

1 Germinal center activity and B cell maturation promote protective antibody responses
2 against *Plasmodium* pre-erythrocytic infection

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19 **Abstract**

20 Blocking *Plasmodium*, the causative agent of malaria, at the asymptomatic pre-erythrocytic
21 stage would abrogate disease pathology and prevent transmission. Rodent-infectious
22 species of *Plasmodium* such as *P. yoelii* (*Py*) serve as key tools to study vaccine efficacy
23 and disease biology in immune-competent experimental animals. Here we evaluated the
24 differences in vaccine-elicited humoral immunity in two widely used, and vastly diverged,
25 inbred mouse strains, BALB/cJ and C57BL/6J, and identified immunological factors
26 associated with protection. We vaccinated with *Py* circumsporozoite protein (CSP), the major
27 surface antigen on the sporozoite, and evaluated protective efficacy after mosquito bite
28 challenge. Vaccination achieved 60% sterile protection and otherwise delayed blood stage
29 patency in BALB/cJ mice, whereas; all C57BL/6J mice were infected similar to controls.
30 Interestingly, protection was mediated by antibodies, and could be passively transferred from
31 immunized BALB/cJ mice into naïve C57BL/6J. Dissection of the underlying immunological
32 features of protection revealed early deficits in antibody titers and polyclonal avidity in
33 C57BL/6J mice. Additionally, *Py*CSP-vaccination in BALB/cJ induced a significantly higher
34 proportion of antigen-specific B-cells and class-switched memory B-cell (MBCs) populations
35 than in C57BL/6J mice. Strikingly, C57BL/6J mice also had markedly fewer germinal center
36 experienced, CSP-specific class-switched MBCs compared to BALB/cJ mice. Analysis of
37 the IgG γ chain repertoires by next generation sequencing in *Py*CSP-specific memory B-cell
38 repertoires also revealed higher somatic hypermutation rates in BALB/cJ mice than in
39 C57BL/6J mice. These findings indicate that BALB/cJ mice achieved higher levels of B cell
40 maturation in response to vaccination with *Py*CSP, which likely enabled the development of
41 protective antibody responses. Overall, our study indicates that germinal center activity and
42 B cell maturation are key processes in the development of vaccine-elicited protective
43 antibodies against CSP.

44 Introduction

45 Malaria remains a major public health crisis, with more than 229 million cases resulting in
46 more than 409K deaths in 2019, concentrated in sub-Saharan Africa and disproportionately
47 affecting women and children (WHO, 2020). After peaking in 2004, cases have steadily
48 declined, but in recent years cases have plateaued or slightly increased, highlighting the
49 urgent need for new counter measures to achieve eradication (WHO, 2019). Vaccines that
50 prevent infection with *Plasmodium* parasites, the causative agents of malaria, offer the best
51 hope to overcome this plateau and facilitate eradication. While vaccines are in development
52 for all stages of the *Plasmodium* life cycle, the pre-erythrocytic (PE) stage is an attractive
53 target, as stopping the parasite at this asymptomatic stage would prevent infection,
54 subsequent disease, and transmission (Burrows et al., 2017). RTS,S, the only malaria
55 vaccine with regulatory approval (Olotu et al., 2016; Stoute et al., 1997; Sun et al., 2003),
56 and the most clinically advanced whole sporozoite vaccine, PfSPZ (Sissoko et al., 2017),
57 both target this stage and result in only partial protection in field trials. Recently, a new PE
58 vaccine candidate, R21, achieved promising results in early clinical field trials (Dattoo et al.,
59 2021). Subunit vaccines, such as RTS,S and R21, induce potent antibody responses against
60 the major surface protein on the sporozoite, the Circumsporozoite Protein (CSP) (Dattoo et
61 al., 2021; Sun et al., 2003). Whole sporozoite vaccines also elicit anti-CSP antibodies, they
62 also produce antibodies to other *Plasmodium* antigens (Camponovo et al., 2020; Mordmuller
63 et al., 2017).

64 The mechanisms by which pre-erythrocytic (PE) vaccines prevent malaria infection
65 are yet to be fully characterized, but both antibodies and CD8⁺ T cells have been implicated,
66 depending on the vaccine modality (Epstein et al., 2011; Ewer et al., 2013; Ewer et al., 2015)
67 Antibodies have been shown to mediate anti-parasitic activity and are thought to work
68 primarily in the skin and interstitial tissues where they interfere with the sporozoite's motility
69 and survival (Aliprandini et al., 2018). This has been confirmed by studies of monoclonal
70 antibodies (mAbs) targeting CSP, which have been shown experimentally to reduce or
71 prevent *Plasmodium falciparum* infection (Aliprandini et al., 2018). The study of anti-CSP
72 mAbs isolated from humans has implicated antibody affinity, epitope specificity, and B cell
73 clonal selection as key factors mediating protective function (Murugan et al., 2018). Together
74 these studies indicate that antibodies are a key mediator of protection in subunit PE
75 vaccines, and that protective antibodies have inherent features that determine neutralizing
76 capacity. Whereas mAbs have been instrumental to our understanding of antibody-mediated
77 protection from malaria, vaccination with CSP induces complex polyclonal responses
78 consisting of a diversity of antibodies. The key features of vaccine-elicited polyclonal
79 antibody responses that determine protection from infection have yet to be fully defined, but

80 their characterization would be a critical milestone toward the development of an effective
81 CSP-based vaccine.

82 Here, we aimed to identify the characteristics of protective antibody responses
83 elicited by full-length CSP vaccination using the rodent malaria, *P. yoelii*. This model of
84 malaria infection enabled us to conduct live vaccination and mosquito bite challenges in a
85 wild type experimental system to dissect the correlates of antibody-mediated protection. To
86 identify features associated with efficacy, we characterized vaccine-elicited B cell responses
87 in two highly diverged mouse strains, BALB/cJ and C57BL/6J, that exhibited differential
88 vaccine-mediated sterilizing immunity. We evaluated serum antibody responses,
89 characterized CSP-specific B cell phenotypes, and compared B cell receptor repertoires
90 between the two strains in response to CSP vaccination. We found that vaccine-elicited anti-
91 CSP antibodies alone are sufficient to achieve sterile protection from infection, and that
92 protection was associated with higher magnitude germinal center (GC) responses and
93 somatic mutation of CSP-specific B cells. These findings implicate B cell maturation as a
94 critical determinant in the development of potent sterilizing antibody-mediated immunity
95 against malaria and indicate that vaccine modalities aimed at inducing mature B cell
96 responses will be necessary to achieve sterilizing immunity in the field.

97

98 Results

99 Immunization with *PyCSP* elicits anti-parasitic antibodies and sterile protection from 100 *P. yoelii* in BALB/cJ, but not C57BL/6J mice

101 The major strains used to study vaccine efficacy and host pathogen interactions in murine
102 models of malaria infection are BALB/cJ and C57BL/6J (Benhnini et al., 2009; Kuipers et al.,
103 2017). In our studies of CSP vaccine-elicited vaccine efficacy, we observed that these two
104 strains exhibited differential levels of protection in a subunit immunization, mosquito bite
105 challenge model, where we routinely failed to achieve sterilizing immunity in C57BL/6J mice.
106 To compare efficacy directly, we immunized animals (n=10 per group) with 20 µg of full
107 length ecto-domain recombinant *PyCSP* in 20% adjuvax adjuvant at weeks 0, 2 and 6
108 (Figure 1A). Control groups received PBS-formulated with 20% adjuvax. At weeks 3 and 7,
109 blood samples were collected to evaluate the antibody responses elicited by the vaccination.
110 At week 7, both mouse strains were subjected to *P. yoelii* 17XNL sporozoite infection by
111 mosquito-bite challenge (15 mosquitoes/mouse) and were monitored for three weeks or until
112 blood-stage patency was reached. All the placebo-injected control mice in both the BALB/cJ
113 and C57BL/6J groups developed blood-stage patency in as early as 5 days post mosquito
114 bite challenge, indicating that the strains were equally susceptible to infection (Figure 1B), as
115 has been reported previously (Miller et al., 2014). All of the *PyCSP*-vaccinated C57BL/6J
116 mice were unprotected and developed blood-stage patency 6 days post mosquito bite
117 challenge. Surprisingly, in contrast to the C57BL/6J mice, *PyCSP*-immunized BALB/cJ mice
118 exhibited sterile protection from infection (60%). Six BALB/cJ mice were parasite free at the
119 end of the experiment, and the remaining mice (40%) exhibited a significant delay in blood
120 stage patency (7 days) (Figure 1B and S1A).

121 To characterize the underlying immune responses mediating sterile protection, we analyzed
122 the anti-parasitic activity of serum polyclonal antibodies (pAbs) *in vitro* and *in vivo*. Anti-CSP
123 serum antibodies from both strains recognized CSP on the surface of the *P. yoelii* sporozoite
124 by immunofluorescence microscopy (Figure 1C), indicating that both strains developed
125 antibodies capable of recognizing surface displayed CSP on the sporozoite. However, only
126 BALB/cJ anti-CSP pAbs induced a CSP reaction on sporozoites (Figure 1C, arrows) in which
127 neutralizing antibodies (Nabs) induce the sporozoite to “shed” its coat of CSP (Balaban et
128 al., 2021; Ghosh and Jacobs-Lorena, 2009; Vanderberg et al., 1969) We then characterized
129 *in vitro* inhibitory activity in the inhibition of Sporozoite Traversal and Invasion (ISTI) assay.
130 *PyCSP*-immunized BALB/cJ and C57BL/6J serum antibodies from Weeks 3 and 7 were pre-
131 incubated with *Py* sporozoites and the percentage invasion and traversal to hepatocytes
132 were analyzed. *PyCSP*-immunized BALB/cJ immune serum from both weeks 3 and 7

133 significantly inhibited sporozoite invasion into hepatocytes compared to controls, whereas
134 *PyCSP*-immunized C57BL/6J immune serum from weeks 3 and 7 did not inhibit invasion
135 above background levels (Figure 1D). Similarly, *PyCSP*-immunized BALB/cJ pAbs
136 significantly reduced sporozoite traversal at weeks 3 and 7 through hepatocytes compared
137 to controls, whereas *PyCSP*-immunized C57BL/6J immune serum did not inhibit traversal
138 above background levels at any time (Figure 1E).

139 To evaluate the differential functional activity *in vivo*, and to assess potential infection
140 differences due to mouse strain, we performed a pAb swap infection challenge and sterile
141 protection experiment. BALB/cJ or C57BL/6J mice were immunized with *PyCSP* as shown in
142 Figure 1A and then protein A purified pAbs from immunized (Post 3rd immunization) animals
143 or naïve controls were passively infused into strain mis-matched naïve mice five days prior
144 to mosquito bite challenge (15 mosquitoes/mouse). Mice were monitored for two weeks or
145 until blood-stage patency was reached. Thus, C57BL/6J immune and naïve serum was
146 infused into naïve BALB/cJ mice prior to challenge, and vice versa. The pAbs were purified
147 from pooled serum and the anti-CSP content was quantified using a standard curve
148 generated with canonical neutralizing anti-*PyCSP* mAb, 2F6. Each animal received an
149 equivalent of 90 µg of polyclonal anti-CSP antibody, with naïve animals receiving 90 µg total
150 IgG. C57BL/6J mice that received BALB/cJ-derived anti-CSP pAbs exhibited delays to
151 patency and 40-60% sterile protection, depending on the experiment (Figure 1F and S1B),
152 whereas BALB/cJ mice infused with C57BL/6J-derived anti-CSP pAbs were not protected
153 and all became infected similar to controls (Figure 1F and S1B). These findings demonstrate
154 that the protection observed in vaccinated BALB/cJ mice is mediated by antibodies alone.
155 Further, it implies that there are specific features of vaccine-elicited antibodies in BALB/cJ
156 mice that are critical to achieving sterile protection, features that likely do not exist in
157 C57BL/6J mice. This differential model of sterile protection from malaria afforded the
158 opportunity to assess what characteristics of the B cell response are associated with
159 protection from infection.

160 ***PyCSP* immunized C57BL/6J mice exhibit early deficits in the development of anti-** 161 **CSP antibodies**

162 To investigate the underlying differences in the protection between *PyCSP*-immunized
163 BALB/cJ and C57BL/6J mice, we analyzed *PyCSP*-specific plasma IgG responses over the
164 course of vaccination. Antibodies are often not measurable after the first immunization but
165 reach high levels with the anamnestic response after the boost immunizations. Thus, we
166 focused on studying samples from after the second and third immunizations, at weeks 3 and
167 7, respectively. At week 3, α-CSP IgG endpoint titers were nearly one log₁₀ higher in

168 BALB/cJ mice than in C57BL/6J mice (Figure 2A). However, by week 7, one week post third
169 immunization and just before mosquito-bite challenge, IgG titers were similar between the
170 strains (Figure 2A). No difference in IgG subclass usage was observed at week 7, and both
171 strains had similar α -CSP titers of IgG1 and IgG2b antibodies (C57BL/6J do not express
172 Ig2a) (Figure S2). Thus, although we detected an early difference in overall binding titers, at
173 the time of challenge the strains had similar levels and subclass distribution of circulating α -
174 CSP IgG antibodies.

