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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

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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^d, 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 γ -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⁺ 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^d-restricted CD8⁺ 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⁺ 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⁺ 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^d-restricted immunodominant epitope
95 of *PbCSP*, plays in protective immunity induced after live attenuated sporozoite immunisation.
96 For this purpose, *PbCSP*^{SIINFEKL} radiation-attenuated sporozoites (RAS), where the SYPSAEKI
97 sequence has been replaced with the H-2-K^b-restricted epitope of ovalbumin, SIINFEKL ((26),
98 Müller and Gibbins et al., unpublished) were used to immunise H-2-K^d-expressing BALB/c
99 mice. To date, there are no other reported strongly immunogenic H-2-K^d-restricted *PbCSP*
100 epitopes identified. Removal of SYPSAEKI, by replacement with an irrelevant epitope, in the
101 *PbCSP*^{SIINFEKL} 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- γ -producing
104 SYIPSAEKI-specific CD8⁺ 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*^{SIINFEKL} RAS parasites elicited no
107 SYIPSAEKI-specific CD8⁺ T cell responses in BALB/c mice.

108

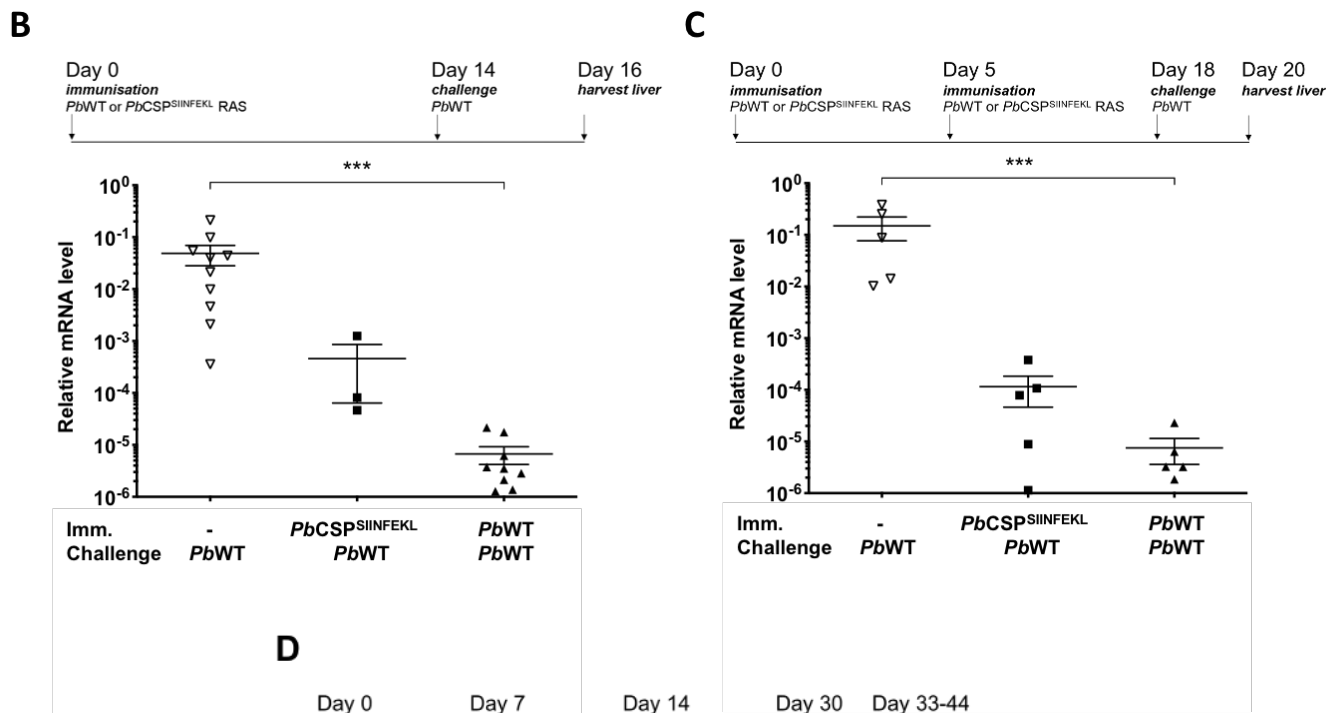
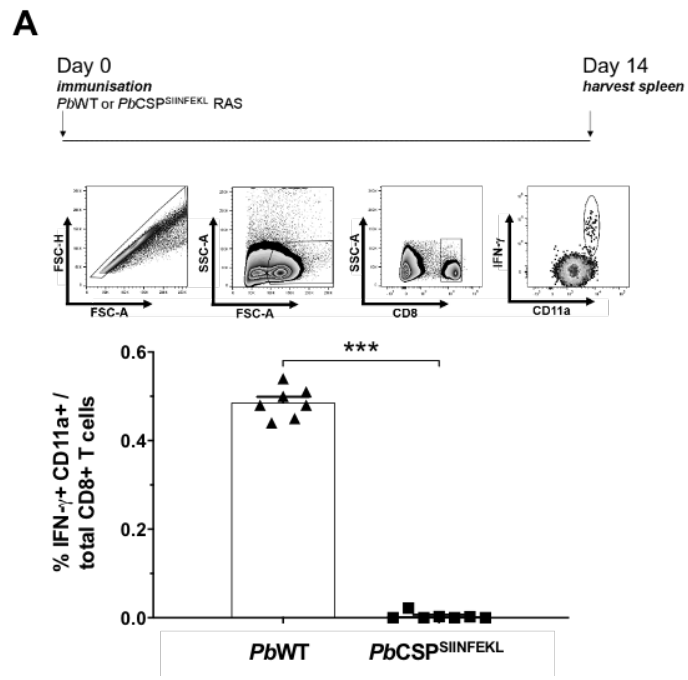
109 To ascertain whether SYIPSAEKI contributes to parasite-induced protection, BALB/c mice
110 were immunised once (Fig 1B) or twice (Fig 1C) with either *PbWT* or *PbCSP*^{SIINFEKL} RAS. Two
111 weeks after immunisation, the mice were challenged with *PbWT* sporozoites and protection
112 was determined 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*^{SIINFEKL} 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

