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## **Importance of the Immunodominant CD8 + T Cell Epitope of Plasmodium berghei Circumsporozoite Protein in Parasite- and Vaccine-Induced Protection**

Matthew Gibbins, Katja Müller, Maya Glover, Jasmine Liu, Elyzana Putrianti, Karolis Bauza, Arturo Reyes-Sandoval, Kai Matuschewski, Olivier Silvie, Julius Clemence R. Hafalla

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1 **The importance of the immunodominant CD8+ T cell epitope of *Plasmodium berghei***  
2 **circumsporozoite protein in parasite- and vaccine-induced protection**

3

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16

17 Running Head: Immunodominant malaria CD8+ T cell epitope

18

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20

21 **ABSTRACT**

22 The circumsporozoite protein (CSP) builds up the surface coat of sporozoites and is the leading  
23 malaria pre-erythrocytic-stage vaccine candidate. CSP has been shown to induce robust CD8+  
24 T cell responses that are capable of eliminating developing parasites in hepatocytes resulting  
25 in protective immunity. In this study, we characterised the importance of the immunodominant  
26 CSP-derived epitope, SYIPSAEKI, of *Plasmodium berghei* in both sporozoite- and vaccine-  
27 induced protection in murine infection models. In BALB/c mice, where SYIPSAEKI is efficiently  
28 presented in the context of the major histocompatibility complex class I (MHC-I) molecule H-  
29 2-K<sup>d</sup>, we established that epitope-specific CD8+ T cell responses contribute to parasite killing  
30 following sporozoite immunisation. Yet, sterile protection was achieved in the absence of this  
31 epitope substantiating the concept that other antigens can be sufficient for parasite-induced  
32 protective immunity. Furthermore, we demonstrated that SYIPSAEKI-specific CD8+ T cell  
33 responses elicited by viral-vectored CSP-expressing vaccines effectively targeted parasites in  
34 hepatocytes. The resulting sterile protection strictly relied on the expression of SYIPSAEKI. In  
35 C57BL/6 mice, which are unable to present the immunodominant epitope, CSP-based  
36 vaccines did not confer complete protection, despite the induction of high levels of CSP-  
37 specific antibodies. These findings underscore the significance of CSP in protection against  
38 malaria pre-erythrocytic stages and demonstrate that a significant proportion of the protection  
39 against the parasite is mediated by CD8+ T cells specific for the immunodominant CSP-derived  
40 epitope.

41

## 42 INTRODUCTION

43 Malaria is caused by a protozoan parasite of the genus *Plasmodium* and remains a major  
44 global health challenge in tropical and subtropical countries (1). A vaccine that diminishes the  
45 burden of disease and prevents malaria transmission remains a decisive goal for malaria  
46 elimination programmes. As a gold standard in malaria vaccination, multiple immunisations of  
47  $\gamma$ -radiation-attenuated *Plasmodium* sporozoites (RAS) can completely protect against wild-  
48 type (WT) sporozoite challenge (2-4). This parasite-induced protection targets the developing  
49 exo-erythrocytic forms in hepatocytes, also called liver stages, and completely abrogates blood  
50 stage infection. Antibodies and T cells have been implicated as important mechanisms of  
51 parasite-induced protection (reviewed in (5)), and CD8<sup>+</sup> T cells are the prime mediators of cell-  
52 mediated protective immunity, as exemplified in murine (6, 7) and non-human primate (8)  
53 infection models.

54

55 The circumsporozoite protein (CSP), the major surface coat protein of the malaria sporozoite,  
56 has been at the forefront of vaccination studies – being the basis of RTS,S/AS01, the most  
57 progressed malaria vaccine candidate to date (9). Immunisation of BALB/c mice  
58 with *Plasmodium berghei* (*Pb*) or *P. yoelii* (*Py*) RAS evokes immunodominant major  
59 histocompatibility complex class I (MHC-I) H-2-K<sup>d</sup>-restricted CD8<sup>+</sup> T cell responses (10, 11)  
60 against distinct CSP epitopes: SYIPSAEKI for *Pb* (12) and SYVPSAEQI for *Py* (13). Indeed,  
61 the measurement of responses to these epitopes has become the standard in fundamental  
62 immunological studies in BALB/c mice and their role in parasite-induced protection from  
63 sporozoite challenge has been repeatedly demonstrated (6, 12, 14). Furthermore, numerous  
64 vaccination studies involving different viral-vectored CSP- or CSP epitope-expressing vaccines  
65 – used alone or in combination as part of prime-boost regimens – have corroborated that CSP  
66 is a highly protective antigen in the BALB/c infection model (15-21). In these studies, elevated  
67 levels of either SYIPSAEKI- or SYVPSAEQI-specific CD8<sup>+</sup> T cell responses correlated with  
68 protection.

69

70 Nonetheless, several studies have interrogated and contested the immunological relevance of  
71 CSP with regard to parasite-induced protection in contrast to vaccine-induced protection.  
72 These studies emanated from observations that in naturally exposed humans T cell responses  
73 to CSP are scarce (22). In murine malaria models, multiple immunisations are required to elicit  
74 CD8+ T cell-dependent protective immunity in various mouse strains, particularly where no  
75 other strongly immunogenic CSP-derived CD8+ T cell epitopes have been identified (23).  
76 Furthermore, in *PyCSP*-transgenic BALB/c mice that are tolerant to *PyCSP*, complete  
77 protection can be achieved by *Py* RAS immunisation (24). In good agreement, BALB/c mice  
78 immunised with *Pb* WT parasites are completely protected when challenged with transgenic  
79 *Pb* parasites where the endogenous CSP has been swapped with the *P. falciparum* CSP (25).  
80 Taken together, these studies indicate that immune responses to CSP are dispensable for  
81 protection, and that other antigens are important to elicit protective immunity.

82

83 In this study, we have extended previous work on the entire CSP by dissecting the relevance  
84 of a single CSP-derived immunodominant epitope in parasite- and vaccine-induced protection.  
85 As the most stringent model system, we utilised transgenic *Pb* parasites lacking SYIPSAEKI  
86 for immunisation and challenge experiments in BALB/c mice. In addition, we have highlighted  
87 the level of protection achieved by CSP-based vaccines in mice expressing the relevant  
88 (BALB/c) or irrelevant (C57BL/6) MHC-I needed to present the CSP-derived immunodominant  
89 epitope.

90

91 **RESULTS**

92 **Sporozoite-induced SYIPSAEKI-specific CD8<sup>+</sup> T cell responses contribute to parasite**  
93 **killing but are dispensable for the development of sterile immunity.**

94 First, we interrogated the role that SYIPSAEKI, the H-2-K<sup>d</sup>-restricted immunodominant epitope  
95 of *PbCSP*, plays in protective immunity induced after live attenuated sporozoite immunisation.  
96 For this purpose, *PbCSP*<sup>SIINFEKL</sup> radiation-attenuated sporozoites (RAS), where the SYPSAEKI  
97 sequence has been replaced with the H-2-K<sup>b</sup>-restricted epitope of ovalbumin, SIINFEKL ((26),  
98 Müller and Gibbins et al., unpublished) were used to immunise H-2-K<sup>d</sup>-expressing BALB/c  
99 mice. To date, there are no other reported strongly immunogenic H-2-K<sup>d</sup>-restricted *PbCSP*  
100 epitopes identified. Removal of SYPSAEKI, by replacement with an irrelevant epitope, in the  
101 *PbCSP*<sup>SIINFEKL</sup> parasites allows unequivocal assignment of critical roles of this  
102 immunodominant *PbCSP*-derived epitope in protection elicited by live sporozoite  
103 immunisations. Two weeks after immunisation, the frequencies of IFN- $\gamma$ -producing  
104 SYIPSAEKI-specific CD8<sup>+</sup> T cell responses in the spleen after gating for CD11a expression  
105 (an activation marker commonly used to identify antigen-experienced cells (27)) were  
106 measured by flow cytometry (Fig. 1A). As expected, *PbCSP*<sup>SIINFEKL</sup> RAS parasites elicited no  
107 SYIPSAEKI-specific CD8<sup>+</sup> T cell responses in BALB/c mice.

