Mapping of safe and early chemo-attenuated live *Plasmodium falciparum* immunization identifies immune signature of vaccine efficacy

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Abstract

Potent protection against malaria can be induced by attenuated live-immunization with *Plasmodium falciparum* (Pf) sporozoites (SPZ). However, a better understanding of the critical processes involved in the establishment of protective immunity is needed. We explored the safety and vaccine efficacy of early chemo-attenuation of PfSPZ under atovaquone-proguanil (AP). AP caused early arrest of *P. berghei* liver stages. Despite the absence of replication, robust protection in mice correlated with parasite-specific effector-memory $CD8^+$ T-cell responses. In a phase I clinical trial a single dose of AP prevented Pf infections in the liver of adult, human subjects who received three doses of $5.12x10^4$ or $1.5x10^5$ PfSPZ by direct venous inoculation combined with oral AP. However, only 2 of 8 (25%) and 2 of 10 (20%), respectively, were protected against controlled human malaria infection (CHMI) 10 weeks after the last vaccine dose, despite levels of IgG antibodies to the Pf circumsporozoite protein (PfCSP) comparable to those achieved in fully protected volunteers after immunization with $5.12x10^4$ PfSPZ with chloroquine chemoprophylaxis active only against subsequent blood stages. We identify lower IgG recognition of the secreted liver stage-specific antigens LISP2 and LSA1 and the multi-stage antigen MSP5 as immune signatures of inferior vaccine efficacy compared to PfSPZ with chloroquine chemoprophylaxis. In conclusion, we show that immune signatures of liver stage antigens, but neither an established rodent malaria model nor concentrations of antibodies against the major surface protein of sporozoites, permit prediction of vaccine efficacy. Thus, this study provides a clear rationale for the development of live sporozoite vaccination protocols that boost exposure to Pf liver stage antigens.

56 **Significance Statement**

57

58 Our research demonstrates that attenuation of liver infection of high doses of *Plasmodium*
59 *falciparum* sporozoites by concomitant single-dose administration of atovaquone-proguanil is
60 safe in humans. However, vaccine efficacy was modest when compared to an identical protocol
61 using chloroquine that acts only on the subsequent blood infection. Immune signatures of
62 secreted *P. falciparum* liver stage antigens, but neither an established rodent malaria model nor
63 concentrations of sporozoite antibodies, permit prediction of vaccine efficacy.

64 **Introduction**

65 An unprecedented global effort to fight malaria in all subtropical and tropical regions of the
66 world has considerably reduced the disease burden. Progress, however, has stalled at an
67 estimated 200 million cases and 450,000 deaths globally per year (1). Further advances towards
68 elimination are likely to be facilitated only by an efficient vaccine that complements the existing
69 conventional control measures such as vector control, long-lasting impregnated bednets and
70 access to rapid diagnosis and chemotherapy (2).

71 Malaria is caused by single-cell eukaryotes of the genus *Plasmodium*. Morbidity and
72 mortality result from a rapid, asexual expansion phase in the blood. In the case of *Plasmodium*
73 *falciparum* (Pf), which accounts for nearly all malaria-related deaths, blood stage parasite biomass
74 can be substantial and can reach up to 10^{12} infected red blood cells. Asexual blood stage infection,
75 however, is preceded by a small mosquito-transmitted inoculum of ~10-400 Pf sporozoites (SPZ),
76 which specifically invade hepatocytes and replicate therein (3). Because this 1-week liver phase
77 of infection (i) is clinically silent and (ii) represents a life-cycle bottleneck, it provides an early target
78 for a malaria vaccine that would prevent blood stage infection with malaria parasites and thereby
79 prevent all disease and transmission stages (4-9).

80 Currently there is no vaccine against malaria parasites or any other eukaryotic human
81 pathogen, which has received marketing authorization (licensure) by the European Medicines
82 Agency (EMA) or the U.S. Food and Drug Administration (FDA). However, a partially effective
83 sub-unit vaccine against malaria (RTS,S/AS01E) (10) has received a favorable scientific opinion
84 by the EMA regarding its quality, safety and short-term efficacy (11).

85 Superior vaccine efficacy (VE) against Pf malaria as compared to results reported for
86 subunit vaccines has been demonstrated by intravenous inoculation of radiation-attenuated,
87 aseptic, purified, cryopreserved PfSPZ, Sanaria® PfSPZ Vaccine (6, 12-15), and intravenous
88 inoculation of infectious, aseptic, purified, cryopreserved PfSPZ (Sanaria® PfSPZ Challenge) with

89 concomitant chemoprophylaxis with chloroquine, Sanaria® PfSPZ-CVac (CQ) (7, 16). Importantly,
90 initial results indicate higher, per-sporozoite VE of PfSPZ-based vaccine protocols that rely on late
91 chemo-attenuation such as those using the chemoprophylactic drug chloroquine (CQ) which is
92 active only against blood stage parasites (7, 17). Based on this, it has been postulated that the
93 parasite expansion in the liver substantially boosts vaccine potency (7, 17), compared to radiation-
94 attenuated parasites that undergo developmental arrest soon after hepatocyte invasion. This
95 strategy however requires that adequate drug concentrations are maintained in the blood beyond
96 the liver stage period in order to kill the parasites when they emerge from the liver, e.g., by weekly
97 administration of the antimalarial drug chloroquine (CQ). It also entails the safety risk of exposing
98 the vaccinee to transient parasitemia and mild symptoms of malaria 7-9 days after immunization.
99 To increase the safety margin of live-immunizations it would be preferable if the parasites never
100 emerged from the liver without compromising VE. Moreover, chemoprophylaxis that acts against
101 liver stages may substantially strengthen a vaccination regimen with concomitant administration
102 of PfSPZ and chemoprophylaxis and thus, would be a significant step towards a simplified,
103 pragmatic PfSPZ immunization protocol.

104 Here, we present the results of pre-clinical experiments and a clinical trial exploring the VE
105 and mechanism of protection of PfSPZ co-administered with the chemoprophylactic drug
106 combination atovaquone-proguanil (AP). This licensed drug combination was used because in
107 contrast to CQ, which kills Pf parasites only in infected red blood cells, AP also kills Pf parasites
108 in the liver before they can emerge into the bloodstream (18-20).
109

110 **Results**

111

112 **Exposure to atovaquone-proguanil leads to early *Plasmodium berghei* liver stage arrest**

113 To initiate our pre-clinical analysis, we determined the *in vitro* effect of a single dose of
114 atovaquone (A) alone or AP when added to cultured hepatoma cells together with *Plasmodium*
115 *berghei* (Pb) sporozoites (Fig. 1A). Quantification of liver stage volume, morphology, and number
116 revealed small, developmentally arrested liver stages (Fig. 1A, Fig. S1B). Notably, A- and AP-
117 treated parasites persisted for several days, indicative of developmental arrest rather than parasite
118 death (Fig. S1A). Hepatocyte invasion appeared to be unaffected by drug exposure since the
119 number of intracellular liver stages was indistinguishable from untreated controls (Fig. S1C).
120 Causal prophylactic activity was confirmed in a murine malaria model (Fig. 1B, C, D).
121 Administration of a single dose of 3 mg/kg atovaquone plus 1.2 mg/kg proguanil or 3 mg/kg
122 atovaquone alone in C57BL/6 mice inoculated with 10^4 Pb sporozoites prevented blood stage
123 infection (Fig. 1B). Quantitative RT-PCR (qRT-PCR) analysis of steady-state levels of Pb18S
124 rRNA and, for comparison, mouse *GAPDH* mRNA in infected livers 42h after Pb sporozoite and
125 drug co-administration (Fig. 1C, D) revealed a >1,000-fold reduced liver stage load in drug-treated
126 mice, in good agreement with our *in vitro* data (Fig. 1A, Fig. S1). Furthermore, when Pb
127 sporozoites were pre-exposed to AP on ice for 2h, all C57BL/6 mice that received 10^4 treated
128 (n=5) or untreated Pb sporozoites (n=3) developed blood stage infection after three days,
129 confirming earlier results of no direct effect on sporozoites (21). Thus, exposure to atovaquone or
130 atovaquone-proguanil exerts prophylactic activity against Pb liver stage parasites, but not Pb
131 sporozoites, allowing maximum hepatocyte invasion followed by robust early liver stage arrest.

132

133

134 **Pb sporozoite immunization under atovaquone (-proguanil) cover induces sterile**
135 **protection and robust IFNγ-secreting CD8$^+$ CD11a$^+$ T-cell responses**

136 As proof of concept, we next immunized three groups of C57BL/6 mice with 10^4 Pb
137 sporozoites and concomitant administration of A or AP at weekly intervals (Fig. 2). Mice were
138 challenged 3-4 weeks after the last immunization dose by intravenous (i.v.) inoculation of 10^4 Pb
139 sporozoites. Three immunizations at weekly intervals with 10^4 Pb sporozoites and atovaquone
140 resulted in sterile protection in all (6/6) mice. Remarkably, only two immunizations with Pb
141 sporozoites and either A or AP still elicited sterile protection in 88% (15/17) of mice and a
142 substantial delay to blood infection in the two mice that were not protected (Fig. 2A). We
143 independently confirmed our findings by quantification of liver stage parasites by qRT-PCR (Fig.
144 2B). After sporozoite challenge parasite liver loads were reduced by at least two orders of
145 magnitude in immunized animals as compared to controls ($p<0.05$) (Fig. 2B). Potential residual
146 effects of drug treatment that could have interfered with assessment of vaccine efficacy (VE) were
147 ruled out in an independent experiment (Fig. S2).

148 We next quantified signatures of effector-memory CD8$^+$ T-cell responses that correlate
149 with protection against challenge with wild-type sporozoites (22, 23) by measuring IFNγ-secretion
150 of CD8$^+$ CD11a$^+$ T-cells after stimulation with the peptides SSP2/TRAP$_{130-138}$ and S20$_{318-326}$, which
151 are recognized in immunized H2-K^b –restricted C57BL/6 mice (24, 25) (Fig. 2C, Figs. S3 and S4).
152 Mice were immunized with two doses of Pb sporozoites and AP or A, and lymphocytes were
153 isolated from spleens three or four weeks after the last immunization, respectively. We detected
154 high levels of antigen-specific IFNγ secretion after both immunization protocols (Fig. 2C). Total
155 numbers of effector memory CD8$^+$ CD62L$^-$ T-cells were also enhanced after immunization (Fig.
156 S4A).

