bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 # Design and assessment of TRAP-CSP fusion antigens as effective malaria
2 # vaccines

3 Chafen Lu[1¶], Gaojie Song[1,3], Kristin Beale[1], Jiabin Yan[1], Emma Garst[1], Emily Lund[2], Flaminia
4 Catteruccia[2] and Timothy A. Springer[1¶]

5 [1] Program in Cellular and Molecular Medicine, Boston Children's Hospital, Harvard Medical
6 School,
7 [2] Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public
8 Health, Boston, MA 02115
9 [3] Current address: Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical
10 Sciences and School of Life Sciences, East China Normal University, Shanghai, 200241, China.

11 [¶] Corresponding authors: lu@crystal.harvard.edu, Springer@crystal.harvard.edu

12 Running title: TRAP-CSP fusion antigens

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

13 # Abstract

14 The circumsporozoite protein (CSP) and thrombospondin-related adhesion protein
15 (TRAP) are major targets for pre-erythrocytic malaria vaccine development. However, the most
16 advanced CSP-based vaccine RTS,S provides only partial protection, highlighting the need for
17 innovative approaches for vaccine design and development. Here we design and characterize
18 TRAP-CSP fusion antigens, and evaluate their immunogenicity and protection against malaria
19 infection. TRAP N-terminal folded domains were fused to CSP C-terminal fragments consisting
20 of the C-terminal αTSR domain with or without the intervening repeat region. Homogenous,
21 monomeric and properly folded fusion proteins were purified from mammalian transfectants.
22 Notably, fusion improved expression of chimeras relative to the TRAP or CSP components
23 alone. Immunization of BALB/c mice with the *P. berghei* TRAP-CSP fusion antigens formulated
24 in AddaVax adjuvant elicited antigen-specific antibody responses. Remarkably, fusion antigens
25 containing the CSP repeat region conferred complete sterile protection against *P. berghei*
26 sporozoite challenge, and furthermore, mice that survived the challenge were completely
27 protected from re-challenge 16 weeks after the first challenge. In contrast, fusion antigens
28 lacking the CSP repeat region were less effective, indicating that the CSP repeat region
29 provided enhanced protection, which correlated with higher antibody titers elicited by fusion
30 antigens containing the CSP repeat region. In addition, we demonstrated that N-linked glycans
31 had no significant effect on antibody elicitation or protection. Our results show that TRAP-CSP
32 fusion antigens could be highly effective vaccine candidates. Our approach provides a platform
33 for designing multi-antigen/multi-stage fusion antigens as next generation more effective malaria
34 vaccines.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

35 # Introduction

36 Malaria remains a global health problem with an estimated 216 million cases of infection
37 and 445,000 deaths worldwide in 2016. Children under age 5 are most vulnerable to malaria
38 infection and suffer high mortality. The most advanced vaccine, RTS,S, reduced infection by
39 27% in infants and 46% in children during the first 18 months, and protection declined thereafter
40 [1, 2], leaving an unmet need for more effective vaccines.
41 Malaria is caused by *Plasmodium* parasites transmitted by *Anopheles* mosquitoes.
42 Infected mosquitoes introduce salivary gland sporozoites into the host during a blood meal.
43 Sporozoites infect hepatocytes, and subsequent infection of red blood cells causes the
44 symptoms of malaria. Most vaccine development has targeted the pre-erythrocytic stage (liver
45 stage infection). Both subunit vaccines and live sporozoites, attenuated by radiation or
46 mutation, or given in combination with chemoprophylaxis, have been studied in preclinical and
47 clinical trials. Although immunization with live sporozoites provides high levels of protection [3-
48 6], the cost of manufacturing sporozoites from infected mosquitos, maintaining them viably, and
49 the requirement for multiple intravenous injections have prevented wide applications in malaria
50 endemic regions.
51 Two major proteins on the surface of sporozoites, the circumsporozoite protein (CSP)
52 and thrombospondin-related adhesion protein (TRAP), are the focus of pre-erythrocytic subunit
53 vaccine development; both are essential for sporozoite motility and liver-stage infection [7, 8].
54 CSP, the most abundant surface protein in sporozoites, is composed of an N-terminal domain
55 (NTD), Region I (RI), where CSP is cleaved during cell invasion [9, 10], a central repeat region,
56 and the C-terminal αTSR domain followed by a glycosylphosphatidylinositol (GPI) membrane
57 anchor (Fig 1A). The RTS vaccine includes the C-terminal portion of the repeat region (R) and
58 the αTSR domain (T) from *P. falciparum* CSP (Fig 1A) fused to the hepatitis B surface antigen
59 (S). TRAP consists of the N-terminal von Willebrand factor type-1 (VWA) or integrin I-domain,

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

60 the thrombospondin type-1 repeat (TSR) domain, C-terminal repeats, and transmembrane and
61 cytoplasmic domains (Fig 1A). TRAP delivered by adenovirus prime and modified vaccinia
62 Ankara virus (MVA) boost regimens achieved 40-95% protection efficacy in murine models [11,
63 12], and 21% sterile protection in one phase I/II trial [13], and in another clinical trial reduced the
64 risk of infection by 67% [14]. With the limited protective efficacy of the RTS,S vaccine and
65 vaccines targeting TRAP in clinical trials, combination vaccination targeting both CSP and
66 TRAP has recently been explored. Although a phase I/II clinical trial combining adjuvanted
67 TRAP and RTS,S showed no benefit of protection from TRAP [15], a recent preclinical study
68 showed that combining a modified RTS,S-like vaccine R21 with viral vectored TRAP
69 significantly enhanced protective efficacy compared to single component vaccines [16].
70 However, immunological interference has been a concern when combining two vaccines.
71 Bauza et al. reported that co-administration of TRAP and CSP vaccines in one immunization
72 regimen resulted in a reduction of CSP antibodies and no improvement of protection over either
73 subunit vaccine alone [12]. In another study, CD8+ T cell interference was observed when
74 combining viral vectored CSP with the blood stage antigen merozoite surface protein 1 (MSP1)
75 [17]. Further work is needed to tailor and refine immunization regimens to achieve the additive
76 benefit of CSP and TRAP co-vaccination.

77 **Figure 1. TRAP-CSP fusion antigen design and expression.** (A and B) Schematic
78 diagrams. SP, signal peptide; TM, transmembrane domain; CTD, cytoplasmic domain;
79 NTD, N-terminal domain; RI, region I; GPI, glycosylphosphatidylinositol membrane
80 anchor. The region included in the RTS component of RTS,S vaccine is shown under
81 the CSP diagram. The dashed line shows the fusion junction between TRAP and CSP.
82 (C) and (D) Expression in 293T transfectants of *P. berghei* constructs (C) and *P.*
83 *falciparum* constructs (D). Supernatants from 293T cells transiently transfected with the
84 indicated constructs or vector alone (mock) were subjected to 10% reducing or

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

85 nonreducing SDS-PAGE and Western blot using antibodies to the His tag or the CSP
86 repeat region as indicated.

87 Here we aimed to design TRAP-CSP fusion antigens to provide the benefit of protection
88 conferred by delivering both antigens as a single protein. TRAP-CSP chimeric constructs
89 containing functional protein domains and regions with protective B and T cell epitopes of both
90 proteins were generated, and monomeric and properly folded chimeric proteins were purified
91 from mammalian transfectants. Immunization with TRAP-CSP fusion antigens elicited strong
92 antigen-specific antibody responses and sterile immunity against *P. berghei* challenge in mice.
93 Our results show that TRAP-CSP fusion antigens offer promise as more effective vaccine
94 candidates.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

95 # Materials and methods

96 ## DNA constructs

97 TRAP and CSP protein sequences are from *P. berghei* strain ANKA (GeneBank accession
98 number AAB63302 for TRAP and CDS44911 for CSP) and from *P. falciparum 3D7* (accession
99 number AAA29775 for TRAP and CAB38998 for CSP). Synthetic cDNAs were codon optimized
100 for mammalian expression by Atum Bio (Newark, CA) for *P. berghei* constructs and by
101 GenScript (Piscataway, NJ) for *P. falciparum* constructs. *P. berghei* chimeras and TRAP-N
102 constructs contain TRAP amino acids Gln^{25}- Pro^{291}, with amino acids 99-119
103 (KRYGSTSKASLRFIIAQLQNN) replaced by the equivalent sequence from *P. falciparum*
104 (HSDASKNKEKALIIIKSLLST, $\alpha2$ and $\alpha3$ helices in VWA domain structure) [18] due to
105 unsuccessful expression of the native *Pb*TRAP sequence. *P. berghei CSP* fusion fragments
106 contain CSP amino acids Asn^{164}- Ser^{318} and Pro^{239}-Ser^{318} for TRAP-N/CSP-RT and TRAP-
107 N/CSP-T chimeras, respectively, with Asn^{280} mutated to Ser to remove the putative N-
108 glycosylation site. *P. falciparum* chimeras contain TRAP amino acids Arg^{26}-Asp^{297} with the
109 nonconserved Cys^{55} and the N-glycosylation site Asn^{132} mutated to Ser and Gln, respectively,
110 and CSP Gln^{96}- Ser^{375} and Asn^{207}- Ser^{375}, for the TRAP-N/CSP-JRT and TRAP-N/CSP-RT,
111 respectively. *P. berghei* constructs were inserted in pLexM vector [18]. *P. falciparum* constructs
112 were cloned into Nhe I-Bam HI sites of the pIRES2-EGFP vector (Takara Bio, formerly
113 Clontech). All constructs contain a modified murine kappa chain secretion signal peptide and C-
114 terminal His tag [18], and were confirmed by DNA sequencing.