175 We next measured the avidity index of the serum IgG to full ectodomain PyCSP over the
176 course of immunization, which is a surrogate measure of the strength of polyclonal antibody
177 binding to the protein. Binding is measured in the presence and absence of thiocyanate
178 chaotrope, and the relative disruption of binding is used to generate an index value (Pullen
179 et al., 1986). Interestingly, C57BL/6J mice had significantly lower avidity for recombinant
180 PyCSP after the second immunization at week 3 (Figure 2E), indicating a potential early
181 deficit in antibody maturation. By week 7 the avidity indices against full length rec-PyCSP
182 were statistically similar. However, this lack of difference in polyclonal avidity at week 7 is
183 likely due to the course measure of the binding characteristics of a diverse mixture of
184 antibodies as a population, where the potential presence of less frequent high affinity clones
185 may not be sufficient to influence the overall avidity index.

186 To determine whether differential protection could be attributed to epitope specificity, we
187 generated repeat peptides corresponding to the major and minor repeat regions (Figure 2C)
188 and assessed IgG binding over the course of immunization. The repeat regions are known to
189 be the target of neutralizing antibodies, whereas the N- and C-terminal domains have not
190 been implicated as targets for neutralizing antibodies (Kisalu et al., 2018; Scally et al., 2018;
191 Tan et al., 2018; Thai et al., 2020; Triller et al., 2017). The repeat region in PyCSP contains
192 two general motifs that make up the longer, N-terminal major repeat region and the shorter,
193 more C-terminal minor repeats (Figure 2B). The minor repeat region is the target of potent α -
194 PyCSP neutralizing antibody activity of mAb 2F6 (Bruna-Romero et al., 2004; Sack et al.,
195 2014; Vijayan et al., 2021) but neutralization targeting the major repeat has not been
196 reported. At week 3, the strains had equivalent IgG titers to the major repeat peptide, but
197 BALB/cJ mice had significantly higher titers to the minor repeat peptide (Figure 2D). At week
198 7, IgG titers to the major repeat remained statistically similar, but, although the difference
199 was not statistically significant, C57BL/6J titers to the minor repeat trended lower (Figure
200 2D). These findings imply that the unprotected phenotype in C57BL/6J mice may be due to
201 dampened antibody responses to the minor repeat, which is known to mediate
202 neutralization.

203 **Both major and minor repeat motifs of *PyCSP* mediate sterile protection from *P. yoelii***
204 **infection**

205 To evaluate this possibility, we investigated whether the major repeat also could be a target
206 for NABs, or whether neutralization is solely mediated through the minor repeat motif as
207 observed for mAb 2F6. We previously characterized a non-neutralizing antibody (nNAb),
208 RAM1, which binds to the major repeat with relatively low affinity, does not induce the CSP
209 reaction, nor protects *in vitro* or *in vivo* (Vijayan et al., 2021). In this study, we isolated a
210 mAb RAM2, which also binds to an epitope in the major repeat motif like RAM1 (Figure 3A
211 and 3D). RAM2 bound to recombinant CSP with a similar EC_{50} to that of 2F6 by ELISA
212 (Figure 3B), and both mAbs had similar binding kinetics measured by Octet-BLI (Figure 3C).
213 In contrast to RAM1, RAM2 induces the CSP reaction (Figure 3E) and blocks *in vitro*
214 infection in ISTI to similar levels the NAb 2F6 (Figure 3F). Unlike RAM1, RAM2 also
215 facilitates sterile protection *in vivo* when administered by passive infusion at survival levels
216 that mirror 2F6 (Figure 3G), indicating that potent neutralization can target either the major
217 or minor repeat motifs, and that the differential protection between mouse strains cannot be
218 fully explained by differential responses to the major and minor repeat motifs.

219 Although RAM1 and RAM2 both target the major repeat domain, they do so with drastically
220 different binding kinetics. RAM2 Fab binds to recombinant CSP with a K_D 7.85×10^{-7} M and
221 with an association and dissociation kinetics values of K_{on} (1.25×10^4 1/Ms) and K_{off} ($9.84 \times$
222 10^{-3} 1/s), whereas RAM1 Fab exhibits little binding to CSP and we were unable to derive a
223 measurable K_D (Figure S3; Vijayan et al., 2021). By comparison, the minor repeat NAb 2F6
224 Fab binds to rec-*PyCSP* with a K_D of 5.24×10^{-7} M (Vijayan et al., 2021). This difference in
225 binding affinity between RAM1 and RAM2 is likely a major contributor to their differential
226 functional activity and implies that there may be a minimum threshold for affinity that is
227 necessary to achieve neutralization. Further, these findings raise the possibility that the lack
228 of neutralization in C57BL/6J mice may be due to a lack of high affinity B cell clones,
229 potentially driven by lower B cell maturation. Taken together, these findings implicate
230 antibody affinity as a potential key feature of protective antibody responses against
231 *Plasmodium*.

232 **Dampened B cell responses elicited by vaccination in C57BL/6J mice**

233 We next evaluated if differential antibody protection between BALB/cJ and C57BL/6J mice
234 was a consequence of nuanced responses to *PyCSP* vaccination in the B cell compartment.
235 To assess the potential immunological origins of the differential protection, we characterized
236 anti-CSP-specific B cell responses in the spleen after vaccination. Splenocytes were
237 harvested at week 3 and 7 or post 1 week after the second and third immunizations,

238 respectively, and stained with markers for flow cytometric analyses, including B220, GL-7,
239 CD38, IgD, IgM, and CD138. Recombinant CSP was tetramerized by conjugation to
240 streptavidin-conjugated APC and APC/Fire750, which were used for column enrichment and
241 dual staining for *PyCSP*-reactive cells. We assessed and quantified the total number of
242 splenic *PyCSP*-specific B-cells (CD3⁻, B220⁺, CSP⁺), IgM memory B-cells (MBCs) (CD3⁻,
243 B220⁺, CD38⁺, CD138⁻, IgM⁺, IgD⁻, CSP⁺), germinal center (GC) experienced MBCs (CD3⁻,
244 B220⁺, GL7⁺, CSP⁺), and class-switched MBCs (CD3⁻, B220⁺, CD38⁺, CD138⁻, IgM⁻, IgD⁻,
245 CSP⁺) (Figure 4 and S4). Both immunized animals and strain-matched placebo immunized
246 animals were analyzed.

247 Interestingly, *PyCSP*-immunized BALB/cJ mice exhibited significantly higher numbers of
248 splenic *PyCSP*-specific B-cells after the 2nd and 3rd immunizations (Figure 4A), indicating a
249 more robust anamnestic response than in vaccinated C57BL/6J mice. This difference was
250 especially apparent in week three, which follows the second immunization, where CSP-
251 specific B cells were nearly 3-fold higher. We did not detect differences in the frequency of
252 CSP⁺ IgM MBCs after either immunization (Figure 4B), although their frequency was
253 relatively low in all samples. However, *PyCSP*-immunized BALB/cJ had more than 3-fold
254 higher numbers of class-switched CSP⁺ MBCs at week three after the second immunization
255 and statistically higher numbers at week 7 (Figure 4C). Strikingly, BALB/cJ mice had
256 approximately 10-fold higher numbers of splenic GL7⁺, GC-experienced CSP-specific B cells
257 than C57BL/6J mice after the second immunization (week 3), and approximately 5-fold more
258 after the third immunization (week 7) (Figure 4D). Thus, except for IgM⁺ MBCs, BALB/cJ
259 mice exhibited comparatively higher *PyCSP*-specific B cell responses, GC activity, and class
260 switch in response to vaccination. As these timepoints measure the anamnestic response,
261 these findings also indicate marked differences in recall responses between the strains. The
262 relative paucity of GC-experienced and class switched MBCs in C57BL/6J mice is intriguing,
263 and likely explains the early differences in IgG titers and binding avidity observed after the
264 second immunization. Overall, the relative deficit GC activity and class switched MBCs may
265 contribute to the inability of vaccinated C57BL/6J mice to produce antibodies that can
266 mediate sterile protection from infection.

267 ***PyCSP*-immunized BALB/cJ mice achieve higher levels of somatic mutation in** 268 **response to vaccination**

269 It is possible that the differences in germinal center activity affect overall maturation and
270 somatic mutation within the CSP-specific B cell receptor (BCR) repertoire, which ultimately
271 drives the generation of high affinity antibodies after vaccination. To assess this, we sorted
272 class-switched CSP- reactive MBCs after either two or three immunizations with

273 recombinant PyCSP and sequenced their IgH heavy chain B cell receptor repertoire by
274 5'RACE next generation sequencing. Class-switched CSP-specific MBCs (CD3⁻, B220⁺,
275 IgM⁻, IgD⁻, CD38⁺, anti-CSP⁺) were stained and enriched as described above, and then
276 sorted into RLT lysis buffer by fluorescent activated cell sorting (FACS). Class switched
277 MBCs from unimmunized animals were also sorted to serve as a reference for sequence
278 analysis. The sorted, CSP-specific MBCs in mice within each strain were combined, and
279 then IgG transcripts were amplified by 5' Rapid amplification of cDNA ends (5'RACE) and
280 sequenced on an Illumina MiSeq instrument, allowing the retrieval of the entire V, D, J
281 rearrangement. The resulting sequences were processed in our in-house bioinformatics
282 pipeline for processing and V/D/J gene annotation and were analyzed to determine the
283 percent identity of each BCR from germline alleles (i.e., rates of somatic mutation) and third
284 complementarity determining region of antibody heavy chains (CDRH3) characteristics.
285 Higher percent identity to germline indicates less mutation, and conversely, lower identity
286 indicates higher rates of mutation. We annotated and estimated the percent sequence
287 identity to V-region germline of the CSP-specific BCR repertoire at weeks three and seven
288 (i.e., one week post two or three immunizations, respectively), as well as the total class
289 switched MBC IgG BCR repertoire from unimmunized animals (Figure 5). The CDRH3 size
290 distributions of the sequenced γ chain repertoires are shown in Figure S5.

291 The average CSP-specific MBC V-region identity after two immunizations in BALB/cJ mice
292 was 98.73%. After three immunizations, identity dropped significantly to an average of
293 97.15%, perhaps an indicator of higher levels of affinity maturation and somatic mutation
294 stimulated by the third immunization (Figure 5A). BCR sequences after three immunizations
295 were detected with as low as ~86% identity, and numerous sequences were identified with
296 less than 92.5% identity. Thus, IgG heavy chains with 7.5-14% somatic mutation were
297 generated in response to vaccination, although these were in the minority in the overall
298 repertoire. The identity to germline of CSP-specific MBCs was statistically higher after two
299 immunizations than the average identity of MBCs in unimmunized BALB/cJ mice (98.73%
300 vs. 98.18%), but lower after three immunizations (98.18% vs 97.15%). Thus, on average,
301 compared to the total MBC repertoire in unimmunized animals, CSP-specific MBCs had less
302 mutation after two immunizations, a possible consequence of BCR clonal selection, but
303 significantly more mutation after three immunizations, suggesting ongoing diversification and
304 affinity maturation.

305 In contrast, the average identity of CSP-specific IgG heavy chains in C57BL/6J mice was
306 99.49% after two immunizations and 98.33% after three (Figure 5B), with only very rare
307 outliers detected with more than 7.5% mutation. Thus, the CSP-specific IgG heavy chain

308 repertoire in C57BL/6J mice after two immunizations remained close to germline identity,
309 possibly related to the lack of GC experienced CSP-specific MBCs detected at this timepoint
310 (Figure 4D). Of note, the non-CSP MBC BCR repertoire in unimmunized mice was more
311 diverse in BALB/cJ mice than in C57BL/6J mice (98.13% vs 99.07), which may be indicative
312 of intrinsic underlying differences between the strains. Taken together, these findings
313 indicate that BALB/cJ mice appear to generate higher levels of somatic mutation in response
314 to vaccination with *PyCSP*. It follows that the higher level of mutation more commonly
315 generated in BALB/cJ mice could be a key factor in their ability to generate high affinity anti-
316 CSP antibodies and achieve sterile protection from infection.

317

318 **Discussion**

319 Defining the features of antibodies that mediate protection from pre-erythrocytic malaria
320 infection is a key milestone that will guide future vaccine development efforts. As RTS,S rolls
321 out more widely, and newer protein-based vaccines are developed, such as R21, it is
322 essential to understand the key characteristics of vaccine-elicited antibody responses that
323 mediate protection from liver stage infection. Here we studied vaccine responses in two
324 strains of mice, BALB/cJ and C57BL/6J, that differ in their sterile protection from infection in
325 response to vaccination with recombinant *PyCSP*. We studied several aspects of the B cell
326 response to vaccination and identified key differences that implicate B cell maturation as a
327 defining factor in the development of protective antibody responses. These findings have
328 direct implications for vaccine development, highlighting the urgent need for vaccine
329 formulations and regimens that drive potent, mature antibody responses.

330 Previous studies have established that binding affinity to CSP is a key factor in the ability of
331 monoclonal antibodies to neutralize infection (Epstein et al., 2011; Jongo et al., 2018;
332 Sissoko et al., 2017; Vijayan et al., 2021). High affinity antibodies are generated through the
333 process of affinity maturation. This occurs in the primary and secondary lymphoid tissues,
334 where antigen-reactive B cells proliferate and migrate into germinal centers. Once in
335 germinal centers, they undergo rounds of somatic hypermutation and clonal selection,
336 ultimately resulting in high affinity B cell clones that secrete antibodies. The effect of affinity
337 on neutralization is exemplified by the mAbs RAM1 and RAM2. RAM2 is protective, whereas
338 RAM1 is not, despite binding to the same peptide repeats. In this scenario, affinity is the
339 major factor driving the difference in neutralization; RAM2 binds with an affinity several
340 orders of magnitude higher than RAM1.