108

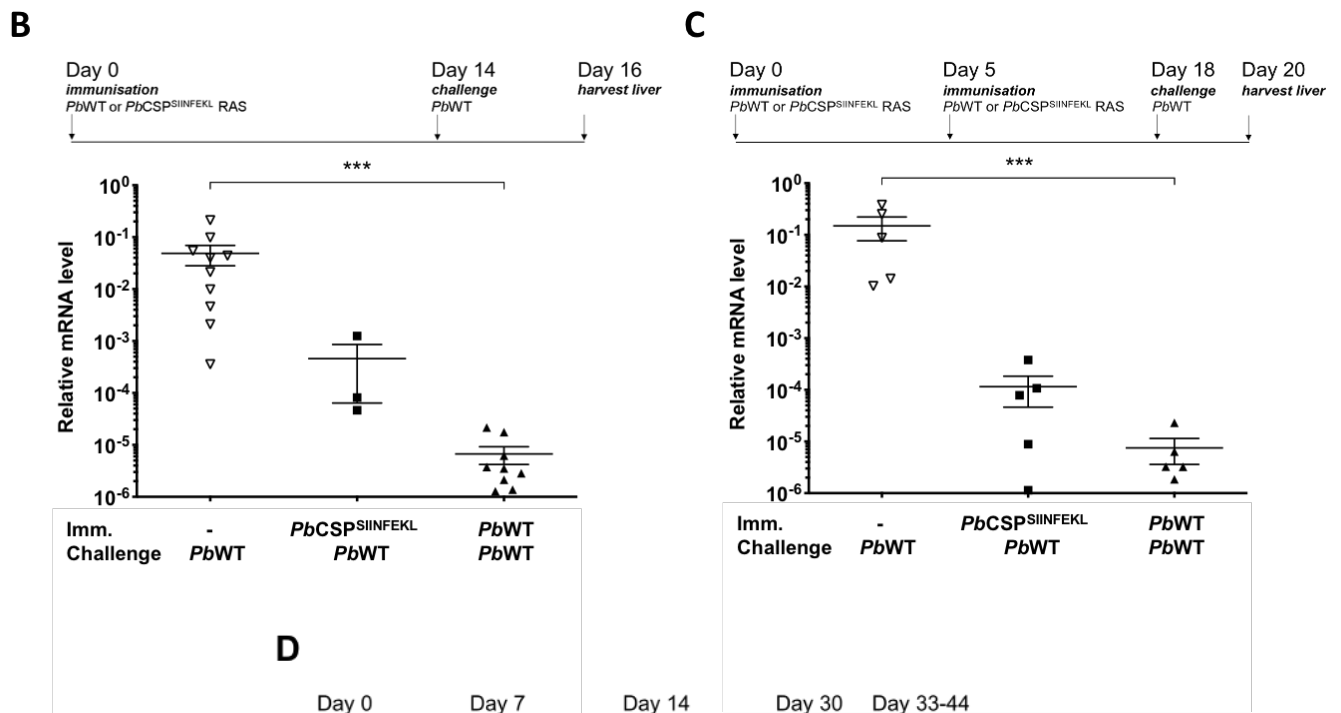
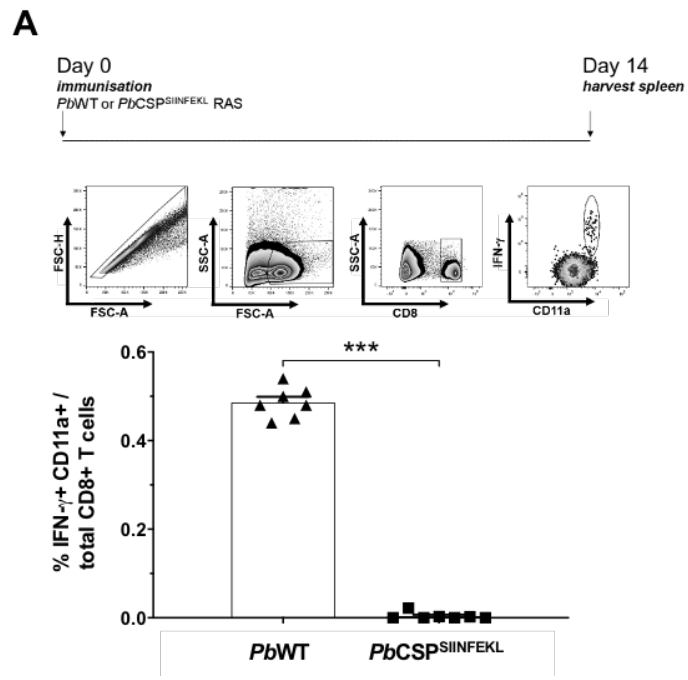
109 To ascertain whether SYIPSAEKI contributes to parasite-induced protection, BALB/c mice  
110 were immunised once (Fig 1B) or twice (Fig 1C) with either *PbWT* or *PbCSP*<sup>SIINFEKL</sup> RAS. Two  
111 weeks after immunisation, the mice were challenged with *PbWT* sporozoites and protection  
112 was determined by measuring the parasite loads in the liver 40 hours later (Fig. 1B and C). A  
113 significant reduction in parasite load – up to four orders of magnitude difference as compared  
114 to naïve mice – was observed in mice immunised with *PbWT* RAS and challenged with *PbWT*  
115 parasites. In contrast, protection was reduced only by approximately two orders of magnitude  
116 (after one immunisation) or three orders of magnitude (after two immunisations) in mice  
117 immunised with *PbCSP*<sup>SIINFEKL</sup> RAS (Fig. 1B and C). These results highlight the notion that  
118 within *PbCSP*, the SYIPSAEKI epitope has a critical and immunodominant contribution to  
119 protecting BALB/c mice after one or two immunisations with RAS.

