

Adjuvants containing aluminium were first discovered in 1926 and currently are the most broadly used in vaccine preparations since they are known to induce a robust immune response. Within hours of Alum administration, innate
inflammatory cells such as neutrophils, eosinophils, dendritic cells and monocytes are recruited at the site of exposure (Marrack et al., 2009). However, a recent study provided evidence that ~80% of the induced cells following Alum injection are eosinophils (Wang and Weller, 2008). The study showed that administration of Alum leads to activation of IL-4-expressing eosinophils that prime splenic B cell responses leading to early generation of antigen specific IgM (Wang and Weller, 2008). This function of early priming (within the first week of administration) of IgM synthesis by Alum may explain why all of the reactive mouse antibodies induced in our work were of the IgM class.

Attempts for developing mouse monoclonal antibodies that target the native and conformational epitopes on the surface of IEs have occasionally been successful due to the presence of large amounts of immunodominant parasite-derived VSAs. These surface antigens can elicit a strong immune response in immunised mice leading to difficulties in the induction of a specific response to desired antigens with a relatively weaker immunogenicity or conformational surface epitopes. Only a few number of mAbs directed against parasite-derived surface antigens on the parasitized erythrocytes surface have been published. In summary, we showed that the mouse immunisation protocol carried out in this work was successful in the production of mAbs and pAbs against recombinant PfEMP1ICAM-1-DBLβ domains. These mouse mAbs and pAbs comprised reactive IgM antibodies that recognised the corresponding DBL domains and cross-reacted with heterologous domains. In addition, most of these IgM antibodies labelled the surface of live erythrocytes infected by ItG P. falciparum isolate. Further work is required to investigate the capacity of these antibodies to recognise the surface of erythrocytes infected by phenotypically and genotypically distinct parasite isolates, and to dissect their functional role in blocking the adhesion of IEs to immobilised receptors or endothelial cells.
2.8 Future work

The biophysical properties of the interactions between IgM antibodies and DBLβ domains can be investigated using Surface Plasmon Resonance (SPR) to obtain kinetic parameters for the IgM-DBLβ interaction. In addition, an epitope mapping assay can be carried out to assess the differences in the fine specificity of IgM binding to an array of different PfEMP1 peptides, compared to binding of control IgM antibodies. This can provide additional details about the PfEMP1 domains, if any, involved in interaction with IgM antibodies. To further determine whether the high-molecular weight PfEMP1 proteins are the targets of mouse anti-sera and labelling mAbs/pAbs, these antibodies can be assessed for their capacity to immunoprecipitate 125I-labelled SDS-stage IE extracts [similar assays described by (Buffet et al., 1999, Avril et al., 2006)].

Many malaria vaccine studies have used rodent models to study the immunogenicity of particular malaria antigens. Although this approach has the advantages of being easily accessible and reasonably priced, it has significant limitations. Malaria isolates that infect humans cannot infect mice and those infecting mice lack several critical antigens of P. falciparum strains. In addition, there are significant differences between humans and mice in terms on the immune and pathogenic response to malaria infection (Richards and Beeson, 2009). These variations have provided a groundwork for the development of alternative approaches for "humanising" mouse mAbs and pAbs that can be used in humans.
Chapter 3

Characterisation of infected erythrocyte-surface labelling by monoclonal and polyclonal antibodies
Chapter 3: Introduction

Antibodies to the polymorphic VSAs on the surface of *P. falciparum* infected erythrocytes, particularly PfEMP1, have been shown to play a crucial role in the acquisition of protective immunity against severe malaria (Giha et al., 2000, Marsh et al., 1989, Bull et al., 1998). An important step in the development of therapeutic interventions based on PfEMP1 is the identification of functional critical ligands of PfEMP1 proteins involved in parasite cytoadhesion, and the investigation of the level of cross-reactivity of these variants with diverse parasite strains. MAbs generated against different domains of PfEMP1 could be a tool of choice (Lekana Douki et al., 2002). However, despite the extensive work to develop mouse mAbs against the native and conformational parasite-derived antigens, only few attempts were successful (discussed in Introduction chapter). This failure to produce specific mAbs was suggested to be due to the large number of parasite-derived immunogenic proteins on the surface of IEs, which makes it extremely difficult to elicit specific immune response to weekly immunogenic surface molecules or conformational epitopes (Lekana Douki et al., 2002).

In this chapter, we used diverse functional assays to characterise the functional properties of mouse mAbs and pAbs. *P. falciparum* isolates of multiple phenotypes expressing variant PfEMP1 molecules were tested for surface staining. We aimed to assess the degree of specificity and/or cross-reactivity of mAbs and pAbs by evaluating their relative abilities to recognise the surface of erythrocytes infected by ICAM-1 binding and non-binding isolates from the IT4 genome, or with *P. falciparum* isolates from distinct genomes. This was challenging since analysis of the surface phenotypes of *P. falciparum* IEs is difficult due to the rapid expression of antigenic and adhesive heterogeneity, even in clonal parasite cultures (Gardner et al. 1996). Our results showed that mAbs and pAbs specifically recognised the surface of erythrocytes infected by homologous and heterologous parasite isolates. We demonstrated that mouse antibodies exhibited extensive cross-reactivity against parasite isolates with distinct binding properties and genomes. We also provided evidence that mAbs and pAbs may partially bind to PfEMP1 on the surface of IEs.
3. Materials and Methods

3.1 Measurement of mouse IgM labelling to the surface of *P. falciparum*-IEs by flow cytometry

MAbs and pAbs were tested for surface reactivity with erythrocytes infected by *P. falciparum* isolates of distinct phenotypes and genotypes. All assays were carried out principally as described in Chapter 2 (sections 2.4.2.1 and 2.7) with some adjustments. Briefly, aliquots of parasite suspension (100 µl, ~2.5-3 × 10⁶ cells) were washed in PBS/1%BSA and the pellet was re-suspended with 50 µl of each mAb/pAb at 0.1-66 µg/ml and incubated for 30 minutes at 37°C.

In a separate assay, cells were incubated with mouse IgM antibodies at gradual dilutions (1:2, 1:4 and 1:8) in PBS/1%BSA to determine the least concentration required for labelling the surface of IEs. All subsequent washings and secondary antibody incubations were carried out as shown in section 2.7. Cells incubated with 20 µg/ml of mouse IgM isotype control (Sigma®, M5909, 200 µg/ml) and stained with ethidium bromide and secondary antibodies were used to exclude background labelling and included in all assays as a negative control. Labelling of > 10% of IE population was considered positive. The cut-off value of 10% for considering positive labelling was determined after several optimization experiments showing that although the background labelling of non-IEs ranged between 1 and 9% for most mAbs/pAbs, higher percentages of background labelling were frequently observed. Therefore, we believe it would be acceptable to set the positive labelling at > 10% of IE population. Data analysis and grading of surface binding were performed as described in section 2.4.2.2. Differences in the binding capacity of mAbs and pAbs against different isolates were confirmed in repeat experiments.

Due to the high percentage of lysed IEs following mouse mAbs/pAbs incubation, assay conditions for testing the surface fluorescence by flow cytometry were optimised to retain a number of IEs sufficient for detecting surface labelling without diluting the binding signal. The number of trophozoite-enriched cells for detecting surface binding of non-lysing antibodies was 0.8-1.0 × 10⁷ cells/ml from which 100 µl was used for the assay (0.8-1.0 × 10⁶ cells).
However, the number of cells was increased to 2.5-3 × 10^6 cells for detecting the binding mAbs and pAbs which caused the lysis of many IEs. Incubation time for mAbs/pAbs was also reduced to 30 minutes, which had no effect on the signal but decreased the amount of lysed cells (data not shown). Despite the remarkable decrease in IE count upon IgM incubation, surface fluorescence was not significantly affected, since 50,000 events were collected for almost all tested antibodies.

3.2 Competition assays

3.2.1 Competition between mouse IgMs and BC6 for surface binding to A4-IEs

The assay was carried out to investigate the effect of pre-incubating A4-IEs with mAb/pAbs on the subsequent binding of the BC6 mAb (20 µg/ml) and vice versa. BC6 is a mouse monoclonal IgG antibody that specifically recognises an exposed epitope on PfEMP1 of erythrocytes infected by A4 P. falciparum isolate (Roberts et al., 1992, Horrocks et al., 2002). Two pools of mouse IgMs that showed positive surface labelling were used in the competition assays including pool 1 (pAb E11, pAb B12, mAb A6A and pAb E4) and pool 2 (mAb E7, pAb B4, pAb D5, mAb G6). For investigating the effect of mouse IgM antibodies on BC6 binding, 50 µl of mouse antibodies (pool 1, pool 2) were pre-incubated with IEs for 30 minutes at 37°C, before the washing twice in PBS/1%BSA and incubation with 50 µl of mAb BC6 (20 µg/ml) for 30 minutes at 37°C. Cells were washed as above and BC6 labelling detected with 100 µl of goat anti-mouse IgG (Fc)-APC conjugated (Thermo Scientific, Product No. 31981) at 1:100 dilution. Cells were analysed for surface labelling using FACSCalibur flow cytometer and data were analysed as described in section 2.4.2.2. The effect of BC6 on subsequent binding by the mAb/pAb pools was assessed following the same procedure, but incubating with mAb BC6 first. Cells were then washed and incubated with mouse IgM antibodies and bound IgM detected with 100 µl of APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech®, Cat. No. 1020-11L, 0.5mg) also at 1:100 dilution. Table 3.1 indicates the order of the first and second
antibody incubations, as well as the aim of each experiment and secondary antibodies used for detecting surface binding.

Competition was assessed in terms of differences in surface labelling of antibodies before and after incubation with the competitor. The percentage of reduction in surface labelling to IEs were quantitated following the formula:

\[ x = 100 - \left( \frac{a}{b} \right) \times 100 \]

Where, \( x \) = the percentage of reduction in surface labelling
\( a \) = percentage of labelled IEs after incubation with the competitor
\( b \) = percentage of labelled IEs without incubation with the competitor (positive control)

### 3.2.2 Competition between IgM mAbs and pAbs and HIS for surface binding to A4-IEs

The assay was performed to examine the effect of pre-incubating A4-IEs with mouse IgMs on the subsequent binding of Malawian hyper immune serum (HIS) and *vice versa*. The same procedure was carried out as in the previous section (3.2.1), except that HIS was used in all incubations at 30 mg/ml (Table 3.1). Antibody pools (pool 1 and pool 2) were used as in section 3.2.1. Binding of HIS was detected with APC-conjugated mouse anti-human IgG-Fc secondary antibody (Southern Biotech®, Cat. No. 9042-11) at 1:100 dilution, whereas mouse IgM binding was detected as previously (section 3.2.1). Cells stained with 50 µl of non-immune sera from UK adults at 1:10 dilution were included as a negative control for HIS.
3.2.3 Competition between IgM mAbs and pAbs and rat anti-
DBL16 antisera for surface binding to ItG-IEs

This assay was carried out to investigate the influence of pre-incubating ItG-IEs with mouse IgMs on the subsequent binding of rat anti-DBL16 antisera and *vice versa*. The same procedure was carried out as in section (3.2.1), except that rat anti-DBL16 antisera were used in all incubations at 1:10 dilution (Table 3.1). Similar to the previous section, only antibody pools (pool 1 and pool 2) were used in this assay. Binding of rat antisera was detected with APC-conjugated goat anti-rat IgG (H+L) secondary antibody (Life Technologies, Cat. No. A10540) at 1:100 dilution, whereas binding of mAbs and pAbs was detected as specified previously (section 3.2.1).
### Table 3.1. Summary of primary and secondary antibody incubations for all competition assays. Eth. Br.: ethidium bromide. CRL: control.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Aim of the assay</th>
<th>First antibody</th>
<th>Second antibody</th>
<th>Secondary detecting antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Competition between mouse IgMs and BC6 for surface binding to A4-IEs</strong></td>
<td>Investigating the effect of BC6 on mouse IgMs binding</td>
<td>BC6</td>
<td>Mouse IgMs</td>
<td>Goat anti-mouse IgM-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Investigating the effect of mouse IgMs on BC6 binding</td>
<td>Mouse IgMs</td>
<td>BC6</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>BC6 positive control</td>
<td>BC6</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Mouse IgMs positive control</td>
<td>Mouse IgMs</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for BC6</td>
<td>-</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for mouse IgMs</td>
<td>Isotype IgM CRL</td>
<td></td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td><strong>Competition between mouse IgMs and HIS for surface binding to A4-IEs</strong></td>
<td>Investigating the effect of HIS on mouse IgMs binding</td>
<td>HIS</td>
<td>Mouse IgMs</td>
<td>Goat anti-mouse IgM-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Investigating the effect of mouse IgMs on HIS binding</td>
<td>Mouse IgMs</td>
<td>HIS</td>
<td>Mouse anti-human IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>HIS positive control</td>
<td>HIS</td>
<td>-</td>
<td>Mouse anti-human IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Mouse IgMs positive control</td>
<td>Mouse IgMs</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for HIS</td>
<td>Non-immune UK sera</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for mouse IgMs</td>
<td>Isotype IgM CRL</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td><strong>Competition between mouse IgMs and rat anti-DBL16 antisera for binding to A4-IEs</strong></td>
<td>Investigating the effect of antisera on mouse IgMs binding</td>
<td>Rat antisera</td>
<td>Mouse IgMs</td>
<td>Goat anti-mouse IgM-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Investigating the effect of mouse IgMs on antisera binding</td>
<td>Mouse IgMs</td>
<td>Rat antisera</td>
<td>Goat anti-rat IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Rat antisera positive control</td>
<td>Rat antisera</td>
<td>-</td>
<td>Goat anti-rat IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Mouse IgMs positive control</td>
<td>Mouse IgMs</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for rat antisera</td>
<td>-</td>
<td>-</td>
<td>Goat anti-rat IgG-APC + Eth. br</td>
</tr>
<tr>
<td></td>
<td>Negative control for mouse IgMs</td>
<td>Isotype IgM CRL</td>
<td>-</td>
<td>Goat anti-mouse IgG-APC + Eth. br</td>
</tr>
</tbody>
</table>
3.3 Binding of antibodies to trypsin-treated IEs

The effect of protease treatment on IE surface binding to mouse IgM was investigated to determine if binding epitopes on the surface of IEs are affected after trypsin treatment. Mature pigmented trophozoites at mid-late stage were enriched from cultures at ~3-8% parasitaemia and a HCT of 1-2%, using plasmagel floatation. Enriched cells were washed twice in PBS and incubated with 1 mg/ml, 100 µg/ml, 10 µg/ml or 1 µg/ml of tosylamido-2-phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma®, product No. T1426, 10 mg/ml) in PBS for 30 minutes at 37°C. Control, undigested samples were incubated with PBS and assayed in parallel with test samples. After incubation, trypsin activity was inhibited by incubating cells with an equivalent volume of soybean trypsin inhibitor at 1 mg/ml (Sigma®, T6414, Lot No. SLBF3888V) for 15 minutes at 37°C. An additional tube including trypsin-undigested cells was incubated with soybean inhibitor and assayed as a negative control. Cells were thoroughly washed (×2) to remove excess trypsin and/or soybean inhibitor and re-suspended in PBS. 100 µl of suspended cells (~1.0-2.5 × 10^6 cells) were then incubated with mouse IgMs and binding investigated by flow cytometry as previously (section 2.4.2.2). The same procedure was used to investigate the effect of trypsin digestion on BC6 (20 µg/ml) or HIS (30 mg/ml) binding with some minor modifications including secondary antibodies used for detection (see sections 3.2.1 and 3.2.2).

The effect of trypsin digestion on antibody binding was assessed in terms of differences in surface labelling of antibodies to trypsin-treated IEs compared to untreated cells. The percentage of reduction in surface labelling to IEs were quantitated following the formula:

\[
\chi = 100 - \left[\left(\frac{a}{b}\right) \times 100\right]
\]

Where, \(\chi\) = the percentage of reduction in surface labelling
\(a\) = percentage of antibody labelling to trypsin-treated IEs
\(b\) = percentage of antibody labelling to trypsin-untreated IEs (incubated with PBS)
3.4 Binding of trypsin-treated ItG-IEs to ICAM-1 receptors

Trypsin sensitivity of ItG-IEs cytoadherence to purified ICAM-1 receptors was assessed by static protein assay as previously described (McCormick et al., 1997). Briefly, binding buffer (RPMI 1640, with 5 mM glucose, pH 7.2) was first prepared and incubated at 37°C prior to the assay. Then, a trophozoite-IE suspension in pre-warm binding buffer and at parasitaemia of 3% and HCT of 1% was prepared for the static assay. Cells were incubated with different concentrations of TPCK-treated trypsin (1000 µg/ml, 100 µg/ml or 10 µg/ml) following the same protocol in section 3.3. After the final wash, trypsin-digested cells or control samples (undigested cells incubated with PBS) were added to petri dishes pre-coated with purified ICAM-1 protein (50 µg/ml) or PBS, and the static protein assay was carried out as described in Chapter 4.

3.5 Localisation of IgM surface labelling using IFA

Binding of mouse antibodies to the external surface of live IEs was visualised and localised using live liquid-phase immunofluorescence assay (L-IFA) as described elsewhere (Ghumra et al., 2008) with minor modifications. MAb BC6 was used as a positive control which specifically recognises an exposed epitope on PfEMP1 of A4-IEs, and shows a distinct punctate staining over the IE (Hughes et al., 2010). A4 trophozoite-IEs, from stable cultures, at 3-8% parasitaemia and a HCT of 1-2%, were enriched using plasmagel floatation. After two rounds of washing in PBS/1%BSA, unfixed cells were incubated (200 µl, 2.5-3 × 10⁷ cells/ml) with 100 µl of mAb BC6 (20 µg/ml in PBS/1%BSA) or 100 µl of mAb E7 (57 µg/ml), for 30 minutes at 37°C. After incubation, cells were washed in PBS/1%BSA and IgM labelling was detected by incubation with Alexa Fluor®488 goat anti-mouse IgM (µ chain) secondary antibody (Molecular Probes, Life technologies, A21042) diluted 1:100 plus 10 µg/ml of 4',6-diamidino-2-phenylindole dihydrochloride [DAPI] (Life technologies, Ca. No. D1306) at 37°C for 60 minutes in darkness. BC6 was detected by incubating cells with Alexa Fluor®488 goat anti-mouse IgG (H+L) secondary antibody.
(Molecular Probes, Life technologies, A11001) at the same conditions. DAPI was used to differentiate IEs from non-IEs, as it only stains nuclei of IEs. IEs were washed as above, mounted on a microscope slide and inspected by Zeiss Axioskop 40 fluorescence microscope fitted with AxioCam MRc digital camera. Cells incubated with 20 µg/ml mouse IgM isotype control (clone MOPC 104E, Sigma-Aldrich®, M5909, 200 µg/ml) and stained with secondary IgM + DAPI or those incubated secondary IgG + DAPI were included as negative controls for E7 and BC6, respectively. A4 parasite culture was selected for BC6 binding to enhance the surface labelling signal. It was established that incubation of BC6 at a range of temperatures (4-37°C) did not affect the pattern of fluorescence (Horrocks et al., 2005). However, all incubations were carried out at 37°C as with flow cytometry.

3.6 IgM-mediated IE lysis

3.6.1 Quantitation of lysed IEs by flow cytometry

Percentage of lysed IEs resulted from mouse mAb/pAb incubation was calculated for all tested parasite isolates using flow cytometry data. As the percentage of IE population was obtained by gating SSC-A vs. FITC-A, differences in this percentage between isotype IgM control and test antibodies were used to assess the percentage of lysed IEs. This was calculated following the formula:

\[ x = 100 - \left[ \left( \frac{a}{b} \right) \times 100 \right] \]

Where, \( x \) = the percentage of lysed IEs
\( a \) = percentage of IE population for test antibodies (mouse IgMs)
\( b \) = percentage of IE population for isotype IgM control
Grading of haemolysis based on the percentage of lysed IEs was set as followed:

- : lysed IEs ≤ 15%
+ : lysed IEs 15.1-40.9%
++ : lysed IEs 41-80.9%
+++ : lysed IEs ≥ 81% of IEs.

We confirmed that mainly IEs were lysed upon antibody incubation by counting 50,000 cells without a stopping gate (i.e. 50,000 IEs and non-IEs were counted) for both IgM control and test sample. The positive lysis of IEs (i.e. ≥ 15%) was set at this percentage since up to ~12% of non-specific IE lysis could be detected with control antibodies.

### 3.6.2 Measurement of lysed IEs by microscopy

20 ml of mixed-stage ItG-IEs from culture was washed twice in PBS/1%BSA, pelleted by centrifugation and 300 µl of this was used to make a thin blood film on a microscopic slide. The smear was air-dried and fixed in 100% methanol for 5-10 seconds, before the film was washed in water and stained with 5% Giemsa stain for 20 minutes. The slide was washed and examined under the microscope using 100x oil immersion objective. 1000 RBCs were counted and the percentage of trophozoites/schizonts was estimated relative to the total number of cells. Then, 1 ml of the same suspension was incubated with 100 µl of pool 2, pAb C2, or PBS for 5, 15, 30 and 60 minutes at 37°C. The percentage of trophozoites/schizonts was quantitated at the end of each time point as described above. The percentage of lysed IEs was then quantitated for control or test samples in relative to the percentage of trophozoites/schizonts before incubation.

### 3.7 Statistical analysis

Spearman's rho correlation was used for correlations between variables using SPSS (version 22) and Spearman's rho correlation coefficient ($r_s$) was used to determine the strength of the association. A two-tailed t-test was used for testing the significance of the correlation, which was considered as statistically significant when $P$ value < 0.05.
3. Results

3.1 Labelling of mouse mAbs and pAbs to the surface of *P. falciparum* IEs

As described in Chapter 2, we generated mAbs and pAbs by immunising mice with four different rDBLβ domains from PfEMP1ICAM-1 variants. We first carried out flow cytometry experiments to identify optimal IgM concentrations of mouse mAbs and pAbs for labelling IEs. Purified mAbs and pAbs were titrated out to compare the relative binding strengths of IgM preparations with the surface of live IEs. This was done by incubating trophozoite-rich suspensions with dilutions of mAbs and pAbs, starting at their original concentrations after purification down to 1:8 dilution. Table 3.1 summarises the binding profile of mAbs and pAbs to the surface of erythrocytes infected with ItG *P. falciparum* isolate. The end titre is identified as the lowest antibody concentration that labels > 10% of IEs in flow cytometry (Figure 3.1, only showing the titration of mAbs E7 and B4 with ItG-IEs). We found that the percentage of IEs recognised by mAbs and pAbs depended on the dose of IgM used. Most antibodies were found to efficiently label IEs at their original concentration and lost the surface binding capacity with increasing dilution except mAbs E7, B4 and G6, which continued to recognise the surface of IEs down to 1:4 dilution but with a significant decrease in the percentage of labelling (Table 3.1). Due to the reduced signal with dilution, all mAbs and pAbs tested in subsequent work were used at concentrations yielded after affinity purification (rather than using diluted IgMs or un-purified supernatants), unless otherwise stated.
<table>
<thead>
<tr>
<th>mAbs/pAbs</th>
<th>Concentration (µg/ml)</th>
<th>IgM titration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neat</td>
</tr>
<tr>
<td>pAb E11</td>
<td>16.9</td>
<td>++</td>
</tr>
<tr>
<td>pAb B12</td>
<td>0.1</td>
<td>+</td>
</tr>
<tr>
<td>mAb A6A</td>
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<td>+</td>
</tr>
<tr>
<td>pAb C2</td>
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<td>-</td>
</tr>
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</tr>
<tr>
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<td>++++</td>
</tr>
<tr>
<td>mAb C9</td>
<td>11.1</td>
<td>+</td>
</tr>
<tr>
<td>pAb 2E10</td>
<td>9.6</td>
<td>++++/+</td>
</tr>
<tr>
<td>pAb B5</td>
<td>59</td>
<td>++++</td>
</tr>
<tr>
<td>pAb D5</td>
<td>31.4</td>
<td>++++/++</td>
</tr>
<tr>
<td>mAb B4</td>
<td>29.7</td>
<td>+++</td>
</tr>
<tr>
<td>mAb G6</td>
<td>66.3</td>
<td>+++</td>
</tr>
<tr>
<td>Control IgM</td>
<td>20</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1. Labelling capacity of mouse mAbs and pAbs to the surface of erythrocytes infected with Ig G P. falciparum isolate at neat, 1:2, 1:4 and 1:8 dilutions. - (labelled IE population ≤ 10%), + (labelled IE population 10.1 to 20%), ++ (labelled IE population 20.1 to 40%), +++ (labelled IE population 41 to 60%), ++++ (labelled IE population > 60.1%). Control IgM: mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®). Results are based on at least two experiments.
Figure 3.1. FACS histograms of gated erythrocytes infected by ItG isolate and labelled by mAbs E7 and B4 at neat concentrations or at 1:2, 1:4 or 1:8 dilutions. Labelling was detected using APC-conjugated goat anti-mouse IgM (μ chain specific) secondary antibody (Southern Biotech) at 1:100 dilution. Cells stained with 20 μg/ml of mouse IgM isotype control from murine myeloma clone MOPC 104E (Sigma®) were included as a negative control and are shown by the red trace. The grey shaded area represents the distribution of IEs labelled by mAbs E7 and B4. Percentages of IEs labelled with reactive IgM antibodies are shown as APC-A-positive subset and were obtained relative to a background labelling of control IgM antibodies. A labelling of > 10% of IE population was considered positive.
Then, we tested the capacity of mouse mAbs and pAbs to label the surface of erythrocytes infected by eight laboratory adapted *P. falciparum* isolates with different genotypes and phenotypes, including isolates from which the recombinant proteins were cloned and with which mAbs/pAbs were raised (Table 3.3). In addition, geometric mean fluorescent intensity (gMFI) values for the labelling of mAbs/pAbs to the surface of erythrocytes infected by eight *P. falciparum* isolates are shown in Table S2 in the Appendix. Values of gMFI and percentages of labelling are consistent for almost all mAbs and pAbs, as mAbs/pAbs that show higher percentages of surface labelling have the highest gMFI values and vice versa.

The origin, dominant transcribed var genes as well as adhesion characteristics of tested *P. falciparum* isolates are summarised in Table 3.2. The var gene transcription profiles of five *P. falciparum* isolates (ICAM-1-binding lines) are shown in Figure S2 in the Appendix. The results show that parasite isolates expressed the expected var transcript more than five fold changes. IT4var16 also expressed another var transcript, IT4var41, besides its corresponding var transcript (IT4var16).

As described in Chapter 2, antibody fractions that showed the highest fluorescence signal in screening assays were selected for all subsequent analyses. The binding profiles for mAbs and pAbs to ICAM-1 binding and non-binding isolates, as well as to parasite isolates of distinct genotypes will be discussed in detail later in this chapter.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Alias</th>
<th>Genome</th>
<th>Dominant transcribed var gene</th>
<th>Ups</th>
<th>Adhesion phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT4var01</td>
<td>3G8 (5)</td>
<td>IT4</td>
<td>var01</td>
<td>UpsC (3,1)</td>
<td>ICAM-1 (1, 5) CD36 (5) Rosetting (2)</td>
</tr>
<tr>
<td>IT4var13</td>
<td>GC503</td>
<td>IT4</td>
<td>var13</td>
<td>UpsB (3)</td>
<td>ICAM-1 (1)</td>
</tr>
<tr>
<td>IT4var14</td>
<td>A4, A4var (1, 2)</td>
<td>IT4</td>
<td>var14</td>
<td>UpsB (3,1)</td>
<td>ICAM-1 (4, 1, 2), CD36 (4, 2)</td>
</tr>
<tr>
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<td>ItG, IT-ICAM (2)</td>
<td>IT4</td>
<td>var16</td>
<td>UpsB (3)</td>
<td>ICAM-1 (4, 1, 2), CD36 (4, 2)</td>
</tr>
<tr>
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<td>2F6</td>
<td>IT4</td>
<td>ND</td>
<td>UpsB (3,1)</td>
<td>ICAM-1 (1) Rosetting (2)</td>
</tr>
<tr>
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<td>P5B6</td>
<td>IT4</td>
<td>var31</td>
<td>UpsB (3,1)</td>
<td>ICAM-1 (1, 2, 5), CD36 (2, 5)</td>
</tr>
<tr>
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<td></td>
<td>IT4</td>
<td>ND</td>
<td>ND</td>
<td>CD36 (4)</td>
</tr>
<tr>
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<td>NF54</td>
<td>3D7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CS2</td>
<td>CS2 var (2)</td>
<td>IT4</td>
<td>ND</td>
<td>ND</td>
<td>CSA, (2, 6), HA (6)</td>
</tr>
</tbody>
</table>

Table 3.2: The origin and properties of nine distinct *P. falciparum* isolates used in the study.