157 Exposure to sporozoite inoculations activates antibody-producing B cells (26-28).
158 Accordingly, the immunization protocols also induced high (~1:10^4) anti-Pb sporozoite antibody
159 titers (Fig. 2D).

160 In conclusion, the preclinical data demonstrate high chemoprophylactic efficacy of AP
161 leading to reliable, early arrest of liver stage development when co-administered with Pb
162 sporozoites. Co-administered AP with intravenous Pb sporozoites as part of an immunization
163 protocol, resulted in parasite-specific effector-memory $CD8^+$ T-cell responses and robust
164 protection against challenge infections. Of note, vaccine efficacy was markedly superior to another
165 early-arrest protocol with primaquine and indeed, comparable to the most potent chemo-
166 attenuation protocols tested so far (29), prompting the design of a clinical trial.

167

168 **Clinical trial of PfSPZ-CVac (atovaquone-proguanil)**

169 Based on the positive pre-clinical results we conducted a randomized, double-blind,
170 placebo-controlled clinical trial of PfSPZ-CVac (AP) from September 2016 to November 2017 at
171 the Institute for Tropical Medicine in Tübingen, Germany (Malaria controlled human infection trial
172 E, MALACHITE; ClinicalTrials.gov Identifier: NCT02858817). The study population was selected
173 to represent healthy, malaria-naïve volunteers aged 18–45 years from Tübingen and the
174 surrounding area. In total, 30 volunteers (15 per dosage/group) were enrolled; 15 in Group A
175 (PfSPZ reference dose) and 15 in Group B (PfSPZ 3-fold higher dose). They were randomly
176 allocated to receive three injections of Sanaria® PfSPZ Challenge (aseptic, purified,
177 cryopreserved PfSPZ of the NF54 strain, n=10 in each group) or normal saline placebo (n=5 in
178 each group) per dosage group (30, 31).

179 In Group A, participants received three doses of 5.12×10^4 PfSPZ by direct venous
180 inoculation (DVI) at 4-week intervals and oral administration of a single dose of AP (1,000 mg/400
181 mg) within one hour before each PfSPZ inoculation. In Group B, participants received 1.5×10^5

182 PfSPZ with the same schedule and chemoprophylactic regimen. Ten weeks after last
183 immunization, participants in both groups underwent controlled human malaria infection (CHMI)
184 by DVI of $3.2x10^3$ PfSPZ of PfSPZ Challenge (NF54).

185 Twenty-seven participants were included in the per-protocol analysis. Three withdrawals
186 occurred, all of them in Group A before CHMI; one requested by the participant and two
187 withdrawals by the investigators based on non-compliance or non-availability for critical study
188 procedures. An additional participant in Group A was lost to follow-up after CHMI and was included
189 in the per-protocol analysis. This volunteer developed parasitemia and started treatment on day
190 12 post-CHMI. Following successful completion of antimalarial treatment, thick blood smear (TBS)
191 and qPCR were negative on day 21 post-CHMI. On day 22 post-CHMI the volunteer was not
192 reachable and refused further follow-up visits. The study flow chart is shown in Fig. S5. Baseline
193 population characteristics are detailed in Table S1.

194 Importantly, during immunization no breakthrough parasitemia by qPCR occurred,
195 demonstrating robust causal prophylactic activity of a single-dose of 1,000 mg/400 mg AP, even
196 with an inoculum of $1.5x10^5$ PfSPZ. This sporozoite dose greatly exceeds the infective dose of
197 $3.2x10^3$ PfSPZ (31, 32) by ~50 times and is estimated to be equivalent to the bites of ~ 200 infected
198 mosquitoes. Of note, safety and tolerability during immunization were similar between placebo
199 controls and vaccinees (Table S1).

200 Upon challenge by CHMI, 6 out of 8 vaccinees in group A and all 4 placebo recipients
201 developed Pf parasitemia (VE, 25%; 95% CI, -12%-50%). Due to the low efficacy and according
202 to protocol, no heterologous repeat CHMI was performed. Subsequently, Group B underwent
203 homologous CHMI with PfSPZ Challenge (NF54), i.e., with the vaccine strain only. In Group B, 8
204 of 10 vaccinees and all 5 placebo controls developed Pf parasitemia (VE, 20%; 95% CI, -9%-
205 41%). Moreover, time to qPCR detectable parasitemia, a marker for partial efficacy, was similar

206 between the groups (Group A; median 7 days; IQR 7-8.5 and Group B; median 7 days; IQR 7-7.5;
207 Kruskal-Wallis H test, p=0.27) (Fig. 3 and Fig. 4).
208

209 **Anti-PfCSP antibodies generated by early arrest of *Pf*SPZ-CVac immunization**

210 The 25% and 20% VE observed after 3 doses of $5.12x10^4$ or $1.5x10^5$ PfSPZ of PfSPZ-
211 CVac (AP), respectively, contrasts sharply with the 100% VE we achieved with 3 doses of
212 $5.12x10^4$ PfSPZ of PfSPZ-CVac (CQ)(7). To better understand the immunological basis for these
213 differences in VE, we first analyzed the levels of IgG antibodies generated by vaccination against
214 the Pf circumsporozoite protein (PfCSP) two weeks after the 3rd dose of vaccine and just prior to
215 CHMI (Fig. 5). The median serum dilution corresponding to an optical density of 1.0, termed net
216 OD 1.0, two weeks after the last dose of PfSPZ-CVac (AP) was 300 (range, 80 to 4,000) for the
217 8 subjects who underwent CHMI in vaccine Group A and 3,800 (range 1,400 to 67,100) for the 9
218 subjects who underwent CHMI in the previous PfSPZ-CVac (CQ) study (7), which used the same
219 dose of $5.12x10^4$ PfSPZ (Table S3)($p < 0.05$, Wilcoxon-Mann-Whitney U Test, 2-tailed).

220 This >10-fold reduction in anti-PfCSP antibody levels in the PfSPZ-CVac (AP) group was
221 unexpected. Since we can firmly exclude batch-to-batch variation during the manufacturing
222 process by GMP-mandated quality control procedures, which includes quantification of sporozoite
223 numbers, motility and invasion capacity, this finding is consistent with a scenario, in which the
224 immune systems of subjects immunized with PfSPZ-CVac (AP) are exposed to less PfCSP than
225 those immunized with PfSPZ-CVac (CQ).

226 Next we assessed anti-PfCSP antibodies in the higher dose group. Increasing the three
227 PfSPZ doses to $1.5x10^5$ PfSPZ of PfSPZ-CVac (AP) significantly increased median anti-PfCSP
228 antibodies two weeks after the 3rd immunization to 7,300 (range, 1,100 to 20,000, $p < 0.01$, Mann-
229 Whitney U Test, 2-tailed) (Fig. 5, Table S3). However, despite the > 20-fold increase in PfCSP
230 antibodies in group B there was no increase in VE. We note that the VE of 20% was significantly

231 lower than the 100% VE after immunization with $5.12x10^4$ PfSPZ of PfSPZ-CVac (CQ) ($p<0.001$,
232 Barnard's test, 2-tailed). The correlation between antibody concentration prior to CHMI and
233 inoculum were similar (Table S3). Strikingly, the higher levels of anti-PfCSP antibody
234 concentrations in Group B were not predictive of VE after CHMI (Fig. 5). These results are
235 consistent with an anti-PfCSP antibody-independent mechanism of protection after immunization
236 with PfSPZ-CVac (CQ).
237

238 **Profiling of IgG antibody responses to sporozoite, liver stage, and asexual blood stage**
239 **antigens**

240 We hypothesized that reduced protection with PfSPZ-CVac (AP) compared to PfSPZ-
241 CVac (CQ) was due to the early arrest of liver stage development by AP. This pharmaceutical
242 arrest is expected to result in significantly reduced exposure to liver stage and blood antigens.
243 To this end, we probed a representative range of IgG antibody responses with a custom protein
244 microarray (33). We detected a striking absence of a few distinct antibody responses in
245 volunteers who received PfSPZ-CVac (AP) compared to PfSPZ-CVac (CQ) (ref.), while the
246 breadth and intensity of immunoreactivity to 216 other antigens was indistinguishable (Fig. 6A,
247 B). In perfect agreement with the ELISA results (Fig. 5A), IgG responses to *Pf*CSP were
248 considerably higher in volunteers with PfSPZ-CVac (AP) compared to PfSPZ-CVac (CQ) (Fig. 5
249 and Fig. 6C), likely reflecting higher exposure to sporozoites in the high-dose group of PfSPZ-
250 CVac (AP) ($1.5x10^5$ vs $5.12x10^4$, respectively). Most importantly, IgG antibody responses to the
251 two well-known secreted liver stage antigens LISP2 and LSA1 were significantly reduced in
252 recipients of PfSPZ-CVac (AP) (Fig. 5B, C and Supplementary Table S4). Together, our IgG
253 profiling data shows that inferior vaccine efficacy of the PfSPZ-CVac (AP) protocol correlates
254 with low responses to liver stage antigens, whereas superior IgG responses to *Pf*CSP are not
255 predictive of protection.

256 **Discussion**

257 Intra-host replication is a key feature of many live attenuated vaccines. This expansion
258 correlates with improved protective immunity against natural exposure (34). For instance, the
259 Sabin poliomyelitis vaccine proved that a polio virus strain capable of replicating in the gut but not
260 the nervous system can generate robust neutralizing anti-viral immunity (34). Similarly, it has been
261 proposed that intra-host replication of live, attenuated, PfSPZ-based vaccines might substantially
262 boost the per-parasite VE (5, 7, 16, 35-38).