120

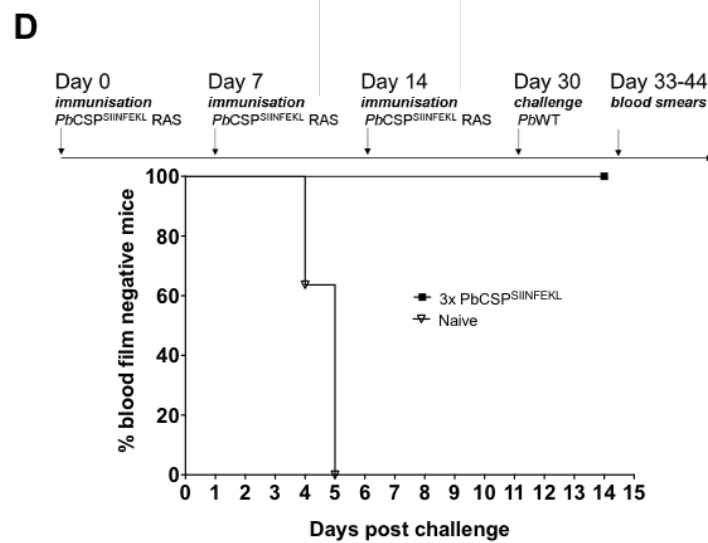
121 However, multiple immunisations with RAS are required to induce sterile protection. To  
122 establish whether the development of sterile immunity is dependent on SYIPSAEKI-specific  
123 CD8+ T cell responses, BALB/c mice were immunised thrice with *PbCSP*<sup>SIINFEKL</sup> RAS one week  
124 apart; two weeks after the last immunisation, mice were challenged with *PbWT* sporozoites  
125 (Fig. 1C). All mice were protected from blood stage infection compared to the naïve controls,  
126 implying that SYIPSAEKI-specific CD8+ T cell responses are not necessary for the  
127 development of sterile immunity.

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143 **FIG 1** SYIPSAEKI is dispensable for RAS immunisation but predominates protection with  
144 fewer immunisations. (A) BALB/c mice were immunised once with 10,000 *PbWT* or  
145 *PbCSP*<sup>SIINFEKL</sup> RAS. Splenocytes were taken after two weeks and restimulated with  
146 SYIPSAEKI peptide. IFN- $\gamma$ -producing cells co-staining with CD11a were assessed by flow  
147 cytometry. Shown are the time course (top), the gating strategy (centre) and proportion of  
148 IFN- $\gamma$ -producing CD11a of total CD8+ T cells (bottom). (B) Groups of BALB/c mice were  
149 immunised once with 15,000 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> RAS. Immunised mice and BALB/c  
150 naïve controls (n=3-10) were challenged with 10,000 *PbWT* parasites two weeks after the  
151 last immunisation. Livers were harvested 40 hours post-challenge and the relative liver  
152 parasite loads were quantified using the  $\Delta\Delta$ Ct method comparing levels of *P. berghei* 18S  
153 rRNA and levels of mouse *GAPDH* mRNA. Mean values ( $\pm$ SEM) are shown and statistics  
154 were calculated using the Kruskal-Wallis test (\*\*\*, p<0.001). (C) Groups of BALB/c mice  
155 were immunised twice with 10,000 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> RAS, 1 week apart. Immunised  
156 mice and BALB/c naïve controls (n=5) were challenged with 10,000 *PbWT* parasites two  
157 weeks after the last immunisation. Livers were harvested 40 hours post-challenge and the  
158 relative liver parasite loads were quantified using the  $\Delta\Delta$ Ct method comparing levels of *P.*  
159 *berghei* 18S rRNA and levels of mouse *GAPDH* mRNA. Mean values ( $\pm$ SEM) are shown and  
160 statistics were calculated using the Kruskal-Wallis test (\*\*\*, p<0.001). (D) BALB/c mice  
161 (n=12) were immunised with three doses of 10,000 *PbCSP*<sup>SIINFEKL</sup> RAS at one-week intervals.  
162 Immunised mice and naïve controls (n=11) were challenged with 5,000 *PbWT* sporozoites 16  
163 days after the last immunisation. Blood smears were taken daily for two weeks after  
164 challenge. Parasitaemia was assessed by microscopic examination of Giemsa-stained  
165 smears. Data shown is a combination of two independent experiments.  
166

167 **Prime-boost vaccination with CSP-expressing viruses induces strong anti-CSP**  
168 **antibody and CD8+ T cell responses and SYIPSAEKI is the key mediator of sterile**  
169 **protection.**

170 Next, we probed the requirement for SYIPSAEKI presentation in protection elicited by viral-  
171 vectored CSP-expressing vaccines administered in a prime-boost regimen. Priming with  
172 adenovirus (Ad) carrying a foreign antigen and boosting with orthopoxvirus modified vaccinia  
173 Ankara (MVA) expressing the same antigen has consistently been shown to induce strong  
174 CD8+ T cell responses with high levels of protective efficacy against intracellular pathogens  
175 including malaria pre-erythrocytic stages (18, 21).

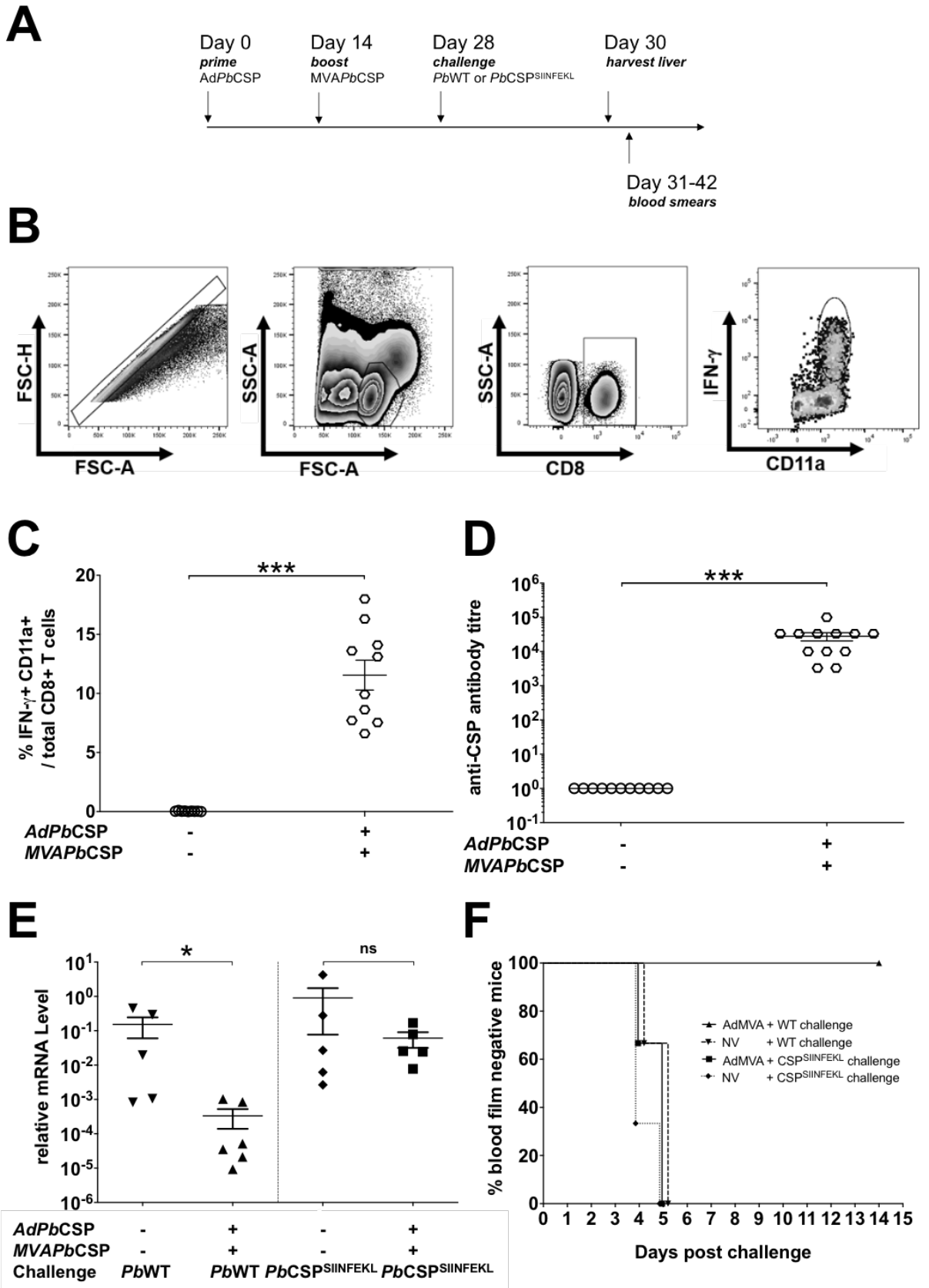
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177 Chimpanzee adenovirus serotype 63 (AdCh63) and MVA vaccines expressing *PbCSP* were  
178 used to vaccinate BALB/c mice with a two-week resting period between priming and boosting  
179 (Fig. 2A). Two weeks after boosting, whole blood was collected and restimulated *ex vivo* with  
180 SYIPSAEKI peptide. The frequencies of IFN- $\gamma$  secreting CD8+ T cells were enumerated by  
181 flow cytometry (Fig. 2B) and Ad-MVA *PbCSP*-vaccinated mice elicited ~12% SYIPSAEKI-  
182 specific circulating CD8+ T cells (Fig. 2C), which is consistent with the level of epitope-specific  
183 CD8+ T cells induced by similar, previously published, viral vectored prime-boost regimens  
184 (19, 21). Serum samples were also collected from the vaccinated animals and were used in  
185 an immunofluorescence assay against air-dried *Pb* sporozoites (Fig. 2D). Ad-MVA *PbCSP*-  
186 vaccinated BALB/c mice induced high anti-CSP antibody titres (1:10<sup>4</sup>). These data indicate that  
187 Ad-MVA *PbCSP* vaccination elicit both high frequencies of SYPSAEKI-specific CD8+ T cells  
188 and high titres of CSP-specific antibodies.

