1 **Title:**

2 **Boosting Live Malaria Vaccine with Cytomegalovirus Vector Can Prolong Immunity**

3 **through Innate and Adaptive Mechanisms**

4

5 **Authors:** Komi Gbedande[1, 2 #a], Samad A. Ibitokou[1, #b], Monique L. Ong[3, 4], Mariapia A. Degli-

6 Esposti[3, 4], Michael G. Brown[5], Robin Stephens[1, 2, 6, *, #a]

7

8 **Affiliation:**

9 [1] Department of Internal Medicine, Division of Infectious Diseases, University of Texas Medical

10 Branch, Galveston, TX 77555-0435

11 [2] Center for Immunity and Inflammation, Rutgers New Jersey Medical School, Cancer Center,

12 205 S. Orange Avenue, Newark, NJ 07103

13 [3] Centre for Experimental Immunology, Lions Eye Institute; Nedlands, Western Australia,

14 Australia

15 [4] Infection and Immunity Program and Department of Microbiology, Biomedicine Discovery

16 Institute, Monash University; Clayton, Victoria, Australia

17 [5] Department of Medicine, Division of Nephrology, and the Beirne B. Carter Center for

18 Immunology Research, University of Virginia, Charlottesville, VA

19 [6] Department of Microbiology and Immunology, University of Texas Medical Branch,

20 Galveston, TX

21 [*] **Corresponding author**

22 [#a] Current Address: RS, KG: Center for Immunity and Inflammation, Rutgers New Jersey Medical

23 School, Cancer Center, 205 S. Orange Avenue, G-1230; Newark, NJ 07103;

1

24   #b Current Address SAI: Fate Therapeutics, 12278 Scripps Summit Drive, San Diego, CA 92131

25   **Short Title:** MCMV Boost Prolongs Malaria Vaccine Protection and Effector T cells

26

27   **Abstract**

28   Vaccines to persistent parasite infections have been challenging, and current iterations lack

29   long-term protection. Cytomegalovirus **(**CMV**)** chronic vaccine vectors drive protection against

30   SIV, tuberculosis and liver-stage malaria correlated with antigen-specific CD8 T cells with a Tem

31   phenotype. This phenotype is likely driven by a combination of antigen-specific and innate

32   adjuvanting effects of the vector, though these mechanisms are less well understood. Sterilizing

33   immunity from live *Plasmodium chabaudi* vaccination lasts less than 200 days. While *P.*

34   *chabaudi*-specific antibody levels remain stable after vaccination, the decay of parasite-specific T

35   cells correlates with loss of challenge protection. Therefore, we enlisted murine CMV as a booster

36   strategy to prolong T cell responses against malaria. To study induced T cell responses, we

37   included *P. chabaudi* MSP-1 epitope B5 (MCMV-B5). We found that MCMV vector alone

38   significantly protected against a challenge *P. chabaudi* infection 40-60 days later, and that

39   MCMV-B5 was able to make B5-specific Teff, in addition to previously-reported Tem, that

40   survive to the challenge timepoint. Used as a booster, MCMV-B5 prolonged protection from

41   heterologous infection beyond day 200, and increased B5 TCR Tg T cell numbers, including both

42   a highly-differentiated Tem phenotype and Teff, both previously reported to protect. B5 epitope

43   expression was responsible for maintenance of Th1 and Tfh B5 T cells. In addition, the MCMV

44   vector had adjuvant properties, contributing non-specifically through prolonged stimulation of

45   IFN-γ. *In vivo* neutralization of IFN-γ, but not IL-12 and IL-18, late in the course of MCMV, led

46   to loss of the adjuvant effect. Mechanistically, sustained IFN-γ from MCMV increased CD8α$^+$

2

47   dendritic cell numbers, and led to increased IL-12 production upon *Plasmodium* challenge. In

48   addition, neutralization of IFN-γ before challenge reduced the polyclonal Teff response to

49   challenge. Our findings suggest that, as protective epitopes are defined, an MCMV vectored

50   booster can prolong protection through the innate effects of IFN-γ.

51

52   **Authors' summary**

53   Malaria represents a challenging target for vaccination. This is in part because of the

54   requirement for CD4 T cell immunity in addition to the standard B cell responses current vaccines

55   induce. However, human malaria vaccine approaches thus far have limited longevity of protection

56   due to decay of T cell responses. This includes the most advanced malaria vaccine, a virus-like

57   particle expressing one recombinant liver-stage antigen (RTS,S), and liver-stage parasites

58   attenuated by radiation (PfSPZ), as well as live vaccination with drug-treatment. Our work seeks

59   to prolong this protection using MCMV, a promising vaccine vector known to promote CD8 T cell

60   responses. We observed that boosting the live malaria vaccine with MCMV including a

61   *Plasmodium* antigen led to longer protection from *P. chabaudi* parasitemia, and can be used to

62   promote maintenance of antigen-specific CD4 T cells. In investigating the mechanisms of the

63   MCMV booster, we found that the cytokine IFN-γ is required for prolonged protection and

64   enhances priming of the innate immune system for prolonged protection from malaria. Our

65   research informs both the quest for a longer-lived malaria vaccine and that to understand

66   mechanisms of protection from persistent infection.