263 The current benchmark in humans for highly protective malaria vaccines is immunization
264 with whole PfSPZ, which relies on radiation-, chemo- or genetic attenuation of live, metabolically
265 active PfSPZ (38-41). Immunization with radiation-attenuated (6) and chemo-attenuated (7)
266 PfSPZ vaccines has induced robust and sustained VE in humans against CHMIs for up to 14
267 months (14, 15) and natural exposure to Pf in the field for at least 6 months (13). Vaccine
268 development has, however, mostly been empirical, and a better understanding of the critical
269 processes involved in the establishment of protective immunity is needed to guide further
270 development of this vaccine strategy.

271 In Pf, the initial massive (20,000-40,000 fold), but clinically silent, liver stage expansion of
272 parasite biomass and antigenic breadth provides ample opportunities for arresting infections prior
273 to the subsequent pathogenic blood stage. Here, we tested the potential of an attenuation protocol
274 based on AP, a licensed antimalarial drug combination with liver stage activity used for the
275 treatment and chemoprophylaxis of Pf malaria. We proposed that this drug partner would improve
276 the *in vivo* chemo-attenuation strategy significantly because: i) chemoprophylaxis would be
277 administered only concomitantly with PfSPZ increasing safety and practicality, whereas CQ
278 prophylaxis starts prior to PfSPZ injection and is continued with weekly doses until after the last
279 vaccination; ii) there would be no egress of the parasites from the liver, and thus, no possibility of
280 malaria symptoms associated with transient asexual erythrocytic stage parasitemia, which occur

281 with doses beyond 10^5 PfSPZ; and iii) AP is better tolerated and safer than 4-aminoquinolines,
282 such as CQ.

283 We demonstrated in the murine malaria model that atovaquone alone and AP led to a
284 complete early arrest of liver stage development. Compared to untreated controls drug-exposed
285 intrahepatic Pb parasites did not begin to replicate as evidenced by single-nucleated liver stage
286 forms observed *in vitro* and by >100-fold reduced parasite liver loads. Of importance, this robust
287 liver arrest was confirmed in the subsequent clinical trial, in which a single dose of AP completely
288 prevented blood stage infections after each of three immunizations with $1.5x10^5$ PfSPZ of PfSPZ
289 Challenge.

290 Previously, full protection was elicited by three doses of $5.12x10^4$ PfSPZ and chemo-
291 attenuation with CQ (7), which only kills Pf parasites that have emerged from the liver and have
292 commenced subsequent intraerythrocytic replication. In stark contrast, only 2 out of 8 volunteers,
293 who received three doses of $5.12x10^4$ PfSPZ with AP chemoprophylaxis, were protected against
294 CHMI with $3.2x10^3$ *Pf*SPZ of *Pf*SPZ Challenge. Even a 3-fold increase in the PfSPZ numbers per
295 dose to three doses of $1.5x10^5$ PfSPZ failed to induce significant protection despite the robust
296 dose-dependency of PfSPZ-based vaccines (6, 7). Levels of anti-PfCSP antibodies on the day of
297 CHMI in volunteers immunized with three doses of $1.5x10^5$ PfSPZ were at least as high as levels
298 induced by three immunizations with $5.12x10^4$ PfSPZ in the PfSPZ-CVac (CQ) group previously
299 reported (7). In marked contrast, we observed a considerable reduction of IgG antibody responses
300 to the two well-characterized liver stage antigens LISP2 and LSA1. Even though these lower
301 antibody responses may mostly reflect reduced exposure, and not necessarily a protective
302 mechanism, in the PfSPZ-CVac (AP) group compared to the PfSPZ-CVac (CQ) group, it is
303 tempting to speculate that T-cell dependent antibody responses could be a surrogate of cellular
304 immunity. Importantly, unlike our earlier study (7), the comparison of similar PfSPZ doses of
305 PfSPZ-CVac (AP) and PfSPZ-CVac (CQ) allowed us to entangle the effects of sporozoite dose

306 and liver stage maturation, resulting in a clear separation of responses that appear to reflect
307 sporozoite exposure (anti-PfCSP IgG) from responses associated with biological processes
308 critical for protection (anti-LISP2 and anti-LSA1 IgG). Taken together, our data indicate that, high-
309 level VE (≥80%) 10 weeks after the last immunizing dose induced by immunization with 3 doses
310 of $5.12x10^4$ to $1.5x10^5$ infectious PfSPZ under chemoprophylaxis appears to depend on intra-
311 hepatic replication of PfSPZ.

312 We cannot formally exclude that AP has heretofore undefined immunosuppressive activity
313 in humans as reported for CQ (42). We consider this notion unlikely since over more than 20 years
314 of clinical use of AP no such evidence has been reported. The observed discrepancy between the
315 VE achieved with sporozoite immunization under AP in the murine and human studies (full *vs.*
316 modest protection, respectively) is likely related to the shorter duration of the Pb liver stage (~2
317 days) compared to the Pf liver stage (~7 days). We also noted a largely reduced anti-PfCSP
318 antibody response in) group A vaccinees in comparison to an identical ($5.12x10^4$) PfSPZ dose in
319 PfSPZ-CVac (CQ) vaccinees (7). A plausible explanation is that PfCSP is produced and acts as
320 an immunogen not only in extracellular PfSPZ but also throughout Pf liver stage development,
321 coherent with robust CSP expression during liver stage maturation in murine malaria models (43,
322 44). Accordingly, the full liver stage maturation achieved by PfSPZ-CVac (CQ) immunization as
323 compared to lack of maturation after PfSPZ-CVac (AP) immunization is a likely cause for the
324 marked differences in anti-PfCSP antibody levels after immunization with the same total number
325 of PfSPZ.

326 We propose that our results indicate an essential role for intrahepatic replication, either
327 through increased amounts of antigen and/or increased numbers of antigens, for achieving
328 maximum protection against CHMI. Furthermore, protection appears to be unrelated to the high
329 anti-PfCSP antibody levels that have been observed previously after high PfSPZ dose
330 immunizations (7). This lack of correlation with antibody responses is supported by data presented

here and in previous murine studies showing that the protection induced by radiation-, chemo-, and genetically -attenuated SPZ immunization is strictly dependent on $CD8^+$ T-cells (5, 45, 46). Although direct activity of human $CD8^+$ T-cells against Pf liver stages in humans has not been demonstrated, our data thus support the hypothesis that cellular immune mechanisms are central to VE of chemo-attenuated PfSPZ vaccines. Further immunological studies are warranted to elucidate the mechanistic basis and identify immune correlates of vaccine-induced protection in humans. It is, of course, conceivable that dependency on intra-host replication can be overcome by administering even higher doses of PfSPZ Challenge with AP, as it has for radiation-attenuated PfSPZ (6, 12, 14, 15), but clinical evaluation of live parasite immunization strategies that combine safe parasite attenuation with superior VE should remain a research priority.

In conclusion, we have demonstrated that generation of full protection against homologous CHMI in humans at an immunizing dose of 5.12×10^4 to 1.5×10^5 PfSPZ of PfSPZ Challenge appears to depend on intra-host expansion of Pf, which correlated with an immune signature of anti-liver stage responses. Neither an established rodent malaria model nor anti-PfCSP antibody levels were predictive of sterilizing immunity.

347 **Methods**

348

349 In vitro *and animal experiments*

350 All animal work was conducted in accordance with the German Animal Welfare Act
351 (Tierschutzgesetz in der Fassung vom 18. Mai 2006, BGBl. I S. 1207), which implements the
352 directive 86/609/EEC from the European Union and the European Convention for the protection
353 of vertebrate animals used for experimental and other scientific purposes. The protocol was
354 approved by the ethics committee of MPI-IB and the Berlin state authorities (LAGeSo Reg#
355 G0469/09, G0294/15). Female C57BL/6 and NMRI mice at the age of 6 to 8 weeks were
356 purchased from Charles River (Sulzfeld, Germany) for sporozoite injections and blood stage
357 passages, respectively.

358

359 *Plasmodium berghei* parasites

360 For all experiments *P. berghei* (Pb) ANKA clone 507, which constitutively expresses the
361 green fluorescent protein (GFP) under control of the *eIF1α* promoter (47), was used. To generate
362 sporozoites, female *Anopheles stephensi* mosquitoes were infected by a bloodmeal on Pb-
363 infected NMRI mice. Starting 17 days after infection, salivary glands were hand-dissected from
364 infected mosquitoes, gently ground, and sporozoites harvested after centrifugation. Freshly
365 dissected Pb sporozoites (10^4) were added to complete DMEM medium (10%FCS, 1% Pen/Strep)
366 containing atovaquone (0.2 μM; Wellvone® Suspension, 750 mg/5ml; GlaxoSmithKline) or
367 Malarone® (0.2 μM atovaquone, 0.08 μM proguanil hydrochloride; GlaxoSmithKline) or the
368 equivalent amounts of DMSO (0.01%) as negative control. Irradiated Pb sporozoites and
369 untreated Pb sporozoites served as control. Sporozoites were added to cultured Huh7 cells in
370 duplicates. Hepatoma cells were incubated for one hour at 37°C for sporozoite sedimentation and
371 for additional 2 hours to permit sporozoite entry. Infected cultures were subsequently washed

372 repeatedly with DMEM complete medium to remove residual drug. Next, infected cells were
373 incubated in complete DMEM medium for 48 h, 69 h or 114 h at 37°C before fixation in 4% para-
374 formaldehyde. Fixed cells were stained with a monoclonal anti-PbHSP70 antibody (48) to visualize
375 parasites, with a rabbit anti-PbUIS4 serum (49) to visualize the parasitophorous vacuole, and with
376 Hoechst 33342 (Invitrogen) that stains nuclei. As secondary antibodies goat Alexa Fluor 488–
377 labeled antibody to mouse immunoglobulin G (IgG) and goat Alexa Fluor 546–labeled antibody to
378 rabbit immunoglobulin G (IgG) (Invitrogen) were used. Image analysis and quantification was
379 performed by fluorescence microscopy using either a Leica DM2500 or a Zeiss Axio Vision
380 microscope. Images were processed with Fiji (Image J, NIH, Bethesda, USA).
381