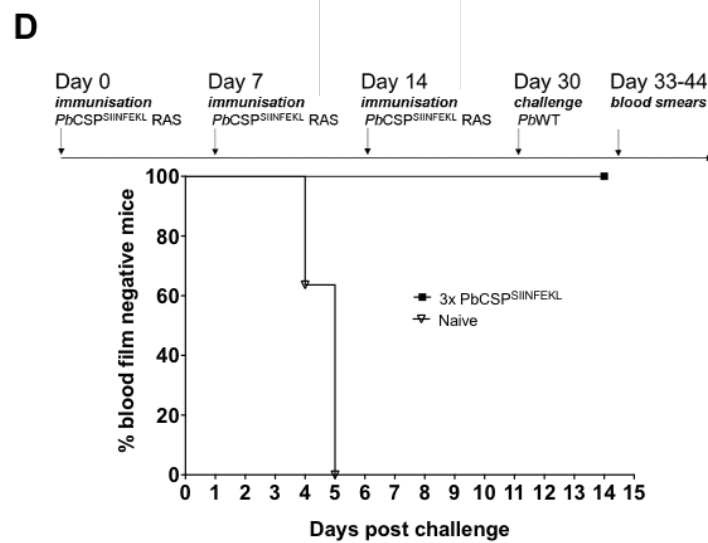
121 However, multiple immunisations with RAS are required to induce sterile protection. To
122 establish whether the development of sterile immunity is dependent on SYIPSAEKI-specific
123 CD8+ T cell responses, BALB/c mice were immunised thrice with *PbCSP*^{SIINFEKL} RAS one week
124 apart; two weeks 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*^{SIINFEKL} RAS. Splenocytes were taken after two weeks and restimulated with
146 SYIPSAEKI peptide. IFN- γ -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- γ -producing CD11a of total CD8+ T cells (bottom). (B) Groups of BALB/c mice were
149 immunised once with 15,000 *PbWT* or *PbCSP*^{SIINFEKL} 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*^{SIINFEKL} 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*^{SIINFEKL} 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).

