Long lived liver-resident memory T cells of biased specificities for abundant sporozoite antigens drive malaria protection by radiation-attenuated sporozoite vaccination

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26 **Short title**

27

28 RAS protects through long-lived sporozoite-specific liver T_{RM} cells

29

30 **Abstract**

31

32 Vaccination with radiation-attenuated sporozoites (RAS) can provide highly effective
33 protection against malaria in both humans and mice. To extend understanding of malaria
34 immunity and inform the development of future vaccines, we studied the protective response
35 elicited by this vaccine in C57BL/6 mice. We reveal that successive doses of *Plasmodium*
36 *berghei* RAS favour the generation of liver $CD8^+$ tissue-resident memory T cells (T_{RM} cells)
37 over circulating memory cells, and markedly enhance their longevity. Importantly, RAS
38 immunisation strongly skews the composition of the liver $CD8^+$ T_{RM} compartment towards
39 cells specific for abundant sporozoite antigens, such as thrombospondin-related adhesive
40 protein (TRAP) and circumsporozoite protein (CSP), which become major mediators of
41 protection. The increased prevalence of sporozoite specificities is associated with limited
42 intrahepatic parasite development and inhibition of naïve T cell responses to all parasite
43 antigens in previously vaccinated mice. This leads to the exclusive expansion of effector T
44 cells formed upon initial immunisation, ultimately reducing the diversity of the liver T_{RM} pool
45 later established. These findings provide novel insights into the mechanisms governing malaria
46 immunity induced by attenuated sporozoite vaccination and highlight the susceptibility of this
47 vaccine to limitations imposed by strain-specific immunity associated with the abundant, yet
48 highly variable sporozoite antigens CSP and TRAP.

49

50

Author Summary

Malaria remains a significant global health challenge. An efficient vaccine could significantly enhance malaria control. Vaccination with radiation-attenuated sporozoites (RAS) can induce highly efficient protection against malaria, and our study brings critical insights into the protective mechanisms elicited by this vaccine. We show that RAS stimulates the formation of parasite-specific cytotoxic memory T cells that permanently reside in the liver (liver T_{RM} cells). These cells are critical mediators of protection. Interestingly, multiple doses of RAS extend the lifespan of these memory cells, potentially improving long term immunity. However, we found that the induced memory T cell response is strongly skewed towards abundant, but highly variable, sporozoite proteins. Thus, this phenomenon exposes a potential limitation of the RAS vaccine against the great parasite diversity in the field, as it focuses the T cell response away from less abundant, but more conserved, parasite antigens.

Keywords

Tissue-resident memory T cells, CD8 T cells, T cell biology, immunodominance, liver immunity, malaria vaccine, radiation-attenuate sporozoites.

69 **Introduction**

70

71 Malaria still kills over 600,000 individuals annually, with 75% of these fatalities occurring
72 among children under five years of age in low income countries [1]. Vaccines stand out as one
73 of the most efficient and cost-effective public health interventions against infectious diseases
74 [2], particularly in resource-limited regions. Radiation attenuated sporozoites (RAS) is one of
75 the most effective malaria vaccines, demonstrating long lived sterilising protection across
76 mice, non-human primates (NHP), and humans against *Plasmodium* spp. sporozoite infection
77 [3-5]. This vaccination approach involves the inoculation of sporozoites attenuated through
78 exposure to either X- or gamma-irradiation. Attenuation is achieved by a carefully calibrated
79 sublethal radiation dose that enables parasites to invade the liver after injection into the host
80 but induces their subsequent arrest in this organ, inhibiting progress to the blood stage [6-8].

81

82 Considerable research has been dedicated to elucidating the protective mechanisms that
83 underlie RAS-induced protection. RAS vaccination requires multiple doses for maximal
84 efficacy [9], and elicits both humoral and cellular immunity. Although the former can
85 contribute to protection [10-13], cellular immunity, mediated by $CD8^+$ T cells, is a pivotal
86 component of RAS-mediated immunity, as demonstrated by the susceptibility to infection of
87 different strains of vaccinated mice, and NHP, when these cells are removed [14-16]. RAS
88 vaccination of mice and NHP was found to induce the accumulation of memory $CD8^+$ T cells
89 in the liver [13, 17-19]. Memory T cells can be subdivided into two major subtypes: circulating
90 memory cells (T_{CIRCM}), in turn separated into central (T_{CM}) and effector (T_{EM}) memory T
91 cells depending on their recirculating pattern, and tissue-resident memory T cells (T_{RM}), which
92 remain in the organ wherein they are formed [20]. Although both types of memory $CD8^+$ T
93 cells are elicited by RAS vaccination and can contribute to protection [21, 22], tissue-resident

94 memory T cells appear to be critical in C57BL/6 mice [22], which are highly susceptible to
95 infection by *P. berghei* and require higher doses of RAS for sterilising immunity than other
96 strains [23, 24]. This notion is also supported in humans, as sterilising protection is still
97 detected in repeatedly vaccinated individuals despite their antibody levels or numbers of
98 circulating memory $CD8^+$ T cells being comparable to those in their unprotected counterparts
99 [13, 19, 25].
100
101 $CD8^+$ T_{RM} cells must find and kill parasites during the brief period of residence in the liver,
102 which lasts 2 days in mice and 7 days in humans [26-28]. An essential determinant of the
103 capacity of $CD8^+$ T_{RM} cells to exert anti-parasitic protection is their antigen specificity, as
104 parasite antigens must be presented via Major Histocompatibility Complex class I (MHC-I)
105 molecules on hepatocytes to enable $CD8^+$ T_{RM} cell recognition of infected cells. *Plasmodium*
106 protein expression vastly changes throughout different stages of the parasite's life cycle [29,
107 30], and T_{RM} cells specific for antigens with contrasting expression patterns therefore differ in
108 their protective capacity. Thus, T_{RM} cells that recognise the 60S ribosomal protein L6 (RPL6),
109 an antigen predominantly expressed during liver stage, exhibit heightened protective efficacy
110 relative to those specific for thrombospondin-related adhesive protein (TRAP) [31], which is
111 mainly expressed by sporozoites [30, 32]. A broad diversity of T cell specificities is thought to
112 promote better protection [33], as late antigens with prolonged expression patterns provide a
113 longer window of opportunity for T cells to find and eliminate parasites before they progress
114 to the blood. Other requirements defining an antigen's immunogenicity and protective capacity
115 are its efficient processing by the proteasome for the generation of peptides that can be loaded
116 onto MHC-I molecules, the strong and stable binding of these peptides to MHC molecules, and
117 the presence of naïve T cells in the endogenous repertoire capable of responding to the
118 peptide/MHC-I complexes generated [34, 35]. Notably, C57BL/6 mice are not known to mount

119 $CD8^+$ T cell responses specific for *P. berghei* circumsporozoite protein (CSP) [36], another
120 major sporozoite antigen that is also expressed during liver stage.
121
122 Dissecting the dynamics of the protective T cell responses elicited by RAS is crucial for
123 understanding immunity to liver stage malaria. In this study, we sought to investigate the
124 landscape of memory T cell specificities triggered by successive *P. berghei* RAS vaccinations
125 in C57BL/6 mice. Our findings reveal a progressive skewing of the liver T_{RM} response towards
126 sporozoite antigen specificities, which undergo robust expansion and become major mediators
127 of protection against live sporozoite challenge. Surprisingly, this immunodominance is
128 established even for T cells targeting the sporozoite antigen TRAP, despite the modest intrinsic
129 protective capacity of these cells. Additionally, repeated immunisations significantly extend
130 the half-life of the parasite-specific T_{RM} cells generated while suppressing naïve T cell
131 responses to any parasite antigen. These elements limit the breadth of the ensuing memory
132 response by largely constraining the induced liver T_{RM} cell pool to cells specific for previously
133 encountered sporozoite antigens.

134 **Results**

135

136 **Liver CD8$^+$ T_{RM} cells protect RAS-vaccinated C57BL/6 mice against *P. berghei* sporozoite**
137 **infection**

138 Using MHC-I restricted, TCR transgenic PbT-I cells [37], specific for the *Plasmodium* antigen
139 RPL6 [31], we previously showed that the protection provided by two doses of RAS,
140 administered 30 days apart, is largely mediated by a liver-resident subset of memory CD8$^+$ T
141 cells [22]. However, the vaccination schedule utilised was suboptimal, only providing ~40%
142 of sterilising protection, and the presence of adoptively transferred PbT-I cells might have
143 altered the endogenous protective responses naturally elicited by this vaccine [38]. Thus, to
144 further characterise the mechanisms responsible for malaria immunity induced by RAS, we
145 injected mice with smaller doses of irradiated parasites (10,000 sporozoites) at shorter intervals
146 (one week apart), as this immunisation strategy is known to provide highly efficient sterilising
147 protection against *P. berghei* sporozoite infection in C57BL/6 mice [39]. Mice were injected
148 with 1, 2 or 3 weekly doses of 10,000 *P. berghei* RAS, and challenged with 200 live, fully
149 infectious *P. berghei* sporozoites 30 days after the last RAS injection. A single injection of
150 RAS (1xRAS) failed to induce sterilising protection against sporozoite infection, but mice in
151 this group displayed significantly reduced parasitemia compared to unvaccinated controls on
152 day 7 after challenge (Figure 1A, B). Protection was improved in mice vaccinated with two
153 doses of RAS (2xRAS), which showed significant sterile protection and further reduced day 7
154 parasitemia. Three RAS injections (3xRAS) conferred even higher sterilising protection
155 against sporozoite challenge (Figure 1A, B). Importantly, in line with previous findings in
156 C57BL/6 mice and non-human primates [14, 16], depletion of CD8$^+$ T cells in 3xRAS
157 vaccinated mice completely removed protection (Figure S1A-C). Furthermore, parasitemia on
158 day 7 in depleted mice was similar to that in unvaccinated controls, underscoring the major

159 role of $CD8^+$ T cells in protection in this system (Figure S1A-C). Next, we sought to define the
160 contribution of liver T_{RM} cells to protection. These cells were selectively depleted in 3xRAS
161 vaccinated mice via treatment with monoclonal antibodies specific for the T_{RM} surface marker
162 CXCR3 [22] prior to challenge with *P. berghei* sporozoites. This treatment efficiently removed
163 endogenous $CD8^+$ T_{RM} cells in the liver, but left numbers of effector memory $CD8^+$ T cells
164 unaltered (Figure S1D-H). Numbers of central memory T cells were moderately reduced in the
165 spleen, as some of these cells also express CXCR3, but not in the liver. As previously observed
166 [22], parasite-specific T_{CM} cells are minimally induced by RAS vaccination in this model and
167 are hence unlikely to play a significant role in protection (Figure S1F-H). Vaccinated mice
168 treated with αCXCR3 mAb and depleted of liver T_{RM} cells became fully susceptible to
169 sporozoite challenge, their parasitemias approaching those of unvaccinated mice (Figure 1C,
170 D). This result strongly indicated that protection in this system is largely $CD8^+$ liver T_{RM}-
171 dependent. Overall, these experiments confirmed that multiple RAS vaccinations can confer
172 highly efficient protection against *Plasmodium* sporozoite infection in C57BL/6 mice and
173 provided strong evidence that this protection is mediated by $CD8^+$ T_{RM} cells.
174
175 **RAS boosting favours generation of liver $CD8^+$ T_{RM} cells specific for abundant sporozoite**
176 **antigens**
177
178 As a whole parasite vaccine, RAS stimulates $CD8^+$ T cell responses of multiple specificities
179 [18, 33]. To better understand the features of the protective $CD8^+$ T cell response elicited by
180 repeated RAS vaccination, we next sought to determine the relative abundance of endogenous
181 memory T cells specific for known parasite antigens generated in 3xRAS vaccinated mice. We
182 focused on antigens with contrasting expression patterns during parasite development in the
183 mouse; i) Thrombospondin-related anonymous protein (TRAP, PBANKA_1349800),

184 containing the PbTRAP$_{130-138}$ epitope [40], which is expressed at high levels by sporozoites
185 [32, 41]; ii) the putative 60S ribosomal protein L6 (RPL6, PBANKA_1351900), an antigen
186 predominantly expressed during liver and blood stage, but less abundantly in sporozoites, that
187 contains the cognate antigen of PbT-I cells, PbRPL6$_{120-127}$ [31, 37]; and iii) the replication
188 protein A1 (RPA1, PBANKA_0416600), originally identified as a blood stage T cell antigen
189 [42] but also expressed during liver stage (Figure S2A), that contains the PbRPA1$_{199-206}$
190 epitope, also known as F4 [42]. Intriguingly, the size and composition of the memory CD8$^+$ T
191 cell compartment generated was markedly different depending on the number of RAS doses
192 administered. Thus, one dose of 10,000 RAS generated similar numbers of RPL6-specific and
193 TRAP-specific liver T_{RM} cells (Figure 2A, S2B), as well as circulating cells of these
194 specificities in the liver and the spleen (Figure S2B). RPA1-specific cells were also expanded,
195 indicating that this antigen is immunogenic during pre-erythrocytic stages, and this was also
196 the case for total numbers of memory CD8$^+$ T cells of undefined specificities (Figure 2A-C,
197 S2B). However, a second injection of RAS profoundly changed the relative abundance of
198 memory T cell specificities. TRAP-specific cells, particularly TRAP T_{RM} cells in the liver,
199 were significantly boosted, with an average 23-fold increase vs a single RAS injection (Figure
200 2A-C, S2B). In comparison, RPL6-specific cells were only increased 3.6-fold, and RPA1-
201 specific cells, or those of undefined specificities, remained largely unchanged (Figure 2C). This
202 trend became more pronounced in mice injected with three doses of RAS (Figure 2A-C, S2B).
203 Numbers of TRAP-specific T_{RM} cells were 77-fold higher in 3xRAS vaccinated mice compared
204 to 1xRAS (Figure 2C) and constituted on average more than 30% of total liver T_{RM} cells
205 (Figure 2B). In contrast, RPL6 and RPA1 specific cells expanded 7.5 to 3.5-fold vs 1xRAS
206 (Figure 2C) and only accounted for 4.5% and 2% of all liver T_{RM} cells respectively (Figure
207 2B). For all specificities, repeated injections of RAS progressively favoured the generation of
208 T_{RM} cells in the liver over circulating memory cells (T_{CIRCM}, comprising T_{EM} and T_{CM} cells),

209 as exemplified by the increasing ratios of liver T_{RM} vs spleen T_{CIRCM} cell numbers upon
210 successive rounds of RAS vaccination (Figure 2D). These results showed that repeated
211 vaccination with RAS favoured the generation of liver-resident memory $CD8^+$ T cells specific
212 for an abundant sporozoite antigen (i.e. TRAP) over those responding to antigens more
213 prominently expressed at later stages.
214
215 To consolidate these findings, we next determined whether a similar bias occurred for T cells
216 specific for sporozoite-associated antigens other than TRAP. We focused on a prototypical,
217 abundant sporozoite antigen, the circumsporozoite protein (CSP). As C57BL/6 mice do not
218 respond to *P. berghei* CSP, we utilised *P. berghei* CS5M parasites (termed CS5M henceforth)
219 for RAS immunisations. In these parasites, CSP was mutated to encode the $OVA_{257-264}$
220 (SIINFEKL) peptide, recognised by endogenous $CD8^+$ T cells in C57BL/6 mice [43]. Mice
221 were vaccinated with 1 or 3 doses of CS5M RAS and the numbers of memory $CD8^+$ T cells
222 specific for OVA, TRAP, RPL6 or RPA1 were measured 30 days later (Figure 2E-H, S3).
223 OVA-specific, as well as TRAP specific T_{RM} cells, expanded to much higher numbers
224 (approximately 46 and 51-fold increases respectively, Figure 2G) than RPL6- or RPA1-specific
225 T_{RM} cells (3.5 and 1.6-fold increase respectively) in 3x vs 1x CS5M RAS vaccinated mice,
226 with T_{RM} cells of other specificities increasing 4.3-fold (Figure 2E-G, S3). The combined
227 frequencies of CS5M-CSP and TRAP-specific cells accounted for close to half of all liver T_{RM}
228 cells (Figure 2F). Interestingly, in this case, OVA-specific T cells formed substantially higher
229 numbers of memory cells (4-fold more on average) than those specific for TRAP (Figure 2E,
230 F, S3), which did not expand to comparable numbers as when WT RAS were used for
231 immunisations (Figure 2A) and hence no known, potent competing sporozoite-specific T cell
232 response was elicited. This suggested that CS5M-CSP specific responses outcompeted those
233 against TRAP, even though both specificities were preferentially expanded in comparison to