189

190 Two weeks after boosting, Ad-MVA *PbCSP*-vaccinated mice were challenged with *PbWT* or  
191 *PbCSP*<sup>SIINFEKL</sup> parasites. Protection was assessed by two complementary assays; (i)  
192 determination of the reduction of parasite load in the liver (Fig. 2E) and (ii) induction of sterile  
193 protection (Fig. 2F). Strikingly, parasite load in the liver of Ad-MVA *PbCSP*-vaccinated mice  
194 was not significantly reduced compared to non-vaccinated mice when challenged with  
195 *PbCSP*<sup>SIINFEKL</sup> sporozoites, in marked contrast to challenge with *PbWT* sporozoites. In perfect

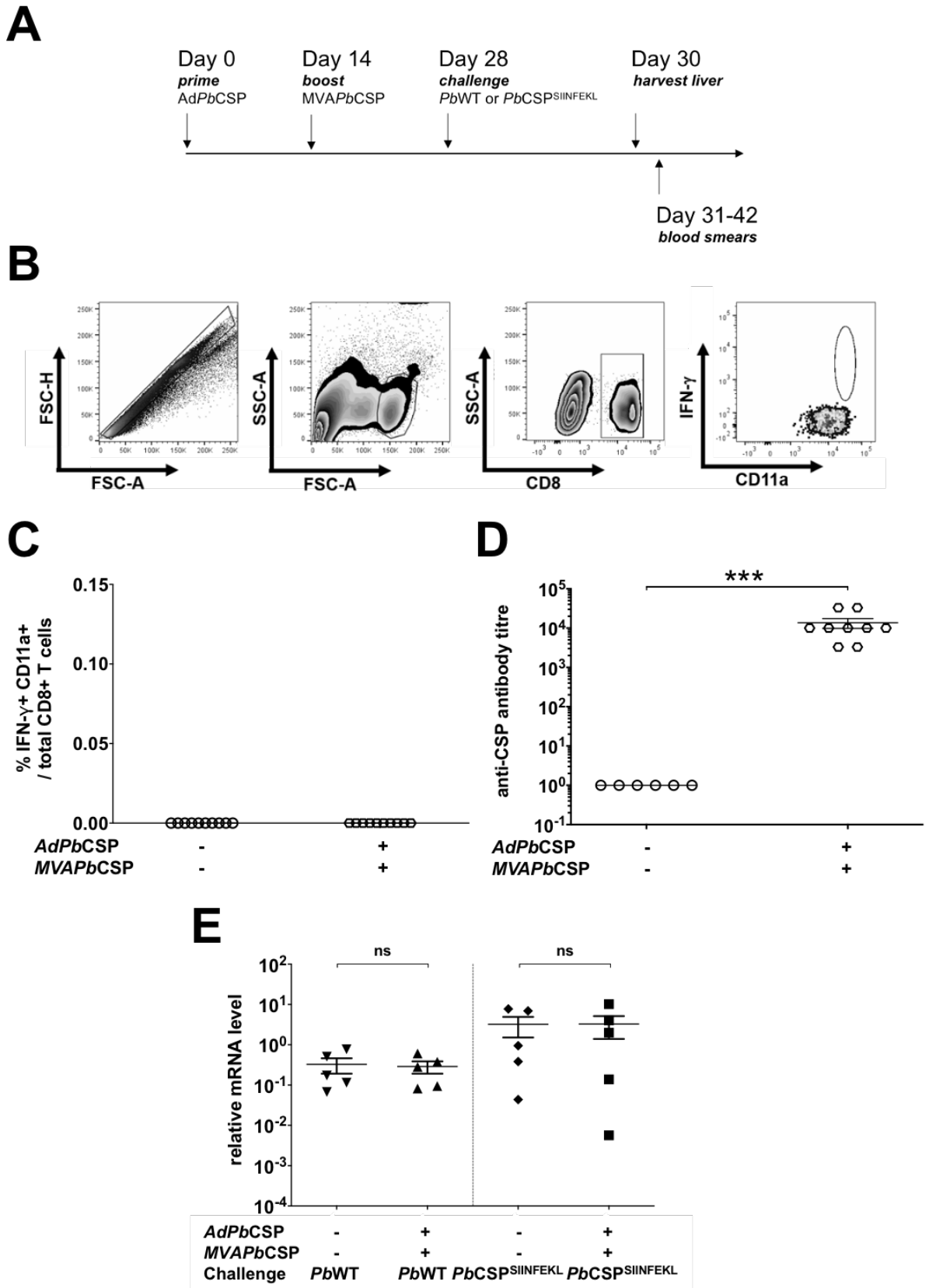
196 agreement, vaccinated mice challenged with *PbCSP*<sup>SIINFEKL</sup> sporozoites were patent for  
197 parasitaemia by day 5, whereas vaccinated mice challenged with *PbWT* sporozoites remained  
198 completely protected. These results denote that vaccine-induced effector SYIPSEAKI-specific  
199 CD8+ T responses efficiently target parasites expressing the cognate epitope. Parasites  
200 lacking the SYIPSAEKI epitope are not eliminated despite high levels of CSP-specific  
201 antibodies evoked by vaccination in this experimental system.  
202