341 Thus, the significant lack of germinal center experienced B cells in C57BL/6J mice is striking,
342 as it indicates that this process is either impaired or happening far less frequently than in
343 BALB/cJ mice. These observations dovetail with our finding that less somatic hypermutation
344 from germline occurred in C57BL/6J mice within the CSP-specific class-switched MBC
345 compartment, indicating that less B cell maturation occurred after vaccination. In fact, the
346 CSP-specific BCR repertoire population average remained close to germline identity, even
347 after the anamnestic response of the second immunization. The natural consequence of less
348 B cell maturation is likely that fewer, less diverse high affinity BCR clones are generated
349 after vaccination, or that overall, the population may be less mutated, as we observed. This
350 is also evident in the lower polyclonal avidity after the second immunization, which may be a
351 significant underlying factor in the lack of antibody protection. Each of these observations
352 support the model where less efficient germinal center activity and class switch (and, in turn,

353 insufficient high-affinity CSP antibodies) in C57BL/6J mice leads to a lack of sterilizing
354 protection, whereas robust affinity maturation activity in BALB/cJ mice, resulting in a subset
355 of high-affinity NAbS like RAM2 and 2F6, leads to sterilizing protection (Figure 6).

356 The underlying immunological origins of reduced germinal center activity in C57BL/6J mice
357 remain unclear and could relate to potential differences in HLA or MHC alleles or subpar T
358 helper responses. However, vaccine protection from *P. berghei* rodent malaria infection has
359 been reported in C57BL/6J mice, especially with adjuvants that strongly drive B cell
360 development, such as Matrix M (Collins et al., 2021). Further, in this study passive transfer
361 of CSP-specific antibodies elicited in BALB/cJ mice achieved sterile protection in C57BL/6J
362 mice. This indicates that it is possible to elicit or passively transfer antibodies in C57BL/6J
363 mice that mediate sterile protection. As such, it is likely that our vaccine regimen was not
364 sufficient to elicit antibodies with the desired characteristics in C57BL/6J, despite eliciting
365 very high titers of anti-*Py*CSP antibodies. Therefore, it is not that C57BL/6J mice were
366 generally hypo-responsive to vaccination or overly sensitive to infection, but rather a deficit
367 in maturing the B response toward ideal neutralization characteristics likely underlies the
368 total lack of protection. Thus, although we do not know the underpinnings of the differential
369 antibody responses in C57/BL6J mice, comparative use of this model allowed the
370 identification of fundamental immunological processes that could be key parameters for
371 achieving durable sterilizing immunity.

372 It is intriguing that C57BL/6J mice lacked any measurable protection from infection,
373 despite statistically equivalent polyclonal antibody avidity indices in the samples taken just
374 prior to mosquito bite challenge (Figure 2E). The avidity index is based on chaotrope
375 disruption of binding in ELISA and is a coarse measure of the binding behavior of antibodies
376 as a population mixture (Alexander et al., 2015; Pullen et al., 1986). This measure is
377 extensively used as a surrogate measure of aggregate B cell maturation. However, this
378 measure can be influenced by nuances in epitope specificity and valency, and may under
379 measure more rare high affinity, highly functional antibodies (Alexander et al., 2015). Much
380 of the antibody response may not be relevant or effective for sterile protection, and it is likely
381 that only a subset of B cell clones will produce antibodies with the desired characteristics.
382 Given the equivalent IgG titers and avidity indices just prior to infection challenge, it is
383 possible that that the protection in BALB/cJ mice is mediated by relatively rare, highly
384 mutated, high affinity B cell clones, rather than through a large population of antibodies that
385 may be less mutated and lower affinity. To deconvolve this will likely require further
386 examination at single B cell level, where direct affinity/functional relationships can be
387 determined, as we have done for RAM1 and RAM2. Indeed, in studies of natural infection

388 and whole sporozoite vaccination, only a tiny fraction of antibodies obtained are functional
389 against the parasite, implying that potent functional antibodies are rare in the overall B cell
390 repertoire (Camponovo et al., 2020).

391 Eliciting durable, potent antibody responses by vaccination is a considerable
392 challenge for the field. RTS,S, which contains the potent adjuvant AS01, elicits high titers of
393 CSP antibodies, but titers and partial protection wanes quickly after vaccination (Dattoo et al.,
394 2021). R21, which is similar to RTS,S, is currently in testing with the potent adjuvant Matrix
395 M, which is hoped to be superior in eliciting high titers of antibody responses (Dattoo et al.,
396 2021). However, our findings imply that high titers of antibodies alone are not sufficient to
397 achieve potent, durable protection from infection. Rather, it is the quality of the antibodies in
398 the response that will determine whether protection can be achieved. A careful examination
399 of B cell responses, including the extent of their hypermutation and germinal center
400 experience, may be critical for extracting meaningful correlates of protection and, ultimately,
401 designing the most potent vaccine regimen against malaria. Novel approaches that
402 emphasize the elicitation of high affinity antibodies to neutralizing epitopes will likely be a
403 more fruitful path forward for vaccine development. Such a vaccine would dramatically
404 impact our ability to curtail malaria infection worldwide and could make a substantial impact
405 in the push towards eradication.

406 **Materials and Methods**

407 **Cloning and production of *PyCSP* and truncation constructs**

408 *P. yoelii* CSP, a 403-amino acid (aa) protein, consisting of an N- and C-terminal domains
409 connected by a central repeat domain (Figure 2B). The two predicted *N*-glycosylation sites
410 (S27A, T348A), one each in the N- and C-terminal domains of the native antigen were
411 mutated to prevent the addition of N-linked glycans in the mammalian expression system
412 and the non-glycosylated protein, *PyCSP* (38.3 kDa), is used in this study. The cartoon
413 representing the *PyCSP*, and different major and minor repeat peptides used in this work are
414 illustrated in Figure 2B and the construction and expression of the protein is published
415 elsewhere (Vijayan et al., 2021). The *PyCSP* antigen was codon optimized for human bias
416 and C-terminally 8X His and Avi-tagged, connected via a GS-linker to the antigen, to
417 facilitate purification and biotinylation. A tPA signal was added at the N-terminus to facilitate
418 protein secretion and the construct was cloned into pcDNA3.4 vector (Thermo Fisher,
419 Waltham, MA, USA) which drives transcription via a CMV promoter. After sequence
420 confirmation the plasmid DNA (500 ng/ml of cells) encoding the antigens were introduced
421 into HEK293 suspension cultures (1 million/ml) by high-density transfection using 2 mg/ml
422 polyethyleneimine (PEI) (1:5 Pei:plasmid DNA). Cells were grown in FreeStyle 293 serum-
423 free media (Thermo Fisher) for five days at 37°C 8% CO₂. Cells were spun down by
424 centrifugation (4000 rpm for 20 min at 4°C) and the supernatant was collected. Sodium azide
425 was added to a final concentration of 0.02% and NaCl was added to a final concentration of
426 500 mM. The antigens were purified by a two-step chromatography protocol. The proteins
427 were captured using Ni-NTA (Qiagen, Germantown, MD, USA), washed in EQ buffer (25 mM
428 Tris pH 8.0, 300 mM NaCl, 0.02% NaN₃), and eluted in EL buffer (25 mM Tris pH 8.0, 300
429 mM NaCl, 200 mM Imidazole, 0.02% NaN₃). The elution fractions were collected, pooled,
430 and concentrated and further purified on a standardized Superdex 200 16/600 (GE
431 Healthcare, Chicago, IL, USA) column running in HBS-E (10 mM HEPES, pH 7, 150 mM
432 NaCl, 2 mM EDTA). Peak fractions were pooled, concentrated, and stored at 4°C until use.

433 **Animal immunization**

434 All animal studies were conducted under protocols reviewed and approved by the
435 Institutional Animal Care and Use Committee (IACUC) at Seattle Children's Research
436 Institute. For regular immunization of 6-8 weeks old female BALB/cJ and C57BL/6J mice, 20
437 µg of *PyCSP* formulated in 20% adjuvax is administered intramuscularly at weeks 0, 2 and
438 6. Blood samples were collected at one week post 2nd and 3rd immunizations i.e., week 3 and
439 week 7, by submandibular or chin bleeds. Placebo-injected mice were used as experimental
440 control. For studying the B-cell responses and NGS of the antigen-specific memory B-cells,
441 mice were harvested at week 7 followed by splenocyte isolation. For pathogen challenge

442 experiments, all the mice were exposed to *P. yoelii* infected mosquito bite challenge (10-15
443 mosquitoes/mouse) in week 7 and followed for 2-3 weeks for blood-stage patency. For
444 serum antibodies swap passive transfer experiment, all the BALB/cJ mice were injected i.p.,
445 with 90 µg PyCSP-antibodies purified from PyCSP-immunized C57BL/6J mice and vice
446 versa followed by mosquito bite challenge (10-15 mosquitoes/mouse) 5 days post passive
447 transfer of antibodies. As a control, purified naïve BALB/cJ serum antibodies (90 µg) were
448 i.p., transferred to C57BL/6J mice and vice versa. All the mice were monitored for 2-3 weeks
449 for blood stage patency.

450 **ELISA**

451 Plasma antibody binding to PyCSP and to different truncation variants and peptides were
452 determined using a Streptavidin-capture ELISA. Mouse plasma samples were heat-
453 inactivated for 30 min at 56°C prior to all assays. All ELISA incubations were done for 1 h at
454 37°C and plates were washed between each ELISA step with PBS, 0.2% Tween-20. To
455 determine antibody binding to the ligand, Immulon 2HB 96-well plates (Thermo Scientific,
456 3455) were coated with 50 ng/well of Streptavidin (NEB, N7021S) in 0.1 M NaHCO₃, pH 9.5,
457 followed by blocking with 3% BSA in PBS. Later the plates were coated with 200 ng/well with
458 biotinylated ligand antigen or peptides followed by a blocking step 10% non-fat milk, 0.3%
459 Tween-20 in PBS. Later mouse plasma was serially diluted in duplicate over a range of
460 1:200 to 1:55,987,200 in PBS with 0.2% BSA. PyCSP mAb, 2F6, was serially diluted on
461 each plate to ensure intra-assay consistency. Bound antibodies were detected using goat
462 anti-mouse IgG Fc-HRP (Southern Biotech) or goat anti-mouse IgG1 Fc-HRP (Southern
463 Biotech) or goat anti-mouse IgG2a Fc-HRP (Southern Biotech) or goat anti-mouse IgG2b
464 Fc-HRP (Southern Biotech) at 1:2000 dilution in PBS with 0.2% BSA. Plates were developed
465 with 50 µl/well of TMB Peroxidase Substrate (SeraCare Life Sciences Inc, 5120-0083) and
466 stopped after 3 min with 50 µl/well of 1 N H₂SO₄. Absorbance at 450 nm was read using a
467 BioTek ELx800 microplate reader. Binding curves were generated by nonlinear regression
468 (log[agonist] vs response [three parameters]) in GraphPad Prism V8 (San Diego, CA).
469 Endpoint titers were defined as the reciprocal of plasma dilution at OD 0.1.

470 **Chaotrope Dissociation ELISA**

471 Antibody avidity of post-2nd and 3rd (Week 3 and week 7) immunization plasma samples to
472 PyCSP were determined using direct immobilization ELISA. Immulon 2HB 96-well plates
473 (Thermo Scientific, 3455) were coated with 50 ng/well of PyCSP overnight at room
474 temperature, then blocked the following day with PBS, 10% non-fat milk, 0.3% Tween-20 for
475 1 h at 37°C. Plates were washed between each step with PBS-containing 0.2% Tween-20.
476 Mouse plasma samples were heat-inactivated for 30 min at 56°C prior to all assays. Plasma

477 was diluted in PBS, 10% non-fat milk, 0.03% Tween-20 over a range of 1:50 to 1:9,331,200
478 and plated in quadruplicate side-by-side on the same plate. After a 1 h incubation with
479 antibodies, plates were washed, and half of the sample wells were treated with 2 M NH₄SCN
480 in PBS, while the other half was treated with PBS alone for 30 min at room temperature.
481 Plates were then washed and goat anti-mouse IgG Fc-HRP (Southern Biotech, 1013-05)
482 was added to the plate at 1:2000 in PBS, 10% non-fat milk, 0.03% Tween-20. Plates were
483 developed as described above. The avidity index was calculated as the ratio of AUC of
484 samples treated with 2 M NH₄SCN over AUC of samples treated with PBS: (AUC_{NH₄SCN} /
485 AUC_{PBS})×100.