115 ## Antibodies

116 Monoclonal antibodies to *P. falciparum* TRAP were generated and characterized as described
117 in the Supplemental Methods and Figures. Antibodies 1G12, 4B3 and 4C2 to *P. falciparum* CSP
118 were kindly provided by Dr. Nicholas J. MacDonald (NIH, Malaria Vaccine Development

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

119 Branch). 3D11 and 2A10 hybridoma lines were obtained from BEI Resources (ATCC), and IgG
120 antibodies were purified from serum-free culture supernatants using protein G affinity
121 chromatography. Secondary antibodies were rabbit polyclonal anti-His (Delta Biolabs),
122 Horseradish peroxidase (HRP)-anti-rabbit and HRP-anti-mouse IgG (GE Healthcare) for
123 Western blot, and HRP-penta-His antibody (Qiagen) and HRP-anti-mouse IgG (Abcam) for
124 ELISA.

125 **Cell culture**

126 293T cells (ATCC) were cultured in DMEM medium supplemented with10% fetal bovine serum
127 (FBS). 293S GnTI- cells (obtained from Philip J. Reeves Laboratory, Departments of Biology
128 and Chemistry, Massachusetts Institute of Technology) [19] and Expi293F cells (Thermo Fisher
129 Scientific) were cultured in suspension in serum-free Ex-Cell 293 medium (Sigma) and Expi293
130 medium (Thermo Fisher Scientific), respectively.

131 **Protein expression and purification**

132 293T cells in 6-well tissue culture plates were transfected using Lipofectamine^{2000} according to
133 manufacturer's instruction (Thermo Fisher Scientific). For scaleup transient transfection of 293S
134 GnTI- cells, suspension cultures were transfected using polyethyleneimine [20]. Culture
135 supernatants were harvested 6 days later. For stable transfection of Expi293F cells, adherent
136 Expi293F cells were transfected in DMEM medium with 10% FBS using Lipofectamine^{2000}
137 transfection. Selection was started 48 hours later by addition of 0.5 mg/ml G418 (final
138 concentration). After 10-12 days, cells were harvested and sorted for top 5% GFP positive cells
139 on a FACSAria machine (BD Biosciences). Cells were sorted a 2nd time to further enrich GFP
140 expressing cells. Sorted cells were expanded in suspension culture in serum-free Expi293
141 medium for protein purification. Proteins were purified from culture supernatants of
142 transfectants by Ni-NTA followed by gel filtration chromatography as described [18]. For
143 endoglycosidase H (Endo H) treatment, Ni-NTA purified materials were buffer exchanged to 50
144 mM sodium acetate, pH 5.5, and 150 mM NaCl, and digested with Endo H at 1:20 mass ratio of

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

145 enzyme:protein at 4°C, overnight. Fractions from gel filtration chromatography were subjected
146 to non-reducing SDS-PAGE and monomeric protein peak fractions were pooled and stored in
147 aliquots at -80°C.

148 **Western blot**

149 Cultural supernatants (10 µl) from transiently transfected 293T cells were mixed with 2.5 µl 5x
150 Laemmli sample buffer containing 25% β-mercaptoethanol or 25 mM N-ethylmaleimide for
151 reducing and non-reducing SDS-polyacrylamide gel electrophoresis (PAGE), respectively.
152 Blotting to PVDF membrane was carried out using Tran-Blot Turbo transfer system (Bio-RAD).
153 Membrane was probed with 0.4 µg/ml primary antibody, followed by incubation with HRP-
154 conjugated 2nd antibodies and chemiluminescence imaging using LAS-4000 system (Fuji Film).
155 ImageJ software was used for quantitation of protein bands.

156 **Enzyme-linked immunosorbent assay (ELISA)**

157 96-well Elisa plates (Costar) were coated with 50 µl of purified antibodies at 5 µg/ml in 50 mM
158 sodium carbonate buffer, pH 9.5, 50 µl/well for 2 hrs at 37°C, and blocked with 3% BSA for 90
159 min at 37°C. His tagged TRAP or TRAP-CSP fusion proteins (50 µl, 0.4 µg/ml) was added and
160 incubated at 4°C overnight. Binding was detected with HRP-anti-His (Penta-His Ab at1:5000
161 dilution) or with biotin-labeled primary antibody at 0.5 µg/ml followed by HRP-streptavidin. 10
162 min after addition of peroxidase substrate (Life Technologies), plates were read at 405 nM on
163 an Emax plate reader (Molecular Devices).

164 **Immunization**

165 10 µg *P. berghei* TRAP/CSP fusion antigens diluted in 100 µl PBS was mixed with 100 µl
166 AddaVax adjuvant (InvivoGen, San Diego), and completely emulsified by pushing the mixture
167 between two glass syringes via a two-way valve. BALB/c mice (The Jackson Laboratory) were
168 injected intraperitoneally (i.p) with 200 µl of antigen and adjuvant emulsion per mouse. Mice
169 were immunized 3 times (prime + 2 boosts) at intervals of three weeks. Control mice received

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

170 PBS and adjuvant emulsion. Tail blood (50-100μl) was collected from each mouse for
171 measuring antibody responses.

172 **Antibody titer measurement**

173 Serum was obtained from tail blood and stored at -80°C until use. 96-well ELISA plates were
174 coated with 50μl of TRAP-N/CSP-RT or TRAP-N/CSP-T antigen at 2.5 μg/ml in sodium
175 carbonate buffer, pH 9.5, overnight at 4°C. Plates were blocked with 3% BSA. Sera were serially
176 diluted 5-fold starting from 1:200, and 50 μl/well added in duplicate and incubated for 2 hrs at
177 room temperature. Similarly, positive control antibody 3D11 to the *P. berghei CSP* repeat region
178 or antibody to the His tag at the C-terminus of the antigens were used at 1 μg/ml. Serum from
179 adjuvant alone immunized mice was diluted and added to each plate as negative control. After
180 incubation with HRP-anti-mouse whole IgG, peroxidase substrate was added, and absorbance
181 at 405 nm was read 10 min later. A semi-logarithmic dilution curve (x-axis: log dilution and y-
182 axis: OD_{405}, subtracted by the negative control) was generated for each serum sample, and a
183 line parallel to the x axis was drawn at half of the OD value of the 3D11 or His tag positive
184 control antibodies. Antibody titer was taken as the dilution factor where the dilution curve
185 intercepted with the line at half of the OD value of the positive control.

186 ***P. berghei-infected* mosquitoes**

187 $PbGFP_{CON}$, a recombinant *Plasmodium berghei* (ANKA strain) that constitutively expresses GFP
188 was used [21]. To infect mosquitoes, $PbGFP_{CON}$-infected mice (3-7% parasitemia) were
189 anaesthetized and laid over a cage of female *Anopheles stephensi* mosquitoes (50-100
190 mosquitoes per cage), which were allowed to feed for 15 min. Successful mosquito infection
191 was confirmed 10 days after blood feeding by dissecting the midgut and examining under a
192 fluorescent microscope for the presence of oocyst. At day 20 after blood feeding, infected
193 mosquitoes (prevalence of infection >80%) were used for challenge.

194 **Challenge by infected mosquito bite**

195 For challenge infection by mosquito bite, immunized or naive mice were anaesthetized and

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

196 exposed to the bites of $PbGFP_{CON}$-infected mosquitoes for 15 min as described above. Mice
197 were monitored for blood stage infection at days 7, 9 and 12 post challenge by Giemsa staining
198 of thin blood smears and microscopic examination, and parasitemia was determined as %
199 infected red blood cells. Mice that remained blood stage parasite-free after 12 days were
200 considered sterilely protected.

201 **Ethics Statement**

202 Animal work was conducted in accordance with and was approved by the Harvard Medical
203 School Institutional Animal Care and Use Committee (IACUC) under protocol #05010. Animals
204 were cared in compliance with the U.S. Department of Agriculture (USDA) Animal Welfare Act
205 (AWA) and the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory
206 Animals.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

207 # Results

208 ## Design of TRAP and CSP fusion antigens

209 TRAP and CSP fusion constructs combined functional domains defined in crystal
210 structures and regions containing protective B and T-cell epitopes from both proteins (Fig 1B).
211 The folded VWA and TSR domains at the N-terminus of TRAP [18] and the αTSR domain at the
212 C-terminus of CSP [22] were left intact in all fusion constructs, which varied in their content of
213 CSP repeats in between the folded domains (Fig 1B). The VWA and TSR domains of TRAP are
214 required for sporozoite motility and host cell invasion [7, 23]. Moreover, the VWA domain is the
215 target of potent protective CD8+ T cell responses elicited by TRAP or whole sporozoite
216 immunization [24-26]. The CSP repeat region contains immunodominant B-cell epitopes
217 recognized by sporozoite neutralizing antibodies, whereas the αTSR domain contains several T-
218 cell epitopes associated with protection [26-30]. The junction (J) between the NTD and the
219 repeat region, which is included in the TRAP-N/CSP-JRT construct and not in the RTS,S
220 vaccine, has recently been identified to contain epitopes for potent protective antibodies [31-33].
221 Fusion constructs from both *P. berghei* and *P. falciparum* were generated for evaluation of
222 protein expression and folding. *P. berghei* fusion proteins contain 4 putative N-glycosylation
223 sites in the TRAP fragment for comparison of glycosylated and N-glycan shaved versions,
224 whereas the single N-glycosylation site in the *P. falciparum* chimeric constructs was removed by
225 mutating TRAP Asn^{132} to Gln.

226 **Figure 2. TRAP-CSP fusion protein purification**. (A) *P. berghei* chimeras were
227 produced by transient transfection of 293S GnTI^{-} cells, purified by Ni-NTA affinity
228 chromatography, treated with or without Endo H, and subjected to reducing SDS-PAGE
229 and Coomassie blue staining. Arrows point to glycosylated (glyco) and Endo H-treated
230 (dNG) chimera and Endo H protein bands. Dotted lines separate lanes run on the same
231 gel and moved. (B) Representative Superdex 200 10/300 GL column purification

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

232 chromatogram, shown TRAP-N/CSP-T sample that had previously been purified by Ni-
233 NTA and treated with Endo H. Insert, nonreducing SDS-PAGE of monomer peak
234 fractions. (C) Nonreducing SDS-PAGE and Coomassie blue staining of *P. berghei* fusion
235 proteins after the Superdex S200 purification step. (dNG) denotes Endo H treatment.
236 Dotted lines divide lanes run on two identical gels and moved. (D) SDS-PAGE and
237 Coomassie blue staining of purified *P. falciparum* TRAP-N/CSP-JRT and TRAP-N/CSP-
238 RT fusion proteins run under nonreducing and reducing conditions.