234 RPL6 or RPA1. As observed previously (Figure 2D), for all specificities, repeated RAS
235 vaccinations biased memory T cell formation towards liver T_{RM} cells over T_{CIRCM} cells (Figure
236 2H). Together, these results verified that multiple RAS vaccinations favoured the generation
237 of resident memory $CD8^+$ T cells specific for abundant sporozoite antigens over those
238 responding to other antigens.
239
240 **Repeated RAS vaccination generates long lived liver T_{RM} cells**
241 Having observed that repeated RAS vaccination altered the memory T cell phenotype by
242 progressively favouring the development of T_{RM} cells, we next aimed to investigate whether
243 boosting could modify other intrinsic properties of these cells. To this end, we assessed the
244 impact of multiple RAS immunisations on the maintenance of liver T_{RM} cells of different
245 specificities over time. Mice were injected with either one dose of $5x10^4$ RAS, or three doses
246 of 10^4 RAS administered weekly, and the numbers of TRAP-, RPL6- and RPA1-specific
247 memory T cells were measured in the liver and spleen at several time points extending up to
248 100 days post-immunisation (Figure 3). Remarkably, T_{RM} cells of all specificities examined in
249 mice vaccinated with 3xRAS displayed markedly increased half-lives (Figure 3A-C), though
250 this trend was not significant for RPA-1 ($p=0.1023$). Specifically, the half-lives of liver T_{RM}
251 cells in 1xRAS vaccinated mice ranged from 47 to 81 days, while those in 3xRAS vaccinated
252 mice extended to nearly 230 days for TRAP-specific cells (Figure 3A), and exceeded a
253 thousand days for the later antigens, RPL6 and RPA1 (Figure 3B, C). Interestingly, this
254 increase in longevity was less pronounced and failed to reach significance for T_{EM} cells. For
255 all specificities, spleen T_{EM} cells displayed much shorter half-lives than liver T_{RM} cells and
256 exhibited moderate increased half-lives in 3xRAS mice, reaching 30-85 days, up from 20-50
257 days in 1xRAS mice (Figures 3D-F). In summary, these results showed that repeated RAS

258 vaccination increased the life span of parasite-specific memory T cells, with liver T_{RM} cells
259 displaying much larger increases.
260
261 **TRAP-specific immunity dominates protection**
262
263 To understand the implications of these findings, we sought to determine the contribution of
264 TRAP-specific T_{RM} cells to protection against challenge with *P. berghei* sporozoites in RAS
265 vaccinated mice. To do this, we suppressed the development of TRAP specific responses by
266 sporozoite vaccination through injection of $PbTRAP_{130-138}$ peptide in the absence of adjuvant,
267 which leads to the removal of T cells specific for this peptide [40, 44]. Mice received 3 doses
268 of TRAP peptide diluted in PBS prior to the first dose of RAS, and then received additional
269 injections a day before administration of the second and third doses of the vaccine (Figure 4A).
270 This resulted in efficient deletion of TRAP-specific cells (Figure S4A). In contrast,
271 administration of an irrelevant peptide (SIINFEKL, i.e. $OVA_{257-264}$) did not alter the generation
272 of TRAP specific memory cells (Figure S4A). Vaccination with three doses of RAS induced
273 strong protection against sporozoite challenge in untreated and SIINFEKL-treated control
274 mice. However, TRAP-tolerised mice displayed markedly reduced levels of sterile protection,
275 comparable to unvaccinated mice, and higher parasitemia 7 days after challenge (Figure 4B,
276 C). Those TRAP-tolerised mice that became infected had significantly lower parasitemias than
277 unvaccinated mice, suggesting that T_{RM} cells of other specificities contributed moderately to
278 protection (Figure S4B). Enumeration of liver T_{RM} cells in challenged mice (Figure 4D)
279 showed that, as expected, 3xRAS vaccination induced substantial numbers of TRAP-specific
280 liver T_{RM} cells, except in the single mouse that remained unprotected (a second unprotected
281 mouse could not be analysed as it developed cerebral malaria and had to be euthanised). TRAP
282 tolerization efficiently impaired formation of TRAP-specific liver T_{RM} cells in unprotected

283 mice, but two of the TRAP-tolerised mice that remained protected had high numbers of TRAP
284 T_{RM} cells, indicating that, in these mice, tolerisation did not work efficiently (Figure 4D). Also,
285 one out of the two non-protected mice in the OVA-tolerised control group tended to have low
286 numbers of TRAP specific cells (similarly to the unprotected mouse in the RAS group),
287 indicating that either RAS immunisation, or expansion of TRAP-specific cells, were
288 suboptimal in this mouse (Figure 4D). Together, these results demonstrated a key role for
289 TRAP-specific T_{RM} cells in protection against sporozoite challenge in C57BL/6 mice
290 vaccinated multiple times with RAS.
291
292 **RAS preferentially boosts previously activated T cells**
293
294 A potential mechanism to explain the T cell memory bias towards sporozoite antigen
295 specificities generated by repeated RAS vaccination was the rapid elimination of incoming
296 irradiated sporozoites by $CD8^+$ T cells induced by prior doses of RAS. This elimination would
297 particularly curtail the expression of late antigens by booster RAS, thereby limiting their
298 immunogenicity compared to the readily available antigens present in the sporozoite [45]. To
299 test whether prior RAS vaccination limited the development of parasites subsequently
300 administered in booster doses, mice were vaccinated with one or two consecutive doses of RAS
301 up to 8 days apart. Then, coinciding with the timing of the final dose in our established RAS
302 vaccination schedule, mice were instead challenged with an equivalent dose (10,000 parasites)
303 of non-irradiated, fully infectious sporozoites. We then assessed protection to evaluate whether
304 the pre-existing immune response interfered with the development of these live sporozoites
305 (Figure 5A). Mice that received a single dose of RAS prior to infection displayed marked
306 decreases in parasitemia on day 7 compared to unvaccinated controls, and some sterilising
307 protection (Figure 5B, C). Moreover, two doses of RAS provided complete sterilising

308 protection (Figure 5B, C). In agreement with previous work [45], these results indicated that
309 the effector response elicited by prior doses of RAS has the capacity to efficiently eliminate
310 incoming sporozoite infections, potentially impairing the generation of T cell responses against
311 liver stage antigens upon administration of subsequent doses of RAS.
312 Given the strong bias towards the development of sporozoite-specific responses observed in
313 figure 2, we reasoned that not all T cell responses elicited by RAS would be equally impaired
314 by this inhibitory mechanism. Particularly, we anticipated that responses targeting abundant
315 antigens in the incoming sporozoites would be less affected by quick parasite killing that those
316 specific for later or less abundant antigens. To test this hypothesis, we sought to define the
317 capacity of RAS to activate naïve T cells of different specificities for parasite antigens, in mice
318 that had been previously vaccinated with RAS, and hence had an ongoing T cell response
319 capable of killing further incoming parasites. In a first series of experiments, we adoptively
320 transferred naive PbT-I cells, i.e. RPL6-specific TCR transgenic T cells [31, 37], into mice that
321 had received no prior RAS vaccination, or one or two doses of RAS one week apart. Mice then
322 received a final dose of RAS one day after PbT-I T cell transfer, and the numbers of memory
323 cells generated by these transgenic T cells were examined 30 days after the last RAS injection
324 (Figure 5D). As expected, when PbT-I cells were transferred one day before a single RAS
325 vaccination, substantial numbers of memory PbT-I cells formed in the spleen and the liver,
326 including T_{RM} cells (Figure 5E, S5A). However, when naïve PbT-I cells were transferred one
327 day before a second RAS vaccination, fewer PbT-I liver T_{RM} cells formed. Furthermore, when
328 transferred prior to the third RAS immunisation, virtually no memory PbT-I cells were detected
329 (Figure 5E, S5A). Importantly, although PbT-I responses were strongly inhibited, endogenous
330 memory $CD8^+$ T cells of the same specificity (i.e. RPL6-specific) increased in numbers upon
331 successive RAS vaccinations (Figure 5F, S5A), and the same occurred for RPA1-specific cells
332 (Figure S5A). As observed before, large numbers of TRAP T_{RM} cells formed in these mice

333 (Figure 5G, S5A). Note that, in agreement with previous reports [37], PbT-I cells did not
334 detectably respond to persisting antigen when adoptively transferred into mice vaccinated with
335 RAS 6 days earlier (Figure S5B), and therefore the observed responses were induced by RAS
336 administered after adoptive T cell transfer. At an early time point (7 days) after the last injection
337 of RAS, PbT-I cells were virtually undetectable in the blood of mice receiving several doses
338 of RAS prior to PbT-I transfer, while numbers of endogenous TRAP- or RPL6-specific T cells
339 increased in the same mice upon administration of every additional dose of RAS (Figure S5C).
340 This indicated that diminished early expansion of PbT-I cells was responsible for the low
341 numbers of memory PbT-I cells subsequently detected. Moreover, this occurred while
342 endogenous $CD8^+$ T cells of the same specificity, activated by prior RAS vaccination,
343 continued expanding.
344
345 To extend these findings and better understand the mechanisms contributing to sporozoite
346 antigen immunodominance, we next sought to determine whether this inhibitory effect also
347 applied to other T cell specificities. To do this, we performed similar experiments as those
348 explained for PbT-I cells, but instead using naïve T cells of a different specificity, i.e. OVA-
349 specific OT-I cells [46]. To provide a target antigen for these cells, we utilised *P. berghei*
350 HsOVA (termed HsOVA henceforth) parasites for vaccination. In these parasites, a C-terminal
351 fragment of OVA (aa 150-386) was fused to the N terminus of a truncated version of the
352 parasite protein Hsp70 (retaining aa 201-398) [47]. This construct is expressed under the
353 control of the Hsp70 promoter, which induces high levels of transcription in sporozoites and
354 during the liver stage (Figure S5D) [30]. However, as mRNA is not necessarily indicative of
355 protein expression [48] or immunogenicity, we first sought to determine the T cell
356 immunogenicity of this antigen in sporozoites. Mice received OT-I cells and were
357 intravenously injected with heat killed sporozoites (HKS), thereby limiting the repertoire of

358 presentable antigens to T cells to those already existing in the sporozoite. Expansion of OT-I
359 cells was assessed in the spleen after 4 days. PbT-I cells were utilised as positive controls, as
360 they can respond to HKS [49]. We found that, indeed, OT-I cells moderately responded to
361 HsOVA HKS (Figure S5E), confirming the immunogenicity of this antigen in sporozoites. We
362 then proceeded to assess whether repeated RAS vaccination resulted in the inhibition of naïve
363 T cell responses specific for Hsp70-OVA. As with the previous experiments with PbT-I cells,
364 mice were vaccinated 1-3 times with HsOVA RAS, and naïve OT-I cells were transferred one
365 day prior to the last HsOVA RAS vaccination. Although OT-I cells generated strong memory
366 in 1xHsOVA RAS vaccinated mice, this response was completely inhibited in mice previously
367 vaccinated once or twice with HsOVA RAS (2xHsOVA RAS and 3xHsOVA RAS
368 respectively) (Figure 5H, S5F), appearing even more strongly inhibited than the response by
369 PbT-I cells (Figure 5E). As expected, endogenous TRAP specific cells formed substantially
370 increasing numbers of T_{RM} cells upon every additional RAS injection (Figure 5I, S5F), with
371 RPL6-specific T_{RM} cells, as well as T_{RM} cells of undefined specificity, displaying more
372 moderate increases (Figure S5F).
373
374 **Preferential boosting of pre-activated T cells extends to abundant sporozoite antigens**
375
376 We hypothesised that suppression of naïve responses in this system would be less pronounced
377 if the adoptively transferred naïve T cells were specific for a readily available, abundant
378 sporozoite antigen, such as CSP (Figure S6A). To test this, we examined the expansion of naive
379 $CD8^+$ T cell responses targeting CSP in mice previously vaccinated with RAS. Here, we again
380 used CS5M RAS, where the OT-I epitope is embedded within CSP and OT-I cells can be used
381 as a readout for responses to *P. berghei* CSP. To demonstrate the immunogenicity of CS5M-
382 CSP expressed on these sporozoites, we adoptively transferred naïve OT-I cells into recipient

383 mice, which were injected with heat killed CS5M sporozoites a day later. Expansion of OT-I
384 cells was then assessed in the spleen after 6 days. CS5M HKS induced substantial OT-I
385 proliferation, indicating that sporozoite-derived surface CS5M-CSP could be captured and
386 presented to responding T cells by host antigen presenting cells (Figure S6B). In a new set of
387 experiments, mice then either received a single injection of CS5M RAS, or three injections,
388 with OT-I cells being transferred one day before the last RAS vaccination. Surprisingly, *de*
389 *novo* OT-I responses in 3xCS5M RAS vaccinated mice were again strongly suppressed (Figure
390 5J), even when endogenous SIINFEKL-specific responses strongly expanded (Figure S6C).
391 TRAP specific, as well as RPL6 and T cells of undefined specificities, formed large numbers
392 of memory cells in 3xRAS vaccinated mice (Figure 5K, S6D). Moreover, mice that, in a
393 separate experiment, received two doses of WT RAS, followed by OT-I transfer before a last
394 dose of CS5M RAS, in which therefore no OVA specific response was generated by prior to
395 CS5M RAS vaccination, also displayed strongly diminished OT-I T_{RM} cell numbers (Figure
396 S7). These results confirmed that naive T cell responses are strongly inhibited by ongoing
397 responses elicited by prior RAS vaccination. This is the case, even for responses specific for
398 an abundant sporozoite antigen that can be cross-presented without requiring liver invasion
399 (Figure S6B), and can even occur when the challenge antigen has not been encountered during
400 previous vaccinations. Furthermore, this inhibition of naïve T cell responses occurs while
401 previously activated T cells of the same specificity continue to respond to each RAS
402 vaccination.
403
404 **Inhibition of naïve T cell activation by RAS boosting does not require TRAP-specific T**
405 **cells**
406