486 **Monoclonal antibodies isolation and production**

487 The method of generation of PyCSP monoclonal antibodies were described earlier
488 (Carbonetti et al., 2017; Vijayan et al 2021). Briefly, six weeks old female BALB/cJ mice
489 were immunized with 20 µg of PyCSP adjuvanted with 20% v/v of Adjuvax at 0, 2 and 6
490 weeks followed by spleens harvest and splenocyte isolation at Week 7. Using EasySep
491 mouse B cell enrichment kit and manufacturer's protocol (StemCell Technologies Inc.,
492 Tukwila, WA, USA), B cells were isolated by negative selection and resuspended in FACS
493 buffer (PBS with 2% Fetal calf serum) followed by staining with anti-mouse CD16/CD32
494 (mouse Fc block; BD Biosciences) and a decoy tetramer (BV510) for 10 mins at room
495 temperature. Later the cells were stained with CD38-APC, IgM-FITC, IgD-AF700, B220-
496 PacBlue (BioLegend, San Diego, CA, USA) and PyCSP tetramer (BV786) for 30 mins at 4°C
497 followed by a wash with FACS buffer. Finally, the cells were resuspended in FACS buffer
498 and filtered by using a 30-micron filter followed by single-cell sorting using a BD FACS Aria II
499 with a 100-µm nozzle running at 20 psi. PyCSP-specific class switched memory B cells
500 (B220+ CD38+ IgM- IgD- antigen+ decoy- cells) were isolated and sorted as single cell per
501 well into 96-well PCR plates. For the cDNA generation and the consequent IgG and IgK
502 variable region amplification a previously described (von Boehmer et al., 2016) protocol was
503 followed and the PCR amplified final heavy and light chain sequences were cloned by
504 Gibson assembly into pcDNA3.4 expression vectors containing the murine IgG1 and IgK
505 constant regions, respectively. The IgG and IgK plasmid DNA sequences were verified for
506 the CDR3 sequences by Sanger sequencing and these plasmid pairs were diluted in PBS
507 followed by addition of polyethyleneimine (Polysciences, Warrington, PA). After 15 mins of
508 incubation at room temperature, the mixture was added to HEK293-F cells at a density of 1
509 million cells per milliliter cultured for 5 days in mammalian FreeStyle™ at 37°C, 5% CO₂ on
510 a shaker platform for mAb expression. The cells were separated from the supernatant by
511 centrifugation at 4000 rpm for 20 mins at room temperature and the cell-free supernatant
512 was passed over a HiTRAP MabSelect Sure column (GE Lifesciences #11003493) followed

513 by wash and elution steps performed as suggested by the manufacturer. The isolated mAbs
514 were buffer exchanged into HBS-E (10 mM HEPES, pH 7, 2 mM EDTA ,150 mM NaCl) and
515 the homogeneity and the size of the antibodies were analyzed by using an analytical
516 Superdex 200 10/300 column (GE Healthcare, Chicago, IL, USA).

517 **Tetramer production**

518 The following tetramers were made for antigen enrichment of splenocytes at an 8:1 ratio of
519 protein to SA-fluorophore, based on protein biotinylation efficiency. Bio-*PyCSP* protein (38.3
520 kDa) at 16 μ M was combined with SA-APC antibody (Biolegend) at 2 μ M. Bio-*PyS23* protein
521 (44.8 kDa) at 16 μ M was combined with SA-APC/Fire750 antibody (Biolegend) at 2 μ M and
522 was used as a decoy. To achieve optimal binding, an antibody was added to protein in two
523 additions with 20 min incubations at room temperature following each addition.

524 **Splenocyte isolation and Antigen-specific cell enrichment**

525 Splenocytes were isolated from fresh spleens by passing the tissue through a 70 μ m strainer
526 and rinsing with splenocyte buffer (1X phosphate buffered saline (PBS) supplemented with
527 2% fetal bovine serum (FBS), 100 μ g/mL DNase I (Sigma) and 5 mM MgCl₂. Collected cells
528 were spun down at 350 x g for 10 min and resuspended in the FACS buffer (1X PBS
529 supplemented with 2% FBS). Fc block (BD Biosciences) was added to each sample at a
530 1:100 dilution with decoy tetramer (Bio-*PyS23*, SA-APC/Fire750) and samples were
531 incubated for 10 min at RT. Then the *PyCSP* tetramer (Bio-*PyCSP*, SA-APC) was added,
532 and samples were incubated for 30 min at 4°C. After the incubation, cells were washed with
533 FACS buffer and spun at 300x g for 10 min at 4°C. Anti-APC microbeads (Miltenyi Biotec) in
534 FACS buffer were added and samples were incubated for 15 min at 4°C. After washing,
535 magnetic separation of cells was performed with LS columns (Miltenyi Biotec) on a
536 quadroMACS separator (Miltenyi Biotec). Cell suspensions were applied to a pre-separation
537 30 μ m filter on separate LS columns. Untetramerized cells without magnetic beads that
538 passed through the column were collected as flow through. Then the columns were removed
539 from the quadroMACS magnet and magnetically labeled cells were flushed out with the
540 FACS buffer and collected. Both the labeled and flow through fractions were spun at 300xg
541 for 10 min and resuspended in the FACS buffer.

542 **Antibody staining and flow cytometry**

543 The following antibody mixture was made and used to stain the flow through and labeled
544 fractions: B220-BV510 (Biolegend) at 1:20, CD38-FITC (Biolegend) at 1:100, IgM-BV786
545 (BD Biosciences) at 1:20, IgD-PerCPy5.5 (Biolegend) at 1:80, CD3-APC/Fire (Biolegend) at
546 1:40, GL7-e450 (ThermoFisher) at 1:40, and CD138-BV605 (BD Biosciences) at 1:80.
547 Samples were incubated on ice for 30 min, washed with FACS buffer and resuspended in

548 cold 1% paraformaldehyde (PFA) in PBS and stored overnight at 4°C until collection of
549 events on the cytometer the following day. For compensation controls, cryopreserved
550 splenocytes were removed from liquid nitrogen storage, quickly thawed at 37°C in water bath
551 for 1 min, washed in RPMI media with DNase (RPMI 1640, Corning +10% FBS + 1:100
552 Pen/strep + 2 mM L-glutamine +100 µg/mL DNase I + 5 mM MgCl₂), passed through a 70
553 µM cell strainer, washed again and finally resuspended in FACS buffer. Single stains
554 controls were made with the following antibodies: B220-BV510 (Biolegend) at 1:20, CD38-
555 FITC (Biolegend) at 1:100, IgM-BV786 (BD Biosciences) at 1:20, IgD-PercpCy5.5
556 (Biolegend) at 1:80, CD3-APC/Fire (Biolegend) at 1:40, B220-APC (Biolegend) at 1:50,
557 B220-e450 (ThermoFisher) at 1:33, and B220-BV605 (Biolegend) at 1:20. Samples were
558 incubated for 25 min at 4°C then washed with the FACS buffer and resuspended in cold 1%
559 PFA in PBS and stored at 4°C overnight. In the flowcytometry experiments to evaluate the
560 number of specific B-cell populations. 1% PFA was added to a small portion of unstained,
561 labeled and flow-through cells and reserved for cell count analysis. These samples were
562 incubated overnight at 4°C followed by the addition of Accucheck beads (ThermoFisher) the
563 following day and the events were collected on the cytometer. For quantification of specific
564 B-cells an LSR II flow cytometer (BD Biosciences) was used, 1.5-2.5 million events were
565 collected for each sample at a threshold of 15,000. A total of 50,000 events were collected
566 for cell count analysis. All the data were analyzed using FlowJo software (FlowJo LLC).

567 **Biolayer interferometry (BLI)**

568 Biolayer interferometry (BLI) is used to test the antigen, antibody interactions using an
569 Octet QK^e instrument (ForteBio Inc., Menlo Park, CA, USA). Streptavidin (SA)
570 biosensors (ForteBio Inc.,) were used to immobilize the biotinylated antigen or
571 peptide, followed by incubation in 10X Kinetics Buffer (PBS+0.1% BSA, 0.02% Tween-20
572 and 0.05% sodium azide). The antigen-derivatized probes were then dipped in indicated
573 concentrations of antibodies in 10X Kinetics Buffer, respectively, followed by an incubation
574 step in 10X Kinetics buffer to test the dissociation of the interactions. The
575 resulting sensograms from the association and dissociation phases were normalized to the
576 buffer values and analyzed by a global fit simple 1:1 binding model using the ForteBio data
577 analysis software (version 7.0.1.5). The K_D was determined from the estimated on- and off-
578 rates of the samples.

579 **Fab generation**

580 The RAM2 Fabs were generated by papain digestion and purified using Immobilized papain
581 agarose resin (Thermofisher Scientific) following manufacturer's protocol. Briefly 1 mg of
582 RAM2 mAb in 400 µl of sample buffer (20 mM Sodium phosphate pH 7.0, 10 mM EDTA) is

583 mixed with 400 µl immobilized papain resin in digestion buffer (20 mM Sodium phosphate
584 pH 7.0, 10 mM EDTA, 20 mM Cysteine.HCl) and incubated overnight on a rotatory shaker at
585 37°C. Later 1.5 ml of Tris-HCl, pH 7.5 is added to the mixture followed by centrifugation at
586 2000 x g for one minute at room temperature. The supernatant containing Fabs is separated
587 from the immobilized agarose resin and is passed through a 0.22 µM spin column. Finally,
588 the Fab fragments were isolated by negative selection on a HiTrap MabSelect SuRe
589 cartridge with 10 column volumes of PBS, pH 7.4 and concentrated on spin column to
590 desired concentration.

591 **Generation of infected *Anopheles stephensi* mosquitoes**

592 *P. yoelii* wild type strain 17XNL (BEI resources) was maintained in Swiss Webster (Envigo,
593 SW) mice. Swiss Webster mice were injected intraperitoneal (i.p.) with 250 µL of infected
594 blood at 3-5% parasitemia. Gametocyte exflagellation rate was checked 4 days post-
595 injection. Mice were then anesthetized with 150 µL Ketamine Xylazine solution (12.5 mg/mL
596 ketamine, 1.25 mg/mL xylazine in PBS) and naïve female *Anopheles stephensi* mosquitoes
597 (3-7 days old) were allowed to feed on them. Mosquitos were maintained at 23°C, 80%
598 humidity with a 12L-12D light cycle. On day ten, the midguts from ten mosquitoes were
599 dissected and the number of oocysts was counted as a measure of infection. On day 15
600 post-infection, the salivary glands of the mosquitoes were dissected to extract sporozoites.

601 **Mosquito Bite Challenge**

602 Mice were anesthetized by IP injection of 150 µL Ketamine Xylazine solution (12.5 mg/mL
603 ketamine, 1.25 mg/mL xylazine in PBS). Once anesthetized pairs of mice were each placed
604 on a carton of 15 infectious mosquitoes and mosquitoes were allowed to bite through the
605 mesh top for 10 min. Every 30 sec mice were rotated among mosquito cartons to ensure
606 equal exposure of mice and to maximize mosquito probing thereby sporozoite transfer. Mice
607 received subcutaneous PBS injections and recovered from anesthesia under a heat lamp.
608 Mice were checked for the presence of blood-stage parasites (patency) beginning four days
609 post-challenge.

610 **Assessment of blood stage patency**

611 Patency was checked daily by blood smear 4 days post-infection by tail snip. Slides were
612 fixed in methanol, dried, and then stained with giemsa (1:5 in H₂O) for 10 min and viewed at
613 100X oil immersion on a compound microscope and twenty fields of view examined for each
614 smear. Mice were considered patent if 2 or more ring stage parasites were observed.

615 **Sporozoite quantification**

616 To quantify the number of sporozoites per mosquito, 10-12 mosquitoes' salivary glands were
617 hand dissected. These glands were then ground with a pestle and spun at 800 rpm to pellet
618 debris. Sporozoites were counted on a hemocytometer.

619 **Invasion and traversal assay**

620 Freshly isolated sporozoites were exposed to 2F6, RAM2, RAM1 or serum antibodies from
621 PyCSP immunized BALB/cJ and C57BL/6J mice for 10 mins at indicated concentrations.
622 5×10^5 Hepa1-6 cells were seeded in each well of a 24-well plate (Corning) and infected with
623 antibody exposed *P. yoelii* sporozoites at a multiplicity of infection (MOI)=0.25 for 90 mins.
624 Cells were co-incubated with high molecular mass Dextran-FITC (70 kDa) (Sigma). After 90
625 mins of infection, cells were harvested with accutase (Life Technologies) and fixed with
626 Cytoperm/Cytofix (BD Biosciences). Cells were blocked with Perm/Wash (BD Biosciences) +
627 2% BSA for 1 h at room temperature then stained overnight at 4°C with antibodies to CSP-
628 Alexafluor 488 conjugate. The cells were then washed and resuspended in PBS-containing 5
629 mM EDTA. Infection rate was measured by flow cytometry (Douglass et al., 2015) on an
630 LSRII (Becton-Dickinson) and analyzed by FlowJo (Tree Star).

631 **Immunofluorescence**

632 Freshly isolated sporozoites were exposed to 2F6, RAM2, RAM1 or serum antibodies from
633 PyCSP immunized BALB/cJ and C57BL/6J mice for 10 mins at indicated concentrations.
634 Sporozoites were spun at 17000g for 5 mins and washed with 1xPBS-EDTA and fixed with
635 3.7% PFA for 20 mins. Sporozoites were transferred to 8-well chambered slides and the
636 images were acquired with a 100x1.4 NA objective (Olympus) on a DeltaVision Elite High-
637 Resolution Microscope (GE Healthcare Life Sciences). The sides of each pixel represent
638 64.5x64.5 nm and z-stacks were acquired at 300 nm intervals. Approximately 5-15 slices
639 were acquired per image stack. For deconvolution, the 3D data sets were processed to
640 remove noise and reassign blur by an iterative Classic Maximum Likelihood Estimation
641 widefield algorithm provided by Huygens Professional Software (Scientific Volume Imaging,
642 The Netherlands).