239 **Expression, purification and folding state of TRAP-CSP fusion proteins**

240 Expression of fusion proteins or fragments of TRAP and CSP alone were tested in
241 mammalian 293 cell transfectants, which were previously used to obtain well-folded,
242 glycosylated *P. falciparum* and *P. vivax* TRAP N-terminal fragments and CSP C-terminal
243 fragments for structure and carbohydrate determination [18, 22, 34]. Equal amounts of culture
244 supernatants from transiently transfected 293 cells were subjected to Western blot using
245 antibodies to the C-terminal His-tag or to the CSP repeat region. TRAP-N protein from both *P.*
246 *berghei* and *P. falciparum* was detected by anti-His, and migrated faster under non-reducing
247 than reducing conditions, as typical of proteins with disulfide bonds (Fig 1C, lanes 2 and 7 and
248 1D, lanes 2 and 8). Lower amount of *P. berghei* CSP-RT fragment was observed (Fig 1C, lanes
249 5 and 10), whereas *P. falciparum* CSP-JRT and CSP-RT fragments were undetectable by anti-
250 His (Fig 1D, top, lane 4 and 6, and lanes 10 and 12). However, a weak *P. falciparum* CSP-RT
251 band of ~27 kD was detected by repeat region antibody 2A10 under reducing condition (Fig 1D,
252 bottom, lane 6). In contrast to TRAP and CSP fragments alone, larger amounts of TRAP-CSP
253 fusion proteins of both *P. berghei* and *P. falciparum* were detected by the His tag antibody and
254 had the expected sizes (Fig 1C top, lanes 8 and 9 vs 7; Fig 1D top, lane 9 and 11 vs 8);
255 quantification of protein bands under non-reducing showed 5- and 3- fold increases of *Pb*TRAP-
256 N/CSP-RT and *Pb*TRAP-N/CSP-T, respectively, relative to *Pb*TRAP-N protein, and 3- and 2-
257 fold increases of *Pf*TRAP-N/CSP-JRT and *Pf*TRAP-N/CSP-RT, respectively, relative to

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

258 *Pf*TRAP-N. Additionally, the chimeras containing the CSP repeat region were recognized by
259 CSP repeat region antibodies (Fig 1C and 1D, bottom). Thus, fusion improved expression of the
260 chimeric proteins relative to the TRAP or CSP components alone.

261 TRAP-CSP fusion proteins were purified for further characterization. *P. berghei* fusion
262 constructs, TRAP-N/CSP-RT and TRAP-N/CSP-T, containing 4 putative N-glycosylation sites,
263 were expressed in 293S GnTI- cells, which lack N-acetylglucosaminyltransferase I (GnTI) and
264 produce glycoproteins with short and homogeneous high mannose-type N-linked glycans that
265 can be removed by endoglycosidase H (Endo H) [19], and purified by Ni-NTA affinity
266 chromatography and treated with Endo H. Endo H removed ~6 kD mass from each of the *P.*
267 *berghei* TRAP-N/CSP-RT and TRAP-N/CSP-T proteins and reduced their mass to 52 kD and 41
268 kD, respectively (Fig 2A, reducing SDS-PAGE). A second purification step, gel filtration
269 chromatography, was utilized to obtain homogenous and monomeric proteins. A representative
270 chromatogram is shown in Fig 2B. The purified *P. berghei* TRAP-N/CSP-RT and TRAP-N/CSP-
271 T chimeras and their respective N-glycan shaved versions (designated dNG) each showed a
272 single band by non-reducing SDS-PAGE (Fig 2C), confirming homogeneity and monomeric
273 state. The *P. falciparum* TRAP-N/CSP-JRT and TRAP-N/CSP-RT fusion proteins, with the
274 single N-glycosylation site mutated, were purified from Expi293F stable transfectants, and
275 monomeric proteins showed mass of ~75 kD and 58 kD, respectively, under reducing condition
276 (Fig 2D).

277 Proper folding of recombinant antigens is required to elicit B cell responses to native
278 proteins on parasites. Purification of monomeric well-behaved material already suggested that
279 the fusion proteins were well folded. We utilized available, well-characterized monoclonal
280 antibodies (mAbs) to *P. falciparum* TRAP and CSP to further probe the folding state of TRAP-
281 CSP fusion proteins. We generated and characterized a panel of mAbs to *Pf*TRAP
282 (Supplemental Material). CL2/1 and CL5/27 mAbs to PfTRAP VWA domain and CL5/22,
283 CL5/42, CL5/46 and CL5/54 mAbs to the TSR domain and β-ribbon (Fig s1) recognized TRAP

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

284 on the surface of transfectants (Fig s2, Table s1) and stained unfixed *P. falciparum* sporozoites
285 (Fig s3, Table s1), suggesting that they recognize native epitopes. Furthermore, the epitopes of
286 mAbs CL2/1, CL5/27, CL5/22, CL5/46 and CL5/54 are sensitive to disulfide reduction (Fig s5).
287 The 4B3, 4C2 and 1G12 mAbs to the αTSR domain of *Pf*CSP have been shown to recognize
288 CSP with intact disulfides and inhibit sporozoite invasion of liver cells *in vitro* [35]. The purified,
289 monomeric *P. falciparum* TRAP-N/CSP-JRT and TRAP-N/CSP-RT fusion proteins bound to the
290 VWA domain mAbs CL2/1 and CL5/27 and mAbs CL5/22, CL5/42, CL5/46 and CL5/54 to the
291 TSR domain and β-ribbon at levels comparable to the TRAP-N alone (Fig 3A). Furthermore, the
292 two fusion proteins reacted with the αTSR domain mAbs 4B3, 4C2 and 1G12 to a level
293 comparable to the His tag antibody and the repeat region antibody 2A10 (Fig 3B). These results
294 indicate that the TRAP VWA and TSR domains and the CSP αTSR domain of the fusion
295 proteins are correctly folded.

296 **Figure 3. Reactivity of fusion proteins to antibodies that recognize correctly**
297 **folded TRAP and CSP.** ELISA plates were coated with the indicated antibodies and
298 incubated with purified *P. falciparum* TRAP-CSP fusion proteins or TRAP-N alone as
299 indicated. Binding to the immobilized antibodies was detected by HRP-penta-His
300 antibody to the C-terminal His tag (A) or biotin-labeled TRAP antibody CL5/42 followed
301 by HRP-streptavidin (B). Results are mean $\pm$ SD of triplicate wells, and representative
302 of 3 independent experiments.

303 These results show that TRAP-CSP chimeras are well expressed in mammalian cells,
304 are properly folded, and yielded homogenous, monomeric proteins after Ni-NTA and gel
305 filtration chromatography.

306

307 **Humoral responses elicited by TRAP-CSP fusion antigens**

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

308 Mice were immunized with *P. berghei* TRAP-N/CSP-T and TRAP-N/CSP-RT fusion
309 antigens to evaluate immunogenicity and protection against *P. berghei* infection. To investigate
310 the effect of the high mannose N-linked glycans on these chimeras, mice were also immunized
311 with the Endo H-shaved versions, TRAP-N/CSP-RT (dNG) and TRAP-N/CSP-T (dNG), in which
312 all but one N-acetylglucosamine residue was removed. Fusion antigens were formulated in
313 AddaVax, a squalene-based oil-in-water emulsion. BALB/c mice were primed with10 μg
314 adjuvanted antigen, followed by two boost immunizations. Sera were collected at weeks 6, 16
315 and 32 post antigen priming to determine antibody responses. Antigen-specific IgG antibody
316 titers, defined as serum dilutions giving half of the absorbance of a positive control antibody, were
317 measured by ELISA.

318 Whether the chimeras contained high-mannose or shaved N-glycans had little effect on
319 antibody responses. Serum IgG antibody titers from the TRAP-N/CSP-RT immunized group
320 were comparable to the TRAP-N/CSP-RT (dNG) antigen group, at all three time points (Fig 4A).
321 Similarly, no significant differences in antibody titers were observed between the TRAP-N/CSP-
322 T and TRAP-N/CSP-T (dNG) groups (Fig 4A and 4B). The results showed that the four putative
323 high mannose type N-linked glycans in the TRAP-N fragment neither enhanced nor
324 compromised antigen-specific antibody responses.

325 **Figure 4. Antibody responses elicited by immunization with *P. berghei* TRAP-CSP**
326 **fusion antigens.** BALB/c mice were immunized with TRAP-N/CSP-RT and TRAP-N/CSP-
327 T antigens and their respective N-glycan shaved versions (dNG) in AddaVax adjuvant. Tail
328 blood was collected at the indicated time points post antigen priming and antigen specific
329 IgG antibody titers were measured by ELISA. Antibody titers of individual mice were
330 measured against the TRAP-N/CSP-RT antigen (A) and the TRAP-N/CSP-T antigen (B).
331 Mean and SD are shown, and analyzed by One-way ANOVA with Sidak's multiple
332 comparisons test (GraphPad Prism 7). *, $P<0.05$; **, $P<0.01$; ns, not significant.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

333 In contrast, the CSP repeats present in TRAP-N/CSP-RT compared to TRAP-N/CSP-T
334 significantly enhanced antibody responses. As shown in Fig 4A, when antibody titers were
335 measured against the TRAP-N/CSP-RT protein containing the repeat region, mice immunized
336 with TRAP-N/CSP-RT and TRAP-N/CSP-RT (dNG) had ~2-fold higher titers than mice
337 immunized with the TRAP-N/CSP-T and TRAP-N/CSP-T (dNG) antigens, 6 weeks and 16
338 weeks post priming (Fig 4A). By comparison, antibody titers against the TRAP-N/CSP-T protein
339 lacking the repeat region were comparable among the four groups of mice (Fig 4B).
340

341 **Protection against *P. berghei* infection**

342 Protection of immunized mice against *P. berghei* infection was assessed. Mice were
343 challenged by bite with *P. berghei* infected *Anopheles stephensi* mosquitoes. Blood stage
344 infection was monitored at days 7, 9 and 12 post challenge. Remarkably, 5 out of 5 mice
345 immunized with TRAP-N/CSP-RT and TRAP-N/CSP-RT (dNG) antigens were completely free of
346 blood stage parasites day 12 after challenge (100% sterile protection) (Table I). In contrast, the
347 TRAP-N/CSP-T and TRAP-N/CSP-T (dNG) antigens each conferred 40% sterile protection; 3 of
348 5 challenged mice in each immunized group showed blood stage infection at each of days 7, 9,
349 and 12 after challenge (Table I).

