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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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17 Running Head: Immunodominant malaria CD8+ T cell epitope
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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.

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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 protection (5), and CD8+ T cells are the prime mediators of cell-mediated protective immunity,
52 as exemplified in murine (6, 7) and non-human primate (8) infection models.
53
54 The circumsporozoite protein (CSP), the major surface coat protein of the malaria sporozoite,
55 has been at the forefront of vaccination studies – being the basis of RTS,S/AS01, the most
56 progressed malaria vaccine candidate to date (9). Immunisation of BALB/c mice
57 with *Plasmodium berghei* (*Pb*) or *P. yoelii* (*Py*) RAS evokes immunodominant major
58 histocompatibility complex class I (MHC-I) H-2-K^d-restricted $CD8^+$ T cell responses against
59 distinct CSP epitopes: SYIPSAEKI for *Pb* (10) and SYVPSAEQI for *Py* (11). Indeed, the
60 measurement of responses to these epitopes has become the standard in fundamental
61 immunological studies in BALB/c mice (12-14). Furthermore, numerous vaccination studies
62 involving different viral-vectored CSP- or CSP epitope-expressing vaccines – used alone or in
63 combination as part of prime-boost regimens – have corroborated that CSP is a highly
64 protective antigen in the BALB/c infection model (12-18). In these studies, elevated levels of
65 either SYIPSAEKI- or SYVPSAEQI-specific CD8+ T cell responses correlated with protection.
66
67 Several studies have interrogated and contested the immunological relevance of CSP in
68 parasite-induced protection. These studies emanated from observations that in naturally
69 exposed humans T cell responses to CSP are scarce (19). In murine malaria models, multiple
70 immunisations are required to elicit CD8+ T cell-dependent protective immunity in various

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71 mouse strains, particularly where no other CSP-derived CD8+ T cell epitopes have been
72 identified (20). Furthermore, in *Py*CSP-transgenic BALB/c mice that are tolerant to *Py*CSP,
73 complete protection can be achieved by *Py* RAS immunisation (21). In good agreement,
74 BALB/c mice immunised with *Pb* WT parasites are completely protected when challenged with
75 transgenic *Pb* parasites where the endogenous CSP has been swapped with the *P. falciparum*
76 CSP (22). Taken together, these studies indicate that immune responses to CSP are
77 dispensable for protection, and that other antigens are important to elicit protective immunity.
78
79 In this study, we have extended previous work on the entire CSP by dissecting the relevance
80 of a single CSP-derived immunodominant epitope in parasite- and vaccine-induced protection.
81 As the most stringent model system, we utilised transgenic *Pb* parasites lacking SYIPSAEKI
82 for immunisation and challenge experiments in BALB/c mice. In addition, we have highlighted
83 the level of protection achieved by CSP-based vaccines in mice expressing the relevant
84 (BALB/c) or irrelevant (C57BL/6) MHC-I needed to present the CSP-derived immunodominant
85 epitope.
86

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87 **RESULTS**

88 **Sporozoite-induced SYIPSAEKI-specific CD8+ T cell responses contribute to parasite**
89 **killing but are dispensable for the development of sterile immunity.**

90 First, we interrogated the role that SYIPSAEKI, the H-2-K^d-restricted immunodominant epitope
91 of *Pb*CSP, plays in protective immunity induced after live attenuated sporozoite immunisation.
92 For this purpose, *Pb*$CSP^{SIINFEKL}$ radiation-attenuated sporozoites (RAS), where the SYPSAEKI
93 sequence has been replaced with the H-2-K^b-restricted epitope of ovalbumin, SIINFEKL ((23),
94 Müller and Gibbins et al., unpublished) were used to immunise H-2-K^d-expressing BALB/c
95 mice. To date, there are no other reported H-2-K^d-restricted *Pb*CSP epitopes identified.
96 Removal of SYPSAEKI, by replacement with an irrelevant epitope, in the *Pb*$CSP^{SIINFEKL}$
97 parasites allows unequivocal assignment of critical roles of this immunodominant *Pb*CSP-
98 derived epitope in protection elicited by live sporozoite immunisations. Two weeks after
99 immunisation, the frequencies of IFN-γ-producing SYIPSAEKI-specific CD8+ T cell responses
100 in the spleen after gating for CD11a expression (an activation marker commonly used to
101 identify antigen-experienced cells (24)) were measured by flow cytometry (Fig. 1A). As
102 expected, *Pb*$CSP^{SIINFEKL}$ RAS parasites elicited no SYIPSAEKI-specific CD8+ T cell responses
103 in BALB/c mice.