382 P. berghei *sporozoite infection, immunization, and challenge experiments*

383 To test whether single dose administrations of atovaquone or atovaquone-proguanil prevent
384 a subsequent Pb blood stage infection and thus, life-threatening pathology, C57BL/6 mice were
385 intravenously (i.v.) infected with 10^4 Pb sporozoites, and one dose of drug was co-administered
386 intraperitoneally (i.p.). Drug doses were 3 mg/kg atovaquone alone or 3 mg/kg atovaquone plus
387 1.2 mg/kg proguanil hydrochloride. Three days later infections were monitored daily by
388 microscopic examination of Giemsa-stained blood films. To test toxicity of atovaquone-proguanil
389 on sporozoites, sporozoites were treated with 1 μM atovaquone-0.4 μM proguanil-hydrochloride
390 in complete DMEM for 2 hours and then washed with RPMI followed by inoculation of 10^4 treated
391 Pb sporozoites to naïve mice. For quantification of pre-erythrocytic parasite development by qRT-
392 PCR livers were removed 42 hours after sporozoite infection and transferred to TRIZOL® for RNA
393 isolation. Complementary DNA (cDNA) synthesis and qRT-PCR were done as described
394 previously (*4*). Briefly, the mean C_t value of the *Pb*18S ribosomal subunit RNA (*18SrRNA*; gene
395 ID: 160641) was normalized to the mean C_t of mouse *GAPDH* mRNA values (gene ID: 281199965)
396 using the $\Delta\Delta C_t$ method. qPCR experiments were performed with the ABI 7500 sequence detection

397 system and done in triplicates. For immunizations, female C57BL/6 mice were inoculated twice or
398 three times at seven-day intervals with 10^4 Pb salivary gland sporozoites i.v. together with one
399 dose of drug administered i.p. as described for the chemoprophylaxis studies. Three to five weeks
400 after the last immunization mice were challenged by intravenous injection of 10^4 Pb salivary gland
401 sporozoites. Naïve, age-matched mice served as infection controls. For determination of sterile
402 protection, blood parasitemia was monitored by microscopic examination of Giemsa-stained blood
403 films daily from day 3 until day 20. Alternatively, the parasite liver load after challenge was
404 quantified by qRT-PCR.
405
406 *Anti-sporozoite antibody titers*
407 For titration of *P. berghei*-specific antibodies in mouse serum, salivary gland-associated
408 sporozoites were deposited on glass slides, air-dried and fixed in aceton. After rehydration in PBS
409 and blocking, mouse serum was titrated by serial dilutions and bound antibodies detected with a
410 secondary goat anti-mouse Alexa Fluor 546-coupled antibody (1:1,000). Nuclei were stained with
411 Hoechst 33342 (1:1,000).
412
413 *Anti-CSP antibody titers*
414 IgG antibodies to the Pf circumsporozoite protein (PfCSP) were assessed by enzyme linked
415 immunosorbent assay (ELISA) as previously described (7). 96-well plates (Nunc Maxisorp
416 Immuno Plate) were coated overnight at 4 °C with 2.0 μg of the recombinant *P. falciparum* (Pf)
417 circumsporozoite protein (rPfCSP, Lot#122006) protein in 50 μL coating buffer (KPL) per well in
418 coating buffer (KPL). Plates were washed three times with 2 mM imidazole, 160 mM NaCl, 0.02%
419 Tween 20, 0.5 mM EDTA and blocked with 1% Bovine Serum Albumin (BSA) blocking buffer (KPL)
420 containing 1% non-fat dry milk for 1 h at 37 °C. Plates were washed three times and serially diluted
421 serum samples (in triplicate) were added and incubated at 37 °C for 1 h. After three washes,

422 peroxidase labelled goat anti-human IgG (KPL) was added at a dilution of 0.1 μg/mL and
423 incubated at 37 °C for 1 h. Plates were washed three times, ABTS peroxidase substrate was
424 added for plate development, and the plates were incubated for 75 mins at 22°C room
425 temperature. The plates were read with a Spectramax Plus384 microplate reader (Molecular
426 Devices) at 405 nm. The data were collected using Softmax Pro GXP v5 and fit to a 4-parameter
427 logistic curve, to calculate the serum dilution at OD 1.0. A negative control (pooled serum from
428 non-immune individuals from a malaria-free area) was included in all assays. Serum from an
429 individual with anti-PfCSP antibodies for PfCSP was used as positive control.
430
431 *Protein microarray and analysis*
432 Microarrays were produced at the University of California Irvine, Irvine, California, USA (33). In
433 total, 262 Pf proteins were expressed using an *E. coli* lysate *in vitro* expression system and spotted
434 on a 16-pad ONCYTE AVID slide, representing 228 important Pf antigens known to frequently
435 appear in sterile and naturally acquired immunity against the parasite (50, 51).
436 For the detection of binding antibodies, secondary IgG antibody (goat anti-human IgG QDot™800,
437 Grace Bio-Labs #110635), secondary IgM antibody (biotin-SP-conjugated goat anti-human IgM,
438 Jackson ImmunoResearch #109-065-043) and Qdot™585 streptavidin conjugate (Invitrogen
439 #Q10111MP) were used. Study serum samples as well as a European control serum pool were
440 diluted 1:50 in 0.05X Super G Blocking Buffer (Grace Bio-Labs, Inc.) containing 10% *E. coli* lysate
441 (GenScript, Piscataway, NJ) and incubated for 30 minutes on a shaker at room temperature (RT).
442 Meanwhile, microarray slides were rehydrated using 0.05X Super G Blocking buffer at RT.
443 Rehydration buffer was subsequently removed and samples added onto the slides. Arrays were
444 incubated overnight at 4°C on a shaker (180 rpm). Serum samples were removed the following
445 day and microarrays were washed using 1X TBST buffer (Grace Bio-Labs, Inc.). Secondary
446 antibodies were then applied at a dilution of 1:200 and incubated for two hours at RT on the shaker,

447 followed by another washing step and a one-hour incubation in a 1:250 dilution of Qdot™585
448 streptavidin conjugate. After a final washing step, slides were dried by centrifugation at 500 g for
449 10 minutes. Slide images were taken using the ArrayCAM® Imaging System (Grace Bio-Labs)
450 and the ArrayCAM 400-S Microarray Imager Software. Microarray data was analyzed in R
451 statistical software package version 3.6.2. All images were manually checked for any noise signal.
452 Each antigen spot signal was corrected for local background reactivity by applying a normal-
453 exponential convolution model (52) using the "saddle" -algorithm for parameter estimation
454 (available in the limma package v3.28.14) (53). Data was $\log_2$-transformed and further normalized
455 by subtraction of the median signal intensity of mock expression spots on the particular array to
456 correct for background activity of antibodies binding to *E. coli* lysate. Differential antibody levels in
457 the different allocated study outcomes (placebo, non-protected and protected vaccinees) were
458 detected by Student's t-test. Antigens with $p<0.05$ and a fold change>2 of mean signal intensities
459 were defined as differentially recognized between the tested sample groups.
460
461 *Quantification of antigen-specific CD8⁺ T-cell responses*
462 For $CD8^+$ T-cell stimulations followed by intracellular cytokine staining (ICS), splenic
463 lymphocytes were stimulated with 10 μM SSP2/$TRAP_{130-138}$ or $S20_{318-326}$ peptides (24) for 5 hours
464 at 37°C in the presence of brefeldin A (1:1,000). Cells were stained with fluorescently-labeled anti-
465 mouse CD8 [53-6.7], CD62L [MEL14], or CD11a [M17/4] antibodies (eBioscience). Following
466 fixation with 4% paraformaldehyde, cells were stained intracellularly with fluorescently-labeled
467 anti-mouse IFN-γ [R4-6A2] antibody (eBioscience) in permeabilization buffer (BD Bioscience).
468 Antibodies were incubated 60 min at 4°C. After washing and transfer to 1% paraformaldehyde/
469 PBS cells were quantified using a LSRII flow cytometer (BD Bioscience). Data analysis was
470 performed using FlowJo (Tree Star Inc., Oregon, USA).
471

472 *Clinical trial*

473 This trial was approved by the ethics committee of the Eberhard Karls University and the
474 University Hospital Tübingen as well as by the Paul Ehrlich Institute (Langen, Germany) and the
475 Regional Council (Regierungspräsidium Tübingen). The study was compliant with the
476 International Council for Harmonisation Good Clinical Practice guidelines and the German
477 Medicinal Product Act (Arzneimittelgesetz, AMG). The trial was registered at ClinicalTrials.gov
478 (NCT02858817).

479 This single center, double-blind, randomized, placebo-controlled phase 1 clinical trial was
480 conducted from September 2016 to November 2017 at the Institute of Tropical Medicine in
481 Tübingen, Germany. The study population was selected to represent healthy, malaria-naïve
482 adults. Volunteers aged 18–45 years from Tübingen and surrounding area with a body-mass index
483 (BMI) between 18 kg/m² and 30 kg/m² were included. Female participants were required to
484 practice effective contraception and to provide a negative pregnancy test. Further inclusion criteria
485 included: being reachable at all times by mobile phone during the whole study, agreement to share
486 medical information about the volunteer with his or her general practitioner, and understanding of
487 study procedures and risks, assessed by a quiz. Additionally, willingness to undergo CHMI with
488 PfSPZ Challenge, to take a curative regimen of antimalarial if necessary, and the ability to comply
489 with all study requirements (in the investigator's opinion) were also required.

490 Exclusion criteria were: a history of malaria or plans to travel to endemic regions during the
491 study, receiving any investigational product in another clinical trial within 90 days before enrolment
492 or planned receipt during the study, previous participation in a malaria vaccine trial, history of
493 serious psychiatric conditions, convulsions, or severe head trauma, any malignancy, and diabetes
494 mellitus. Moreover, falling in moderate risk or higher categories for fatal or non-fatal cardiovascular
495 event within 5 years (54), prolonged QTc interval (>450 ms), or any other clinically significant
496 abnormalities in the electrocardiogram, breast feeding, or intention to become pregnant, HIV,

497 hepatitis B or C virus infection, alcohol or drug abuse, any suspected immunodeficient state,
498 history of splenectomy, and haemoglobinopathies also prevented participation. A complete list of
499 eligibility criteria is available as an online supplement. Eligibility criteria were assessed after written
500 informed consent was given.