204 **FIG. 2** Prime-boost vaccination with viral vectored CSP-expressing vaccines induces strong  
205 anti-CSP antibody and CD8<sup>+</sup> T cell responses, and SYIPSAEKI-specific CD8<sup>+</sup> T cell  
206 responses are essential for protection. (A) BALB/c mice were vaccinated with AdCh63 and  
207 MVA vaccines expressing *PbCSP* (Ad*PbCSP* and MVA*PbCSP*) and challenged with 10,000  
208 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> sporozoites as shown. (B) Flow cytometry gating strategy used to  
209 determine proportions of IFN- $\gamma$ <sup>+</sup> CD11a<sup>+</sup> CD8<sup>+</sup> T cells. (C) Proportion of IFN- $\gamma$ -producing  
210 CD11a of total CD8<sup>+</sup> T cells. Blood was drawn from the tail from naïve (n=9) and vaccinated  
211 mice (n=10) two weeks after boost and restimulated with SYIPSAEKI and stained for CD8 and  
212 CD11a surface markers, and IFN- $\gamma$  for flow cytometric analysis. (D) Reciprocal antibody titers  
213 of mouse serum reactive to whole sporozoites. Serum from naïve (n=11) and vaccinated mice  
214 (n=12) was isolated two weeks after boost and CSP specific antibody titres were measured by  
215 immunofluorescent antibody assay. (E) Livers from vaccinated mice (+) challenged with *PbWT*  
216 (n=6) or *PbCSP*<sup>SIINFEKL</sup> sporozoites (n=5) and non-vaccinated mice (-) challenged with *PbWT*  
217 (n=5) or *PbCSP*<sup>SIINFEKL</sup> sporozoites (n=5) were harvested 42 hours post-challenge and relative  
218 liver parasite levels were quantified using the  $\Delta\Delta$ Ct method comparing levels of *P. berghei* 18S  
219 rRNA and levels of mouse *GAPDH* mRNA. (F) Groups of vaccinated and non-vaccinated mice  
220 (n=6) were challenged with 5,000 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> sporozoites. Vaccinated mice  
221 challenged with *PbWT* (triangles) or *PbCSP*<sup>SIINFEKL</sup> (squares) and non-vaccinated mice  
222 challenged with *PbWT* (inverted triangles) or *PbCSP*<sup>SIINFEKL</sup> (diamonds) had daily tail smears  
223 taken from day 3-14 post challenge. Slides were stained with Giemsa and parasitaemia was  
224 assessed by microscopy. (C-E) Each data point represents one mouse with mean values  
225 ( $\pm$ SEM) shown and statistics were calculated using the Mann-Whiney test (\*, p<0.05; \*\*\*,  
226 p<0.001).  
227

228 **CSP-based vaccines do not elicit protective immunity in C57BL/6 mice.**

229 To further investigate the requirement of SYIPSAEKI as the indispensable protective epitope  
230 of CSP, mice unable to present this epitope were vaccinated with the *PbCSP* prime-boost  
231 regimen with an interval of two weeks between vaccines, followed by challenge with either  
232 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> parasites (Fig 3A). C57BL/6 mice were used because SYIPSAEKI is  
233 an H-2-K<sup>d</sup> restricted epitope, and this mouse strain does not express the relevant MHC-I allele.  
234 Thus, SYIPSAEKI would fail to be presented by infected hepatocytes. As before, blood and  
235 serum were derived two weeks after boost. As expected, SYIPSAEKI-specific CD8<sup>+</sup> T cells  
236 (Fig. 3B) were not detectable in Ad-MVA *PbCSP*-vaccinated C57BL/6 mice (Fig. 3C), but  
237 strong anti-CSP antibody titres (1:10<sup>4</sup>) were elicited (Fig. 3D). Ad-MVA CSP-vaccinated  
238 C57BL/6 mice challenged with either *PbWT* or *PbCSP*<sup>SIINFEKL</sup> parasites had comparable  
239 parasite load in the liver (Fig. 3E), indicative of full liver stage development in all groups.  
240



242 **FIG 3** Prime-boost vaccination with CSP expressing viruses does not protect C57BL/6 mice,  
243 irrespective of induced antibody titres.

244 (A) C57BL/6 mice were vaccinated with AdCh63 and MVA vaccines *PbCSP* and challenged  
245 with 10,000 *PbWT* or *PbCSP*<sup>SIINFEKL</sup> sporozoites as shown. (B) Flow cytometry gating strategy  
246 used to determine proportions of IFN- $\gamma$ <sup>+</sup> CD11a<sup>+</sup> CD8<sup>+</sup> T cells. (C) Proportion of IFN- $\gamma$ -  
247 producing CD11a of total CD8<sup>+</sup> T cells. Blood was drawn from the tail from naïve (n=10) and  
248 vaccinated mice (n=10) two weeks after boost was restimulated with SYIPSAEKI and stained  
249 for CD8 and CD11a surface markers, and IFN- $\gamma$  for flow cytometric analysis. (D) Reciprocal  
250 antibody titres of mouse serum reactive to whole sporozoites. Serum from naïve (n=6) and  
251 vaccinated mice (n=9) was isolated two weeks after boost and CSP specific antibody titres  
252 were measured by immunofluorescent antibody assay. (E) Livers from groups of 5 mice per  
253 condition were harvested 42 hours post-challenge and relative liver parasite levels were  
254 quantified using the  $\Delta\Delta$ Ct method comparing levels of *P. berghei* 18S rRNA and levels of  
255 mouse *GAPDH* mRNA. None of the differences were significant ( $p>0.05$ ). (C-E). Each data  
256 point represents one mouse with mean values ( $\pm$  SEM) shown and statistics were calculated  
257 using the Mann-Whiney test (\*\* $p<0.001$ ).

258



259 **DISCUSSION**

260 Our findings lend full support to the notion that CSP is an immunodominant sporozoite-derived  
261 antigen (24). A single epitope, SYIPSAEKI, is the immunodominant CD8+ T cell epitope of  
262 CSP, and we show that it is responsible for the antigen's protective capacity against parasites  
263 in the liver in the BALB/c model. Following RAS immunisation, CD8+ T cell responses to  
264 SYIPSAEKI contribute to the reduction in parasite load in the liver following sporozoite  
265 challenge, as shown herein. When RAS-immunised mice are challenged with *PbCSP*<sup>SIINFEKL</sup>,  
266 transgenic parasites lacking SYIPSAEKI, reduced anti-*Plasmodium* activity in the liver is  
267 observed. Nonetheless, complete protection is achievable in the absence of SYIPSAEKI-  
268 specific CD8+ T cell responses, demonstrating that responses to other, yet unidentified, H-2-  
269 K<sup>d</sup>-restricted epitopes contribute to parasite killing. It is conceivable that these epitopes are  
270 encoded by the hundreds of other *Plasmodium* genes expressed in malaria pre-erythrocytic  
271 stages, some of which might be shared with blood stage antigens (28).

272

273 Our findings also emphasise the importance of SYIPSAEKI-specific CD8+ T cell responses for  
274 promoting protective immunity when using CSP-based viral vaccines in the BALB/c model.  
275 These vaccines are aimed at generating high levels of epitope-specific memory CD8+ T cells  
276 but rely on the expression of relevant MHC-I in the vaccinated host and the presence of the  
277 cognate epitope in the parasite used for challenge (29). Notably, despite high levels of  
278 antibodies against whole sporozoites elicited following Ad-MVA *PbCSP* vaccination, sterile  
279 protection was not achieved following challenge of C57BL/6 mice. These mice cannot present  
280 SYIPSAEKI, fully supporting the notion that the protective efficacy of CSP strictly depends on  
281 the expression of the immunodominant epitope. These findings were independently  
282 corroborated by the lack of protection in mice, either BALB/c or C57BL/6, immunised with  
283 transgenic sporozoites lacking SYIPSAEKI.