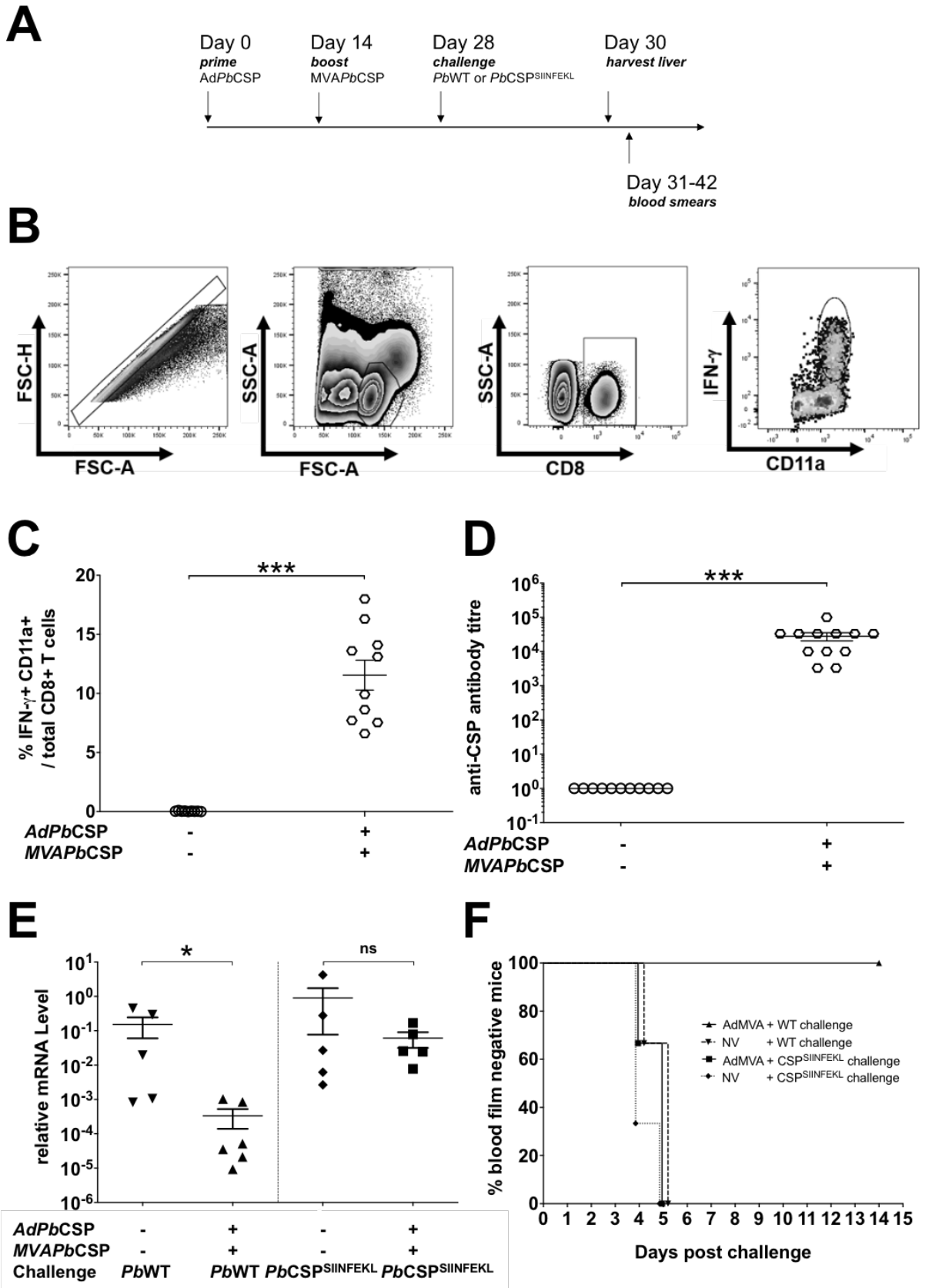
176

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- γ 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 (1:10⁴). 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*^{SIINFEKL} 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*^{SIINFEKL} sporozoites, in marked contrast to challenge with *PbWT* sporozoites. In perfect

