

1 Multivalency drives the neutralizing activity of antibodies against the  
2 *Plasmodium falciparum* circumsporozoite protein

3

4 Short Title: Antibody responses to the *Plasmodium* circumsporozoite protein

5

6 Camilla R. Fisher<sup>1¶</sup>, Joe A. Kaczmarek<sup>1¶</sup>, Henry J. Sutton<sup>2¶</sup>, Ben Clifton<sup>1</sup>, Joshua  
7 Mitchell<sup>1</sup>, Yeping Cai<sup>2</sup>, Johanna N. Dups<sup>2</sup>, Nicholas J. D'Arcy<sup>2</sup>, Mandeep Singh<sup>2</sup>,  
8 Hayley A. McNamara<sup>2</sup>, Aaron Chuah<sup>2</sup>, Tom Peat<sup>3</sup>, Colin J. Jackson<sup>1\*</sup> and Ian A.  
9 Cockburn<sup>2\*</sup>

10

11 1. Research School of Chemistry, The Australian National University, Canberra, ACT  
12 2601, Australia

13

14 2. John Curtin School of Medical Research, The Australian National University,  
15 Canberra, ACT 2601, Australia

16

17 3. CSIRO Materials, Science and Engineering, Parkville, Victoria, Australia

18

19 \* Corresponding authors

20 E-mail: [colin.jackson@anu.edu.au](mailto:colin.jackson@anu.edu.au)

21 E-mail: [ian.cockburn@anu.edu.au](mailto:ian.cockburn@anu.edu.au) (lead contact).

22

23 <sup>¶</sup> These authors contributed equally to this work

24

25

## 26    **Abstract**

27

28    The repeat region of the *Plasmodium falciparum* circumsporozoite protein (CSP) is a  
 29    major vaccine antigen because it can be targeted by parasite neutralizing antibodies;  
 30    however, little is known about this interaction. We used isothermal calorimetry and  
 31    X-ray crystallography to analyze the binding of the *Plasmodium*-neutralizing 2A10  
 32    antibody to CSP. Strikingly, we found that the repeat region of CSP is bound by  
 33    multiple antibodies and that this multivalent interaction drives the affinity of this  
 34    antibody. Because the CSP protein can cross-link multiple B cell receptors (BCRs) we  
 35    hypothesized that the B cell response might be T-independent. However, by  
 36    sequencing the BCRs of CSP-repeat specific cells we found that these cells underwent  
 37    somatic hypermutation and affinity maturation indicative of a T-dependent response.  
 38    Interestingly, the BCR repertoire of responding B cells was limited suggesting that the  
 39    structural simplicity of the repeat may limit the breadth of the immune response.

40

41

## 42 **Author Summary**

43

44 Vaccines aim to protect by inducing the immune system to make molecules called  
 45 antibodies that can recognize molecules on the surface of invading pathogens. In the  
 46 case of malaria, our most advanced vaccine candidates aim to make antibodies that  
 47 recognize the circumsporozoite protein molecule on the surface of the invasive  
 48 parasite stage called the sporozoite. In this report we use X-ray crystallography to  
 49 determine the structure of CSP-binding antibodies at the atomic level. We use other  
 50 techniques such as isothermal titration calorimetry to examine how this antibody  
 51 interacts with the CSP molecule. Strikingly, we found that each CSP molecule could  
 52 bind 6 antibodies. This finding has implications for the immune response and may  
 53 explain why high titers of antibody are needed for protection. Moreover because the  
 54 structure of the CSP repeat is quite simple we determined that the number of different  
 55 kinds of antibodies that could bind this molecule are quite small. However those  
 56 antibodies can become quite high affinity as a result of a process called affinity  
 57 maturation that allows the body to learn how to make improved antibodies specific for  
 58 pathogen molecules. These data show that while it is challenging for the immune  
 59 system to recognize and neutralize CSP, it should be possible to generate viable  
 60 vaccines targeting this molecule.

61

## 62    **Introduction**

63

64            Malaria caused by *Plasmodium falciparum* causes the deaths of around  
65    430,000 people each year [1]. The most advanced vaccine candidate is currently the  
66    RTS,S/AS01 vaccine which consists of a truncated version of the sporozoite-surface  
67    circumsporozoite protein (CSP), packaged in a Hepatitis C core virus-like particle  
68    delivered in AS01 - a proprietary liposome based vaccine [2]. Phase II and Phase III  
69    clinical trials have repeatedly demonstrated that the vaccine is capable of giving  
70    around 50% protection against clinical malaria in field settings for the first year  
71    following vaccination [3]. The bulk of protection is attributed to antibodies targeting  
72    the CSP repeat epitope included within the vaccine, with some contribution from  
73    CD4+ T cells [4]. It is still unclear why the antibody response to CSP is only partially  
74    protective. We lack structural information about how neutralizing antibodies bind to  
75    CSP and knowledge on the breadth and nature of the B cell response elicited.

76

77            Antibodies to CSP were first identified as potential mediators of protection  
78    following seminal studies that showed that immunization with irradiated sporozoites  
79    could induce sterile protection against live parasite challenge [5,6]. In the early 1980s,  
80    monoclonal antibodies (mAbs) isolated from mice immunized with sporozoites were  
81    found to be capable of inducing the neutralization of sporozoites (known as the  
82    circumsporozoite reaction) and were used to clone CSP, one of the first malaria  
83    antigens identified [7,8]. In all *Plasmodium* species CSP contains 3 domains: an N-  
84    terminal domain and a C-terminal domain, separated by a repeat region, which was  
85    the target of all the original mAbs identified [7,9,10]. In the 3D7 reference strain of *P.*  
86    *falciparum* the CSP repeat has 38 NANP-repeats interspersed with 4 NVDP repeats

87 especially towards the N-terminus [11] though different numbers of repeats have been  
88 observed [12]. One of the most effective *P. falciparum* sporozoite neutralizing  
89 antibodies identified in these early studies was 2A10 which can neutralize sporozoite  
90 infectivity *in vitro* [13] and in *in vivo* mouse models utilizing rodent *P. berghei*  
91 parasites expressing the *P. falciparum* CSP repeat region [14,15].

92  
93 While CSP binding antibodies have been shown to be able to neutralize  
94 sporozoites and block infection, it has also been proposed that CSP is an  
95 immunological “decoy” that induces a suboptimal T-independent immune response  
96 perhaps because of the CSP repeat cross-linking multiple B cell receptors (BCRs)  
97 [16]. Nonetheless this hypothesis has not been tested and it remains unknown if the  
98 repetitive regions of CSP can cross-link multiple BCRs as they are not as large as  
99 typical type-II T-independent antigens [17]. Furthermore, the very little published  
100 data on the sequences of CSP binding antibodies does not convincingly support  
101 activation of a broad B cell repertoire: a small study of five *P. falciparum* CSP mouse  
102 monoclonal antibodies (mAbs) identified some shared sequences [18]. In humans, a  
103 study that generated mAbs from three individuals who received RTS,S found that the  
104 three antibodies studied had distinct sequences though these all used similar heavy  
105 chains [19].

106  
107 Given these gaps in our understanding of the antibody response to CSP we  
108 undertook a comprehensive biophysical characterization of the 2A10 sporozoite-  
109 neutralizing antibody that binds to the CSP repeat. Using rigorous biophysical  
110 methodology we found that this antibody binds with a higher affinity than expected,  
111 in the nano-molar range. Previous studies using competition ELISAs with peptides

112 predicted a micro-molar affinity [20,21]. Strikingly, isothermal titration calorimetry  
 113 and structural analyses revealed that the CSP repeat can be bound by around six  
 114 antibodies suggesting that it may require large amounts of antibody for neutralization,  
 115 and that the repeat may potentially crosslink multiple BCRs on the surface of a B cell.  
 116 However, analysis of CSP-specific B cells revealed that CSP-specific B cells can  
 117 enter germinal centers and undergo affinity maturation contradicting the notion that  
 118 the response to CSP is largely T-independent. Moreover, we found that the BCR  
 119 repertoire of CSP-binding B cells is quite limited which may restrict the size and  
 120 effectiveness of the immune response.

## 121     **Results**

122

### 123     **Characterization of the thermodynamics of 2A10-antigen binding**

124

125             We began our analysis by performing isothermal titration calorimetry (ITC) to  
 126 understand the interaction between 2A10 and CSP. For ease of expression we used a  
 127 recombinant CSP (rCSP) construct described previously which was slightly truncated  
 128 with 27 repeats [22]. ITC experiments were run on the purified 2A10 antibody and the  
 129 purified 2A10 antigen-binding fragment ( $F_{AB}$ ) fragment to test the thermodynamic  
 130 basis of the affinity of 2A10  $F_{AB}$  towards CSP. Experiments were also performed on  
 131 the 2A10  $F_{AB}$  fragment with the synthetic peptide antigen (NANP)<sub>6</sub>, which is a short  
 132 segment of the antigenic NANP-repeat region of CSP (**Table 1; Fig. 1**). The binding  
 133 free energies ( $\Delta G$ ) and dissociation constants ( $K_D$ ) were found to be -49.0 kJ/mol and  
 134 2.7 nM for the full 2A10 antibody with CSP, -40 kJ/mol and 94 nM for the 2A10  $F_{AB}$   
 135 with CSP, and -36.4 kJ/mol and 420 nM for the 2A10  $F_{AB}$  with the (NANP)<sub>6</sub> peptide.

136             Surprisingly, we did not observe a typical 1:1 antibody/ $F_{AB}$  domain:antigen  
 137 binding stoichiometry (Table 1). We found that each (NANP)<sub>6</sub> peptide was bound to  
 138 by ~2  $F_{AB}$  fragments (2.8 repeats per  $F_{AB}$  domain). With the rCSP protein we  
 139 observed that  $10.8 \pm 0.7$   $F_{AB}$  fragments were able to bind to each rCSP molecule, (2.5  
 140 repeats per  $F_{AB}$  domain. Finally, when the single-domain  $F_{AB}$  fragment is replaced by  
 141 the full 2A10 antibody (which has two  $F_{AB}$  domains), we observe binding of 5.8  
 142 antibodies per rCSP molecule (4.7 repeats per antibody), i.e. all complexes exhibit  
 143 approximately the same binding stoichiometry of two  $F_{AB}$  fragments/domains per ~5  
 144 repeat units. These results suggest that the antigenic region of CSP constitutes a  
 145 multivalent antigen and that repeating, essentially identical, epitopes must be

146 available for the binding of multiple  $F_{AB}$  domains.