284

285 Together, these results have important implications for the development of next generation  
286 malaria vaccines. We have demonstrated the significance of a single epitope of CSP in  
287 mediating protective CD8+ T cell responses while also recapitulating that protection can be

288 achieved in the absence of responses to the entire CSP antigen (24, 25). In BALB/c mice,  
289 SYIPSAEKI-specific CD8+ T cell responses offered protection. However, to achieve complete  
290 sterile protection either multiple sporozoite immunisations or viral vaccines, which induced  
291 large populations of SYIPSAEKI-specific CD8+ T cells, were required. Multiple immunisations  
292 likely induced a broad range of immune responses and multiple high-dose immunisations with  
293 RAS in humans have been shown to induce dose-dependent anti-sporozoite CD8+ T cell  
294 responses in addition to dose dependent anti-sporozoite antibody and CD4+ T cell responses  
295 (4). It will be important in the future to determine how the magnitude of SYIPSAEKI-specific  
296 CD8+ T cell responses modulates, after consecutive immunisations, but also the breadth of  
297 responses to other CSP epitopes (B and T cell) and their effect on protection compared to  
298 other antigens. In line with this, our findings also show that protection can be achieved in the  
299 absence of responses to immunodominant epitopes, leading us to suggest that future pre-  
300 erythrocytic malaria vaccine research should not only focus on inducing strong CD8+ T cell  
301 responses against one or multiple antigens but should try to target a broad array of antigens  
302 and cover diverse MHC to offer the best protection possible. The identification of novel  
303 antigens and epitopes that contribute to protection in H-2-K<sup>d</sup>-restricted BALB/c mice, and  
304 ultimately in human populations with broad MHC haplotypes, will aid this development. In  
305 C57BL/6 mice pre-erythrocytic immunity is mounted irrespective of CSP-specific CD8+ T cell  
306 responses, and recent genome-wide epitope profiling returned multiple sporozoite antigens  
307 and epitopes (30-32). RTS,S/AS01, the leading subunit malaria vaccine based on CSP, seems  
308 to offer some protection against *P. falciparum* re-infection (9). Partial and short-lived protection  
309 is likely primarily mediated by the action of transitory anti-sporozoite antibodies (33-35).  
310 Strikingly, peripheral blood CD8+ T cell responses were not identified to provide a role  
311 following sporozoite challenge in this candidate vaccine. Together with previous findings (7,  
312 17, 19, 24) our data underscore efforts to improve the most advanced candidate malaria  
313 vaccine, RTS,S/AS01, by eliciting CD8+ T cells against CSP or other immunodominant  
314 antigens.

315

## 316 MATERIALS AND METHODS

317 **Ethics and animal experimentation.** Animal procedures were performed in accordance with  
318 the German 'Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)' which  
319 implements the directive 2010/6 3/EU from the European Union. The protocol was approved  
320 by the ethics committee of the Berlin state authority ('Landesamt für Gesundheit und Soziales  
321 Berlin', permit number G0469/09). Animal experiments at London School of Hygiene and  
322 Tropical Medicine were conducted under license from the United Kingdom Home Office under  
323 the Animals (Scientific Procedures) Act 1986. CD-1 mice were bred in-house at LSHTM, while  
324 NMRI, C57BL/6 and BALB/c laboratory mouse strains were purchased from either Charles  
325 River Laboratories (Margate, UK or Sulzfeld, Germany) or Janvier (Saint Berthevin, France).  
326 Female mice of 6-8 weeks of age were used in the experiments.

327

328 **Plasmodium parasites and immunisation.** The transgenic *P. berghei* ANKA CSP<sup>SIINFEKL</sup>  
329 (*PbCSP*<sup>SIINFEKL</sup>) parasite was generated with the immunodominant CSP CD8+ T cell epitope  
330 SYIPSAEKI (252-260aa) being replaced with the H-2<sup>b</sup> restricted *Gallus gallus* ovalbumin  
331 CD8+ T cell epitope SIINFEKL (258-265aa) via double homologous recombination ((26),  
332 Müller and Gibbins *et al.*, unpublished). Wild-type *Plasmodium berghei* ANKA (clone c115cy1)  
333 (*PbWT*) and *PbCSP*<sup>SIINFEKL</sup> were maintained by continuous cycling between murine hosts  
334 (NMRI or CD-1) and *Anopheles stephensi* mosquitos. Infected mosquitos were kept in  
335 incubators (Panasonic and Mytron) at 80% humidity and 20°C temperature. Sporozoites were  
336 isolated from the salivary glands and attenuated by  $\gamma$ -irradiation at  $1.2 \times 10^4$  cGy. Mice were  
337 immunised with 10,000 sporozoites administered intravenously with multiple doses given one  
338 week apart unless otherwise stated. For challenge infections, 5,000 or 10,000 sporozoites were  
339 administered intravenously to assess sterile protection and parasite load in the liver,  
340 respectively.

341

342 **Viral-vectored CSP-expressing vaccines.** AdCh63 and MVA vaccines expressing the  
343 mammalian codon-optimised fragment of *PbCSP* were constructed and propagated based on  
344 previously published viral vectors (36, 37). The viral vectors were administered intramuscularly

345 in endotoxin-free PBS at a concentration of  $10^5$  viral particles for AdPbCSP for the prime  
346 immunisation and  $10^6$  viral particles MVAPbCSP for the boost immunisation.

347

348 **Immunofluorescent antibody assay.** 10,000 sporozoites were spotted onto epoxy coated  
349 glass slides with marked rings (Medco), dried at room temperature and stored at  $-20^{\circ}\text{C}$ .  
350 Thawed slides were fixed in acetone, dried and rehydrated with PBS before incubation in 10%  
351 FCS supplemented DMEM (Gibco) for 1 hour at  $37^{\circ}\text{C}$  in a humid chamber. Serum at  
352 concentrations  $1:10^3$ ,  $1:3.3 \times 10^3$ ,  $1:10^4$ ,  $1:3.3 \times 10^4$ ,  $1:10^5$  (and, additionally,  $1:3.3 \times 10^5$  and  $1:10^6$   
353 for C57BL/6 serum) were added to the ring wells and incubated for 1 hour at  $37^{\circ}\text{C}$  in a humid  
354 chamber. Slides were washed and stained with a mouse anti-CSP (38) primary antibody.  
355 Hoechst33342 was added as the nuclear stain together with a respective fluorescently labelled  
356 anti-mouse secondary antibody for a further one-hour incubation. Slides were washed and  
357 mounted with 'Fluoromount-G' (Southern Biotech) and analysed by fluorescent microscopy  
358 (Zeiss Axio Observer).