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

350 **Table I. Protection against *P. berghei* challenge**

P. berghei fusion antigen Immunization	1st challenge (16 weeks post antigen priming)				Re-challenge of protected mice (32 weeks post antigen priming)			
	Number infected/ challenged mice	Parasitemia (%)[a] (day post challenge)			Number infected/ challenged mice	Parasitemia (%)[a] (day post challenge)		
		Day 7	Day 9	Day 12		Day 7	Day 9	Day 12
TRAP-N/ CSP-RT	0/5	0	0	0	0/5	0	0	0
TRAP-N/ CSP-RT (dNG)	0/5	0	0	0	0/5	0	0	0
TRAP-N/ CSP-T	3/5	3.5 2.9 2.3	3.8 4.7 3.9	8.4 10.4 8.5	2/2	2.4 3.1	3.8 4.6	9.1 10.5
TRAP-N/ CSP-T (dNG)	3/5	2.6 2.4 1.6	3.2 2.8 3.1	10.9 12.5 13.9	2/2	2.7 2.9	3.2 3.7	13.9 12.6
None (naïve)	1/1	3.1	8.0	18.9	1/1	2.3	3.8	13.8

351 Mice were immunized in AddaVax adjuvant and challenged as described in the Methods. Blood
352 stage infection was monitored at days 7, 9 and 12 post each challenge. Mice free of blood
353 stage infection 12 days after challenge were determined as sterilely protected. Mice sterilely
354 protected from the 1st challenge were re-challenged 16 weeks after the 1st challenge.
355 [a] Data shown are individual infected mice; "0", no infection of the entire group.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

356 To evaluate maintenance of protection, sterilely protected mice were re-challenged 16
357 weeks after the 1st challenge (32 weeks after antigen priming). Strikingly, the TRAP-N/CSP-RT
358 and TRAP-N/CSP-RT (dNG) immunized groups were completely protected from re-challenge
359 (Table I). In contrast, the 2 mice each immunized with TRAP-N/CSP-T and TRAP-N/CSP-T
360 (dNG) antigens that were protected at week 16 were not protected at week 32 and showed
361 blood stage infection (Table I).

362 These results show that immunization with TRAP-N/CSP-RT and its N-glycan shaved
363 (dNG) version conferred sustained sterile protection, whereas TRAP-N/CSP-T and its dNG
364 version, lacking the CSP repeat region, were less protective. Thus, the CSP repeat region
365 enhanced protection, which correlated with higher antibody titers elicited by the TRAP-N/CSP-
366 RT and TRAP-N/CSP-RT (dNG) fusion antigens (Fig 4A).

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

367 # Discussion

368 The benefit of protection against malaria infection by co-immunization with CSP and
369 TRAP was first demonstrated in 1991 by Khusmith *et al*., who showed that immunization of mice
370 with a mixture of *P. yoeli* CSP and TRAP-expressing cells provided complete protection,
371 whereas immunization with either cell type alone only provided partial protection [36]. Despite
372 this result, pre-erythrocytic vaccine development has focused on CSP and TRAP single subunit
373 vaccines over the last two decades and clinical trials to date with CSP and TRAP subunit
374 vaccines have achieved only partial protection. We show here that combining TRAP and CSP
375 fragments into a single fusion protein that contained well-folded protein domains and regions
376 with protective T- and B-cell epitopes from each fusion partner yielded a vaccine that stimulated
377 sterilizing immunity.

378 The TRAP-N/CSP-RT fusion antigen containing from *Pb*TRAP the VWA and TSR
379 domains and from *Pb*CSP the C-terminal half of the repeat region and the αTSR domain
380 conferred durable and complete sterile protection against *P. berghei* infection in BALB/c mice, a
381 significant improvement over the 50% sterile protection obtained in BALB/c mice with three
382 immunizations of adjuvanted full-length CSP protein [37]. By comparison, the *Pb*TRAP-N/CSP-
383 T antigen that lacked the CSP repeat region was less protective (40% sterile protection),
384 showing that the repeat region provided the enhanced protection, which correlated with higher
385 antibody responses elicited by the TRAP-N/CSP-RT antigen. The same results on sterilizing
386 immunity and antibody responses were obtained with chimeras containing high mannose or
387 Endo H-shaved N-glycans. It is well known that the CSP repeats elicit strong sporozoite
388 neutralizing antibody responses; several potent protective antibodies have been isolated and
389 characterized [38-41]. On the other hand, TRAP delivered by viral vectors primarily stimulates
390 CD8+ cytotoxic T cell responses that eliminate infected hepatocytes [11, 13, 42]. Further
391 studies are required to understand how the VWA and TSR domains of TRAP and the repeats

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

392 and αTSR domain of CSP additively or synergistically contribute to the sterile protection
393 conferred by the TRAP-N/CSP-RT fusion antigen.

394 We expressed the TRAP-CSP fusion proteins in a mammalian system that allows proper
395 disulfide formation and glycosylation. For protein-based subunit vaccines, proper folding of
396 recombinant antigens to elicit antibody responses to native protein epitopes is important, and
397 correct disulfide formation to stabilize antigen native conformation is critical. Many targets of
398 malaria vaccine candidates, including TRAP and CSP, contain multiple disulfide bonds, which
399 pose challenges to obtain antigens with correct disulfide formation from bacterial expression.
400 Yeast and insect cell expression systems have achieved some success [35, 43, 44]. However,
401 RTS,S is expressed in the yeast cytoplasm where disulfide bond formation is not promoted, and
402 RTS has not been demonstrated to be monomeric or to have a natively folded CSP αTSR
403 domain [45-48]. We showed that mammalian expressed, purified *P. falciparum* TRAP-CSP
404 fusion proteins are properly folded by demonstrating their reactivity to a panel of antibodies that
405 recognize correctly folded VWA and TSR domains of *Pf*TRAP and the αTSR domain of *Pf*CSP.
406 We expect the purified monomeric *P. berghei* TRAP-CSP fusion proteins are properly folded
407 likewise.

408 Importantly, mammalian cells mannosylate and fucosylate TSR domains similarly to
409 *Plasmodium* sporozoites. X-ray crystallography and mass spectrometry have shown that the
410 TSR domains of *Plasmodium* TRAP or its orthologue in *Toxoplasma gondii*, when expressed in
411 mammalian cells, bear a C-linked mannose on tryptophan and O-linked fucose on threonine [18,
412 49]. Both modifications are also found on TRAP on the surface of *P. falciparum* sporozoites [34].
413 Furthermore, the αTSR domain of CSP expressed in mammalian cells is fucosylated [22], as is
414 the αTSR domain of intact CSP on the surface of *P. falciparum* sporozoites [34]. Yeast lacks the
415 gene required for fucosylation of TSR domains and the αTSR domain expressed in *Pichia* was
416 not fucosylated [22]. Fucosylation and mannosylation of TSR domains in Apicomplexans have

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

417 only recently been recognized [22] [18, 34, 49] and are little considered if at all in the malaria
418 vaccine literature. However, these modifications may be of considerable importance in
419 stabilizing folding and enhancing production of components of subunit vaccines and also may
420 constitute important portions of both B cell folded antigenic epitopes and T cell peptide epitopes.

421 Less is known about N-glycosylation in *Plasmodium.* In contrast to N-glycans in yeast,
422 insect and mammalian cells, *Plasmodium* makes severely truncated N-glycans composed of
423 one or two N-acetylglucosamine (GlcNAc) residues [50]. Glycans can shield native epitopes
424 from immune responses, as shown by the impact of the HIV-1-glycan shield on antibody
425 elicitation [51, 52]. Efforts to overcome glycosylation differences between native and
426 recombinant malarial antigens have been mostly restricted to mutations to remove putative N-
427 glycosylation sites [43, 44]. We compared immune responses in mice to *P. berghei* TRAP-
428 N/CSP-RT and TRAP-N/CSP-T fusion proteins with high mannose N-glycans to chimeras with
429 all but one N-acetylglucosamine residue removed by Endo H (dNG). We found comparable
430 antibody responses and no effect on protection in our *P. berghei* challenge model. Datta *et al*
431 reported that N-glycosylation of the *Pf*s25 antigen delivered as DNA vaccine did not significantly
432 affect antibody response and malaria transmission blocking efficacy [53]. In contrast, un-
433 glycosylated *P. falciparum* MSP1 antigen derived from transgenic mice and glycosylated high
434 mannose MSP1 from baculovirus expression both protected monkeys from *P. falciparum*
435 challenge, whereas glycosylated MSP1 antigen from mice conferred no protection [54]. These
436 results suggest that the types of N-glycans, i.e. complex type with terminal sialic acids from
437 mammalian cells vs. high mannose type from insect cells, contributed to the difference in
438 protection. Therefore, the impact of N-glycosylation of malarial antigens on protection efficacy
439 may vary both among antigens and the types of glycans.