67

68   **Introduction**

3

69   Compared to acute and localized infections, persistent systemic infections are the most challenging

70   vaccines to develop [1, 2]. Despite impressive progress for malaria vaccine development, and

71   using emerging approaches including live or attenuated parasites, and recombinant protein

72   constructs targeting different stages in the parasite life cycle, none of the current vaccine

73   candidates provides both highly effective and long-lived immunity. Both natural parasite infection

74   and malaria vaccines generate immunity to parasitemia on re-infection that wanes quickly, and

75   this is shown to be due to a progressive loss of specific T cells [3, 4]. Key insights to decay of

76   protection from persistent systemic infections has come from the study of immunity to parasite

77   infections with *Leishmania* and *Plasmodium spp.,* which decays over time in the absence of

78   infection [3, 5, 6]. Protection from recurrent infection in nature is likely mediated by sustained

79   generation of otherwise short-lived Th1 Teff while infection persists [7, 8]. We and others have

80   shown that both short-lived effector T cells and long-lived effector memory T cells are generated

81   in persistent infection. It is unclear if long-lived memory T cells (Tmem) play an important role,

82   or could be used to improve protection from potentially persistent systemic infections. Persistent

83   parasite infection, including *Leishmania major* or *P. chabaudi,* generate recirculating Tcm that do

84   not protect as well as activated Teff or Tem, defined as previously divided $CD4^+CD62L^{lo}$ Tem/Teff

85   [9], or distinguished as $CD127^-$ Teff and $CD127^{hi} CD44^{hi} CD62L^{lo} CD27^-$ Tem [10]. In *P. chabaudi*

86   infection, we showed that inducing a pre-activated MSP1-specific B5 T cell receptor transgenic

87   (TCR Tg) T cell response, along with a B cell response to MSP-1 p19, before infection with *P.*

88   *chabaudi*, was an effective way to promote an effective reduction of the parasitemia of re-infection.

89   These data suggest an important role for effector T cells in protection. It should be noted that while

90   B5 T cells protect immunocompromised recipients from death, they do not eliminate parasite in

91   the absence of B cells [11]. By defining Teff as $CD127^-$, an early marker of T cell activation that

92   is transient and not present on Tem, we were able to determine that adoptively transferred Teff are

93   the most protective T cells in systemic, blood-stage *P. chabaudi* infection [8, 10]. However, short-

94   lived CD4 T cell immunity has been identified as the crucial variable resulting in short-term

95   protection from persistent *P. chabaudi* infection or vaccination [3, 12]. However, standard

96   vaccines do not generate durable Teff responses, leading to the problem at hand of what vaccine

97   vector to use to prolong protection, if the cell type that protects is short-lived.

98   Therefore, we investigated the potential of CMV to increase the duration of the T cell

99   response to effectively prolong vaccine-induced immunity to *Plasmodium* infection as a booster

100   vector. CMV has emerged as a promising long-lasting vaccine vector, for example, it provided

101   lasting immunity over a year against mucosal Simian Immunodeficiency virus (SIV) challenge in

102   Rhesus macaques [13]. CMV is a potent CD8 T cell-inducing vector with efficacy against SIV,

103   ebolavirus, tuberculosis, cancer and liver-stage malaria [14-18]. CMV infection can induce

104   sustained, or inflationary CD8 T cell responses with an effector memory phenotype. Th1-type

105   effector function is also generated [19-21], but the effect of persistent CMV on CD4 T cells is less

106   well understood. CMV also has non-specific effects suggesting a useful adjuvant effect of the

107   vector. For example, MCMV provides non-specific protection to *Listeria monocytogenes*, *Yersinia*

108   *pestis* and influenza virus, particularly in the latent phase of infection [22, 23]. Previous vaccine

109   studies all have used CMV in the priming phase of the vaccine response, and it has not been tested

110   as a vector for boosting existing responses, nor has it been studied extensively in the generation of

111   CD4 T cell responses. In addition, CMV generates effector/memory T cells, but the specific

112   phenotype has not been determined, and extending the duration of an effector response will be

113   critical to the fight against persistent infection.