407 Having established that TRAP-specific T cells dominate the protective response elicited by
408 multiple WT *P. berghei* RAS vaccinations in C57BL/6 mice, we sought to determine whether
409 these cells were also responsible for the observed inhibition of naïve T cell responses. To do
410 this, we transferred naïve PbT-I cells into mice vaccinated with 1x, 2x or 3xWT RAS, one day
411 before the last RAS vaccination, as done before, but in this case, vaccinated mice were tolerised
412 for TRAP or OVA prior to RAS vaccination as done before (Figure 4A). Memory formation
413 by PbT-I cells was measured 30 days after the last RAS vaccination (Figure 5L, M, SF8). Naïve
414 PbT-I cells were impeded from forming memory (Figure 5L, S8A), despite TRAP tolerisation
415 having efficiently removed the TRAP-specific response in most mice (Figure 5M, S8B). As
416 observed in previous experiments (Figure 4D), TRAP tolerization did not work efficiently in
417 all mice, yet a clear inhibition of PbT-I responses could be observed in those mice in which
418 TRAP tolerization was efficiently achieved (Figure S8C, D). T_{RM} cells of undefined
419 specificities (other than RPL6 or TRAP) progressively increased in numbers after each dose of
420 RAS (Figure S8E, F), independently of the presence or absence of TRAP specific cells. These
421 results show that, although TRAP specific cells contribute decisively to protection, inhibition
422 of naïve T cell responses in 3xRAS mice was not exclusively mediated by effector T cells of
423 this specificity. Taken together, these data suggested that immunity generated after the initial
424 RAS immunisation hindered the boosting of effector $CD8^+$ T cell responses to late epitopes,
425 and dramatically inhibited the priming of new T cell responses to any antigen, either sporozoite
426 or liver stage derived. Overall, this resulted in a strong bias of the T cell response towards
427 sporozoite-derived immunogenic antigens associated with the primary exposure to RAS.
428
429 **Liver T_{RM} cells specific for abundant sporozoite antigens can confer potent protection**
430

431 To better understand the implications of the skewed T_{RM} specificities towards sporozoite
432 proteins, we next sought to explore the protective capacity of liver T_{RM} cells specific for the
433 major sporozoite antigen CSP, and benchmarked this protection with that provided by T_{RM}
434 cells specific for Hsp70. This was achieved by evaluating the ability of OT-I T_{RM} cells to
435 protect against challenges with either CS5M or HsOVA parasites. This experimental design
436 allowed us to directly compare the efficacy of T_{RM} cells of the same specificity (SIINFEKL)
437 against two modified antigens containing this epitome, namely CS5M-CSP and Hsp70-OVA.
438 As reported above, Hsp70 is present in the sporozoite and, although it is moderately
439 immunogenic at this stage (Figure S5D), it is prominently transcribed during liver stage
440 (Figure S5C) and can be targeted for protection [50]. In turn, CSP combines abundant
441 expression and immunogenicity in sporozoites (Figure S6A) with prolonged expression during
442 liver stage [51]. To ensure protective responses were only specific for the relevant OVA
443 peptide, SIINFEKL, expressed by HsOVA and CS5M parasites, and not for other parasite
444 antigens, we generated OVA-specific responses using our previously developed prime-and-
445 trap (P&T) approach [22]. Mice either received OT-I cells or not (and hence relied on
446 endogenous SIINFEKL-specific $CD8^+$ memory T cells for protection), and were challenged
447 with CS5M or HsOVA parasites. Large numbers of OT-I T_{RM} (Figure 6A-C), or SIINFEKL-
448 specific endogenous cells (Figure 6D-F), were generated in mice receiving the complete P&T
449 vaccine. When these mice were challenged with CS5M or HsOVA parasites, high levels of
450 sterile protection were achieved (Figure 6G, H). As T_{RM} cell numbers generated with this
451 vaccination strategy provided exceedingly efficient protection against both parasites, we
452 decided to vaccinate mice suboptimally, to better gauge the numbers of T_{RM} cells required for
453 protection against these parasites. Thus, we injected mice with a low dose of Clec9A-OVA
454 (0.5 μg) plus 5 nmol CpG B-P adjuvant. This induced lower numbers of liver OT-I T_{RM} cells,
455 averaging $5x10^4$ cells (Figure 6I). These mice displayed substantial sterile protection against

456 CS5M CSP, which was superior to that of Hsp70-OVA (Figure 6J). This result showed that
457 T_{RM} cells specific for sporozoite antigens, particularly those abundantly expressed, can provide
458 highly efficient protection against infection. Overall, this supported the view that bias towards
459 sporozoite-specific T_{RM} cells induced by repeated RAS vaccination provided highly efficient
460 protection against infection.
461
462 Together, this study shows that repeated RAS vaccination enhances the development of long-
463 lived liver-resident memory $CD8^+$ T cells of biased specificity for abundant sporozoite
464 antigens, some of them highly protective, but fails to fully exploit the protective potential of T
465 cells specific for less abundant or later antigens.

466 **Discussion**

467

468 RAS can induce efficient protection against malaria across a diverse range of species, including
469 humans [9, 13], non-human primates, and mice. The rodent model provides the opportunity to
470 expand our understanding of this vaccine, as well as to investigate fundamental immunology
471 processes, to inform the design of more effective malaria vaccines.

472

473 Once inside hepatocytes, malaria parasites become targets of $CD8^+$ T cell immunity. The
474 pivotal role of memory $CD8^+$ T cells in protecting mice [10, 14, 15] and NHP [16] during the
475 liver stage of malaria has been well established. However, not all types of memory $CD8^+$ T
476 cells are equally efficient at providing protection. We here show that liver T_{RM} cells, which
477 afford highly focused and efficient responses to infection [22], are critical mediators of the
478 immunity to *P. berghei* conferred by this vaccination strategy in C57BL/6 mice. Existing data
479 also supports a prominent protective role for liver $CD8^+$ T_{RM} cells in primates. $CD8^+$ T cells
480 had been observed to accumulate in the liver in NHP vaccinated with RAS [19], prior to the
481 identification of liver T_{RM} cells. In humans, protection was maintained in RAS-vaccinated
482 individuals a year after vaccination, a time when numbers of circulating T cells and antibodies
483 had declined to background levels [13]. Further studies showed that numbers of parasite-
484 specific circulating $CD8^+$ T cell numbers generated by RAS are low, and do not discriminate
485 between protected and non-protected individuals [19, 25, 52]. The results presented here mirror
486 these observations, as repeated RAS vaccination in mice does not prominently increase
487 numbers of circulating memory cells but comparatively enhances liver T_{RM} numbers and
488 protection. Additionally, protection increases with successive RAS injections, mirroring
489 observations in humans [9]. This mouse model hence presents strong analogies with humans
490 and is a valuable tool to study immunity to pre-erythrocytic malaria in the liver.

491

492 T_{RM} cells are more abundant than circulating memory T cells [53], and have been identified
493 and comprehensively characterised in humans as analogous to those in mice [54]. These cells
494 express high levels of cytokines and cytotoxicity mediators [22, 55, 56] and exert rapid effector
495 function [57, 58] for potent, protective responses against infection [22, 59-62]. The significant
496 protective capacity of liver T_{RM} cells against malaria has been previously demonstrated [22,
497 63, 64], although circulating, effector memory $CD8^+$ T cells also infiltrate the liver and were
498 shown to contribute to protection against *P. berghei* infection in CB6F1 mice [21]. In C57BL/6
499 mice, which are highly susceptible to infection by *P. berghei* [23], liver T_{RM} cells appear to be
500 critical for protection, with T_{CIRCM} cells failing to provide substantial protection even at
501 considerable numbers [22]. In line with these findings, we here show that depletion of T_{RM}
502 cells rendered RAS vaccinated mice devoid of sterile protection, and the parasitemia developed
503 by depleted mice, although slightly lower, was statistically comparable to that of unvaccinated
504 mice. Additionally, the increase in protection observed upon administration of successive doses
505 of RAS was associated with a comparably more pronounced increase in T_{RM} cells than T_{CIRCM}
506 cells, as exemplified by the improved ratio of the former cells vs the latter. As T cell responses
507 derived from naïve T cells are strongly inhibited in repeatedly vaccinated mice (discussed
508 below), the observed increase in the T_{RM}/T_{CIRCM} ratio may be due to local proliferation of liver
509 T_{RM} cells upon antigen encounter [65, 66], or to conversion of T_{CIRCM} cells into T_{RM} cells [67,
510 68]. In line with our findings, repeated immunisation with *Listeria monocytogenes* has been
511 previously found to progressively increase the accumulation of specific memory T cells in the
512 liver [69]. Together, our findings showcase the capacity of RAS to generate T_{RM} immunity and
513 underscore a major role of these cells in the induced protection against malaria.

514

515 Despite their intrinsic ability for efficient pathogen control, T_{RM} cells are constrained by their
516 antigen specificity in their capacity to provide protection against infection. Thus, defining the
517 specificities that drive immunity in RAS vaccinated mice is critical to understand the
518 mechanism of protection of this vaccine, and can be invaluable for antigen selection for subunit
519 vaccine development. Upon invasion of the liver as sporozoites, parasites proliferate massively
520 within hepatocytes and progressively develop towards the merozoite form, able to invade
521 erythrocytes. This process entails pronounced variations in protein expression [41, 51, 70], and
522 therefore in available immune targets. Vaccination with genetically modified parasites
523 engineered to interrupt their intrahepatic development at a late stage after hepatocyte invasion
524 induces more protective T cell responses than early arresting parasites, which express a more
525 limited antigen breadth [33]. Similarly, as RAS parasites die quickly upon hepatocyte invasion
526 [8], the repertoire of antigens they present to the immune system is limited [33]. In addition to
527 inducing responses of comparably lower protective quality, the logical consequence of this
528 phenomenon, as we have observed, is that the expansion of T cells specific for early antigens,
529 such as those present in the sporozoite, is favoured, introducing a bias for these specificities in
530 the T_{RM} cell repertoire generated. Our results parallel prior observations in RAS-vaccinated
531 BALB/c mice [45], where responses specific for a sporozoite antigen (CSP), but not those
532 recognising a liver stage antigen, were enhanced through repeated *P. yoelii* short-interval RAS
533 immunisations. Importantly, *P. yoelii* CSP features an immunodominant K^d-restricted epitope
534 in BALB/c mice [71], which hence becomes a clear target of T cell immunity, whereas
535 C57BL/6 mice do not mount $CD8^+$ T cell responses to *P. berghei* CSP [36]. As we have found
536 here, the response elicited by RAS in this mouse strain is instead dominated by T cells specific
537 for TRAP, another major sporozoite antigen [41, 72]. Although expected on the basis of the
538 evidence presented above, this is particularly striking because TRAP is poorly recognised by
539 the $CD8^+$ T cell compartment of C57BL/6 mice, with only about 1 cell per million naïve $CD8^+$

540 T cells specific for its single known MHC-I-restricted epitope [31, 73]. Indeed, when *P. berghei*
541 CSP is modified to contain SIINFEKL, a much more immunogenic antigen in C57BL/6 mice
542 [35], and therefore both CSP- and TRAP-derived $CD8^+$ T cell antigens are concurrently present
543 in the same, early sporozoite stage at approximately similar levels [41], then CSP-specific cells
544 clearly outcompete TRAP specific cells (Figure 2D, E), evidencing the comparative weakness
545 of the latter antigen. Nevertheless, TRAP-specific cells expand to much greater numbers than
546 RPL6-specific cells in mice vaccinated with three doses of WT RAS, even though the
547 frequency of the latter cells in the naïve repertoire is about 100-fold higher [31]. These results
548 underline the surprising strength of the bias towards T cells specific for early antigens induced
549 by RAS.
550
551 A straightforward explanation for this phenomenon is that proliferation of parasite-specific T
552 cells is strongly dictated by early antigen availability, whereby T cells specific for abundant
553 sporozoite antigens are preferentially expanded. Premature death, or quick elimination, of RAS
554 parasites after hepatocyte invasion hence disfavours the expression and immunogenicity of
555 later epitopes. Indeed, Murphy *et al.* monitored parasite mRNA expression in mice repeatedly
556 vaccinated with RAS and found reduced mRNA expression of non-sporozoite antigens [45].
557 In line with these findings, we found that existing immunity generated during initial RAS
558 vaccination strongly reduced the infectivity of subsequent sporozoite infections, potentially
559 limiting the immunogenicity of later doses of RAS and likely reinforcing the T cell bias towards
560 readily available early antigens. Possible sources of sporozoite antigens for $CD8^+$ T cell
561 immunity at early stages of infection are the parasite proteins that leak to the cytoplasm of the
562 invaded hepatocyte and are presented via MHC-I molecules, as well as dying parasites that do
563 not reach the liver and are captured by antigen presenting cells in lymphoid organs [74].
564 Although parasites can partially develop and express later antigens in these organs [32], we

565 injected RAS intravenously, and therefore parasite development outside the liver is unlikely to
566 have occurred [75]. Additionally, several sporozoite proteins, including CSP and TRAP, are
567 cleaved and released as the parasites traverse hepatocytes [76, 77], generating a "gliding trail"
568 of potential antigens that could potentially be captured by antigen presenting cells, or presented
569 via MHC-I by the traversed hepatocytes. Moreover, those sporozoites that either die while
570 traversing hepatocytes, or inside the final hepatocyte in which they settle for further
571 development [78], may also become sources of additional antigen. We and others observed that
572 HKS immunisation is markedly less immunogenic than RAS [19, 49, 74] and, although heat-
573 induced damage to sporozoite proteins may explain these results, it is also possible that the
574 simple cross-presentation of antigen that is readily available in sporozoites is not sufficient to
575 induce potent immunogenicity. Liver invasion, or the presence of metabolically active
576 parasites, may be required for maximal expansion of sporozoite-specific $CD8^+$ T cell
577 responses.
578
579 As mRNA levels can be poor indicators of immunogenicity, we attempted an alternative
580 method to more accurately measure the immunogenicity of T cell antigens of interest with
581 contrasting expression patterns. We did this by evaluating the memory formation capacity of
582 adoptively transferred naïve TCR transgenic T cells in mice previously vaccinated with RAS.
583 This led to our identification of another factor strongly contributing to sporozoite-specific T_{RM}
584 cell bias, namely the strong inhibition of naïve T cell responses by prior RAS immunisation.
585 This inhibition occurred progressively and was broad. It affected responses to all antigens
586 examined, including those targeting abundant sporozoite antigens available for cross-
587 presentation such as CSP, even when these antigens had not been encountered previously.
588 Moreover, removing TRAP-specific responses, although significantly impacting protection,
589 did not prevent inhibition of naïve T cell expansion. Notably, while naïve PbT-I cell activation

590 was strongly inhibited, endogenous cells of the same specificity (RPL6), activated after the
591 first RAS injection, continued to expand, albeit moderately, upon subsequent RAS injections
592 (Figure S5A). Similarly, naïve OT-I cell expansion was hindered in CS5M RAS vaccinated
593 mice (figure 3C) while the endogenous SIINFEKL-specific response continued to grow (Figure
594 S6C). These findings suggested that this inhibition was not necessarily exerted by sporozoite-
595 specific T cells over those specific for late antigens, but potentially by effector cells activated
596 upon previous RAS injections, which outcompeted naïve T cells. Naïve $CD8^+$ T cells have the
597 capacity to proliferate massively over a few days following activation [38]. However, as
598 pronounced changes in the naïve T cell, including a significant increase in size and a switch to
599 glycolytic metabolism, must occur to enable such fast expansion, a period of about 24 hours is
600 needed before proliferation commences [79]. This places naïve T cells at a disadvantage
601 compared to already activated and expanding effector T cells. Additionally, activated T cells
602 require factors such as IL-2 that enable further proliferation and subsequent formation of
603 memory [80], and the more numerous effector T cells likely outcompete naïve T cells for these
604 factors. Our results mirror those obtained by Hafalla *et al*, who observed that naïve $CD8^+$ T
605 cells specific for *P. yoelii* CSP expanded poorly when injected into mice immunised with a
606 single dose of RAS 1-4 days earlier [81]. Subsequent work showed that competition for antigen
607 presenting cells was another mechanism whereby effector T cells outcompeted naïve T cells
608 [82]. Interestingly, this work observed that inhibition of naïve T cell responses by effector T
609 cells only occurred when both responses were of the same specificity. However, we here
610 present evidence that this suppression is broad and not restricted to specific antigens. In
611 contrast to the scenario presented here, Hafalla *et al* utilised two different infection systems to
612 create a competition between effector and naïve T cells (namely RAS and influenza infection).
613 These systems may differ in parameters such as antigen availability or strength or quality of
614 inflammatory signals, which could influence whether a *de novo* response can form. In