147 It is not possible to separate affinity from avidity in this system, although it is  
148 apparent that there is a substantial benefit to the overall strength of binding between  
149 the antibody and antigen through the binding of multiple  $F_{AB}$  domains. The  $F_{AB}$ :rCSP  
150 complex and the 2A10:rCSP complex had similar enthalpy and entropy of binding  
151 (**Table 1**), but the lower enthalpy of binding for the  $F_{AB}$  fragment:(NANP)<sub>6</sub> peptide  
152 complex suggests that there are additional stabilizing interactions when larger  
153 numbers of  $F_{AB}$  domains can bind to the rCSP protein. The observation that this  
154 antibody-antigen (Ab-Ag) interaction is primarily enthalpically driven is consistent  
155 with the general mechanism of Ab-Ag interactions [23]. Altogether, these data  
156 suggest that the binding of multiple antibodies to the repeat region of CSP stabilizes  
157 the interaction and the multivalent nature of this interaction increases the affinity of  
158 binding.

159  
160 **Table 1. Thermodynamic parameters for interactions between 2A10  $F_{AB}$ , 2A10**  
161 **and antigens.**

|                              | (NANP) <sub>6</sub> : $F_{AB}$ | rCSP: $F_{AB}$                | rCSP:2A10                   |
|------------------------------|--------------------------------|-------------------------------|-----------------------------|
| $K_a$ (M <sup>-1</sup> )     | $(2.37 \pm 0.91) \times 10^6$  | $(1.07 \pm 0.39) \times 10^7$ | $(3.6 \pm 2.7) \times 10^8$ |
| $K_d$ (nM)                   | $420 \pm 160$                  | $94 \pm 34$                   | $2.7 \pm 2.1$               |
| $\Delta H$ (kJ/mol complex)  | $-113 \pm 5$                   | $-1245 \pm 112$               | $-1175 \pm 44$              |
| $T\Delta S$ (kJ/mol complex) | $-76.6 \pm 4.9$                | $-1205 \pm 112$               | $-1126 \pm 44$              |
| $\Delta G$ (kJ/mol complex)  | $-36.4 \pm 1.0$                | $-40.0 \pm 0.9$               | $-49.0 \pm 1.9$             |
| $n$ ( $F_{AB}$ /2A10: Ag)    | $2.16 \pm 0.06$                | $10.8 \pm 0.7$                | $5.8 \pm 0.1$               |

162 Parameters were determined by ITC at 25 °C. Errors for  $n$  (Ag :  $F_{AB}$ ),  $K_a$  and  
163  $\Delta H$  (complex) are 95% confidence intervals estimated from a single titration; errors  
164 for other parameters were propagated.



165

## 166 **Structural analysis of the (NANP)-repeat region and the 2A10 F<sub>AB</sub>**

167

168       To better understand the molecular basis of the multivalent interaction  
169 between 2A10 and rCSP, we performed structural analysis of the components.  
170 Previous work has indicated that the NANP-repeat region of CSP adopts a flexible  
171 rod-like structure with a regular repeating helical motif that provides significant  
172 separation between the N-terminal and the C-terminal domains [24]. Here, we  
173 performed far-UV circular dichroism (CD) spectroscopy to investigate the structure of  
174 the (NANP)<sub>6</sub> peptide. These results were inconsistent with a disordered random coil  
175 structure (**S1 Fig.**). Rather, the absorption maximum around 185 nm, minimum  
176 around 202 nm and shoulder between 215 and 240 nm, is characteristic of intrinsically  
177 disordered proteins that can adopt a spectrum of states [25].

178

179       The lowest energy structures of the (NANP)<sub>6</sub> repeat were predicted using the  
180 PEP-FOLD *de novo* peptide structure prediction algorithm [26]. The only extended  
181 state among the lowest energy structures that was consistent with the reported spacing  
182 of the N-and C-terminal domains of CSP [24], and which presented multiple  
183 structurally similar epitopes was a linear, quasi-helical structure, which formed a  
184 regularly repeating arrangement of proline turns (**Fig. 2A**). The theoretical CD  
185 spectrum of this conformation was calculated (**S1 Fig.**), qualitatively matching the  
186 experimental spectra: the maximum was at 188 nm, the minimum at 203 nm and there  
187 was a broad shoulder between 215 and 240 nm. To investigate the stability of this  
188 conformation, we performed a molecular dynamics (MD) simulation on this peptide,  
189 which showed that this helical structure could unfold, and refold, on timescales of

190    tens of nanoseconds, supporting the idea that it is a low-energy, frequently sampled,  
191    configuration in solution (**S1 Mov.**, **S2 Fig.**). We also observed the same  
192    characteristic hydrogen bonds between a carbonyl following the proline and the amide  
193    nitrogen of the alanine, and the carbonyl group of an asparagine and a backbone  
194    amide of asparagine three residues earlier, that are observed in the crystal structure of  
195    the NPNA fragment [27]. Thus, this configuration, which is consistent with  
196    previously published experimental data, is a regular, repeating, extended  
197    conformation that would allow binding of multiple F<sub>AB</sub> domains to several  
198    structurally similar epitopes.

199

200           To better understand the interaction between the 2A10 and the (NANP)-repeat  
201    region, we solved the crystal structure of the 2A10 F<sub>AB</sub> fragment in two conditions (**S1**  
202    **Table**), yielding structures that diffracted to 2.5 Å and 3.0 Å. All of the polypeptide  
203    chains were modeled in good quality electron density maps (**Fig. 2B**), except for  
204    residues 134-137 of the light chain. This loop is located at the opposite end of the F<sub>AB</sub>  
205    fragment to the variable region and not directly relevant to antigen binding. The 2.5 Å  
206    structure contained a single polypeptide in the asymmetric unit, whereas the 3.0 Å  
207    structure contained three essentially identical chains. Superimposition of the four  
208    unique F<sub>AB</sub> fragments from the two structures revealed that the variable antigen  
209    binding region is structurally homogeneous, suggesting that this region might be  
210    relatively pre-organized in the 2A10 F<sub>AB</sub>. This is consistent with the observation that  
211    antibodies typically undergo relatively limited conformational change upon epitope  
212    binding [23]. Indeed, a recent survey of 49 antibody:antigen complexes revealed that  
213    within the binding site, CDR-H3 was the only element that showed significant  
214    conformational change upon antigen binding and even this was only observed in one

215 third of the antibodies [28].

216

217 Attempts to obtain a crystal structure of a complex between 2A10 F<sub>AB</sub> and the  
 218 (NANP)<sub>6</sub> peptide were unsuccessful; unlike binary Ab-Ag interactions, in which the  
 219 Ab will bind to a single epitope on an antigen and produce a population of structurally  
 220 homogeneous complexes that can be crystallized, in this interaction we are dealing  
 221 with an intrinsically-disordered peptide, the presence of multiple binding sites  
 222 (epitopes) on the peptide and the possibility that more than one 2A10 F<sub>AB</sub> domain can  
 223 bind the peptide, i.e. it is difficult to obtain a homogeneous population of complexes,  
 224 which is a prerequisite for crystallization. Attempts to soak the (NANP)<sub>6</sub> peptide into  
 225 the high-solvent form of 2A10 F<sub>AB</sub>, in which there were no crystal packing  
 226 interactions with the binding-loops, caused the crystals to dissolve, again suggesting  
 227 that the heterogeneity of the peptide and the presence of multiple epitopes produces  
 228 disorder that is incompatible with crystal formation.

229

### 230 **Modeling the interaction of the 2A10 F<sub>AB</sub> with the NANP-repeat region and** 231 **testing the model through site-directed mutagenesis**

232

233 Although it was not possible to obtain a crystal structure of the 2A10-  
 234 (NANP)<sub>6</sub> peptide complex, the accurate structures of the 2A10 F<sub>AB</sub> fragment, the  
 235 (NANP)<sub>6</sub> peptide, and the knowledge that antibodies seldom undergo significant  
 236 conformational changes upon antigen binding [28], allowed us to model the  
 237 interaction, which we tested using site directed mutagenesis. Computational modeling  
 238 of Ab-Ag interactions has advanced considerably in recent years and several  
 239 examples of complexes with close to atomic accuracy have been reported in the

240 literature [29]. Using the SnugDock protein-protein docking algorithm [29], we  
 241 obtained an initial model for binding of the peptide to the CDR region of the 2A10  
 242 F<sub>AB</sub> fragment (**Fig. 2C**). We then performed, in triplicate, three 50 ns molecular  
 243 dynamics simulations on this complex to investigate whether the interaction was  
 244 stable over such a time period (**S2 Mov.**, **S3 Fig.**). These simulations confirmed that  
 245 the binding mode that was modeled is stable, suggesting that it is a reasonable  
 246 approximation of the interaction between these molecules. To experimentally verify  
 247 whether our model of the 2A10 F<sub>AB</sub>:(NANP)<sub>6</sub> peptide interaction was plausible, we  
 248 performed site directed mutagenesis of residues predicted to be important for binding.  
 249 Our model predicted that the interaction with (NANP)<sub>6</sub> would be mainly between  
 250 CDR2 and CDR3 of the light chain and CDR2 and CDR3 of the heavy chain (**Fig.**  
 251 **2C**).

252  
 253 In the light chain (**Fig. 3A,B**), Y38 is predicted to be one of the most  
 254 important residues in the interaction; it contributes to the formation of a hydrophobic  
 255 pocket that buries a proline residue and is within hydrogen bonding distance, *via* its  
 256 hydroxyl group, to a number of backbone and side-chain groups of the peptide. Loss  
 257 of this side-chain abolished binding. Y56 also forms part of the same proline-binding  
 258 pocket as Y38, and loss of this side-chain also resulted in an almost complete loss of  
 259 binding. R109 forms a hydrogen bond to an asparagine residue on the side of the  
 260 helix; mutation of this residue to alanine results in a partial loss of binding. Y116 is  
 261 located at the center of the second proline-binding pocket; since loss of the entire  
 262 side-chain through an alanine mutation would lead to general structural disruption of  
 263 the F<sub>AB</sub> fragment, we mutated this to a phenylalanine (removing the hydroxyl group),  
 264 which led to a significant reduction in binding. Finally, S36A was selected as a

265 control: the model indicated that it was outside the binding site, and the ELISA data  
266 indicated that had no effect on (NANP)<sub>n</sub> binding.