501 Fifteen volunteers per group were enrolled and randomized to receive Sanaria® PfSPZ
502 Challenge for immunization or normal saline placebo with an allocation ratio of 2:1 for
503 vaccine:placebo. In Group A, participants received 5.12×10^4 PfSPZ of PfSPZ Challenge (NF54)
504 by DVI three times at four week intervals and a single dose of AP (1,000 mg/400 mg) administered
505 orally within one hour before each immunization. In Group B participants received 1.5×10^5 PfSPZ
506 of PfSPZ Challenge (NF54) by DVI with the same scheduling and chemoprophylactic regimen.

507 Ten weeks after the last immunization, the first CHMI was performed in both groups for
508 vaccine efficacy (VE) testing. CHMI was done by DVI of 3.2×10^3 PfSPZ of PfSPZ Challenge
509 (NF54). Active follow-up of the participants was conducted through 56 days after the injection of
510 PfSPZ Challenge for CHMI. The protocol stipulated two successive CHMIs, the first at 10 weeks
511 and the second at 16-44 weeks. The sequence of CHMI was planned to be PfSPZ Challenge
512 (NF54, homologous clone) followed by PfSPZ Challenge (7G8, heterologous clone) for Group A.
513 The sequence for Group B was to be based on VE following first CHMI in Group A: NF54 followed
514 by 7G8 when VE against homologous CHMI was <75%, 7G8 followed by 7G8 when VE was ≥75%.
515 Due to the low efficacy of the first CHMI also in group B a second CHMI was not performed in
516 Group B. First CHMI was thus performed with PfSPZ Challenge (NF54) for both groups.

517 PfSPZ Challenge (NF54) is comprised of aseptic, purified, cryopreserved NF54 PfSPZ,
518 produced by Sanaria Inc. (Rockville, US). PfSPZ Challenge was stored and transported in liquid
519 nitrogen vapor phase at -150 to -196°C. Formulation and reconstitution was made in Tübingen on
520 the day of infection. Volunteers were inoculated within 30 minutes after thawing of PfSPZ
521 Challenge. Sterile isotonic normal saline, identical in appearance to PfSPZ Challenge was used

522 as placebo. A volume of 0.5 mL of vaccine or placebo was injected into an arm vein by DVI through
523 a 25 gauge needle. After each immunization, participants were monitored for at least 60 minutes
524 before leaving the clinic for local and systemic adverse events. Participants were assessed on site
525 for safety and to measure parasitemia on days 1, 5, 7, 10, 14 and 21 after each immunization, and
526 on day 1, 6-21, 28, 56 after CHMI. Medically qualified study personnel were available continually
527 for unscheduled visits. Antimalarial treatment, according to the German guidelines (55) for the
528 treatment of uncomplicated Pf malaria (AP or artemether-lumefantrine as first-line drugs) was to
529 be initiated, in the event of breakthrough parasitemia with symptoms during immunization or in the
530 case of parasitemia following CHMI. Breakthrough parasitemia was defined as microscopically
531 detectable parasitemia during immunization with at least two symptoms consistent with malaria
532 for 2 days despite chemoprophylaxis. Protection was defined as the absence of parasites in the
533 peripheral blood for 28 days following CHMI. Parasitemia after CHMI was assessed on daily basis
534 from day 6 to day 21 and again on day 28 via thick blood smear (TBS) and quantitative PCR.
535 Treatment was administered upon occurrence of three consecutive positive PCR results one of
536 them at least 100 Pf parasites/ml from samples taken at least 12 hours apart or the first TBS
537 positivity. An additional follow-up visit for safety was conducted on day 56. Participants were
538 encouraged to immediately report adverse events between the scheduled follow-up visits.

539 If a volunteer was withdrawn from the study after receiving a dose of PfSPZ Challenge at
540 one or more of the three immunizations or at CHMI, a full, appropriate, curative course of
541 antimalarial therapy was administered.

542 In both groups 10 volunteers were randomly allocated to receive immunizations with PfSPZ
543 Challenge and 5 volunteers to receive normal saline placebo (PfSPZ Challenge (NF54):placebo
544 = 2:1). Group membership was allocated using a Mersenne-Twister random number generator
545 implemented in R. A third party outside the study team generated and distributed the
546 randomization list. A dedicated member of the formulation team, who was not involved in volunteer

547 management or diagnostic activities, kept the randomization envelopes and dosing schedule.

548 The primary aim of the study was to assess the safety and VE of repeated immunization by
549 DVI of PfSPZ Challenge under AP chemoprophylaxis in malaria naïve adults. The primary VE
550 endpoint was the proportion of protected volunteers.

551 Protection was defined as the absence of parasites in the peripheral blood for 28 days
552 following first CHMI with PfSPZ Challenge. To assess safety outcomes, Grade 3 and 4 adverse
553 events (AEs) and serious adverse events (SAEs) were captured from time of first administration
554 of A/P until the end of the study. Functional characterization of humoral and cellular immune
555 responses were exploratory endpoints.

556

557 *Statistical analysis and power calculation of clinical trial*

558 To be able to show, with a power of 80% and a two-tailed alpha of 5%, that 25% or less of
559 immunized volunteers and 95% of controls, allocated in a 2:1 ratio became infected by CHMI, 10
560 immunized and 5 placebo-treated volunteers per group were required. Hence, a total of 30 (10
561 each for 5.12×10^4 and 1.5×10^5 *Pf*SPZ Challenge (NF54) with AP and 10 placebo) volunteers were
562 required. The sample size was calculated using the nBinomial function in the gsDesign package.

563 No formal hypothesis testing was done for safety and tolerability data. Safety and tolerability
564 data are presented as descriptive analyses in listings and graphically. VE was calculated by
565 comparison of proportions between immunized and placebo-treated volunteers using an
566 unconditional exact test (Boschloo's test) and time-to-parasitemia using a log-rank (LR) test.
567 Multiple non-parametric group comparisons were performed by using the Kruskal-Wallis (KW) H
568 test. The level of significance was set at a two-tailed type 1 error alpha <5%. All statistical analyses
569 were performed using R version 3.4.4 and GraphPad Prism 5. Kaplan-Meier curves were
570 compared by a log-rank (Mantel Cox) test. Statistical significance of parasite sizes, qPCR data,
571 and flow cytometric analyses were assessed using the Mann-Whitney U test for nonparametric

572 test samples. P values of $P < 0.05$ were considered as significant.

573

574

575

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736 **Figure legends**

737

738 **Fig. 1. Early arrest of *Plasmodium berghei* liver stage development after co-administration**
739 **of live sporozoites and atovaquone or atovaquone-proguanil.**

740 (A) Composite fluorescence micrographs of *Plasmodium berghei* (Pb) liver stages in cultured
741 hepatoma cells. Shown are representative images of liver stages 48h after infection with
742 sporozoites. During the first 3h cultures were exposed to atovaquone (A), atovaquone-proguanil
743 (AP) or buffer only. Parasites were visualized by fluorescent staining of the cytoplasm (green; anti-
744 PbHSP70 antibody), the parasitophorous vacuolar membrane (red; anti-PbUIS4 anti-serum), and
745 nuclei (blue; Hoechst 33342). Scale bars: 10 μm.

746 (B) Kaplan-Meier analysis of the proportion of C57BL/6 mice that remained blood film-negative
747 after a single intravenous dose of 10^4 *P. berghei* sporozoites without drug (black line; n=5) or co-
748 administration of 3 mg/kg A (blue line) or 3/1.2 mg/kg AP (red line). Shown are cumulative data
749 from two (A co-administration) and five (AP co-administration) independent experiments ($n \geq 5$ mice
750 each).

751 (C, D) Liver parasite load 42h after infection of C57BL/6 mice with 10^4 sporozoites and co-
752 administration of (C) 3 mg/kg A (blue circles) or (D) 3/1.2 mg/kg AP (red circles) or no drug (white
753 circles). Shown are mean values (±S.D.) of relative RNA levels of Pb18S rRNA normalized to
754 mouse *GAPDH* ($n \geq 5$). **, $p < 0.01$ (Mann-Whitney U test)

755

756
757 **Fig 2. Robust protection against sporozoite challenge infections and antigen-specific**
758 **immune responses after sporozoite/atovaquone (-proguanil) immunization**

759 (A) Kaplan-Meier analysis of protection in mice immunized by co-administration of sporozoites
760 and a single dose of atovaquone (A; 3 mg/kg i.p.) or atovaquone-proguanil (AP; 3/1.2 mg/kg i.p.).
761 Mice were either immunized twice (AP co-administration, red line; n=11; A co-administration, blue

762 line; *n*=6) or three times (A co-administration, dark blue line; *n*=6). Naïve mice served as controls
763 (black line; *n*=14). Sporozoite challenge was done by i.v. injection of 10^4 sporozoites three to four
764 weeks after the last immunization.
765 (B) Quantification of parasite liver loads after challenge infection. Mice were immunized twice (A
766 co-administration, *n*=5, blue circles; AP co-administration; *n*= 5, red circles) and three times (A co-
767 administration, *n*= 6, dark blue circle) as in (B). Naïve mice served as controls (*n*=6 and 4,
768 respectively, white circles). Challenge infection was done by i.v. injection of 10^4 sporozoites at
769 least three weeks after the last immunization. Livers were harvested 42h later and parasite RNA
770 quantified by RT-PCR. Relative RNA levels of Pb18S rRNA were normalized to mouse *GAPDH*.
771 Shown are mean values (±S.D.). **: $p < 0.01$ (Mann-Whitney U test).
772 (C) Quantification of SSP2/$TRAP_{130-138}$ peptide-specific IFNγ-secretion by $CD8^+$ $CD11a^+$ T-cells
773 from spleens of immunized or control mice ($n \geq 5$ each). Shown are mean values (±S.D.). **, $p <$
774 0.01 (Mann-Whitney U Test).
775 (D) Quantification of anti-sporozoite antibody titers from serum of immunized or control mice ($n \geq 5$
776 each). Shown are mean values (±S.D.). **, $p < 0.01$(Mann-Whitney U Test).
777
778 **Fig. 3. qPCR-assessed Pf parasitemia kinetics in clinical trial participants after CHMI.**
779 Shown is the quantification of Pf blood stage parasite load (log_{10} per ml blood) by qPCR over time
780 after CHMI in the 9 individuals from the placebo control group (top), the 6 non-protected individuals
781 in group A (reference PfSPZ vaccine dose; center) and the 8 non-protected individuals in group B
782 (high PfSPZ vaccine dose; bottom). Curves in different colors depict parasite densities over time
783 in individual participants. Treatment was initiated upon reaching the pre-defined parasitemia
784 endpoint.
785
786