440 Given the highly encouraging protection efficacy conferred by the *P. berghei* TRAP-
441 N/CSP-RT fusion antigens in this study, TRAP-CSP chimeras offer promise as effective pre-
442 erythrocytic vaccines and as fusion partners for multistage chimeric vaccines. Recently

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

443 identified potent protective and "dual specificity" antibodies to *Pf* CSP bind to the NANP repeats
444 and the junction (J) region [32, 33]. This region has not been included in our *P. berghei*
445 constructs or RTS,S. However, the *P. falciparum* chimeric construct containing this region,
446 TRAP-N/CSP-JRT, was expressed even better than the TRAP-N/CSP-RT construct (Fig 1D). It
447 would be very interesting to compare protection efficacy of TRAP-N/CSP-JRT with the TRAP-
448 N/CSP-RT chimera in a *P. falciparum* challenge model. A chimeric antigen of *P. vivax* CSP and
449 the transmission-blocking vaccine target s25 conferred 43% protection against parasite infection
450 and 82% transmission blocking efficacy [55]. Recent crystal structures of HAP2, a gamete
451 fusion protein that is also a transmission blocking target, offer further promise for rational design
452 of TRAP-CSP-HAP2 fusion antigens with dual efficacy [56-59].
453 In conclusion, we have shown that TRAP-CSP fusion antigens could be highly effective
454 vaccine candidates. Chimeric antigens provide a platform for development of multi-
455 antigen/multi-stage vaccines to elicit dual immunity to prevent infection of humans and block
456 transmission of infection by mosquitos using a single immunization regimen.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

457 # Acknowledgments

458 We thank Dr. Shahid Khan and Dr. Chris J. Janse (Leiden Malaria Research Group) for
459 providing transgenic *Plasmodium berghei* lines.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

460 # References

461 1. Rts SCTP. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after
462 vaccination: a phase 3 randomized, controlled trial in children and young infants at 11
463 African sites. PLoS Med. 2014 Jul;11(7):e1001685.

464 2. Rts SCTP. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster
465 dose in infants and children in Africa: final results of a phase 3, individually randomised,
466 controlled trial. Lancet. 2015 Jul 4;386(9988):31-45.

467 3. Epstein JE, Paolino KM, Richie TL, Sedegah M, Singer A, Ruben AJ, et al. Protection
468 against Plasmodium falciparum malaria by PfSPZ Vaccine. JCI Insight. 2017 Jan
469 12;2(1):e89154.

470 4. Butler NS, Vaughan AM, Harty JT, Kappe SH. Whole parasite vaccination approaches for
471 prevention of malaria infection. Trends Immunol. 2012 May;33(5):247-54.

472 5. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection
473 against malaria by intravenous immunization with a nonreplicating sporozoite vaccine.
474 Science. 2013 Sep 20;341(6152):1359-65.

475 6. Mordmuller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, et al.
476 Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature.
477 2017 Feb 23;542(7642):445-9.

478 7. Sultan AA, Thathy V, Frevert U, Robson KJH, Crisanti A, Nussenzweig V, et al. TRAP is
479 necessary for gliding motility and infectivity of Plasmodium sporozoites. Cell.
480 1997;90(3):511-22.

481 8. Nussenzweig V, Nussenzweig RS. Circumsporozoite proteins of malaria parasites. Cell.
482 1985;42(2):401-3.

483 9. Coppi A, Pinzon-Ortiz C, Hutter C, Sinnis P. The Plasmodium circumsporozoite protein is
484 proteolytically processed during cell invasion. J Exp Med. 2005 Jan 3;201(1):27-33.

485 10. Coppi A, Natarajan R, Pradel G, Bennett BL, James ER, Roggero MA, et al. The malaria
486 circumsporozoite protein has two functional domains, each with distinct roles as
487 sporozoites journey from mosquito to mammalian host. J Exp Med. 2011 Feb
488 14;208(2):341-56.

489 11. Reyes-Sandoval A, Berthoud T, Alder N, Siani L, Gilbert SC, Nicosia A, et al. Prime-boost
490 immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the
491 durability and polyfunctionality of protective malaria CD8+ T-cell responses. Infect
492 Immun. 2010 Jan;78(1):145-53.

493 12. Bauza K, Atcheson E, Malinauskas T, Blagborough AM, Reyes-Sandoval A. Tailoring a
494 Combination Preerythrocytic Malaria Vaccine. Infect Immun. 2015 Dec 14;84(3):622-34.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

495 13. Ewer KJ, O'Hara GA, Duncan CJ, Collins KA, Sheehy SH, Reyes-Sandoval A, et al.
496 Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-
497 MVA immunisation. Nature communications. 2013;4:2836.

498 14. Ogwang C, Kimani D, Edwards NJ, Roberts R, Mwacharo J, Bowyer G, et al. Prime-boost
499 vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP
500 provides partial protection against Plasmodium falciparum infection in Kenyan adults. Sci
501 Transl Med. 2015 May 6;7(286):286re5.

502 15. Kester KE, Gray Heppner D, Jr., Moris P, Ofori-Anyinam O, Krzych U, Tornieporth N, et
503 al. Sequential Phase 1 and Phase 2 randomized, controlled trials of the safety,
504 immunogenicity and efficacy of combined pre-erythrocytic vaccine antigens RTS,S and
505 TRAP formulated with AS02 Adjuvant System in healthy, malaria naive adults. Vaccine.
506 2014 Nov 20;32(49):6683-91.

507 16. Collins KA, Snaith R, Cottingham MG, Gilbert SC, Hill AVS. Enhancing protective
508 immunity to malaria with a highly immunogenic virus-like particle vaccine. Sci Rep. 2017
509 Apr 19;7:46621.

510 17. Forbes EK, Biswas S, Collins KA, Gilbert SC, Hill AV, Draper SJ. Combining liver- and
511 blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell
512 interference. J Immunol. 2011 Oct 1;187(7):3738-50.

513 18. Song G, Koksal AC, Lu C, Springer TA. Shape change in the receptor for gliding motility
514 in plasmodium sporozoites. Proc Natl Acad Sci U S A. 2012 December
515 26;109(52):21420-5.

516 19. Reeves PJ, Callewaert N, Contreras R, Khorana HG. Structure and function in rhodopsin:
517 high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a
518 tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable
519 mammalian cell line. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13419-24.

520 20. Zhou YF, Springer TA. Highly reinforced structure of a C-terminal dimerization domain in
521 von Willebrand factor. Blood. 2014 Mar 20;123(12):1785-93.

522 21. Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, van der Linden
523 R, et al. A Plasmodium berghei reference line that constitutively expresses GFP at a high
524 level throughout the complete life cycle. Mol Biochem Parasitol. 2004 Sep;137(1):23-33.

525 22. Doud MB, Koksal AC, Mi LZ, Song G, Lu C, Springer TA. An unexpected fold in the
526 circumsporozoite protein target of malaria vaccines. Proc Natl Acad Sci U S A.
527 2012;109(20):7817-22.

528 23. Wengelnik K, Spaccapelo R, Naitza S, Robson KJ, Janse CJ, Bistoni F, et al. The A-
529 domain and the thrombospondin-related motif of Plasmodium falciparum TRAP are
530 implicated in the invasion process of mosquito salivary glands. EMBO J. 1999 Oct
531 1;18(19):5195-204.

532 24. Bauza K, Malinauskas T, Pfander C, Anar B, Jones EY, Billker O, et al. Efficacy of a
533 Plasmodium vivax malaria vaccine using ChAd63 and modified vaccinia Ankara

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

534 expressing thrombospondin-related anonymous protein as assessed with transgenic
535 Plasmodium berghei parasites. Infect Immun. 2014 Mar;82(3):1277-86.

536 25. Hafalla JC, Bauza K, Friesen J, Gonzalez-Aseguinolaza G, Hill AV, Matuschewski K.
537 Identification of targets of CD8(+) T cell responses to malaria liver stages by genome-
538 wide epitope profiling. PLoS Pathog. 2013 May;9(5):e1003303.

539 26. Doll KL, Pewe LL, Kurup SP, Harty JT. Discriminating Protective from Nonprotective
540 Plasmodium-Specific CD8+ T Cell Responses. J Immunol. 2016 May 15;196(10):4253-
541 62.

542 27. Cohen J, Nussenzweig V, Nussenzweig R, Vekemans J, Leach A. From the
543 circumsporozoite protein to the RTS, S/AS candidate vaccine. Hum Vaccin. 2010
544 Jan;6(1):90-6.

545 28. Dups JN, Pepper M, Cockburn IA. Antibody and B cell responses to Plasmodium
546 sporozoites. Front Microbiol. 2014;5:625.

547 29. Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, et al. A CD4(+) T-cell
548 immune response to a conserved epitope in the circumsporozoite protein correlates with
549 protection from natural Plasmodium falciparum infection and disease. Nat Med. 2004
550 Apr;10(4):406-10.

551 30. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, et al.
552 The circumsporozoite protein is an immunodominant protective antigen in irradiated
553 sporozoites. Nature. 2006;444(7121):937-40.

554 31. Espinosa DA, Gutierrez GM, Rojas-Lopez M, Noe AR, Shi L, Tse SW, et al. Proteolytic
555 Cleavage of the Plasmodium falciparum Circumsporozoite Protein Is a Target of
556 Protective Antibodies. J Infect Dis. 2015 Oct 1;212(7):1111-9.

557 32. Tan J, Sack BK, Oyen D, Zenklusen I, Piccoli L, Barbieri S, et al. A public antibody
558 lineage that potently inhibits malaria infection through dual binding to the
559 circumsporozoite protein. Nat Med. 2018 May;24(4):401-7.

560 33. Kisalu NK, Idris AH, Weidle C, Flores-Garcia Y, Flynn BJ, Sack BK, et al. A human
561 monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on
562 the parasite. Nat Med. 2018 May;24(4):408-16.

563 34. Swearingen KE, Lindner SE, Shi L, Shears MJ, Harupa A, Hopp CS, et al. Interrogating
564 the Plasmodium Sporozoite Surface: Identification of Surface-Exposed Proteins and
565 Demonstration of Glycosylation on CSP and TRAP by Mass Spectrometry-Based
566 Proteomics. PLoS Pathog. 2016 Apr 29;12(4):e1005606.