615 summary, the effector T cell response generated upon primary RAS exposure constrains the
616 breadth of the parasite-specific T_{RM} compartment generated by this vaccine, contributing to the
617 establishment of a strong immunodominance biased towards early antigens that influences the
618 quality of the protective liver T_{RM} response elicited by multiple RAS vaccinations.
619
620 Our work establishes sporozoite proteins as dominant antigens for naturally occurring T cell
621 immunity to malaria in the liver. Notably, removing the TRAP response in 3xRAS vaccinated
622 mice results in loss of protection. This finding was unexpected, given that $2x10^5$ TRAP T_{RM}
623 cells generated by prime-and-trap vaccination, similar in number to those in 3xRAS mice, only
624 conferred around 15% sterile protection against challenge with 200 *P. berghei* sporozoites in
625 C57BL/6 mice [31]. This discrepancy could be attributed to the fact that RAS vaccination
626 generates T_{RM} cells of multiple specificities [33], which can collectively contribute to
627 protection to variable extents (Figure S4B), hence lowering the numeric requirements for
628 TRAP-specific cells for sterilising protection. When a dominant specificity such as TRAP is
629 removed, then the total number of protective T_{RM} cells is reduced below the threshold required
630 for sterile protection, rendering mice susceptible to infection. Our results contrast with
631 observations that substitution of *P. berghei* CSP for its ortholog in *P. falciparum*, which
632 removes an immunodominant K^d-restricted epitope in the rodent parasite [83], does not
633 abrogate protection of BALB/c mice against challenge with WT sporozoites [84]. BALB/c
634 mice are inherently more resistant that C57BL/6 mice to *P. berghei* sporozoite infection [18,
635 23], and can be efficiently protected by a comparably lower number of parasite-specific $CD8^+$
636 T cells [18]. Hence, a potential explanation is that the pool of minor T_{RM} specificities suffice
637 to exert protection in these mice.
638

639 Regarding further antigens, we found SIINFEKL-specific responses targeting Hsp70-OVA to
640 be highly protective. This aligns with previous reports comparing vaccination targeting OVA
641 expressed under the liver stage UIS4 promoter, and CSP, which found both antigens to be
642 protective [85], yet we could determine by suboptimal vaccination that CS5M CSP responses
643 induced more efficient protection than those against Hsp70-OVA. Comparatively strong
644 protection by $CD8^+$ T cells targeting sporozoite antigens has also been found by other groups
645 [86]. However, the intrinsic protective capacity of an antigen depends on several factors, and
646 not exclusively on its expression pattern. Thus, other immunogenic antigens with diverse
647 expression patterns, including in the sporozoite, such as GAP50 or S20, or the liver stage, such
648 as RPL3, were found not to be protective [40, 45, 86], a critical element for some of these
649 antigens being antigen availability for MHC-I presentation in the hepatocyte [86].
650
651 Another important aspect of repeated RAS vaccination identified in this work is the extension
652 of the lifespan of liver T_{RM} cells. Our results align with those obtained by van Braeckel-
653 Budimir *et al.* on lung T_{RM} cells, whose lifespan is also significantly enhanced upon repeated
654 influenza infection [68]. In that model, T_{EM} cells convert into T_{RM} cells after infection [67],
655 themselves progressively decreasing in numbers [68], and consequently maintaining the T_{RM}
656 compartment. Conversion of T_{EM} cells into T_{RM} cells has also been observed in the skin of
657 Herpes Simplex virus infected mice [65], and a decrease in the lifespan of T_{EM} cells was also
658 detected upon repeated stimulation with attenuated *Listeria monocytogenes* [87]. Our
659 observation that lifespan extension is induced in T_{RM} cells, but not in circulating memory cells,
660 is compatible with the occurrence of a similar phenomenon in our system. As naïve T cell
661 responses are strongly inhibited by repeated RAS vaccination, this potential source of new T_{RM}
662 cells is likely minor. In addition to recruitment from circulating memory cells, expansion of
663 the T_{RM} compartment can also be due to *in situ* proliferation in the tissue in response to repeated

664 antigen encounter [65]. Finally, repeated antigen stimulation induces intrinsic changes in
665 memory T cells [69], including moderate changes in molecules involved in survival [68].
666 Access to antigen in the liver by T_{RM} cells might foster preferential antigen stimulation leading
667 to the expression of pro-survival genes in these cells.
668
669 Although our results demonstrate that sporozoite-specific liver T_{RM} cells can provide highly
670 efficient protection against infection, the preferential development of sporozoite specific T cell
671 responses by RAS may have some drawbacks. Firstly, certain late antigens such as RPL6,
672 which are highly protective [31], are not expanded as much upon RAS vaccination, and their
673 full protective potential is therefore missed. This issue may be more prominent in human
674 infections, where the liver stage lasts longer than in mice (7 days vs 2 days), and therefore T_{RM}
675 cells targeting parasites during intrahepatic development may be comparably more relevant for
676 protection. Secondly, reducing the generation of late-stage antigen-specific T cells may impair
677 immunity against parasites with delayed development in the liver. Sporozoite-specific $CD8^+$ T
678 cells, such as those specific for CSP, are less efficient at protecting against *P. yoelii* than *P.*
679 *berghei*, and this is associated to strongest late replication of the former parasite [24].
680 Moreover, unlike *P. berghei* RAS, repeated long interval *P. yoelii* RAS immunisation fails to
681 improve protection in C57BL/6 mice [18]. Thirdly, we and others have observed that TRAP is
682 highly variable across *P. falciparum* field isolates [31], and this is also true for CSP [88, 89].
683 Sporozoite surface antigens such as CSP or TRAP are directly exposed to immune attack and
684 hence subjected to intense immune selection, which likely results in increased variability.
685 Biasing the immune response towards highly polymorphic sporozoite antigens may increase
686 the strain-specificity of RAS-mediated protection (or, similarly, of immunity from natural
687 infection), reducing the capacity of memory T_{RM} cells generated to recognise and combat new
688 infections. Administration of high doses of RAS, however, may aid initial expansion of

689 protective T cells specific for less abundant sporozoite antigens and increase cross-strain
690 immunity [25]. And fourthly, we have observed that the lifespan of liver T_{RM} cells generated
691 by repeated RAS vaccination is strongly increased. This could potentially perpetuate the
692 suppression of naïve responses to new antigens, not only further hindering the generation of T
693 cell responses to liver stage antigens upon natural infection, but also to new sporozoite antigens
694 from genetically diverse parasite haplotypes [90]. As we have seen here, those T cells are still
695 able to respond to the new parasite, even when unable to exert protection. This could prevent
696 the generation of immunity to novel epitopes, thereby reducing the breadth of immunity
697 generated against newly encountered parasite strains. Together, these elements may establish
698 a potent screen that prevents the generation of T_{RM} cell immunity of maximal efficacy against
699 malaria parasite infection in the liver.

700 **Materials and Methods**

701

702 **Mice, mosquitos, parasites and infections**

703 All procedures were performed in strict accordance with the recommendations of the
704 Australian code of practice for the care and use of animals for scientific purposes. The protocols
705 were approved by the Melbourne Health Research Animal Ethics Committee, University of
706 Melbourne (ethic project IDs: 1112347, 1814522, 20088).

707 Female C57BL/6 (B6), GFP [91], OT-I [46] and PbT-I [37] mice were used between 6-12
708 weeks of age and were bred and maintained at the Department of Microbiology and
709 Immunology, The University of Melbourne. Animals used for the generation of the sporozoites
710 were 4-5-week-old male Swiss Webster mice purchased from the Monash Animal Services
711 (Melbourne, Victoria, Australia) and maintained at the School of Botany, The University of
712 Melbourne, Australia. *Anopheles stephensi* mosquitoes (strain STE2/MRA-128 from The
713 Malaria Research and Reference Reagent Resource Center) were reared and infected with *P.*
714 *berghei* ANKA (*P. berghei*) as described [92]. *P. berghei* ANKA WT, *P. berghei* ANKA
715 HsOVA [47] and *P. berghei* ANKA CS5M [43] sporozoites were dissected from mosquito
716 salivary glands and resuspended in cold PBS. Freshly dissected *P. berghei* sporozoites were
717 injected intravenously (i.v.) as indicated in the figure legends. Parasitemia was assessed by
718 microscopic analysis of blood smears or by flow cytometry. Mice showing no evidence of
719 blood-stage infection by day 11 after infection were considered sterilely protected. For blood
720 stage infections, mice were injected i.v. with the indicated amount of *P. berghei* infected red
721 blood cells (iRBC).

722 Heat killing of sporozoites was done by incubating freshly isolated sporozoites at 56°C for 45
723 minutes.

724

725 **Adoptive transfer of CD8$^+$ T cells**

726 PbT-I and OT-I CD8$^+$ T cells were negatively enriched from the spleens and lymph nodes of
727 mice from various genetic crosses as described [93]. 50,000 purified PbT-I cells in 0.2 mL PBS
728 were injected i.v. into recipient mice. CellTrace^{TM} violet (CTV, Thermofisher) was used to
729 coat PbT-I and OT-I cells following manufacturer's instructions.

730

731 **Prime-and-trap vaccination**

732 B6 mice were injected i.v. with the indicated doses of rat anti-Clec9A (clone 24/04-10B4)
733 genetically fused to OVA (containing the $OVA_{257-264}$ epitope) via a 4 Alanine linker to make
734 the αClec9A-OVA mAb construct [94]. Recombinant adeno-associated virus (rAAV-OVA)
735 was prepared and purified in house at the Centenary Institute or by the Vector and Genome
736 Engineering Facility (at the Children Medical Research Institute, Sydney, Australia) over
737 cesium chloride (CsCl)-density gradient centrifugation followed by dialysis as previously
738 described (. This vector expresses a membrane bound form of OVA protein bicistronically with
739 green fluorescent protein (GFP). αClec9A was injected with 5 nmol of a CpG oligonucleotide
740 (CpG) generated by linking (5' to 3') CpG-2006 to CpG-21798 [95] (Integrated DNA
741 Technologies, Coralville, IA, USA). For P&T vaccination, mice were injected i.v. with Clec9A
742 mAb and the indicated vector gene copies (vgc) of rAAV-OVA on the same day.

743

744 **Organ processing for T cell analysis**

745 Tissues were harvested from mice at different time points after immunization and finely
746 chopped using curved scissors to generate single cell suspensions. For spleen cell preparations,
747 red blood cells were lysed, and remaining cells were filtered through a 70 μm mesh. Liver cell
748 suspensions were passed through a 70 μm mesh and resuspended in 35% isotonic Percoll. Cells

749 were then centrifuged at 500g for 20 min at room temperature (RT), the pellet harvested, and
750 then red cells lysed before further analysis.
751
752 **Flow cytometry**
753 CD11a (2D7), CD8α (53-6.7) mAb were purchased from BD; CD44 (IM7), CD62L (MEL-
754 14), CD69 (H1.2F3), from ThermoFisher Scientific (Waltham, MA, USA); CXCR3
755 (CXCR3-173), CXCR6 (SA05D1), CX3CR1 (SA011F11), from BioLegend (San Diego, CA,
756 USA); H2-K^b-PbRPL6$_{120-127}$, H2-K^b-PbRPA1$_{199-206}$, H2-K^b-OVA$_{257-264}$ and H2-D^b-
757 PbTRAP$_{130-138}$ tetramers were made in house. Dead cells were excluded by propidium iodide
758 (PI) staining. For the analysis of memory $CD8^+$ T cell populations in the spleen and the liver,
759 tetramer$^+$, PbT-I or OT-I $CD8^+$ $CD44^{hi}$ cells were subdivided into T_{CM}, T_{EM} or T_{RM} based on
760 CD69 and CD62L expression (T_{CM} $CD62L^+$ $CD69^-$, T_{EM} $CD62L^-$ $CD69^-$ and T_{RM} $CD62L^-$
761 $CD69^+$, see Figure S1H). Parasitaemia was assessed by incubating ~2µl tail blood with a 5
762 pg/mL Hoechst 33258 solution (ThermoFisher Scientific) in FACS buffer for 1 hour at 37°C.
763 Parasites were discriminated from uninfected RBC using a 405 violet laser and a 450/50 filter.
764 Cells were analyzed by flow cytometry on a FACS Canto, Fortessa or Fortessa X20 (BD
765 Immunocytometry Systems, San Jose, CA, USA), using FACSDiva (BD Immunocytometry
766 Systems) or FlowJo software (Tree Star, Ashland, OR, USA).
767
768 **Depletion of liver T cells**
769 To deplete $CXCR3^+$ cells, mice were intravenously injected with 2 doses (200 µg and then
770 100 µg) of anti-CXCR3 antibody (CXCR3-173, eBioscience) or Armenian Hamster IgG
771 isotype control (eBio299Arm, eBioscience) 3 and 1 days before challenge with 200 live
772 sporozoites [22]. To deplete $CD8^+$ cells, mice were intravenously injected with 100 µg of anti-
773 CD8 antibody (clone 2.43) or isotype control (GL117, IgG2a) one day before challenge.

774

775 **CD8 T cell tolerisation**

776

777 Removal of TRAP specific T cells was achieved by injection of $PbTRAP_{130-138}$ peptide in the
778 absence of adjuvant [40, 44]. Mice received 3 intravenous doses of TRAP peptide diluted in
779 PBS on days 7, 4 and 1 prior to the first dose of RAS, and then received additional injections
780 a day before administration of subsequent doses of the vaccine. The first peptide dose was
781 300μg, and the rest were 100μg.

782

783 **Statistical analyses**

784 Figures were generated using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA).
785 Data are shown as mean values ± standard error of the mean (SEM). Statistical analyses were
786 performed using GraphPad Prism 10. Unless otherwise stated, statistical comparisons of cell
787 numbers in different groups were performed by log-transforming the data and using a Student's
788 t-test (2 groups) or one-way ANOVA followed by Tukey's multiple comparisons test (>2
789 groups). Cell number values equal to 0 were converted to 1 to enable log transformation.
790 $P<0.05$ was considered to indicate statistical significance. *, $P<0.05$; **, $P<0.01$; ***,
791 $P<0.001$; ****, $P<0.0001$; n.s., not significant ($P>0.05$). Asterisks directly over groups denote
792 statistical differences with the unvaccinated control group. Rates of sterile protection were
793 compared using Fisher's exact tests.

794

795 **Declaration of generative AI and AI-assisted technologies in the writing process**

796 The authors used the AI-powered language models Perplexity AI and Microsoft Copilot for
797 editorial suggestions to assist with improving the language and readability of this manuscript.

The authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Author contributions

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Data availability

All relevant data are within the manuscript and its supporting information files

817 **Figure legends**

818

819 **Figure 1. Repeated RAS vaccination of C57BL/6 mice provides efficient protection**
820 **against *P. berghei* sporozoite infection that is dependent on CD8$^+$ T_{RM} cells. (A-B)** Mice
821 were vaccinated with 1, 2 or 3 doses of 10,000 RAS (1xRAS, 2xRAS and 3xRAS respectively),
822 one week apart and were challenged with 200 *P. berghei* sporozoites 30 days after the last dose
823 of RAS. (**A**) Rates of sterile protection. Numbers above columns denote numbers of protected
824 mice / total numbers of mice per group (**B**) Parasitemia at day 7 post-challenge. (**C-D**) Mice
825 were vaccinated using three doses of 5,142-10,000 RAS, one week apart, and treated with
826 αCXCR3 mAb 3 and 1 days prior to challenge with 200 live sporozoites, which was performed
827 on day 30-34 after the last vaccination. Parasitemia was monitored to evaluate protection. (**C)**
828 Rates of sterilising protection. (**D)** Parasitemia on day 7 after sporozoite infection. Data were
829 pooled from 2 independent experiments. Comparisons of sterile protection rates were done
830 using Fisher's exact tests. Parasitemia data were log-transformed and compared using one-way
831 ANOVA and Tukey's multiple comparisons test.

832

833 **Figure 2. Abundance of liver CD8$^+$ T_{RM} cells specific for known *Plasmodium* antigens in**
834 **mice vaccinated multiple times with WT RAS. A-D.** CD8$^+$ T_{RM} cell responses specific for
835 TRAP, RPL6, RPA1, or other specificities (tetramer-negative T_{RM} cells) in the livers of mice
836 vaccinated with 1, 2 or 3 rounds of 10,000 RAS, one week apart. Cell numbers were assessed
837 30 days after the last round of immunisation. Data were log-transformed and compared using
838 two-way ANOVA and Tukey's multiple comparisons test. **A.** Liver T_{RM} cell numbers. Data
839 were log transformed and statistically compared using two-way ANOVA and Tukey's multiple
840 comparisons test. **B.** Frequencies of liver T_{RM} cells of different specificities amongst all T_{RM}
841 cells in the liver. **C.** Fold change in the number of T_{RM} cells of the indicated specificities

842 compared to those in 1xRAS vaccinated mice. Data were log transformed and statistically
843 compared using one-way ANOVA and Tukey's multiple comparisons test. Data in A-C were
844 pooled from two independent experiments. **D.** Ratios of T_{RM} cell numbers in the liver vs
845 numbers of circulating memory T cells (T_{CIRCM}, calculated by adding T_{CM} and T_{EM} cells) in the
846 spleen. Data were compared using one-way ANOVA and Tukey's multiple comparisons test.
847 **E-H.** $CD8^+$ T_{RM} cells specific for mutated CSP (SIINFEKL), TRAP, RPL6, RPA1, or other
848 specificities in the livers of mice vaccinated with 1 or 3 rounds of 5,050-7,700 CS5M RAS,
849 4-8 days apart. Cell numbers were assessed 30-63 days after the last round of immunisation.
850 **E.** Liver T_{RM} cell numbers. Data were log transformed and statistically compared using two-
851 way ANOVA and Tukey's multiple comparisons test. **F.** Percentages of liver T_{RM} cells of
852 different specificities amongst all T_{RM} cells in the liver. **G.** Fold change in the number of T_{RM}
853 cells of the indicated specificities compared to those in 1xCS5M RAS vaccinated mice. Data
854 were log transformed and statistically compared using one-way ANOVA and Tukey's multiple
855 comparisons test. Data in D-F were pooled from two independent experiments. **H.** Ratios of
856 T_{RM} cell numbers in the liver vs numbers of circulating memory T cells (T_{CIRCM}, calculated by
857 adding T_{CM} and T_{EM} cells) in the spleen. Data were log-transformed and compared using
858 unpaired Student's t-tests.
859
860 **Figure 3. Repeated RAS vaccination generates long lived memory T cells**. Numbers of
861 $CD8^+$ T_{RM} cells in the liver (**A-C**) or T_{EM} cells in the spleen (**D-F**) specific for TRAP (**A, D**),
862 RPL6 (**B, E**) and RPA1 (**C, F**) in mice vaccinated with one dose of 50,000 RAS (dotted line)
863 or three doses of 10,000 RAS (solid line), one week apart. Cell numbers were assessed up to
864 day 101 after the last round of immunisation. Data were pooled from 5 independent
865 experiments for 1xRAS and 8 independent experiments for 3xRAS. Data were log-2

866 transformed and linear regression analyses of the log-transformed data were performed. Slopes
867 were compared using F-tests.
868
869 **Figure 4. TRAP-specific liver T_{RM} cells substantially contribute to protection induced by**
870 **3xWT RAS in C57BL/6 mice. A.** Experimental design. Red arrows denote intravenous
871 injection of $PbTRAP_{130-138}$ or $OVA_{257-264}$ peptide dissolved in PBS. Numbers denote days after
872 the first RAS vaccination. **B.** Sterile protection to live sporozoite challenge in 3xRAS
873 vaccinated mice in which TRAP-specific cells (3xRAS-TRAPtol), or T cells specific for an
874 irrelevant antigen (3xRAS-OVAtol) were removed (tol=tolerated). Data were compared using
875 Fisher's exact test. **C.** Parasitemia on day 7. Data were log-transformed and compared using
876 one-way ANOVA and Tukey's multiple comparisons test. **D.** Number of TRAP-specific liver
877 $CD8^+$ T_{RM} cells in protected vs non-protected mice. Data were log-transformed and compared
878 using two-way ANOVA and uncorrected Fisher's LSD test. Data in this figure were pooled
879 from two independent experiments.
880
881 **Figure 5. Mechanisms contributing to T cell specificity bias towards sporozoite antigens.**
882 **A-C.** Protective capacity of effector responses elicited by RAS against incoming sporozoites.
883 **A.** Representative experimental design. **B-C.** Mice received one or two doses of $5\text{-}10 \times 10^3$ RAS
884 4-8 days apart, and 8-9 days later were injected with 1×10^4 live sporozoites. Emergence of
885 parasitemia was measured to evaluate protection. Data were pooled from two independent
886 experiments. **B.** Parasitemia on day 5 after liver sporozoite challenge. Data were log-
887 transformed and compared using one-way ANOVA and Tukey's multiple comparisons test. **C.**
888 Sterile protection. Data were compared using Fisher's exact test. Numbers above columns
889 denote numbers of protected mice / total numbers of mice per group. **D-K.** Inhibition of naïve
890 $CD8^+$ T cell responses by prior RAS vaccination. **D.** Representative experimental design. Red

891 arrows denote the time point in which mice were intravenously injected with $5x10^4$ naïve
892 transgenic T cells (PbT-I cells in D-F; OT-I cells in F-L). **E-G.** Inhibition of naïve RPL6-
893 specific (PbT-I) T cell responses by prior RAS vaccination. Distribution of memory PbT-I (**E**)
894 and endogenous, RPL6- (**F**) and TRAP-specific (**G**) T cells in the liver 30-35 days after the
895 last RAS injection. Mice received 1-3 doses of $5\text{-}10x10^3$ RAS. Data were pooled from two
896 independent experiments. Statistical analyses, denoted by dark green and purple asterisks,
897 denote comparisons of T_{RM} numbers. **H-I.** Inhibition of naïve HsOVA-specific (OT-I) T cell
898 responses by prior HsOVA RAS vaccination. Distribution of memory OT-I (**H**) and
899 endogenous, TRAP-specific (**I**) T cells in the liver 30 days after the last HsOVA RAS injection.
900 Mice received 1-3 doses of $5.9\text{-}10x10^3$ HsOVA RAS. Data were pooled from two independent
901 experiments. Statistical analyses, denoted by brown and purple asterisks, denote comparisons
902 of T_{RM} numbers. **J-K.** Inhibition of naïve CSP-specific (OT-I) T cell responses by prior CS5M
903 RAS vaccination. Distribution of memory OT-I (**J**) and endogenous, TRAP-specific (**K**) T
904 cells in the liver 30 days after the last CS5M RAS injection. Mice received 1 or 3 doses of 5.9-
905 $10x10^3$ CS5M RAS. Data were pooled from two independent experiments. Statistical analyses,
906 denoted by brown and purple asterisks, denote comparisons of T_{RM} numbers. **L-M**. Naïve
907 $CD8^+$ T cell responses to RPL6 are inhibited in the absence of TRAP-specific T cells. Mice
908 were vaccinated with $1\text{-}3$ doses of $5\text{-}10x10^3$ WT RAS and received intravenous injections of
909 either $PbTRAP_{130\text{-}138}$ (TRAPtol) or $OVA_{257\text{-}264}$ peptide (OVAtol) dissolved in PBS as
910 explained in Figure 4A. PbT-Is were transferred 1 day before the last RAS vaccination and, on
911 day 36 after the last RAS vaccination, mice were euthanised and numbers of memory PbT-I or
912 TRAP-specific memory $CD8^+$ T cells were examined in the liver. **L.** Number of memory PbT-I
913 cells in the liver. **M.** Numbers of TRAP-specific memory T cells in the liver. Data were pooled
914 from two independent experiments, log-transformed and analysed using two-way ANOVA and
915 Tukey's multiple comparisons test.

916

917 **Figure 6. Memory T cell generation of prime-and-trap targeting OVA, and protection**
918 **against challenge with parasites expressing SIINFEKL under the Hsp70 promoter or**
919 **within CSP. A-H**. Mice received 50,000 naïve OT-I/uGFP cells (**A-C, G, H**) or not (**D-F, G,**
920 **H**) and were vaccinated with either 2 μg Clec9A-OVA plus 5 nmol CpG B-P, 10^9 rAAV-OVA,
921 or all components combined. OT-I (**A-C**) or SIINFEKL-specific memory cells (**D-F**) were
922 enumerated in the liver and the spleen 35 days later. Data were pooled from two independent
923 experiments, log transformed and compared using one-way ANOVA and Tukey's multiple
924 comparisons test. Mice vaccinated with full P&T-OVA were challenged with 200 HsOVA (**G**)
925 or CS5M (**H**) sporozoites on day 35 after vaccination, and rates of sterile protection were
926 determined. Data were pooled from two independent experiments and compared using Fisher's
927 exact test. Asterisks over columns denote comparisons with the unvaccinated control group.
928 Numbers over columns denote numbers of protected mice vs total numbers of mice. **I-J.**
929 Generation of OVA-specific memory T cells in suboptimally vaccinated mice. Mice received
930 50,000 naïve OT-I/uGFP cells and were vaccinated with a low dose of 0.5 μg Clec9A-OVA
931 plus 5 nmol CpG B-P. Numbers of OT-I T_{RM} cells were measured in the liver 30 days later (**I**).
932 Separate cohorts of mice were challenged with 200 HsOVA or CS5M sporozoites (**J**), and rates
933 of sterile protection were determined. Data were pooled from two independent experiments
934 and compared using Fisher's exact test.

935

936 **Supplementary figures**

937

938 **Figure S1. Related to figure 1. $CD8^+$ T cell depletion removes protection conferred by**
939 **repeated RAS vaccination.** Mice vaccinated thrice with 10,000 RAS, one week apart, were
940 treated with anti-CD8 antibodies on day 27 after the third RAS immunisation and were

941 challenged with 200 live *P. berghei* sporozoites on day 30. (**A**) Rates of sterile protection.
942 Numbers above columns denote numbers of protected mice / total numbers of mice per group.
943 (**B**) Parasitemia at day 7 post-challenge. (**C**) Mice were bled on day 29 after the third RAS
944 vaccination (i.e. 2 days after αCD8 treatment) and percentages of $CD4^+$ and $CD8^+$ T cells
945 (showed as numbers above the $CD8^+$ T cell gate) were measured in the blood using flow
946 cytometry to verify $CD8^+$ T cell depletion. An example of $CD8^+$ T cell percentages in an
947 untreated (top) and a treated (bottom) mouse are shown. One experiment was performed.
948 Comparisons of sterile protection rates were done using Fisher's exact tests. Parasitemia data
949 were log-transformed and compared using one-way ANOVA and Tukey's multiple
950 comparisons tests. (**D-H)** Related to figure 1C, D. Distribution of memory T cell populations
951 in the liver (**D, F)** and the spleen (**E, G)** in mice treated with αCXCR3 or isotype control mAb.
952 **D** and **E** show total memory $CD8^+$ T cells, whereas **F** and **G** show $PbTRAP_{130-138}$ tetramer-
953 specific memory $CD8^+$ T cells. Cell numbers were log-transformed and compared using
954 unpaired t-tests. Dark purple, pale purple and grey stats over the columns denote comparisons
955 of T_{RM}, T_{EM} and T_{CM} numbers respectively. (**H)** Representative gating strategy of liver cells,
956 including lymphocytes, single cells, live $CD8^+$ T cells ($CD8^+$ Propidium Iodide [PI]$^-$), memory
957 T cells ($CD44^{high}$), TRAP-tetramer$^+$ cells (gated from memory T cells) and total (middle row)
958 or TRAP-specific (bottom row) memory T cell subsets T_{CM} $CD62L^+$ $CD69^-$, T_{EM} $CD62L^-$
959 $CD69^-$ and T_{RM} $CD62L^-$ $CD69^+$. Panels titled "Isotype" and "αCXCR3" show total (middle
960 row) or TRAP-specific (bottom row) memory T cells in the livers of a representative, isotype-
961 treated and αCXCR3-treated mouse respectively. Numbers beside gates represent percentages
962 over total cells in the plot.
963
964 **Figure S2. Related to figure 2. Abundance of liver CD8 T_{RM} cells specific for known**
965 ***Plasmodium* antigens in mice vaccinated multiple times with WT RAS. A.** Expression of

966 TRAP, RPL6 and RPA1 proteins in salivary gland sporozoites (sgSpz), injected sporozoites
967 (bbSpz), exo-erythrocytic forms (EEF), merozoite and ring forms of *P. berghei*, as per the
968 Malaria Cell Atlas [30]. **B.** Related to Figure 2A-C. Detailed distribution of memory $CD8^+$ T
969 cells of known (tetramer-positive, as indicated) or unknown (tetramer-negative) specificities
970 in the spleen and the liver. Data were compared using one-way ANOVA and Tukey's multiple
971 comparisons test. The statistical analysis performed on liver data (dark asterisks) compared
972 numbers of T_{RM} cells, and that in spleen data (pale asterisks) compared numbers of T_{EM} cells.
973
974 **Figure S3. Related to figure 2E-H. Detailed distribution of memory $CD8^+$ T cells of known**
975 **specificities in the spleen in mice vaccinated with 1x CS5M RAS or 3x CS5M RAS.** Data
976 were compared using one-way ANOVA and Tukey's multiple comparisons test. The statistical
977 analysis performed on liver data (dark asterisks) compared numbers of T_{RM} cells.
978
979 **Figure S4. Related to figure 4. Tolerisation of TRAP-specific $CD8^+$ T cells in RAS**
980 **vaccinated mice. A.** Related to figure 4B-D. Representative flow cytometry charts showing
981 depletion efficacy of TRAP specific cells. **B.** Related to figure 4C. Comparison of the
982 parasitemias of those mice that were not sterilely protected.
983
984 **Figure S5**. **Related to figure 5. Distribution of TCR transgenic and endogenous T cells in**
985 **RAS vaccinated mice. A.** Related to figure 5D-G. Distribution of PbT-I and endogenous,
986 TRAP-, RPL6- and RPA1-specific memory T cells in the spleen and the liver as indicated.
987 Numbers of T_{EM} cells were statistically compared in the spleen (pale green or purple asterisks),
988 and numbers of T_{RM} cells were compared in the liver (dark asterisks). PbT-I and TRAP specific
989 cell data were pooled from two independent experiments, and RPL6 and F4 cell data come
990 from one experiment. Data were log-transformed and compared using one-way ANOVA and