267

268         Within the heavy chain (**Fig. 3C,D**), mutation of N57 to alanine led to  
269 complete loss of binding, which is consistent with it forming a hydrogen bond to a  
270 side-chain asparagine but also being part of a relatively well packed region of the  
271 binding site that is mostly buried upon binding. T66 is located on the edge of the  
272 binding site and appears to provide hydrophobic contacts through its methyl group  
273 with the methyl side-chain of an alanine of the peptide; mutation of this residue  
274 resulted in a partial loss of binding. Interestingly, mutation of E64, which is location  
275 in an appropriate position to form some hydrogen bonds to the peptide resulted in a  
276 slight increase in binding, although charged residues on the edge of protein:protein  
277 interfaces are known to contribute primarily to specificity rather than affinity [30].  
278 Specifically, the cost of desolvating charged residues such as glutamate is not  
279 compensated for by the hydrogen bonds that may be formed with the binding partner.  
280 Y37 is located outside the direct binding site in the apo-crystal structure; the loss of  
281 affinity could arise from long-range effects, such as destabilization of the position of  
282 nearby loops. In general, the effects of the mutations are consistent with the model of  
283 the interaction.

284

## 285 **The multivalency of the CSP repeat region**

286

287         As shown in **Fig. 3A** and **3C** the binding mode of the F<sub>AB</sub> fragment to the  
288 (NANP)<sub>6</sub> peptide is centered on two proline residues from two non-adjacent NANP-  
289 repeats. These cyclic side-chains are hydrophobic in character and are buried deeply

290 in the core of the F<sub>AB</sub> antigen binding site, into hydrophobic pockets formed by Tyr38  
291 and Tyr56 of the light chain and the interface between the two chains. In contrast, the  
292 polar asparagine residues on the sides of the helix are involved in hydrogen binding  
293 interactions with a number of polar residues on the edge of the binding site, such as  
294 N57 of the heavy chain. Due to the twisting of the (NANP)<sub>6</sub> repeat, the binding  
295 epitope of the peptide is 2.5-3 alternate NANP repeats, with a symmetrical epitope  
296 available for binding on the opposite face (**Fig. 4A**). Thus, this binding mode is  
297 consistent with the stoichiometry of the binding observed in the ITC measurements,  
298 where we observed a stoichiometry of two 2A10 F<sub>AB</sub> fragments per (NANP)<sub>6</sub> peptide.  
299 To investigate whether this binding mode was also compatible with the indication  
300 from ITC that ~10.7 2A10 F<sub>AB</sub> fragments, or six antibodies (containing 12 F<sub>AB</sub>  
301 domains) could bind the CSP protein (**Table 1**), we extended the peptide to its full  
302 length. It is notable that the slight twist in the NANP helix results in the epitope being  
303 offset along the length of the repeat region, thereby allowing binding of ten 2A10 F<sub>AB</sub>  
304 fragments (**Fig. 4B**). Six 2A10 antibodies can bind if two antibodies interact by a  
305 single F<sub>AB</sub> domain and the other four interact with both F<sub>AB</sub> domains. The observation  
306 that the F<sub>AB</sub> fragments bind sufficiently close to each other to form hydrogen bonds  
307 also explains the observation from the ITC that the complexes with rCSP, which  
308 allow adjacent F<sub>AB</sub> fragment binding, have more favorable binding enthalpy, i.e. the  
309 additional bonds formed between adjacent F<sub>AB</sub> fragments further stabilize the  
310 complex and lead to greater affinity (**Table 1**). Thus, the initially surprising  
311 stoichiometry that we observe through ITC appears to be quite feasible based in the  
312 structure of the NANP-repeat region of the rCSP protein and the nature of the rCSP-  
313 2A10 complex. It is also clear that the effect of antibody binding to this region would  
314 be to prevent the linker flexing between the N- and C-terminal domains and

maintaining normal physiological function, explaining the neutralizing effect of the antibodies.

# **Identification of endogenous (NANP)<sub>n</sub> specific B cells to determine the BCR repertoire**

We next set out to determine the implications of our structure for the B cell response to CSP. In particular we wanted to know if the repeat structure drove a diverse T-independent response resulting in a broad antibody response or if B cells specific for CSP were able to enter the germinal center and undergo affinity maturation. To test this hypothesis we used (NANP)<sub>n</sub>-based tetramers to identify antigen specific B cells in BALB/C mice immunized with *P. berghei* sporozoites expressing the repeat region of the *P. falciparum* CSP (*P. berghei* CS<sup>Pf</sup>) [15]. We then used high throughput BCR sequencing to identify the BCR sequences of the (NANP)<sub>n</sub> specific cells. The tetramers are formed by the binding of 4 biotinylated (NANP)<sub>9</sub> repeats with streptavidin conjugated phycoerythrin (PE) or allophycocyanin (APC). We used BALB/C mice as this was the strain originally used to generate 2A10. To validate our tetramer approach, mice were immunized with either *P. berghei* CS<sup>Pf</sup> or another line of *P. berghei* with a mutant CSP (*P. berghei* CS<sup>5M</sup>) that contains the endogenous (*P. berghei*) repeat region, which has a distinct repeat sequence (PPPPNPND)<sub>n</sub>. (NANP)<sub>n</sub>-specific cells were identified with two tetramer probes bound to different conjugates to exclude B cells that are specific for the PE or APC components of the tetramers which are numerous in mice [31]. We found that mice immunized with *P. berghei* CS<sup>Pf</sup> sporozoites developed large tetramer double positive populations, which had class switched (**Fig. 5A and B**). In contrast, the number of

340 tetramer double positive cells in mice receiving control parasites was the same as in  
 341 unimmunized mice; moreover these cells were not class switched and appeared to be  
 342 naïve precursors indicating that our tetramers are identifying bona-fide (NANP)<sub>n</sub>-  
 343 specific cells (**Fig. 5B** and **C**). Further analysis of the different populations of B cells  
 344 showed that most B cells present at this time-point were GL7<sup>+</sup> CD38<sup>-</sup> indicating that  
 345 they are germinal center B cells (**Fig. 5B** and **D**). Given that T cells are required to  
 346 sustain germinal center formation beyond ~3 days these data indicate that a T-  
 347 dependent response can develop to CSP following sporozoite immunization [32].

348

### 349 **A restricted repertoire of BCRs can bind to the (NANP)<sub>n</sub> repeat**

350

351       We next set out to determine the diversity of the B cell response to CSP.  
 352 While the repeat structure of CSP is hypothesized to induce a broad polyclonal  
 353 response [33], an alternative hypothesis is that the antigenically simple structure of  
 354 the repeat epitope might only be recognized by a small number of naïve B cells. To  
 355 examine the BCR usage of (NANP)<sub>n</sub>-specific B cells we sorted (NANP)<sub>n</sub>-specific  
 356 cells 35 days post immunization with sporozoites. We then prepared cDNA from the  
 357 cells and amplified the heavy and kappa chain sequences using degenerate primers as  
 358 described previously [34,35]. Heavy and light chain libraries were prepared from 4  
 359 immunized mice as well as from 3 naïve mice from which we bulk sorted B cells as  
 360 controls. We obtained usable sequences from 3 of the 4 mice for both the heavy chain  
 361 and kappa chain. Analysis of the heavy chain revealed that in each mouse 3 or 4 V  
 362 regions dominated the immune response (**Fig. 6A**). The V regions identified (IGHV1-  
 363 20; IGHV1-26; IGHV1-34 and IGHV5-9) were generally shared among the mice. As  
 364 a formal measure of the diversity of our V region usage in the (NANP)<sub>n</sub> specific cells



and the bulk B cells from naïve mice we calculated the Shannon entropy for these populations. This analysis formally demonstrated that the diversity of the antigen specific B cells was significantly lower than the diversity of the repertoire in naïve mice (**Fig. 6B**). We further found that each V region was typically associated with the same D and J sequences even in different mice. For example, IGHV1-20 was typically associated with J4, IGHV5-9 with J4 while in different mice IGHV1-34 was variously paired with J1 or J4 (**Fig. 6C**). Similar results were obtained for the kappa chain with the response dominated by IGKV1-135; IGKV5-43/45; IGKV1-110; IGKV1-117 and IGKV14-111 (**Fig. 6D and E**). The V regions were typically paired with the same J regions even in different mice (**Fig. 6F**), for example IGKV5.43/45 was typically paired with IGKJ5 or IGKJ2 and IGKV1-110 was typically paired with IGKJ5, although IGKV1-135 was typically more promiscuous. One limitation of our high throughput sequencing approach is that the degenerate primers only amplified ~70% of the known IGHV and IGKV sequences in naïve mice, suggesting that we may not capture the full diversity of the response. However, comparison with the 5 published antibody sequences (**S2 and S3 Table**) that include IGHV-1-20, IGKV5-45 and IGKV1-110 reveals that we are likely capturing the bulk of the antibody diversity. Together these data suggest that the number of B cell clones responding to CSP may be limited, potentially reducing the ability of the immune system to generate effective neutralizing antibodies.

385

### 386 **CSP-binding antibodies undergo somatic hypermutation to improve affinity**

387

388 While it is clear that CSP is the target of neutralizing antibodies it has been  
389 suggested that CSP might induce large T-independent responses at the expense of

390 potentially more useful T-dependent germinal center responses that can result in  
 391 robust B cell memory [33]. We therefore examined our deep sequencing data to  
 392 determine if CSP-specific antibodies had undergone somatic hypermutation (SHM)  
 393 that would be indicative of B cells specific for CSP entering the germinal center.  
 394 Taking advantage of the fact that our kappa chain primers capture the entire V-J  
 395 sequences of the antibodies we sequenced we asked: 1) if the kappa chains shared  
 396 between immune animals differed from the germline (providing evidence of SHM)  
 397 and 2) if the mutations were conserved between different mice indicative of directed  
 398 selection. Analysis of the reads from the kappa chains of the three immune mice  
 399 showed that these had a much higher degree of mutation than bulk B cells from naïve  
 400 mice, demonstrating SHM in the CSP-specific antibodies (**Fig. 7A**). We further  
 401 examined each specific common kappa chain in turn (IGVK1-110; IGKV1-135;  
 402 IGVK5-53/54) comparing the sequences obtained from naïve B cells and (NANP)<sub>n</sub>  
 403 specific cells in immune mice. This analysis showed that while, as expected,  
 404 sequences from naïve mice contained few mutations, the sequences from immune  
 405 mice had much higher levels of SHM. Importantly mutations were found to be  
 406 concentrated in the CDR loops, and were frequently shared by immunized mice  
 407 providing strong circumstantial evidence for affinity maturation (**Fig. 7B**; data for  
 408 IGVK1-110 only shown).