643 **Next-generation sequencing (NGS)**

644 CSP-specific MBCs were sorted into RLT buffer supplemented with 2-mercaptoethanol and
645 then IgG gamma chain was amplified by 5' Rapid amplification of cDNA ends (5'RACE)
646 followed by sequencing on an Illumina MiSeq instrument generating the entire V, D, J
647 rearrangements (Doepker et al., 2020; Simonich et al., 2019; Vigdorovich et al., 2016).
648 Briefly, cell lysate was homogenized using QIAshredder columns (Qiagen, #79654). RNA
649 was extracted from homogenized lysate containing a range of 3×10^3 to 1×10^5 cells per
650 sample using a RNeasy Mini kit (Qiagen, #80204). RNA was purified and

651 concentrated using RNAClean XP beads (Beckman Coulter, #A63987). The concentration
652 and quality of the RNA was determined by an Agilent 2100 Bioanalyzer with the Agilent RNA
653 6000 Pico Kit (Agilent Technologies, #5067-1513). The oligonucleotides used for the NGS
654 were tabulated (Table 1). Up to 1 µg of RNA was mixed with a gamma chain reverse primer
655 (vv-534- TGCATTTGAACTCCTTGCC) and incubated at 72°C for 3 min to denature the
656 RNA, then cooled to 42°C to anneal the synthesis primer. cDNA was generated by mixing 5x
657 First-Strand Buffer, DTT (20 mM), dNTP mix (ThermoFisher, #18427-013, 10 mM), a
658 template switch adaptor with UMI tag (vv-877-
659 AAGCAGUGGTAUCAACGCAGAGNNNNUNNNNUNNNNUNNNNUCTTGrGrG),
660 Recombinant RNase Inhibitor (Takara Bio/Clontech, #2313A, 40 U/ul), and SMARTScribe
661 RT (Takara Bio/Clontech, #639536, 100 U/ul) with the denatured RNA and incubating for 90
662 min at 42°C then heating to 70°C for 10 min. Uracil-DNA glycosylase (NEB, #M0280S, 5
663 U/ul) was added and the reaction was incubated for 1 h at 37°C. RNAClean XP beads were
664 used to purify and concentrate the reaction. A polymerase chain reaction (PCR) was
665 performed to generate variable libraries using cDNA with primers vv-869-
666 AAGCAGTGGTATCAACGCAG, vv-870-KKACAGTCACTGAGCTGCT, vv-872-
667 TACAGTCACCAAGCTGCT, and Q5 2x Master Mix (NEB, #M0492S). The reaction was
668 incubated for 30 sec at 98°C, then cycled 18 times at 98°C for 10 sec, 63°C for 30 sec, and
669 72°C for 30 sec, and finally incubated at 72°C for 5 min. A cleanup step was performed with
670 SPRI beads (Beckman Coulter, #B23318). The second PCR was performed with primers
671 vv873-CACTCTATCCGACAAGCAGTGGTATCAACG, vv874-GGGCCAGTGGATAGAC,
672 vv876-GGGACCAAGGGATAGAC and Q5 Master Mix. The reaction was incubated using
673 the same conditions as the first PCR, but with an annealing temperature of 60°C and only 8-
674 15 amplification cycles. Another clean up step was performed with SPRI beads. After the
675 second PCR, the NEB Library Prep protocol of the manufacturer was followed and samples
676 were adaptor ligated using the NEBNext End Prep kit (NEB, #E7645). The adaptor-ligated
677 DNA fragments were indexed with the NEBNext Multiplex Oligos for Illumina kit (NEB,
678 E7335). Adapted and indexed libraries were quantified using a KAPA Library Quantification
679 Kit (Roche, KK4873) on a QuantStudio3 Real Time PCR System (ThermoFisher, #A28567).
680 Quantified libraries were pooled to a concentration of 4 nM and denatured following the
681 MiSeq Guide (2). Libraries were denatured in 0.2 N NaOH and diluted to 20 pM. As a
682 sequencing control, PhiX Control V3 (Illumina, #FC-110-3001) was denatured and diluted to
683 20 pM and was added to the pooled library sample at a 1% spike in. The pooled library
684 sample plus PhiX was sequenced using the MiSeq Reagent Kit v3 (Illumina, #MS-102-3003)
685 on the Illumina MiSeq system.

686 Raw Illumina MySeq reads were processed using an approach similar to the one previously
687 described (Vigdorovich et al., 2016) with several modifications to utilize unique molecular
688 identifier (UMI)-based error correction (Turchaninova et al., 2016). Briefly, following the
689 amplicon reconstruction and oligonucleotide trimming, UMI sequences were identified and
690 used to collect sequences into molecular identifier groups (MIGs), representing PCR-
691 amplified mRNA molecules. Consensus sequence for each MIG was then determined using
692 the approach adapted from the MIGEC pipeline (Shugay et al., 2014), in which the MIG is
693 first represented by a position frequency matrix, followed by base calling and calculation of a
694 cumulative quality score for each position. Resulting sequence sets were annotated using
695 IgBLAST (version 1.11.0) (Ye et al., 2013) against a custom database of mouse germline
696 sequences obtained from the IMGT/GENE-DB collection (Giudicelli et al., 2005)
697 (www.imgt.org) to determine segment boundaries (e.g., to define CDR3 regions), identify
698 closest germline matches and derive sequence-identity-to-germline values. In order to
699 eliminate multiple identical transcripts likely originating from the same B cell, sequence set
700 deduplication was carried out using VSEARCH (Rognes et al., 2016) (version 2.9.1) at 100%
701 sequence identity. In the finalized deduplicated data sets, only sequences that were
702 supported by ≥ 10 raw reads were used in further analysis. The visualizations were
703 generated using the ggstatsplot (version 0.7.2) R package.

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709 **Author Contributions**

710 GRRV, KV, RC, OT, SLW, VV, AY, AR, AW, WS, MZ, and ND conducted experiments and
711 analyzed data. RC, GRRV, KV, MZ, AK, and DNS developed the experimental plan and
712 designed experiments. GRRV, KV, AK, and DNS wrote the manuscript, and all authors
713 edited initial drafts. AK and DNS conceived of the study and obtained funding.

714

715 **Figure Legends**

716 **Figure 1. The *PyCSP*-immunization in BALB/cJ and C57BL/6J mice delays blood stage**
717 **patency and confers protection only in the prior. A.** The immunization and challenge
718 regimen are illustrated. 6-8 weeks old female BALB/cJ (n=10) and C57BL/6J (n=10) mice
719 were intramuscularly immunized with 20 µg of *PyCSP* formulated with 20% adjuvax on
720 weeks 0, 2 and 6 (arrows) followed by *Py* sporozoites-bearing mosquito bite challenge (15
721 mosquitoes/mouse) on week 7. Post challenge the blood smears were collected from the tail
722 vein for up to 3 weeks to monitor blood-stage patency by Giemsa stain. Submandibular
723 bleeds were performed in Week 3 and on week 7 (before challenge). **B.** BALB/cJ and
724 C57BL/6J were immunized with *PyCSP* at 0, 2 and 6 weeks. A week post final boost, mice
725 were challenged with *P. yoelii* infected mosquitoes and patency was assessed from day 3
726 through day 21. The Kaplan-Meier Survival plots represent percentage parasitemia free mice
727 over time. Data are from 10 mice /condition across 2 independent experiments. The control,
728 *PyCSP*-immunized BALB/cJ and C57BL/6J mice are indicated in green, orange, blue and
729 red, respectively. The numeric indicates that 6 out of 10 *PyCSP*-immunized BALB/cJ mice
730 were parasitemia free. **C.** Representative immunofluorescent images of *P. yoelii* sporozoites
731 incubated with purified serum antibodies from post week 7- *PyCSP*-immunized BALB/cJ and
732 C57BL/6J mice for 10 min. *PyCSP* monoclonal antibody, 2F6, is used as control for CSP
733 trailing assay and DAPI is used to stain the nucleus of the sporozoites. The arrowhead
734 points to the regions of CSP reaction or shedding (CSPR). **(D and E)** Freshly isolated
735 sporozoites were pre-incubated with *PyCSP*-immunized BALB/cJ and C57BL/6J serum
736 antibodies were incubated with *Py* sporozoites (1:1 dilution) for 20 mins. Normal mouse
737 serum (NMS) is used as infection control for *in vitro* assays. Hepa 1-6 cells were infected
738 with antibody-treated sporozoites for 90 min to assess hepatocyte entry. The bar graph here
739 represents the percentage of hepatocytes that were CSP-positive as evaluated by flow
740 cytometry **(D)**. Antibody-treated sporozoites were exposed to HFF-1 cells in the presence of
741 Dextran-FITC for 30 mins. The bar graph represents the percentage of cells traversed as
742 assessed by dextran positive cells **(E)**. For **(D)** and **(E)**, data represents mean values ± SE
743 from three independent experiments: n=3. p values were determined by comparing each
744 treatment to untreated using one-way ANOVA for multiple comparisons tests. **F.** Polyclonal
745 antibodies from *PyCSP*-immunized BALB/cJ and C57BL/6J mice sera were purified and
746 *PyCSP*-specific antibodies were quantified by ELISA. Naïve BALB/cJ (n=5) and C57BL/6J
747 (n=9) were passively transferred (i.p.) with 90 µg of *PyCSP* antibodies from *PyCSP*-
748 immunized C57BL/6J (blue) and BALB/cJ (red) mice, respectively. Post five days of antibody
749 passive transfer mice were challenged with mosquito-bites from 15 *Py*-infected mosquitoes
750 and the blood stage patency is monitored for 2 weeks. Naïve BALB/cJ (n=5) and C57BL/6J

751 (n=5) that were passively transferred with purified polyclonal antibodies from naïve
752 C57BL/6J (light blue) and BALB/cJ (orange) mice, respectively were used as controls. Data
753 analyzed by Two-way ANOVA and p values were obtained by Tukey's multiple comparison
754 test. *** $p < 0.0004$; ** $p < 0.005$; * $p < 0.0005$; ns- not significant.

755 **Figure 2. Comparison of antibody responses after immunization of PyCSP at week 3**
756 **and 7 time points in BALB/cJ and C57BL/6J mice. A.** 6-8 weeks old female BALB/cJ
757 (n=5) (blue) and C57BL/6J (n=5) (red) mice were intramuscularly immunized with 20 μ g of
758 PyCSP with 20% adjuvax on weeks 0, 2 and 6 and the blood samples were collected on
759 week 3 and week 7. ELISA was performed to analyze the PyCSP-specific total IgG titers. **B.**
760 Cartoon representing the domain organization of mature PyCSP ectodomain. The N- and C-
761 terminal domains are represented as NT and CT, respectively. The TSR (RII) domain is in
762 CT. The major and minor repeats of the repeat domain are colored blue and green,
763 respectively and their respective amino acid repeat sequences are illustrated. The QEPP
764 amino acid sequence connecting the major and minor repeat regions is indicated in red. The
765 binding epitopes of anti-PyCSP mAbs RAM1, RAM2 and 2F6 are indicated with arrows. **C**
766 and **D.** PyCSP-specific IgG titers of PyCSP-immunized BALB/cJ (blue) and C57BL/6J (red)
767 mice to the major and minor repeats at Week 3 (**2C**) and Week 7 (**2D**) were analyzed. **E.**
768 The avidity of the PyCSP-immunization elicited antibodies to the PyCSP antigen at weeks 3
769 and 7 in BALB/cJ (blue) and C57BL/6J (red) mice were analyzed. Avidity index is calculated
770 by (AUC of NH₄SCN wells)/ (AUC of PBS wells)*100. Data analyzed by Two-way ANOVA
771 and p values were obtained by Tukey's multiple comparison test. **** $p < 0.0001$; *** $p < 0.0004$
772 ** $p < 0.005$; ns- not significant.

773 **Figure 3. PyCSP mAb RAM2 avidly binds to major repeat regions of PyCSP and**
774 **provides sterile liver stage protection from the pathogen. A.** ELISA showing the binding
775 of RAM2 to the PyCSP full length protein (black), the major repeat peptide (dark green) but
776 not to the minor repeat peptide (light green). **B.** ELISA showing the EC₅₀ of RAM2 (orange)
777 and 2F6 (pink) binding to PyCSP full length protein **C.** Biotinylated-PyCSP (5 μ g) derivatized
778 streptavidin biosensors were incubated in different mAbs (RAM1 (cyan), RAM2 (orange) 2F6
779 (pink) and 50C1 (black), 5 μ g each) and the association and dissociation kinetics were
780 assayed by Octet-BLI **D.** Biotinylated-major repeat peptide (5 μ g) loaded streptavidin
781 biosensors were dipped in 5 μ g each of mAbs (RAM1-cyan, RAM2-orange, 2F6-pink and
782 50C1-black) and the association and dissociation kinetics were assayed by Octet-BLI. **E.**
783 Representative immunofluorescent images of *P. yoelii* sporozoites incubated with mAb RAM2
784 (10 μ g) for 10 min. PyCSP mAb, 2F6, is used as control for CSP trailing assay and DAPI is
785 used to stain the nucleus of the sporozoites. The arrowhead points to the regions of CSP

786 reaction or shedding (CSPR). **F.** Freshly isolated sporozoites were pre-incubated with (10 µg)
787 of RAM2 (orange), 2F6 (pink) and normal mouse serum (black) antibodies for 10 mins. Hepa
788 1-6 cells were infected with antibody-treated sporozoites for 90 min to assess hepatocyte
789 entry. The bar graph here represents the percentage of hepatocytes that were CSP-positive
790 as evaluated by flow cytometry. The data represents mean values ± SE from three
791 independent experiments: n=3. p values were determined by comparing each treatment to
792 untreated using one-way ANOVA for multiple comparisons tests. **G.** BALB/cJ were injected
793 with 150 µg of RAM2 (orange) or 2F6 (pink) intraperitoneally. Naïve BALB/cJ (black) mice
794 injected with PBS is used as control. After 24 h, mice were challenged with bites from fifteen
795 *P. yoelii* infected mosquitoes and patency was assessed from day 4 through day 14. Kaplan-
796 Meier survival plot represents percentage parasitemia free mice over time, including 10 mice
797 from 2 independent experiments.