References: 1 (Howell et al., 2008); 2 (Kraemer et al., 2007); 3 (Avril et al., 2012); 4 (Gray et al., 2003); 5 (Janes et al., 2011); 6 (Maier et al., 2007). ND: no data.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Domain</th>
<th>Specificity</th>
<th>3G8</th>
<th>GC503</th>
<th>A4</th>
<th>ItG</th>
<th>P5B6</th>
<th>C24</th>
<th>3D7</th>
<th>CS2</th>
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<tbody>
<tr>
<td>E11</td>
<td>DBL13</td>
<td>Polyclonal</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>B12</td>
<td>DBL13</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A6A</td>
<td>DBL13</td>
<td>Monoclonal</td>
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<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E4</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A4</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
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<td>+++</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
<td>+++</td>
<td>+</td>
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<td>Polyclonal</td>
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<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
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<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
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<td>Monoclonal</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
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Table 3.3. The reactivity of monoclonal and polyclonal anti-DBLβ antibodies to the surface of erythrocytes infected by phenotypically and genotypically distinct *P. falciparum* isolates as measured by flow cytometry. Two experiments were carried out to confirm reactivity. - (labelled IE population ≤ 10%), + (labelled IE population 10.1-20%), ++ (labelled IE population 20.1-40%), +++ (labelled IE population 41-60%), ++++ (labelled IE population > 60.1%). ND: no data.
3.1.1 Measurement of IgM binding to the surface of ICAM-1 binding P. falciparum-IEs

Mouse mAbs and pAbs were characterised for their capacity to recognise the surface of RBCs infected by five ICAM-1 binding P. falciparum isolates from the IT4 genotype (3G8, GC503, A4, ItG and P5B6). All mAbs and pAbs, except pAb C2, exhibited variable levels of IgM surface recognition of erythrocytes infected by at least one ICAM-1 binding isolate with a labelling capacity ranging from 10.1% to 67.6% of total IEs (Table 3.3). All antibodies raised to rDBL41 (pAbs B5 and D5, and mAbs B4 and G6) and a mAb raised to rDBL31 (E7), were the most effective in surface recognition. On the contrary, there was little or no binding with pAbs B12, C2, E4, 2E10 and mAb C9 against all tested isolates. There was no clear pattern of strain-specific recognition by mAbs and pAbs to erythrocytes infected by homologous parasite isolates (GC503 and P5B6), as all positive mAbs and pAbs equally recognised homologous and heterologous isolates. However, pAb A4 recognised erythrocytes infected by its corresponding isolate (P5B6) more specifically than heterologous ICAM-1 binding strains. A representative flow cytometry surface labelling by mAbs and pAbs to erythrocytes infected by the strong ICAM-1 binder ItG isolate is shown in Figure 3.2.
Figure 3.2. FACS histograms of gated erythrocytes infected by ItG isolate and labelled by mAbs and pAbs. 50 µl of mouse IgM antibodies were incubated with plasmagel-enriched trophozoite-IEs and labelling was detected using APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech® at 1:100 dilution). Shaded histograms show IEs populations labelled by mouse mAbs/pAbs. Negative controls including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in blue and those only stained with ethidium bromide and secondary antibodies shown in red. Percentages of IEs labelled with reactive IgM antibodies are shown as APC-A-positive subset and were obtained relative to a background labelling of control IgM antibodies. A labelling of > 10% of IE population was considered positive.
3.1.2 Measurement of IgM binding to the surface of non-ICAM-1 binding *P. falciparum* IEs

We next tested whether the surface-reactive antibodies could also recognise the surface of erythrocytes infected with the non-ICAM-1 binding C24 *P. falciparum* isolate from the IT4 genome (Figure 3.3). Overall, the labelling profile resembled that of ICAM-1 binding *P. falciparum* isolates and IgMs that strongly label ICAM-1 binding IEs also labelled C24-IEs with a similar pattern (Table 3.3). The only exception was observed with DBL13-specific pAbs (E11 and B12), which recognised the surface of erythrocytes infected by ICAM-1-binding isolates but did not react with those infected with C24 (Fig 3.3). This indicates that most mAbs and pAbs lack phenotype specificity and were equally cross-reactive with non-ICAM-1 binding isolates despite being raised to ICAM-1 binding DBLβ variants.

The next question addressed was whether mAbs and pAbs had the ability to recognise erythrocytes infected by 3D7, a genotypically distinct *P. falciparum* isolate, to further characterise the specificity of mouse IgM antibodies against heterologous strains (Figure 3.4). We detected IgM surface-fluorescence levels with 3D7 strain similar to those observed with IT4 isolates (Table 3.3). These results may suggest that *P. falciparum* isolates of distinct genotypes share common surface epitopes that can be recognised by our mAbs and pAbs. It is not yet known whether shared epitopes are indeed located on the native PfEMP1, however, the fact that mAbs and pAbs only label IEs indicates that labelling is specific for surface-exposed parasite-derived antigens. It is noteworthy that trophozoite-enrichment of 3D7 strain was not achievable with the plasmagel floatation procedure used for all tested isolates, as overgrown 3D7 used in this assay may have lost surface knobs. Therefore, trophozoites were enriched over a magnetic field and the elution (enriched pellet) contained almost 90-95% of trophozoites.
Figure 3.3. The reactivity of mouse mAbs and pAbs with parasite-derived proteins on the surface of C24-IEs as measured by flow cytometry. 50 µl of mouse IgM antibodies was incubated with plasmagel-enriched trophozoite-IEs and labelling was detected using APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech®) at 1:100 dilution. Shaded histograms show IEs populations labelled by mouse mAbs/pAbs. Negative controls including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in blue and those only stained with ethidium bromide and secondary antibodies shown in red. Percentages of IEs labelled with reactive IgM antibodies are shown as APC-A-positive subset and were obtained relative to a background labelling of control IgM antibodies. A labelling of > 10% of IE population was considered positive.
Figure 3.4. MAbs and pAbs surface reactivity with 3D7 *P. falciparum*-IEs as measured by flow cytometry. Trophozoites were enriched by exposure to strong magnetic field and 50 µl of mouse IgM antibodies was incubated with trophozoite-enriched IEs. Labelling was detected using APC-conjugated goat anti-mouse IgM (μ chain specific) secondary antibody (Southern Biotech®) at 1:100 dilution. The grey shaded area represents the IE population labelled by mAbs/pAbs. Negative controls including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in blue and those only stained with ethidium bromide and secondary antibodies shown in red. Percentages of IEs labelled with reactive IgM antibodies are shown as APC-A-positive subset and were obtained relative to a background labelling of control IgM antibodies. A labelling of > 10% of IE population was considered positive.
3.1.3 Measurement of IgM binding to the surface of non-IEs

Mouse IgMs were then investigated for their ability to recognise the surface of non-IEs for all *P. falciparum* isolates used in screening assays (Table 3.4). This was carried out to assess whether mAbs and pAbs only recognised parasite-derived VSAs on the surface of IEs or were cross-reactive with other ligands on the RBC surface. In general, the results showed that only some mAbs and pAbs that exhibited higher capacity for labelling the surface of IEs also exhibited variable degrees of cross-reactivity to non-IEs, whereas antibodies that moderately recognised the surface of IEs or non-labelling antibodies were not reactive with non-IEs. However, labelling to some *P. falciparum* isolates (i.e. GC503, 3D7 and CS2) was mainly specific to the surface of IEs for all tested mouse IgMs, whereas binding to non-IEs was negligible. The capacity of mAbs and pAbs to significantly label the surface of erythrocytes infected by GC503, 3D7 and CS2 isolates, but not non-IE populations, indicates that mouse IgMs were specific for a parasite-derived VSA on the surface of IEs, and confirms that non-specific labelling was only related to some *P. falciparum* isolates.

Labelling to non-IEs was mainly observed with mAb A6A, mAb E7, pAb B5, mAb B4 and mAb G6. These mAbs and pAbs were the most effective in IE surface recognition among all mouse antibodies. However, labelling to non-IEs for these mAbs and pAbs was not observed with all *P. falciparum* isolates. For instance, mAb E7 only showed a high background labelling of non-IEs with only 3 out of the 8 tested *P. falciparum* isolates, and pAb B5 labelled the surface of non-IEs for 4 *P. falciparum* isolates. Taken together, these results indicate that mouse mAbs and pAbs are mainly specific for the surface of IEs, and the background labelling observed with some mAbs/pAbs may suggest that mouse antibodies recognise a protein with the homologous structure present both on IE and non-IEs. A protein with such characteristics is parasite-modified band 3. Band 3 is a protein found abundantly on the surface of erythrocytes and acts as an anion transporter (Smith and Craig, 2005). Parasite-modified band 3 is abundantly found on the surface of IEs and has been suggested as an immune target and a cytoadhesion ligand for IE binding to TSP and CD36 (Winograd et al., 2004). Further studies are required to investigate the capacity of mAbs and pAbs to bind to band 3 and its parasite-mediated modified form.
Table S3 in the Appendix shows geometric mean fluorescent intensity (gMFI) values for the labelling of mAbs/pAbs to the surface of non-IEs for all *P. falciparum* isolates. Values of gMFI in Table S1 and percentages of labelling in Table 3.4 are consistent for almost all mAbs and pAbs, as mAbs/pAbs that show higher percentages of surface labelling have the highest gMFI values and vice versa.
<table>
<thead>
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<th>Antibody</th>
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<th>Specificity</th>
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<td></td>
<td></td>
<td></td>
<td>3G8</td>
</tr>
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Table 3.4. The reactivity of monoclonal and polyclonal anti-DBLβ antibodies to the surface of non-infected erythrocytes. Percentages of labelled RBC population were obtained from gated population of non-IEs and percentages of APC-A-positive cells were used in the table.
3.1.4 Correlation of IgM surface labelling among *P. falciparum* isolates

To compare the surface binding profiles of all tested isolates with each other, we calculated correlation coefficients between the labelling scores by 13 mAbs/pAbs for a single isolate with those for the other isolates using Spearman's rho \((r_s)\) correlation test. This enabled the identification whether surface labelling to erythrocytes infected by a particular isolate was correlated with labelling to the other tested *P. falciparum* strains. We found that all tested isolates, except CS2 and 3D7, showed a significant positive correlation with each other \((r_s = 0.56 - 0.95; P < 0.05; n = 13)\), i.e. if IgM binding to a particular isolate was detected, then IgM to the other isolate was also observed especially when higher levels of IgM binding were detected (Table 3.5). Labelling of CS2-IEs showed no significant correlation with all tested isolates \((P > 0.05)\) whereas binding to 3D7-IEs was significantly correlated with all tested isolates except C24 \((r_s = 0.52, P = 0.07)\) and CS2 \((r_s = 0.297, P = 0.33)\). Some individual mAbs/pAbs exhibited either substantial binding to distinct isolates or significant binding to other isolates which explains the variation in correlation results. These results indicate each individual mAb or pAb possesses an overlapping cross-reactivity with *P. falciparum* isolates, regardless of their binding phenotype or genotype. However, since the numbers used in this correlation analysis and in subsequent analyses in this study are not large enough to say much about the presence or absence of significant correlations, interpretation of correlations that did not reach statistical significance must be carried out with extra caution.
Table 3.5. Spearman's rho correlation of the surface labelling profiles of thirteen mAbs/pAbs to erythrocytes infected by eight *P. falciparum* parasite isolates. Binding of IgM to the surface of erythrocytes infected by 3G8, GC503, A4, ItG, P5B6, C24, 3D7 or CS2 was determined using flow cytometry and surface labelling was expressed as percentage of IEs labelled by mAbs and pAbs relative to control IgM binding. Values of Spearman's rho correlation coefficient ($r_s$): 0 (no correlation between variables), 0.1 to 0.3 (weak positive correlation), 0.3 to 0.5 (moderate positive correlation), 0.5 to 1.0 (strong positive correlation). Correlation is statistically significant when $P$ value $<$ 0.05. *: correlation is significant at the 0.05 level. **: correlation is significant at the 0.001 level.

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<th>Spearman's rho</th>
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<td>.791**</td>
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<td>.923**</td>
<td>.841**</td>
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<td>.544</td>
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3.1.5 Correlation between IgM reactivity to recombinant domains and IE surface labelling

The association between IgM reactivity to recombinant domains in ELISA and surface labelling to IEs in flow cytometry was investigated to determine whether recognition of recombinant proteins used in immunisations correlated with the binding to live infected cells. The analysis revealed a strong positive correlation between surface labelling of all tested isolates and the reactivity to recombinant DBL31 proteins, indicating that IgM antibodies that showed higher capacity of surface labelling to IEs also exhibited higher levels of reactivity to recombinant domains (Table 3.6). A statistically-significant correlation was observed with all tested isolates ($r_s \geq 0.56$, $P < 0.05$) except for GC503 and CS2 isolates, which showed $r_s$ values $\leq 0.53$ ($P > 0.05$).

<table>
<thead>
<tr>
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<th>ItG</th>
<th>PFB6</th>
<th>C24</th>
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<td>0.593</td>
<td>0.74</td>
<td>0.7</td>
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<td>0.46</td>
</tr>
<tr>
<td>$P$ value</td>
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<td>0.019*</td>
<td>0.033*</td>
<td>0.004*</td>
<td>0.007*</td>
<td>0.047*</td>
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</table>

Table 3.6. Correlation between surface labelling of 13 mAbs and pAbs to erythrocytes infected by eight *P. falciparum* isolates and reactivity to rDBL31. Binding of mouse IgM to the surface of erythrocytes infected by all isolates was expressed as percentage of labelled IEs, whereas values of recognition to recombinant DBL31 domains were expressed as the mean OD values of each mAb/pAb subtracted from (OD value of the control + 3 SDs). The strength of correlation was determined as described in Table 3.4. Correlation is statistically significant when $P$ value $< 0.05$. *: correlation is significant at the 0.05 level.
3.2 Competition assays

A flow cytometric analysis was carried to investigate the influence of pre-incubating IEs with mAbs and pAbs on subsequent binding of well-characterised surface-labelling antibodies to further characterise the surface epitopes recognised by mouse IgM antibodies. Competition was defined as an alteration or complete inhibition in the relative ability of BC6, anti-DBL16 rat antisera or HIS to bind to the surface of IEs pre-incubated with mouse IgM antibodies and vice versa. Two pools of mouse IgMs that showed positive surface labelling were mainly used in the competition assays including pool 1 (pAb E11, pAb B12, mAb A6A and pAb E4) and pool 2 (mAb E7, pAb D5, mAb B4 and mAb G6). Antibody pools were primarily used at this point of study due to lack of materials available to do all experiments with individual antibodies.

3.2.1 The effect of pre-incubation of A4-IEs with mAbs and pAbs on BC6 surface binding

We tested the ability of mouse IgMs to interfere with the interaction between mAb BC6 and PfEMP1 on the surface of erythrocytes infected with A4 isolate to investigate the specificity of mouse IgMs to bind surface PfEMP1 molecules. BC6 is a mouse monoclonal IgG antibody that specifically recognises an exposed epitope on PfEMP1 of erythrocytes infected by A4 P. falciparum isolate (Roberts et al., 1992, Horrocks et al., 2002). Analysis of BC6 binding to A4-IEs by flow cytometry showed that 60% (Adams et al., 2000) and up to 80% (Hughes et al., 2010) of IEs are recognised by mAb BC6. We therefore hypothesised that inhibition of BC6-PfEMP1 interaction may indicate that mouse IgMs fully or partially bind to PfEMP1 on the surface of IEs. In the first assay, A4-IEs were pre-incubated with mAb BC6, then washed and co-incubated with mouse IgMs and tested by flow cytometry for surface fluorescence (Figure 3.5). The competition results showed that pre-incubating IEs with BC6 reduced the subsequent binding of pool 1 and pool 2 mouse IgM antibodies by 25% and 20%, respectively. On the other hand, pre-incubating IEs with pool 1 reduced the subsequent binding of BC6 by 45%, whereas pool 2 only inhibited 10% of IEs labelling (Figure 3.5).
Figure 3.5. Flow cytometry analysis of the effect of pre-incubating A4-IEs with mouse mAbs and pAbs on the subsequent binding of mAb BC6 (20 µg/ml) and *vice versa*. Shaded histograms represent IE populations labelled by mouse pools or BC6 antibodies. Negative controls for mouse pools including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in red, or those only stained with ethidium bromide and anti-mouse IgG secondary antibodies shown in green (for BC6). A: binding of pool 1 (left), the subsequent binding of pool 1 after pre-incubation with BC6 (right), binding of pool 2 (bottom left) and the subsequent binding of pool 2 after pre-incubation with BC6 (bottom right). B: binding of mAb BC6 (left), the subsequent binding of BC6 after pre-incubation with pool 1 (middle) or pool 2 (right). Labelling was detected using APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech®) for mouse pools or with APC-conjugated goat anti-mouse IgG secondary antibody (Thermo Scientific) for mAb BC6. IgG or IgM surface labelling were expressed as percentage of APC-positive labelled IEs (grey shades) compared to background labelling (red or green). A labelling of > 10% of IE population was considered positive. Pool 1 (14 µg/ml) includes pAb E11, pAb B12, mAb A6A and pAb E4 and pool 2 (51.6 µg/ml) includes mAb E7, pAb D5, mAb B4 and mAb G6.
3.2.2 The effect of pre-incubation of A4-IEs with mAbs and pAbs on HIS surface binding

We next investigated whether mouse IgMs can compete with pooled Malawian hyper-immune sera (HIS) for IE surface binding and vice versa. HIS comprises IgG class antibodies and demonstrates a high level of cross-reactivity against *P. falciparum* isolates of different phenotypes and genotypes (data not shown). We first investigated the surface fluorescence of HIS at different concentrations (5 mg/ml, 10 mg/ml, 30 mg/ml and 60 mg/ml) and found that surface labelling was relatively unaffected by increasing the concentration from 30 to 60 mg/ml (Figure 3.6), suggesting saturation of binding by HIS at 30 mg/ml. Although HIS at 10 or 30 mg/ml demonstrated similar levels of surface labelling, repeating assays confirmed that the latter concentration has a higher capacity for surface recognition and therefore 30 mg/ml was used in all subsequent assays.

We then tested the competition profile between our mAbs/pAbs and HIS and found that pre-incubating IEs with pool 1 significantly reduced the subsequent binding of HIS by 53%, whereas pool 2 inhibited IE binding by 57% (Figure 3.7). On the reverse experiment, pre-incubating IEs with HIS has reduced the binding of pool 1 and pool 2 by 45% and 37%, respectively. This interference of binding between mouse IgMs and HIS suggests that the binding site on the surface of IEs is closely related between mouse IgMs and HIS.
Figure 3.6. The reactivity of HIS against the surface of A4-IEs as measured by flow cytometry. 50 µl of HIS at 5 mg/ml, 10 mg/ml, 30 mg/ml or 60 mg/ml was incubated with plasmagel-enriched trophozoite-IEs and the labelling was detected using APC-conjugated mouse anti-human IgG-Fc secondary antibody (Southern Biotech) at 1:100 dilution. Shaded histograms show IEs populations labelled by IgG antibodies. Negative controls including cells stained with 50 µl of non-immune UK sera at 1:10 dilution shown in blue and those stained with ethidium bromide and secondary antibodies alone shown in red. A labelling of > 10% of IE population was considered positive.
Figure 3.7. Flow cytometry histograms of the effect of pre-incubating A4-IEs with mouse IgM pools on the subsequent binding of HIS (30 mg/ml) and vice versa. Shaded histograms show IE populations labelled by IgM pools or HIS. Negative controls including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in red or those stained with 50 µl of non-immune UK sera at 1:10 dilution shown in blue. A: binding of pool 1 (left), the subsequent binding of pool 1 after pre-incubation with HIS (right), binding of pool 2 (bottom left) and the subsequent binding of pool 2 after pre-incubation with HIS (bottom right). Binding of HIS (left), the subsequent binding of HIS after pre-incubation with pool 1 (middle) or pool 2 (right). IgG or IgM surface labelling expressed as percentage of APC-positive labelled IEs (grey shades) compared to background labelling (red or blue). Labelling was detected using APC-conjugated mouse anti-human IgG-Fc secondary antibody (Southern Biotech®) for HIS or with APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech®) for mouse IgM pools. A labelling of > 10% of IE population was considered positive. Pool 1 (14 µg/ml) includes pAb E11, pAb B12, mAb A6A and pAb E4 and pool 2 (51.6 µg/ml) includes mAb E7, pAb D5, mAb B4 and mAb G6.
3.2.3 The effect of pre-incubation of ItG-IEs with mAbs and pAbs on surface labelling of rat anti-DBL16 antisera

Two rats were immunised by recombinant DBL16 domains at Severn Biotech Ltd® (Worcestershire, United Kingdom) and anti-DBL16 antisera were tested for surface fluorescence with erythrocytes infected by the homologous ItG *P. falciparum* isolate. Preliminary flow cytometry scanning assays indicated that anti-DBL16 anti-sera specifically recognised ~70-78% of IE population when used at 1:10 dilution. On the other hand, other rat anti-sera raised to different domains (DBL13, DBL27, DBL31 and DBL41) showed little if any reactivity with ItG-IEs which suggests anti-DBL16 antisera specifically bind to ItG-IEs. Then we tested the competition between rat anti-sera and mouse IgMs in terms of surface labelling to ItG-IEs. Our results demonstrated that both IgM pools (pool 1 and pool 2) reduced subsequent surface labelling of rat anti-DBL16 antisera by ~20% when pre-incubated with IEs. On the other hand, rat antisera significantly interfered with mouse IgM binding with an inhibition percentage of 55% and 36.2% for pool 1 and pool 2 surface binding, respectively as would be expected for polyclonal anti-sera (Figure 3.8).
Figure 3.8. Flow cytometry histograms of the effect of pre-incubation of IgG-IEs with mouse IgM pools on the subsequent labelling of rat anti-DBL16 antisera at 1:10 dilution and vice versa. Shaded histograms show IE populations labelled by mouse pools or anti-DBL16 antisera. Negative controls including cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in red or those stained with 50 µl of rat pre-immune sera at 1:10 dilution shown in green. A: binding of pool 1 (left), the subsequent binding of pool 1 after pre-incubation with rat anti-DBL16 antisera (right), the binding of pool 2 (bottom left) and the subsequent binding of pool 2 after pre-incubation with rat anti-DBL16 antisera (bottom right). B: the binding of rat anti-DBL16 antisera (left), the subsequent binding of rat anti-DBL16 antisera after pre-incubation with pool 1 (middle) or pool 2 (right). Labelling was detected using APC-conjugated goat anti-rat IgG secondary antibody for rat anti-DBL16 antisera or with APC-conjugated goat anti-mouse IgM (µ chain specific) secondary antibody (Southern Biotech®) for mouse pools. IgG or IgM surface labelling expressed as percentage of APC-positive labelled IEs (grey shades) relative to background labelling (in red and green). A labelling of > 10% of IE population was considered positive. Same pools were used as described in Figures 3.5 and 3.7.
3.3 Mouse IgM binding to trypsin-treated IEs

The effect of protease digestion on IE surface proteins on mouse IgM binding was investigated to determine if binding epitopes on the surface of IEs are affected after trypsin treatment. In addition, this experiment was carried out to assess the contribution of PfEMP1, an extremely trypsin-sensitive protein, in the binding of mouse mAbs and pAbs. *P. falciparum* IEs were incubated with 1 mg/ml, 100 µg/ml or 10 µg/ml of TPCK-treated trypsin and tested for surface fluorescence. Firstly, the effect of trypsin treatment on intact IEs was evaluated either with static adhesion assay (for ItG-IEs, Figure 3.9) or flow cytometry (for A4-IEs with mAb BC6, Figure 3.10).

In the static adhesion assay, trypsin treated or untreated ItG-IEs were investigated for static adhesion to purified ICAM-1 proteins (50 µg/ml) immobilised on plastic dishes. We found that ICAM-1 binding with this parasite line is highly sensitive to cleavage by trypsin as ItG-IEs binding to ICAM-1 receptors was almost completely abolished at all tested concentrations (Figure 3.9). At a concentration of 10 µg/ml, the mean inhibition of adhesion was 98.3%, at 100 µg/ml and 1 mg/ml, the mean inhibition was 99%. This indicates that lower concentrations of trypsin (down to 10 µg/ml) were effective in cleaving all the PfEMP1 ligand involved in ICAM-1 binding.
Figure 3.9. Adhesion of trypsin-treated ItG-IEs to immobilised ICAM-1 protein. ItG-IEs were pre-treated with 1 mg/ml, 100 µg/ml or 10 µg/ml TPCK-treated trypsin (Sigma) diluted in PBS (100 µl) then added to petri dishes pre-coated with spots of ICAM-1 protein (50 µg/ml) or PBS. Dishes were washed, fixed and stained as described in Materials and Methods. Bound IEs to protein or control spots were visualised and counted under the microscope. Data represent 36 readings obtained from 6 ICAM-1 spots, from two independent dishes. Values expressed as mean number of bound IEs per mm² ± Std. Dev. No trypsin = IEs incubated with PBS (control).

The effect of trypsin digestion on intact IEs was further evaluated by flow cytometry in terms of binding of the well-characterised mAb BC6 to A4-IE surface. As noted before, mAb BC6 binds an exposed epitope on PfEMP1 of A4-IEs (Roberts et al., 1992, Horrocks et al., 2002) and therefore the least trypsin concentration required for inhibiting BC6 binding was indicative for cleaving the entire PfEMP1, or at least a functional epitope on this large molecule. We found that treating IEs with 1 µg/ml of TPCK-treated trypsin caused a comparatively modest reduction in BC6 surface binding by about 39% relative to control (Table 3.7, Figure 3.10). However, the binding epitope for BC6 on PfEMP1 was almost cleaved at ≥ 10 µg/ml trypsin concentrations (at 10 µg/ml, 100 µg/ml and 1 mg/ml, percentage of IE labelling was reduced by 97.9%, 98.9% and 98.6%, respectively). This supports previous findings of ICAM-1 adhesion that only 10 µg/ml of trypsin was required for cleaving functional binding epitopes on PfEMP1, if not the entire molecule.
<table>
<thead>
<tr>
<th>Trypsin concentration (µg/ml)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of IEs labelled by BC6</td>
<td>67.9%</td>
<td>41.3%</td>
<td>1.37%</td>
<td>0.73%</td>
<td>0.98%</td>
</tr>
<tr>
<td>Percentage of reduction relative to positive control</td>
<td>-</td>
<td>39.2%</td>
<td>97.9%</td>
<td>98.9%</td>
<td>98.6%</td>
</tr>
</tbody>
</table>

Table 3.7. Labelling of mAb BC6 to trypsin-treated A4-IEs as measured by flow cytometry. IEs were treated with 1 mg/ml, 100 µg/ml, 10 µg/ml or 1 µg/ml of TPCK-treated trypsin (Sigma) diluted in PBS and tested for binding with mAb BC6 (20 µg/ml). Cells incubated with PBS and labelled by BC6 were included as a positive control. Surface labelling was indicated in terms of the percentage of IE population labelled by mAb BC6.

Table 3.10. Flow cytometry histograms of mAb BC6 labelling to trypsin-treated A4-IEs. Trophozoite-enriched cultures were pre-treated with 1000 µg/ml, 100 µg/ml, 10 µg/ml or 1 µg/ml of TPCK-treated trypsin and tested with BC6 mAb (20 µg/ml) for surface labelling. BC6 labelling was detected using APC-conjugated goat anti-mouse IgG secondary antibody (Thermo Scientific) at 1:100 dilution. IEs stained with ethidium bromide and anti-mouse IgG secondary antibodies were used to exclude the non-specific background labelling of IEs and shown as APC-negative IE population (subset). BC6 surface labelling expressed as percentage of APC-positive IE population compared to background labelling of the control. Cells incubated with PBS and labelled by BC6 were included as a positive control for trypsin digestion. A labelling of > 10% of IE population was considered positive.
We then tested the binding of pAb B5, mAb G6 and pool 1 of mouse IgMs to the surface of trypsin-treated ItG-IEs to investigate the effect of trypsin digestion on mouse IgM labelling (Table 3.8, Figure 3.11). Malawian HIS was also tested as it resembled the mouse IgM antibodies in terms of cross-reactivity with heterogeneous *P. falciparum* isolates. The results indicated that surface labelling of mAb G6 to ItG-IEs was relatively more sensitive to trypsin digestion at all concentrations compared to pAb B5, and labelling was greatly reduced as the concentration of trypsin increased. A similar sensitivity to trypsin digestion, albeit to a lower extent, was observed with the HIS and pool 1. On the other hand, labelling of pAb B5 was mostly unaffected at 10 µg/ml and 100 µg/ml trypsin treatment but significantly reduced with 1 mg/ml trypsin digestion. Correlation analyses of the parameters revealed a statistically significant strong negative correlation between trypsin concentration and percentage of labelled IEs by mAb G6, pool 1 and HIS ($r_s = -1.0, P < 0.0001$). The correlation was strong but not significant with pAb B5 ($r_s = -0.8, P > 0.05$).