409

410 To directly test if CSP-binding antibodies undergo affinity maturation we  
 411 expressed the predicted germline precursor to the 2A10 antibody (2A10 gAb) in  
 412 HEK293T cells. We identified the predicted germline precursors of the 2A10 heavy  
 413 and light chains using the program V-quest (**Figs. S4 and S5**). This analysis identified  
 414 the heavy chain as IGHV9-3; IGHD1-3; IGHJ4 and the light chain as IGKV10-

415 94;IGKJ2, with the monoclonal antibody carrying 6 mutations in the heavy chain and  
416 7 in the light chain. The 2A10 gAb had considerably lower binding in ELISA assays  
417 compared to the 2A10 mAb itself (**Fig. 7C**) indicative that affinity maturation has  
418 taken place in this antibody. To determine the relative contribution of mutations in the  
419 heavy and light chain to enhancing binding we also made hybrid antibodies consisting  
420 of the mAb heavy chain and the gAb light chain and vice versa. Interestingly  
421 mutations in the light chain were almost entirely sufficient to explain the enhanced  
422 binding by the mAb compared to the gAb (**Fig. 7C**).

423

424 To identify the specific mutations that were important we introduced the  
425 mutations individually into the gAb light chain construct. We prioritized mutations  
426 that were shared with the 27E antibody which has previously been found to be  
427 clonally related to 2A10 having been isolated from the same mouse and which shares  
428 the same germline heavy and light chains as the 2A10 mAb [18]. We found that two  
429 mutations (L114F and T117V) in the CDR3 of the light chain appeared to account for  
430 most of the gain in binding (**Fig. 7C**). The effect of these antibodies appeared to be  
431 additive rather than synergistic as revealed by experiments in which we introduced  
432 these mutations simultaneously (**Fig. 7D**). A further mutation close to the light chain  
433 CDR2 (H68Y) also caused a modest increase in binding. As expected mutations in the  
434 heavy chains appeared generally less important for increasing binding though M39I,  
435 N59I and T67F all gave modest increases in binding (**Fig. 7E**). Collectively our data  
436 suggest that CSP repeat antibodies can undergo somatic hypermutation in germinal  
437 centers resulting in affinity maturation, however the antibody response may be limited  
438 by the number of naïve B cells that can recognize and respond to this antigen.

439

## 440 Discussion

441

442 Here we provide an analysis of the structure of a *Plasmodium falciparum*  
 443 sporozoite-neutralizing antibody (2A10). We further model the binding of this  
 444 antibody with its antigen target, the repeat region of CSP, and provide a  
 445 thermodynamic characterization of this interaction. Finally, we used novel tetramer  
 446 probes to identify and sort antigen specific B cells responding to sporozoite  
 447 immunization in order to measure the diversity and maturation of the antibody  
 448 response. We found that the avidity of 2A10 for the rCSP molecule was in the  
 449 nanomolar range, which was much higher than the affinity previously predicted from  
 450 competition ELISAs with small peptides [20,21]. This affinity is a consequence of the  
 451 multivalent nature of the interaction, with up to 6 antibodies being able to bind to each  
 452 rCSP molecule. To spatially accommodate this binding the antibodies must surround  
 453 the CSP in an off-set manner, which is possible due to the slight twist in the helical  
 454 structure that the CSP can adopt. It is notable that the twisted, repeating arrangement  
 455 of the CSP linker is the only structure that would allow binding in the stoichiometry  
 456 observed through the ITC. We further found that the diversity of the antibody  
 457 repertoire to the CSP repeat was limited, perhaps due to the relative simplicity of the  
 458 target epitope. However, these antibodies have undergone affinity maturation to  
 459 improve affinity, potentially allowing protective immune responses to develop.

460

461 Using ITC we determined the affinity of 2A10 for rCSP to be 2.7 nM, which  
 462 is not unusual for a mouse mAb. However it is a much higher affinity than that  
 463 predicted from competition ELISAs that predicted a micro-molar affinity [20,21].  
 464 However, these competition ELISAs were performed with short peptides rather than

465 rCSP. Indeed, when we performed ITC with a short peptide and F<sub>AB</sub> fragments we too  
 466 obtained an affinity in the micro-molar range (0.42  $\mu$ M). The difference in avidity  
 467 between the F<sub>AB</sub> binding to the peptide or full-length CSP and that of the antibody  
 468 appears to be driven by a more favorable enthalpy of binding. It is likely that  
 469 additional stabilizing interactions between adjacent F<sub>AB</sub> domains, which is consistent  
 470 with the structural model, contribute to this. One caveat of these data is that we used a  
 471 slightly truncated repeat, however it is likely that longer repeats will have further  
 472 stabilization of the interaction that could result in even higher affinity interaction  
 473 between CSP and binding antibodies.

474

475 Our data provide important insights into the requirements for sporozoite-  
 476 neutralization by CSP binding antibodies. The finding that each rCSP molecule can be  
 477 bound by ~ 6 antibody molecules is consistent with the finding that relatively large  
 478 amounts of antibody are required for protection against sporozoites [14,19,36].  
 479 Indeed, this may be an underestimate and it may be that full-length CSP can  
 480 accommodate additional antibody binding. If on the one hand, the surface of the  
 481 sporozoite provides multiple binding sites for CSP binding antibodies this may allow  
 482 the parasites to be relatively easily opsonized and phagocytized. On the other hand,  
 483 this may not be an important mechanism of action as anti-CSP F<sub>AB</sub> fragments have  
 484 previously been shown to be sufficient for blocking sporozoite infectivity in vivo  
 485 [36]. Moreover CSP is readily shed from the surface of the sporozoite as the  
 486 sporozoites undergo migration, which may act as a means of sloughing off bound  
 487 antibody to evade this response [37]. It has also been suggested that the CSP repeat  
 488 might act as a hinge allowing the N-terminal domain to mask the C-terminal domain  
 489 that is believed to be important for binding to and invading hepatocytes [9]. Antibody

490 binding as observed here may disrupt this hinge perhaps resulting in the premature  
491 exposure of the C-terminal domain and the loss of sporozoite infectivity.

492

493 Our results uncovering how neutralizing antibodies bind to CSP has several  
494 implications for understanding the development of the immune response to CSP.  
495 Notably the finding that the CSP molecule can be bound by multiple antibodies/B cell  
496 receptors raises the possibility that this molecule can indeed crosslink multiple BCRs  
497 and potentially act as a type-II T independent antigen [17]. Such antigens typically  
498 induce large but relatively short-lived immune responses [17]. Interestingly, the  
499 RTS,S/AS01 vaccine based on that contains 18 CSP repeats and does appear to induce  
500 high, but relatively short-lived, titers of anti-CSP antibodies [4,38], which would be  
501 consistent with it inducing a type-II T-independent response. Nonetheless, we also  
502 detected extensive somatic hypermutation and affinity maturation, not only from  
503 established monoclonal antibodies but also from CSP-specific B cells following a  
504 single sporozoite immunization. Thus, there is a T-dependent germinal center  
505 component to the antibody response too. The relative contributions of short-lived  
506 antibody production and long-term B cell memory to protection is an area for future  
507 investigation.

508

509 The finding of a limited repertoire in the BCR sequences specific for the  
510 (NANP)<sub>n</sub> repeat contradicts previous suggestions that the response to CSP might be  
511 broad and polyclonal [33]. One explanation for this limited antibody diversity is that  
512 the antigenic simplicity of the CSP repeat region limits the range of antibodies that are  
513 capable of responding. A prior example of this is the antibodies to the Rhesus (Rh) D  
514 antigen. The RhD antigen differs from RhC by only 35-36 amino acids, resulting in

515 the creation of a minimal B cell epitope [39]. The repertoire of antibodies that can  
516 bind this epitope are accordingly limited and mainly based on the VH3-33 gene  
517 family [40]. Another potential explanation for a limited antibody repertoire could be  
518 that the (NANP)<sub>n</sub> repeat shares structural similarity with a self-antigen as is  
519 speculated to happen with meningococcus type B antigens [41], however it is not  
520 clear what this self-antigen might be. One potential outcome of this finding is that if  
521 each B cell clone has a finite burst size this may limit the magnitude of the overall B  
522 cell response.

523

524 One area for future investigation is to determine the binding modes and  
525 sporozoite neutralizing capacities of other antibodies in the response. It is clear that  
526 not all CSP-repeat binding antibodies have the same capacity for sporozoite  
527 neutralization [13]. As such the finding of a limited repertoire of responding B cells  
528 may lead to the possibility that some people have holes in their antibody repertoires  
529 limiting their ability to make neutralizing antibodies. This may explain why, while  
530 there is a broad correlation between ELISA titres of antibodies to the CSP repeat and  
531 protection following RTS,S vaccination, there is no clear threshold for protection [4].

532

533 While our work has been performed with mouse antibodies, there are major  
534 similarities between mouse and human antibody loop structure [42]. The main  
535 difference between the two species is the considerably more diverse heavy chain  
536 CDR3 regions that are found in human antibodies [43]. In terms of our sequence data,  
537 it may be that humans may have a more diverse antibody repertoire, not least because  
538 as larger individuals they may have a greater diversity of naïve B cells specific CSP.  
539 However, it is notable that all 4 human monoclonal antibodies described to date from

540 different volunteers share the use of the IGHV3-30 gene family [19,20], suggesting  
541 that in humans as well as mice there is a constrained repertoire of responding B cells.

542

543 Overall our data provide important insights into how the antibody response to  
544 CSP develops. Our results also help explain why relatively large amounts of  
545 antibodies are required for sporozoite neutralization and suggest that the ability to  
546 generate an effective B cell response may be limited by the very simplicity of the  
547 repeat epitope. These data support previous suggestions that CSP may be a  
548 suboptimal target for vaccination. However, we also find that CSP binding antibodies  
549 can undergo somatic hypermutation and reach high affinities. This suggests if we can  
550 develop vaccination strategies to diversify the repertoire of responding B cells and  
551 favor the germinal center response it may be possible to generate long-term protective  
552 immunity targeting this major vaccine candidate antigen.

553

554



## 555 **Methods**

556

## 557 **Ethics statement**

558 All animal procedures were approved by the Animal Experimentation Ethics  
559 Committee of the Australian National University (Protocol numbers: A2013/12;  
560 A2014/62 and A2015/76). All research involving animals was conducted in  
561 accordance with the National Health and Medical Research Council's (NHMRC)  
562 Australian Code for the Care and Use of Animals for Scientific Purposes and the  
563 Australian Capital Territory Animal Welfare Act 1992.