104

105 To ascertain whether SYIPSAEKI contributes to parasite-induced protection, BALB/c mice
106 were immunised once with either *Pb*WT or *Pb*$CSP^{SIINFEKL}$ RAS. Two weeks after immunisation,
107 the mice were challenged with *Pb*WT sporozoites and protection was determined by
108 measuring the parasite loads in the liver 40 hours later (Fig. 1B). A significant reduction in
109 parasite load – up to four orders of magnitude difference as compared to naïve mice – was
110 observed in mice immunised with *Pb*WT RAS and challenged with *Pb*WT parasites. In contrast,
111 protection was reduced only by approximately two orders of magnitude in mice immunised with
112 *Pb*$CSP^{SIINFEKL}$ RAS (Fig. 1B). These results highlight the notion that within *Pb*CSP, the
113 SYIPSAEKI epitope has a critical and immunodominant contribution to protecting BALB/c mice
114 after one or two immunisations with RAS.

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116 However, multiple immunisations with RAS are required to induce sterile protection. To
117 establish whether the development of sterile immunity is dependent on SYIPSAEKI-specific
118 CD8+ T cell responses, BALB/c mice were immunised thrice with *Pb*CSP^{SIINFEKL} RAS one week
119 apart; two weeks after the last immunisation, mice were challenged with *Pb*WT sporozoites
120 (Fig. 1C). All mice were protected from blood stage infection compared to the naïve controls,
121 implying that SYIPSAEKI-specific CD8+ T cell responses are not necessary for the
122 development of sterile immunity.
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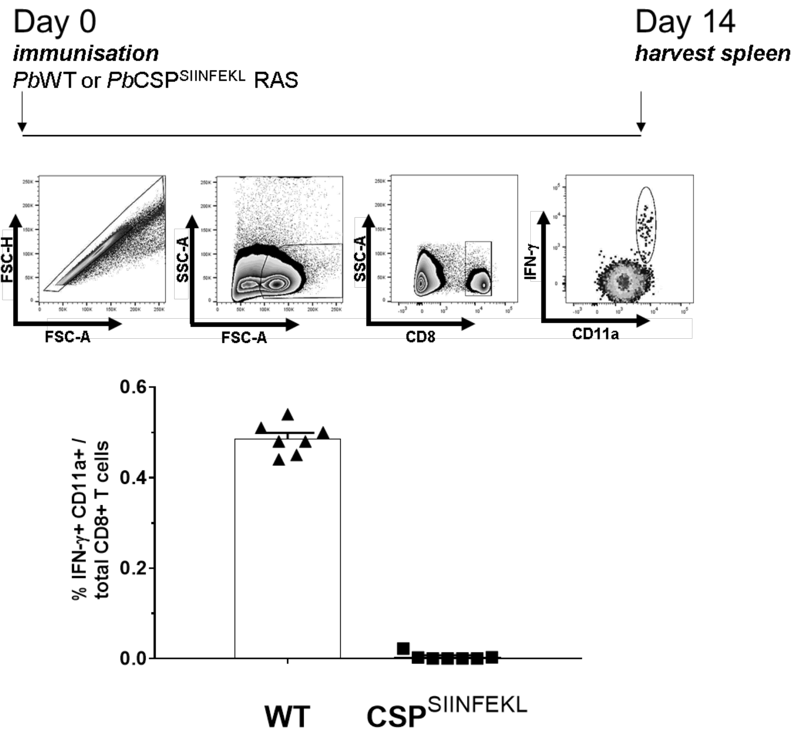