Table S4 in the Appendix shows geometric mean fluorescent intensity (gMFI) values for the labelling of pAb B5, mAb G6, pool 1 and HIS to the surface of trypsin-treated and non-treated ItG-IEs. Values of gMFI in Table S4 and percentages of labelling in Table 3.8 are consistent for all mAbs/pAbs and HIS, as antibodies that show higher percentages of surface labelling have the highest gMFI values and vice versa.
Figure 3.11. Flow cytometry histograms of mAb G6, pAb B5, pool 1 of mouse IgMs and HIS labelling to trypsin-treated ItG-IEs. Trophozoite-enriched cells were pre-treated with 1000 µg/ml, 100 µg/ml or 10 µg/ml of TPCK-treated trypsin and tested with mAbs/pAbs or HIS for surface labelling. Cells incubated with PBS and labelled by IgGs or IgMs were included as a positive control for surface labelling. Cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in red or those only stained with ethidium bromide and secondary antibody (green) were included as negative controls for mouse IgM antibodies. IgG or IgM surface labelling expressed as percentage of APC-positive labelled IEs (grey shades) relative to background labelling (red or green). A labelling of > 10% of IE population was considered positive.
<table>
<thead>
<tr>
<th>Trypsin concentration (µg/ml)</th>
<th>PBS</th>
<th>1000</th>
<th>100</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of IEs labelled by mAb G6</td>
<td>65.2%</td>
<td>24.5%</td>
<td>30.1%</td>
<td>41.7%</td>
</tr>
<tr>
<td>Percentage of reduction compared to IgM control</td>
<td>-</td>
<td>62.4%</td>
<td>53.8%</td>
<td>36%</td>
</tr>
<tr>
<td>B5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of IEs labelled by pAb B5</td>
<td>53.8%</td>
<td>24.5%</td>
<td>52.4%</td>
<td>55.3%</td>
</tr>
<tr>
<td>Percentage of reduction compared to IgM control</td>
<td>-</td>
<td>54.5%</td>
<td>2.6%</td>
<td>0</td>
</tr>
<tr>
<td>Pool 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of IEs labelled by pool 1 of mAbs</td>
<td>42.7%</td>
<td>18.3%</td>
<td>29.7%</td>
<td>30.5%</td>
</tr>
<tr>
<td>Percentage of reduction compared to IgM control</td>
<td>-</td>
<td>57.1%</td>
<td>30.4%</td>
<td>28.6%</td>
</tr>
<tr>
<td>HIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of IEs labelled by HIS</td>
<td>49.2%</td>
<td>23.7%</td>
<td>28.5%</td>
<td>30.3%</td>
</tr>
<tr>
<td>Percentage of reduction compared to control serum</td>
<td>-</td>
<td>51.8%</td>
<td>42.1%</td>
<td>38.4%</td>
</tr>
</tbody>
</table>

Table 3.8. Labelling of mAb G6, pAb B5, pool 1 of mouse IgMs and HIS to ItG-IEs treated with 10 µg/ml, 100 µg/ml or 1000 µg/ml of TPCK-treated trypsin as measured by flow cytometry. Percentage of labelling was calculated as shown in Figure 3.12.

### 3.4 Localisation of IgM surface labelling using liquid-phase immunofluorescence assay (L-IFA)

Binding of mouse IgM antibodies to the outer surface of live IEs was visualised using L-IFA. Flow cytometry is a sensitive method for quantitating the number of IEs labelled by particular antibodies, however, the localisation of surface binding can only be visualised by imaging techniques including IFA. Fluorescent microscopy scanning with the strain-specific mAb BC6 revealed a punctate fluorescent pattern distributed across the external surface of A4-IEs (Figure 3.12), a characteristic pattern of PfEMP1-specific labelling similar to
that observed in a published work (Hughes et al., 2010). A similar surface immunolabelling was observed mAb E7 though with less surface distribution and fluorescence intensity (Figure 3.13). The labelling was absent with mouse IgM control antibody indicating that the labelling was specific for mouse mAbs. The assay was carried out using a representative mouse mAb that showed a significant labelling by flow cytometry with all tested isolates (i.e. mAb E7). The surface labelling was imaged qualitatively by IFA and the percentage of IEs showing positive fluorescence signal by tested antibodies was not quantitated.

Figure 3.12. L-IFA staining of the surface of live A4-IEs with mAb BC6 (20 µg/ml). A: fluorescent images of staining parasite nuclei with 10 µg/ml of DAPI (left), staining of the sides of IEs (middle) or the top (right) by mAb BC6 using Alexa Fluor® goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes). B: fluorescent images of staining IEs with secondary antibody alone (negative control) showing stained parasite nuclei with DAPI (left) and with Alexa Fluor® goat anti-mouse IgG (H+L) secondary antibody (right). DAPI staining was carried out to differentiate IEs from non-IEs.
Figure 3.13. L-IFA for staining the surface of live A4-IEs with mAb E7. A: fluorescent images of staining parasite nuclei with 10 µg/ml of DAPI (left), staining of the sides of IEs (middle) or the top (right) by mAb E7 (49.9 µg/ml) using Alexa Fluor® goat anti-mouse IgM (µ chain) secondary antibody (Molecular Probes). B: fluorescent images of staining IEs with 20 µg/ml of mouse IgM isotype control (negative control) showing stained parasite nuclei with DAPI (left) or with Alexa Fluor® goat anti-mouse IgM (µ chain) secondary antibody (right). DAPI staining was carried out to differentiate IEs from non-IEs.
3.5 Mouse IgM-mediated IE lysis

3.5.1 Quantitation of lysed IEs by flow cytometry

We showed that mouse mAbs and pAbs recognise the surface of erythrocytes infected by distinct *P. falciparum* isolates. However, during flow cytometry analyses we observed a remarkable reduction in counts of IEs upon incubation with mAbs and pAbs (Table 3.9), whereas the number of normal RBCs was mostly unaffected. 69.2% (n=9) of all mAbs and pAbs showed variable levels of haemolysis (ranging from 17.2% to 96.2% of total IEs) with all tested *P. falciparum* isolates. Of these lysing antibodies, six (66.7%) showed positive surface labelling with all tested parasite isolates. On the other hand, the ability to lyse IEs was less frequent among non-labelling IEs. For instance, pAb C2, the least labelling mouse IgM antibody, only caused a little if any lysis among tested parasite isolates (Table 3.9). Figure 3.14 represents surface labelling to C24-IEs by E11, C2, A4, E7 and G6 showing the percentage of lysed IEs against a control IgM mAb.
Figure 3.14. IgM-mediated IE lysis caused by binding of mouse mAbs/pAbs to C24-IEs as indicated by flow cytometry. Trophozoite-enriched cells were incubated with 50 µl of mAbs/pAbs for surface labelling. Cells stained with 20 µg/ml mouse IgM isotype control (Sigma) shown in blue or those only stained with ethidium bromide and secondary IgM antibody alone (red) were included as negative controls. Population of IEs lysed after mouse IgM incubation was obtained from the FITC-positive channel (ethidium bromide labelled cells), and the percentage of lysed IEs was calculated relative to the percentage of mouse isotype IgM control.

<table>
<thead>
<tr>
<th>mAbs/ pAbs</th>
<th>Surface Labelling</th>
<th>SSC vs. FITC</th>
<th>Percentage of lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td>64.1%</td>
</tr>
<tr>
<td>C2</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td>27.3%</td>
</tr>
<tr>
<td>A4</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td>20.2%</td>
</tr>
<tr>
<td>E7</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td>77.8%</td>
</tr>
<tr>
<td>G6</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td>78.2%</td>
</tr>
<tr>
<td>IgM control</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
<td>0%</td>
</tr>
<tr>
<td>Antibody</td>
<td>DBL</td>
<td>specificity</td>
<td>3G8</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>E11</td>
<td>DBL13</td>
<td>Polyclonal</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(90.9%)</td>
</tr>
<tr>
<td>B12</td>
<td>DBL13</td>
<td>Polyclonal</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(77.2%)</td>
</tr>
<tr>
<td>A6A</td>
<td>DBL13</td>
<td>Monoclonal</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(93.4%)</td>
</tr>
<tr>
<td>C2</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.3%)</td>
</tr>
<tr>
<td>E4</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(94.6%)</td>
</tr>
<tr>
<td>A4</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6.9%)</td>
</tr>
<tr>
<td>E7</td>
<td>DBL31</td>
<td>Monoclonal</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(78.7%)</td>
</tr>
<tr>
<td>C9</td>
<td>DBL31</td>
<td>Monoclonal</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(38.6%)</td>
</tr>
<tr>
<td>2E10</td>
<td>DBL31</td>
<td>Polyclonal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.8%)</td>
</tr>
<tr>
<td>B5</td>
<td>DBL41</td>
<td>Polyclonal</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(91.7%)</td>
</tr>
<tr>
<td>D5</td>
<td>DBL41</td>
<td>Polyclonal</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(94.7%)</td>
</tr>
<tr>
<td>B4</td>
<td>DBL41</td>
<td>Monoclonal</td>
<td>+++</td>
</tr>
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<td></td>
<td></td>
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<td>(85.4%)</td>
</tr>
<tr>
<td>G6</td>
<td>DBL41</td>
<td>Monoclonal</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(69.1%)</td>
</tr>
</tbody>
</table>

Table 3.9. Summary of IgM-mediated IE lysis caused by binding of thirteen mAbs and pAbs to the surface of erythrocytes infected by eight phenotypically and genotypically distinct *P. falciparum* isolates as measured by flow cytometry. Grading of haemolysis based on the percentage of lysed IEs was determined as followed: - (lysed IEs ≤ 15%), + (lysed IEs 15.1-40.9%), ++ (lysed IEs 41-80.9%), +++ (lysed IEs ≥ 81% of IEs).
3.5.2 Estimating the parasitaemia after IgM incubation

To further tackle the haemolysis phenomenon, we estimated the parasitaemia level of ItG-IEs pre-incubated with mouse IgMs. The number of mid-late trophozoites or schizonts was counted over time (5, 15, 30 and 60 minutes) by counting 1000 cells of Giemsa stained suspension with/without IgM incubation under the microscope (Figure 3.15, Table 3.10). The results showed that the percentage of trophozoites/schizonts in cell suspension pre-incubated with pool 2 was dramatically decreased over time compared to pAb C2 or antibody-free suspensions. At 15, 30 and 60 minutes of incubation, the percentage of trophozoites/schizonts incubated with pool 2 was decreased by 27.3%, 77.3% and 83.3%, respectively relative to pre-incubation percentage (Figure 3.15, Table 3.10). On the other hand, percentage of ItG-trophozoites/schizonts was not significantly reduced following pAb C2 incubation compared to control suspension. These results indicated that haemolysis was initiated after 15 minutes of antibody incubation, and confirmed previous findings that IE lysis was associated with incubation with mouse IgMs that showed positive surface labelling. The antibody-free suspension was included in the assay to exclude non-specific IE lysis and confirmed that time-dependent cell destruction mediated by irrelevant factors was not observed throughout the assay. The absence of significant levels of lysed IEs incubated with pAb C2 indicated that cell lysis was not caused by buffer-related factors.
Figure 3.15. Estimating the percentage of IE lysis after incubation with mouse IgMs over time.
A: 1 ml of ItG-IE suspension containing mixed-stage parasites was incubated with pAbs C2 or pool 2, or tested without adding pAbs (no IgM). The percentage of trophozoites/schizonts was firstly estimated microscopically before adding pAb, and then re-estimated at 5, 15, 30 and 60 minutes after antibody incubation. B: Corresponding Eppendorf tubes containing the IE suspension with/without IgM incubation at 60 minutes, showing signs of haemolysis in IE suspension incubated with pool 2.
Table 3.10. Estimating the percentage of IE lysis with/without incubation with mouse IgM antibodies (C2 or pool 2) at 5, 15, 30 and 60 minutes of incubation. The percentage of trophozoites was firstly estimated microscopically before adding antibodies, and then estimated at 5, 15, 30 and 60 minutes following IgM incubation. Percentage of trophozoite reduction over time was calculated relative to percentage of trophozoites before adding mouse IgMs.

3.5.3 Correlation between IE surface labelling and lysis

We then asked the question whether IgM labelling to erythrocytes infected by a particular isolate was significantly correlated with haemolysis to that isolate. We determined the association between IgM binding, in terms of percentage of surface labelling and percentage of IE lysis using Spearman's rho Correlation (Table 3.11). We found that 3G8, A4, ItG and P5B6 parasite isolates showed a moderate positive correlation between surface labelling and haemolysis ($r_s = 0.37-0.49$, $P > 0.05$), i.e. if IgM surface binding to a particular isolate was detected, then IE lysis was also observed in that isolate. A statistically significant positive correlation was only observed with GC503 ($r_s = 0.77$, $P < 0.05$) and 3D7 ($r_s = 0.92$, $P < 0.001$). Figure 3.16 represents the correlation between IE surface labelling and lysis for 3D7 isolate with a scatter plot demonstrating the significant linear relationship between both parameters.
**Correlations**

<table>
<thead>
<tr>
<th></th>
<th>3D7 surface labelling</th>
<th>3D7 lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman's rho</td>
<td>1.000</td>
<td>.923**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>3D7 lysis</td>
<td>1.000</td>
<td>.923**</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed).**

Figure 3.16. Output of Spearman's rho correlation between surface labelling and IE lysis for 3D7 parasite strain as indicated by SPSS (version 22). A: Spearman's rho correlation ($r_{s}$) showing a significant positive correlation between the surface labelling of 13 mAbs/pAbs and IE lysis to 3D7 isolate. B: Scatter plot showing a positive linear relationship between surface binding of 3D7-IEs and cell lysis as indicated by the line of best fit. $R^{2}$: coefficient of determination.
### Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>3G8</th>
<th>GC503</th>
<th>A4</th>
<th>ItG</th>
<th>PFB6</th>
<th>C24</th>
<th>3D7</th>
<th>CS2</th>
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<td>0.002*</td>
<td>0.098</td>
<td>0.17</td>
<td>0.09</td>
<td>0.067</td>
<td>0.0001**</td>
<td>0.409</td>
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Table 3.11. Correlation between surface labelling and IE lysis for eight *P. falciparum* isolates as indicated by SPSS (version 22). Values of Spearman's rho correlation coefficient (*r_s*): 0 (no correlation between variables), 0.1 to 0.3 (weak positive correlation), 0.3 to 0.5 (moderate positive correlation), 0.5 to 1.0 (strong positive correlation), -0.1 to -0.3 (weak negative correlation), -0.3 to -0.5 (moderate negative correlation), -0.5 to -1.0 (strong negative correlation). Correlation is statistically significant when *P* value < 0.05. **: Correlation is significant at the 0.001 level, *: correlation is significant at the 0.05 level.

### 3.6.3 Effect of IgM dilution on IE lysis

We then investigated whether diluting the concentration of IgM affected the amount of lysis observed. B4 and G6 mAbs were tested for surface labelling with ItG-IEs at neat concentrations or at 1:2, 1:4 or 1:8 dilution (Figure 3.17). We found that the percentage of lysed cells was noticeably reduced with decreasing concentrations of IgM. When subjected to further analysis, IgM concentration and percentage of lysed IEs were significantly correlated, with correlation coefficients (*r_s*) of 1.0 (*P* < 0.0001) for B4 and G6. The percentage of haemolysis returned to normal levels (< 15%) at 1:4 dilution when the surface labelling signal also disappeared. These findings confirm that IE lysis was only observed with IgM labelling to the surface of IEs, and cell lysis was not detected when the surface binding of the tested IgM was lost.
Figure 3.17. Association between IgM concentration and percentage of ItG-IEs lysis by mAbs B4 and G6 as indicated by flow cytometry. Trophozoite-enriched cells were incubated with 50 µl of mAbs at neat concentrations or at 1:2, 1:4 or 1:8 dilutions in PBS/1%BSA for surface labelling. Cells stained with 20 µg/ml mouse IgM isotype control (Sigma) were included as negative controls for mouse IgM binding and shown in red. Population of IEs lysed after mouse IgM incubation was obtained from the FITC-positive channel (ethidium bromide labelled cells), and the percentage of lysed IEs (shown in red) was calculated relative to the percentage of lysis by mouse isotype IgM control.
3.7 Discussion

With the aim of defining smaller regions from the PfEMP1 protein capable of inducing functional antibodies that block the adhesion to ICAM-1 receptors, we used recombinant DBLβ domains from four ICAM-1 binding PfEMP1 variants for mouse immunisation. Only three domains (DBLβ13, DBLβ31 and DBLβ41) elicited functional mAbs and pAbs that recognised the corresponding DBL domains and cross-reacted with heterologous domains. In the present chapter, we used different immunological methods to further characterise the functional properties of these antibodies. In general, our results demonstrated that labelling was not limited to homologous ICAM-1 binding strains, as antibodies also recognised erythrocytes infected by C24, a non-ICAM-1 binding isolates. This indicated that induced antibodies were not phenotypically-specific, since DBL domains used in mouse immunisation were obtained from ICAM-1 binding PfEMP1 proteins.

Our results also showed that mAbs and pAbs labelled the surface of 3D7 strain-infected RBCs, which further extended the level of cross-reactivity to a heterologous strain with a distinct genotype. We also demonstrated that mAbs and pAbs did not show any increased specific labelling for their homologous isolates. There was no clear pattern of variant-specific recognition by mouse antibodies raised to DBL13 and DBL31 against erythrocytes infected by their corresponding parasite isolates (GC503 and P5B6, respectively), as almost all positive mAbs and pAbs equally recognised homologous and heterologous isolates. For instance, mAb E7 was raised to DBL31 from IT4VAR31, but gave stronger labelling with 3G8 and A4 than the homologous strain (P5B6). However, pAb A4 was an exception for this observation as it recognised erythrocytes infected by its corresponding isolate (P5B6) more specifically than heterologous parasites. In terms of specificity, we found that the strongest labelling antibodies were mainly monoclonal antibodies. In fact, all mAbs except C9 were comparatively most effective in labelling the surface of all tested IEs. On the other hand, antibodies of polyclonal specificity varied in their capacities to recognise parasitized cells. Variations in labelling the surface of IEs
among monoclonal and polyclonal antibodies indicate that non-reactive clones, particularly of polyclonal specificity, could not produce antibodies at the right fine specificity to recognise surface proteins. It may also demonstrate that immunogenic epitopes are conformational and only accessible to antibodies with certain specificities.

An interesting finding of this study was that mAbs and pAbs raised to DBL41 were comparatively the most effective in all assays. This was surprising, since this protein showed the weakest reactivity with homologous and heterologous mouse immune sera in ELISA and Western Blotting. E7 was the only mAb from DBL31-specific antibodies that showed significant labelling profiles with all tested isolates. Other antibodies raised to the same antigen including C2, C9 and 2E10 showed the least functional reactivity with the surface of parasitized erythrocytes. These results suggest that recombinant DBL41 domains expressed in *E. coli* contained the functional components required to elicit a broadly reactive immune response. Therefore, this domain might give rise to novel therapeutics and development of cross-reactive malaria vaccine.

Investigating the pattern of individual mouse IgM binding has led to the observation that mAbs and pAbs exhibited similar patterns of IgM surface labelling among parasite isolates. All tested parasites, except the CSA-binding CS2, showed a significant positive correlation between each other in terms of surface labelling to diverse parasites. This suggests that reactive mAbs and pAbs comprised a repertoire of cross-reactive IgM antibodies that capable of recognising erythrocytes infected by diverse *P. falciparum* strains. The higher level of cross-reactivity strongly suggests the presence, among parasites of different genotypes, of conserved epitopes within the PfEMP1 or in another parasite-derived protein on the surface of infected erythrocytes that are accessible to polymeric IgM antibodies. Cross-reactivity cannot be attributed to the presence of a mixture of antibodies with multiple specificities, since most reactive antibodies were of monoclonal specificities.

Production of cross-reactive mAbs or pAbs that recognise RBCs infected by distinct *P. falciparum* isolates has been shown in several published studies. For
instance, Lekana Douki et al. (2002) produced mAbs in mice to DBL-γ3CSA of the FCR3 strain and the produced mAbs, of which 70.8% were IgMs, labelled the surface of IEγ3CSA and cross-reacted with CSA-binding parasites from distant geographic areas. Some of the produced antibodies labelled the surface of other parasites with other adhesive phenotypes (i.e. ICAM-1 or CD36 binding). In another work, Gamain et al. (2001) raised mAbs to CIDR1 region of PfEMP1 and found that two mAbs, targeting different regions of CIDR1, reacted with up to 90% of tested *P. falciparum* strains expressing variant PfEMP1s. In addition, pre-adsorption of serum samples obtained from semi-immune African adults with recombinant DBL-1α domains has resulted in the reduction of total anti-VSA antibody response, suggesting that conserved domains of PfEMP1 may induce cross-reactive antibodies (Abdel-Latif et al., 2004). However, the cross-reactivity of antibodies raised to VAR2CSA or to the head structure (CIDR1α-DBL1α) is mainly attributed to the relatively higher degree of sequence conservation in these domains, and to our knowledge, no mAbs were raised to DBLβ domains similar to those raised in the present study.

Our data indicated that almost all purified mAbs and pAbs efficiently recognised recombinant domains as indicated by ELISA, but not all antibodies recognised the surface of live IEs. There was a positive correlation between reactivity of all mouse IgMs to rDBL31 and surface labelling to erythrocytes infected by all tested isolates. For example, mAb E7 and all anti-DBL41 antibodies showed the highest levels of reactivity in ELISA and also recognised the surface of most tested parasite strains. On the contrary, pAbs E4 and 2E10 and mAb C9 showed a higher reactivity against recombinant proteins in ELISA but exhibited the weakest profiles of IE surface fluorescence. In addition, pAb C2 significantly recognised the recombinant protein but almost failed to recognise parasite isolates. The lack of surface reactivity in some antibodies despite their reactivity in ELISA likely suggests the importance of conformational 3D epitopes in surface labelling, and that critical epitopes that were exposed and easily recognisable in linear recombinant proteins were hidden in the native structure and only accessible to specific antibodies. Our findings also indicated that the presence of IgM per se in a particular mAb/pAb does not account for surface
labelling. Some antibodies (e.g. pAb C2) comprised higher amounts of IgM but exhibited weak if any surface binding, which indicates that surface reactivity was only dependent on the presence of specific antibodies that can recognise target epitopes regardless of IgM abundance in purified yields. This was supported by the ability of pAb B12 to recognise IEs although this antibody had not efficiently purified and only comprised trivial amounts of IgM in the purified yield.

We assumed that the surface protein recognised by mouse IgM antibodies was DBLβ domains of the native PfEMP1, a major immunogen exported to the surface of IEs and is responsible for at least some of the adhesive properties of IEs (Rowe et al., 2009). However, ICAM-1-binding DBLβ domains only share 46% sequence identity with the other six domains from the IT4 genome (Brown et al., 2013), and this extensive sequence diversity among different PfEMP1 variants suggests that antibody cross-reactivity between different P. falciparum isolates could be explained by the recognition of another conserved, cross-reactive epitope within different PfEMP1 proteins. Despite the geographic variation and enormous diversity between HB3, IT4 and 3D7 parasite genomes, some examples of segmental sequence similarity were observed between var genes of different isolates. For instance, DBL1α of the head structure DBL1α-CIDR1α tend to have the most similarity between PfEMP1 of HB3, IT4 and 3D7 parasite genes, although there are regions of similarity in some CIDRI domains (Kraemer et al., 2007). This semi-conserved head structure plays a functional role in mediating the cytoadherence to multiple independent host receptors (Chen et al., 2000) and may act as a candidate for mouse IgM binding.

Although surface labelling was mainly specific for IEs, we could not confirm that labelling was specific for the native PfEMP1 as other parasite-derived proteins exported on the surface of IEs also can act as surface epitopes for binding. P. falciparum-induced modifications to the host cell surface might have resulted in the expression of a novel antigen containing IgM binding epitopes, but that antigen has not yet been identified. For instance, parasite-derived surface proteins encoded by the rif or stevor multigene families have been suggested to play a role in host-parasite interaction and antigenic properties
(Beeson et al., 2006, Niang et al., 2009, Rowe et al., 2009). Although their biological significance and role in natural immunity are largely unknown (Chan et al., 2012), the higher expressed level of these genes by *P. falciparum* parasites suggests their important role in parasite contact with host cells (Smith and Craig, 2005). They co-express with PfEMP1 proteins on the surface of IEs, despite the transcriptional findings that *rif* genes expressed after *var* genes, only for a limited time from late rings to early pigmented trophozoites (Kyes et al., 2000). In addition, human immune sera agglutinated IEs treated with trypsin at conditions (>100 µg/ml) such that the highly trypsin-sensitive PfEMP1 proteins are mostly removed but RIFINs detected, suggesting the existence of immunogenic epitopes in this family of proteins targeted by agglutinating antibodies (Fernandez et al., 1999). Another study showed that RIFIN recombinant proteins were well recognised by a large panel of human immune sera suggesting the capacity of these proteins to induce a strong immune response in *P. falciparum* infected adults in malaria endemic regions (Abdel-Latif et al., 2002). Furthermore, it has been demonstrated that acquisition of specific anti-RIFIN antibodies was associated with protection against *P. falciparum* malaria (Abdel-Latif et al., 2003). Recently, Goel et al. (2015) have demonstrated that RIFINs are expressed on the surface of IEs, bind to RBCs (preferentially of blood group A) to form large rosettes and mediate microvascular adhesion of IEs. Taken together, these studies highlighted the immunogenicity of RIFINs and suggested that these proteins can act as binding ligands for our mouse mAbs and pAbs.

Unlike PfEMP1 and RIFIN, data presenting evidence that that STEVOR proteins are exported to external surface of IEs are limited. A recently published study demonstrated that STEVOR proteins are expressed on the surface of IEs, using a combination of live IFA and flow cytometry, and this expression was only localised on erythrocytes infected by schizont parasite stage (Niang et al., 2009). Additionally, the similarity between STEVOR and RIFIN in terms of shared conserved sequences (Smith and Craig, 2005), and the peak expression of STEVOR during late trophozoites or early schizonts (Niang et al., 2009) make them potential candidates for surface binding. Modified host proteins such band
3 have also been proposed as immune targets since they are trypsin-resistant, located on the surface of IEs and were immunoprecipitated by mAbs to band 3 (Gardner et al., 1996). In addition, these highly abundant proteins on the surface of IEs were suggested to be involved in adhesion to various cells through CD36 receptors (Smith and Craig, 2005). Whether or not mAbs and pAbs bind to any of the surface proteins noted above is still unknown, and further characterisation analyses are needed for defining the surface molecule(s) incorporated in IgM binding.

In competition assays we investigated whether mouse IgMs out-compete BC6, HIS or anti-DBL16 rat antisera for IE surface adhesion and vice versa. BC6 and anti-rat antisera specifically recognise erythrocytes infected by their corresponding parasite isolates (A4 and ItG, respectively) whereas HIS cross-reacted with different *P. falciparum* isolates. Based on the assumption that strain-specific antibodies (BC6 and anti-DBL16 antisera) label the native PfEMP1, we hypothesised that reduction or complete loss of surface labelling competed by mAbs/pAbs may indicate that these mouse IgMs fully or partially bind to PfEMP1 on the surface of IEs. Our results demonstrated that mAb BC6 moderately interfered with the subsequent binding of mouse IgMs, which may suggest that mAbs and pAbs partially bind to PfEMP1 on the surface of IEs and that binding epitopes were partly masked by BC6. The extent of interference by BC6 on mouse IgM subsequent binding is a crucial marker for mapping the IgM-binding epitopes, since mAb BC6 is an IgG class antibody that only recognise an exposed epitope on surface PfEMP1 of erythrocytes infected by A4 *P. falciparum* isolate (Roberts et al., 1992, Horrocks et al., 2002). Failure to completely block mouse IgM binding does not necessarily indicates that IgMs do not bind PfEMP1, as the binding activity of BC6 may lie distal to the mouse IgM binding sites on PfEMP1 protein which explains the diversity between BC6 and mouse IgMs in labelling trypsin-digested IEs. Our findings also suggested that the binding sites for mouse IgMs and rat anti-DBL16 antisera are partially overlapped on the surface of IEs, as revealed by the remarkable reduction of mouse IgM subsequent binding to IEs pre-incubated with anti-DBL16 rat antisera. Although mouse IgMs had a relatively lower effect on rat antisera
binding, the results noted above support our suggestions that mAbs and pAbs partially bind to the surface PfEMP1 molecules since rat anti-DBL16 antisera comprise strain-specific antibodies capable of labelling ItG-PfEMP1 variants.