114   In this study, we found that the MCMV vector boosted protection generated by a live

115   malaria vaccine against challenge infection with heterologous parasite. In addition to showing that

116   we can induce epitiope-specific effector T cells 40-60 days post-boost, we identified a significant

117   adjuvanting effect of boosting with MCMV. We showed that IFN-γ produced during the persistent

118   phase of MCMV increases CD8α$^+$ dendritic cell numbers. Upon challenge, MCMV boosted

119   animals had increased serum IL-12 and a larger CD4 polyclonal effector T cell response, which

120   were reduced by neutralization of IFN-γ before infection. This work informs our basic

121   understanding of the immunology and vaccinology of persistent infections, and suggests that

122   persistent stimulation of innate and adaptive mechanisms can promote persistence of protective

123   immunity.

124

125   **Results**

126   **MCMV as a vaccine vector with adjuvant properties against *P. chabaudi***

127   In order to evaluate an MCMV vaccine vector and observe vector- and *Plasmodium*-

128   specific CD4 T cell phenotypes, we generated an MCMV vector carrying the *P. chabaudi* MSP-1

129   B5 CD4 T cell epitope (MCMV-B5) using bacterial artificial chromosome (BAC) recombineering.

130   The B5 epitope was inserted in the intermediate early 1 locus with a linker to facilitate processing,

131   as diagrammed in **S1A Fig**. As MCMV has been shown to induce more sustained CD4 T cell

132   numbers in BALB/c, than in C57BL/6 mice [24], we developed a BALB/c system. Although *P.*

133   *chabaudi* MSP-1 B5 CD4$^+$ T cells prevent immunodeficient animals from dying, B5 T cells alone

134   do not affect *P. chabaudi* parasitemia significantly unless they are pre-activated, and in the

135   presence of parasite-specific B cells [11]. Several aspects of the virology and immunology of the

136   two constructs were compared for rigor of the study. The kinetics and tissue tropism of MCMV-

137   B5 infection was confirmed to be the same as the parental virus, MCMV-BAC using plaque assay

138   (**S1B Fig**) and PCR (**S1C Fig**). The virus is largely cleared by day 30, however low recrudescence

139   can be seen at later timepoints. Proportions of polyclonal $CD4^+CD127^-$ Teff, and $CD4^+$

140    $CD44^{hi}CD127^{hi}$ Tmem were similar for MCMV-BAC and MCMV-B5 at day 15 p.i. (**S1D Fig**).

141   MCMV-specific $CD4^+$ T cells were identified using m78 peptide/I-A^d tetramers, using CLIP/I-A^d

142   as non-specific tetramer control, and MCMV-specific $CD8^+$ T cells were detected with the m45/D^d

143   -tetramer [20, 21]. Both MCMV-BAC and MCMV-B5 induced an MCMV m78-specific CD4 T

144   cell response at day 15 p.i. (**S1E Fig**). A large MCMV m45-specific CD8 T cell response to

145   MCMV-B5 was sustained near a million T cells per spleen from day 11 to 60 p.i. (**S1F Fig**).

146   MCMV is an excellent vaccine vector that showed early promise against liver-stage

147   malaria [16] and other infections fought by CD8 T cells. However, the mechanisms by which the

148   vector contributes to the adjuvanting protective effect are not well understood. To test for a

149   protective effect of the low chronic phase of MCMV infection on *P. chabaudi* challenge,

150   BALB/cByJ mice were infected with MCMV-B5 or MCMV-BAC or aged, and challenged 60 days

151   later with *P. chabaudi* AS, as shown schematically in **Fig 1A**. Groups given either MCMV-BAC

152   or MCMV-B5 had strongly reduced parasite growth upon *P. chabaudi* infection compared to a

153   primary infection age-matched control (**Fig 1B**). Pathology from malaria, as indicated by weight

154   loss, was also significantly inhibited by prior MCMV-BAC or MCMV-B5 infection (**Fig 1C**). This

155   strong, non-specific effect reduced parasitemia and pathology by about half, so not as effectively

156   as prior infection, or live vaccination [3].

157

158   **MCMV stimulates and maintains *Plasmodium*-specific T cell responses**

159   Using B5 TCR Tg T cells, we were able to distinguish the non-specific adjuvant effects

160   from epitope-specific effects on CD4$^+$ T cells [11]. To determine effects of the MCMV vector on

161   epitope-specific T cells, we adoptively transferred B5 Tg T cells (2 x 10^6) and infected the

162   recipients with MCMV-BAC or MCMV-B5, as illustrated in **Fig 2A**. The B5 CD4 T cell response

163   in the spleen 15 and 55 days post-MCMV infection was assessed by flow cytometry. The

164   proliferated B5 T cell (CTV$^-$ Thy1.2$^+$) number was specifically increased by MCMV-B5, which