787 **Fig. 4. Time to patency after CHMI of participants in groups A and B.**
788 Shown are Kaplan-Meier curves of time to initiation of treatment upon reaching a pre-defined
789 parasitemia endpoint. CHMI was done at week 10 after the last immunization in all volunteers,
790 with the exception of one volunteer in Group A (verum) and B (placebo) each, who underwent
791 CHMI at 17 weeks and 14 weeks, respectively. Both were treated for blood infections on day 10
792 and 12 after CHMI and were included in the graph.
793
794 **Fig. 5. Antibodies to PfCSP 2 weeks after the third dose of vaccine in the current trial**
795 **(Malachite = PfSPZ-CVac [AP]) compared to data from a previous trial (TÜCHMI-002 =**
796 ***PfSPZ*-CVac [CQ]) (7).** Lines represent the median and 25th and 75th quartile levels. Filled circles
797 represent individuals, who did not develop Pf parasitemia (protected), and open circles represent
798 individuals who did develop Pf parasitemia (unprotected). Note that anti-PfCSP antibody levels
799 did not predict VE during CHMI. OD 1.0 is the serum dilution at which the optical density was 1.0.
800 Net OD 1.0 is the OD 1.0 2 weeks after the third dose of vaccine minus the OD 1.0 prior to
801 immunization.
802
803 **Fig. 6. Antibody response measured by protein microarrays comparing reactivities of the**
804 **two vaccination regimen PfSPZ-CVac (AP) and PfSPZ-CVac (CQ).** Sera from all volunteers
805 collected before immunization (baseline, D-1) and one day before challenge (C-1) were applied
806 on protein microarrays at a 1:50 dilution containing 262 Pf proteins representing 228 unique
807 antigens. Analysis was performed on C-1 data after subtraction of the individual baseline
808 reactivity. (A) Bar charts of mean reactivity in the two vaccine regimen, ordered by descending
809 signal intensities for PfSPZ-CVac (CQ) and subset of highest mean changes (> 2-fold change)
810 from both vaccine protocols. Array data were normalized, log_2-transformed and baseline
811 reactivity was subtracted. (B) Volcano plot to analyze differential immunoreactivity in the two

812 trials. Antigen reactivity in verum donors of PfSPZ-CVac [AP] (to the right) was compared to the
813 verum donors of PfSPZ-CVac [CQ] (dose 5.12×10^4 PfSPZ) (to the left). Differentially recognized
814 antigens (p value < 0.05 and fold change > 2) are depicted in red. (C) Box plot of signature IgG
815 responses in PfSPZ-CVac (AP) (stratified by vaccine dose) and PfSPZ-CVac (CQ) (dose
816 5.12×10^4 PfSPZ). Asterisks indicate statistical significance (Rolf/Freya: please indicate p values
817 or remove double and triple asterisks).

818 **Figure 1.**

819 **A**

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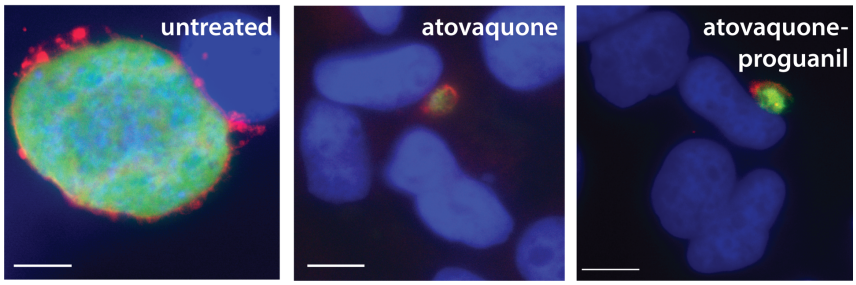

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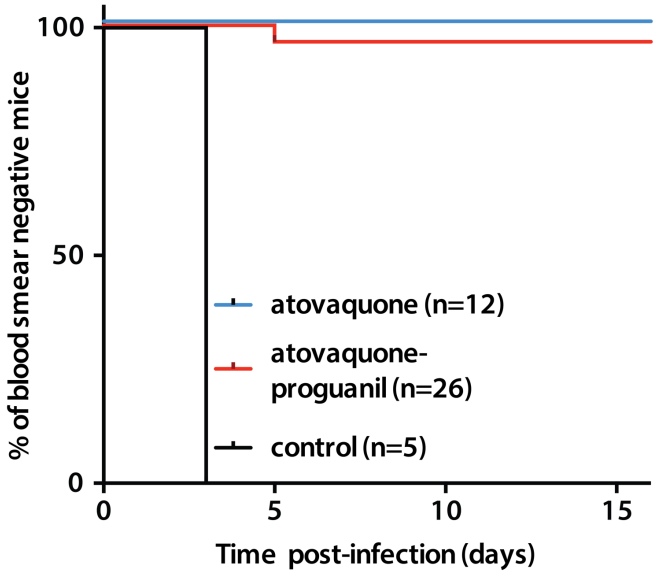

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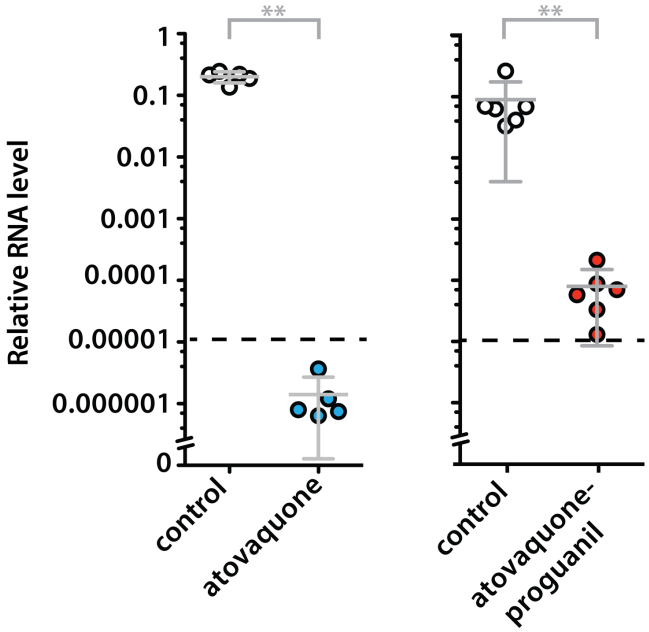

821 **Figure 2.**

822 **A**

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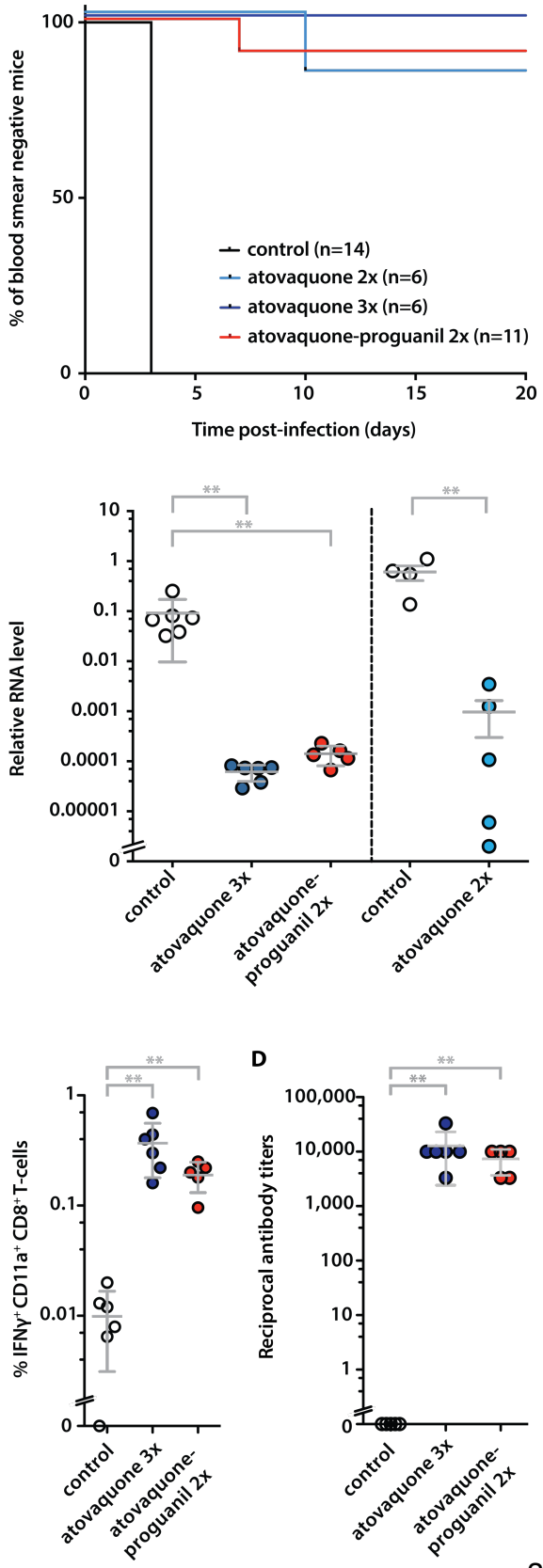