An alternative explanation of the observations discussed above is that mouse IgMs bind to epitopes different from those of BC6 or anti-rat sera on the surface of IEs and the influence of mouse IgMs on BC6 or antisera could be explained by the binding of the large polymeric IgM molecules to parasite-derived surface proteins. This binding may have unintentionally evolved to partly shield functional epitopes involved in BC6 and rat anti-DBL16 antisera on PfEMP1 upon binding to other surface antigens, which explains the limited ability of mAbs and pAbs in disrupting specific BC6 and rat antisera binding. This confirms that the surface of parasitized erythrocytes must contain separate functional domains that mediate binding to variant antibodies and can have antigenically distinct epitopes.

We found that mAbs and pAbs had a significant role in interference with the subsequent binding of the cross-reactive HIS, and the latter also remarkably reduced the binding of mouse IgMs. This two-sided inhibitory effect can be related to cross-reactivity of mouse IgMs and Malawian HIS to the surface of erythrocytes infected by diverse parasite isolates. Mouse IgMs could either mask a residue important for HIS binding or alter the conformation of the binding epitope in such a way to destabilise HIS interaction. However, the fact that HIS has a similar effect on mouse IgM binding suggests that at least part of the binding site may be common for the HIS and mouse IgMs. A study showed that pre-incubating IEsCSA with a human IgM control antibodies or a pool of sera from non-immune adults had not affected on anti-VAR2CSA mouse IgM antibodies specific for var2csa-PfEMP1, whereas a decrease in IE recognition by 42.8% was observed after pre-incubation with immune sera from multigavidae women (Avril et al., 2006). This indicates that the binding region of cross-reactive immune sera and specific mAbs may overlap and polyclonal IgG antibodies in immune sera can partially interfere with the binding of specific IgM antibodies, which is consistent with our findings. To sum up, competition between mouse IgMs and tested Abs appears to be inconclusive. The notable
influence of mAbs and pAbs on the binding of the cross-reactive HIS compared to their relatively moderate interference with the strain specific BC6 or anti-DBL16 antisera may suggest that mouse IgMs bind to non strain-specific but cross-reactive epitopes shared between variant *P. falciparum* isolates. Further studies are needed to investigate the kinetics of IgM binding to surface receptors and to determine the functionality of these cross-reactive, broadly expressed binding receptors.

Controlled proteolytic digestion of intact IEs has been widely utilised to explore the expression of antigens and determine the potential relationship between of VSAs expressed on IEs and adhesion phenotype (Gardner et al., 1996, Fernandez et al., 1999). We took advantage of the differential sensitivities of parasite-derived VSAs to controlled trypsin treatment for characterising the surface candidates involved in the binding of mAbs and pAbs. Unlike other surface proteins, the high-molecular-weight PfEMP1 is highly sensitive to low concentrations of trypsin and can be cleaved from the surface of intact IEs at ≥10 µg/ml trypsin treatment (Leech et al., 1984). This was consistent with our preliminary data demonstrating that treating IEs with trypsin at 10 µg/ml was extremely effective in abolishing binding ligands for ICAM-1 and BC6 on PfEMP1, though the former epitope was abolished at a lower trypsin concentration (1 µg/ml). This was shown by the complete loss of BC6 binding to PfEMP1 on A4-IEs or binding of ItG-IEs to immobilised ICAM-1 receptors upon trypsin digestion. The binding of mouse IgMs was substantially reduced with trypsin treatment, i.e. the higher the concentration of trypsin the lower the binding detected. However, the binding was not completely ablated as observed with mAb BC6. This may propose that mouse IgMs don’t completely bind to PfEMP1 as indicated by the sustained binding to IEs at higher trypsin concentrations, and suggests a separate or partially-overlapping binding sites for mouse IgMs and BC6. Mouse IgMs responded differently to trypsin digestion. For example, binding of mAb G6 was reduced by 36% at 10 µg/ml trypsin digestion, the suggested concentration required for cleaving PfEMP1, and the binding was dramatically reduced with 1 mg/ml trypsin treatment (62%). This sensitivity indicates that G6 partially recognises a trypsin-sensitive surface
antigen with the characteristics of PfEMP1, with the majority of binding to a trypsin-resistant epitope. On the contrary, the binding regions for pAb B5 were more resistant to trypsin cleavage at 10 and 100 µg/ml, suggesting its specificity to bind a trypsin-resistant ligand expressed on the cell surface. The binding profile for HIS to trypsin digested IEs principally resembled that of mouse IgMs, particularly mAb G6. This is consistent with previous suggestions in the present work that both mouse IgMs and HIS bind the same surface protein or to a largely overlapped epitope. However, the naturally acquired immune response in human HIS is predominantly variant specific as evidenced from mixed agglutination assays of pooled and individual sera in endemic areas (Newbold et al., 1992). On the contrary, the nature of the immune response of mouse mAbs and pAbs appears to be cross-reactive rather than variant specific, since antibodies with monoclonal specificities (mAbs) recognise all tested *P. falciparum* isolates, and showed no specificity for particular genotypes or phenotypes. Several trypsin-resistant parasite-derived surface proteins, such as RIFINs and modified band 3, can act as binding ligands for mouse IgMs and HIS, but the identity of such ligands is not yet identified.

Despite the higher sensitivity of PfEMP1 to trypsin digestion, protease-resistant adhesion to ICAM-1 was observed with some parasite lines from the IT4 line (Gardner et al., 1996). This may be explained by the rapid antigenic switching in *P. falciparum* parasite isolates that resulted in the existence of a phenotypically heterologous protease-resistant subpopulation of IEs within cultures (Gardner et al., 1996). It has been established that not all PfEMP1 proteins exported to outer membrane of IEs are digested with trypsin (Waterkeyn et al., 2000). A study showed that the ~300 kDa band of intact PfEMP1 was observed following the treatment with trypsin, suggesting that either not all of the PfEMP1 is exposed and accessible to trypsin, or only some portions of the protein are sensitive to trypsin (Beeson et al., 2006). Although the ICAM-1 binding site in PfEMP1 was localised within the trypsin cleavage region in Gardner et al. (1996) model for PfEMP1 domain proteases digestion, mAbs and pAbs could partially bind to a trypsin-resistant constant region of PfEMP1 located close to the membrane, with
the majority of antibody population binding to a trypsin-resistant parasite-derived surface protein.

In the present study, we detected a notable reduction in IE population after incubation with mouse mAbs and pAbs. We further investigated whether i) this lysis was IgM-dependant, ii) there was a correlation between surface labelling and IE lysis and iii) whether human serum components in culture contributed to the lysis phenomenon. Our findings indicated that haemolysis was resulted from an immunological interaction between mouse IgMs and a parasite-derived component on the surface of live infected erythrocytes as confirmed by three observations. First, mainly IEs were lysed upon mouse IgM incubation, whereas non-IEs were largely unaffected. Second, the majority of mouse IgMs that caused IE lysis were highly efficient in recognising the surface of erythrocytes infected by all tested isolates, whereas haemolysis was less observed or absent with non-labelling mAbs and pAbs. We observed a moderate to strong correlation between surface labelling in terms of percentage of surface recognition and percentage of IE lysis. All antibodies raised to DBL41 domains (B5, D5, B4 and G6) and anti-DBL31 mAb E7 that demonstrated that highest levels of surface recognition with all tested isolates also lysed most IEs of the corresponding parasite lines. Furthermore, the concentration of IgM in purified mAbs and pAbs was not a significant factor contributing to IE lysis, since antibodies with higher IgM concentrations (e.g. C2 and C9) were comparatively ineffective in lysing cells compared other antibodies at lower concentrations (E4 and B4). Third, diluting the signal of surface labelling was significantly correlated with decreasing the amount of lysed IEs, and the percentage of lysed cells was almost returned to normal levels with the loss of labelling signal of tested antibodies. These findings indicated that IE lysis resulted from an IgM-mediated binding to a parasite-derived protein on the surface of IEs.

All mAbs and pAbs were purified and neutralised at the same buffer conditions at pH 7.0-8.0, therefore, variations in the capacity to destroy IES among mAbs and pAbs indicated that haemolysis was resulted from factors related to IgM-binding rather than alteration in the buffer conditions. We further investigated the effect of temperature on IE lysis by incubating IEs with mAbs/pAbs on ice
or at 4°C rather than 37°C. No significant change was observed in terms of surface labelling or IE lysis (data not shown), indicating that temperature conditions did not influence cell lysis. Although the definite mechanism of IE lysis is not yet understood, IgM-dependent complement mediated lysis could be the potential mechanism underlying this phenomenon. Binding of IgM to a surface epitope, potentially PfEMP1 may result in the activation of the classical complement cascade through C1q binding leading to cell lysis. This is possible since mouse IgM antibodies bind human complement component C1 (Boullanne et al., 1987). In addition, complement activation is one of the earliest markers of immune response to malaria (Roestenberg et al., 2007). The polymeric structure of IgM antibodies principally benefits these molecules to efficiently agglutinate and neutralise pathogens or pathogen-infected cells (Czajkowsky et al., 2010). This sole structure makes IgM extremely efficient at activating the classical cascade, as a single IgM molecule is capable of activating the complement system leading to the lysis of a single RBC (Czajkowsky et al., 2010). Upon binding to the surface epitope on IEs, IgM may undergo a conformational change favouring the binding of C1q (Czajkowsky and Shao, 2009) which facilitates downstream components terminating in the assembly of membrane attack complexes (MAC) that lyse the cell. In order to investigate the involvement of serum complement components in IE lysis, we tested surface labelling of mouse IgMs with IEs cultured in media supplemented with heat-inactivated human serum compared to those cultured in normal serum. We aimed to eliminate complement components present in traces of human serum in parasite cultures by heating the serum at 56°C for 30 minutes. The results indicated that IE lysis was also detected in heat-inactivated culture media and there was no difference in terms of haemolysis between both parasite cultures (data not shown). Moreover, parasites were maintained in culture media devoid of human serum (supplemented with albumax) and the same finding was observed. If cell lysis was mediated through complement activation, our data suggest that complement factors are present on the parasite-infected cells rather than circulating in the human serum. Although no published evidence confirming that C1q in particular is present on the surface of IEs (Czajkowsky et al., 2010), an early study demonstrated that components of complement cascade are fixed on the surface of *P. falciparum* IEs (Wiesner et al., 1997). This study
also suggested that the parasite relies solely on the host cell to restrict complement activation and cell lysis. Thus, binding of IgM may has resulted in the disruption of a parasite-derived regulatory protein that suppresses complement activation and contributes to establishment of chronic disease.

Despite the significant role of PfEMP1-specific antibodies in acquiring protection against severe malaria, the extensive diversity of PfEMP1 variants limits their potential in the development of a universal vaccine (Bull et al., 1999, Nielsen et al., 2004). However, some structural conservations that limit the diversity of PfEMP1 must exist to maintain its function as an adhesion protein (Bull et al., 1999, Gamain et al., 2001). It was suggested that common "basic types" of PfEMP1 are expressed during P. falciparum infection. These "basic types" tend to be immunogenic and induce antibody response (Giha et al., 1999). Immune sera collected from people living in different African countries agglutinated parasitized erythrocytes from distal regions, suggesting the presence of cross-reactive epitopes expressed by several parasite isolates (Aguiar et al., 1992). Another study showed that antibodies in plasma collected from geographically distant and epidemiologically distinct regions recognised VSAs of different parasite isolates independent of the geographical origin of those parasites, suggesting that the repertoire of immunologically distinct VSAs are restricted and geographically-conserved among diverse populations (Nielsen et al., 2004). Another study showed that parasite isolates from individuals with severe malaria were agglutinated very frequently by heterologous plasma antibodies, which provided evidence for the existence of dominant subset of antigens on the surface of IEIs recognised by prevalent antibodies (Bull et al., 1999). Moreover, a study conducted on Kenyan children showed that the agglutination frequency among parasite isolates causing severe malaria was significantly higher than the agglutination frequency of those causing mild disease, suggesting that parasites causing severe disease express a prevalent subset of VSAs (Bull et al., 2000). These studies are consistent with earlier suggestions that the large repertoire of isolate-specific surface antigens may comprise antigenically cross-reactive antigens among diverse geographically distant parasites (Aguiar et al., 1992). Indeed, our experimentally induced mouse antibodies are similar in function to protective antibodies present in immune
adults in that they recognise the surface of diverse parasite isolates, making them suitable candidates for universal vaccine development.

In conclusion, our study demonstrated that single domains of *E. coli*-expressed DBLβ from PfEMP1ICAM-1 could elicit broadly cross-reactive, IE-lysing mouse antibodies against different strains of heterologous phenotypes and genotype. Such dual activity could be highly beneficial in the development of therapeutic antibodies. Indeed, cross-reactivity against diverse parasite population is encouraging since polymorphisms of PfEMP1 variants and the ability of *P. falciparum* isolates to switch surface PfEMP1 to other variants with different phenotypes make it difficult to develop strain-specific vaccine candidates based on targeting single DBL domains. An effective vaccine would elicit broadly reactive antibodies that cross-react with most, if not all binding isolates, or would contain a mixture of components each recognise a different isolate. However, further studies are required to further characterise the binding sites for mAbs and pAbs on the surface of IEs and to assess their capacity for blocking the adhesion of IEs to immobilised receptors or endothelial cells lines. In addition, more work is needed to elucidate the molecular mechanism of parasitized erythrocyte lysis by mouse IgMs and blood or antibody factors that underlie this phenomenon.
3.8 Limitations and Future work

Several functional assays have not yet been carried out due to limitations of time and amount of purified antibodies resulting from IgM purifications. Some of these significant assays include the investigation of surface reactivity of mAbs and pAbs against PfEMP1-knockout *P. falciparum* isolates. Whether or nor our mAbs and pAbs are capable of labelling these transgenic isolates relative to their corresponding parental lines is significant in characterising the surface ligand(s) labelled by mouse IgM. Recently, we have successfully cultured a CS2 *P. falciparum* isolate with disrupted *P. falciparum* skeleton binding protein 1 (*PfSBP1*) gene. This gene was shown to be required for the transport of PfEMP1 to the surface of IEs (Maier et al., 2007). Assessing the capacity of mAbs and pAbs to label the surface of this CS2ΔSBP1 transgenic line may further identify the surface binding epitopes. Since the present work has evaluated the reactivity of mouse IgMs against lab-adapted parasite isolates, mAbs and pAbs can be investigated for surface reactivity with fresh patient isolates.

The definite mechanism for IgM-mediated IE lysis is not yet understood. We suggested that IgM-dependent complement mediated lysis is a potential mechanism underlying this phenomenon. It has been shown that components of complement cascade are fixed on the surface of *P. falciparum* IEs (Wiesner et al., 1997). Further assays can be carried out to investigate the presence of C1q or other complement components on the surface of IEs, such as the use of different detecting mAbs directed to these components.
Chapter 4

Investigating the capacity of mAbs and pAbs to block IE cytoadhesion
Chapter 4: Introduction

Our understanding about the interaction between IEs and host receptors comes essentially from in vitro studies. IE Adhesion assays under static conditions or in laminar flow systems are amongst the major assays used to study the dynamics and kinetics of the binding between IEs and their receptors (Cooke and Nash, 1995). Several in vitro models have been used to study parasite cytoadhesion including those investigating the interaction of IEs with purified proteins immobilised on plastic (Roberts et al., 1985, Barnwell et al., 1989, Ockenhouse et al., 1989, Ockenhouse et al., 1992b, Tse et al., 2004, Craig et al., 2000, Madkhali et al., 2014), cDNA transfected cells such as CHO (Berendt et al., 1989), amelanotic melanoma cells (Barnwell et al., 1989, Ockenhouse et al., 1989), human ECs such as HUVEC and human dermal microvascular endothelial cells [HDMEC] (Madkhali et al., 2014, Gray et al., 2003, McCormick et al., 1997) or human tissues such as placenta cryosections (Avril et al., 2006). Parasite cytoadhesion under static conditions measures immobilization of IEs on coated proteins, whereas flow assays attempt to mimic the in vivo physiological system and assess strength or avidity of parasite adhesion under the pressure of blood flow (Cooke and Nash, 1995, Ochola et al., 2011). Although both the static and flow adhesion systems have provided us with valuable information about the interaction between the parasite and its ligands, these information should be observed in parallel to advance our knowledge about these procedures (Cooke and Nash, 1995).

The suitability of in vitro models for studying the in vivo sequestration can be judged by the following criteria: adherence of IEs to receptors on cells or tissues must be specific for erythrocytes infected by trophozoites or schizonts; parasite adhesion must occur at any level of parasitaemia; IE adhesion must be mediated by the direct interaction between protein ligands on the surface of IEs and the target cell or tissue; and IE adhesion must be subjected to inhibition or reversal by suitable antibodies (Udeinya, 1990). Therefore, the practicality of a particular in vitro model can be determined by the closeness of the observations obtained
from that model to the results of *in vivo* sequestration and follow the above criteria (Udeinya, 1990).

Most studies that investigated the cytoadhesion of *P. falciparum* parasite isolates have been carried out under static conditions. In the present chapter, we investigated the capacity of mAbs and pAbs to block parasite binding under both static and physiological flow conditions, as results taken individually from either assays could give contrasting information about interactions between receptors and parasite ligands (Chakraborty and Craig, 2005). We evaluated the capacity of mouse mAbs and pAbs to block the adhesion of IEs to immobilised receptors (ICAM-1 or CD36) or endothelial cell lines under static or physiological flow conditions. Our results provided evidence that mAbs and pAbs produced in this work not only cross-reacted with different *P. falciparum* parasite isolates, but also cross-inhibited the adhesion of different parasites to immobilised receptors and HUVEC line. However, further work is needed to elucidate the mechanism of blockade and to evaluate the capacity of inhibition with a diverse range of parasite isolates against different proteins and cell lines.
4. Materials and Methods

4.1 Purified proteins used for static and flow adhesion assays

Recombinant protein chimeras of wild-type ICAM-1, consisting of five extracellular domains linked to the Fc portion of human IgG1, were prepared in our laboratory by Tadge Szestak (Liverpool School of Tropical Medicine, UK) following a protocol described by (Craig et al., 1997). NanoDrop spectrophotometer was used to estimate the concentration of soluble purified proteins (ranging from 200-700 µg/ml), before proteins were aliquoted and stored at -80°C for further use. CD36 proteins were provided commercially as soluble recombinant proteins (R&D systems, UK), which was stored at -20°C. ICAM-1 and CD36 proteins were used in all assays at 50 µg/ml in Dulbecco’s phosphate buffer saline (PBS).

4.2 Monoclonal antibodies

MAbs used in adhesion-blocking assays were anti-ICAM-1 mAb 15.2, purchased from AbD serotec® (MCA1615T, mouse anti-human CD54, IgG1), and anti-CD36 mAb IVC7 which was kindly provided by Prof. Ellen van der Schoot (Sanquin, Amsterdam). All mAbs were incubated with IE suspensions at 5 µg/ml in inhibition assays. Mouse monoclonal and polyclonal IgM antibodies were prepared as described in Chapter 2.

4.3 Cytoadhesion assays under static conditions

4.3.1 IE adhesion to immobilised protein receptors

Static protein binding assay was carried out as previously described (McCormick et al., 1997) with minor modifications. All P. falciparum isolates used in the adhesion assays were obtained from cultures maintained for 1-2 weeks after selection, since most isolates in continuous cultures lose the capacity
to bind after a short period (~ 24–43 days), although some continue to bind after longer periods (Udeinya et al., 1983). In addition, the use of parasite isolates cultured for longer periods as a model for cytoadherence in vivo could be misleading (Biggs et al., 1989). Briefly, multiple circles (~10 mm in diameter) were drawn on the base of 60 × 15 mm Falcon Petri dishes (Cat. No. 08-757-100B, Fisher Scientific). Two dishes were assayed for each parasite isolate. Then, spots (2 µl) of soluble ICAM-1-Fc in PBS (50 µg/ml), CD36 (50 µg/ml) or PBS were adsorbed in triplicates onto the circles and incubated in a humidified chamber at 37°C for 120 minutes to allow proteins to adsorb onto the dishes (Figure 4.1). These concentrations had previously been demonstrated to be within the dynamic range for observing variations in receptor-adhesion interactions (Gray et al., 2003). Protein spots were aspirated off and uncoated sites were subsequently blocked overnight at 4°C in 2 ml of PBS/1%BSA. Dishes were incubated for 60 minutes at 37°C prior to adhesion assays. Blocking solution was removed prior to adding 1.25 ml of IE suspension in binding buffer (RPMI 1640 [Sigma®, R4130] supplemented with 2% glucose at pH 7.2) at a parasitaemia of 3% and a HCT of 1% to each dish. Plates were incubated at 37°C for 60 minutes with gentle resuspension every 10 minutes. Unbound IEs and non-IEs were removed by 6-8 gentle washes with binding buffer and successful washing was monitored using inverted microscope. Bound IEs were fixed onto the dishes by the addition of 1% glutaraldehyde in PBS for 20 minutes and then stained with 10% Giemsa for another 20 minutes at room temperature. Stained IEs were viewed using high-power microscope (Eclipse T 200, Nikon) under 20 × 15 (×300) magnification. Photos of the remaining adherent cells were then captured using Nikon camera equipped to the microscope, analysed using HC Image software (Sewickley, USA) and counted using Image-Pro Plus Image analyser software version 7.0 (Rockville MD, USA).

Data presented correspond to the mean number of bound IEs of 36 readings (6 protein spots) from two independent dishes (18 readings from each plate) and expressed as the mean percentage of binding of IEs incubated with IgM.
antibodies relative to binding of IEs incubated in buffer alone (normalised to 100%).

![Diagram of ICAM-1 and CD36 receptors coating on bacteriological petri dishes]

Figure 4.1. Coating of ICAM-1 and CD36 receptors onto bacteriological petri dishes for static adhesion assays. PBS spots were included in all assays as negative controls.

4.3.2 Blockade of IE adhesion under static conditions

To investigate the capacity of mouse mAbs and pAbs to block IE adhesion to ICAM-1 or CD36 receptors, the same adhesion assay was carried out as described in the previous section with slight modifications. Briefly, IE suspension for two dishes (2.5 ml) was centrifuged for 3 minutes at 1800 rpm, before the pellet was incubated separately with 100 µl of each of the mouse IgM antibodies (pAb C2, pAb A4, mAb E7, pAb B5, mAb B4 and mAb G6) for 30 minutes at 37°C. After incubation, cells were re-suspended in 2.5 ml binding buffer and 1.25 ml was added to each dish (×2), then the assay was continued as described in the previous section. MAbs that block parasite adhesion to ICAM-1 (mAb 15.2) or CD36 (mAb IVC7) were added to IE suspension at 5 µg/ml prior to addition to the protein-coated dishes and the same procedure were followed as shown above. Percentage of IE adhesion-blocking was determined by the difference in the count of IEs assayed in the presence of IgM or IgG antibodies relative to IEs incubated in binding buffer alone. The results were expressed as percentage of binding of test samples relative to control, normalised as 100%.
4.4  Cytoadhesion assays under physiological flow conditions

4.4.1  IE adhesion to immobilised receptors

4.4.1.1  Adhesion to ICAM-1 under flow conditions

Flow-based protein adhesion assays were carried out using a modified laminar flow system as described elsewhere (Gray et al., 2003) with minor modifications. *P. falciparum* IE adhesion to ICAM-1 was determined using 3-aminopropyl triethoxysilane (APES)-treated VenaFluoro+™ biochips (cat no. V8CF-400-100-02, Cellix Ltd, Dublin, Ireland) shown in Figure 4.2. Channels were coated with ICAM-1 (50 µg/ml) and incubated in a humidified chamber at 37°C for 120 minutes before they were blocked with 1%BSA/PBS and incubated overnight at 4°C. Micro-slides were connected to tubing system on a microscopic stage enclosed within a plastic chamber that maintains the temperature at 37°C. IEs were adjusted to 3% parasitaemia and 2% HCT in 500 µl binding buffer and flowed through the micro-slides using the VenaFlux Platform (Cellix) for 5 minutes at a flow rate yielding a wall shear stress of 0.05 Pa (0.186 ml/min) calculated based on the formula described by Cooke et al. (1994). The wall shear stress was calculated to mimic the wall stress in the microvessels and temperature of the system was adjusted to 37°C throughout the assay. Binding buffer was then perfused for 2 minutes to remove unbound cells. The number of stationary adherent IEs was immediately counted in six random fields of view on the micro-slide under 20 × 15 (×300) magnification while the buffer is still running. Values were then normalised to the number of bound cells per square millimetre. Results expressed as the mean percentage of binding of IEs incubated with IgM antibodies relative binding of IEs incubated buffer alone (normalised to 100%). The assay was run in duplicate per single experiment to assess the consistency of the results. Bound IEs were removed from the slides by flowing binding buffer at a high level of shear stress over the slides, and removal was confirmed by examining the slides under the microscope. Micro-slides were washed with water then with binding buffer and stored at 4°C to be re-used in
future experiments. Each slide can be used up to six times and a positive control was carried out to ensure the validity of the slides.

Figure 4.2. Vena8 Fluoro+TM biochips (Cellix Ltd, Dublin, Ireland).

4.4.1.2 Blockade of IE adhesion to ICAM-1 under flow conditions

In assays that assess the ability of mouse mAbs and pAbs to inhibit parasite binding to immobilised ICAM-1 receptors, the same procedure was followed as shown above with some modifications. IE suspension (500 µl) designated for each antibody was firstly centrifuged for 3 minutes at 1800 rpm, before the pellet was incubated with 100 µl of mAbs or pAbs for 30 minutes at 37°C. After incubation, cells were pelleted and re-suspended in 500 µl of binding buffer, and then the assay was continued as shown in the previous section. To block specific adhesion to ICAM-1, mAb 15.2 was added to the suspension at 5 µg/ml prior to start of the assay. To minimise variations that effect the concentration of coated proteins during the assay, the same slide was used for cultures with or without antibodies. Percentage of IE adhesion blocking was determined by the difference in the count of IEs assayed in the presence of IgM antibodies to IEs incubated in binding buffer alone. The results were expressed as percentage of binding of test samples relative to control, normalised as 100%.
4.4.2 IE adhesion to HUVEC

4.4.2.1 HUVEC culture

HUVEC was purchased from PromoCell (C-12200, 500,000 cryopreserved cells) and cultured in endothelial cell growth medium (PromoCell, C-22120) supplemented with endothelial cell growth medium supplement Mix (PromoCell, C-39215) following standard conditions. Cells were allowed to attach to the internal surface of gelatine-treated ventilated T25 culture flasks containing 5 ml warm culture media for 2-3 hours at 37°C in CO₂ incubator. Then, the media was discarded and replaced by the same volume of warm media before the flask was incubated for 48 hours at 37°C in CO₂ incubator. Cells were checked for growth and confluence every alternate day and fresh culture medium was added.

Confluent cultures were split by trypsinisation using PromoCell detach kit (C-41220). The kit contains HEPES balanced salt medium (HEPES-BSS), trypsin / EDTA solution (0.04%/0.03%) and trypsin neutralising solution (TNS). Sub-culturing of cells was carried out as followed: culture medium was firstly removed from the flasks, then 1.5 ml of HEPES-BSS was added to wash off remaining media before HEPES-BSS was removed and trypsin / EDTA solution was added to detach adherent cells from plastic surfaces and detachment of cells was confirmed by inverted microscope. This was immediately followed by the addition of TNS to stop trypsin digestion. The suspension was centrifuged at 300 x g for 3 minutes before the pellet of cells was re-suspended in fresh warm media. Culture medium was changed every alternate day, and stage of confluence was monitored using inverted microscope. HUVECs were cultured to confluence (6th-7th passage) 24 hours prior to experimentation. On the day of the assay, Accutase® (Sigma-Aldrich®, A6964) was used to detach cells rather than trypsin.
4.4.2.2 Analysis of receptor expression on HUVEC by flow cytometry

Surface expression of ICAM-1 or CD31 receptors on monolayers of HUVEC line was investigated by flow cytometry. Briefly, HUVECs from stable cultures were assayed with or without activation by 10 ng/ml of TNF-α at 18-24 hours prior to flow cytometry analysis. After activation, ECs were detached by treatment with Accutase® (Sigma-Aldrich®, A6964) and neutralised with culture medium. Cells were centrifuged at 300×g for 3 minutes, re-suspended with 100 µl of PBS at 1 × 10^6 cells/ml and incubated with APC-conjugated mouse anti-human CD54 (ICAM-1) IgG antibodies (BD biosciences, Ltd.) for ICAM-1 or with FITC-conjugated mouse anti-human CD31 IgG antibodies (BD biosciences, Ltd) for CD31, at 1:100 dilution for 60 minutes at 37°C. Cells incubated with APC-conjugated human IgG isotype control were included as a negative control for ICAM-1 expression and used to exclude the background signal, whereas cells incubated with FITC-conjugated human IgG isotype control were included as a negative control for CD31 expression.