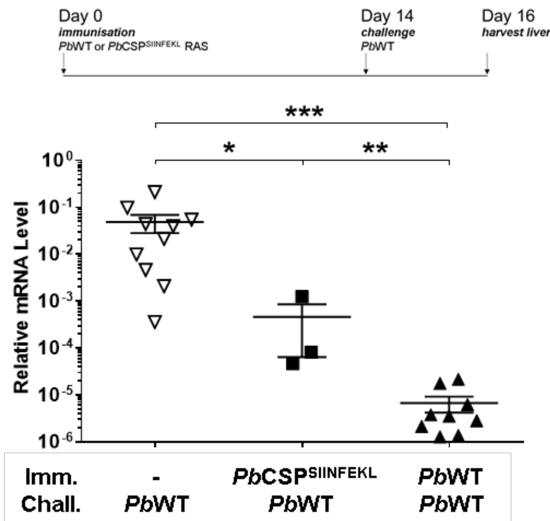

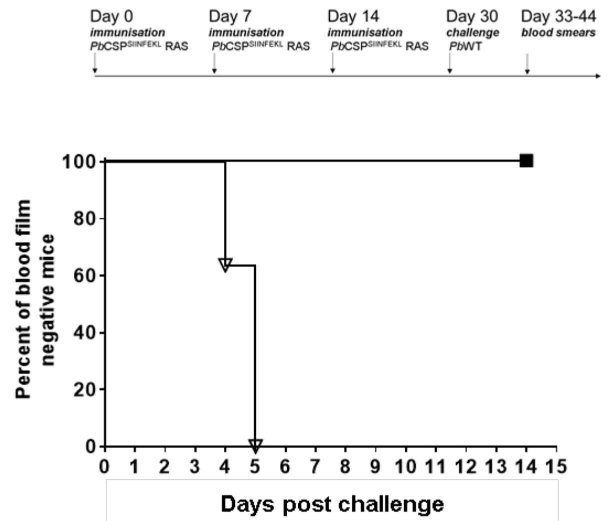

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143 **FIG 1** SYIPSAEKI is dispensable for RAS immunisation but predominates protection with fewer
144 immunisations. (A) BALB/c mice were immunised once with 10,000 *Pb*WT or *Pb*CSP^{SIINFEKL}
145 RAS. Splenocytes were taken after two weeks and restimulated with SYIPSAEKI peptide. IFN-
146 γ-producing cells co-staining with CD11a were assessed by flow cytometry. Shown are the
147 time course (top), the gating strategy (centre) and proportion of IFN-γ-producing CD11a of total
148 CD8+ T cells (bottom). (B) Groups of BALB/c mice were immunised once with 15,000 *Pb*WT
149 or *Pb*CSP^{SIINFEKL} RAS. Immunised mice and BALB/c naïve controls (n=3-10) were challenged
150 with 10,000 *Pb*WT parasites two weeks after the last immunisation. Livers were harvested 40
151 hours post-challenge and the relative liver parasite loads were quantified using the ΔΔCt
152 method comparing levels of *P. berghei* 18S rRNA and levels of mouse *GAPDH* mRNA. Mean
153 values (±SEM) are shown and statistics were calculated using the Mann-Whitney U-test (*,
154 $p<0.05$; **, $p<0.01$; ***, $p<0.001$). (C) BALB/c mice (n=12) were immunised with three doses
155 of 10,000 *Pb*CSP^{SIINFEKL} RAS at one-week intervals. Immunised mice and naïve controls (n=11)
156 were challenged with 5,000 *Pb*WT sporozoites 16 days after the last immunisation. Blood
157 smears were taken daily for two weeks after challenge. Parasitaemia was assessed by
158 microscopic examination of Giemsa-stained smears. Data shown is a combination of two
159 independent experiments.
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161 **Prime-boost vaccination with CSP-expressing viruses induces strong anti-CSP**
162 **antibody and CD8+ T cell responses and SYIPSAEKI is the key mediator of sterile**
163 **protection.**
164 Next, we probed the requirement for SYIPSAEKI presentation in protection elicited by viral-
165 vectored CSP-expressing vaccines administered in a prime-boost regimen. Priming with
166 adenovirus (Ad) carrying a foreign antigen and boosting with orthopoxvirus modified vaccinia
167 Ankara (MVA) expressing the same antigen has consistently been shown to induce strong