798 **Figure 4. PyCSP-specific B-cell responses of BALB/cJ and C57BL/6J mice at weeks 3**
799 **and 7.** 6-8 weeks old female BALB/cJ (n=5) and C57BL/6J (n=5) mice were immunized
800 intramuscularly with 20 µg of PyCSP adjuvanted with 20% adjuvax on weeks 0, 2 (Week 3)
801 or weeks 0, 2 and 6 (week 7) and the splenocytes were isolated either on week 3 or week 7
802 as described in the materials and methods. Cartoon representing the general workflow in B-
803 cell population analysis is illustrated (Figure S4). B-cells responses of different B-cell
804 populations per spleen were quantified for PyCSP-specific B-cells (**A**), IgM memory B-cells
805 (MBCs) (**B**), class-switched (sw) Ig+MBCs (**C**), and Germinal center (GC) B-cells (**D**). Data
806 analyzed by Two-way ANOVA and p values were obtained by Tukey's multiple comparison
807 test. **** $p < 0.0001$.

808 **Figure 5. PyCSP-immunized BALB/cJ mice employ more highly diverse V-gene**
809 **rearrangements than C57BL/6J.** Somatic hypermutations in the V-gene of IgG heavy
810 chains of PyCSP-specific mouse memory B-cells at Week 3 (orange, post 2nd) and 7 (purple,
811 post 3rd) performed by high throughput cell sorting and NGS in BALB/cJ (**A**) and C57BL/6J
812 (**B**) mice. The unimmunized mice (green) in a similar age group of the week 7 vaccinated
813 mice were used as controls. The results are representative of three independent
814 experiments (n=5/group). Each data point represents a sequence containing deduplicated
815 members (i.e., 100% identity) and supported by at least 10 raw reads. The visualizations
816 were analyzed and generated using the ggstatsplot (version 0.7.2) R package.

817 **Figure S1. A.** Graphics showing the statistical significance of PyCSP-immunized BALB/cJ
818 (blue), C57BL/6J (red) and their respective placebo controls in green and yellow. **B.**
819 Statistics showing the significance of the delay in blood stage patency in swapped PyCSP-
820 pAbs (90 µg)-infused BALB/cJ (blue), C57BL/6J (red) and their respective placebo controls

821 in green and yellow. The days to patency are measured and the number of mice that are
822 sterile protected in the *PyCSP*-immunized BALB/cJ mice **(A)** and *PyCSP*-immunized
823 BALB/cJ mice pAbs infused naïve C57BL/6J mice **(B)** were indicated. Data analyzed by
824 Two-way ANOVA and p values were obtained by Tukey's multiple comparison test. ***
825 $p < 0.0004$; ** $p < 0.005$; * $p < 0.0005$; ns- not significant

826 **Figure S2. IgG subclass differences between the *PyCSP*-immunized BALB/cJ and**
827 **C57BL/6J mice.** The plasma IgG1, IgG2a (only for BALB/cJ) and IgG2b antibody responses
828 of *PyCSP*-immunized BALB/cJ (black circles) and C57BL/6J (black squares) mice at week 7
829 (pre-challenge) were measured by ELISA using biotinylated *PyCSP* as ligand. Data
830 analyzed by Two-way ANOVA and p values were obtained by Sidak's multiple comparisons
831 test. **** $p < 0.0001$

832 **Figure S3. RAM2 Fab association and dissociation kinetics.** Streptavidin biosensors
833 loaded with *N*-terminally biotinylated-major repeat peptides were incubated in different
834 concentrations of RAM2 Fab ranging from 7500 nM to 625 nM and the association and
835 dissociation kinetics were analyzed. The resulting association and dissociation sensorgrams
836 were analyzed by a global fit 1:1 binding model using the ForteBio data analysis software
837 (version 7.0.1.5) generating K_D as estimated from the on- and off-rates.

838 **Figure S4.** Summary of the workflow showing the different steps involved in the
839 quantification of CSP-specific B-cell responses as described in the Materials and Methods
840 section.

841 **Figure S5.** The CDRH3 amino acids (aa) lengths and density of **(A)** *PyCSP*-specific MBCs γ
842 chain sequences from naïve animals (lavender), and after two (peach) or three (green)
843 immunizations in BALB/cJ mice, and **(B)** from C57BL/6J mice. The results are representative
844 of three independent experiments (n=5/group).

845 **Table 1. Oligonucleotides used in the NGS experiment**

Primer Name	Sequence
vv-534	TGCATTTGAACTCCTTGCC
vv-877	AAGCAGUGGTAUCAACGCAGAGNNNUNNNUNNNUNNNUNNNUNNNUCTTrGrGrG
vv-869	AAGCAGTGGTATCAACGCAG
vv-870	KKACAGTCACTGAGCTGCT
vv-872	TACAGTACCAAGCTGCT
vv-873	CACTCTATCCGACAAGCAGTGGTATCAACG
vv-874	GGGCCAGTGGATAGAC
vv-876	GGGACCAAGGGATAGAC

846

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984

Figure 1

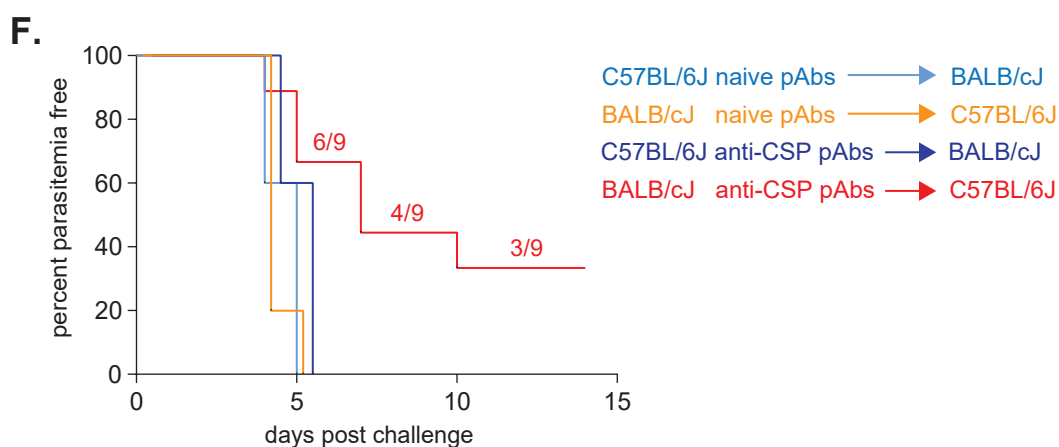
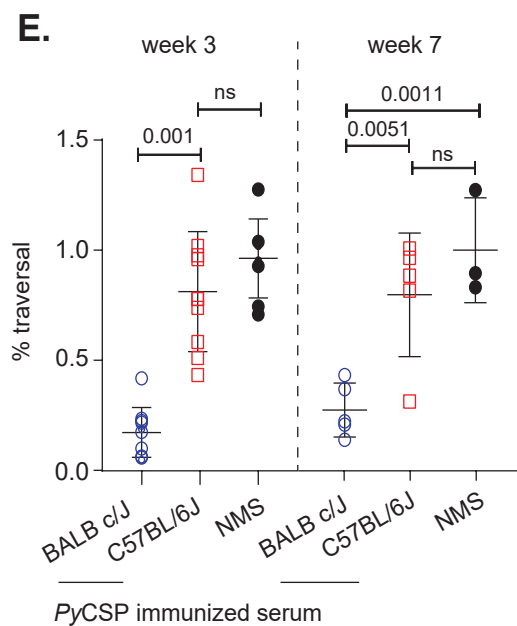
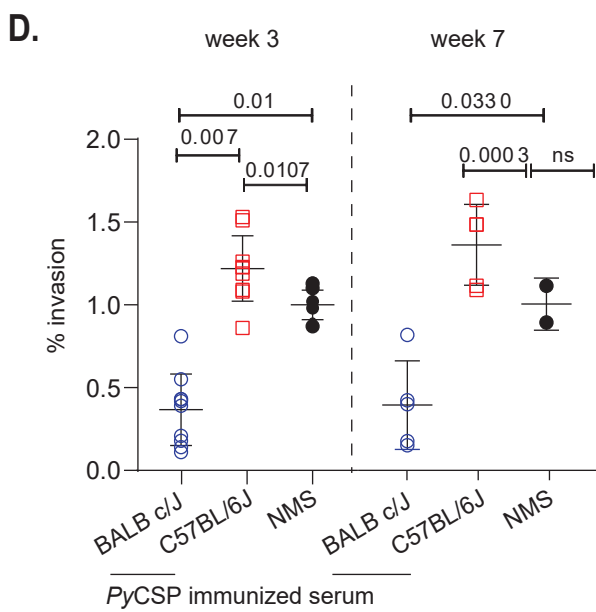
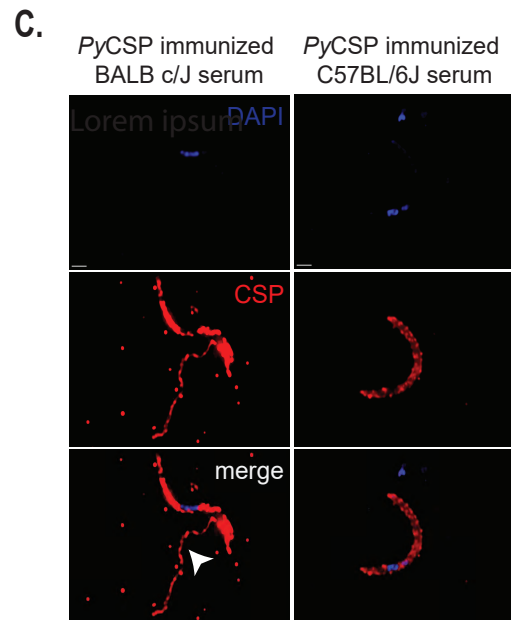
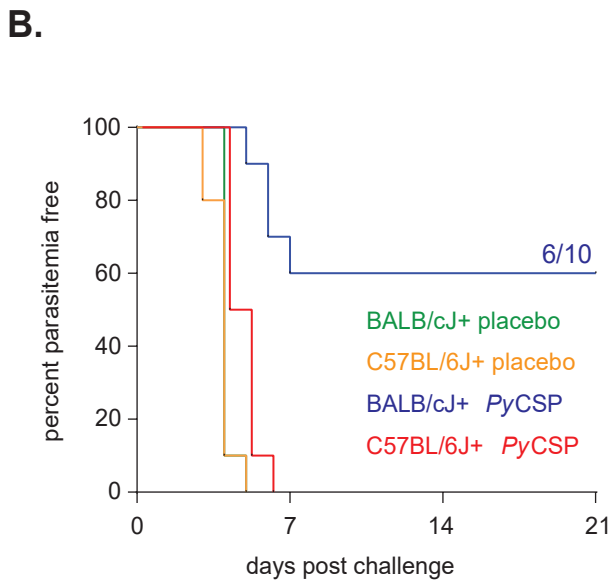
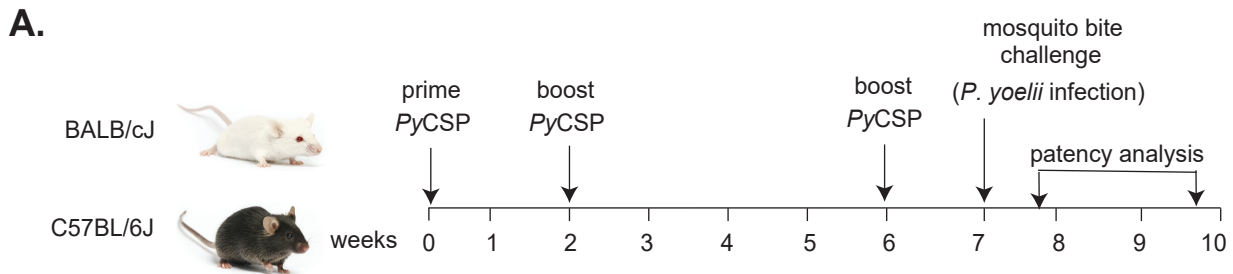
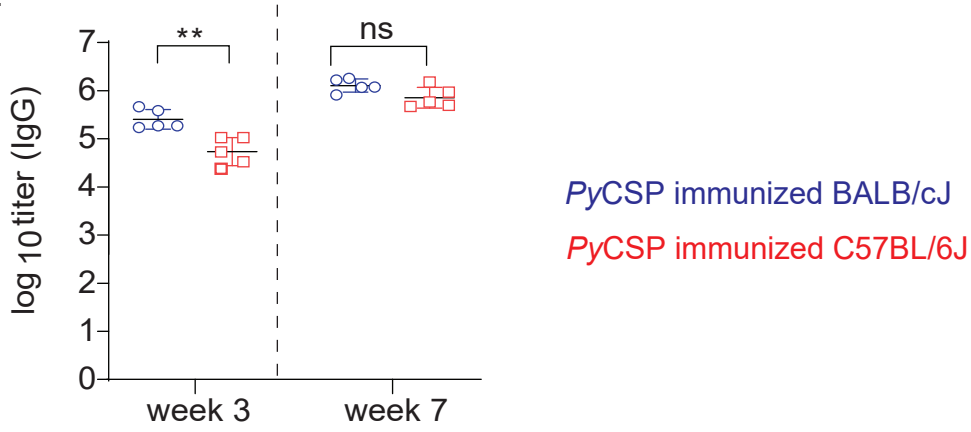
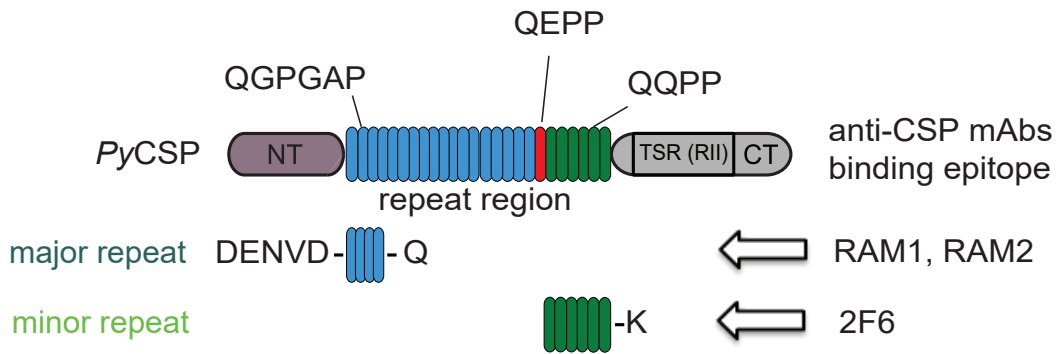


Figure 2.