After incubation, cells were washed in PBS, re-suspended in 400 µl Cell Wash and analysed using Becton-Dickinson FACSCalibur flow cytometer (BD LSR11). 10,000 events were collected and analysed using FlowJo software (10.0, San Carlos, CA, USA). Gating on FSC-A vs. APC-A was carried out to recognise HUVEC population expressing ICAM-1 receptors whereas gating on FSC-A vs. FITC-A was used to identify CD31-positive cells. Data were presented as histograms showing the percentage of ICAM-1- or CD31-positive populations relative to corresponding negative controls. Corrected mean fluorescence intensity (MFI) of ICAM-1 expression for resting and activated HUVEC was calculated by subtracting the MFI values of the isotype control from that of positive samples and expressed as clustered columns.

4.4.2.3 IE adhesion to HUVEC under flow conditions

Flow cell adhesion was designed to mimic the conditions of blood flow through microvasculature by allowing IEs to flow through micro-slides coated with ECs at a flow rate of 0.24 ml/min (0.05 Pa) using the Cellix system. The assay was
carried out as described by Gray et al. (2003) with some modifications. Adhesion of A4-IEs was assessed on HUVECs which constitutively express ICAM-1 receptors as indicated by flow cytometry (Figure 4.5 in the Results section). VenaEC™ biochips (8-channels, Cellix, Dublin, Ireland) were coated with 100 µg/ml fibronectin (12 µl) to promote adhesion of cells to the chips and incubated in a humidified chamber overnight at 4°C. HUVECs were allowed to reach confluence (6th-7th passage) and activated with 10 ng/ml of TNF-α 18-24 hours prior to performing the adhesion assay. After activation, ECs were detached by treatment with Accutase® (Sigma-Aldrich®, A6964) and neutralised with culture medium. Cells were centrifuged at 300× g for 3 minutes and resuspended with appropriate volume of culture medium to obtain 1.5 × 10⁶ cells/ml. Cells (5 µl) were seeded onto pre-warmed VenaEC™ micro-slides and incubated in CO₂ incubator at 37°C. HUVECs were allowed to adhere to the micro-slides and fed every 30 minutes with HUVEC culture medium until reaching confluence (~2-3 hours). IE suspension was prepared essentially as for flow protein assay, and suspensions were perfused through confluent micro-slides following the same protocol of VenaFlux Platform (Cellix). The number of stationary adherent IEs was then counted in 6 random fields on the micro-slide under 20 × 15 (×300) magnification and expressed as the number of bound cells per square millimetre. Results expressed as the mean percentage of binding of IEs incubated with IgM antibodies relative binding of IEs incubated buffer alone (normalised to 100%). The assay was run in duplicate in single experiments to assess the consistency of the results.

4.4.2.4 Blockade of IE adhesion to HUVEC under flow conditions

Antibody incubations and washing for assays that investigate the capacity of IgM antibodies to block parasite adhesion to HUVEC were carried following the same procedure described in section 4.4.1.2.
4.5 IE adhesion reversal to immobilised protein receptors under static conditions

Reversal of IE adhesion to ICAM-1 receptors by mAbs and pAbs or anti-ICAM-1 mAb 15.2 was investigated. Briefly, ItG-IEs were allowed to bind to immobilised ICAM-1 (50 µg/ml) on 35 × 10 mm bacteriological Petri dishes (Falcon 1058, Becton Dickinson). Adhesion to purified receptors was carried out following the same static protein binding assay. Prior to fixation, plates were incubated in the absence or presence of 800 µl of pAb C2, mAb E7, pAb G6 or anti-ICAM-1 mAb 15.2 (5 µg/ml) for 30 minutes at 37°C, with gentle resuspension every 10 minutes. Control dishes where only binding buffer was added were assayed in parallel throughout the reversal assay. Antibody suspension was aspirated, non-adherent cells were removed by repeated washing with binding buffer and the remaining bound IEs were fixed with 1% glutaraldehyde and stained with 10% Giemsa stain. Stained IEs were viewed and counted as described in static protein assay. Data presented represent the mean number of bound IEs of 30 readings from two independent dishes (3 spots each) and expressed as the number of IEs bound per mm² (per square millimetre). Percentage of adhesion reversal was determined by comparing the number of adherent IEs in presence of mouse IgM Abs relative to number of those incubated in the absence of antibodies.

4.6 Statistical analysis

Spearman's rho correlation was used for indicating the correlation between variables using SPSS and Spearman's rho correlation coefficient ($r_s$) was used to determine the strength of the correlation. Two-tailed $t$-test was used for indicating the significance of the correlation, which was considered as statistically significant when $P$ value $< 0.05$. One-way ANOVA test with Daunett $t$-test (two-sided) were used to measure the difference in IE adhesion. The difference was considered significant when $P$ value $< 0.05$. All statistical analyses were carried out using SPSS version 22.
4. Results

4.1 Adhesion blocking of ItG-IEs to ICAM-1 and CD36 receptors under static conditions

4.1.1 Blocking ItG-IE binding to ICAM-1 by mAbs and pAbs

ItG *P. falciparum* isolate maintained in laboratory culture was tested for adhesion to immobilised ICAM-1 receptors after incubation with mouse IgMs that strongly label the surface of live ItG-IEs (e.g. pAb B5, mAbs E7, B4 and G6 – see chapter 3). We hypothesised that cross-reactive mAbs/pAbs would block PfEMP1 regions involved in ICAM-1 binding. A non-labelling mouse antibody (pAb C2, raised against rDBL31) was included in the assays as a negative control and to investigate the association between surface labelling and adhesion blocking. To confirm the specificity of ItG-IE adhesion to ICAM-1, we tested the anti-ICAM-1 mAb 15.2 for its ability to inhibit the interaction (control). MAb 15.2 maps to ICAM-1 domain 1 and completely abrogates ItG-IE interaction with ICAM-1 (Berendt et al., 1992, Cooke et al., 1994, Adams et al., 2000). Thus, it was used as a positive control for adhesion-inhibition in the assays. We also compared the level of binding between IEs incubated in binding buffer alone and those incubated with an irrelevant IgM isotype control from murine myeloma clone MOPC 104E (Sigma®), and no significant difference was observed between both IE suspensions (data not shown). Therefore, cells incubated in buffer alone were assayed as positive controls for adhesion in all assays.

We took into considerations the lysis of substantial amounts of IEs following incubation with mouse mAbs and pAbs during adhesion blocking assays. First, we optimised the incubation time for mAbs/pAbs with IEs such that the least amounts of IEs are lysed while not affecting mouse IgM binding, and we found that an incubation of 30 minutes is appropriate for IgM binding with a relatively less destructive effect on IEs (data not shown). In a separate assay, we evaluated the blocking capacity of mAbs after incubation with IEs for a short period (~2-5
minutes). Although no lysis of IEs was observed when incubated for this period, no blocking effect was observed indicating that the incubation was not sufficient for IgM binding (data not shown).

Similar to previous studies (Ockenhouse et al., 1991, Gray et al., 2003), our results showed that ItG-IEs bound strongly to purified immobilised ICAM-1 receptors (Figure 4.1), and this adhesion was almost completely inhibited by pAb B5 and mAbs E7, B4 and G6 (97.8% - 99.9% of inhibition compared to binding of IEs incubated with binding buffer alone, $P < 0.001$). This was comparable to IE adhesion blocking by anti-ICAM-1 mAb 15.2, which blocked 98.9% of IE adhesion to ICAM-1 ($P < 0.001$). Pre-incubation of IEs with pAb A4 inhibited 56.9% of their adhesion to ICAM-1 compared to control ($P < 0.001$), whereas pAb C2 did not significantly affect the IE adhesion to ICAM-1 receptors ($P > 0.05$).
Figure 4.1. Adhesion inhibition of ItG-IEs to purified ICAM-1 receptors under static conditions. Binding of IEs (3% parasitaemia, 1% HCT) to ICAM-1 receptors (50 µg/ml) was observed after pre-incubating IEs with pAb A4 (56.6 µg/ml), mAb E7 (49.9 µg/ml), pAb B5 (59 µg/ml), mAb B4 (29.7 µg/ml), mAb G6 (66.3 µg/ml), pAb C2 (43 µg/ml) or with anti-ICAM-1 mAb 15.2 (5 µg/ml). The graphs represent the mean percentage of binding to ICAM-1 relative to the binding of ItG-IEs incubated in buffer alone (normalised to 100%). Error bars represent SDs of two independent dishes (6 spots, 36 readings). Control experiments include binding of ItG-IEs to PBS spots immobilised onto the same plates, which showed no binding (data not shown). The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding without mouse IgM incubation is represented by two asterisks ($P < 0.001$). The results correspond to one experiment.
4.1.2 Blocking ItG-IEs binding to CD36 by mAbs and pAbs

To further characterise the adhesion blocking of mouse mAbs and pAbs, ItG-IEs were tested for binding to immobilised CD36 receptors with or without mouse IgM incubation (Figure 4.2). Essentially, similar adhesion-blocking patterns were observed with CD36 compared to ICAM-1 receptors. For instance, pre-incubating ItG-IEs with the four antibodies that blocked ICAM-1 binding (pAb B5 and mAbs E7, B4 and G6) significantly inhibited ≥ 98.9% of IE stationary binding to CD36 compared to control ($P < 0.001$). IEs bound to CD36 at ~15% of control levels after incubation with pAb A4 ($P < 0.001$), indicating that adhesion-blocking activity of pAb A4 was more specific against CD36 compared to ICAM-1 receptors. No significant difference in binding to CD36 was observed after incubating IEs with pAb C2 or anti-ICAM-1 15.2 ($P > 0.05$). This is consistent with ICAM-1 adhesion-blocking findings that non-labelling IgM antibodies (pAb C2) had no effect on IE adhesion to immobilised receptors under static conditions, and also consistent with pAb C2 not lysing IEs. Failure to block the adhesion by the anti-ICAM-1 mAb 15.2 indicated its selective specificity for ICAM-1 purified receptors.
Figure 4.2. Adhesion inhibition of ItG-IEs to purified CD36 receptors under static conditions. Binding of IEs to CD36 receptors (50 µg/ml) was observed after pre-incubating IEs with mouse IgMs at the same conditions described in Figure 4.1. The graphs represent the mean percentage of binding to CD36 relative to the binding of ItG-IEs incubated in the absence of antibodies (normalised to 100%). Error bars are SDs of two independent dishes (6 spots, 36 readings). Control experiments include binding of ItG-IEs to PBS spots immobilised onto the same plates, which showed no binding (data not shown). The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding without IgM incubation is represented by two asterisks ($P < 0.001$ One-way ANOVA). The results correspond to one experiment.
4.2 Adhesion blocking of C24-IEs to CD36 receptors under static conditions

Adhesion of *P. falciparum* C24-IEs to CD36 purified receptors under static conditions was assayed following incubation with mouse IgM antibodies, to further investigate the cross-inhibitory effect of mAbs and pAbs (Figure 4.3). C24 *P. falciparum* is an IT4 isolate that strongly binds to CD36 receptors, but not ICAM-1 (Roberts et al., 1992, Gray et al., 2003). MAb IVC7 was included in the assay as a control for adhesion-inhibition, which significantly blocks IEs adhesion to CD36 (Berendt et al., 1989, Cooke et al., 1994). Our results demonstrated that mAbs E7, B4, G6 and pAbs A4 and B5 significantly blocked C24-IEs binding to CD36 with percent inhibition of 91.5, 91, 93.7, 94.6 and 95.2%, respectively, compared to binding of control (*P* < 0.001). Mouse IgMs were more efficient at blocking the adhesion to CD36 than anti-CD36 mAb IVC7, which inhibited 76.3% of IE adhesion (*P* < 0.001). Although C24-IEs adhesion was only reduced by 29% after incubation with pAb C2, the inhibition was considered statistically significant (*P* < 0.001). This blockade can be attributed to the ability of pAb C2 to label the surface of C24-IEs (27.3% of surface recognition). Profiles of adhesion blocking to CD36 receptors were similar for both ItG- and C24-IEs; if a particular IgM blocked the binding of one isolate it also inhibited the adhesion of the other isolate. This may suggest that mAbs/pAbs block an essential region of PfEMP1 involved in CD36 binding, i.e. CIDRα domains, in both parasite isolates.
Figure 4.3. Adhesion inhibition of C24-IEs to CD36 receptors under static conditions. Binding of IEs to CD36 receptors (50 µg/ml) was observed after pre-incubating IEs with mouse IgMs at the same conditions described in Figure 4.1. Graphs shown represent the mean percentage of binding to CD36 relative to the binding of C24-IEs incubated in the absence of antibodies (normalised to 100%). Error bars are SDs of two independent dishes (6 spots, 36 readings). The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding without IgM incubation is represented by two asterisks ($P < 0.001$, One-way ANOVA). The results correspond to one experiment.
4.3 Adhesion blocking of ItG-IEs to ICAM-1 receptors under physiological flow conditions

Next we investigated the ability of mouse IgMs to inhibit adhesion of erythrocytes infected by ItG under flow conditions to determine whether changing the static conditions of IE binding had any effect on the capacity of mouse antibodies to block parasite adhesion. ItG-IEs have been shown to bind preferentially to ICAM-1 receptors under flow conditions at physiologically relevant wall shear stress of 0.05 Pa (Cooke et al., 1994, Adams et al., 2000). In the present work, we only counted the number of IEs that formed stationary attachment to immobilised receptors or ECs, whereas IEs that formed rolling adhesion were neglected.

Overall, similar trends of flow-based adhesion blocking were observed but at slightly lower levels to those seen under static conditions. Percentages of inhibition when compared to control ItG were 91.1, 96.6, 98.4 and 93.3% for mAb E7, pAb B5, mAb B4 and mAb G6, respectively ($P < 0.001$) [Figure 4.4]. These inhibition levels were relatively similar to blocking obtained with the anti-ICAM-1 mAb 15.2 (98.7% of blocking, $P < 0.001$). Pre-incubating IEs with pAb A4 produced a statistically significant reduction in adhesion to ICAM-1 by 63.3% ($P < 0.001$), which is comparatively similar to its effect on ICAM-1 adhesion under static conditions. Although ItG-IEs bound to ICAM-1 at 75% of control levels after incubation with pAb C2, the difference in adhesion level was significant ($P < 0.001$) indicating that pAb C2 was more efficient at blocking ItG-IE adhesion to ICAM-1 under flow than static conditions. Comparison of percentages of static-based blockade to ICAM-1 with those under flow conditions revealed a statistically significant positive correlation ($r_s = 0.94$, $P < 0.05$, $n = 6$), indicating that mouse IgMs have the capacity to block parasite adhesion regardless of the state of binding.
Figure 4.4. Effect of antibody blockade on ICAM-1-mediated ItG-IEs adhesion at an inflow shear stress of 0.05 Pa. IE suspension (3% parasitaemia, 2% HCT) was perfused over ICAM-1-coated (50 µg/ml) micro-slides after pre-incubation with pAb A4 (56.6 µg/ml), mAb E7 (49.9 µg/ml), pAb B5 (59 µg/ml), mAb B4 (29.7 µg/ml), mAb G6 (66.3 µg/ml), pAb C2 (43 µg/ml) or with anti-ICAM-1 mAb 15.2 (5 µg/ml). The graphs represent the mean percentage of binding to ICAM-1 relative to the binding of ItG-IEs incubated in the absence of antibodies (normalised to 100%). Error bars are SDs of the means of six readings from random fields. Blocking capacity was confirmed with repeating assay on another micro-slide. The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding without IgM incubation is represented by two asterisks ($P < 0.001$). The results correspond to one experiment.
4.4 Adhesion blocking of A4-IEs to HUVEC under physiological flow conditions

To substantiate the *in vivo* relevance of static assay findings, we used the HUVEC to assess the ability of mAbs and pAbs to inhibit A4-IEs cytoadhesion under flow conditions. A4-IEs bind to HUVEC via ICAM-1 ligands since these ECs express ICAM-1 receptors, but not CD36, upon stimulation with cytokines such as TNF-α (Dobbie et al., 1999, Gray et al., 2003). Confluent HUVEC monolayers were firstly investigated by flow cytometry for expression of surface receptors (ICAM-1 and CD31) at resting state or at 18-24 hours after activation with TNF-α (Figure 4.5). As expected, surface expression of ICAM-1 was highly upregulated after TNF-α activation (74% of total ECs), although non-activated HUVECs also expressed ICAM-1 on 50.2% of cells. MFI data demonstrated that ICAM-1 expression was upregulated ~10-fold higher after TNF-α activation, similar to previous findings showing a 9-fold up-regulation of ICAM-1 expression in TNF-α stimulated cells (Dobbie et al., 1999). CD31 is a surface marker that constitutively expressed on the surface of ECs (McCormick et al., 1997, Gray et al., 2003) and showed positive surface expression levels of 71.7% and 54.1% for activated and non-activated HUVECs, respectively.

Adhesion blocking of mAbs and pAbs was only assessed with TNF-α stimulated HUVECs, since cytokine-mediated ICAM-1 expression was shown to significantly correlate with increased adhesion to ICAM-1 receptors (Gray et al., 2003). Our findings demonstrated that binding of A4-IEs to activated HUVECs was almost completely abrogated by pAb B5 and mAb G6 (93.6% and 94.5%, respectively) under physiological flow conditions (Figure 4.6). MAbs E7 and B4 were comparatively less efficient at blocking IE adhesion, as they reduced IE binding to ~25% and 35% of control levels, respectively (*P* < 0.001). Surprisingly, pAb C2, a non-labelling IgM, significantly inhibited 54.9% of IE adhesion (*P* < 0.001). Furthermore, pre-incubation with pAb A4 significantly affected binding to stimulated HUVECs (*P* < 0.05).
Figure 4.5. Representative flow cytometry analysis of the expression of ICAM-1 and CD31 surface receptors on monolayers of HUVEC with or without activation by 10 ng/ml TNF-α at 18-24 hours prior to the assay. A: expression of CD31 surface marker on non-activated (left panel) or activated HUVECs (right panel). B: expression of ICAM-1 receptors on non-activated (left panel) or activated HUVECs (right panel). C: the corrected MFI calculated by subtracting the MFI values of the negative control from that of positive samples. Shaded histograms represent the percentage of cells expressing the corresponding receptors relative to isotype control signal shown in blue trace for CD31 and red trace for ICAM1. Expression of ICAM-1 was detected using APC-conjugated mouse anti-human CD54 (ICAM-1) IgG antibodies (BD biosciences, Ltd.), whereas CD31 expression was detected with FITC-conjugated mouse anti-human CD31 IgG antibodies (BD biosciences, Ltd) at 1:100 dilution. Cells incubated with APC-conjugated IgG isotype control were included as a negative control for ICAM-1 expression (red trace) and used to exclude the background negative signal, and cells incubated with FITC-conjugated IgG isotype control were included as a negative control for CD31 expression (blue trace). The results correspond to one experiment.
Figure 4.6. Effect of antibody blockade on A4-IEs adhesion to TNF-α stimulated HUVEC under flow conditions at a wall shear stress of 0.05 Pa. Confluent HUVEC monolayers on flow micro-slides were stimulated 18-24 hours prior to the assay with 5 ng/ml TNF-α. Then, IEs (3% parasitaemia, 2% HCT) were perfused over the micro-slides after pre-incubation with pAb A4 (56.6 µg/ml), mAb E7 (49.9 µg/ml), pAb B5 (59 µg/ml), mAb B4 (29.7 µg/ml), mAb G6 (66.3 µg/ml) or pAb C2 (43 µg/ml). Graphs shown represent the mean percentage of binding to HUVEC relative to the binding of A4-IEs incubated in the absence of antibodies (normalised to 100%). Error bars are SDs of the means of six readings from random fields. Blocking capacity was confirmed with repeating assay on another HUVEC-coated channel. The difference in binding was considered significant if \( P < 0.05 \), calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding without IgM incubation is represented by one asterisk \( (P < 0.05) \) or two asterisks \( (P < 0.001, \text{One-way ANOVA test}) \). The results correspond to one experiment.
4.5 Adhesion blocking of ItG-IEs to ICAM-1 receptors after incubation with different concentrations of mAb G6

We next investigated whether adhesion blocking to immobilised receptors was dependent on the concentration of tested antibodies. MAb G6, which significantly blocked the adhesion of ItG-IEs to ICAM-1 receptors, was used in the assay at concentrations yielded on elution from affinity columns (neat) or at 1:2 or 1:10 dilution (Figure 4.7). The results indicated that mAb G6 inhibited ItG-IE adhesion to ICAM-1 in a dose-dependent manner with almost complete blocking of IE observed at 66.3 μg/ml (98.8%), inhibited by 45% at 1:2 dilution (33.2 μg/ml) and almost unaffected at 1:10 dilution (6.6 μg/ml). A statistically significant reduction in the total number of bound IEs was only observed after incubation with 66.6 or 33.2 μg/ml IgM concentrations ($P < 0.001$), whereas no significant difference was detected between 6.6 μg/ml (1:10 dilution) and control ($P > 0.05$). In addition, there was a strong negative correlation between antibody concentration and number of bound IEs ($r_s = -1.0$, $P < 0.001$, n=3). These results indicate that the maximal effect of adhesion blocking was observed at original antibody concentrations yielded from the purification and consistent with previous findings demonstrating that the highest levels of surface labelling were observed with antibodies tested at these concentrations.
Figure 4.7. Adhesion blocking of ItG-IEs to ICAM-1 receptors under static conditions. The same procedure described in the legend of Figure 4.1 was carried out except that G6 was incubated with IEs at neat concentration (66.3 µg/ml), or at 1:2 dilution (33.2 µg/ml) or 1:10 dilution (6.6 µg/ml). The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion blocking compared to binding without IgM incubation is represented by two asterisks ($P < 0.001$, One-way ANOVA test). The results correspond to one experiment.
4.6 Adhesion reversal of ItG-IEs to ICAM-1 receptors under static conditions

The capacity of mouse mAbs and pAbs to outcompete bound IEs to ICAM-1 receptors was investigated. ItG-IEs were allowed to bind to immobilised ICAM-1 receptors under static conditions before mouse IgMs were added in an attempt to reverse parasitized erythrocyte adhesion (Figure 4.8). The results show that mAbs E7 and G6 could significantly reverse (55.2% and 52.2% respectively) binding of IEs to ICAM-1 ($P < 0.001$). These levels were comparable to adhesion reversal obtained with the anti-ICAM-1 mAb 15.2 (55.3%, $P < 0.001$). Although pAb C2 only reversed 19.5% of parasite binding, this reversal was considered significant ($P < 0.001$).

The ability of antibodies to inhibit almost all IE adhesion to immobilised ICAM-1 receptors while only reversing ~50% of bound parasites confirms the high-avidity interaction between ICAM-1 and ItG-IEs with a low off-rate. This is consistent with the ability of 15.2 to only reverse 55% of bound IEs despite its higher capacity to mask IE-binding sites on ICAM-1. Although ICAM-1 is considered as a rolling receptor in interactions that occur under flow conditions, the adhesion of this receptor to IEs has been shown to be an avid one, and once bound; IEs remained in contact with ICAM-1 receptors even under high wall shear stress (Cooke et al., 1994). Taken together, our data indicate that mAbs can efficiently block IE binding sites when pre-incubated before the firm interaction between ICAM-1 and IEs takes place, while only possessing a limited ability for outcompeting bound IEs after that interaction occurs.
Figure 4.8. Reversal of ItG-IEs binding to purified ICAM-1 receptors with mouse mAbs under static conditions. ItG-IEs (3% parasitaemia, 1% HCT) were allowed to bind to ICAM-1 receptors (50 µg/ml), then unbound cells were washed in binding buffer and plates were incubated in the presence or absence of mAb E7 (19 µg/ml), mAb G6 (22 µg/ml), pAb C2 (14.3 µg/ml) or anti-ICAM-1 mAb 15.2 (5 µg/ml). Graphs shown represent the mean percentage of binding to ICAM-1 relative to the binding of ItG-IEs incubated in buffer alone (normalised to 100%). Error bars represent SDs of 30 readings from independent protein spots. Control experiments include binding of ItG-IEs to PBS spots immobilised onto the same plates, which showed no binding (data not shown). The difference in binding was considered significant if \( P < 0.05 \), calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion reversal is represented by two asterisk \( (P < 0.001) \). The results correspond to one experiment.
4.7 Correlation between surface reactivity, IE lysis and adhesion blocking

We next investigated the statistical correlation between adhesion blocking under static or flow conditions with IgM surface labelling in terms of the percentage of labelled IEs or with percentages of lysed IEs for all tested mAbs and pAbs (n=6). At the first analysis, correlation coefficients (r_s) were calculated for the association between the percentage of blocking of ItG-IE binding to ICAM-1 or CD36 receptors under static or flow conditions with ItG-IE lysis or surface labelling mediated by the six mAbs and pAbs. The analyses revealed a strong positive correlation, though not significant, between ItG-IE surface labelling and adhesion blocking to ICAM-1 under static conditions (r_s = 0.77, P > 0.05), to ICAM-1 under flow conditions (r_s = 0.6, P > 0.05) or to CD36 under static conditions (r_s = 0.66, P > 0.05). This indicates that the higher the percentage of labelled IEs by a particular antibody, the higher the percentage of blocked IEs by that antibody and vice versa (Table 4.1). A strong positive correlation was also observed between percentages of lysed IEs and adhesion blocking to ICAM-1 and CD36 receptors under static or flow conditions (r_s ranging from 0.67 to 0.899). The correlation was statistically significant for ItG-IE lysis with adhesion blocking to ICAM-1 under static or flow conditions P ≤ 0.05).

Another correlation analysis was carried out to determine the association between surface labelling to C24-IEs, percentage of lysed cells and blocking of IE adhesion to CD36 receptors under static conditions (Table 4.1). There was a moderate positive correlation between percentage of blocking and C24-IE lysis by the six mAbs and pAbs (r_s = 0.43, P > 0.05). The correlation was stronger and statistically significant between C24-IE lysis and surface labelling (r_s = 0.83, P < 0.05). The last analysis measured the correlation coefficient for the relationship between A4-IEs surface labelling or haemolysis with percentages of blocked IEs from binding to HUVEC under flow conditions (Table 4.1). We detected a statistically significant positive association between A4-IE lysis and adhesion blocking (r_s = 0.83, P < 0.05) and between surface labelling and adhesion blocking to HUVEC (r_s = 0.83, P < 0.05).
### Table 4.1. Output of Spearman's rho correlation between surface labelling, IE lysis and adhesion blocking for ItG, C24 and A4 parasite isolates.

**A**

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<th>ItG CD36 static</th>
<th>ItG ICAM-1 flow</th>
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**B**

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**C**

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<td>.042</td>
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**Correlation**

- **Correlation is significant at the 0.01 level**, *: correlation is significant at the 0.05 level.
4.8 Discussion

Sequestration of parasitized erythrocytes to ECs lining the microvasculature of a variety of host organs is thought to be the pathogenic basis of severe malaria disease (Miller et al., 2002, Autino et al., 2012). The main parasite-derived ligand that mediates IE cytoadhesion is the antigenic variant PfEMP1 protein expressed on the surface of parasitized erythrocytes (Baruch et al., 1995, Su et al., 1995; Smith et al., 1995), which facilitates parasite adhesion to various endothelial receptors such as ICAM-1 (Berendt et al., 1989) and CD36 (Ockenhouse et al., 1989). Strategies aiming to block PfEMP1 domains responsible for cytoadhesion have been hypothesised to reduce the severity of \textit{P. falciparum} malaria by reducing or suppressing the ability of IEs to sequester to the host microvasculature (Chattopadhyay et al., 2004). The development of mAbs that target different adhesive domains of PfEMP1 is an intervention of intense interest. Antibody-mediated adhesion-blocking/reversal methods were shown not only to reduce ability of IEs to sequester, but also to have a role in the clearance of parasitized erythrocytes from the circulation by the host spleen (Pasloske and Howard, 1994, Rowe et al., 2009). In the case of CM, inhibition of PfEMP1 interaction with ICAM-1 receptors in the brain micro-vessels may contribute to prevention of IE sequestration and hence preventing the severe consequences of cerebral disease.