567 35. Plassmeyer ML, Reiter K, Shimp RL, Jr., Kotova S, Smith PD, Hurt DE, et al. Structure of
568 the Plasmodium falciparum circumsporozoite protein, a leading malaria vaccine
569 candidate. J Biol Chem. 2009 Sep 25;284(39):26951-63.

570 36. Khusmith S, Charoenvit Y, Kumar S, Sedegah M, Beaudoin RL, Hoffman SL. Protection
571 against malaria by vaccination with sporozoite surface protein 2 plus CS protein. Science.
572 1991 May 3;252(5006):715-8.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

573 37. Noe AR, Espinosa D, Li X, Coelho-Dos-Reis JG, Funakoshi R, Giardina S, et al. A full-
574 length Plasmodium falciparum recombinant circumsporozoite protein expressed by
575 Pseudomonas fluorescens platform as a malaria vaccine candidate. PLoS One.
576 2014;9(9):e107764.

577 38. Oyen D, Torres JL, Wille-Reece U, Ockenhouse CF, Emerling D, Glanville J, et al.
578 Structural basis for antibody recognition of the NANP repeats in Plasmodium falciparum
579 circumsporozoite protein. Proc Natl Acad Sci U S A. 2017 Nov 28;114(48):E10438-E45.

580 39. Triller G, Scally SW, Costa G, Pissarev M, Kreschel C, Bosch A, et al. Natural Parasite
581 Exposure Induces Protective Human Anti-Malarial Antibodies. Immunity. 2017 Dec
582 19;47(6):1197-209 e10.

583 40. Fisher CR, Sutton HJ, Kaczmarski JA, McNamara HA, Clifton B, Mitchell J, et al. T-
584 dependent B cell responses to Plasmodium induce antibodies that form a high-avidity
585 multivalent complex with the circumsporozoite protein. PLoS Pathog. 2017
586 Jul;13(7):e1006469.

587 41. Imkeller K, Scally SW, Bosch A, Marti GP, Costa G, Triller G, et al. Antihomotypic affinity
588 maturation improves human B cell responses against a repetitive epitope. Science. 2018
589 Jun 22;360(6395):1358-62.

590 42. Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, et al.
591 Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of
592 malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nat Med. 1998
593 Apr;4(4):397-402.

594 43. Lee SM, Wu CK, Plieskatt J, McAdams DH, Miura K, Ockenhouse C, et al. Assessment
595 of Pfs25 expressed from multiple soluble expression platforms for use as transmission-
596 blocking vaccine candidates. Malar J. 2016 Aug 11;15(1):405.

597 44. Hjerrild KA, Jin J, Wright KE, Brown RE, Marshall JM, Labbe GM, et al. Production of full-
598 length soluble Plasmodium falciparum RH5 protein vaccine using a Drosophila
599 melanogaster Schneider 2 stable cell line system. Sci Rep. 2016 Jul 26;6:30357.

600 45. Rutgers T, Gordon D, Gathoye AM, Hollingdale M, Hockmeyer W, Rosenberg M, et al.
601 Hepatitis B Surface Antigen as Carrier Matrix for the Repetitive Epitope of the
602 Circumsporozoite Protein of Plasmodium Falciparum. Biotechnology (N Y). 1988
603 09/01/online;6:1065.

604 46. Jacobs E, Rutgers T, Voet P, Dewerchin M, Cabezon T, de Wilde M. Simultaneous
605 synthesis and assembly of various hepatitis B surface proteins in Saccharomyces
606 cerevisiae. Gene. 1989 Aug 15;80(2):279-91.

607 47. Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, et al. Safety,
608 immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum
609 circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J Infect Dis. 1995
610 Jun;171(6):1576-85.

611 48. Heppner DG, Jr., Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, et al.
612 Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria:

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

613 progress at the Walter Reed Army Institute of Research. Vaccine. 2005 Mar 18;23(17-
614 18):2243-50.

615 49. Song G, Springer TA. Structures of the Toxoplasma gliding motility adhesin. Proc Natl
616 Acad Sci U S A. 2014 Mar 17;111:4862-7.

617 50. Bushkin GG, Ratner DM, Cui J, Banerjee S, Duraisingh MT, Jennings CV, et al.
618 Suggestive evidence for Darwinian Selection against asparagine-linked glycans of
619 Plasmodium falciparum and Toxoplasma gondii. Eukaryot Cell. 2010 Feb;9(2):228-41.

620 51. Zhou T, Doria-Rose NA, Cheng C, Stewart-Jones GBE, Chuang GY, Chambers M, et al.
621 Quantification of the Impact of the HIV-1-Glycan Shield on Antibody Elicitation. Cell
622 reports. 2017 Apr 25;19(4):719-32.

623 52. Dubrovskaya V, Guenaga J, de Val N, Wilson R, Feng Y, Movsesyan A, et al. Targeted
624 N-glycan deletion at the receptor-binding site retains HIV Env NFL trimer integrity and
625 accelerates the elicited antibody response. PLoS Pathog. 2017 Sep;13(9):e1006614.

626 53. Datta D, Bansal GP, Kumar R, Ellefsen B, Hannaman D, Kumar N. Evaluation of the
627 Impact of Codon Optimization and N-Linked Glycosylation on Functional Immunogenicity
628 of Pfs25 DNA Vaccines Delivered by In Vivo Electroporation in Preclinical Studies in
629 Mice. Clin Vaccine Immunol. 2015 Sep;22(9):1013-9.

630 54. Stowers AW, Chen Lh LH, Zhang Y, Kennedy MC, Zou L, Lambert L, et al. A
631 recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys
632 from a lethal challenge with Plasmodium falciparum. Proc Natl Acad Sci U S A. 2002 Jan
633 8;99(1):339-44.

634 55. Mizutani M, Iyori M, Blagborough AM, Fukumoto S, Funatsu T, Sinden RE, et al.
635 Baculovirus-vectored multistage Plasmodium vivax vaccine induces both protective and
636 transmission-blocking immunities against transgenic rodent malaria parasites. Infect
637 Immun. 2014 Oct;82(10):4348-57.

638 56. Feng J, Dong X, Pinello J, Zhang J, Lu C, Iacob RE, et al. Fusion surface structure,
639 function, and dynamics of gamete fusogen HAP2. eLife. 2018 Oct 3;7.

640 57. Fedry J, Forcina J, Legrand P, Pehau-Arnaudet G, Haouz A, Johnson M, et al.
641 Evolutionary diversification of the HAP2 membrane insertion motifs to drive gamete
642 fusion across eukaryotes. PLoS Biol. 2018 Aug;16(8):e2006357.

643 58. Angrisano F, Sala KA, Da DF, Liu Y, Pei J, Grishin NV, et al. Targeting the Conserved
644 Fusion Loop of HAP2 Inhibits the Transmission of Plasmodium berghei and falciparum.
645 Cell reports. 2017 Dec 5;21(10):2868-78.

646 59. Fedry J, Liu Y, Pehau-Arnaudet G, Pei J, Li W, Tortorici MA, et al. The Ancient Gamete
647 Fusogen HAP2 Is a Eukaryotic Class II Fusion Protein. Cell. 2017 Feb 23;168(5):904-15
648 e10.

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

649 # Supporting information

650 S1 Text. Methods for generation and characterization of monoclonal antibodies to *P.*
651 *falciparum* TRAP

652 S2 Text. Supplemental figure legends.

653 S3 Fig s1. Antibody epitope mapping.

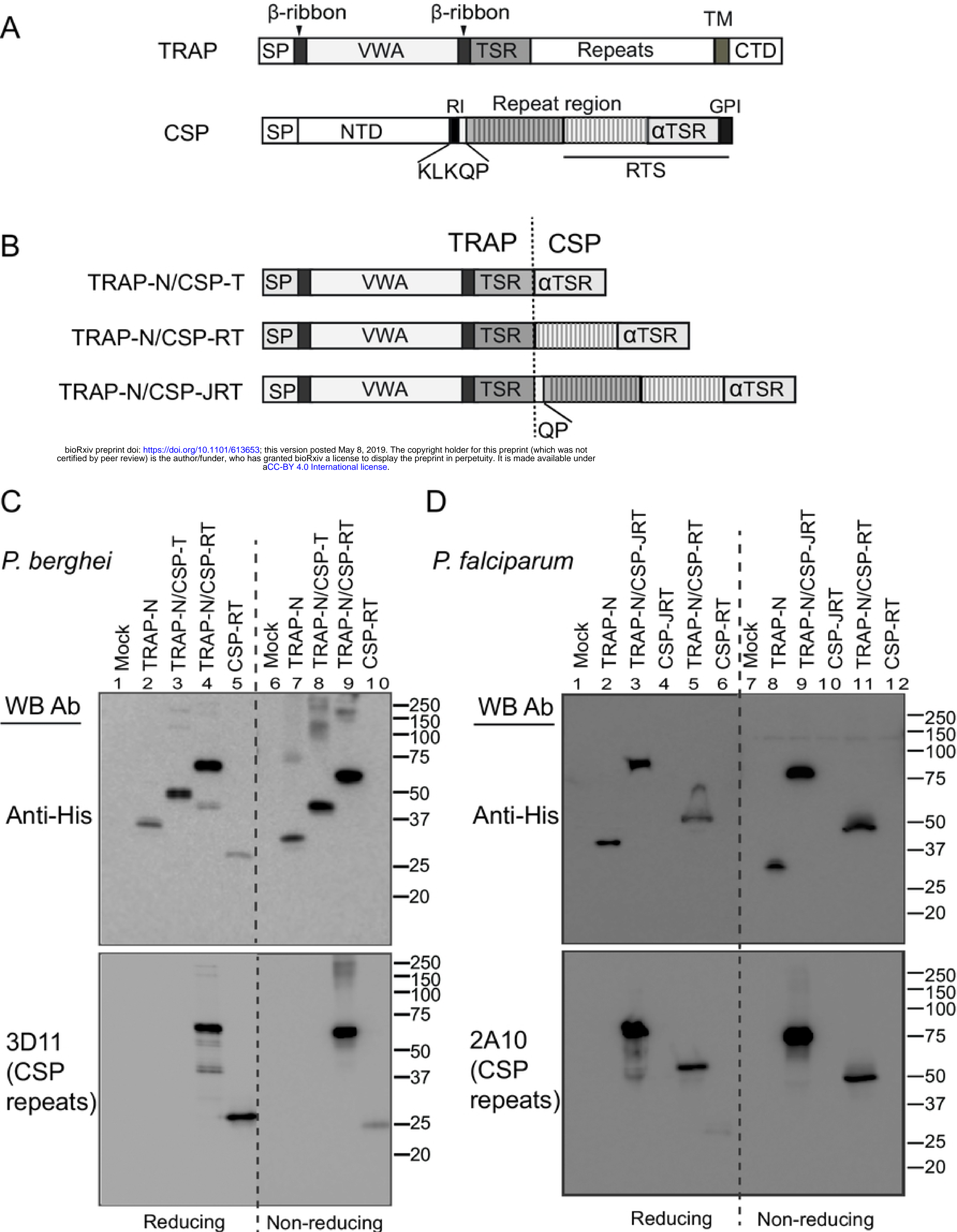
654 S4 Fig s2. Flow cytometry analysis of antibody binding to TRAP transfectants.