991 Tukey's multiple comparisons test. **B.** Memory PbT-I cells in the liver on day 30 after transfer
992 of 50,000 naïve PbT-I cells into mice that had been vaccinated with 10,000 RAS 6 days earlier
993 (RAS + PbT-I), or one day later (PbT-I+RAS). Data were pooled from two independent
994 experiments. **C.** Number of PbT-I cells, TRAP- and RPL6-specific $CD8^+$ T cells in the blood
995 on day 7 after the last RAS vaccination. Data come from one experiment and were log-
996 transformed and compared using one-way ANOVA and Tukey's multiple comparisons test. **D-**
997 **F.** Related to figure 5H, I. **D.** Expression of Hsp70 across different life stages of the parasite,
998 as per the Malaria Cell Atlas [30]. sgSpz, salivary gland sporozoites; bbSp, injected
999 sporozoites; EEF, exo-erythrocytic forms. **E.** OT-I and PbT-I cell expansion after HsOVA
1000 HKS injection. Mice received $5x10^5$ naïve CellTrace Violet-coated OT-I and PbT-I cells one
1001 day before injection of $5.2\text{-}8x10^4$ HsOVA HKS, and numbers of divided OT-I and PbT-I cells
1002 were quantified in the spleen 4 days later. Data were pooled from two independent experiments,
1003 log-transformed and analysed using unpaired Student's T-tests. **F.** Distribution of memory T
1004 cells of the indicated specificities in the liver and the spleen. Data were pooled from two
1005 independent experiments, log-transformed and analysed using two-way ANOVA and Tukey's
1006 multiple comparisons test.
1007
1008 **Figure S6. Related to figure 5. Distribution of TCR transgenic and endogenous T cells in**
1009 **CS5M RAS vaccinated mice. A.** Expression of CSP across different life stages of the parasite,
1010 as per the Malaria Cell Atlas [30]. **B.** OT-I and PbT-I cell expansion after CS5M HKS injection.
1011 Mice received $5x10^5$ naïve CellTrace Violet-coated OT-I and PbT-I cells one day before
1012 injection of $4.2\text{-}4.5x10^4$ CS5M HKS, and numbers of divided OT-I and PbT-I cells were
1013 quantified in the spleen 6 days later. Data were pooled from two independent experiments, log-
1014 transformed and analysed using unpaired Student's T tests. **C-D.** Related to figure 5I, J.
1015 Distribution of OVA-specific endogenous memory T cells (**C**) and other specificities as

1016 indicated (**D**) in the liver and the spleen. Data were pooled from two independent experiments,
1017 log-transformed and analysed using one-way ANOVA and Tukey's multiple comparisons test.
1018 The statistical analysis performed on liver data compared numbers of T_{RM} cells
1019
1020 **Figure S7. Related to figure 5. Distribution of TCR transgenic and endogenous T cells in**
1021 **mice vaccinated with WT and CS5M RAS.** Memory $CD8^+$ T cells were enumerated in the
1022 liver and the spleen of mice vaccinated with 2 doses of $5x10^3$ and $10x10^3$ RAS 4 days apart,
1023 then transferred with $50x10^3$ naïve OT-I cells and given a final dose of $5.1x10^3$ CS5M RAS 8
1024 days later, or control mice receiving OT-I cells and one dose of $5.1x10^3$ CS5M RAS. Mice
1025 were euthanised on day 62 after the last sporozoite injection. Data were generated in one
1026 experiment, log-transformed and analysed using one-way ANOVA and Tukey's multiple
1027 comparisons test. The statistical analysis performed on liver data compared numbers of T_{RM}
1028 cells.
1029
1030 **Figure S8. Related to figure 5K, L. Distribution of TCR transgenic and endogenous**
1031 **memory T cells in RAS-vaccinated mice that were efficiently or suboptimally tolerised**
1032 **for $PbTRAP_{130-138}$. A.** Memory PbT-I cells in the spleen. **B.** TRAP-specific memory $CD8^+$ T
1033 cells in the spleen. **C-D**. Numbers of TRAP-specific (**C**) and PbT-I (**D**) memory cells in the
1034 liver as in figure 5K, L, but mice in the 3xRAS group in which TRAP tolerisation worked
1035 efficiently (effT) or suboptimally (subT) were separated into different columns. **E.** Endogenous
1036 memory $CD8^+$ T cells of undefined specificities (non-TRAP) in the liver. **F.** Endogenous
1037 memory $CD8^+$ T cells of undefined specificities (non-TRAP) in the spleen.
1038

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1401 antigen to mouse dendritic cells via Clec9A induces potent CD4 T cell responses biased toward
1402 a follicular helper phenotype. J Immunol. 2011;187(2):842-50. Epub 2011/06/17. doi:
1403 10.4049/jimmunol.1101176. PubMed PMID: 21677141.
1404 95. Li J, Panetta F, O'Keeffe M, Leal Rojas IM, Radford KJ, Zhang J-G, et al. Elucidating
1405 the Motif for CpG Oligonucleotide Binding to the Dendritic Cell Receptor DEC-205 Leads to

1406 Improved Adjuvants for Liver-Resident Memory. The Journal of Immunology.
1407 2021:ji2001153. doi: 10.4049/jimmunol.2001153.
1408

Figure 1

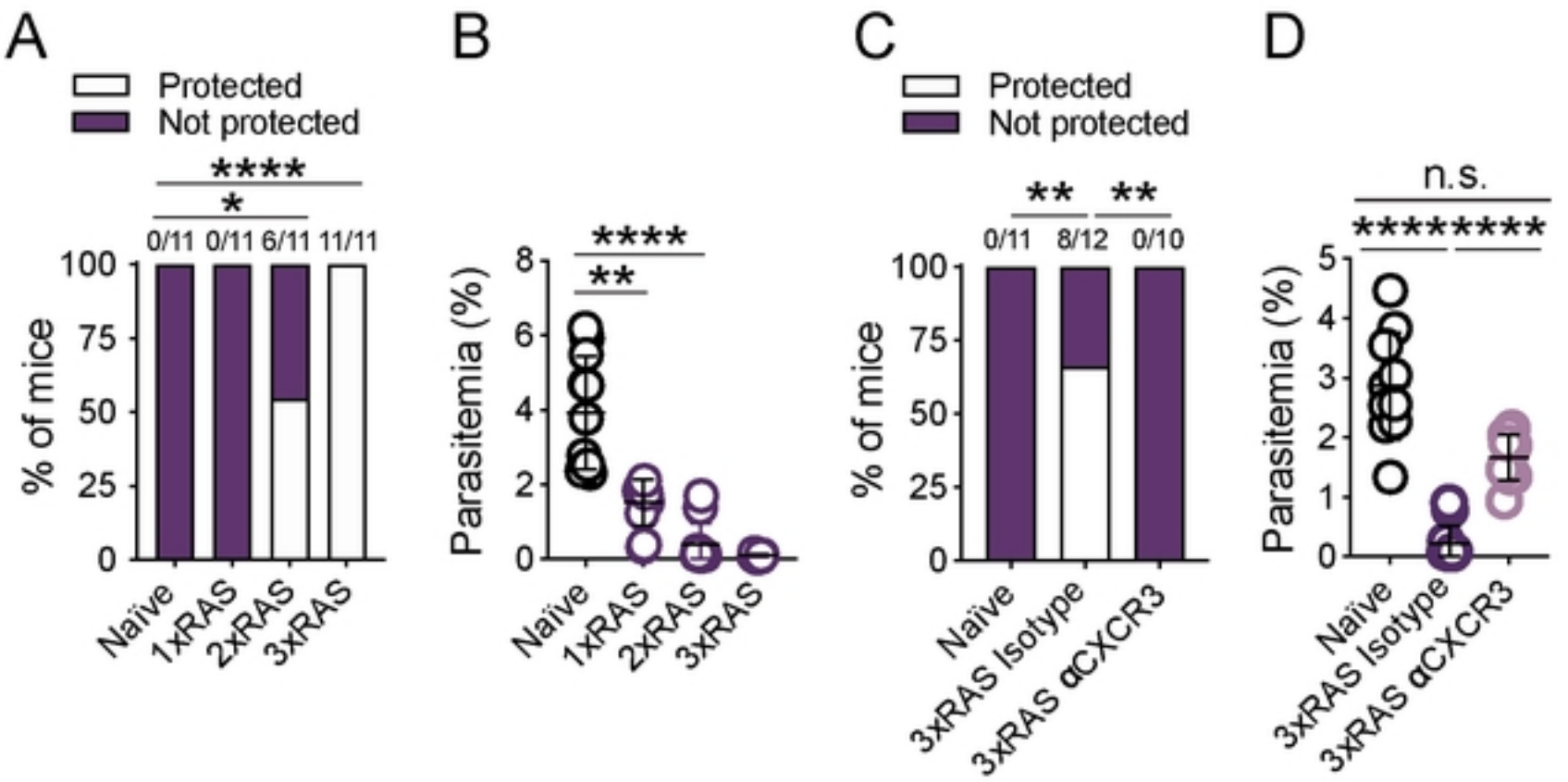
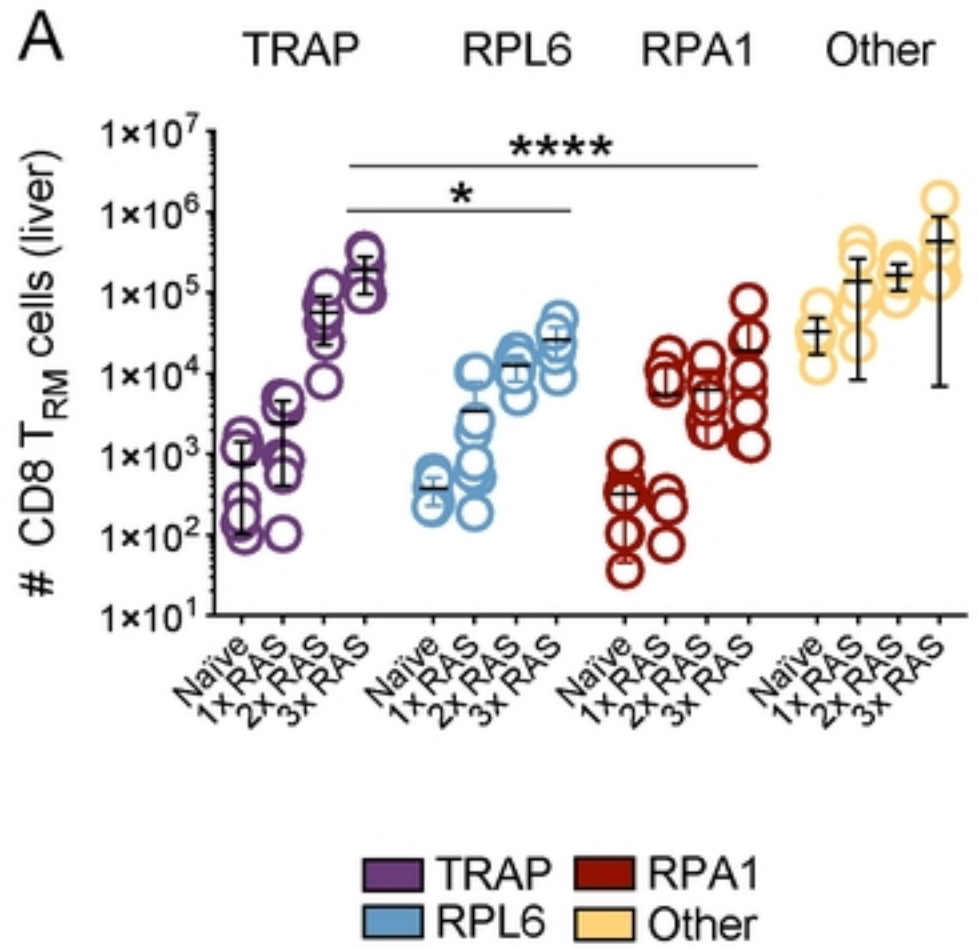
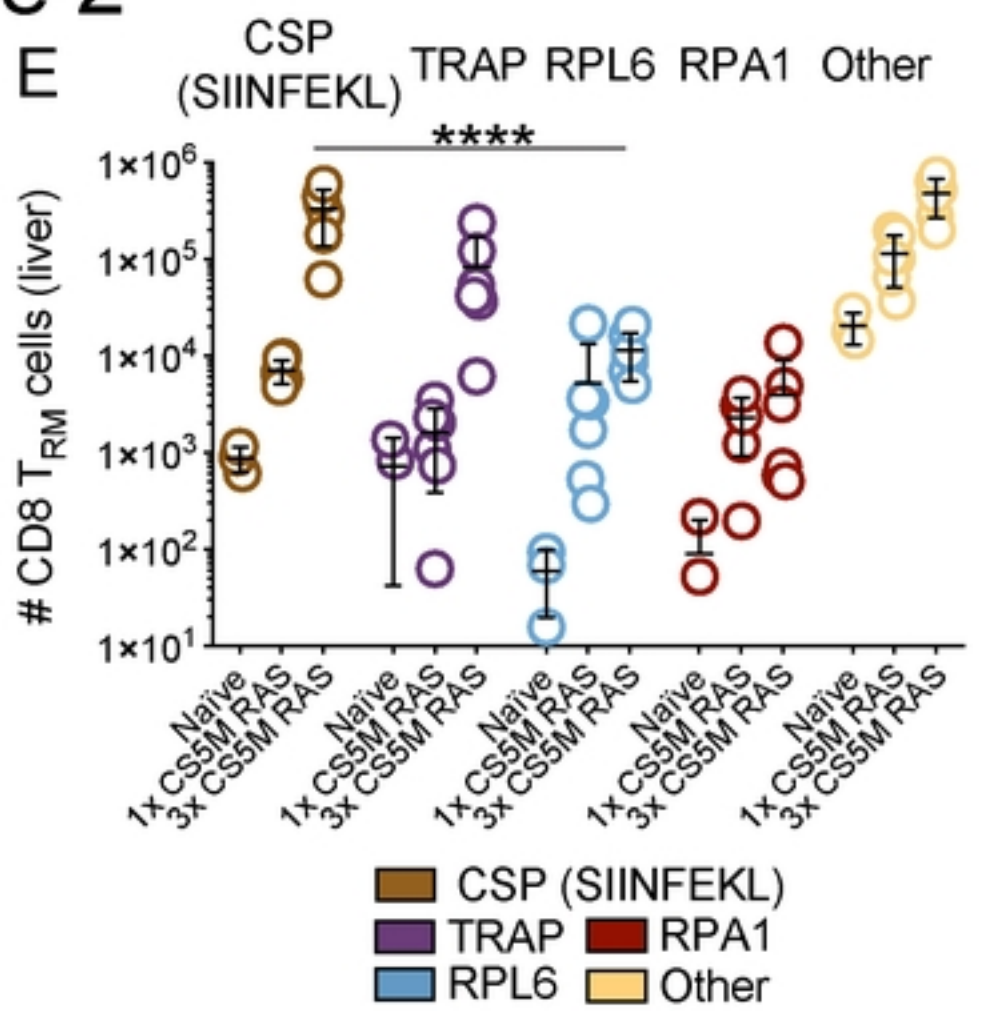
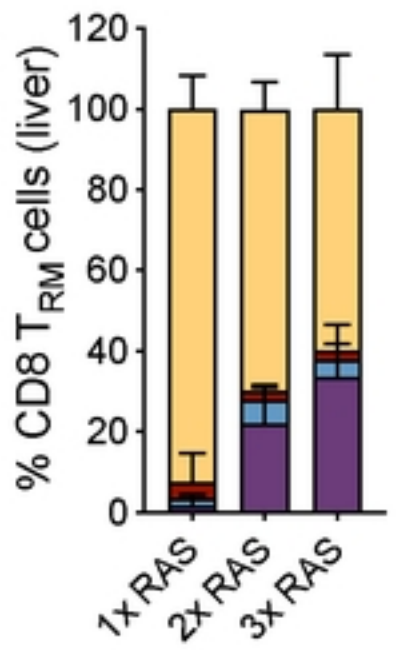
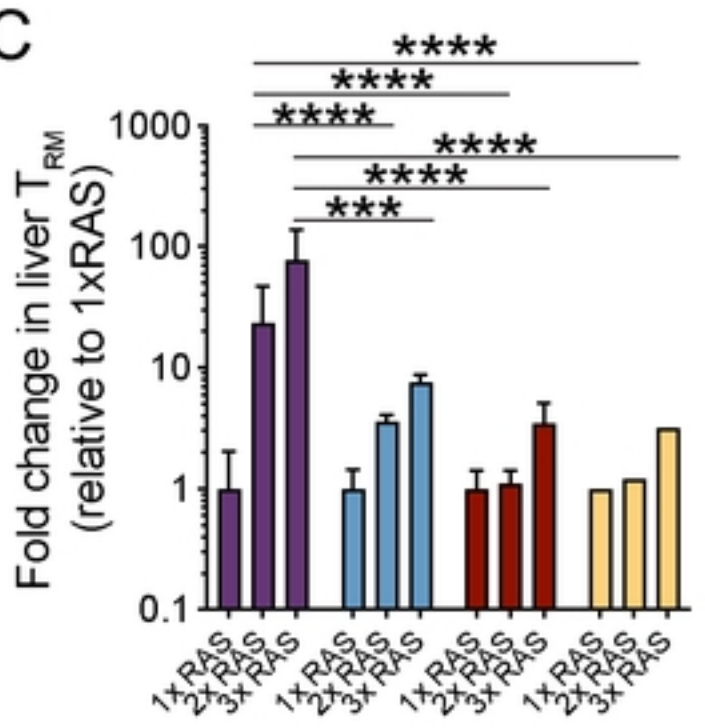