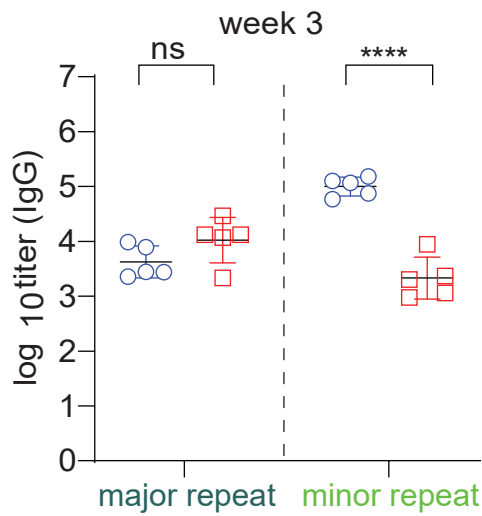
A.



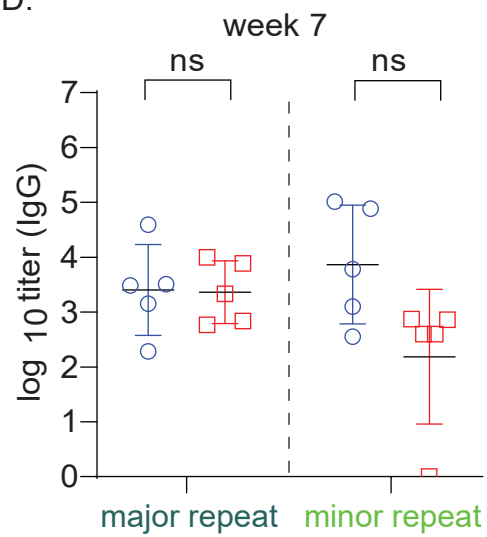
B.



C.



D.



E.

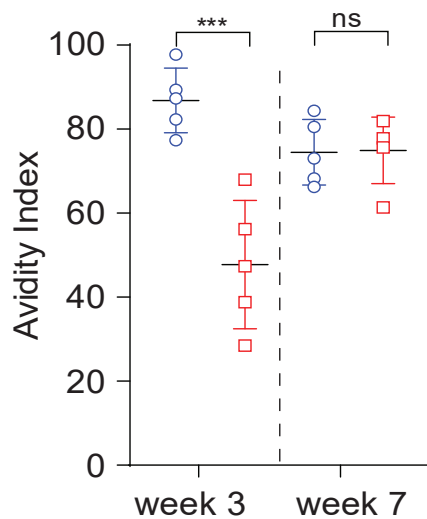


Figure 3

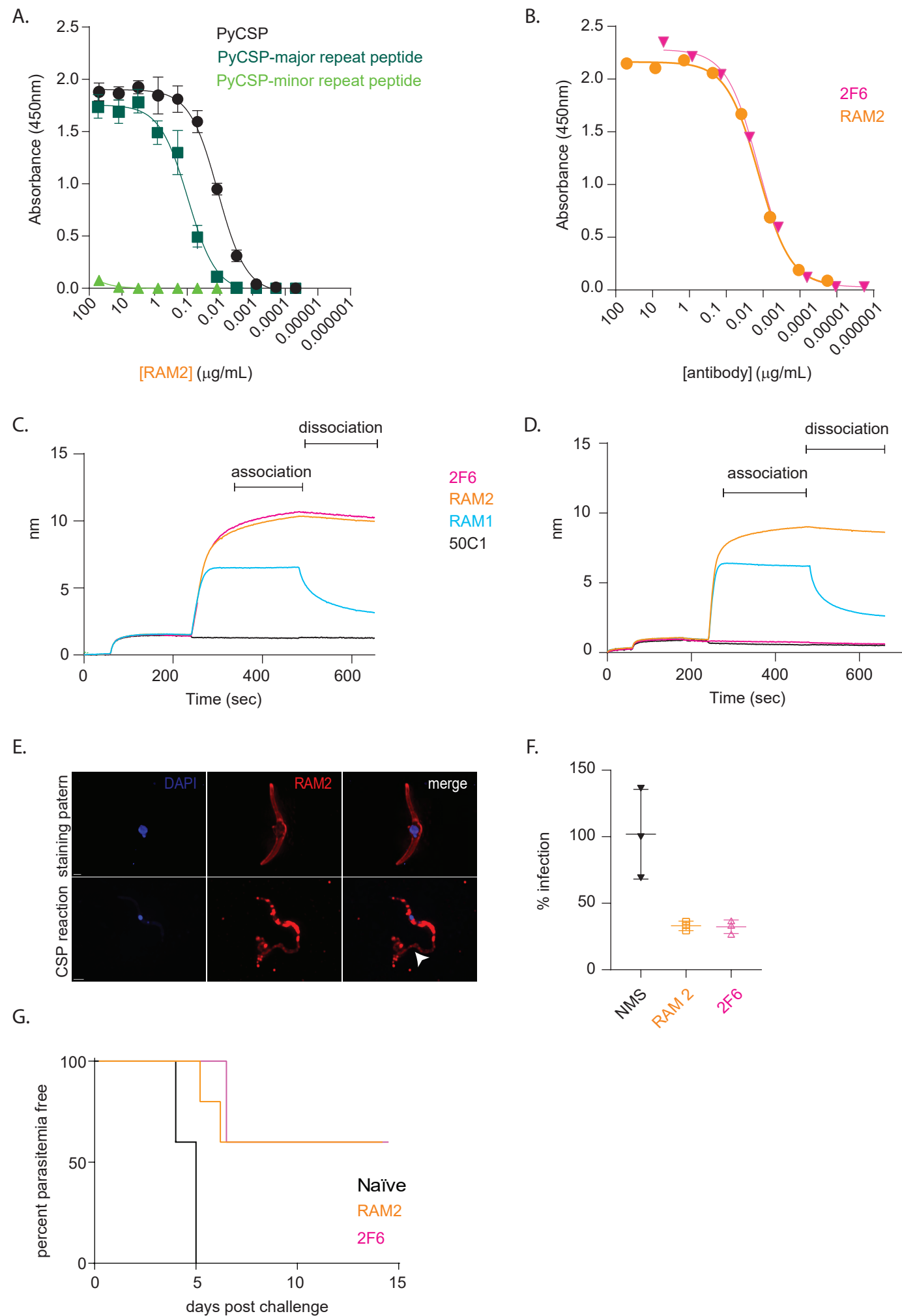


Figure 4.

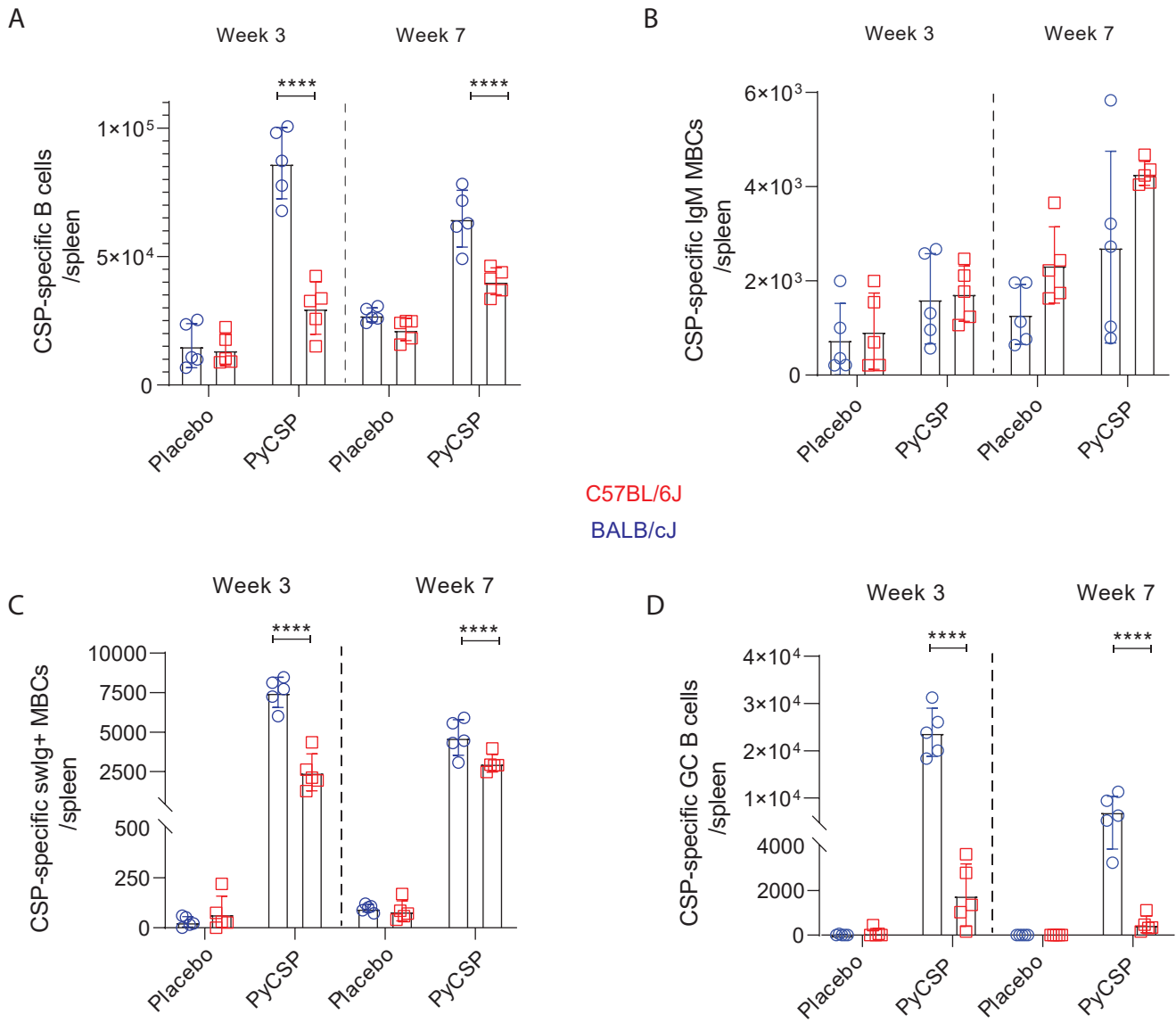
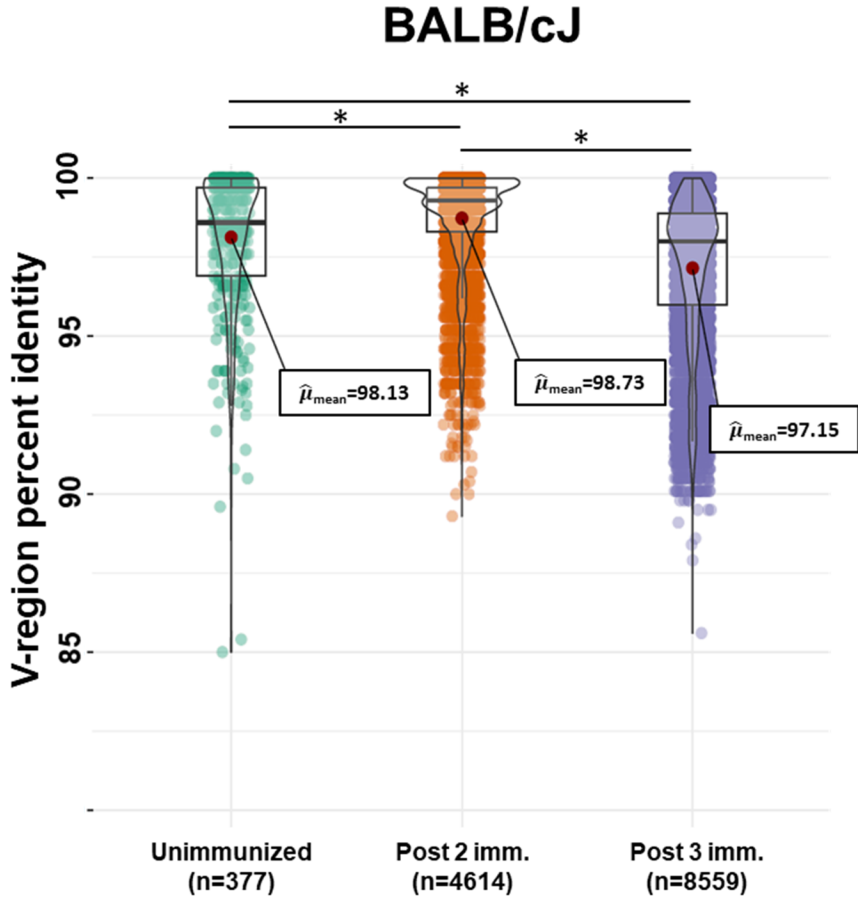
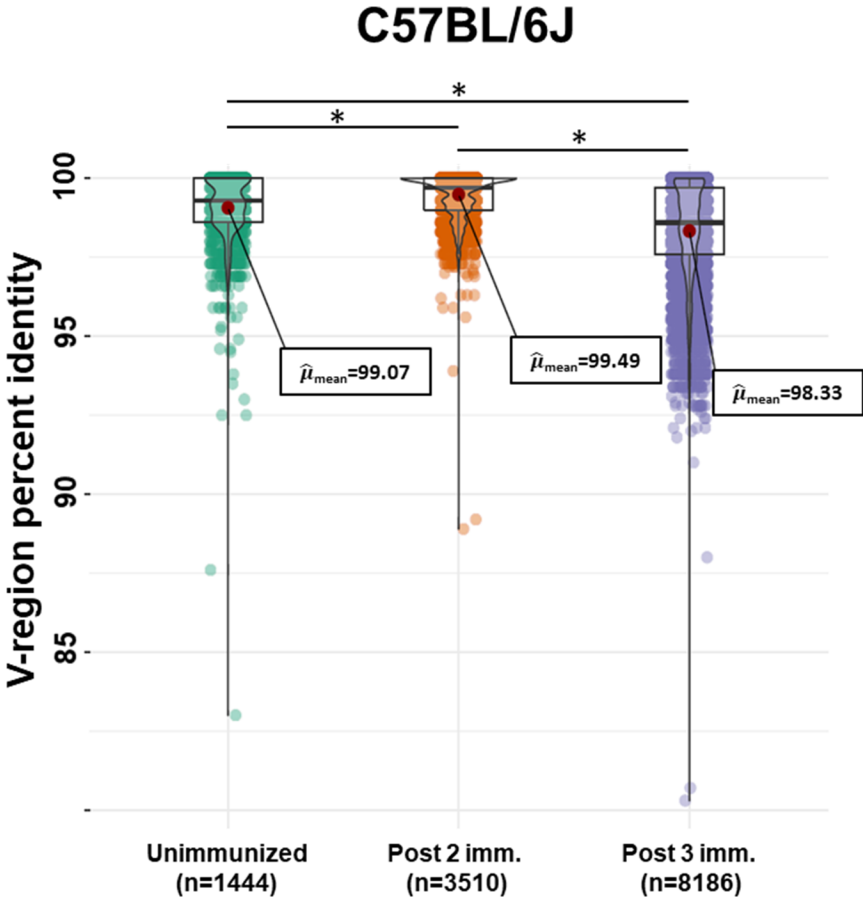