825 **Figure 3.**

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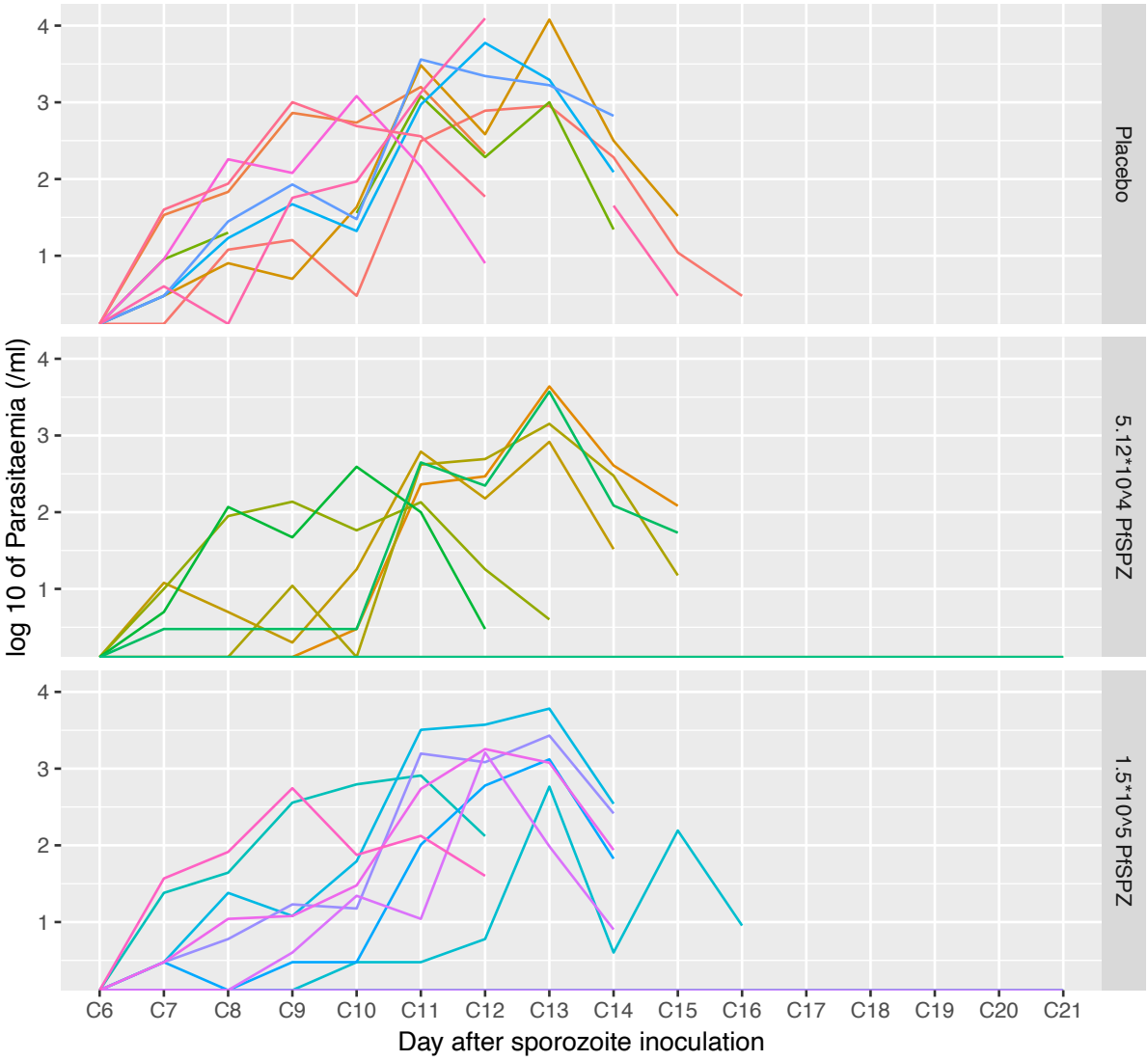

832 **Figure 4.**

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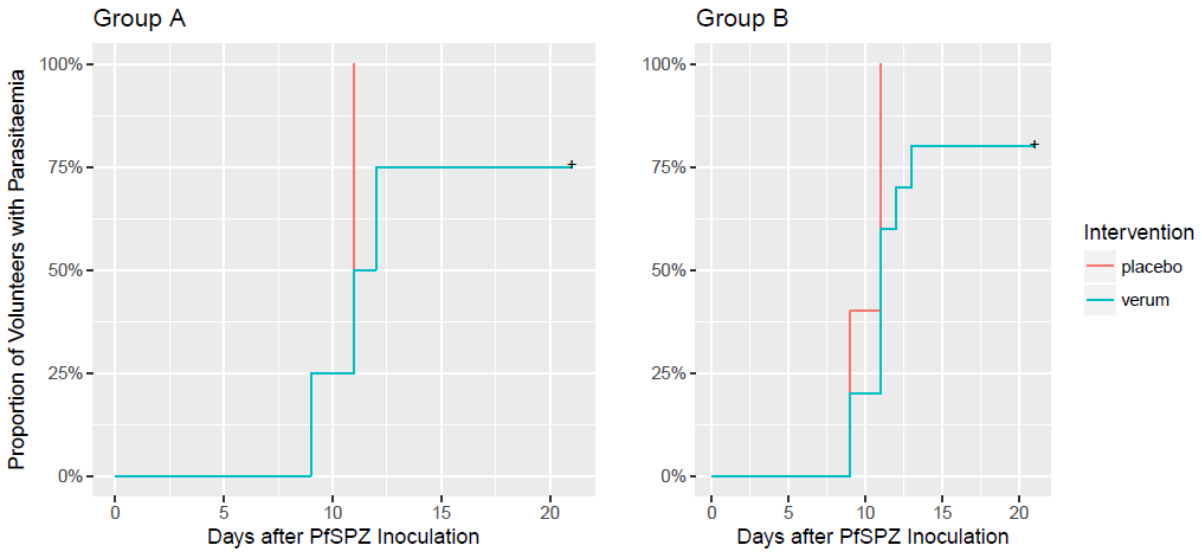

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836 **Figure 5.**

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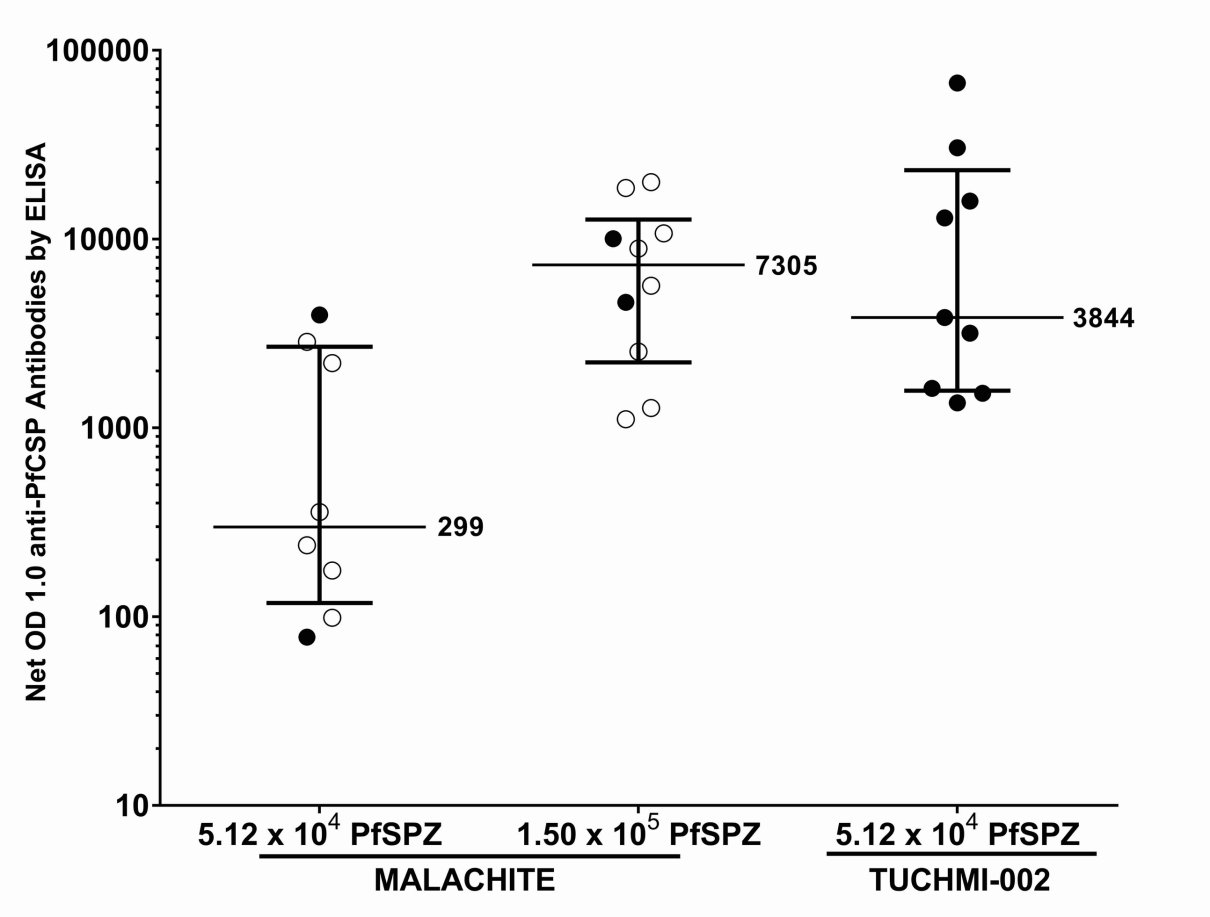