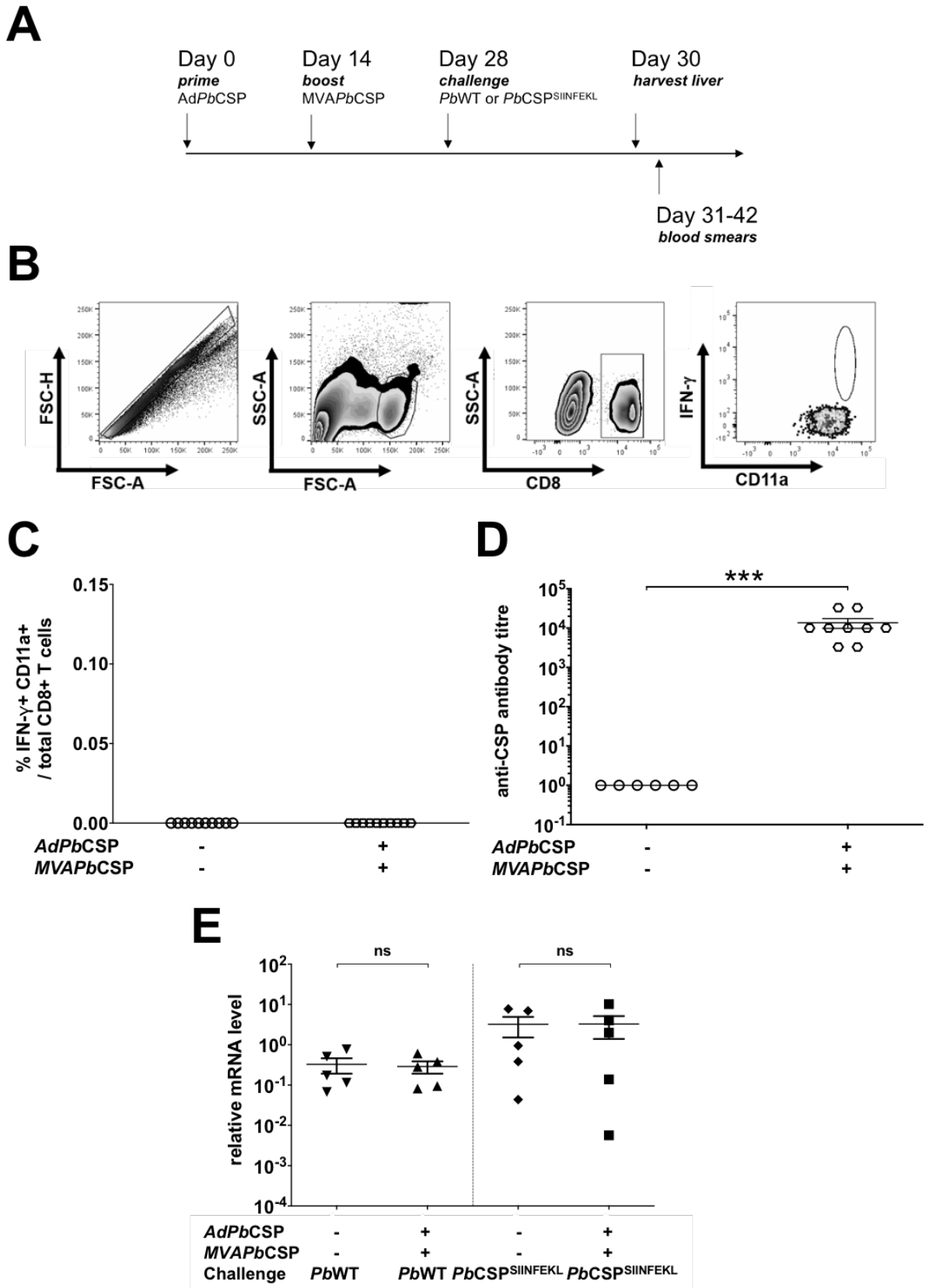
196 agreement, vaccinated mice challenged with *PbCSP*^{SIINFEKL} sporozoites were patent for
197 parasitaemia by day 5, whereas vaccinated mice challenged with *PbWT* sporozoites remained
198 completely protected. These results denote that vaccine-induced effector SYIPSEAKI-specific
199 CD8+ T responses 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⁺ T cell responses, and SYIPSAEKI-specific CD8⁺ 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*^{SIINFEKL} sporozoites as shown. (B) Flow cytometry gating strategy used to
209 determine proportions of IFN- γ ⁺ CD11a⁺ CD8⁺ T cells. (C) Proportion of IFN- γ -producing
210 CD11a of total CD8⁺ 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- γ 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*^{SIINFEKL} sporozoites (n=5) and non-vaccinated mice (-) challenged with *PbWT*
217 (n=5) or *PbCSP*^{SIINFEKL} 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*^{SIINFEKL} sporozoites. Vaccinated mice
221 challenged with *PbWT* (triangles) or *PbCSP*^{SIINFEKL} (squares) and non-vaccinated mice
222 challenged with *PbWT* (inverted triangles) or *PbCSP*^{SIINFEKL} (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*^{SIINFEKL} parasites (Fig 3A). C57BL/6 mice were used because SYIPSAEKI is
233 an H-2-K^d 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⁺ 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 (1:10⁴) were elicited (Fig. 3D). Ad-MVA CSP-vaccinated
238 C57BL/6 mice challenged with either *PbWT* or *PbCSP*^{SIINFEKL} 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*^{SIINFEKL} sporozoites as shown. (B) Flow cytometry gating strategy
246 used to determine proportions of IFN- γ ⁺ CD11a⁺ CD8⁺ T cells. (C) Proportion of IFN- γ -
247 producing CD11a of total CD8⁺ 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- γ 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*^{SIINFEKL},
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^d-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^d-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^{SIINFEKL}
329 (*PbCSP*^{SIINFEKL}) parasite was generated with the immunodominant CSP CD8+ T cell epitope
330 SYIPSAEKI (252-260aa) being replaced with the H-2^b 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*^{SIINFEKL} 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 γ -irradiation at 1.2×10^4 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°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°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°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 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). 2×10^6 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°C and 5% CO_2 . For staining
370 of cell surface markers and intracellular cytokines, cells were incubated for 1 hour at 4°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- γ (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^{SIINFEKL}; 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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