655 S5 Fig s3. Antibody staining of sporozoites.

656 S6 Fig s4. Antibody competition.

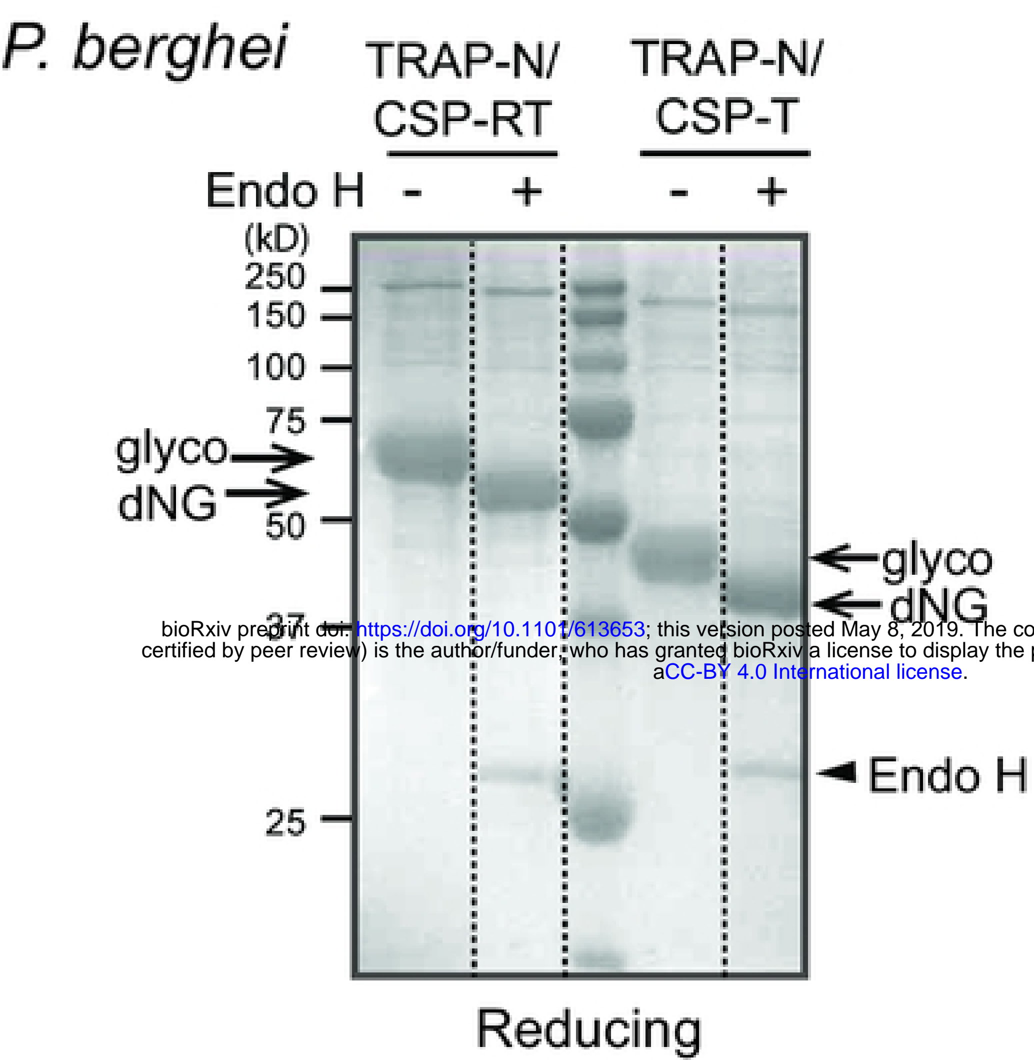
657 S7 Fig s5. Effect of antigen disulfide reduction on antibody reactivity.

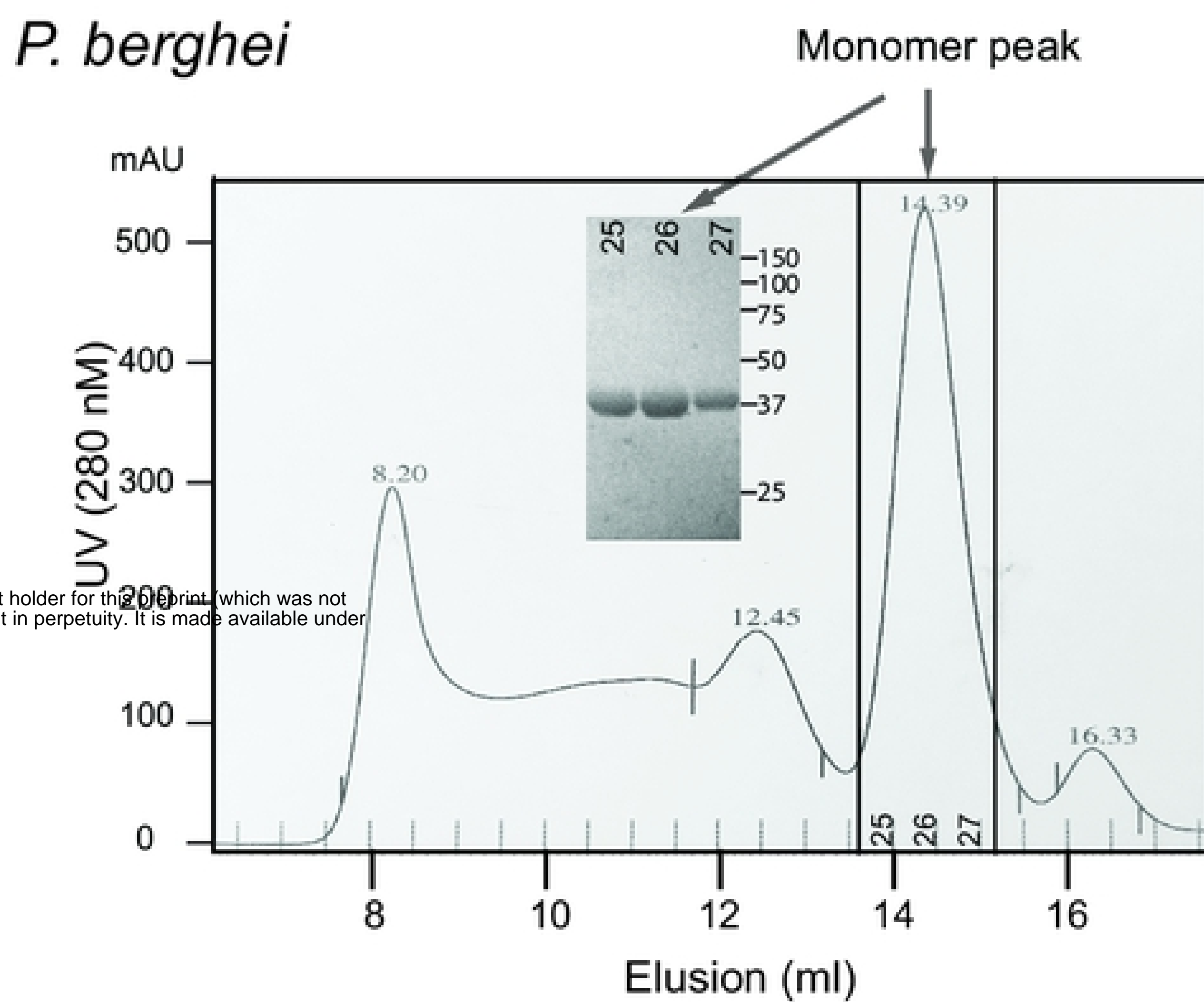
658 S8 Table s1. Summary of antibodies to P. falciparum TRAP