564

## 565 **Mice and Immunizations**

566 BALB/C mice (bred in house at the Australian National University) were immunized  
567 IV with  $5 \times 10^4$  *P. berghei* CS<sup>SM</sup> sporozoites expressing mCherry [44] or  $5 \times 10^4$  *P.*  
568 *berghei* CS<sup>Pf</sup> sporozoites dissected by hand from the salivary glands of *Anopheles*  
569 *stephensi* mosquitoes. Mice were then treated with 0.6mg chloroquine IP daily for 10  
570 days to prevent the development of blood stage infection.

571

## 572 **Flow Cytometry and sorting**

573 Single cell preparations of lymphocytes were isolated from the spleen of immunized  
574 mice and were stained for flow cytometry or sorting by standard procedures. Cells  
575 were stained with lineage markers (anti-CD3, clone 17A2; anti-GR1, clone RB6-8C5  
576 and anti-NKp46, clone 29A1.4) antibodies to B220 (clone RA3-6B2), IgM (clone  
577 II/41), IgD (11-26c2a) and (NANP)<sub>9</sub> tetramers conjugated to PE or APC. Antibodies  
578 were purchased from Biolegend while tetramers were prepared in house by mixing  
579 biotinylated (NANP)<sub>9</sub> peptide with streptavidin conjugated PE or APC (Invitrogen) in

the a 4:1 molar ratio. Flow-cytometric data was collected on a BD Fortessa flow cytometer (Becton Dickinson) and analyzed using FlowJo software (FlowJo). Where necessary cells were sorted on a BD FACs Aria I or II machine.

583

#### 584 **Sequencing of (NANP)<sub>n</sub> specific cells and BCR analysis**

585 Single cell suspensions from the spleens of immunized mice were stained with  
586 (NANP)<sub>n</sub> tetramers and antibodies to B cell markers as described in the supplemental  
587 experimental procedures. Antigen specific cells were sorted on a FACS ARIA I or II  
588 instrument prior to RNA extraction with the Arturus Picopure RNA isolation kit  
589 (Invitrogen) and cDNA preparation using the iScript cDNA synthesis kit (BioRad).  
590 BCR sequences were amplified using previously described heavy and kappa chain  
591 primers including adaptor sequences allowing subsequent indexing using the Nextera  
592 indexing kit (Illumina). Analysis was performed in house using R-scripts and the  
593 program MiXCR as described in supplemental experimental procedures.

594

#### 595 **Binding of antibody variants**

596 Variants of the 2A10 antibody were expressed in HEK293 T cells (a kind gift of  
597 Carola Vinuesa, Australian National University) as described in the supplemental  
598 experimental procedures. Binding to the CSP repeat was tested by ELISA and ITC  
599 using standard techniques as described in the supplemental experimental procedures.

600

#### 601 **Data Deposition**

602 Sequence data generated in this paper is deposited at the NCBI BioProject database  
603 accession number PRJNA352758. Atomic coordinates and related experimental data

604 for structural analyses are deposited in the Protein Data Bank (PDB) with PDB codes  
605 5ZSF and 5T0Y.

606    **Acknowledgments**

607

608    We thank the C3 Crystallisation Centre at CSIRO for help with crystal formation and  
609    the Australian Synchrotron and beamline scientists for help with data collection. We  
610    thank Michael Devoy, Harpreet Vohra and Catherine Gillespie of the Imaging and  
611    Cytometry Facility at the John Curtin School of Medical Research for assistance with  
612    flow cytometry and multi-photon microscopy.

613

## 614     **References**

615

- 616     1. World Health Organization (2016) World Malaria Report 2016. Geneva: World  
617             Health Organization.
- 618     2. Casares S, Brumeanu TD, Richie TL (2010) The RTS,S malaria vaccine. Vaccine  
619             28: 4880-4894.
- 620     3. RTS,S Clinical Trials Partnership (2015) Efficacy and safety of RTS,S/AS01  
621             malaria vaccine with or without a booster dose in infants and children in  
622             Africa: final results of a phase 3, individually randomised, controlled trial.  
623             Lancet 386: 31-45.
- 624     4. White MT, Bejon P, Olotu A, Griffin JT, Riley EM, et al. (2013) The relationship  
625             between RTS,S vaccine-induced antibodies, CD4(+) T cell responses and  
626             protection against Plasmodium falciparum infection. PLoS One 8: e61395.
- 627     5. Nussenzweig RS, Vanderberg J, Most H, Orton C (1967) Protective immunity  
628             produced by the injection of x-irradiated sporozoites of plasmodium berghei.  
629             Nature 216: 160-162.
- 630     6. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, et al. (2013) Protection  
631             against malaria by intravenous immunization with a nonreplicating sporozoite  
632             vaccine. Science 341: 1359-1365.
- 633     7. Dame JB, Williams JL, McCutchan TF, Weber JL, Wirtz RA, et al. (1984)  
634             Structure of the gene encoding the immunodominant surface antigen on the  
635             sporozoite of the human malaria parasite Plasmodium falciparum. Science  
636             225: 593-599.

- 637 8. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M (1980)  
638 Hybridoma produces protective antibodies directed against the sporozoite  
639 stage of malaria parasite. Science 207: 71-73.
- 640 9. Coppi A, Natarajan R, Pradel G, Bennett BL, James ER, et al. (2011) The malaria  
641 circumsporozoite protein has two functional domains, each with distinct roles  
642 as sporozoites journey from mosquito to mammalian host. J Exp Med 208:  
643 341-356.
- 644 10. Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V (1983)  
645 Circumsporozoite proteins of malaria parasites contain a single  
646 immunodominant region with two or more identical epitopes. J Exp Med 157:  
647 1947-1957.
- 648 11. Gardner MJ, Hall N, Fung E, White O, Berriman M, et al. (2002) Genome  
649 sequence of the human malaria parasite Plasmodium falciparum. Nature 419:  
650 498-511.
- 651 12. Zeeshan M, Alam MT, Vinayak S, Bora H, Tyagi RK, et al. (2012) Genetic  
652 variation in the Plasmodium falciparum circumsporozoite protein in India and  
653 its relevance to RTS,S malaria vaccine. PLoS One 7: e43430.
- 654 13. Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS (1984)  
655 Inhibition of entry of Plasmodium falciparum and P. vivax sporozoites into  
656 cultured cells; an in vitro assay of protective antibodies. J Immunol 132: 909-  
657 913.
- 658 14. Espinosa DA, Gutierrez GM, Rojas-Lopez M, Noe AR, Shi L, et al. (2015)  
659 Proteolytic Cleavage of the Plasmodium falciparum Circumsporozoite Protein  
660 Is a Target of Protective Antibodies. J Infect Dis 212: 1111-1119.

- 661 15. Persson C, Oliveira GA, Sultan AA, Bhanot P, Nussenzweig V, et al. (2002)  
662 Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines:  
663 rodent parasites bearing a hybrid Plasmodium falciparum circumsporozoite  
664 protein. J Immunol 169: 6681-6685.
- 665 16. Schofield L, Uadia P (1990) Lack of Ir-Gene Control in the Immune-Response to  
666 Malaria .1. A Thymus-Independent Antibody-Response to the Repetitive  
667 Surface Protein of Sporozoites. Journal of Immunology 144: 2781-2788.
- 668 17. Defrance T, Taillardet M, Genestier L (2011) T cell-independent B cell memory.  
669 Current Opinion in Immunology 23: 330-336.
- 670 18. Anker R, Zavala F, Pollok BA (1990) VH and VL region structure of antibodies  
671 that recognize the (NANP)<sub>3</sub> dodecapeptide sequence in the circumsporozoite  
672 protein of Plasmodium falciparum. Eur J Immunol 20: 2757-2761.
- 673 19. Foquet L, Hermsen CC, van Gemert GJ, Van Braeckel E, Weening KE, et al.  
674 (2014) Vaccine-induced monoclonal antibodies targeting circumsporozoite  
675 protein prevent Plasmodium falciparum infection. J Clin Invest 124: 140-144.
- 676 20. Chappel JA, Rogers WO, Hoffman SL, Kang AS (2004) Molecular dissection of  
677 the human antibody response to the structural repeat epitope of Plasmodium  
678 falciparum sporozoite from a protected donor. Malar J 3: 28.
- 679 21. Zavala F, Tam JP, Hollingdale MR, Cochrane AH, Quakyi I, et al. (1985)  
680 Rationale for development of a synthetic vaccine against Plasmodium  
681 falciparum malaria. Science 228: 1436-1440.
- 682 22. Cerami C, Frevert U, Sinnis P, Takacs B, Clavijo P, et al. (1992) The basolateral  
683 domain of the hepatocyte plasma membrane bears receptors for the  
684 circumsporozoite protein of Plasmodium falciparum sporozoites. Cell 70:  
685 1021-1033.

- 686 23. Braden BC, Poljak RJ (1995) Structural features of the reactions between  
687 antibodies and protein antigens. *FASEB J* 9: 9-16.
- 688 24. Plassmeyer ML, Reiter K, Shimp RL, Jr., Kotova S, Smith PD, et al. (2009)  
689 Structure of the Plasmodium falciparum circumsporozoite protein, a leading  
690 malaria vaccine candidate. *J Biol Chem* 284: 26951-26963.
- 691 25. Kelly SM, Jess TJ, Price NC (2005) How to study proteins by circular dichroism.  
692 *Biochim Biophys Acta* 1751: 119-139.
- 693 26. Shen Y, Maupetit J, Derreumaux P, Tuffery P (2014) Improved PEP-FOLD  
694 Approach for Peptide and Miniprotein Structure Prediction. *J Chem Theory*  
695 *Comput* 10: 4745-4758.
- 696 27. Ghasparian A, Moehle K, Linden A, Robinson JA (2006) Crystal structure of an  
697 NPNA-repeat motif from the circumsporozoite protein of the malaria parasite  
698 Plasmodium falciparum. *Chem Commun (Camb)*: 174-176.
- 699 28. Sela-Culang I, Alon S, Ofra Y (2012) A systematic comparison of free and  
700 bound antibodies reveals binding-related conformational changes. *J Immunol*  
701 189: 4890-4899.
- 702 29. Sircar A, Gray JJ (2010) SnugDock: paratope structural optimization during  
703 antibody-antigen docking compensates for errors in antibody homology  
704 models. *PLoS Comput Biol* 6: e1000644.
- 705 30. Davis SJ, Davies EA, Tucknott MG, Jones EY, van der Merwe PA (1998) The  
706 role of charged residues mediating low affinity protein-protein recognition at  
707 the cell surface by CD2. *Proc Natl Acad Sci U S A* 95: 5490-5494.
- 708 31. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK (2011) Different B cell  
709 populations mediate early and late memory during an endogenous immune  
710 response. *Science* 331: 1203-1207.