359

360 **Quantification of SYIPSAEKI-specific CD8+ T cell responses.** Spleens were harvested and  
361 lymphocytes were derived by passing spleens through  $40\mu\text{m}$  cell strainers (Corning).  
362 Peripheral blood was drawn from the tail vein and collected in  $\text{Na}^+$  heparin capillary tubes  
363 (Brand) and assayed in 96-well flat bottom plates (Corning). Red blood cells were lysed using  
364 PharmLyse (BD) and lymphocytes were resuspended in 10% FCS, 2% Penicillin-Streptomycin  
365 and 1% L-glutamine supplemented RPMI 1640 (Gibco). Splenocytes were counted using a  
366 40x dilution with Trypan Blue (ThermoFisher Scientific) and a Neubauer 'Improved'  
367 haemocytometer (Biochrom).  $2 \times 10^6$  splenocytes and the lysed blood samples were prepared  
368 in 96 well plates and incubated with a final concentration of  $10\mu\text{g/ml}$  of SYIPSAEKI peptide in  
369 in the presence of Brefeldin A (eBioScience) for 5-6 hours at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . For staining  
370 of cell surface markers and intracellular cytokines, cells were incubated for 1 hour at  $4^{\circ}\text{C}$  for  
371 each staining. Cells were stained for CD8 (53-6.7) and CD11a (M17/4) (eBioscience). Splenic  
372 cells were fixed with 4% paraformaldehyde and peripheral blood cells were fixed with 1%

373 paraformaldehyde before staining for IFN- $\gamma$  (XMG1.2) (eBioscience) in the presence of  
374 Perm/Wash buffer (BD) for intracellular staining. Data was acquired by flow cytometry using  
375 an LSRFortessa or LSRII (BD) and analysed using Flowjo9.5.2 (Tree Star, Inc.).

376

377 **Quantification of parasite load in the liver.** Livers were harvested 40-42 hours after  
378 sporozoite challenge and total RNA was extracted following homogenisation using TRIzol  
379 (ThermoFisher Scientific). cDNA was generated using the RETROScript Kit (Ambion).  
380 Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System  
381 and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative liver parasite levels  
382 were quantified using the  $\Delta\Delta C_t$  method comparing levels of *P. berghei* 18S rRNA using specific  
383 primers and normalised to levels of mouse *GAPDH* mRNA (39).

384

385 **Assessment of parasitaemia.** Sterile protection was assessed by daily blood smears, taken  
386 from mice 3-14 days after sporozoite challenge, stained with Giemsa (improved solution; VWR)  
387 to microscopically determine the presence of blood stage parasites.

388

389 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism v7 (GraphPad  
390 Software, Inc.). Statistics were calculated using the Mann-Whitney U test.

391

392 **AUTHOR CONTRIBUTIONS**

393 O.S. and J.C.R.H. designed the experiments in the laboratory of K.Matuschewski; O.S.  
394 generated the transgenic parasites CSP<sup>SIINFEKL</sup>; M.P.G., K.Müller., M.G., J.L. and E.D.P.  
395 performed experiments and analysed data; K.B. and A.R.-S. generated the CSP-expressing  
396 viruses AdPbCSP and MVAPbCSP; M.P.G. and J.C.R.H. wrote the paper. All authors  
397 commented on and approved the paper.

398

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414   **REFERENCES**

415

- 416   1.     WHO. 2017. World Malaria Report 2017 World Health Organisation, Geneva, Switzerland.
- 417   2.     Nussenzweig RS, Vanderberg J, Most H, Orton C. 1967. Protective immunity produced by the  
418       injection of x-irradiated sporozoites of *Plasmodium berghei*. Nature 216:160-2.
- 419   3.     Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M,  
420       Paul C, Gordon DM, Stoute JA, Church LW, Sedegah M, Heppner DG, Ballou WR, Richie TL.  
421       2002. Protection of humans against malaria by immunization with radiation-attenuated  
422       *Plasmodium falciparum* sporozoites. J Infect Dis 185:1155-64.
- 423   4.     Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER,  
424       Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben  
425       AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH,  
426       Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M,  
427       Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK,  
428       Ledgerwood JE, Graham BS, Hoffman SL, Team VRCS. 2013. Protection against malaria by  
429       intravenous immunization with a nonreplicating sporozoite vaccine. Science 341:1359-65.
- 430   5.     Doll KL, Harty JT. 2014. Correlates of protective immunity following whole sporozoite  
431       vaccination against malaria. Immunol Res 59:166-76.
- 432   6.     Doolan DL, Hoffman SL. 2000. The complexity of protective immunity against liver-stage  
433       malaria. J Immunol 165:1453-62.
- 434   7.     Schmidt NW, Podyminogin RL, Butler NS, Badovinac VP, Tucker BJ, Bahjat KS, Lauer P, Reyes-  
435       Sandoval A, Hutchings CL, Moore AC, Gilbert SC, Hill AV, Bartholomay LC, Harty JT. 2008.  
436       Memory CD8 T cell responses exceeding a large but definable threshold provide long-term  
437       immunity to malaria. Proc Natl Acad Sci U S A 105:14017-22.
- 438   8.     Weiss WR, Jiang CG. 2012. Protective CD8+ T lymphocytes in primates immunized with  
439       malaria sporozoites. PLoS One 7:e31247.
- 440   9.     RTS SCTP. 2015. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster  
441       dose in infants and children in Africa: final results of a phase 3, individually randomised,  
442       controlled trial. Lancet 386:31-45.
- 443   10.    Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V. 1987.  
444       Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites.  
445       Nature 330:664-6.
- 446   11.    Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF. 1988. CD8+ T cells  
447       (cytotoxic/suppressors) are required for protection in mice immunized with malaria  
448       sporozoites. Proc Natl Acad Sci U S A 85:573-6.
- 449   12.    Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F. 1989. Cloned  
450       cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against  
451       malaria. Nature 341:323-6.
- 452   13.    Weiss WR, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good MF, Berzofsky JA, Miller LH,  
453       Hoffman SL. 1990. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on  
454       malaria-infected hepatocytes. J Exp Med 171:763-73.
- 455   14.    Rodrigues MM, Cordey AS, Arreaza G, Corradin G, Romero P, Maryanski JL, Nussenzweig RS,  
456       Zavala F. 1991. CD8+ cytolytic T cell clones derived against the Plasmodium yoelii  
457       circumsporozoite protein protect against malaria. Int Immunol 3:579-85.
- 458   15.    Li S, Rodrigues M, Rodriguez D, Rodriguez JR, Esteban M, Palese P, Nussenzweig RS, Zavala F.  
459       1993. Priming with recombinant influenza virus followed by administration of recombinant  
460       vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. Proc Natl  
461       Acad Sci U S A 90:5214-8.
- 462   16.    Rodrigues EG, Zavala F, Eichinger D, Wilson JM, Tsuji M. 1997. Single immunizing dose of  
463       recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity  
464       against malaria. J Immunol 158:1268-74.