168 CD8+ T cell responses with high levels of protective efficacy against intracellular pathogens
169 including malaria pre-erythrocytic stages (15, 18).
170
171 Chimpanzee adenovirus serotype 63 (AdCh63) and MVA vaccines expressing *Pb*CSP were
172 used to vaccinate BALB/c mice with a two-week resting period between priming and boosting
173 (Fig. 2A). Two weeks after boosting, whole blood was collected and restimulated *ex vivo* with
174 SYIPSAEKI peptide. The frequencies of IFN-γ secreting CD8+ T cells were enumerated by
175 flow cytometry (Fig. 2B) and Ad-MVA *Pb*CSP-vaccinated mice elicited ~12% SYIPSAEKI-
176 specific circulating CD8+ T cells (Fig. 2C). Serum samples were also collected from the
177 vaccinated animals and were used in an immunofluorescence assay against air-dried *Pb*
178 sporozoites (Fig. 2D). Ad-MVA *Pb*CSP-vaccinated BALB/c mice induced high anti-CSP
179 antibody tires (1:10^4). These data indicate that Ad-MVA *Pb*CSP vaccination elicit both high
180 frequencies of SYPSAEKI-specific CD8+ T cells and high titres of CSP-specific antibodies.
181
182 Two weeks after boosting, Ad-MVA *Pb*CSP-vaccinated mice were challenged with *Pb*WT or
183 *Pb*CSP^{SIINFEKL} parasites. Protection was assessed by two complementary assays; (i)
184 determination of the reduction of parasite load in the liver (Fig. 2E) and (ii) induction of sterile
185 protection (Fig. 2F). Strikingly, parasite load in the liver of Ad-MVA *Pb*CSP-vaccinated mice
186 was not significantly reduced compared to non-vaccinated mice when challenged with
187 *Pb*CSP^{SIINFEKL} sporozoites, in marked contrast to challenge with *Pb*WT sporozoites. In perfect
188 agreement, vaccinated mice challenged with *Pb*CSP^{SIINFEKL} sporozoites were patent for
189 parasitaemia by day 5, whereas vaccinated mice challenged with *Pb*WT sporozoites remained

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190 completely protected. These results denote that vaccine-induced effector SYIPSEAKI-specific
191 CD8+ T responses efficiently target parasites expressing the cognate epitope. Parasites
192 lacking the SYIPSAEKI epitope are not eliminated despite high levels of CSP-specific
193 antibodies evoked by vaccination in this experimental system.
194