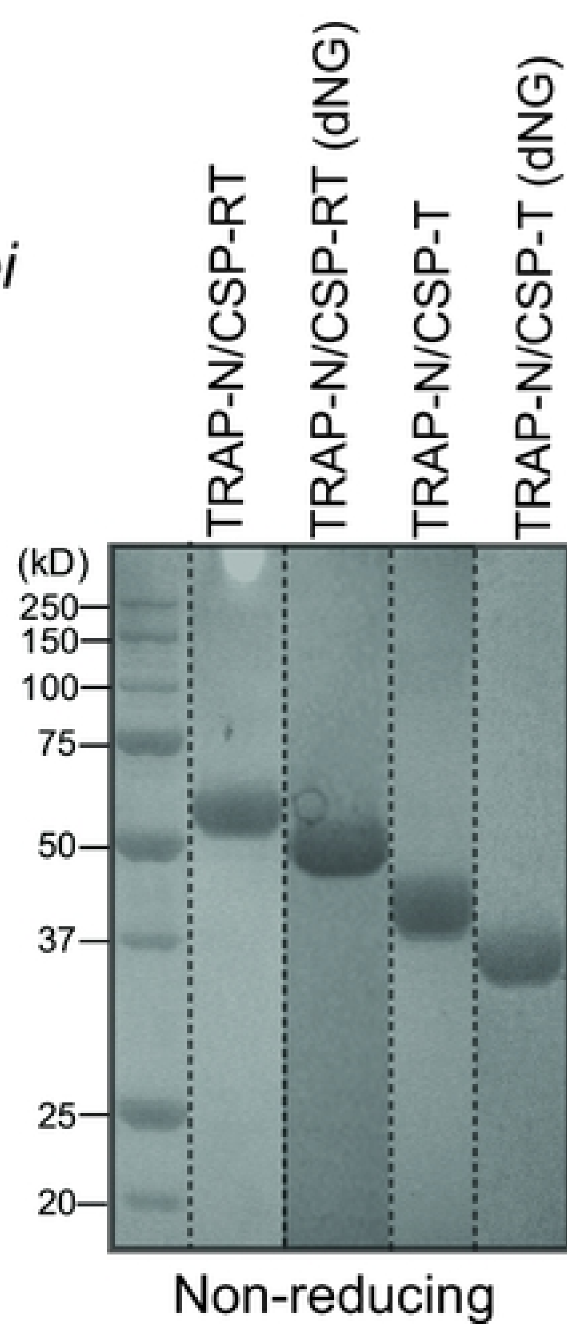
bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figure 1





bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



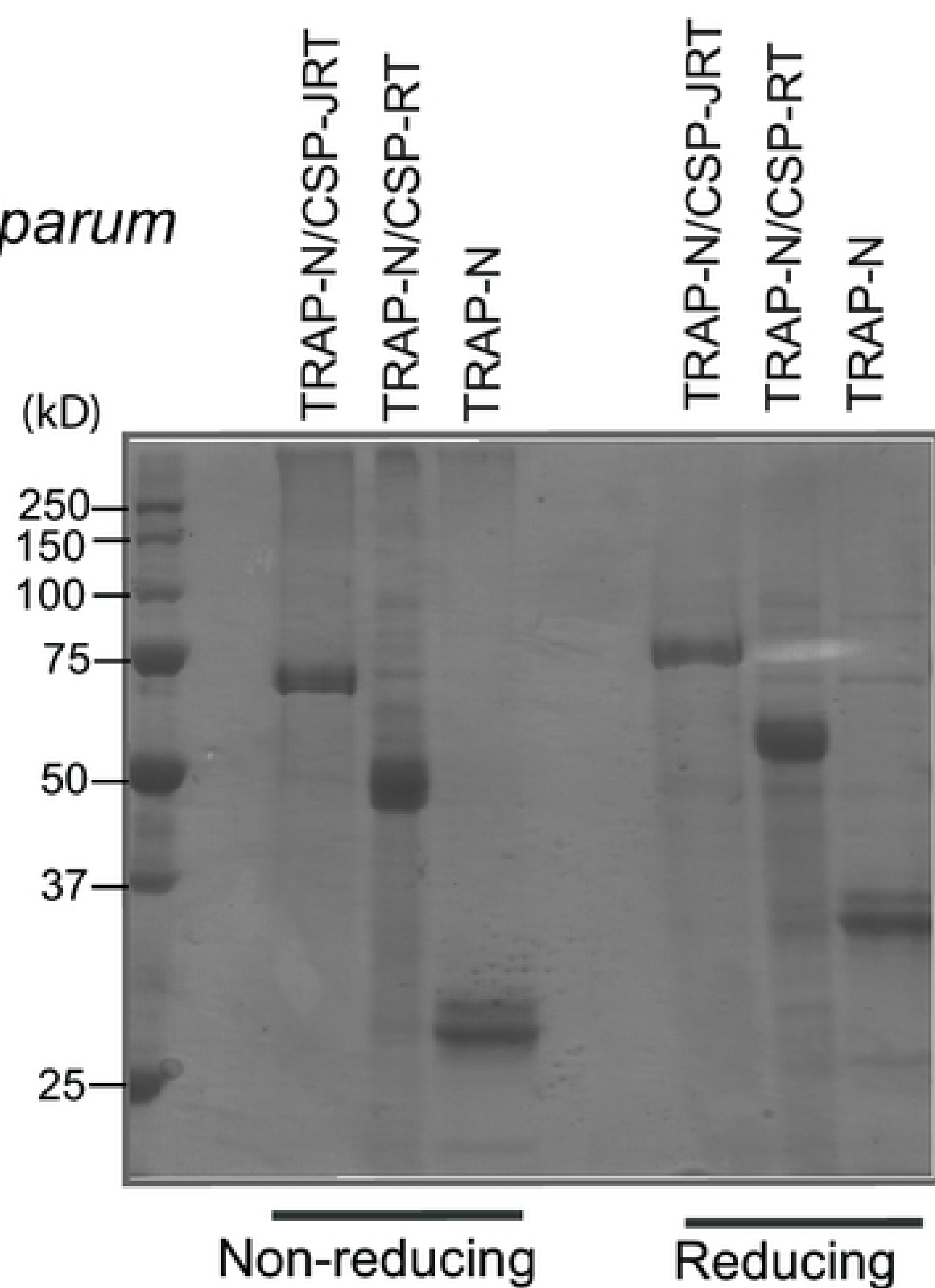


Figure 2

A

TRAP-N
TRAP-N/CSP-JRT
TRAP-N/CSP-RT

Absorbance
2.0
1.5
1.0
0.5
0.0

CL2/1
CL5/27
CL5/22
CL5/42
CL5/46
CL5/54
2A10

PfTRAP (VWA)
PfTRAP (TSR+β-ribbon)
PfCSP (repeats)

bioRxiv preprint doi: https://doi.org/10.1101/613653; this version posted May 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

B

TRAP-N/CSP-JRT
TRAP-N/CSP-RT

Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Anti-His
2A10
4B3
4C2
1G12

PfCSP (αTSR)

Figure 3

Fig 4

A

TRAP-N/CSP-RT titer (x10^4)
6
5
4
3
2
1
0
**
*
ns
ns
*
*
ns
ns
ns
ns
6 weeks
16 weeks
32 weeks
Time post antigen priming

P. berghei fusion antigen
TRAP-N/CSP-RT
TRAP-N/CSP-RT (dNG)
TRAP-N/CSP-T
TRAP-N/CSP-T (dNG)

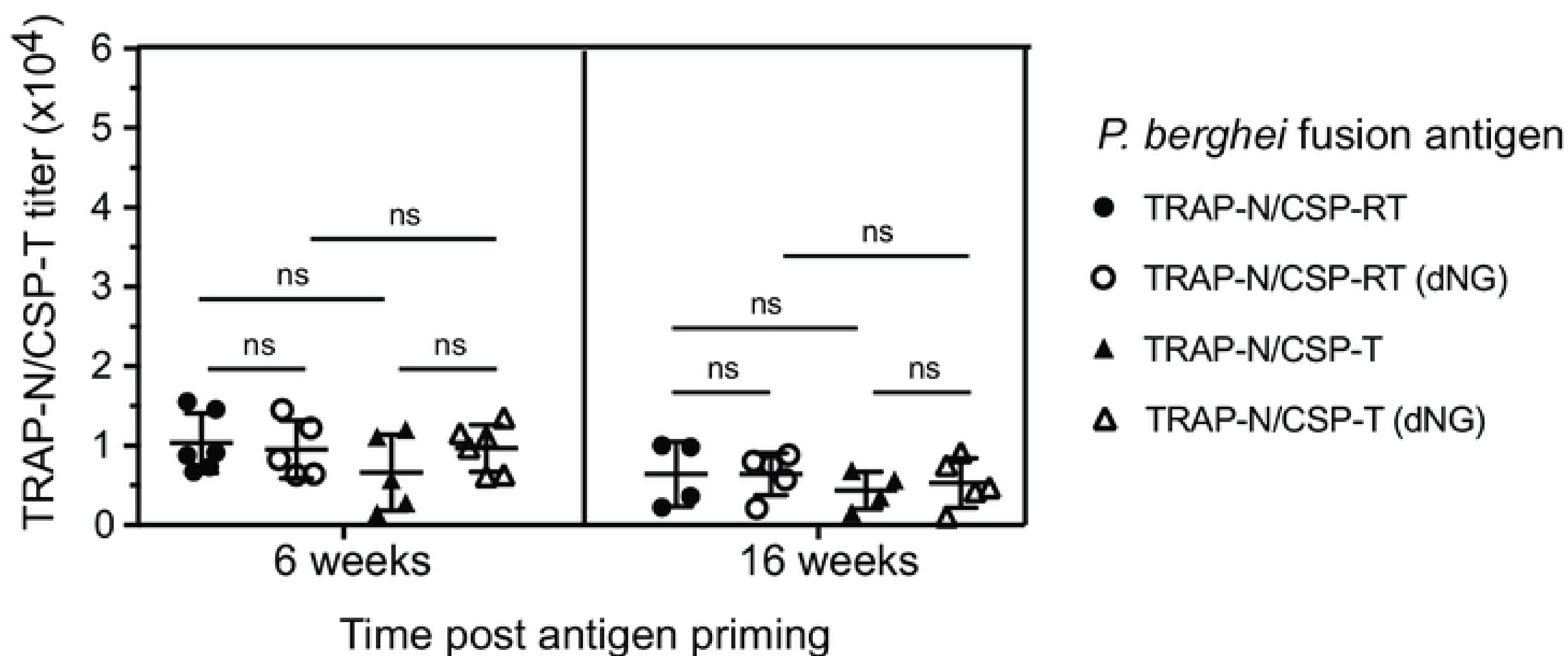


Figure 4