- 465 17. Bruna-Romero O, Gonzalez-Aseguinolaza G, Hafalla JC, Tsuji M, Nussenzweig RS. 2001.  
466 Complete, long-lasting protection against malaria of mice primed and boosted with two  
467 distinct viral vectors expressing the same plasmodial antigen. Proc Natl Acad Sci U S A  
468 98:11491-6.
- 469 18. Gilbert SC, Schneider J, Hannan CM, Hu JT, Plebanski M, Sinden R, Hill AV. 2002. Enhanced  
470 CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a  
471 recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. Vaccine  
472 20:1039-45.
- 473 19. Gonzalez-Aseguinolaza G, Nakaya Y, Molano A, Dy E, Esteban M, Rodriguez D, Rodriguez JR,  
474 Palese P, Garcia-Sastre A, Nussenzweig RS. 2003. Induction of protective immunity against  
475 malaria by priming-boosting immunization with recombinant cold-adapted influenza and  
476 modified vaccinia Ankara viruses expressing a CD8+ T-cell epitope derived from the  
477 circumsporozoite protein of *Plasmodium yoelii*. J Virol 77:11859-66.
- 478 20. Reyes-Sandoval A, Sridhar S, Berthoud T, Moore AC, Harty JT, Gilbert SC, Gao G, Ertl HC,  
479 Wilson JC, Hill AV. 2008. Single-dose immunogenicity and protective efficacy of simian  
480 adenoviral vectors against *Plasmodium berghei*. Eur J Immunol 38:732-41.
- 481 21. Reyes-Sandoval A, Berthoud T, Alder N, Siani L, Gilbert SC, Nicosia A, Colloca S, Cortese R, Hill  
482 AV. 2010. Prime-boost immunization with adenoviral and modified vaccinia virus Ankara  
483 vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell  
484 responses. Infect Immun 78:145-53.
- 485 22. Doodoo D, Hollingdale MR, Anum D, Koram KA, Gyan B, Akanmori BD, Ocran J, Adu-  
486 Amankwah S, Geneshan H, Abot E, Legano J, Banania G, Sayo R, Brambilla D, Kumar S, Doolan  
487 DL, Rogers WO, Epstein J, Richie TL, Sedegah M. 2011. Measuring naturally acquired immune  
488 responses to candidate malaria vaccine antigens in Ghanaian adults. Malar J 10:168.
- 489 23. Schmidt NW, Butler NS, Badovinac VP, Harty JT. 2010. Extreme CD8 T cell requirements for  
490 anti-malarial liver-stage immunity following immunization with radiation attenuated  
491 sporozoites. PLoS Pathog 6:e1000998.
- 492 24. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, Nussenzweig V.  
493 2006. The circumsporozoite protein is an immunodominant protective antigen in irradiated  
494 sporozoites. Nature 444:937-40.
- 495 25. Gruner AC, Mauduit M, Tewari R, Romero JF, Depinay N, Kayibanda M, Lallemand E, Chavatte  
496 JM, Crisanti A, Sinnis P, Mazier D, Corradin G, Snounou G, Renia L. 2007. Sterile protection  
497 against malaria is independent of immune responses to the circumsporozoite protein. PLoS  
498 One 2:e1371.
- 499 26. Cockburn IA, Tse SW, Radtke AJ, Srinivasan P, Chen YC, Sinnis P, Zavala F. 2011. Dendritic cells  
500 and hepatocytes use distinct pathways to process protective antigen from plasmodium in  
501 vivo. PLoS Pathog 7:e1001318.
- 502 27. Rai D, Pham NL, Harty JT, Badovinac VP. 2009. Tracking the total CD8 T cell response to  
503 infection reveals substantial discordance in magnitude and kinetics between inbred and  
504 outbred hosts. J Immunol 183:7672-81.
- 505 28. Muller K, Gibbins MP, Matuschewski K, Hafalla JCR. 2017. Evidence of cross-stage CD8+ T cell  
506 epitopes in malaria pre-erythrocytic and blood stage infections. Parasite Immunol 39.
- 507 29. Silvie O, Amino R, Hafalla JC. 2017. Tissue-specific cellular immune responses to malaria pre-  
508 erythrocytic stages. Curr Opin Microbiol 40:160-167.
- 509 30. Hafalla JC, Bauza K, Friesen J, Gonzalez-Aseguinolaza G, Hill AV, Matuschewski K. 2013.  
510 Identification of targets of CD8(+) T cell responses to malaria liver stages by genome-wide  
511 epitope profiling. PLoS Pathog 9:e1003303.
- 512 31. Lau LS, Fernandez-Ruiz D, Mollard V, Sturm A, Neller MA, Cozijnsen A, Gregory JL, Davey GM,  
513 Jones CM, Lin YH, Haque A, Engwerda CR, Nie CQ, Hansen DS, Murphy KM, Papenfuss AT,  
514 Miles JJ, Burrows SR, de Koning-Ward T, McFadden GI, Carbone FR, Crabb BS, Heath WR.  
515 2014. CD8+ T cells from a novel T cell receptor transgenic mouse induce liver-stage immunity  
516 that can be boosted by blood-stage infection in rodent malaria. PLoS Pathog 10:e1004135.



- 517 32. Pichugin A, Zarling S, Perazzo L, Duffy PE, Ploegh HL, Krzych U. 2018. Identification of a Novel  
518 CD8 T Cell Epitope Derived from Plasmodium berghei Protective Liver-Stage Antigen. *Front*  
519 *Immunol* 9:91.
- 520 33. Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, Lang T, Gould J, Dubois MC,  
521 Jongert E, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S,  
522 Mturi N, Drakeley CJ, Savarese B, Villafana T, Lapierre D, Ballou WR, Cohen J, Lemnge MM,  
523 Peshu N, Marsh K, Riley EM, von Seidlein L, Bejon P. 2011. Efficacy of RTS,S/AS01E malaria  
524 vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in  
525 children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet*  
526 *Infect Dis* 11:102-9.
- 527 34. White MT, Verity R, Griffin JT, Asante KP, Owusu-Agyei S, Greenwood B, Drakeley C, Gesase  
528 S, Lusingu J, Ansong D, Adjei S, Agbenyega T, Ogutu B, Otieno L, Otieno W, Agnandji ST, Lell B,  
529 Kreamsner P, Hoffman I, Martinson F, Kamthunzu P, Tinto H, Valea I, Sorgho H, Onoko M,  
530 Otieno K, Hamel MJ, Salim N, Mtoro A, Abdulla S, Aide P, Sacarlal J, Aponte JJ, Njuguna P,  
531 Marsh K, Bejon P, Riley EM, Ghani AC. 2015. Immunogenicity of the RTS,S/AS01 malaria  
532 vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a  
533 phase 3 randomised controlled trial. *Lancet Infect Dis* 15:1450-8.
- 534 35. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, Schwenk R,  
535 Nielsen RA, Debebe Z, Pinelis E, Juompan L, Williams J, Dowler M, Stewart VA, Wirtz RA,  
536 Dubois MC, Lievens M, Cohen J, Ballou WR, Heppner DG, Jr., Rts SVEG. 2009. Randomized,  
537 double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in  
538 malaria-naive adults: safety, efficacy, and immunologic associates of protection. *J Infect Dis*  
539 200:337-46.
- 540 36. O'Hara GA, Duncan CJ, Ewer KJ, Collins KA, Elias SC, Halstead FD, Goodman AL, Edwards NJ,  
541 Reyes-Sandoval A, Bird P, Rowland R, Sheehy SH, Poulton ID, Hutchings C, Todryk S, Andrews  
542 L, Folgori A, Berrie E, Moyle S, Nicosia A, Colloca S, Cortese R, Siani L, Lawrie AM, Gilbert SC,  
543 Hill AV. 2012. Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new  
544 vaccine vector. *J Infect Dis* 205:772-81.
- 545 37. Reyes-Sandoval A, Wyllie DH, Bauza K, Milicic A, Forbes EK, Rollier CS, Hill AV. 2011. CD8+ T  
546 effector memory cells protect against liver-stage malaria. *J Immunol* 187:1347-57.
- 547 38. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V. 1980. Monovalent fragments (Fab)  
548 of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against  
549 malarial infection. *J Exp Med* 151:1504-13.
- 550 39. Muller K, Matuschewski K, Silvie O. 2011. The Puf-family RNA-binding protein Puf2 controls  
551 sporozoite conversion to liver stages in the malaria parasite. *PLoS One* 6:e19860.  
552