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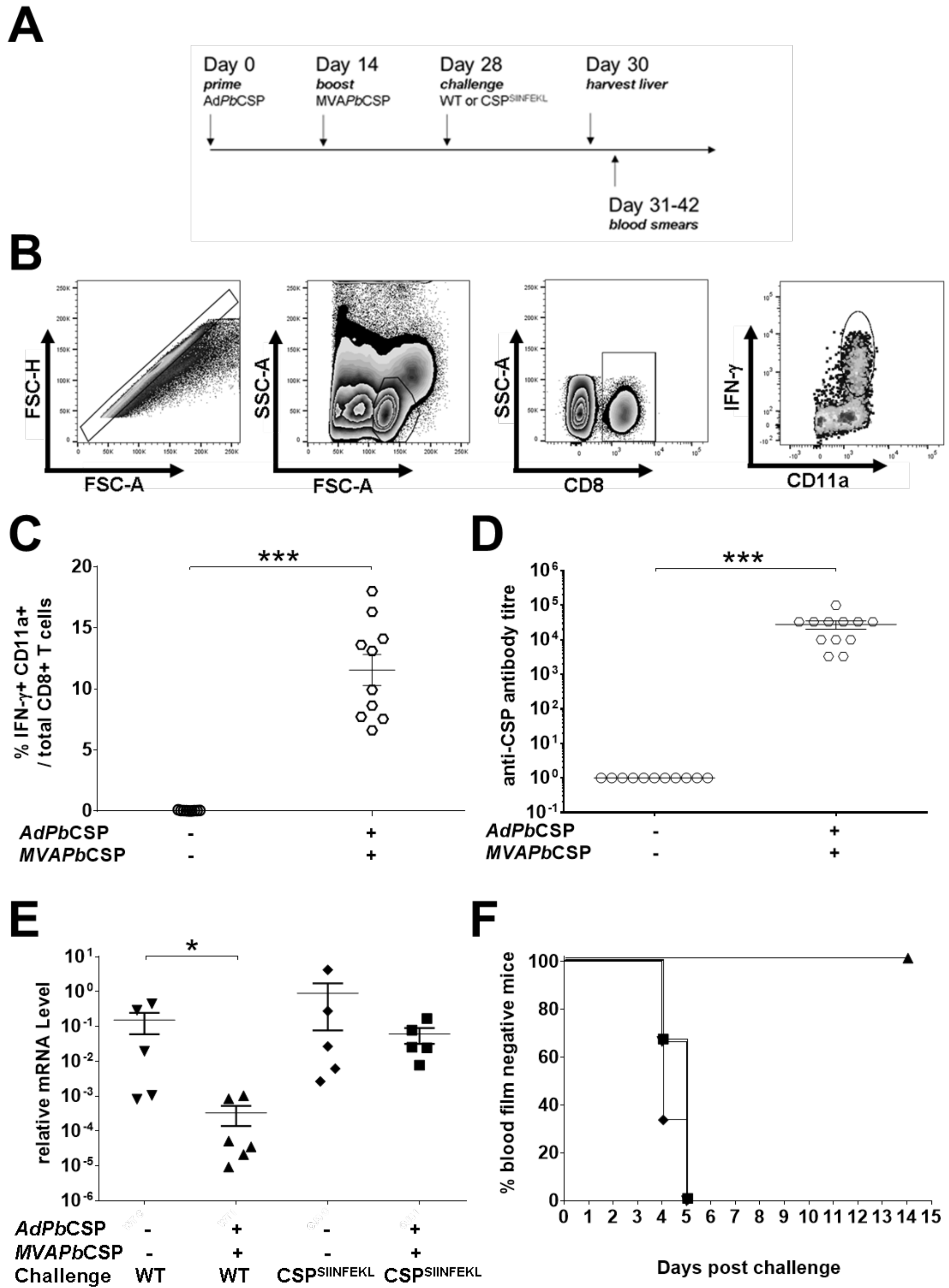

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200 **FIG. 2** Prime-boost vaccination with viral vectored CSP-expressing vaccines induces strong
201 anti-CSP antibody and CD8+ T cell responses, and SYIPSAEKI-specific CD8+ T cell
202 responses are essential for protection. (A) BALB/c mice were vaccinated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

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224 **CSP-based vaccines do not elicit sterile immunity in C57BL/6 mice.**

225 To further investigate the requirement of SYIPSAEKI as the indispensable protective epitope
226 of CSP, mice unable to present this epitope were vaccinated with the *Pb*CSP prime-boost
227 regimen with an interval of two weeks between vaccines, followed by challenge with either
228 *Pb*WT or *Pb*CSP^{SIINFEKL} parasites (Fig 3A). C57BL/6 mice were used because SYIPSAEKI is
229 an H-2-K^{d} restricted epitope, and this mouse strain does not express the relevant MHC-I allele.
230 Thus, SYIPSAEKI would fail to be presented by infected hepatocytes. As before, blood and
231 serum were derived two weeks after boost. As expected, SYIPSAEKI-specific CD8+ T cells
232 (Fig. 3B) were not detectable in Ad-MVA *Pb*CSP-vaccinated C57BL/6 mice (Fig. 3C), but
233 strong anti-CSP antibody titres (1:10^{4}) were elicited (Fig. 3D). Ad-MVA CSP-vaccinated
234 C57BL/6 mice challenged with either *Pb*WT or *Pb*CSP^{SIINFEKL} parasites had comparable
235 parasite load in the liver (Fig. 3E), indicative of full liver stage development in all groups. In
236 perfect agreement, all mice from any groups (15/15) developed blood infections.