Figure 5

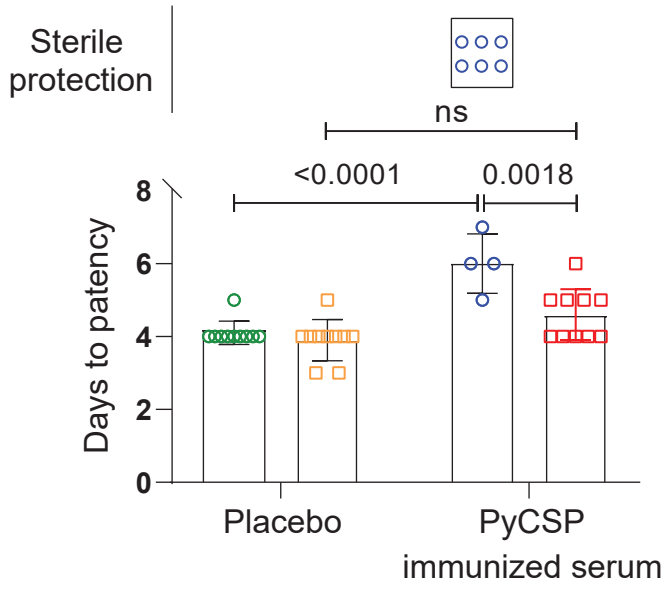
A.



B.



A related to Fig 1B.



B related to Fig 1F.

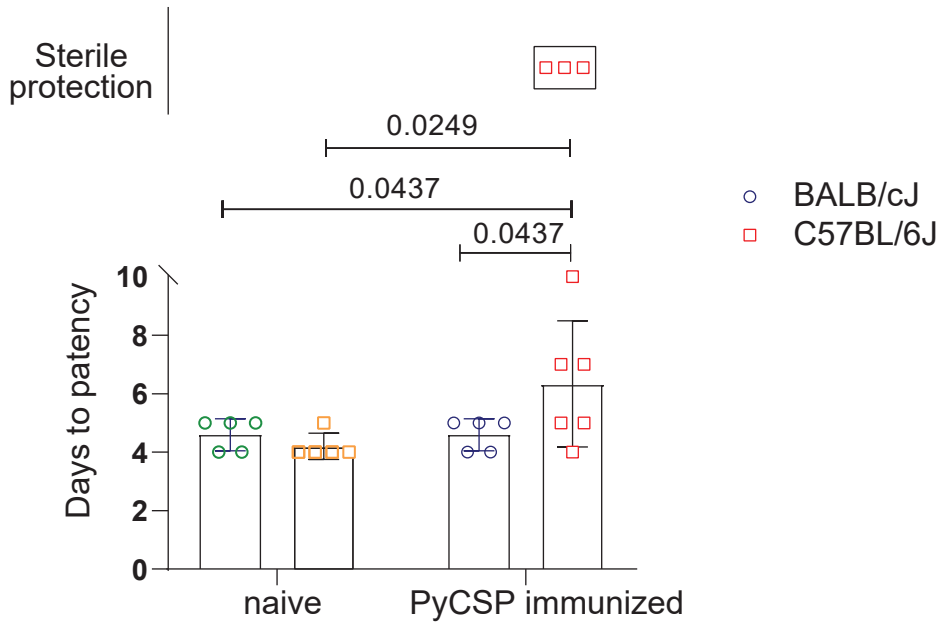


Figure S2.

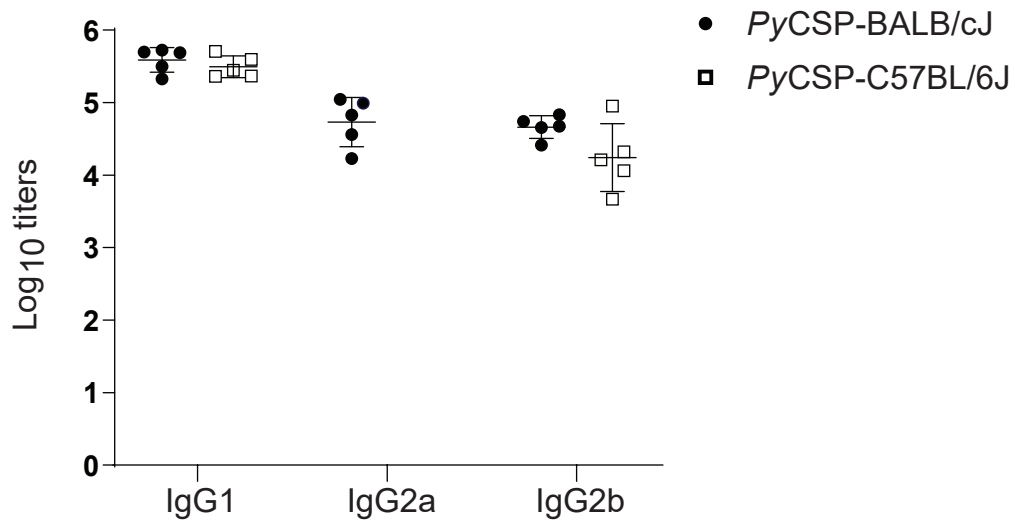


Figure S3.

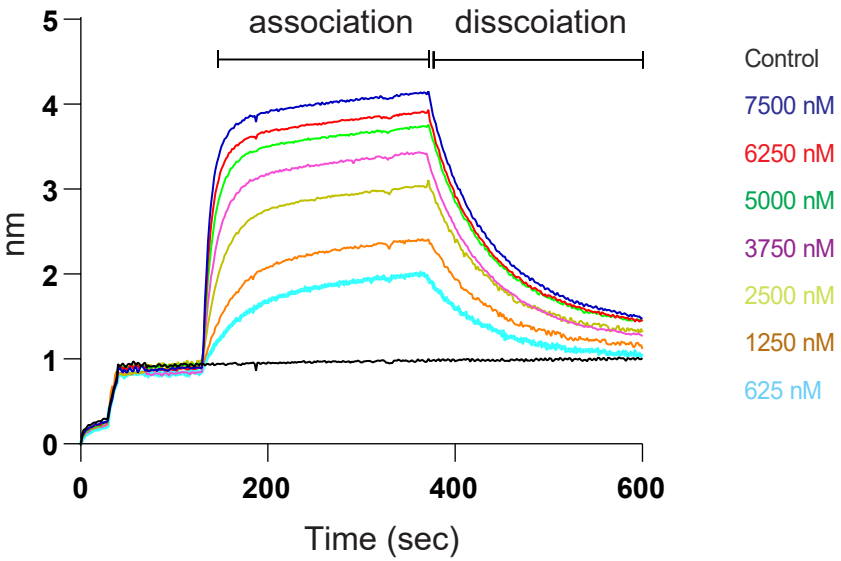


Figure S4.

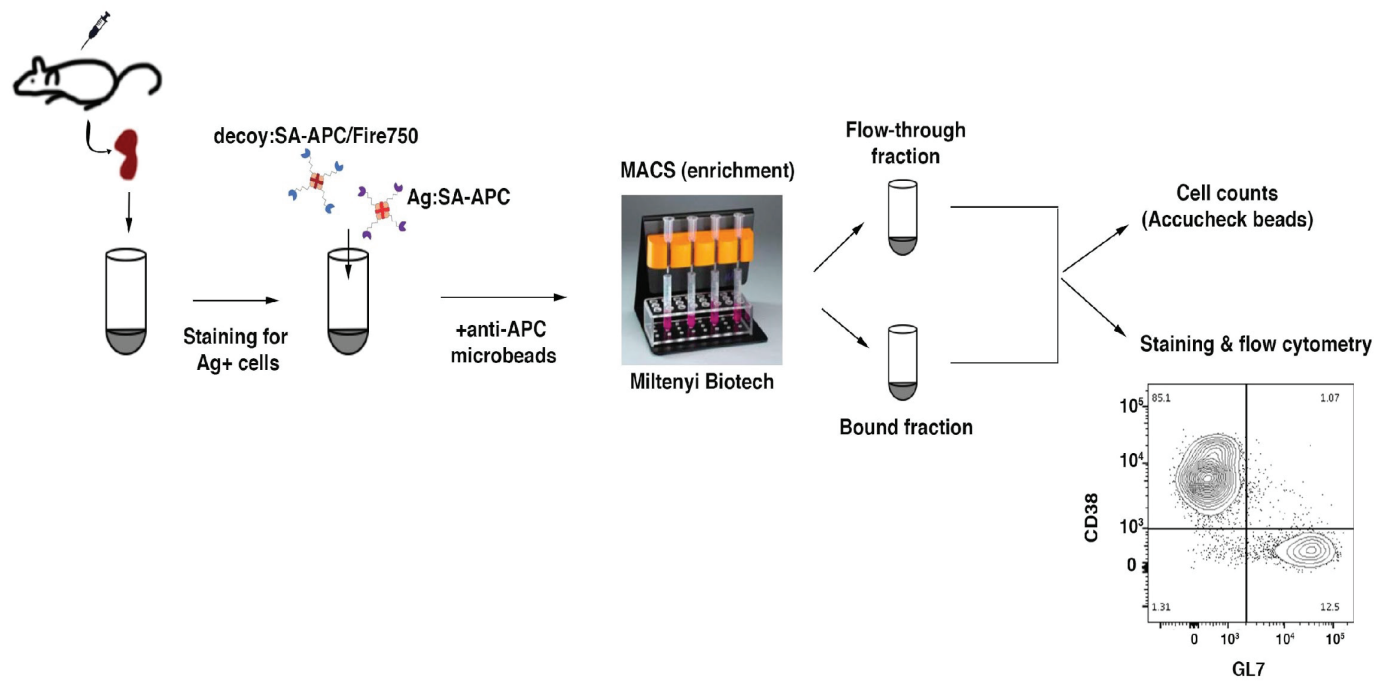


Figure S5