A
Protected
Not protected
****
*
0/11 0/11 6/11 11/11
% of mice
100
75
50
25
0
Naïve
1xRAS
2xRAS
3xRAS
B
****
**
Parasitemia (%)
8
6
4
2
0
Naïve
1xRAS
2xRAS
3xRAS
C
Protected
Not protected
** **
0/11 8/12 0/10
% of mice
100
75
50
25
0
Naïve
3xRAS Isotype
3xRAS αCXCR3
D
n.s.
****
Parasitemia (%)
5
4
3
2
1
0
Naïve
3xRAS Isotype
3xRAS αCXCR3

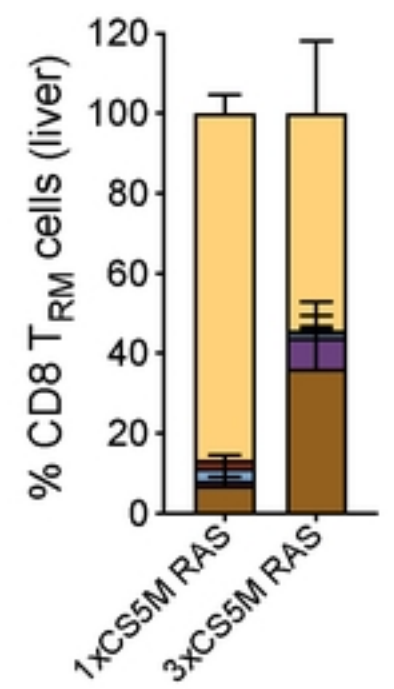
Figure 2

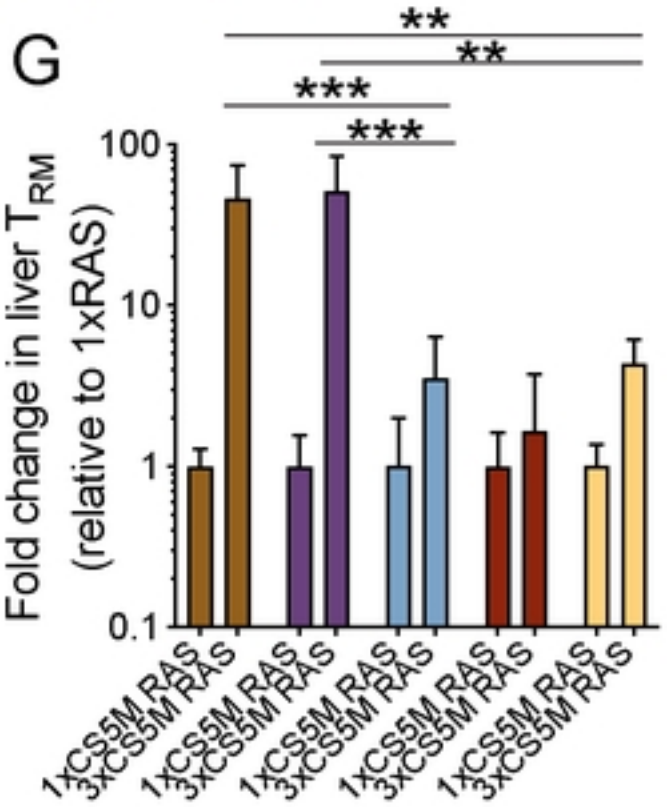

A
TRAP
RPL6
RPA1
Other
CD8 T_RM cells (liver)
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
1×10^1
****
*
Naïve
1x RAS
2x RAS
3x RAS
TRAP
RPA1
RPL6
Other

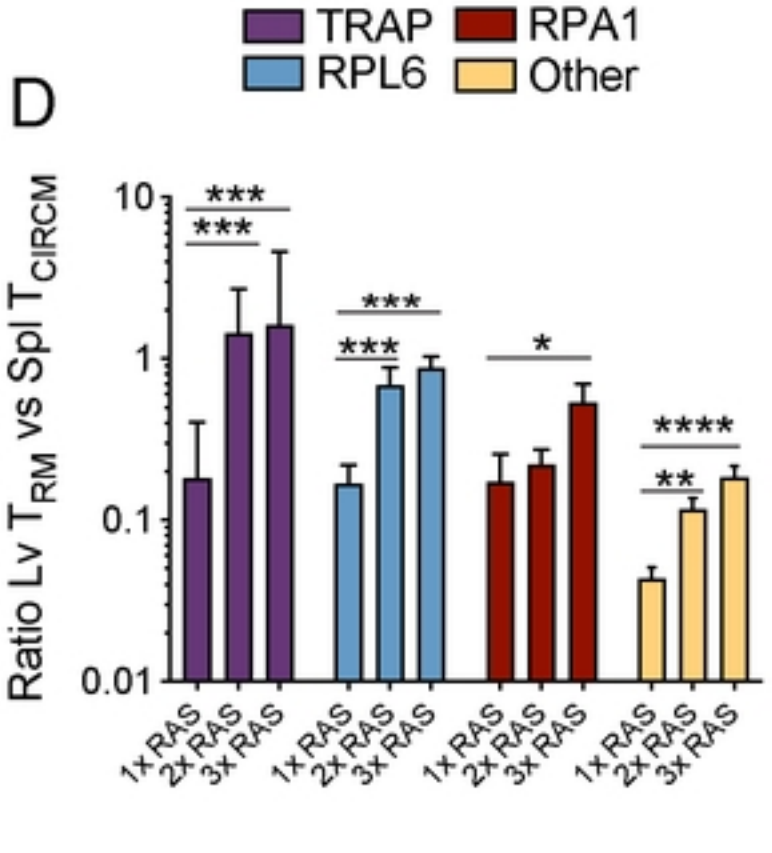

E
CSP (SIINFEKL)
TRAP
RPL6
RPA1
Other
****
CD8 T_RM cells (liver)
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
1×10^1
Naïve
1x CS5M RAS
3x CS5M RAS
CSP (SIINFEKL)
TRAP
RPA1
RPL6
Other


B
% CD8 T_RM cells (liver)
120
100
80
60
40
20
0
1x RAS
2x RAS
3x RAS


C
Fold change in liver T_RM (relative to 1xRAS)
1000
100
10
1
0.1
****
****
****
****
****
***
1x RAS
2x RAS
3x RAS


F
% CD8 T_RM cells (liver)
120
100
80
60
40
20
0
1xCS5M RAS
3xCS5M RAS


G
Fold change in liver T_RM (relative to 1xRAS)
100
10
1
0.1
**
**
***
***
1xCS5M RAS
3xCS5M RAS


TRAP
RPA1
RPL6
Other
D
Ratio Lv T_RM vs Spl T_CIRCM
10
1
0.1
0.01
***
***
***
***
*
****
**
1x RAS
2x RAS
3x RAS

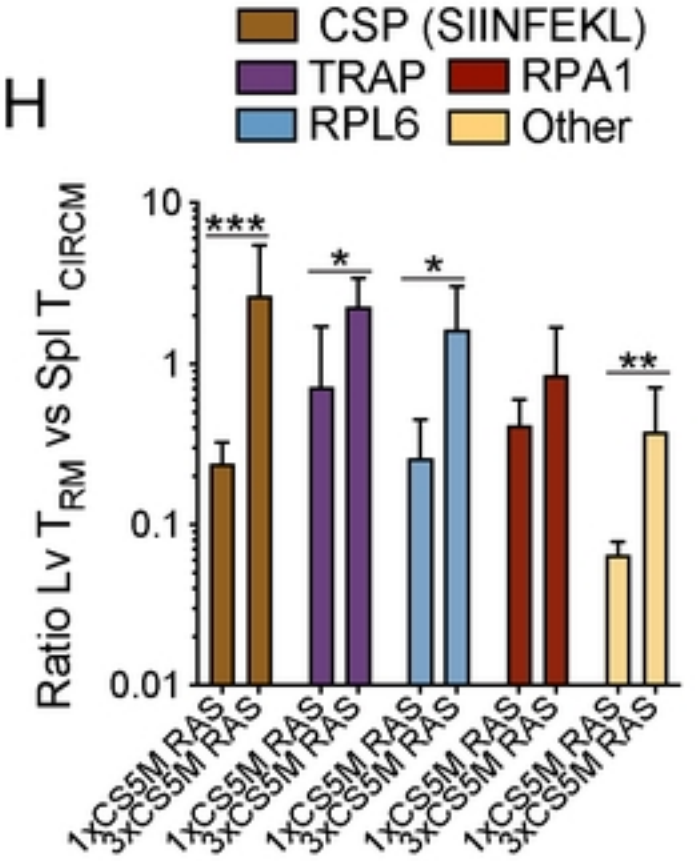

CSP (SIINFEKL)
TRAP
RPA1
RPL6
Other
H
Ratio Lv T_RM vs Spl T_CIRCM
10
1
0.1
0.01
***
*
*
**
1xCS5M RAS
3xCS5M RAS

Figure 3

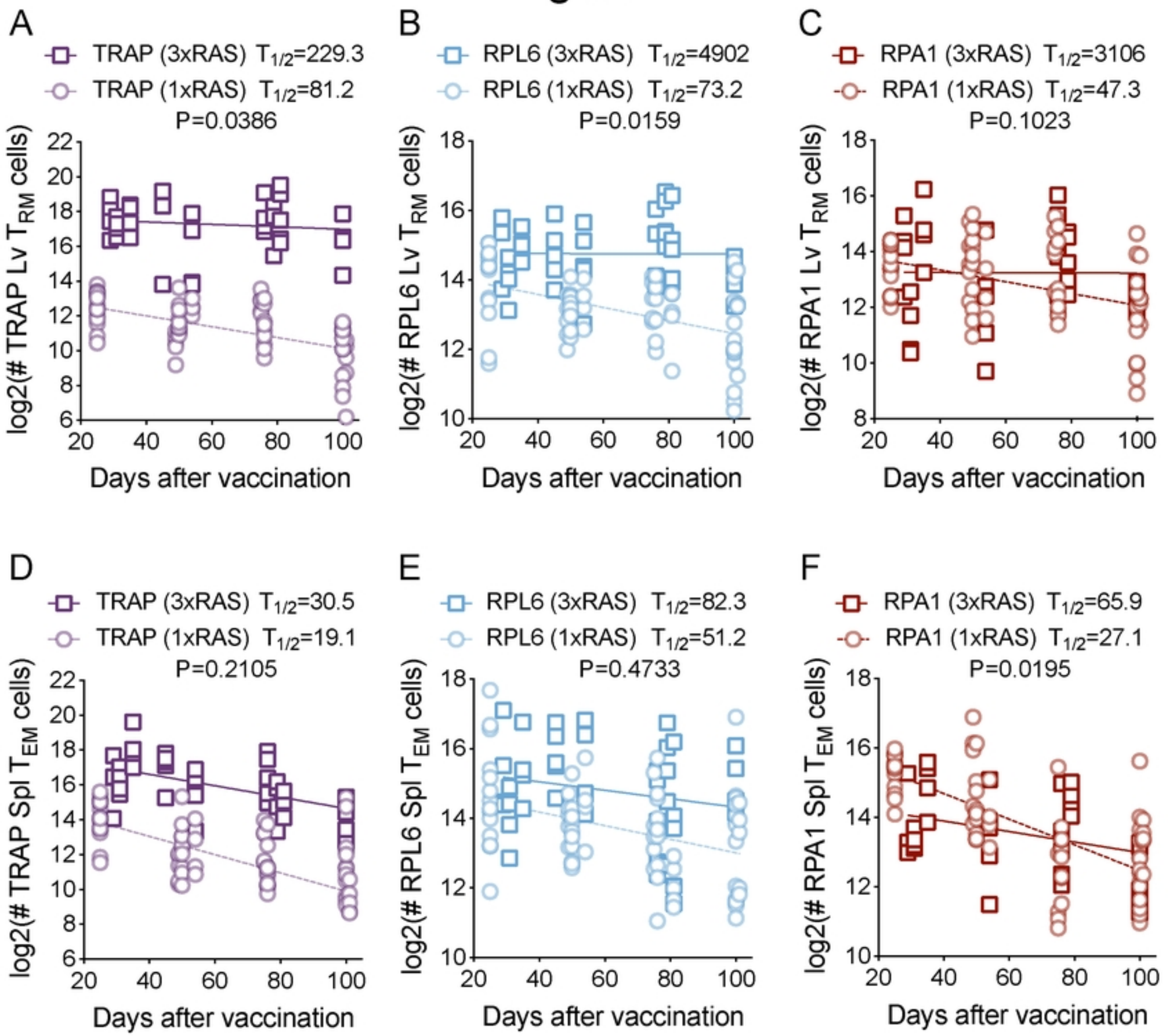

A
TRAP (3xRAS) $T_{1/2}$=229.3
TRAP (1xRAS) $T_{1/2}$=81.2
P=0.0386
log2(# TRAP Lv T_{RM} cells)
Days after vaccination
B
RPL6 (3xRAS) $T_{1/2}$=4902
RPL6 (1xRAS) $T_{1/2}$=73.2
P=0.0159
log2(# RPL6 Lv T_{RM} cells)
Days after vaccination
C
RPA1 (3xRAS) $T_{1/2}$=3106
RPA1 (1xRAS) $T_{1/2}$=47.3
P=0.1023
log2(# RPA1 Lv T_{RM} cells)
Days after vaccination
D
TRAP (3xRAS) $T_{1/2}$=30.5
TRAP (1xRAS) $T_{1/2}$=19.1
P=0.2105
log2(# TRAP Spl T_{EM} cells)
Days after vaccination
E
RPL6 (3xRAS) $T_{1/2}$=82.3
RPL6 (1xRAS) $T_{1/2}$=51.2
P=0.4733
log2(# RPL6 Spl T_{EM} cells)
Days after vaccination
F
RPA1 (3xRAS) $T_{1/2}$=65.9
RPA1 (1xRAS) $T_{1/2}$=27.1
P=0.0195
log2(# RPA1 Spl T_{EM} cells)
Days after vaccination