711 32. de Vinuesa CG, Cook MC, Ball J, Drew M, Sunners Y, et al. (2000) Germinal  
712 centers without T cells. *J Exp Med* 191: 485-494.

713 33. Schofield L (1990) The circumsporozoite protein of Plasmodium: a mechanism of  
714 immune evasion by the malaria parasite? *Bull World Health Organ* 68 Suppl:  
715 66-73.

716 34. Arnaout R, Lee W, Cahill P, Honan T, Sparrow T, et al. (2011) High-resolution  
717 description of antibody heavy-chain repertoires in humans. *PLoS One* 6:  
718 e22365.

719 35. Busse CE, Czogiel I, Braun P, Arndt PF, Wardemann H (2014) Single-cell based  
720 high-throughput sequencing of full-length immunoglobulin heavy and light  
721 chain genes. *Eur J Immunol* 44: 597-603.

722 36. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V (1980) Monovalent  
723 fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen  
724 (Pb44) protect mice against malarial infection. *J Exp Med* 151: 1504-1513.

725 37. Stewart MJ, Vanderberg JP (1988) Malaria Sporozoites Leave Behind Trails of  
726 Circumsporozoite Protein during Gliding Motility. *Journal of Protozoology*  
727 35: 389-393.

728 38. White MT, Bejon P, Olotu A, Griffin JT, Bojang K, et al. (2014) A combined  
729 analysis of immunogenicity, antibody kinetics and vaccine efficacy from  
730 phase 2 trials of the RTS,S malaria vaccine. *BMC Med* 12: 117.

731 39. Avent ND, Madgett TE, Lee ZE, Head DJ, Maddocks DG, et al. (2006) Molecular  
732 biology of Rh proteins and relevance to molecular medicine. *Expert Rev Mol*  
733 *Med* 8: 1-20.

- 734 40. Chang TY, Siegel DL (1998) Genetic and immunological properties of phage-  
735 displayed human anti-Rh(D) antibodies: implications for Rh(D) epitope  
736 topology. Blood 91: 3066-3078.
- 737 41. Finne J, Leinonen M, Makela PH (1983) Antigenic similarities between brain  
738 components and bacteria causing meningitis. Implications for vaccine  
739 development and pathogenesis. Lancet 2: 355-357.
- 740 42. North B, Lehmann A, Dunbrack RL (2011) A New Clustering of Antibody CDR  
741 Loop Conformations. Journal of Molecular Biology 406: 228-256.
- 742 43. Stanfield RL, Wilson IA (2014) Antibody Structure. Microbiology spectrum 2.
- 743 44. Cockburn IA, Tse SW, Zavala F (2014) CD8+ T cells eliminate liver stage  
744 Plasmodium parasites without detectable bystander effect. Infect Immun.  
745  
746  
747

## 748    **Supporting Information Legends**

749

### 750    **S1 Fig. Theoretical (A) and experimental (B) CD spectra of the (NANP)<sub>6</sub> peptide.**

751    The computational prediction of the spectra (A) was performed using DichroCalc [9],  
752    the experimental spectra was measured at 222 nm at 25 °C. A peak at 185 nm,  
753    minimum at 205 nm and shoulder between 215 and 240 nm are consistent with an  
754    intrinsically disordered, but not random coil, structure.

755

### 756    **S2 Fig. Cluster analysis for MD simulations of (NANP)<sub>6</sub> peptide.** Conformations

757    were clustered by concatenating the trajectory and performing a Jarvis-Patrick  
758    analysis. The clusters are sorted by their RMSD from the first cluster (starting  
759    geometry). As shown, Run 2 is stable in the starting geometry for several ns, while  
760    Run 3 diverged, then reconverged to the starting geometry, where it was stable for  
761    several ns. These data suggest the quasi-helical structure observed from the ab initio  
762    calculations is stable, and can be spontaneously sampled, on a timescale of several ns.

763

### 764    **S3 Fig. Cluster analysis for MD simulations of (NANP)<sub>6</sub> peptide.** Molecular

765    dynamics simulation of the (NANP)<sub>6</sub>:F<sub>AB</sub> complex. Root mean square deviation  
766    (RMSD) of the (NANP)<sub>6</sub>:F<sub>AB</sub> complex as a function of time, independent simulations  
767    are shown in green, black and red.

768

### 769    **S4 Fig: Alignment of 2A10 heavy chain and the predicted germline sequence**

770    Residues that are mutated away from the predicted germline sequence in more one or  
771    more other antibody heavy chain (2E7 or 3D6) are highlighted in red, mutations that  
772    are predicted to be involved in binding to CSP are highlighted in blue.

773

774 **S5 Fig: Alignment of 2A10 heavy chain and the predicted germline sequence**

775 Residues that are mutated away from the predicted germline sequence in both 2A10  
776 and the related 2E7 antibody are highlighted in red, mutations that are predicted to be  
777 involved in binding to CSP are highlighted in blue.

778

779 **Movie S1: Molecular Dynamics simulation of the solution structure of the**  
780 **(NANP)<sub>6</sub> peptide**

781 Excerpt from (NANP)<sub>6</sub> run 3. Trajectory was fitted to minimize alpha-carbon RMSD  
782 and then passed through a low-pass filter with filter length 8 frames to reduce  
783 temporal aliasing.

784

785 **Movie S2: Molecular Dynamics simulation of the interaction of the (NANP)<sub>n</sub>**  
786 **repeat with the 2A10 FAB**

787 Excerpt from 2A10:(NANP)<sub>6</sub> run 3. Trajectory was fitted to minimize alpha-carbon  
788 RMSD and then passed through a low-pass filter with filter length 8 frames to reduce  
789 temporal aliasing.

790

## 791 **Figure Legends**

792

793 **Fig. 1. ITC data for interactions between 2A10 F<sub>AB</sub> and antigens.** (A) Titration of  
794 2A10 F<sub>AB</sub> with (NANP)<sub>6</sub>. (B) Titration of 2A10 F<sub>AB</sub> with rCSP. (C) Titration of 2A10  
795 with rCSP. The upper panels represent baseline-corrected power traces. By  
796 convention, negative power corresponds to exothermic binding. The lower panels  
797 represent the integrated heat data fitted to the independent binding sites model.

798

799 **Fig 2. Structures of the (NANP)<sub>6</sub> peptide (A), the 2A10 F<sub>AB</sub> fragment (B) and the**  
800 **model of the F<sub>AB</sub> fragment-(NANP)<sub>6</sub> complex (C).** (A) The calculated structure of  
801 the (NANP)<sub>6</sub> peptide is a helical structure containing the same hydrogen bonds  
802 between a carbonyl following the proline and the amide nitrogen of the alanine, and  
803 the carbonyl group of an asparagine and a backbone amide of asparagine 3 residues  
804 earlier (highlighted in red) that are observed in [27]. (B) Electron density (blue mesh;  
805 2mF<sub>o</sub>-dF<sub>c</sub> at 1 σ) of the 2A10 F<sub>AB</sub> fragment viewed from above the antigen-binding  
806 site. Light chain is shown as yellow sticks, heavy chain as cyan. (C) A calculated  
807 model of the (NANP)<sub>6</sub>:2A10 F<sub>AB</sub> fragment complex. The CDR2 regions of each chain  
808 are shown in red, the CDR3 regions of each chain are shown in blue.

809 **Fig 3. Detailed view of the (NANP)<sub>6</sub>:2A10 F<sub>AB</sub> interface and site directed**  
810 **mutagenesis.** (A) A model of the light chain:(NANP)<sub>6</sub> interface. (B) ELISA results  
811 showing the effect of mutating light chain interface residues; error bars are based on  
812 technical replicates from one of two independent experiments. (C) A model of the  
813 heavy chain:(NANP)<sub>6</sub> interface. (D) ELISA results showing the effect of mutating  
814 heavy chain interface residues; error bars are based on technical replicates from one

815 of two independent experiments.

816 **Fig 4. The multivalency of the NANP repeat region of the CSP protein.** (A) An  
 817 (NANP)<sub>6</sub> peptide results in the presentation of two symmetrical epitopes, formed by  
 818 alternating repeats (cyan and magenta), allowing binding by two F<sub>AB</sub> domains, in  
 819 keeping with the stoichiometry observed by ITC. (B) The full 27-mer repeat region  
 820 results in the presentation of at least 10 separate epitopes and the twist of the helix  
 821 results in displacement along the length of the repeat region, which allows binding of  
 822 up to 10 separate F<sub>AB</sub> fragments, consistent with 4 antibodies bound by both F<sub>AB</sub>  
 823 domains, and two bound by a single F<sub>AB</sub> domain.

824

825 **Fig 5. CSP-specific B cells enter the germinal center following sporozoite**  
 826 **immunization.** BALB/C Mice were immunized with either  $5 \times 10^4$  *P. berghei* CS<sup>5M</sup>  
 827 (expressing the endogenous *P. berghei* CSP repeat) or  $5 \times 10^4$  *P. berghei* CS<sup>Pf</sup>  
 828 (expressing the circumsporozoite protein from *P. falciparum*). 12 days later the B cell  
 829 response was analyzed by flow cytometry and putative (NANP)<sub>n</sub>-specific cells were  
 830 identified using PE and APC conjugated tetramers. (A) Representative flow  
 831 cytometry plots showing the identification of (NANP)<sub>n</sub>-specific (Tetramer+) cells. (B)  
 832 Representative flow cytometry plots showing the proportion of Tetramer+ cells that  
 833 have class switched and entered a germinal center. (C) Quantification of the number  
 834 of class switched Tetramer+ cells under different immunization conditions. (D)  
 835 Quantification of the number of germinal center Tetramer+ cells under different  
 836 immunization conditions.