In the present chapter, we evaluated the capacity of anti-PfEMP1\textsuperscript{DBLβ} mAbs and pAbs to block the adhesion of parasitized RBCs to immobilised receptors (ICAM-1 or CD36) or endothelial cell lines under static or physiological flow conditions. We aimed to investigate whether the cross-reactive, surface-exposed VSAs recognised by mAbs and pAbs are critical to molecular function and have a role in IE cytoadhesion. This was essential to elucidate since cross-reactive IgM antibodies may predominantly recognise epitopes on the surface of IEs that are not involved in receptor adhesion. We found that IgM antibodies that demonstrated higher levels of cross-reactivity with different parasite isolates were able to abrogate binding of erythrocytes infected by different parasite isolates to immobilised receptors or ECs, indicating their specificity to target
receptor-binding molecules on the surface of IEs. Our data indicated that mAbs and pAbs might have a potential therapeutic role through the development of anti-adhesion drugs.

Parasite adhesion is specific for the trophozoite or schizont stage of parasite development, as ring-stage parasites do not bind receptors such as CD36 (Ockenhouse et al., 1991). As discussed in the previous chapter, significant percentages of trophozoites/schizonts in parasite suspensions were lysed following incubation with mouse IgMs and this lysis varied among different IgM antibodies and different parasite isolates. Since most mAbs and pAbs used in adhesion blocking assays cause IE lysis, we took into consideration that this lysis may largely contribute to blockade of IE adhesion to immobilised receptors or ECs. Correlation analyses between surface labelling, IgM-mediated lysis and adhesion blocking generally indicated that inhibition of parasite binding to immobilised receptors or ECs under flow or static conditions was directly associated with the ability of antibodies to recognise the surface of IEs, and also correlated with the amount of lysed cells. Antibodies raised to DBL41 domains (pAb B5, mAb B4 and mAb G6) and anti-DBL31 mAb E7, which exhibited the highest capacities for surface recognition, also demonstrated higher specificities in blocking the adhesion of IEs. Surprisingly, pAb C2 that showed low if any surface-labelling activity also demonstrated also blocked IE adhesion to immobilised receptors under static or flow conditions, though at relatively lower capacity compared to mAb E7, pAb B5, mAb B4 and mAb G6. Blockade of adhesion also correlated with IgM-dependent IE lysis; as antibodies that caused lysis of significant amounts of IEs were highly effective in blocking the adhesion of erythrocytes infected with the same parasite isolate.

In spite of the relatively higher amounts of lysed IEs upon antibody incubation, a significant proportion of parasitized cells are not lysed. For instance, incubation of ItG-IEs with mAb E7 resulted in an average IE lysis of 64.6%, and adhesion inhibition assays demonstrated that mAb E7 inhibits 97.8% and 91.1% of ItG-IE binding to ICAM-1 receptors under conditions of static and flow, respectively, and 99.1% of static-based binding to CD36. This indicates that 33.2% and
26.5% of ItG-IEs were not-lysed but were prevented from binding to ICAM-1 receptors under static and flow conditions, respectively, whereas 34.5% of IE binding to CD36 receptors were inhibited after incubation with mAb E7. In another example, pAb A4 only caused lysis of 20.2% of C24-IEs and significantly inhibited 94.6% of C24-IEs from binding to CD36 receptors under static conditions. This indicates that pAb A4 has the capacity to block ~74% of non-lysed C24-IEs. However, this argument should be interpreted with caution since correlation data argued against it and indicated that lysis is associated with blockade of adhesion. The C24 data are more convincing but weird in that we would not expect CD36 blocking. Taken together, these data may indicate that mAbs and pAbs have the capacity to block IE binding through a dual effect of masking receptor-adhesion surface ligands and interfering with parasite survival through destruction of parasite-invaded RBCs.

Our results showed that mAbs and pAbs specifically inhibited parasite adhesion to both ICAM-1 and CD36 receptors. Blocking of ItG-IE binding to ICAM-1 was significantly correlated with adhesion-inhibition to CD36 receptors ($r_s = 0.89$, $P < 0.05$). In addition, mouse IgM antibodies that significantly blocked CD36 binding for ItG-IEs also blocked C24 binding, with a comparatively less capacity of inhibition. The ICAM-1 binding sites are mapped to DBLβ domains, which are clearly distinct from the CIDR domains involved in CD36 binding. Therefore, we were surprised that mouse IgMs that blocked ICAM-1 binding equally well inhibited CD36 binding. Two suggestions can be attributed to this cross-inhibition phenomenon. First, despite the fact that mouse IgMs were raised to ICAM-1 binding DBLβ domains, the large polymeric IgM molecules may have fully or partially masked critical PfEMP1 epitopes also involved in CD36 binding. Possible steric hindrance generated after binding of IgM molecules to PfEMP1 or adjacent ligands may have blocked the interaction between receptors and PfEMP1 domains involved in adhesion. Second, IgM binding may have resulted in destabilisation of the basic structure of PfEMP1 leading to loss of adhesion capacity of functional domains. These findings suggest that binding
regions for mouse IgMs overlap, or localised at close proximity to those for ICAM-1 and CD36 binding on the native PfEMP1 protein.

Most studies that investigated the cytoadhesion of clinical parasite isolates have been carried out under static conditions. In this work we tested the capacity of mouse antibodies to inhibit parasite binding under both static and physiological flow conditions, as results taken individually from either assays could give disparate information about interactions between receptors and parasite ligands (Chakravorty and Craig, 2005) such as the disparity between ItG and A4 parasite isolates in terms of binding to HUVEC under flow conditions (Gray et al., 2003). In addition, results acquired from static assays alone can be deceptive when interpreted on interactions that take place in the blood circulation under flow conditions (Adams et al., 2000). Flow-based assays can be useful in demonstrating variations in the characteristics and kinetics of ligand-receptor interactions that are influenced by the shear pressure induced by flowing blood (Cooke et al., 1995, Adams et al., 2000). Comparison of mouse IgM-mediated adhesion blocking to plastic-adsorbed ICAM-1 under static conditions with inhibition under physiological flow conditions revealed a coefficient of correlation of 0.94, indicating that blocking under static and flow conditions was positively correlated. These findings indicate that blocking of IgM antibodies was not influenced by state of binding (flow or static) although blockade was relatively greater under static condition to plastic-adsorbed ICAM-1 receptors than inhibition under flow conditions.

A variety of primary EC lines from different tissues can be used as models for studying cytoadhesion such as human dermal microvascular endothelial cells (HDMEC) and HUVEC (Gray et al., 2003, Phiri et al., 2009). HUVEC can be studied as a model for investigating brain microvascular endothelium as both cell lines constitutively express ICAM-1 receptors, but not CD36, when stimulated with proinflammatory cytokines such as TNF-α (Dobbie et al., 1999, Gray et al., 2003). Previous work has demonstrated that binding of some parasite isolates to HUVEC is primarily dependent on ICAM-1 receptors under static (Berendt et al., 1989) and flow conditions (Cooke et al., 1994). Based on these
characteristics, we hypothesised that blockade of parasite binding to HUVEC after incubation with mouse IgMs can be related to their blockade to ICAM-1 receptors under similar flow conditions. Our findings indicated that mAbs and pAbs that blocked parasite adhesion to immobilised ICAM-1 receptors under flow conditions also inhibited binding of A4-IEs to activated monolayers of HUVECs but at relatively reduced capacity of blocking. An exception for this observation was the inhibition profile for pAbs C2 and A4, as the former strongly inhibited IE binding whereas pAb A4 did not significantly affect parasite adhesion. Although pAb C2 neither labelled the surface of A4-IEs nor lysed infected cells, we suggest that a small population of pAb C2 antibodies had the required specificity to block binding ligands to HUVEC despite the failure of that population to produce a recognisable surface labelling signal. Alternatively, HUVECs may express surface receptors that act as ligands for mouse IgM binding, and binding of mAbs/pAbs to these receptors may interfere with the interaction between IEs with HUVECs. Previous work has shown that human IgM binds to HUVEC (Mold and Morris, 2001), but whether mouse IgM binds to these cells or not is still unknown.

The comparatively reduced capacity for IE blocking to HUVEC compared to inhibition of binding to immobilised ICAM-1 receptors under flow could be due to differences in the length of PfEMP1 molecules expressed by A4 and ItG parasite isolates. PfEMP1 proteins expressed by A4 parasites tend to be relatively longer than those expressed by ItG isolates (Gray et al., 2003), which makes it more difficult for mAbs to access and mask critical epitopes involved in cytoadhesion under flow conditions. Although IEs primarily bind to HUVEC through ICAM-1, other surface receptors expressed at lower levels such as CD36 may support the adhesion of parasitized erythrocytes to HUVEC surface (McCormick et al., 1997). Furthermore, other factors such as receptor mobility within the surface of HUVEC may have enhanced binding under flow conditions, compared to binding to immobilised ICAM-1 receptors. These data suggest that the specificity of mouse IgMs to block binding sites on A4-PfEMP1 is relatively lower than for ItG, or level of receptor expression on HUVEC and binding
avidity of this cell line to A4-IEs may have influenced binding of IgM-coated IEs.

A significant milestone in the development of PfEMP1-based drugs is through the production of adhesion-blocking antibodies. Such antibodies would inhibit the sequestration of parasitized erythrocytes and consequently lead to clearance of parasitized RBCs from peripheral circulation by the spleen (Rowe et al., 2009). The majority of work in anti-adhesive therapies was carried out on placental malaria and CD36-mediated cytoadherence, in which binding regions have been characterised and antibodies to soluble proteins based on these domains have been shown to be successful in blocking parasite adhesion (Tse et al., 2004). To our knowledge, no studies for the development of antibodies targeting ICAM-1-binding DBL domains have been published despite the potential role of ICAM-1 binding in cerebral malaria. In this work, we presented evidence that anti-DBLβ mAbs and pAbs not only cross-reacted with diverse P. falciparum parasite isolates but also cross-inhibited binding of IEs to immobilised receptors and EC lines. Although we provided evidence that blocking can also be mediated by surface labelling, the fact there is no specific inhibition to a particular receptor or a parasite isolate, as blocking mAbs and pAbs inhibited IE adhesion to all receptors tested, makes it convincible to believe that inhibition of IE adhesion is mainly due to IgM-mediated lysis. Since a large proportion of IEs are lysed upon mouse IgM incubation, and the effect of IgM on non-lysed cells is not yet known nor the reasons why not all IEs are lysed, are additional factors that may support the role of lysis in adhesion inhibition. Furthermore, the number of samples in the correlation experiments is not large enough and interpretation of any positive correlation, such as the correlation of surface labelling and inhibition, must be carried out with extra caution especially for those that did not reach statistical significance.

From a therapeutic development point of view, it is promising that cross-reactive antibodies possess anti-adhesive properties, regardless of the mechanism of inhibition. In fact, effective anti-adhesive antibodies should target all receptor-binding sites or at least multiple sites at the same time to interfere with parasite
adhesion. Therefore, we suggest that mouse IgM antibodies may have therapeutic values and could play a significant role in protection against severe malaria through blockade of sequestration and lysis of parasite-invaded erythrocytes. Identification of the mechanism of IE lysis as well as binding sites for mAbs and pAbs on the surface of IEs remains an important subject of research, particularly due to the possibility that IgM binding regions would seem to be of a significant interest in the quest to develop PfEMP1-based therapies.
4.9 Limitations and future work

Due to limitations of the amount of IgM antibodies yielded from the purification, we could not repeat the adhesion-blocking experiments for several times to confirm preliminary results. Although most assays were repeated using mAb G6 and the results were consistent with preliminary findings (data not shown), it is recommended that the amount of antibodies in future work be expanded to higher volumes for obtaining the required confidence of data.
Chapter 5

Characterisation of semi-immune sera collected from female adults living in Kenya
Chapter 5: Introduction

In endemic areas where malaria transmission is usually high and stable, repeated exposure to malaria infections results in the gradual development of a clinical immunity, which limits the parasite-related health consequences (Gupta et al., 1999). Protective immunity acquired this way is primarily mediated by IgG antibodies (Nielsen et al., 2004). The risk of severe and life-threatening disease in these areas is usually highest during early childhood, after which the occurrence and severity of the disease rapidly decrease over several years, as individuals acquire natural protective immunity (Bull et al. 1998, Gupta et al. 1999).

Several studies have provided evidence that VSAs on *P. falciparum*-IEs are important targets of naturally acquired immunity, and antibodies directed against these highly immunogenic antigens are associated with protection against homologous parasite isolates (Giha et al., 2000, Marsh et al., 1989, Bull et al., 1998). Immunological profiling of sera for the existence of reactive antibodies against VSAs is a widely utilised method for evaluating acquired immunity and recognising potential candidates for vaccine development (Oleinikov et al., 2009). Agglutination has been used in several studies for studying immune responses and antigenic variation of PfEMP1 molecules, as switches in the agglutination phenotype are associated with switches in expression of var genes or PfEMP1 molecules (Smith et al., 1995, Chan et al., 2014). Because this technique is time-consuming, only provides semi-quantitative results and rosettes can be counted by mistake as agglutinates in some conditions, most of studies now use other quantitative methods such as flow cytometry for studying the antibody response against antigens on the surface of IEs to overcome these limitations (Staalose et al., 1999, Giha et al., 2000). Flow cytometry provides efficient, precise and quantitative measurement of different combinations of parasite strains and serum samples (Ofori et al., 2002). In addition, this technique has an important advantage over agglutination assay including the ability to measure antibody reactivity to parasite isolates that do not agglutinate.
IEs (Giha et al., 1999, Ofori et al., 2002). However, in some situations such as studying the adhesion blocking capacity of semi-immune sera, the use of both agglutination and flow cytometry is important to elucidate the mechanism of inhibition.

In this chapter, we describe the use of several functional assays for characterizing the functional antibody response of eight serum samples obtained from adult female donors living in Junju sub-location, Kilifi County, Kenya. We aimed to evaluate the reactivity of these sera against recombinant PfEMP1 domains using ELISA and investigate the ability of IgG to recognise the surface of erythrocytes infected by diverse *P. falciparum* isolates of differing genomes and PfEMP1 phenotypes. In addition, we investigated the presence of IgG antibodies that have the capacity to agglutinate IEs and block the adhesion of IEs to immobilised receptors and HUVECs. Our findings demonstrated that semi-immune sera recognised all tested recombinant domains and exhibited variable levels of surface reactivity against tested parasite isolates. In addition, some sera exhibited higher capacity at blocking parasite adhesion to immobilised ICAM-1/CD36 receptors and HUVEC and comprised agglutinating antibodies. These findings suggest that some of the semi-immune sera can have therapeutic implications and used for further vaccine development research.

There is no direct relation between the present and previous chapters. However, since all assays carried out in this chapter are the same as those in previous chapters and the general aims of investigating the immune response towards the surface of IEs and assessing the capacity of adhesion blocking are similar to those in previous chapters, we decided that this chapter is added to the present thesis.
5. Materials and Methods

5.1 Serum samples

Human serum samples used in the present study were kindly provided by Dr. Britta Urban, LSTM. The samples were originally collected from eight female adults living in Junju sub-location, Kilifi County, Kenya who were between 31 and 52 years old (median age, 34.5 ± 6.5 years [Table 5.1]). The definition of semi-immune individual is an adult living in an area of malaria endemicity and does not have the symptoms at the time of sample collection even if he/she was previously infected with *P. falciparum* malaria (Oguariri et al., 2001). All samples were kept at 4°C throughout the period of the study.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age</th>
<th>Sex</th>
<th>Malaria Pigment</th>
<th>Slide results</th>
<th>PCR</th>
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<td>False</td>
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<td>False</td>
<td>False</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 5.1. Characteristics of semi-immune serum samples used in the present study. Sera were collected from female adults living in Kilifi County, Kenya. Blood samples from each donor were firstly screened microscopically for presence of parasites (parasitaemia) and malaria pigments. Samples were also screened using polymerase chain reaction (PCR) for the molecular detection of malaria.
5.2 **Indirect ELISA for screening semi-immune sera against recombinant DBL domains**

The same ELISA assay was carried out following the procedure described in Chapter 2 (section 2.4.1.1) with minor modifications. Briefly, 96-well microtitre plates (Nunc®) were coated with 100 µl of recombinant domains (rDBL13, rDBL16, rDBL27, rDBL31, rDBL41 and rDBL4ε) dissolved in 0.1 M carbonate bicarbonate buffer at 5 µg/ml and incubated overnight at 4°C. Subsequent blocking and washing steps were performed as described. After washing, plates were incubated with human semi-immune sera (JA221 – JA254) diluted 1:50 in complete medium for 2 hours at room temperature with gentle shaking. Malawian HIS at 1:50 dilution was used as a positive control. Complete medium was included in the assay as a negative control. After incubation, wells were washed and incubated at room temperature for 1 hour with 100 µl of HRP-conjugated goat anti-human IgG antibodies (Sigma) at 1:500 dilution in complete medium. Subsequent washing, developing and reading were carried out as described in section 2.4.1.1. Recognition was assessed in terms of the response indicated as OD values and shown as the average of triplicate readings with SD values expressed as error bars. The results were considered positive if the mean absorbance value obtained from the semi-immune sera was more than the cut-off value described as 3 SDs above the mean of control absorbance values.

5.3 **Analysis of semi-immune sera by flow cytometry**

The capacity of human semi-immune sera to recognise the surface of erythrocytes infected by diverse lab-adapted *P. falciparum* isolates was assessed by flow cytometry following the same protocol described in Chapter 2 (section 2.4.2) with some modifications. Briefly, mature pigmented trophozoites at mid-late stage were enriched as described, and 3 µl of the enriched pellet was added to 1 ml of PBS/1%BSA to prepare IE suspension. Aliquots of the suspension (100 µl, 0.8-1 × 10⁶ cells) were washed in 1.5 ml Eppendorf tubes and the pellet
was re-suspended with semi-immune sera or non-immune sera from UK adults at 1:25 dilution in PBS/1%BSA and incubated for 60 minutes at 37°C. After incubation, cells were washed and bound IgG antibodies were detected by incubation with 1:100 dilution of APC-conjugated mouse anti-human IgG-Fc secondary antibody (Southern Biotech) plus 10 µg/ml ethidium bromide. Cells were incubated in the dark for 60 minutes at 37°C. After incubation, cells were washed twice, re-suspended with 400 µl of Cell Wash and analysed using Becton-Dickinson FACSCalibur flow cytometer (BD LSR11). All washes and antibody dilutions were carried out in PBS/1%BSA. Cells incubated with non-immune sera from UK adults and stained with ethidium bromide and secondary antibodies or those only incubated with secondary antibodies and ethidium bromide were included as negative controls. Labelling of uninfected cells with human IgG antibodies was found at a very low range. Differences in the binding capacity of semi-immune sera against tested isolates were further confirmed by repeating experiments (n=2).

Data were acquired using FACSCalibur flow cytometer and 50,000 events were collected and analysed with FlowJo software. Specific surface staining for IEs was determined in terms of percentage of APC-positive IE population labelled by test antibodies relative to control antibody. In all assays, a labelling of \( \geq 10\% \) of IE population was considered positive for surface labelling, whereas \( \leq 10\% \) was considered negative. Grading of capacity of surface recognition was set as followed:

\[
\begin{align*}
+: & \quad \text{if labelled IE population was within a range of 10.1 - 20}\% \\
++: & \quad \text{if labelled IE population was within a range of 20.1 - 40}\% \\
+++: & \quad \text{if labelled IE population was within a range of 40.1 - 60}\% \\
++++: & \quad \text{if labelled IE population was } > 60.1\% .
\end{align*}
\]
5.4 Adhesion-blocking of IEs to ICAM-1 or CD36 receptors under static conditions

Blockade of adhesion under static conditions was carried out as described in chapter 4 (section 4.3) with some differences. Briefly, IE suspension for each dish (1.25 ml) was centrifuged, before the pellet was incubated separately with 100 µl of semi-immune female sera at 1:25 dilution for 60 minutes at 37°C. After incubation, cells were re-suspended in 1.25 ml binding buffer and added to each dish (2×), and then the assay was continued as described in section 4.3. Previously characterised control mAbs that block IE adhesion to ICAM-1 (mAb 15.2) or CD36 (mAb IVC7) were added to IE suspension at 5 µg/ml prior to addition to the protein-coated dishes and the same procedure were followed as shown above. Percentage of IE adhesion-inhibition was determined by the difference in the count of IEs assayed in the presence of semi-immune sera relative to IEs incubated with UK control sera. The results were expressed as percentage of binding of test samples relative to control, normalised as 100%.

5.5 Adhesion-blocking of IEs to ICAM-1 receptors under physiological flow conditions

In assays that assess the ability of human semi-immune sera to block parasite binding to immobilised ICAM-1 receptors under flow conditions, the same procedure was followed as described in chapter 4 (section 4.4.1) with minor modifications. Briefly, IE suspension (500 µl) designated for each serum sample was firstly centrifuged, before the pellet was incubated with 100 µl of semi-immune sera at 1:25 dilution for 60 minutes at 37°C. After incubation, cells were centrifuged and re-suspended in 500 µl of binding buffer, and then the assay was continued as described in section 4.4.1. To block IE binding to ICAM-1, mAb 15.2 was added to the suspension at 5 µg/ml prior to beginning of the assay. Percentage of IE adhesion blocking was determined by the difference in the count of IEs assayed in the presence of semi-immune sera to IEs incubated with UK control serum. The results were expressed as percentage of binding of test samples relative to control, normalised as 100%.
5.6 Adhesion-blocking of IEs to HUVEC under physiological flow conditions

HUVEC culture and investigation of IE adhesion to these cells under conditions of physiological blood flow were carried out as described in chapter 4 (Section 4.4.2). For assessing the capacity of semi-immune sera to block parasite adhesion to HUVEC, the same protocol was followed as described in the previous section.

5.7 Agglutination assay

Semi-immune sera were tested in agglutination assay using trophozoite-IEs (parasitaemia of 1-5% and haematocrit of 5%) as described by Bull et al., 1999 with some modifications. Cells were washed in serum-free RPMI 1640, and non-infected washed RBCs were added, if necessary, to adjust HCT and parasitaemia levels. After washing, cells were re-suspended in serum-free RPMI 1640 and ethidium bromide was added to a final concentration of 10 µg/ml to label IEs. A 25 µl reaction volume was prepared for test or control sera at a final dilution of 1:25 (1 µl of sera added to 24 µl of stained suspension) and added to U-bottomed 96-wells plate. Then, the plate was sealed using an adhesive film and incubated at room temperature on a rotating wheel for 60 minutes in darkness. The 25 µl reaction volume was split into two, gently spread onto two pre-labelled slides and covered with 22 mm coverslips. Slides were examined using light and fluorescent microscopes for the presence of agglutinates at 300× (20 × 15) magnification. 10 fields were examined, and samples were scored positive on the basis of the observation of 15 or more agglutinates within all scanned fields. Agglutinating scores (based on the size of agglutinates) were also counted to provide additional measurements. Five scores based on the number of IEs in each agglutinate were included: 3-9, 10-20, 21-40, 41-60 and ≥ 61. To confirm that agglutinates were clumps of IEs and not rosettes of non-IEs, all agglutinates were stained with ethidium bromide as described above and examined under fluorescent microscope.
5.8 **Statistical analysis**

Spearman's rho correlation was used for assessing the correlation between variables using SPSS and Spearman's rho correlation coefficient ($r_s$) was used to determine the strength of the correlation. Two-tailed $t$-test was used for determining the significance of the correlation, which was considered as statistically significant when $P$ value $< 0.05$. One-way ANOVA test with Daunett $t$-test (two-sided) was used to measure the difference in IE adhesion. The difference was considered significant when $P$ value $< 0.05$. All statistical analyses were carried out using SPSS version 22.

5.9 **Ethics approval**

This study was carried out under protocol No. 1131: Integrated studies for the development of natural immunity to malaria in children in Kilifi district, Kenya. The above protocol was discussed during the 136th Meeting of the KEMRI/National Ethical Review Committee held on the 22nd of August, 2006, and an approval was granted for the protocol. A copy of the revised protocol is provided in the Appendix, together with a copy of the approval granted by Oxford Tropical Research Ethics Committee (OXTREC) on the 4th of October 2006 for studies under the same protocol.
5. Results

5.1 Indirect ELISA for investigating the reactivity of semi-immune sera against recombinant DBL domains

Serum samples used in the present study were collected from eight female adults living in Junju sub-location, Kilifi County, Kenya. In order to investigate the natural human response to malaria antigens, semi-immune sera were tested for reactivity with a panel of five recombinant DBLβ domains from PfEMP1 ICAM-1 variants of the IT4 genotype (rDBL13, rDBL16, rDBL27, rDBL31 and rDBL41) or with VAR2CSA-DBL4ε domain using indirect ELISA. The reactivity against recombinant proteins with serum IgG or IgM were analysed, and frequencies of IgG recognition of recombinant proteins with all sera are shown in Figure 5.1 and Table 5.1. Since no reactivity of serum IgM was observed (data not shown), all results will discuss IgG reactivity henceforth. All sera exhibited a broad range of immune responses against DBL proteins above the calculated cut-off values, with rDBL41 showing the highest levels of reactivity. On the other hand, the immune response towards DBL4ε was comparatively weaker for all tested sera. It is noteworthy that serum JA249 demonstrated the highest response with almost all coated proteins. Thus, each adult is likely to have a repertoire of antibodies to different DBL antigens, probably due to repeated exposure to malaria infection.
Figure 5.1. The reactivity of serum samples collected from eight female adults living in Kilifi, Kenya against recombinant DBL domains as measured by indirect ELISA. Plates were coated with 100 µl of rDBL proteins (rDBL13, rDBL16, rDBL27, rDBL31, rDBL41 and rDBL4ε) dissolved in 0.1 M carbonate bicarbonate buffer at 5 µg/ml and incubated overnight at 4ºC. Plates were washed, blocked and incubated with 1:50 dilution of adult sera (JA221 - JA254) or CM alone and incubated for 2 hours at room temperature with gentle shaking. Malawian HIS at 1:50 dilution was used as a positive control. CM was included in the assay as a negative control. Reactive IgG antibodies were detected with HRP-conjugated goat anti-human IgG antibodies (Sigma) at 1:500 dilution. Recognition was assessed in terms of the response indicated as OD values and shown as the average of triplicate readings with SD values expressed as error bars.
<table>
<thead>
<tr>
<th>Semi-immune sera</th>
<th>Recombinant DBL domains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBL13</td>
</tr>
<tr>
<td>JA221</td>
<td>++</td>
</tr>
<tr>
<td>JA225</td>
<td>+++</td>
</tr>
<tr>
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<td>++++</td>
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<td>++++</td>
</tr>
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</tr>
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<td>+++</td>
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<td>JA249</td>
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<td>JA254</td>
<td>++++</td>
</tr>
<tr>
<td>HIS</td>
<td>++++</td>
</tr>
</tbody>
</table>

Table 5.1. Investigating the presence of reactive IgG antibodies in serum samples collected from eight female adults living in Kilifi, Kenya against recombinant DBL domains as measured by indirect ELISA. The results were considered positive if the mean absorbance value for the semi-immune sera was more than the cut-off value described as 3 SDs above the mean of control absorbance values. Capacity of recognition was as followed: +: > 3 SD values above the mean of control absorbance value (i.e. 0.19) to OD value of 0.29, ++: OD values of 0.30 to OD values of 0.39, +++: OD values of 0.40 to OD values of 0.49, ++++: OD values of 0.50 to OD values of 0.59, ++++++: > OD values of 0.60.
5.2 Measurement of IgG binding to the surface of *P. falciparum*-IEs

Semi-immune sera were then used for comparisons of IgG binding to the surface of live erythrocytes infected by A4, ItG, C24, 3D7 and CS2 *P. falciparum* isolates using flow cytometry (Table 5.2). Parasite isolates used in the screening were phenotypically distinct; including isolates that bind ICAM-1 and CD36 from the IT4 genotype (A4 and ItG), non-ICAM-1 binding isolate from the same genome (C24), CSA-binding isolate from the IT4 genome (CS2) and strain NF54 from the distinct 3D7 genotype. Representative flow cytometry binding profiles for semi-immune sera with erythrocytes infected by ItG and 3D7 isolates are shown in Figures 5.2 and 5.3, respectively. ItG was the most specifically recognised *P. falciparum* isolate by all semi-immune sera followed by 3D7, whereas A4 was relatively the least recognised parasite. Overall, the variation seen with the reactivity against live IEs was greater than the reactivity with recombinant proteins. IgG of most sera bound to the surface of IEs at variable levels among all samples (range of the percentage of IE recognition was 14.2 - 79.5%). Sera JA235, JA239, JA249 and JA254 recognised the surface of erythrocytes infected by all tested parasite isolates, whereas JA221 only recognised 50% (n=3) of parasite lines. No pattern of strong surface labelling was detected for a particular serum although JA235 demonstrated an overall higher capacity of recognition with all isolates compared to the other semi-immune sera. In order to establish appropriate parameter values for the surface labelling with flow cytometry and adhesion blocking assays, we first examined the binding at different dilutions of semi-immune sera (1:25 or 1:50). We found that 1:25 dilution was optimal for surface recognition (data not shown). Therefore, it was used in all subsequent surface labelling experiments and adhesion blocking assays.
Figure 5.2. FACS analysis of ItG-IE surface reactivity with semi-immune sera from female adults living in Kilifi, Kenya. 50 µl of each test serum at 1:25 dilution was incubated with trophozoite-enriched IEs and labelling was detected using APC-conjugated mouse anti-human IgG-Fc secondary antibody (Southern Biotech) at 1:100 dilution. Grey shaded histograms represent IE populations labelled by IgG antibodies. Negative controls including cells stained with 50 µl of non-immune sera from UK adults at 1:25 dilution shown in blue and those only stained with ethidium bromide and secondary antibodies shown in red. Percentages of labelling were calculated relative to non-immune UK serum control. A labelling of > 10% of IE population was considered positive. Uninfected cells were shown to be negative for labelling with semi-immune sera (data not shown).
Figure 5.3. FACS analysis of 3D7-IE surface reactivity with semi-immune sera collected from female adults living in Kilifi, Kenya. The same assay was carried out as described in Figure 5.2.
Table 5.2. The reactivity of semi-immune serum samples collected from female adults living in Kilifi, Kenya with the surface of erythrocytes infected by phenotypically and genotypically distinct *P. falciparum* isolates as indicated by flow cytometry. - (labelled IE population ≤ 10%), + (labelled IE population 10.1 - 20%), ++ (labelled IE population 20.1 - 40%), +++ (labelled IE population 41 - 60%), ++++ (labelled IE population > 60%).