Figure 4

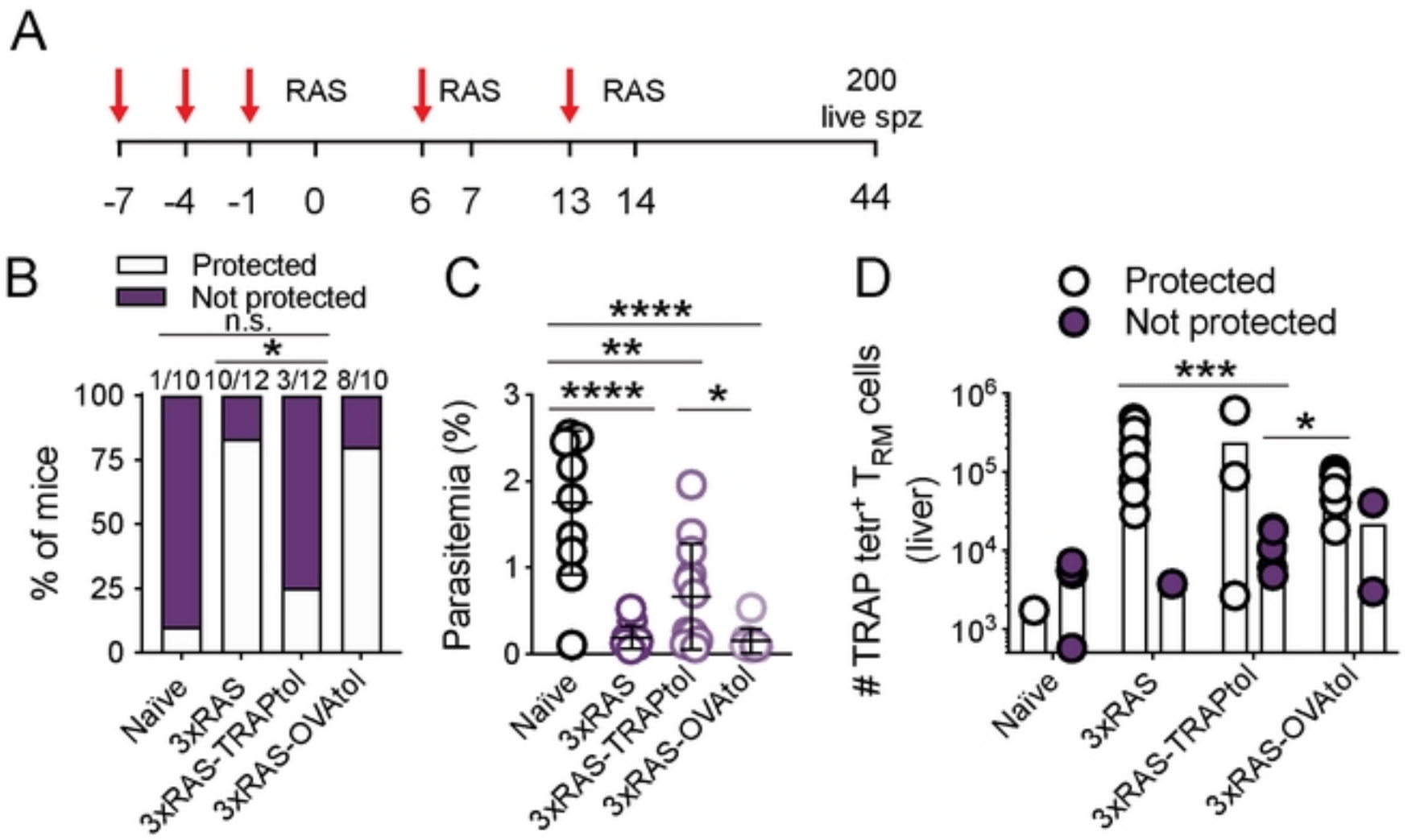

A
RAS
RAS
RAS
200
live spz
-7 -4 -1 0 6 7 13 14 44
B
Protected
Not protected
n.s.
*
1/10 10/12 3/12 8/10
% of mice
100
75
50
25
0
Naïve
3xRAS
3xRAS-TRAPtol
3xRAS-OVAtol
C
****
**
****
*
Parasitemia (%)
3
2
1
0
Naïve
3xRAS
3xRAS-TRAPtol
3xRAS-OVAtol
D
Protected
Not protected
***
*
TRAP tetr+ TRM cells
(liver)
10^6
10^5
10^4
10^3
Naïve
3xRAS
3xRAS-TRAPtol
3xRAS-OVAtol

Figure 5

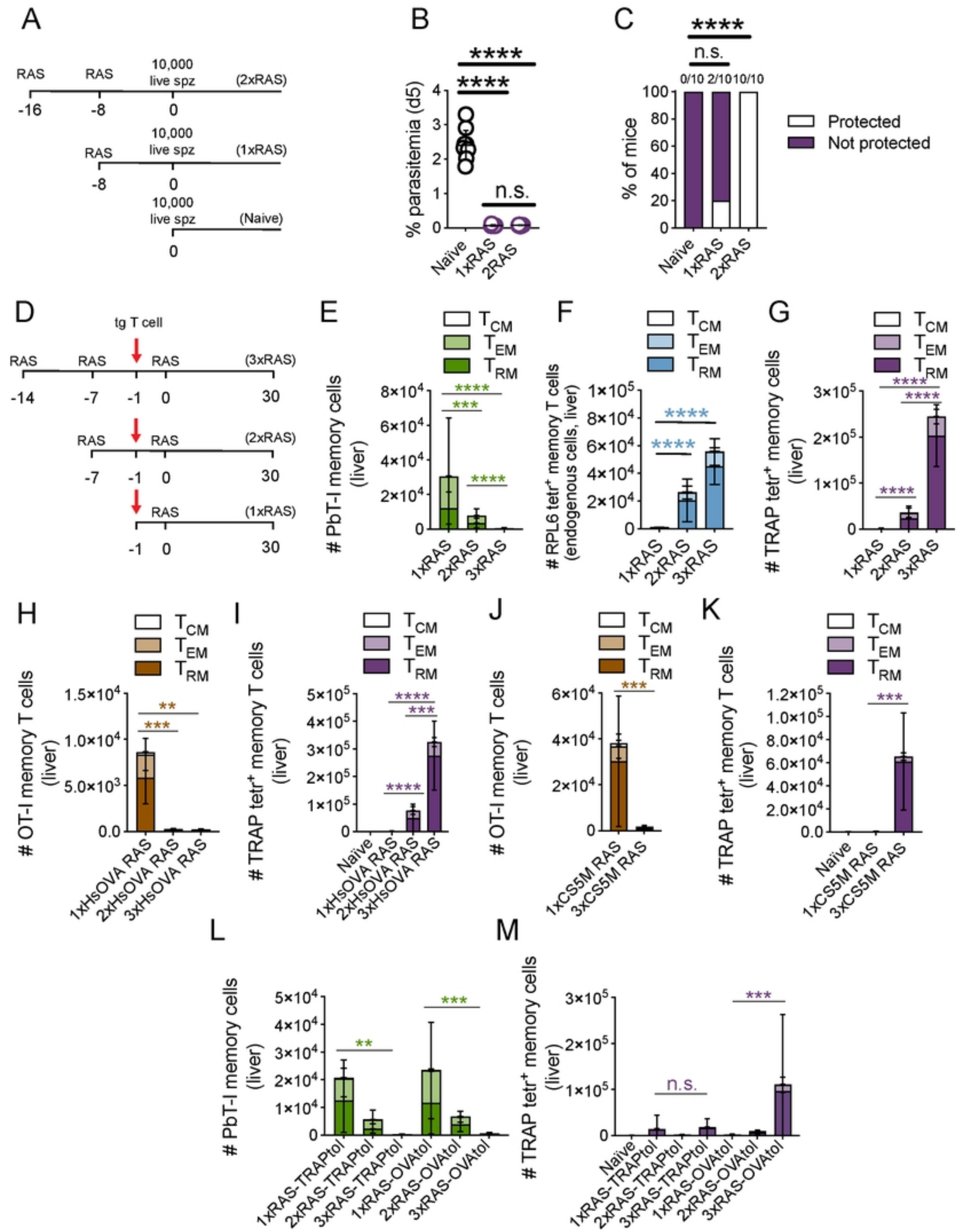
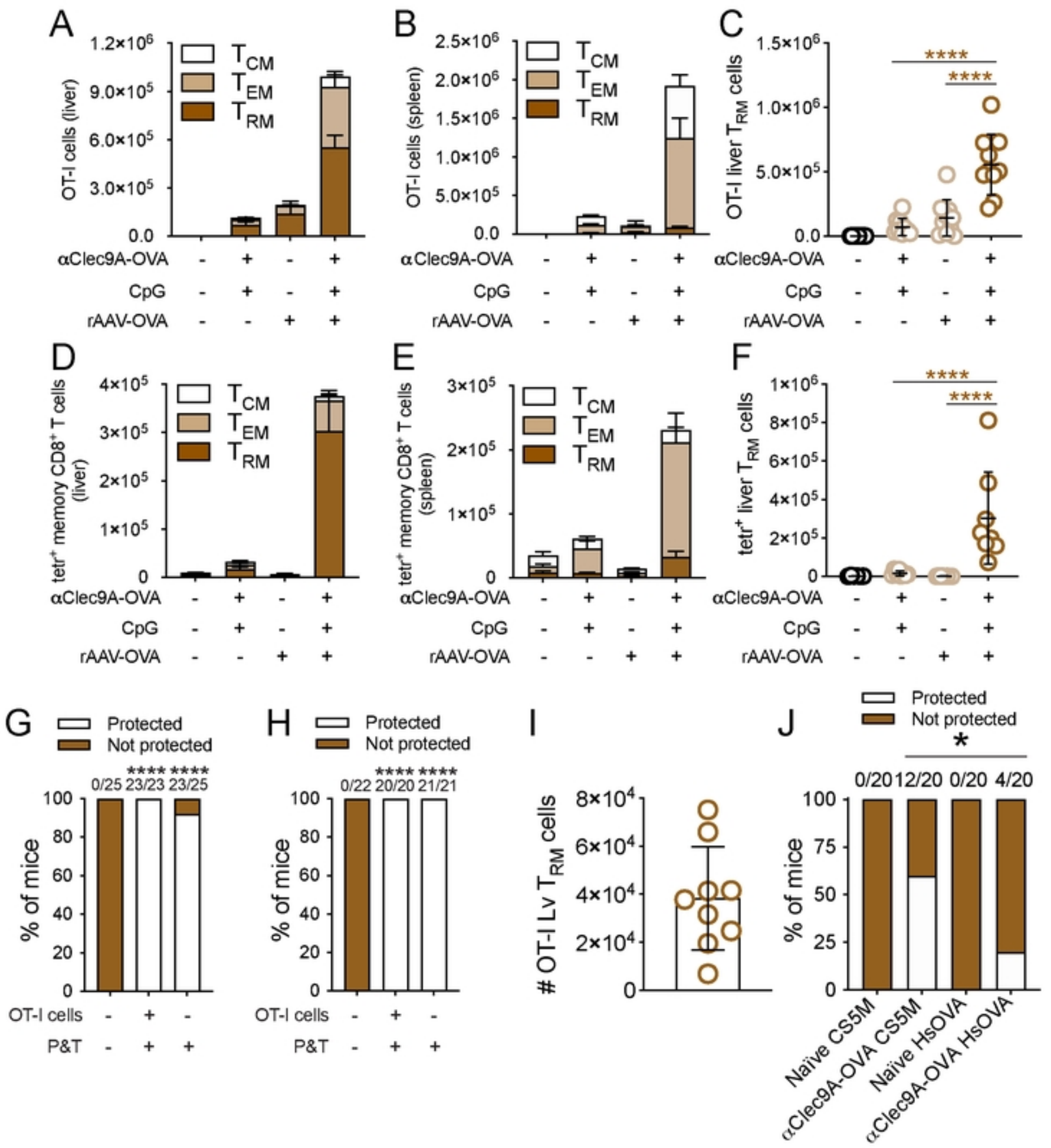

A
RAS
RAS
10,000 live spz
(2xRAS)
-16
-8
0
RAS
10,000 live spz
(1xRAS)
-8
0
10,000 live spz
(Naive)
0
B
****
****
n.s.
% parasitemia (d5)
Naïve
1xRAS
2RAS
C
****
n.s.
0/10 2/10 10/10
% of mice
Protected
Not protected
Naïve
1xRAS
2xRAS
D
tg T cell
RAS
RAS
RAS
(3xRAS)
-14
-7
-1
0
30
RAS
RAS
(2xRAS)
-7
-1
0
30
RAS
(1xRAS)
-1
0
30
E
T_{CM}
T_{EM}
T_{RM}
PbT-I memory cells (liver)
****
***
****
1xRAS
2xRAS
3xRAS
F
T_{CM}
T_{EM}
T_{RM}
RPL6 tetr+ memory T cells (endogenous cells, liver)
****
****
1xRAS
2xRAS
3xRAS
G
T_{CM}
T_{EM}
T_{RM}
TRAP tetr+ memory cells (liver)
****
****
****
1xRAS
2xRAS
3xRAS
H
T_{CM}
T_{EM}
T_{RM}
OT-I memory T cells (liver)
**
***
1xHsOVA RAS
2xHsOVA RAS
3xHsOVA RAS
I
T_{CM}
T_{EM}
T_{RM}
TRAP tetr+ memory T cells (liver)
****
***
****
Naïve
1xHsOVA RAS
2xHsOVA RAS
3xHsOVA RAS
J
T_{CM}
T_{EM}
T_{RM}
OT-I memory T cells (liver)
***
1xCS5M RAS
3xCS5M RAS
K
T_{CM}
T_{EM}
T_{RM}
TRAP tetr+ memory T cells (liver)
***
Naïve
1xCS5M RAS
3xCS5M RAS
L
PbT-I memory cells (liver)
**
***
1xRAS-TRAPtol
2xRAS-TRAPtol
3xRAS-TRAPtol
1xRAS-OVAtol
2xRAS-OVAtol
3xRAS-OVAtol
M
TRAP tetr+ memory cells (liver)
n.s.
***
Naïve
1xRAS-TRAPtol
2xRAS-TRAPtol
3xRAS-TRAPtol
1xRAS-OVAtol
2xRAS-OVAtol
3xRAS-OVAtol

Figure 6


A
B
C
D
E
F
G
H
I
J
OT-I cells (liver)
OT-I cells (spleen)
OT-I liver T_RM cells
tetr+ memory CD8+ T cells (liver)
tetr+ memory CD8+ T cells (spleen)
tetr+ liver T_RM cells
T_CM
T_EM
T_RM
αClec9A-OVA
CpG
rAAV-OVA
Protected
Not protected
% of mice
OT-I cells
P&T
0/25 23/23 23/25
0/22 20/20 21/21
OT-I Lv T_RM cells
0/20 12/20 0/20 4/20
Naïve CS5M
αClec9A-OVA CS5M
Naïve HsOVA
αClec9A-OVA HsOVA