837

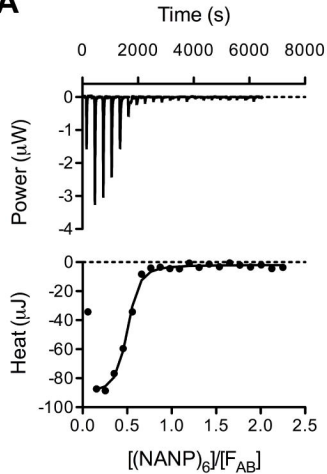
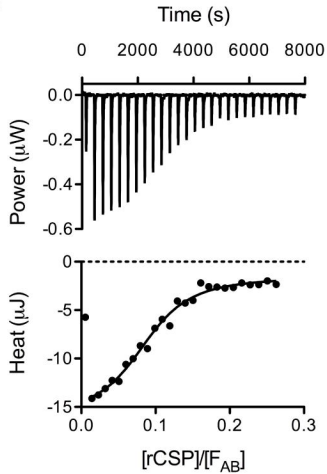
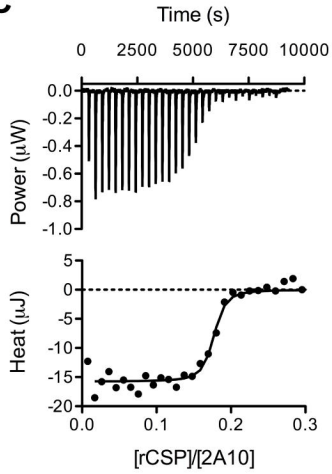
838 **Fig 6. Limited diversity of (NANP)<sub>n</sub> specific antibodies.** BCR sequences were  
 839 amplified from Tetramer+ cells sorted from BALB/C mice 35 days after

840 immunization with *P. berghei* CS<sup>Pf</sup> as well as bulk B cells from naïve BALB/C mice  
 841 (A) IGHV gene usage from among B cells from a representative naïve mouse (grey  
 842 bars) and Tetramer+ cells from immune mice (red, blue and yellow bars). (B)  
 843 Shannon's diversity calculated for the diversity of IGHV region usage among bulk B  
 844 cells and Tetramer+ cells. (C) Circos plots showing the IGHV-IGHJ pairings in a  
 845 representative naïve mice and 3 immune mice. (D) IGKV gene usage from among B  
 846 cells from a representative naïve mouse (grey bars) and Tetramer+ cells from immune  
 847 mice (red, blue and green bars). (E) Shannon's diversity calculated for the diversity of  
 848 IGKV region usage among bulk B cells and Tetramer+ cells. (F) Circos plots showing  
 849 the IGKV-IGKJ pairings in a representative naïve mouse and 3 immune mice.

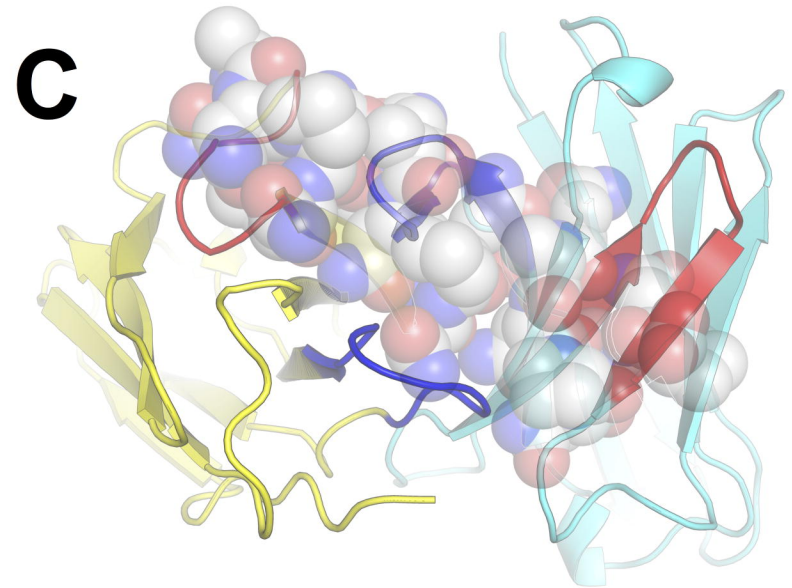
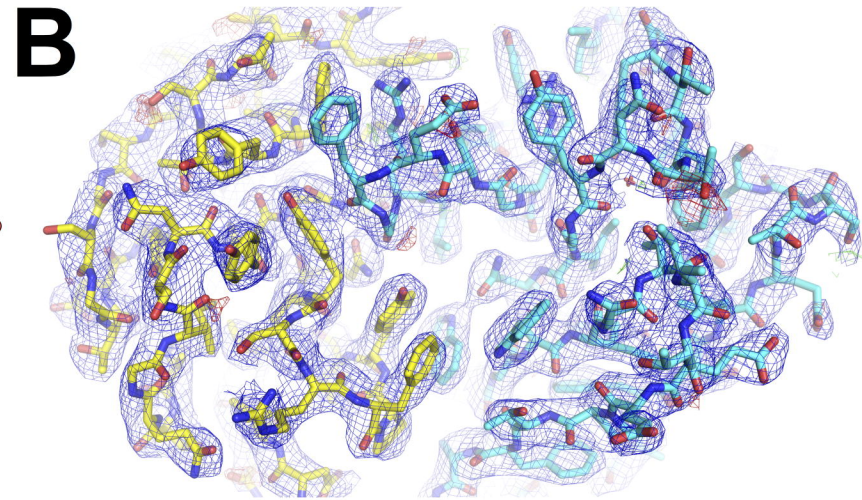
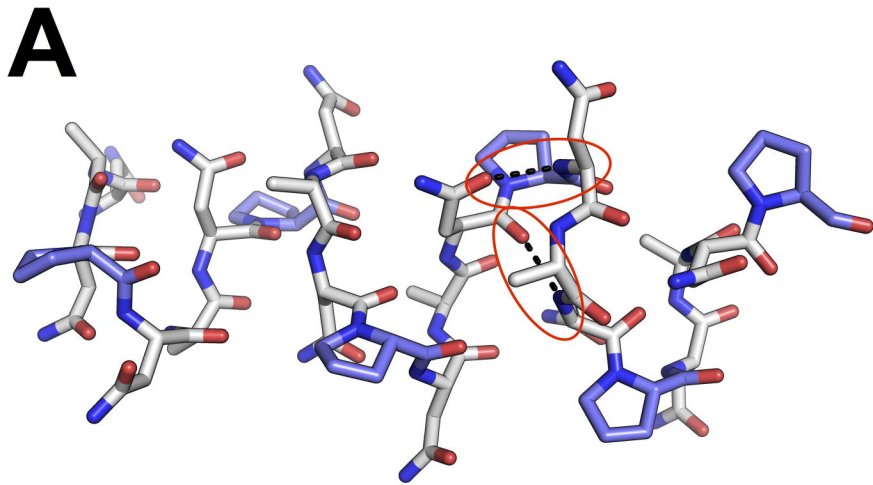
850