<table>
<thead>
<tr>
<th>Serum</th>
<th>P. falciparum isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A4</td>
</tr>
<tr>
<td>JA221</td>
<td>+</td>
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<td>-</td>
</tr>
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<td>++</td>
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<tr>
<td>JA254</td>
<td>+</td>
</tr>
<tr>
<td>UK control</td>
<td>-</td>
</tr>
</tbody>
</table>

5.3 Correlation of IgG surface labelling between *P. falciparum* parasite isolates

The eight semi-immune serum samples obtained from Kenyan female adults were used for comparisons in terms of IgG binding to the surface of A4, ItG, C24, 3D7 and CS2-IEs using Spearman’s rho correlation test (Table 5.3). This was to investigate whether surface labelling by a particular serum to erythrocytes infected by a given isolate was correlated with labelling to the other tested isolates. For the same serum samples, IgG binding was only significantly correlated between ItG and A4 ($r_s = 0.85$, $P < 0.05$), and between ItG and C24 parasite isolates ($r_s = 0.79$, $P < 0.05$) indicating that serum samples labelling the surface of ItG-IEs also recognised A4- and C24-IEs particularly when higher
IgG response binding were detected. It also suggests that antibody repertoires with similar labelling properties were acquired in semi-immune individuals after exposure to *P. falciparum* infection. The comparison also revealed a strong positive correlation, although not statistically significant, between A4 and C24 ($r_s = 0.62, P > 0.05$) and between 3D7 and CS2 ($r_s = 0.595, P > 0.05$). The strong significant correlation between parasite isolates expressing different PfEMP1 variants indicate that semi-immune serum samples are broadly cross-reactive, as indicated by the significant association between an ICAM-1 binder (ItG) and a non-binding isolate (C24), and between two isolates from different genomes (CS2 and 3D7).

<table>
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<tr>
<th>Correlations</th>
<th>ItG</th>
<th>A4</th>
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<th>3D7</th>
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<td>.786</td>
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<td>.021</td>
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<tr>
<td>A4 Correlation Coefficient</td>
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<td>.933</td>
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Table 5.3. Spearman's rho correlation of surface labelling profiles of eight semi-immune serum samples obtained from Kenyan adult females to erythrocytes infected by five *P. falciparum* parasite isolates. Binding of IgG to the surface of erythrocytes infected by A4, ItG, C24, 3D7 or CS2 was determined using flow cytometry and surface labelling was expressed as percentage of IEs labelled by semi-immune sera relative to binding of control serum. Values of Spearman’s rho correlation coefficient ($r_s$): 0 (no correlation between variables), 0.1 to 0.3 (weak positive correlation), 0.3 to 0.5 (moderate positive correlation), 0.5 to 1.0 (strong positive correlation). Correlation is statistically significant when $P$ value < 0.05. **: correlation is significant at the 0.01 level. *: correlation is significant at the 0.05 level.
5.4 Adhesion blocking studies with semi-immune sera

5.4.1 Blockade of ItG-IE adhesion to ICAM-1 receptors under static conditions

Semi-immune sera that showed high levels of cross-reactivity with variant recombinant DBLβ proteins and recognised the surface of erythrocytes infected by diverse parasite lines were tested for their capacity to inhibit IE adhesion to immobilised receptors under static or flow conditions. Adhesion assays were essentially carried out following the same methods described in Chapter 4 with some modifications. Serum samples obtained from non-immune UK adults were included in all assays and as negative controls. The first assay was performed to assess the capacity of semi-immune Kenyan sera to block ItG-IE binding to purified ICAM-1 under static conditions (Figure 5.4). Out of 8 tested sera, only two (JA225 and JA235) strongly blocked binding to ICAM-1 by 90.4% and 93.9%, respectively compared to UK non-immune adult serum ($P < 0.001$). On the other hand, JA238 and JA254 moderately inhibited IE adhesion by 30.2% and 17%, respectively ($P < 0.001$). With regard to JA221, JA239, JA242 and JA249 sera, no appreciable anti-adhesive effect was observed.
Figure 5.4. Adhesion blocking of ItG-IEs to purified ICAM-1 receptors under static conditions. Binding of IEs (3% parasitaemia, 1% HCT) to ICAM-1 receptors (50 µg/ml) was observed after incubating IEs with semi-immune sera collected from female adults living in Kilifi, Kenya at 1:25 dilution (100 µl). Graphs shown represent the mean percentage of binding to ICAM-1 relative to the binding of ItG-IEs incubated with UK control sera at 1:25 dilution (normalised to 100%). Error bars represent SDs of two independent dishes (6 spots, 36 readings). Control assays include binding of ItG-IEs to PBS spots immobilised onto the same plates, which showed no binding (data not shown). The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding with UK non-immune sera is represented by one asterisk ($P < 0.05$) or two asterisks ($P < 0.001$).
5.4.2 Blockade of C24-IE adhesion to CD36 receptors under static conditions

Semi-immune sera were then tested for inhibition of C24-IEs to immobilised CD36 receptors under static conditions (Figure 5.5). C24 P. falciparum-IEs strongly bind to CD36 receptors, but not ICAM-1 (Roberts et al., 1992, Gray et al., 2003). Contrary to the results observed with ICAM-1, stationary binding to CD36 was not greatly affected after incubation with all semi-immune sera except JA254 which only blocked 23% of C24-IE binding ($P < 0.001$). Although JA249 caused a reduction of adhesion by ~13%, this effect was no statistically significant ($P > 0.05$). These results suggest that naturally acquired antibodies in semi-immune sera lack the capacity to effectively block PfEMP1 domains involved in parasite binding to CD36 receptors. This is interesting since binding to CD36 is a common adhesion phenotype and most wild and culture-adapted P. falciparum isolates have the capacity to bind purified CD36 receptors (Chilongola et al., 2009, Newbold et al., 1997, Rogerson et al., 1999). Thus, exposure of individuals in endemic areas to this binding phenotype is huge yet they did not develop blocking antibodies. However, we should evaluate the blocking capacity of sera against different CD36-binding isolates to support these results.
Figure 5.5. C24-IE adhesion blocking to CD36 receptors with semi-immune sera under static conditions. C24-IEs were pre-incubated with semi-immune sera or UK non-immune sera at 1:25 dilution then allowed to bind with CD36 immobilised on plastic dishes at 50 µg/ml. Graphs shown represent the mean percentage of binding to CD36 relative to the binding of C24-IEs incubated with UK control sera (normalised to 100%). Error bars indicate SDs of 36 readings collected from two independent dishes (6 spots). Control assays include binding of C24-IEs to PBS spots immobilised onto the same plates, which showed no binding (data not shown). The difference in binding was considered significant if \( P < 0.05 \), calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding with UK non-immune sera is represented by one asterisk \( (P < 0.05) \) or two asterisks \( (P < 0.001) \).
5.4.3 Blockade of ItG-IE adhesion to ICAM-1 receptors under physiological flow conditions

To validate the in vivo relevance of static-based findings, we tested whether pre-incubating ItG-IEs with adult pre-immune sera affected their adhesion to ICAM-1 receptors under physiological flow conditions (Figure 5.6). Similar to blockade of ItG-IE adhesion under static conditions, JA225 and JA235 were the most effective in inhibiting parasitized erythrocyte binding to ICAM-1 under flow conditions, with inhibition percentages of 68% and 46%, for JA225 and JA235 sera respectively compared to inhibition of UK control serum ($P < 0.001$). Although JA242, JA249 and JA254 sera only inhibited ~18% of ItG-IE adhesion, their blockade was considered significant ($P < 0.05$).
Figure 5.6. Blockade of Ig-IE adhesion to ICAM-1 at an inflow shear stress of 0.05 Pa. IE suspension (3% parasitaemia, 2% HCT) was flowed over ICAM-1-coated (50 µg/ml) microslides after incubation with semi-immune sera or UK control serum at 1:25 dilution. Graphs shown represent the mean percentage of binding to ICAM-1 relative to the binding of UK control sera (normalised to 100%). Error bars represent SDs of the means of 6 readings from randomly selected fields. Blocking capacity was confirmed with repeating assay on another micro-slide. The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding of UK control sera is represented by one asterisk ($P < 0.05$) or two asterisks ($P < 0.001$).
5.4.4 Blockade of A4-IE adhesion to HUVEC under physiological flow conditions

We then tested the capacity of semi-immune sera to inhibit parasite binding to HUVEC under flow conditions at a physiologically relevant shear stress (0.05 Pa). As stated previously, A4-IEs adhere preferentially to HUVEC via ICAM-1 ligands, as these ECs express ICAM-1, but not CD36, upon TNF-α activation (Dobbie et al., 1999, Gray et al., 2003). A4-IEs were firstly incubated with semi-immune or control sera at 1:25 dilution, before they were tested for adhesion to confluent monolayers of TNF-α-stimulated HUVEC (Figure 5.7). Surprisingly, all semi-immune sera have significantly reduced IE adhesion to HUVEC ($P < 0.001$), with JA225 and JA235 demonstrating the highest capacity of inhibition ($\sim 86\%$) followed by JA238 which blocked $\sim 80\%$ of IE binding ($P < 0.001$). JA221, JA239, JA249 and JA254 exhibited similar levels of blocking ($\sim 70\%$) whereas JA242 had the least blocking capacity compared to the other sera, with an inhibition percentage of 60% relative to UK control serum ($P < 0.001$).
Figure 5.7. Blockade of A4-IE adhesion by semi-immune sera to TNF-α-stimulated HUVEC under flow conditions at a wall shear stress of 0.05 Pa. Confluent HUVEC monolayers on flow micro-slides were stimulated 18-24 hours prior to the assay with 5 ng/ml TNF-α. Then, IEs (3% parasitaemia, 2% HCT) were flowed over the micro-slides after incubation with semi-immune sera or UK non-immune sera at 1:25 dilution. Graphs shown represent the mean percentage of binding to HUVEC relative to the binding of UK control sera (normalised to 100%). Error bars are SDs of the means of six readings from randomly selected fields. Inhibition capacities were confirmed by flowing IE suspensions through another HUVEC-coated channel. The difference in binding was considered significant if $P < 0.05$, calculated by One-way ANOVA test with Daunett t-test (two-sided). Statistically significant adhesion inhibitory capacity compared to binding of UK control sera is represented by two asterisks ($P < 0.001$).
5.5 Correlation between surface labelling and adhesion blocking

IE labelling by semi-immune sera was tested for correlation with adhesion-blocking to immobilised receptors or HUVEC (Table 5.4). At the first analysis, correlation coefficients ($r_s$) were calculated for the association between blocking percentages of ItG-IEs to ICAM-1 receptors under static or flow conditions with ItG-IE surface labelling for all semi-immune sera. The analysis revealed a strong positive correlation, though not significant, between adhesion-inhibition of ItG-IEs under static and flow conditions ($r_s = 0.59$, $P > 0.05$), indicating that semi-immune sera which strongly blocked parasite adhesion under static conditions also blocked the binding under flow. There was a moderate to strong positive correlation between ItG-IE surface labelling and adhesion blocking to ICAM-1 under static conditions ($r_s = 0.551$, $P > 0.05$), i.e. the higher the percentage of labelled IEs by a particular individual serum, the higher the percentage of blocked erythrocytes by that serum and *vice versa*. On the other hand, there was no correlation between surface recognition and adhesion blocking under flow conditions (Table 5.4).

Analysis of the association between surface labelling to C24-IEs by semi-immune sera and blockade of static-based binding to CD36 revealed a moderate, not-significant positive correlation between both variables ($r_s = 0.36$, $P > 0.05$), whereas no correlation was detected between surface recognition of A4-IEs and adhesion blocking ($r_s = 0.11$, $P > 0.05$).
Table 5.4. Output of Spearman’s rho correlation between surface labelling and adhesion blocking for ItG, C24 and A4 isolates. 

A: Spearman’s rho correlation coefficients (rs) for the association between surface labelling of eight semi-immune sera to ItG-IEs and adhesion blocking of ItG-IEs to ICAM-1 receptors under static or flow conditions. B: Correlation between surface labelling to C24-IEs adhesion blocking of C24-IEs to CD36 receptors under static conditions. C: Correlation between surface labelling to A4-IEs and adhesion blocking of A4-IEs to HUVEC under flow conditions. Values of Spearman’s rho correlation coefficient (rs): 0 (no correlation between variables), 0.1 to 0.3 (weak positive correlation), 0.3 to 0.5 (moderate positive correlation), 0.5 to 1.0 (strong positive correlation). Correlation is statistically significant when P value < 0.05.
5.6 Agglutination assay

We calculated the agglutination score and frequency of A4- and ItG-IEs incubated with each of the semi-immune Kenyan serum to determine whether blockade of parasite adhesion was indirectly associated with IE agglutination (Tables 5.5 and 5.6). Agglutination score was determined by counting the number of IEs in each agglutinate and categorised into five groups based on the size of agglutinates, whereas agglutination frequency was estimated by quantitating the number agglutinates in 10 random fields under 300× (20 × 15) magnification. Despite being sampled from adult females of a similar age and living in the same area, the agglutination reactivity of the sera used in this work was highly variable. However, all sera that agglutinate A4-IEs also comprised agglutinating antibodies to ItG-IEs, but the frequency of agglutinates was noticeably higher with A4 than ItG. Out of all tested sera, five samples (JA225, JA235, JA238, JA249 and JA254) comprised antibodies that agglutinated A4- and ItG-IEs. For A4-IEs, Agglutinates containing 10-20 IEs were the most frequent among positive samples, whereas agglutinates that comprised ≥ 61 cells were the least observed. On the other hand, the most frequently detected agglutinates with ItG-IEs were those containing 3-9 IEs, whereas agglutinates containing 41-60 IEs were the least counted.

Serum samples obtained from non-immune UK donors were included as negative controls and showed no signs of IE agglutination. This indicated that parasite isolates used in this assay did not form non-specific IE agglutinates. To establish that agglutinates only comprised IEs and were not rosettes resulting from the binding of two or more non-IEs to one IE, parasite suspensions were stained with ethidium bromide and visualised under fluorescent microscope (Figure 5.8). We found that all agglutinates were positive for staining with the fluorescent dye, which confirms that observed clumps comprised IEs alone.
<table>
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<tr>
<th>Sera</th>
<th>3-9</th>
<th>10-20</th>
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<td>-</td>
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</tr>
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<td>3</td>
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<tr>
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<td>-</td>
<td>-</td>
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Table 5.5. Agglutination score and frequency for A4-IEs after incubation with eight semi-immune Kenyan sera or UK control serum. * Total number of IE agglutinates per 10 fields under 300× (20 × 15) magnification. Samples were scored positive on the basis of observing ≥ 15 agglutinates in all scanned fields. HS CRL: control serum from UK donors.

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<th>Sera</th>
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<th>21-40</th>
<th>41-60</th>
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<th>Agglutination</th>
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<td>-</td>
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<td>Negative</td>
</tr>
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</table>

Table 5.6. Agglutination score and frequency for ItG-IEs after incubation with eight semi-immune Kenyan sera or UK control serum. * Total number of IE agglutinates per 10 fields under 300× (20 × 15) magnification. Samples were scored positive on the basis of observing ≥ 15 agglutinates in all scanned fields. HS CRL: control serum from UK donors.

272
Figure 5.8. A4-IE agglutination mediated by semi-immune adult sera. IE agglutinates (red arrows) were visualised under light microscopy (A, B and C) or fluorescent microscope (D and E). Representative A4-IE clumps formed after incubation with JA235 (A) or JA249 (B) semi-immune sera are indicated by red arrows. A4-IEs did not form clumps after incubation with UK non-immune serum control (C). Aggregates of IEs were visualised under fluorescent microscope after staining with 10 μg/ml ethidium bromide (D and E) and clumps with non-IEs (rosettes) were not observed.
5.7 Correlation between adhesion blocking and agglutination

We then tested the statistical correlation between adhesion inhibition under static or flow conditions with IE agglutination for all tested sera (n=8). At the first analysis, correlation coefficients ($r_s$) were calculated for the association between inhibition percentages of A4-IE binding to HUVEC under flow conditions with agglutination of the same parasite isolate after incubation with semi-immune sera (Table 5.7). The analysis revealed a moderate positive correlation between blockade of A4-IE adhesion to HUVEC and agglutination scores ($r_s = 0.34$, $P > 0.05$). However, this correlation was not significant since all semi-immune sera blocked A4-IEs adhesion to HUVEC whereas only five (JA225, JA235, JA238, JA249 and JA254) comprised agglutinating antibodies.

The association between ItG-IE agglutination following incubation with all semi-immune sera and blockade of adhesion to ICAM-1 under static or flow conditions was then investigated. There was a strong correlation between agglutination and adhesion blocking under static conditions ($r_s = 0.66$, $P = 0.07$) and under flow conditions ($r_s = 0.51$, $P > 0.05$). This strong correlation, particularly for blocking under static conditions, may suggest that adhesion blocking was mostly mediated by agglutination, as agglutinates can clearly affect IE binding.
### Table 5.7

Output of Spearman's rho correlation between adhesion blocking and agglutination for A4 and ItG parasite isolates.

#### A

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</tr>
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#### B

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<th>ItG blocking static</th>
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<td>Sig. (2-tailed)</td>
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<td>Sig. (2-tailed)</td>
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Table 5.7. Output of Spearman's rho correlation between adhesion blocking and agglutination for A4 and ItG parasite isolates. A: Spearman's rho correlation coefficients ($r_s$) for the association between adhesion blocking of A4-IEs to HUVEC under flow conditions and agglutination of A4-IEs mediated by eight semi-immune sera. B: Spearman's rho correlation coefficients ($r_s$) for the association between adhesion blocking of ItG-IEs to ICAM-1 receptors under static or flow conditions and agglutination of ItG-IEs.
5.8 Discussion

Several published studies have provided evidence supporting the significance of antibodies directed to the surface of IEs in protection against severe malaria disease (Bull et al., 1998, Giha et al., 2000). Immunological profiling of sera for the existence of reactive antibodies against VSAs is a widely utilised method for evaluating acquired immunity and recognising potential candidates for vaccine development (Oleinikov et al., 2009). In this chapter, we describe the use of different functional assays including ELISA, flow cytometry, adhesion blocking and agglutination for characterizing the functional antibody response of eight serum samples obtained from adult female donors living in Junju sub-location, Kilifi County, Kenya. We aimed to assess the reactivity of the semi-immune sera to bacterially synthesised PfEMP1 domains using ELISA and investigate the capacity of IgG to recognise diverse \textit{P. falciparum} isolates of differing genomes and PfEMP1 phenotypes. The capacity of serum IgG to block IE adhesion to immobilised receptors and ECs was also assessed, as well as investigating the presence of agglutinating antibodies in semi-immune sera. Our results demonstrated that semi-immune sera recognised all tested recombinant domains and exhibited variable degrees of surface reactivity against tested parasite isolates. In addition, some sera exhibited higher efficiency at blocking parasite adhesion to immobilised ICAM-1 receptors and HUVEC and comprised agglutinating antibodies. These findings suggest that some of the semi-immune sera can be used for the development of antibody-based therapeutic implications. For instance, antibody-based fragments produced by manipulating the variable regions of the antibody genes can be generated and used to design Igs with defined class and specificity, directed against functional epitopes on the surface of IEs. The Ig variable domain of reactive IgGs could be cloned and engrailed onto human constant region genes, for expression in mammalian cells.

The results obtained from this work clearly demonstrate that all recombinant domain proteins were specifically recognised by semi-immune sera with inter-variable levels of recognition. This may suggest the presence of cross-reactive epitopes in the linear domains that were easily recognised by serum IgG.
antibodies. The pattern of seroreactivity with all recombinant domains can also be explained by the presence, in all tested sera, of IgG populations with different specificities capable of recognising distinct regions of the recombinant proteins. This was supported by the capacity of serum IgG to recognise the distinct VAR2CSA DBL4ε domain. In a relatively similar study, semi-immune sera obtained from Gabonese individuals were tested against nine DBL-α domains. The study revealed that most recombinant domains were recognised by 50 to 80% of sera from semi-immune individuals, and found that the majority of the antibody response was directed to variable regions of DBL-α domains with a minor population of antibodies targeting conserved epitopes (Oguariri et al., 2001). However, the cross-reactive immune response of Gabonese sera was expected to some extent since the reactivity was evaluated against the relatively conserved DBL-α domains, whereas the response of Kenyan sera in our study was assessed against the comparatively diverse DBLβ domains. It is not known whether VAR2CSA DBL4ε or PfEMP1ICAM-1 DBLβ domains in this work were expressed by a parasite isolate that previously infected the Kenyan female adults, but the very strong reactivity of serum IgG indeed suggests a previous and possibly frequent exposure of individuals to these domains.

Two previously described methods were broadly used for the detection of antibodies to parasite-derived surface antigens; these include flow cytometry and IE agglutination (Oguariri et al., 2001, Newbold et al., 1992, Piper et al., 1999). Flow cytometry provides efficient, precise and quantitative measurement of different combinations of parasite strains and serum samples (Ofori et al., 2002). In addition, this technique has an important advantage over agglutination assay including the ability to measure antibody reactivity to parasite isolates that do not agglutinate IEs (Ofori et al., 2002, Giha et al., 1999). We used standard flow cytometry assay to test semi-immune sera for IgG reactivity against the surface of erythrocytes infected by diverse P. falciparum isolates from the IT4 and 3D7 genomes. Sera were investigated at 1:25 dilution throughout the assays as increasing the dilution resulted in substantial reduction in IgG reactivity (data not shown). The selective labelling of IgG antibodies to IEs, but not non-IEs,
indicates that antibodies recognise PfEMP1 or other parasite-derived protein exported to the surface of IEs. All of the eight adult sera that we tested were classified as positive for IgG surface binding to at least 80% of the parasite isolates used in the assay except JA221 which only showed positive labelling with 3 out of the 5 tested parasite lines (60%). We found that JA235, JA239, JA249 and JA254 sera had antibodies to all tested parasites, suggesting that they may had been previously exposed to related parasite variants.

The possible explanations for the higher proportion of cross-reactivity with all tested isolates are: (a) that individual serum samples comprised populations of IgG antibodies of multiple specificities, in accordance with previous conclusions (Newbold et al., 1992, Gamain et al., 2001), or (b) that antibodies in the sera recognised cross-reactive, probably conserved epitopes on PfEMP1 or other VSAs consistent with previous suggestions that antibodies recognising conserved regions in VSAs (Marsh and Howard, 1986) or in PfEMP1 (Staalso et al., 1998) are induced upon exposure to malaria. An early study conducted in a Sudanese village demonstrated that although parasite isolates circulating in the region were genetically varied and express distinct PfEMP1 variants, they also possessed a significant overlap in their PfEMP1 repertoires (Giha et al., 1999).

The capacity of serum IgG to recognise "common" regions is promising since parasites expressing commonly recognised VSA have been associated with severe disease (Bull et al., 1999, Bull et al., 2000). Such antibodies with a broader specificity range would be acquired after multiple exposures to P. falciparum parasites, probably including isolates with similar determinants to those used in this study. The weaker or absent reactivity of some semi-immune sera to particular parasite isolates while demonstrating higher response to other isolates is in agreement with the "hole in the anti-VSA antibody repertoire" hypothesis (Ofori et al., 2002, Nielsen et al., 2002, Bull et al., 1998). Although these "holes" would be expected in plasma of younger children due to lower exposure to infection (Ofori et al., 2002), adult individuals showing low or absent reactivity in the present study may have not been infected with the same or antigenically-related P. falciparum isolates to that they fail to recognise. This
is consistent with previous findings that serum samples obtained from immune Gambian adults failed to agglutinate a significant number of serotypes, suggesting that multiple exposure to infection does not necessarily lead to production of protective immune response (Newbold et al., 1992).

In the present study, we found that some semi-immune sera were efficient at blocking the adhesion of parasitized erythrocytes to immobilised receptors or primary cell lines. This capacity of inhibition can be attributed to two contributors; a) surface masking of the adhesion ligands of PfEMP1 proteins by specific IgG antibodies in the sera or b) agglutination of infected cells which leads to massive reduction in the overall capacity of erythrocyte binding. IgG-mediated IE lysis was not observed with Kenyan serum samples as detected with mouse IgM antibodies in the previous chapters, and therefore was not considered as a factor contributing in adhesion blocking.

The higher capacity of JA225 and JA235 sera, and the relatively moderate efficacy of JA238 to block IE adhesion to ICAM-1 under static conditions while failing to inhibit the binding to CD36 under the same conditions suggests that these sera comprise a population of antibodies capable of masking residues on PfEMP1 involved in ICAM-1 binding, i.e. DBLβ domains, or alter the conformation of the native PfEMP1 protein leading to disruption of ICAM-1 binding domains while leaving CD36 binding sites unaffected. The strength of reactivity with recombinant IT4VAR16 DBLβ domains as indicated by ELISA was not related to adhesion blocking, since JA225 and JA235 sera which strongly blocked ICAM-1 binding to ItG-IEs were the least reactive sera compared to the other semi-immune sera. On the other hand, some individual sera that showed higher reactivity levels with recombinant domains failed to block IE adhesion to ICAM-1 receptors. This lack of association is expected since recombinant domains will have non-physiological epitopes not seen on the IE. However, the surface recognition for JA225 and JA235 sera to ItG-IEs was considerably high, particularly for JA235 which exhibited the highest magnitude of surface labelling with live IEs. These data indicated that recognition of the linear recombinant domains involved in ICAM-1 binding does not necessarily
correlate with blockade of parasite adhesion to the same receptors, and suggest that ICAM-1 binding ligands on PfEMP1 molecules are conformational with critical binding sites being only accessible to a sub-population of specific antibodies. On the other hand, serum obtained from JA225 donor had a significant, though moderate blocking capacity to ICAM-1 receptors, suggesting that a proportion of antibodies partially shielded receptor adhesion ligands on PfEMP1 proteins.

Our findings demonstrated that all semi-immune sera have significantly reduced IE adhesion to HUVEC (\(P < 0.001\)), with JA225 and JA235 demonstrating the highest magnitude of inhibition followed by JA238 serum. A4-IEs bind to activated HUVEC through ICAM-1 ligands, since this cell line expresses ICAM-1 but not CD36 upon TNF-\(\alpha\) activation (Dobbie et al., 1999, Gray et al., 2003). We found that the results of adhesion blocking to HUVEC are in agreement blocking to ICAM-1 receptors under static conditions, which revealed a similar higher magnitude of adhesion blocking by JA225 and JA235 sera.