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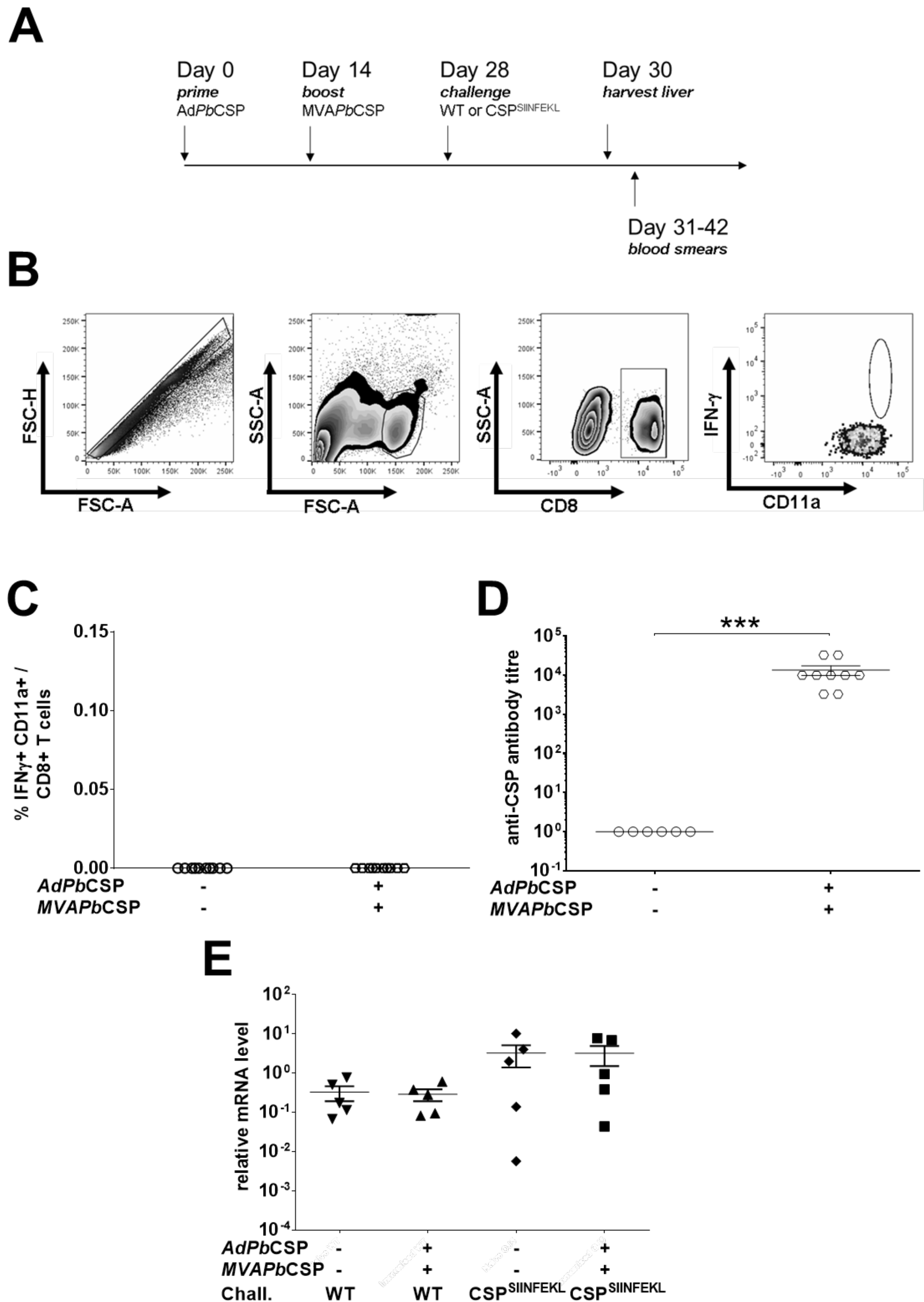