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846 **Figure 6A.**

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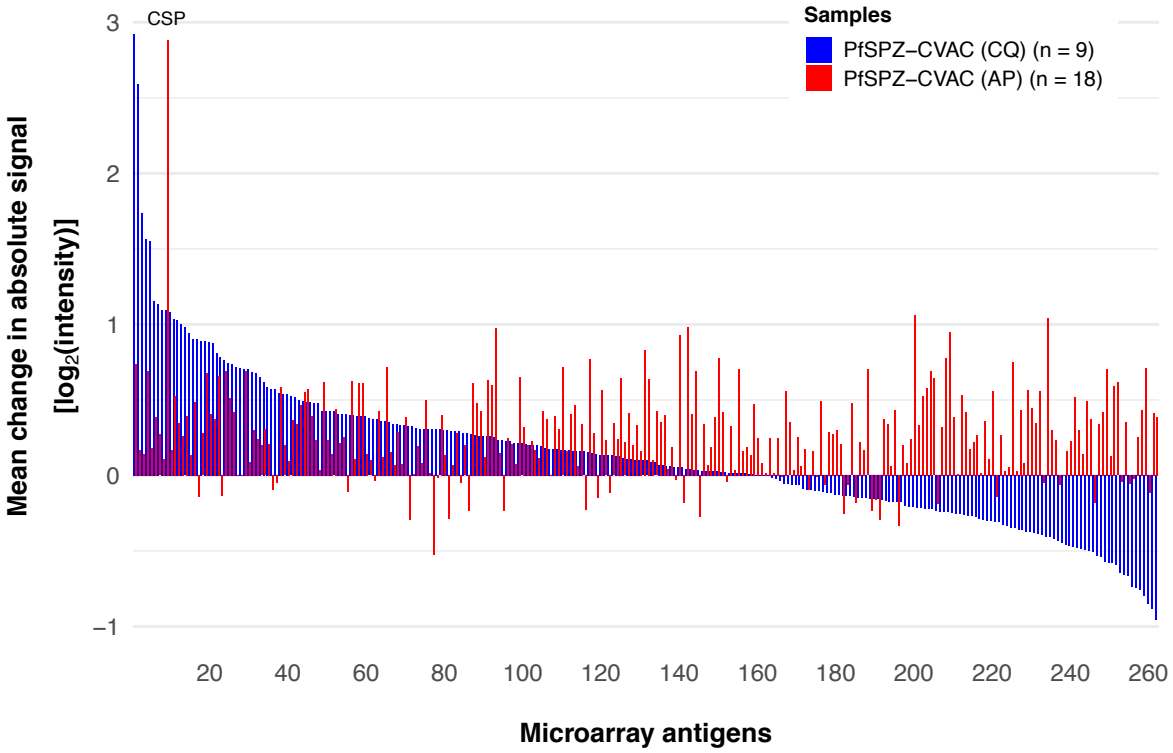

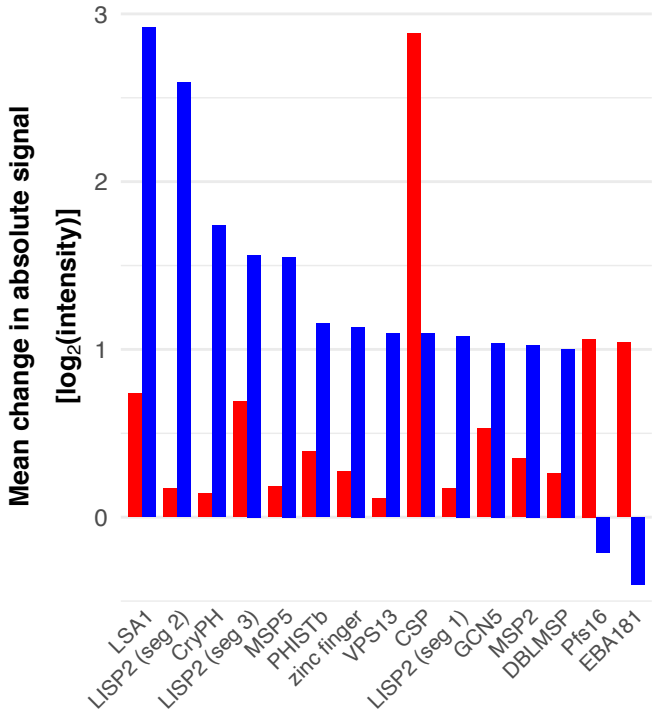

848 **Figure 6B.**

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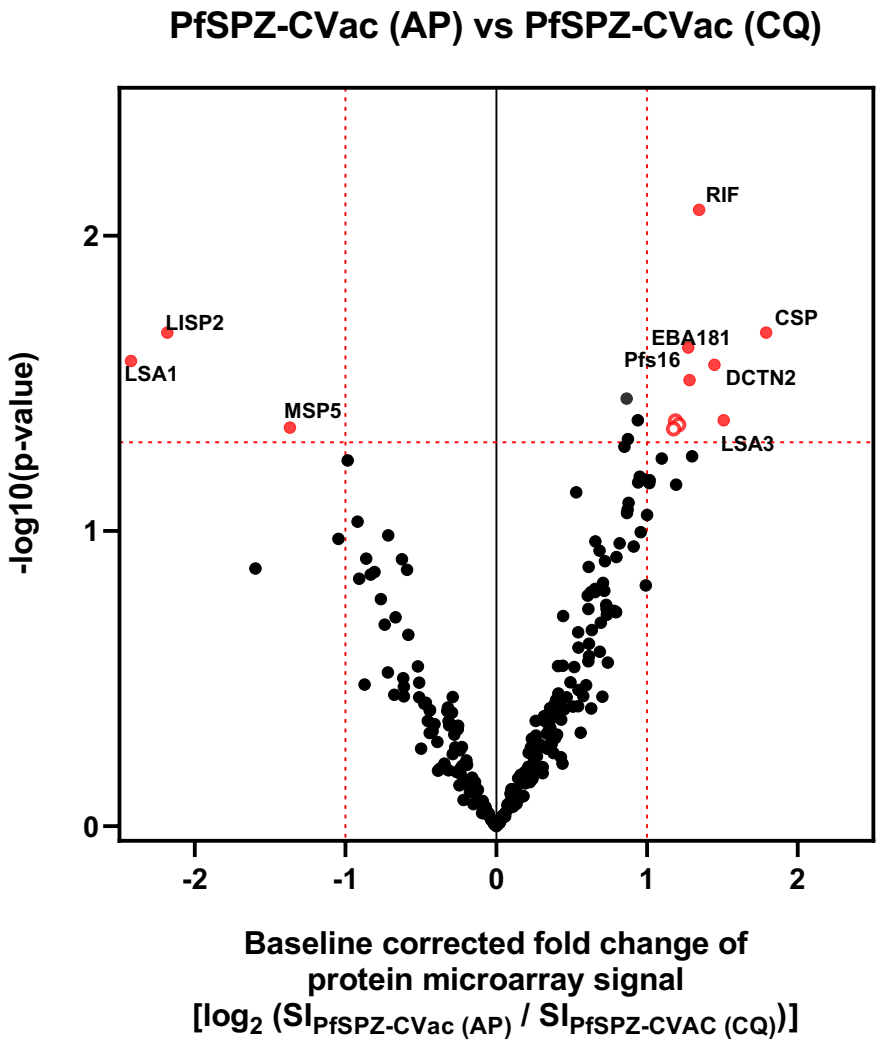

851 **Figure 6C.**

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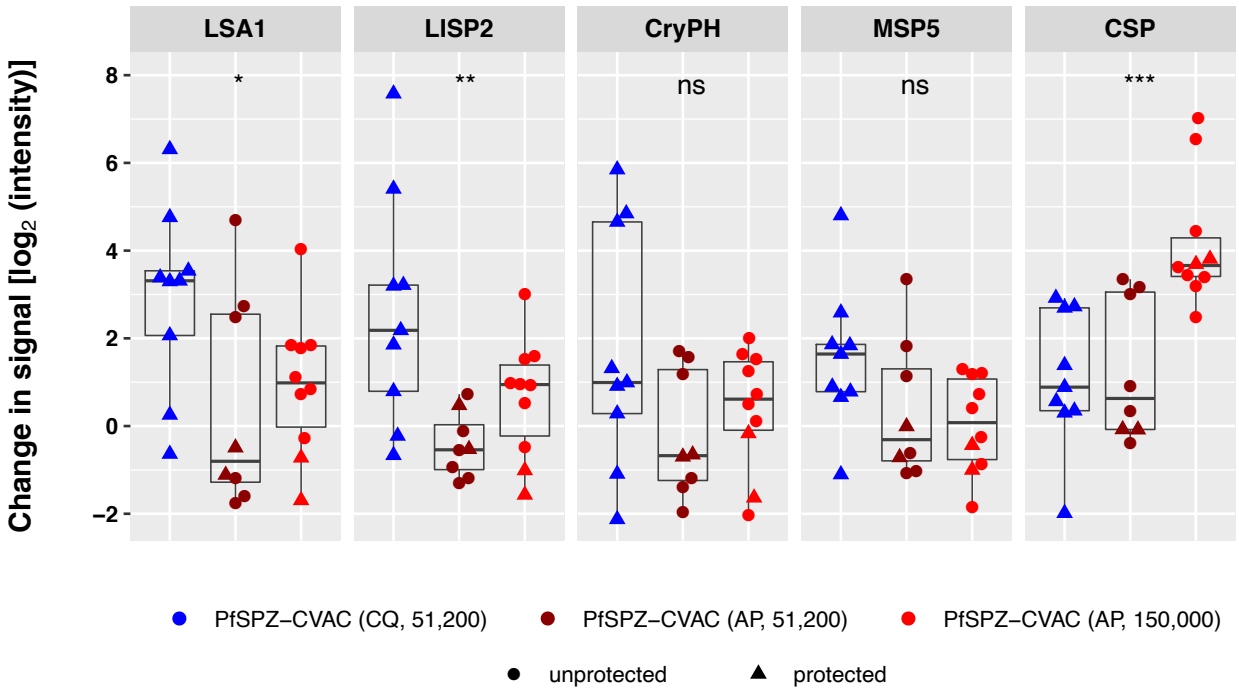
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