There was a moderate positive correlation between surface recognition of A4-IEs and adhesion blocking to HUVEC under flow conditions (\(r = 0.34, P > 0.05\)). The lack of significance may has resulted from the fact that some semi-immune sera (i.e. JA225 and JA242) significantly blocked the A4-HUVEC adhesion but failed to label the surface of IEs. This can be explained by the presence of low levels of highly specific antibodies in the semi-immune sera that blocked IE adhesion, similar to an observation in a previously published study (Beeson et al., 2004). Total antibodies to the parasite-derived VSAs may primarily bind to distinct epitopes on the surface of IEs, rather than adhesion blocking antibodies that bind more specifically to sites on PfEMP1 involved in receptor adhesion (Beeson et al., 2004). Therefore, our findings provide evidence that a limited subset of antibody population that failed to produce a signal of surface fluorescence can block the adhesion of IEs to cell lines, suggesting that adhesion blocking depends on the quality, and not just the quantity of antibodies. This is consistent with previous findings suggesting that adhesion inhibitory antibodies and those recognising VSAs on the surface of IEs
have distinct and overlapping epitopes, possibly were acquired independently during infection and have various roles for protection (Beeson et al., 2004). Another possible explanation for the overall reduced binding of A4-IEs to HUVEC is the ability of serum IgG to bind receptors expressed on activated HUVEC, such as ICAM-1, leading to reduced or blocked interaction between PfEMP1 and HUVEC receptors.

Agglutination and flow cytometry assays seem to principally investigate the same set of antibodies, with flow cytometry mostly being more sensitive (Ofori et al., 2002, Giha et al., 1999). Using the agglutination assay, we addressed the question of whether IE clumping mediated by semi-immune sera would give an indication of the extent of the contribution of agglutination to the capacity of IE adhesion blocking. In addition, we aimed to investigate the general associations or differences between measures of antibodies that agglutinate IEs with those which block the parasite adhesion or label the surface of IEs, since it has been established that samples showing strong reactivity by flow cytometry and agglutination inhibited parasite adhesion more efficiently than samples with no noticeable reactivity (Beeson et al., 2004). The results of adhesion blocking of A4-IEs to HUVEC demonstrated that all semi-immune serum samples comprised antibodies that significantly blocked 60-86% of A4-HUVEC adhesion under flow conditions. This was not significantly correlated with agglutination profiles for all semi-immune sera with A4-IEs since only five sera agglutinated A4-IEs ($r_s = 0.34, P > 0.05$). In addition, some semi-immune sera such as JA221 and JA239 were efficient at blocking A4-IE adhesion and labelled the surface of the same parasite isolate but did not contain agglutinating antibodies. This provided evidence that blockade of parasite binding to HUVEC was not entirely attributed to IgG-mediated IE agglutination, and other factors such as surface labelling may have contributed in blockade of adhesion. This also indirectly suggests that the parasite-derived protein on the surface of IEs recognised by semi-immune sera is PfEMP1, as it an essential candidate involved in parasite adhesion to protein receptors or EC lines.
Comparison of the IgG response to the surface of A4-IEs with the agglutination profiles of erythrocytes infected by the same isolate revealed a strong positive correlation between the two antibody measures. This is in line with previous suggestions that antibodies recognising the surface of live IEs in flow cytometry were the same nature as agglutinating antibodies (Ofori et al., 2002, Giha et al., 1999). Several published studies were consistent with this suggestion and indicated that the recognised proteins by immune sera using surface labelling or agglutination were PfEMP1 proteins (e.g. Chattopadhyay et al., 2003).

In a recent study, a novel approach was developed using genetically modified *P. falciparum* isolates with suppressed PfEMP1 expression to elucidate the importance of PfEMP1 and other VSAs as targets for protective antibody response among serum samples collected from residents in malaria endemic area in Eastern Africa (Chan et al., 2012). The study revealed that binding of IgG antibodies to the surface of erythrocytes infected by the transgenic line was significantly reduced compared to wild type isolate that expresses PfEMP1, suggesting that acquired antibody response to the surface of IEs mainly targets PfEMP1 molecules. However, a proportion of antibody response to transgenic *P. falciparum* isolates was observed suggesting the role of other VSAs as antibody targets (Chan et al., 2012). In fact, some published studies suggested that other parasite-derived VSAs such as RIFINs and STEVORs might play a significant role in immunity against malaria. For instance, a study revealed that human immune sera agglutinated IEs treated with trypsin at conditions such that PfEMP1 proteins are mostly cleaved off the surface of IEs, indicating that RIFINs can be targeted by agglutinating antibodies (Fernandez et al., 1999). Another study carried out in an endemic area with intense malaria transmission rate in Gabon showed that the majority of adult serum population exhibited a higher antibody response to recombinant RIFIN proteins, suggesting that this family of proteins is naturally immunogenic (Abdel-Latif et al., 2002). The higher levels of anti-RIFIN antibodies in serum samples obtained from 60 children living in the same hyperendemic area in Gabon significantly correlated with parasite clearance from the circulation, suggesting that these antibodies
may have a protective role against progression of malaria disease (Abdel-Latif et al., 2003). Furthermore, pre-adsorption of antibodies from semi-immune adult plasma from the same area in Gabon to recombinant RIFIN proteins resulted in the reduction of the level of IgG reactivity with VSAs on the surface of erythrocytes infected with different of P. falciparum isolates as measured by flow cytometry (Abdel-Latif et al., 2004). The influence of pre-adsorption to RIFIN proteins was even higher than for recombinant DBL-1α on the overall IgG subsequent binding to VSAs (Abdel-Latif et al., 2004). A cross-sectional study of immune adults living in malaria endemic region in Ghana demonstrated higher levels of serum IgG antibodies to recombinant STEVOR antibodies. Although conclusions regarding the role of anti-STEVOR antibodies in protection against severe disease have not been drawn from this study, the results indicated that STEVOR proteins could act as targets for antibody-mediated immunity (Schreiber et al., 2008). We conclude that semi-immune sera may comprise antibodies that predominantly bind different VSAs, with the majority of the immune response directed to PfEMP1 protein family.

In the present study, semi-immune sera were tested against a panel of genotypically and phenotypically variant lab-adapted P. falciparum isolates to extend the diversity rate of expressed var genes and to mimic parasites isolates in areas with high malaria endemicity, which have high sequence diversity. The higher prevalence of cross-reactive antibodies among semi-immune sera to diverse parasite populations (e.g. ItG and 3D7) suggests that different P. falciparum isolates share restricted antigenic determinants recognised by IgG antibodies in serum pools. Based on the assumption that the target of the agglutinating antibodies is PfEMP1 (Baruch et al., 1995), the ability of most sera in our study to agglutinate A4- and ItG-IEs would suggest the existence of a considerable overlap between PfEMP1 repertoires in variant parasite lines, in agreement with previous propositions (Giha et al., 1999).

In summary, our findings indicate that semi-immune female sera comprise antibodies that cross-reacted with diverse P. falciparum isolates and blocked IE adhesion to protein receptors and primary ECs. We suggest that the antibody
repertoire predominantly bind different VSAs, with the majority of the immune response directed to PfEMP1 protein family. Our results may help to guide future work required to identify the role of cross-reactive and cross-inhibitory antibodies in protection from severe diseases and possible development of preventive of therapeutic interventions. Understanding the targets of the naturally acquired immune response in individuals living in endemic areas is significant for advancing the development of malaria vaccines. However, there are key important gaps in our knowledge about other VSAs and their significance in protective immunity against severe disease, and more studies using multiple approaches are therefore required to elucidate the importance of these antigens in immunity.

5.9 Limitations and future work

The present dataset is limited and it would be premature to make decisive conclusions. Because of the limited amount of the serum samples available in this work, there were frequently insufficient quantities for repeating assays of adhesion blocking and surface reactivity to validate preliminary data. With an expanded dataset, we can correlate functional antibody responses with parasite samples collected from patients with severe malaria or with a larger panel of laboratory-adapted isolates. We can also test the adhesion blocking capacity of semi-immune sera to different protein receptors (e.g. CSA) or cell lines (e.g. HDMEC and HBEC). Future work will also include investigation of the reactivity against other PfEMP1 recombinant proteins including the conserved CIDR1α-DBL1α head structure.
Chapter 6: General conclusions

Sequestration of IEs to ECs lining the post-capillary venules of different host organs is a characteristic feature in the virulence of \textit{P. falciparum} infection (Cooke et al., 1995, Pasloske and Howard, 1994). Among host endothelial ligands that mediate cytoadhesion, ICAM-1 was linked to severe forms of malaria diseases, including CM (Newbold et al., 1997, Turner et al., 1994). PfEMP1 is a key parasite-derived protein ligand exported to the surface of IEs and is associated with the adhesion of IEs to host endothelium (Cooke et al., 1995). The binding domains of ICAM-1 on PfEMP-1 have been identified as DBLβ domains (Smith et al., 2000a, Oleinikov et al., 2009, Howell et al., 2008, Brown et al., 2013). In the present work, we selected four recombinant PfEMP1–ICAM-1–DBLβ domains for mouse immunisations. Sera were collected from immunised mice and screened for reactivity against recombinant domains and live IEs. We found that antisera raised to all recombinant domains, except DBL41, had higher IgG titres against corresponding immunising antigens. Anti-rDBL41 antiserum failed to recognise its own domain but cross-reacted with heterologous antigens. Moreover, all antisera exhibited variable degrees of cross-reactivity with the other heterologous DBL domains but almost failed to recognise rDBL41 antigen. These results are consistent with Western Blotting data and suggest that rDBL41 protein used in the present work either has not been correctly expressed and purified, or has been degraded during expression or after purification.

Our FACS data showed that antisera raised to DBL27, DBL31 and DBL41 comprised IgM antibodies that showed variable reactivities with the surface of erythrocytes infected by ItG and outgrown A4 isolates, but almost none of these sera contained reactive IgGs. On the other hand, DBL13-specific antiserum comprised cross-reactive IgM antibodies that labelled the surface of erythrocytes infected by homologous and heterologous isolates. However, it is uncertain whether these sera were cross-reactive to conserved epitopes on the surface of IEs or alternatively they comprised a mixture of antibodies of multiple
specificities. Although surface-labelling was only specific for IEs, we could not confirm that the surface protein recognised by antisera was DBLβ or another variant epitope in the native PfEMP1, since several immunogenic VSAs co-express with PfEMP1 on the IE surface and can act as binding ligands.

Further work has resulted in the production of thirteen hybridoma clones that secrete reactive IgM antibodies against DBL13, DBL31 and DBL41 domains. The induced mAbs and pAbs elicited not only recognised their corresponding antigens, but also efficiently cross-reacted with heterologous domains with a similar capacity of recognition. The higher level of cross-reactivity observed in ELISA may propose that all recombinant domains share conserved immunogenic epitopes, despite their lower level of sequence identity (46%), that were easily recognisable and accessible to IgM antibodies. It was surprising that all positive hybridoma clones only secreted reactive IgM antibodies, and none of the clones comprised reactive IgG antibodies despite the higher IgG titre of mouse antisera. The definitive reason for this is not completely understood. We suggest that the adjuvant system used for driving the immune response (i.e. Alum) may has contributed in priming of IgM synthesis.

Further FACS analyses demonstrated that labelling of mAbs and pAbs was not limited to homologous ICAM-1-binding strains or isolates from the corresponding genome, as antibodies also recognised erythrocytes infected by C24, a non-ICAM-1 binding isolates, and a genotypically-distinct strain (3D7). This indicated that induced antibodies were not specific for a particular binding phenotype or genotype, although DBL domains used in mouse immunisation were obtained from ICAM-1-binding PfEMP1 variants from the IT4 genome. In terms of specificity, we found that the strongest labelling antibodies were mainly mAbs, and those raised to DBL41 showed the highest capacity of surface binding in all assays in addition to one mAb raised to DBL31 (E7). This was surprising, since DBL41 showed the weakest reactivity with homologous and heterologous mouse immune sera in ELISA and Western Blotting. These results suggest that recombinant DBL41 domains expressed in E. coli contained the functional components required to elicit a broadly reactive immune response.
Therefore, this domain can give rise to novel therapeutics and development of cross-reactive malaria vaccine.

Investigating the pattern of individual IgM labelling has led to the finding that mAbs and pAbs exhibited similar patterns of IgM surface recognition among parasite isolates. The higher level of cross-reactivity strongly suggests the presence, among parasites of different genotypes, of conserved epitopes within the PfEMP1 or in another parasite-derived protein on the surface of IEs that are accessible to polymeric IgM antibodies. Our findings also indicated that the presence of IgM per se in a particular mAb/pAb does not account for surface labelling, since some antibodies (e.g. pAb C2) comprised higher amounts of IgM but exhibited weak if any surface binding. This indicates that surface reactivity was only dependent on the presence of specific antibodies that can recognise target epitopes regardless of IgM abundance in purified yields. Although surface labelling was mainly specific for IEs, we could not confirm that labelling was specific for the native PfEMP1 as other parasite-derived proteins exported on the surface of IEs also can act as surface epitopes for binding. For instance, RIFINs, STEVORs and modified band 3 could act as binding ligands for our mouse mAbs and pAbs.

In competition assays we investigated whether mouse IgMs out-compete BC6, HIS or anti-DBL16 rat antisera for IE surface adhesion and vice versa. We provided evidence that mAb BC6 moderately interfered with the subsequent binding of mouse IgMs, which may suggest that mAbs and pAbs partially bind to PfEMP1 on the surface of IEs and that binding epitopes were partly masked by BC6. Our findings also suggested that the binding sites for mouse IgMs and rat anti-DBL16 antisera are partially overlapped on the surface of IEs, as revealed by the remarkable reduction of mouse IgM subsequent binding to IEs pre-incubated with anti-DBL16 rat antisera. Alternatively, mouse IgMs may bind to surface ligands distinct from those of BC6 or anti-rat sera on the surface of IEs and the influence of mouse IgMs on BC6 or antisera could be explained by the binding of the large polymeric IgM molecules to parasite-derived surface proteins. This binding may have evolved to partly mask functional epitopes.
involved in BC6 and rat anti-DBL16 antisera on PfEMP1 upon binding to other surface antigens, which explains the limited ability of mAbs and pAbs in disrupting specific BC6 and rat antisera binding. With regard to HIS, we found that mAbs and pAbs had a significant role in interference with the subsequent binding of the HIS, and the latter also remarkably reduced the binding of mouse IgMs. This two-sided inhibitory effect can be related to cross-reactivity of mouse IgMs and HIS to the surface of erythrocytes infected by diverse parasite isolates. Mouse IgMs could either mask a residue important for HIS binding or alter the conformation of the binding epitope in such a way to destabilise HIS interaction. Further studies are required to investigate the kinetics of IgM binding to surface receptors and to determine the functionality of these cross-reactive binding epitopes.

In our study, we took advantage of the differential sensitivities of parasite-derived VSAs to controlled trypsin digestion for characterising the surface candidates involved in the binding of mAbs and pAbs. Consistent with existing data, we demonstrated that PfEMP1 is sensitive to trypsin digestion down to 10 µg/ml, a concentration that was extremely effective in abolishing binding ligands for ICAM-1 and BC6 on PfEMP1. The binding of mouse IgMs (mAb G6, mAb B5, pool 1) was substantially reduced with trypsin treatment, i.e. the higher the concentration of trypsin the lower the binding detected. However, the binding was not completely ablated as observed with mAb BC6. This may propose that mouse IgMs partially recognise a trypsin-sensitive surface antigen with the characteristics of PfEMP1. However, the sustained binding to IEs at higher trypsin concentrations may suggest that a repertoire of mouse IgM antibodies bind trypsin-resistant epitopes on the IE surface. Several trypsin-resistant parasite-derived surface proteins, such as RIFINs and modified band 3, can act as binding ligands for mouse IgMs, but the identity of such ligands is not yet identified.

Analysis of surface labelling following incubation with mAbs and pAbs has revealed a unique phenomenon associated IgM binding, lysis of IEs. We provided evidence that haemolysis was resulted from an immunological
interaction between mouse IgMs and a parasite-derived component on the surface of live IEs. Only IEs were lysed upon mouse IgM incubation, whereas non-IEs were largely unaffected. In addition, the majority of mouse IgMs that caused IE lysis were highly effective in recognising the surface of erythrocytes infected by all tested isolates, whereas haemolysis was less observed or absent with non-labelling mAbs and pAbs. The concentration of IgM in mAbs and pAbs was not a significant factor contributing to IE lysis, since antibodies with higher IgM concentrations (e.g. C2 and C9) were comparatively ineffective in lysing cells compared other antibodies at lower concentrations (E4 and B4). These findings indicated that IE lysis resulted from an IgM-mediated binding to a parasite-derived protein on the surface of IEs. Although we investigated the effect of possible factors such as temperature and serum complement components in culture media, the definite mechanism(s) of IE lysis and key factors contributing to this phenomenon are not yet understood.

The capacity of mAbs and pAbs to block the adhesion of IEs to immobilised receptors (ICAM-1 or CD36) or EC lines under static or physiological flow conditions was investigated. We showed that mAbs and pAbs induced in our work not only cross-reacted with diverse *P. falciparum* isolates but also cross-inhibited binding of IEs to immobilised receptors and EC lines. Since most mAbs and pAbs used in adhesion blocking assays cause IE lysis, we took into consideration that this lysis may largely contribute to blockade of IE adhesion to immobilised receptors or ECs. Our analyses revealed that blockade of adhesion correlated with IgM-dependent IE lysis; as antibodies that caused lysis of significant amounts of IEs were highly effective in blocking the adhesion of erythrocytes infected with the same parasite isolate. However, despite the relatively higher amounts of lysed IEs upon antibody incubation, a significant proportion of parasitized cells are not lysed following incubation with mAbs and pAbs. We provided evidence that some mAbs and pAbs, such as pAb A4, prevented a significant proportion of non-lysed IEs to CD36 receptors under static conditions. This may suggest that blockade of adhesion is not entirely...
attributed to IE lysis, and other factors such as masking epitopes involved in parasite adhesion may contribute in adhesion blocking.

The ICAM-1 binding sites are mapped to DBLβ domains, which are obviously distinct from the CIDR domains involved in CD36 binding. Therefore, we were astonished that mouse IgMs that blocked ICAM-1 binding equally well blocked CD36 binding. We suggest that the large polymeric IgM molecules may have fully or partially masked important PfEMP1 epitopes involved in CD36 binding. Possible steric hindrance generated after binding of IgM molecules to PfEMP1 or adjacent ligands may have blocked the interaction between receptors and PfEMP1 domains involved in adhesion. Alternatively, IgM binding may have resulted in destabilisation of the basic structure of PfEMP1 leading to loss of binding capacity of adhesion domains. These results suggest that binding regions for mouse IgMs overlap, or localised at close proximity to those for ICAM-1 and CD36 binding on the native PfEMP1 protein.

In the last experimental chapter, we described the use of different functional assays to characterise the immunological response of eight serum samples collected from adult female donors living in Junju sub-location, Kilifi County, Kenya. Our findings indicated that all recombinant domain proteins used for reactive IgG screening were specifically recognised by semi-immune sera with variable levels of recognition. Standard FACS assays showed that all adult sera comprised reactive IgG antibodies that labelled the surface of at least 80% of tested parasite isolates except JA221, which only showed positive labelling with 60% parasite strains. The higher level of cross-reactivity with all tested isolates suggests that individual serum samples comprised sub-populations of IgG antibodies of multiple specificities, or that antibodies in the sera recognised cross-reactive, probably conserved epitopes on PfEMP1 or other VSAs.

In the present study, we found that some semi-immune sera were efficient at blocking the adhesion of parasitized erythrocytes to immobilised receptors or primary cell lines. Of particular significance, two serum samples (JA225 and JA235) significantly blocked IE adhesion to ICAM-1 under both static and flow conditions. However, all sera except JA254 failed to significantly block parasite
adhesion to CD36 receptors. This suggests that blocking sera comprise a population of antibodies capable of masking residues on PfEMP1 involved in ICAM-1 binding, i.e. DBLβ domains, or alter the conformation of the native PfEMP1 protein leading to disruption of ICAM-1 binding domains while leaving CD36 binding sites unaffected. This is intriguing since binding to CD36 is a common adhesion phenotype and most field and culture-adapted P. falciparum isolates bind CD36 receptors (Chilongola et al., 2009, Newbold et al., 1997, Rogerson et al., 1999). Thus, exposure of individuals in endemic areas to this binding phenotype is huge yet they did not develop blocking antibodies. However, we should evaluate the blocking capacity of sera against different CD36-binding isolates to support these findings.

Using the agglutination assay, we addressed the question of whether IE agglutination mediated by semi-immune sera contributes to the capacity of IE adhesion blocking. Our data have demonstrated that IE agglutination did not exclusively contributed in adhesion blocking, since some sera were efficient at blocking A4-IE adhesion and labelled the surface of the same parasite isolate but did not contain agglutinating antibodies. Thus, other mechanisms such as IgG-mediated surface labelling and blocking of critical binding epitopes may have contributed in the inhibition of IE binding. This may indirectly suggest that the labelled protein on the on the surface of IEs by semi-immune sera is PfEMP1.

Advances in technical approaches highlighted the possibility for synthesising antibodies targeting essential malaria antigens and could be used in therapeutic interventions. These antibodies could be designed to act as drugs and kill the parasite or could have a protective role from infections (Pleass and Holder, 2005). In addition, antibodies can be used to identify the structure and function of critical antigens, and recognise epitopes that could be used for the development of vaccines. Due to the lack of good animal models for P. falciparum malaria, antibodies can provide an unlimited source of measures to provide in vitro associates for development of vaccines (Pleass and Holder, 2005). Most of the work on human malaria has been focused on antibodies purified from immune sera. However, sera comprise a mixture of antibodies with
different classes and specificities, and can contain antibody classes that may trigger unwanted immune responses (Pleass and Holder, 2005). Therefore, the use of antibodies with monoclonal specificities directed to desired antigens could be ideal for therapeutic interventions.

In the present work, we generated mouse mAbs and pAbs that have the capacity to recognise the surface of erythrocytes infected by diverse parasite isolates, block IE adhesion to different receptors and cell lines and selectively destroy IEs while leaving non-IEs largely unaffected. Such multiple activity could be highly beneficial in the development of therapeutic antibodies against malaria disease. However, passive administration of these mouse IgM antibodies to human hosts might not be appropriate for many significant reasons (reviewed in Pleass and Holder, 2005). Therefore, genetic engineering approaches could be used in the future to provide a "humanised" form of our mAbs and pAbs to be used for targeting functional parasitic epitopes in human hosts. Antibody-based fragments produced by manipulating the variable regions of the antibody genes can be generated and used to design Igs with defined class and specificity, directed against functional epitopes on the surface of IEs (Pleass et al., 2003). This approach can be used to circumvent the problems of using whole antibodies, especially for the large IgM molecules. The Ig variable domain from a mouse IgM can be cloned and engrafted onto human constant region genes, for expression in mammalian cells. This approach can also be applied on reactive semi-immune sera (e.g. JA235) by cloning variable domains from an antibody repertoire phage library generated from semi-immune sera and linking them to constant domains in appropriate expression vectors (reviewed in Pleass and Holder, 2005).

In summary, our work is the first to demonstrate that single domains of *E. coli*-expressed DBLβ from PfEMP1^ICAM-1^ could elicit broadly cross-reactive, IE lysing and adhesion blocking mouse antibodies against different strains of heterologous phenotypes and genotypes. Indeed, cross-reactivity against diverse parasite populations is important since polymorphisms of PfEMP1 variants and the ability of *P. falciparum* isolates to switch surface PfEMP1 to other variants
with different phenotypes makes it difficult to develop strain-specific therapeutic candidates based on targeting single DBL domains. Therapeutic antibodies should be broadly reactive and cross-react with most, if not all isolates, or would contain a mixture of components each recognise a different isolate. It is significant that cross-reactive antibodies possess anti-adhesive properties, regardless of the mechanism of inhibition. In fact, effective anti-adhesive antibodies should target all receptor-binding sites or at least multiple sites at the same time to interfere with parasite adhesion. Therefore, we suggest that mouse IgM antibodies may have therapeutic values and could play a significant role in protection against severe malaria. However, further studies are required to further characterise the binding sites for mAbs and pAbs on the surface of IEs and to assess their capacity for blocking the adhesion of IEs to other receptors or EC lines. In addition, more work is needed to elucidate the molecular mechanism of IE lysis by mouse IgMs and blood or antibody factors that underlie this phenomenon.
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Publication

The thesis material has been previously presented in local and international conferences. However, none of the materials has been published. Publication will be carried out once the remaining experiments are done.
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300


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Appendix

Figure S1. Indirect ELISA for investigating the efficiency of affinity purification of mAbs and pAbs against different coating antigens. The reactivity of purified antibody yield, culture supernatant prior to purification and the resulted flow-through media of pAb A4 were tested against recombinant DBL31 or VAR2CSA-DBL4ε (5 μg/ml). 50 μl purified mAbs/pAbs, 200 μl of hybridoma culture supernatants before purification or flow-through fluid resulted from the purification were incubated with coated antigens. Reactive IgM antibodies were detected with 100 µl HRP-conjugated goat anti-mouse IgM (Southern Biotech®) at 1:500 dilution. Incubation of Culture medium (CM) or mouse IgM isotype control with recombinant proteins were included in the assay as negative controls, whereas anti-DBL31 mouse serum (50 μl, 1:50 dilution) was used as a positive control. Recognition was measured in terms of the magnitude of the response demonstrated as OD values and shown as the average of duplicate wells with SD values expressed as error bars.
Figure S2: var gene transcription profiles of five *P. falciparum* isolates used in the present study.

For each parasite isolate, RNA was harvested from ring-stage parasites and expression profiles of dominant var genes was determined by RT-qPCR using primers for six var transcripts (*var01, var13, var14, var16, var31* and *var41*). The results show that isolates expressed the expected var transcript more than five fold changes. IT4var16 also expressed another var transcript, IT4var41, besides its corresponding var transcript (IT4var16). IT4var01 = 3G8; IT4var13 = GC503; IT4var14 = A4; IT4var16 = ItG; IT4var31 = P5B6.
Table S2. Geometric mean fluorescent intensity (gMFI) values for IEs upon incubation with mAbs and pAbs. RBC population was firstly gated to exclude debris, and then IEs (Ethidium bromide positive cells) were carefully obtained by a closer gating from the FITC channel (FITC positive cells). gMFI values for IEs in the APC channel were then obtained and added in the table.
<table>
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Table S3. Geometric mean fluorescent intensity (gMFI) values for non-IEs. RBC population was firstly gated to exclude debris, and then non-IEs (Ethidium bromide negative cells) were carefully obtained by a closer gating from the FITC channel (FITC negative cells). gMFI values for non-IEs in the APC channel were then obtained and added in the table.
Table S4. Labelling of mAb G6, pAb B5, pool 1 of mouse IgMs and HIS to ItG-IEs treated with 10 µg/ml, 100 µg/ml or 1000 µg/ml of TPCK-treated trypsin as measured by flow cytometry. Geometric mean fluorescent intensity (gMFI) values for IEs upon incubation with mAbs and pAbs are shown in the table. RBC population was firstly gated to exclude debris, and then IEs (Ethidium bromide positive cells) were carefully obtained by a closer gating from the FITC channel (FITC positive cells). gMFI values for IEs in the APC channel were then acquired and added in the table.

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</table>
KEMRI/RES/7/3/1

Dr. K. Marsh
CGMR-C,
KILIFI.

Thro’
Director,
CGMR-C,
KILIFI.

Dear Sir,

Re: SSC Protocol No. 1131 (Revised) — Integrated studies of the development of natural immunity to malaria in children in Kilifi district, by K Marsh et al

During the 136th Meeting of the KEMRI/National Ethical Review Committee held on the 22nd of August 2006 the above protocol was discussed.

Thank you for a well written and informative proposal that aims to establish an epidemiological framework in which to carry out longitudinal studies of the development of immune responses to malaria.

Due consideration has been given to ethical issues and therefore the protocol is granted approval. You are responsible for reporting to the Ethical Review Committee any changes to the protocol or in the Informed Consent Document. This includes changes to research design or procedures that could introduce new or more than minimum risk to human subjects. In addition it is requirement of this Committee to include the contact of the KEMRI/NERC which is Secretary, KEMRI/NERC, +254-02-2722541.

Kindly remit a copy of the revised protocol for our records.

R.C.M. Kithinji
For: Secretary,
KEMRI/National Ethical Review Committee

In Search of Better Health
Oxford Tropical Research Ethics Committee
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4 October 2006

Professor Kevin Marsh
KEMRI
GCMRC
PO Box 230
Kilifi
Kenya

Dear Kevin

OXTREC 030-06: Integrated studies of the development of natural immunity to malaria in children in Kilifi district

Thank you for submitting this application form which the committee considered on 28 September 2006. It was very well put together and explained clearly the reasons for the various projects and the issues concerning sample acquisition and consent. We thought you should change the ‘tick box’ aspect of the consent. It may be better in these circumstances to have a ‘yes’ or ‘no’ to circle or to mark as it may be confusing if a tick means yes and a cross means no in the boxes. The OXTREC number should appear on the information and consent forms.

We are happy to give approval for this group of studies. Approval is given in the first instance for three years subject to an annual review form being submitted by the investigators.

Good luck with the studies.

Yours sincerely

Dr Chris Conlon
Chairman

cc Dr Britta Urban