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242 **FIG 3** Prime-boost vaccination with CSP expressing viruses does not protect C57BL/6 mice,
243 irrespective of induced antibody titres.
244 (A) C57BL/6 mice were vaccinated with AdCh63 and MVA vaccines *Pb*CSP and challenged
245 with 10,000 *Pb*WT or *Pb*CSP^{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 ΔΔCt 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 (± SEM) shown and statistics were calculated
257 using the Mann-Whiney test (***p<0.001).
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259 **DISCUSSION**

260 Our findings lend full support to the notion that CSP is an immunodominant sporozoite-derived
261 antigen (21). 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 *Pb*CSP^{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 (25).
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 (26). 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

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288 achieved in the absence of responses to the entire CSP antigen (21, 22). 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). In line with this, our findings lead us to suggest that future pre-erythrocytic malaria vaccine
296 research should not only focus on inducing strong CD8+ T cell responses against one or
297 multiple antigens, but should try to target a broad array of antigens covering diverse MHC to
298 offer the best protection possible. The identification of novel antigens and epitopes that
299 contribute to protection in H-2-K^d-restricted BALB/c mice, and ultimately in human populations
300 with broad MHC haplotypes, will aid this development. In C57BL/6 mice pre-erythrocytic
301 immunity is mounted irrespective of CSP-specific CD8+ T cell responses, and recent genome-
302 wide epitope profiling returned multiple sporozoite antigens and epitopes (27-29).
303 RTS,S/AS01, the leading subunit malaria vaccine based on CSP, seems to offer some
304 protection against *P. falciparum* re-infection (9). Partial and short-lived protection is likely
305 primarily mediated by the action of transitory anti-sporozoite antibodies (30-32). Strikingly,
306 peripheral blood CD8+ T cell responses were not identified to provide a role following
307 sporozoite challenge in this candidate vaccine. Together with previous findings (7, 14, 16, 21)
308 our data underscore efforts to improve the most advanced candidate malaria vaccine,
309 RTS,S/AS01, by eliciting CD8+ T cells against CSP or other immunodominant antigens.
310

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311 **MATERIALS AND METHODS**

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

322

323 ***Plasmodium* parasites and immunisation.** The transgenic *P. berghei* ANKA $CSP^{SIINFEKL}$
324 (*Pb*$CSP^{SIINFEKL}$) parasite was generated with the immunodominant CSP CD8+ T cell epitope
325 SYIPSAEKI (252-260aa) being replaced with the $H\text{-}2\text{-}^{b}$ restricted *Gallus gallus* ovalbumin
326 CD8+ T cell epitope SIINFEKL (258-265aa) via double homologous recombination ((23),
327 Müller and Gibbins *et al.*, unpublished). Wild-type *Plasmodium berghei* ANKA (clone c115cy1)
328 (*Pb*WT) and *Pb*$CSP^{SIINFEKL}$ were maintained by continuous cycling between murine hosts
329 (NMRI or CD-1) and *Anopheles stephensi* mosquitos. Infected mosquitos were kept in
330 incubators (Panasonic and Mytron) at 80% humidity and 20°C temperature. Sporozoites were
331 isolated from the salivary glands and attenuated by γ-irradiation at $1.2x10^{4}$cGy. Mice were
332 immunised with 10,000 sporozoites administered intravenously with multiple doses given one
333 week apart unless otherwise stated. For challenge infections, 5,000 or 10,000 sporozoites were
334 administered intravenously to assess sterile protection and parasite load in the liver,
335 respectively.

336

337 **Viral-vectored CSP-expressing vaccines.** AdCh63 and MVA vaccines expressing the
338 mammalian codon-optimised fragment of *Pb*CSP were constructed and propagated based on
339 previously published viral vectors (33, 34). The viral vectors were administered intramuscularly