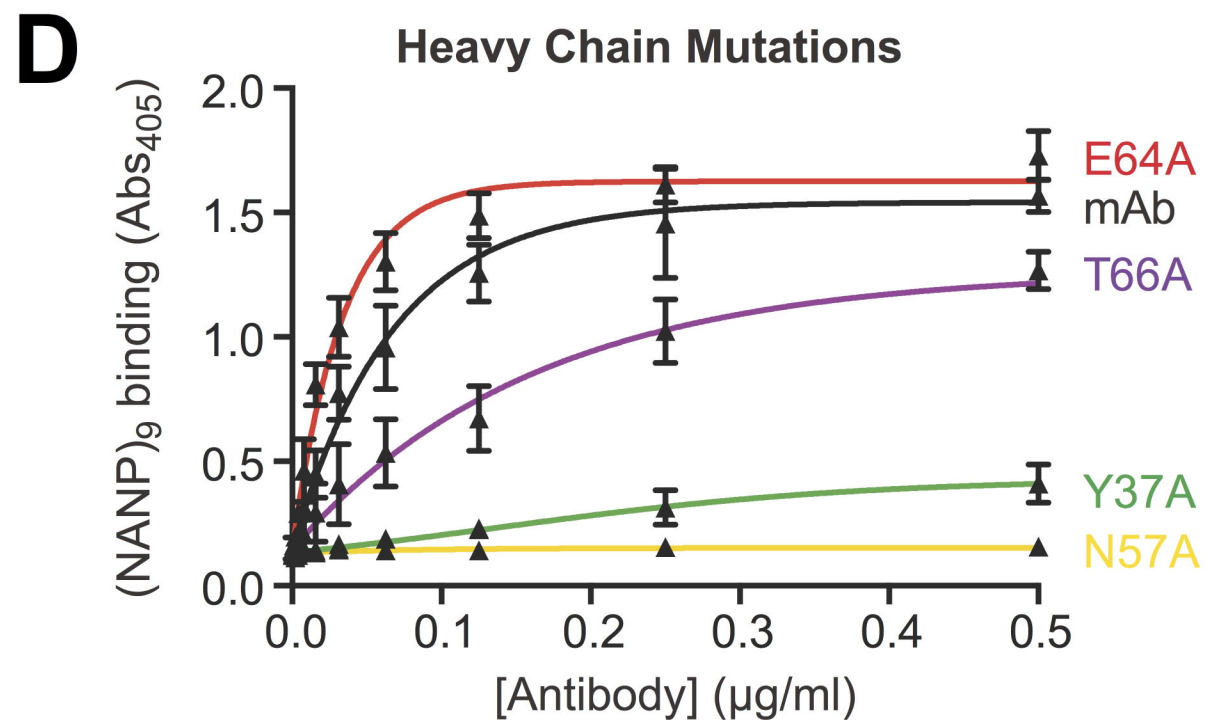
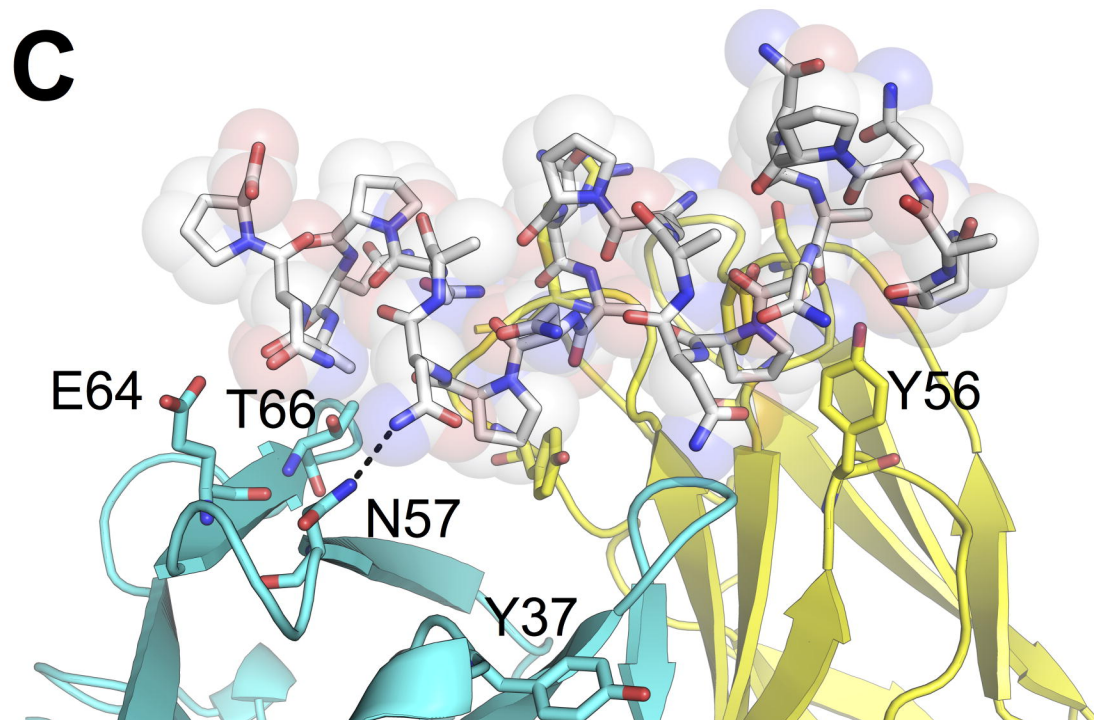
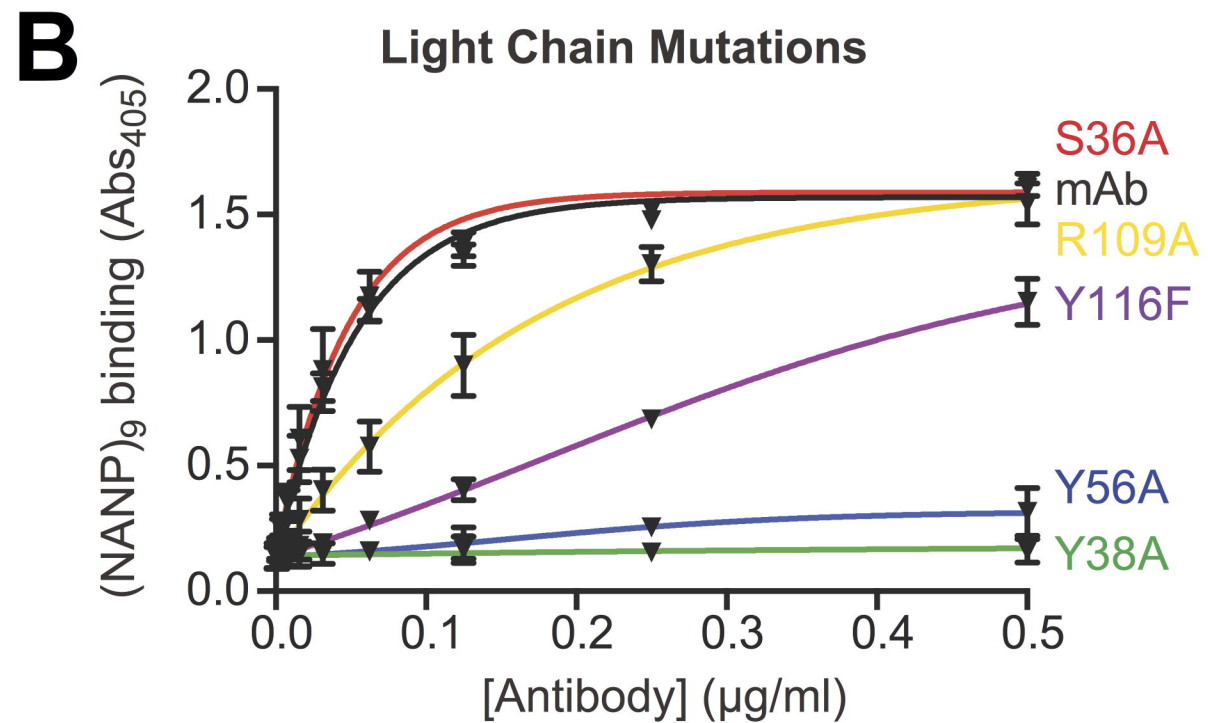
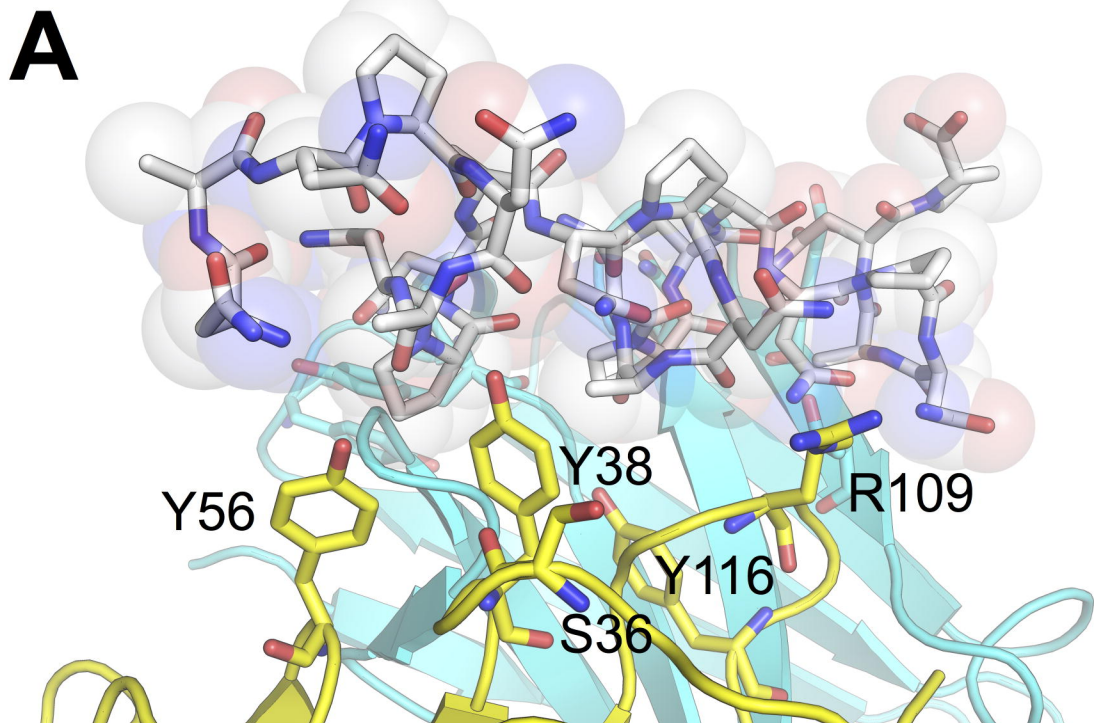
# **Fig 7: CSP-binding antibodies undergo somatic hypermutation and affinity**

852 **maturation** (A) Violin plots showing the number of mutations per read from bulk B  
 853 cells from 3 individual naïve mice and sorted (NANP)<sub>n</sub> specific B cells from  
 854 sporozoite immunized mice (B) Skyscraper plots showing the location of mutations  
 855 away from germline in the IGKV1-110 gene in a naïve mouse and in sorted (NANP)<sub>n</sub>  
 856 specific cells in three sporozoite immunized mice. (C) ELISA binding to the (NANP)<sub>9</sub>  
 857 peptide of recombinant antibodies corresponding to the 2A10 mAb, the predicted  
 858 germline precursor, and hybrid antibodies containing the 2A10 heavy chain (mHC)  
 859 paired with the germline light chain (gLC) and the 2010 light chain (mLC) paired  
 860 with germline heavy chain (gHC). (D) Predicted mutations in the gLC were  
 861 introduced to the germline precursor and their effect on binding to (NANP)<sub>9</sub> measured  
 862 by ELISA (E) Predicted mutations in the gHC were introduced to hybrid antibodies  
 863 consisting of the mLC and the gHC heavy chain and their effect on binding to  
 864 (NANP)<sub>9</sub> measured by ELISA.

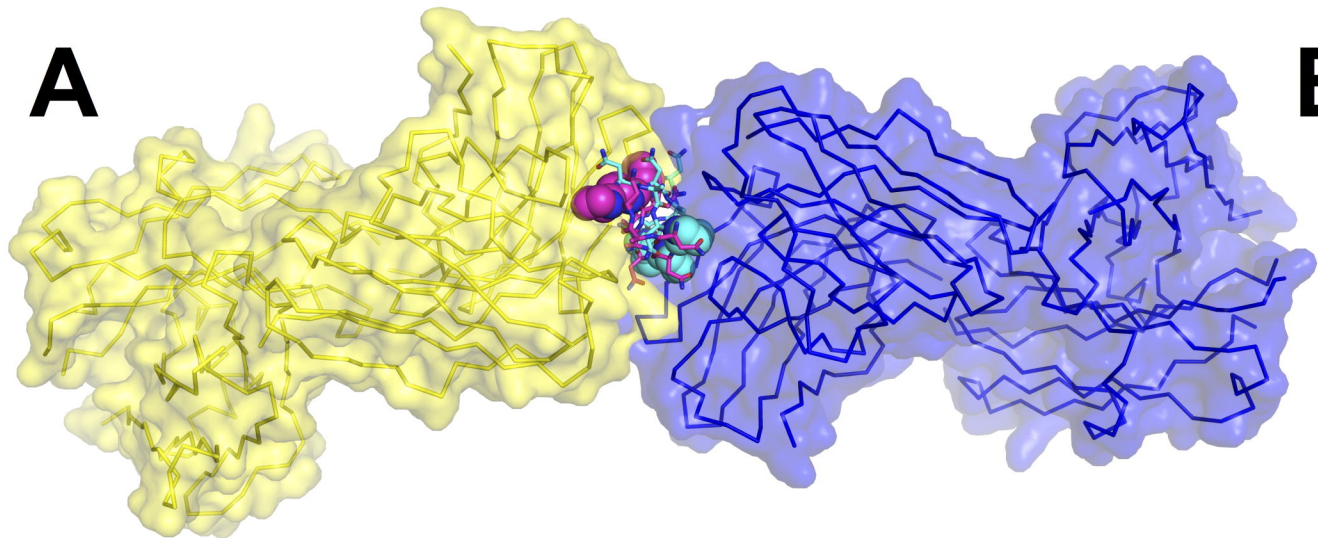
**A****B****C**









**A****B**