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340 in endotoxin-free PBS at a concentration of 10^5 viral particles for Ad*Pb*CSP for the prime
341 immunisation and 10^6 viral particles MVA*Pb*CSP for the boost immunisation.
342
343 **Immunofluorescent antibody assay.** 10,000 sporozoites were spotted onto epoxy coated
344 glass slides with marked rings (Medco), dried at room temperature and stored at -20°C.
345 Thawed slides were fixed in acetone, dried and rehydrated with PBS before incubation in 10%
346 FCS supplemented DMEM (Gibco) for 1 hour at 37°C in a humid chamber. Serum at
347 concentrations $1:10^3$, $1:3.3x10^3$, $1:10^4$, $1:3.3x10^4$, $1:10^5$ (and, additionally, $1:3.3x10^5$ and $1:10^6$
348 for C57BL/6 serum) were added to the ring wells and incubated for 1 hour at 37°C in a humid
349 chamber. Slides were washed and stained with a mouse anti-CSP (35) primary antibody.
350 Hoechst33342 was added as the nuclear stain together with a respective fluorescently labelled
351 anti-mouse secondary antibody for a further one-hour incubation. Slides were washed and
352 mounted with 'Fluoromount-G' (Southern Biotech) and analysed by fluorescent microscopy
353 (Zeiss Axio Observer).
354
355 **Quantification of SYIPSAEKI-specific CD8+ T cell responses.** Spleens were harvested and
356 lymphocytes were derived by passing spleens through 40μm cell strainers (Corning).
357 Peripheral blood was drawn from the tail vein and collected in Na^+ heparin capillary tubes
358 (Brand) and assayed in 96-well flat bottom plates (Corning). Red blood cells were lysed using
359 PharmLyse (BD) and lymphocytes were resuspended in 10% FCS, 2% Penicillin-Streptomycin
360 and 1% L-glutamine supplemented RPMI 1640 (Gibco). Splenocytes were counted using a
361 40x dilution with Trypan Blue (ThermoFisher Scientific) and a Neubauer 'Improved'
362 haemocytometer (Biochrom). $2x10^6$ splenocytes and the lysed blood samples were prepared
363 in 96 well plates and incubated with a final concentration of 10μg/ml of SYIPSAEKI peptide in
364 in the presence of Brefeldin A (eBioScience) for 5-6 hours at 37°C and 5% CO_2. For staining
365 of cell surface markers and intracellular cytokines, cells were incubated for 1 hour at 4°C for
366 each staining. Cells were stained for CD8 (53-6.7) and CD11a (M17/4) (eBiosience). Splenic
367 cells were fixed with 4% paraformaldehyde and peripheral blood cells were fixed with 1%

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368 paraformaldehyde before staining for IFN-γ (XMG1.2) (eBioscience) in the presence of
369 Perm/Wash buffer (BD) for intracellular staining. Data was acquired by flow cytometry using
370 an LSRFortessa or LSRII (BD) and analysed using Flowjo9.5.2 (Tree Star, Inc.).
371
372 **Quantification of parasite load in the liver.** Livers were harvested 40-42 hours after
373 sporozoite challenge and total RNA was extracted following homogenisation using TRIzol
374 (ThermoFisher Scientific). cDNA was generated using the RETROScript Kit (Ambion).
375 Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System
376 and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative liver parasite levels
377 were quantified using the ΔΔCt method comparing levels of *P. berghei 18S* rRNA using specific
378 primers and normalised to levels of mouse *GAPDH* mRNA (36).
379
380 **Assessment of parasitaemia.** Sterile protection was assessed by daily blood smears, taken
381 from mice 3-14 days after sporozoite challenge, stained with Giemsa (improved solution; VWR)
382 to microscopically determine the presence of blood stage parasites.
383
384 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism v7 (GraphPad
385 Software, Inc.). Statistics were calculated using the Mann-Whitney U test.
386

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387 **AUTHOR CONTRIBUTIONS**

388 O.S. and J.C.R.H. designed the experiments in the laboratory of K.Matuschewski; O.S.
389 generated the transgenic parasites $CSP^{SIINFEKL}$; M.P.G., K.Müller., M.G., J.L. and E.D.P.
390 performed experiments and analysed data; K.B. and A.R.-S. generated the CSP-expressing
391 viruses Ad*Pb*CSP and MVA*Pb*CSP; M.P.G. and J.C.R.H. wrote the paper. All authors
392 commented on and approved the paper.

393

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