165   leading to a specific increase that lasted until day 55 (**Fig 2B**). MCMV-BAC infected mice also

166   maintained proliferated CTV$^-$ B5 T cell numbers slightly above background to day 55. In order to

167   test the MCMV-driven generation of effector T cells, they were stained for the IL-7Rα (CD127),

168   which is downregulated on recently activated T cells. The majority of CTV$^-$ B5 TCR Tg T cells

169   on day 15 are CD127$^-$ Teff (**Fig 2C**). On day 55, the fraction of Tmem increases, but a significant

170   number of Teff also are present, as clearly seen in the flow cytometry plot of B5 T cells recovered

171   at this timepoint (**Fig 2D**). In addition, we observed that MCMV-B5, but not MCMV-BAC,

172   specifically promoted the differentiation of MCMV-B5 epitope-specific GC Tfh (CXCR5^{hi}PD-1^{hi},

173   **Fig 2E**).

174   We also detected some MCMV-specific CD4 T cell responses using MCMV m78 tetramer

175   at 11 and 60 days after MCMV-B5 infection (S2A **Fig**). MCMV-B5 infection induced MCMV

176   m78-specific CD4$^+$ T cell responses that persisted for two months (S2B **Fig**). Phenotypically,

177   MCMV vector-specific T cells were predominantly Teff (Tet$^+$ CD127$^-$) early in infection (day 11),

178   with re-upregulation of CD127 into a Tmem phenotype at day 60 (S2C **Fig**), similar to the B5-

179   specific response to MCMV-B5. Proliferation of transiently transferred B5 T cells in response to

180   MCMV-B5 was tested over various five-day windows. Congenic recipients (Thy1.1) were infected

181   with MCMV-BAC or MCMV-B5 or uninfected, and CTV-labelled CD4-purified B5 TCR Tg T

182   cells were adoptively transferred for the last five days of infection, testing presentation of B5

183   peptide between days 5-10, 25-30, or 55-60 of MCMV infection. Splenocytes were analyzed for

184   proliferation (%CTV-) of B5 T cells in each experimental window (**S2D Fig**). The MCMV-B5

185   vector, and not the MCMV-BAC control, led to strong specific proliferation of epitope-specific

186   B5 Tg T cells during acute infection (days 5-10) (**S2E Fig**). Despite the presence of B5 TCR Tg

187   Teff on day 55, there was no detectable proliferation in this assay at later timepoints. This may be

188   due to background proliferation, even in uninfected animals.

189

190   **MCMV boosts long-term protection of live malaria vaccine**

191   As the protection of malaria vaccines tested in mice and humans decays after one to two

192   years [25, 26], we sought to test a vaccine booster strategy to prolong immunity. *Plasmodium spp*.

193   infection followed by drug treatment is an effective vaccination protocol in C57BL/6 mice, and

194   also in human studies; however, this protection shows measurable decay by day 200 in mice [3,

195   27]. Using the protocol published by Freitas do Rosario et al, where mice are infected with *P*.

196   *chabaudi* AS followed by sub-curative dose of chloroquine each time parasitemia reaches 1%, we

197   tested sterilizing immunity with heterologous reinfection (**S3A Fig**). This experiment confirmed

198   that *P. chabaudi* infection induced sterilizing immunity for two months, and that a measurable

199   decay in protection was evident by day 200 in BALB/c mice (**S3B Fig**), as in B6. We also

200   confirmed that there was not a measurable decay in antibody concentration between 2 and 6.5

201   months p.i., as shown previously in B6 mice (**S3C Fig**), leading us to test a known T cell-response-

202   promoting vector to attempt to prolong T cell responses and protection through as yet unknown

203   adjuvant effects.

204   To test the potential of an MCMV-based booster vaccine, mice were vaccinated with live

205   *P. chabaudi* AS infection followed by sub-curative doses of chloroquine and then boosted with

206   MCMV-BAC or MCMV-B5 140 days later, as schematically depicted in **Fig 3A**. As vaccine-

207   induced protection decays by day 200, we tested MCMV boosters for protection from heterologous

208   re-infection with *P. chabaudi* AJ at day 200, which corresponds to 60 days after boosting. This

209   timing was chosen on the basis that neither immunity nor the T cell response to live vaccination

210   has completely decayed by day 120 [3, 12], and that the MCMV infection is below detectable by

211   day 60 [24], though we expected it to show prolonged stimulatory capacity at this timepoint [22].

212   Age- and sex-matched *Plasmodium*-vaccinated mice, with or without the MCMV-booster, were

213   challenged with heterologous *Plasmodium* infection. The booster prolonged live vaccine induced

214   protection, as evidenced by the data shown in **Fig 3B** that mice that were boosted with MCMV

215   had a lower parasitemia on challenge on day 200 post-vaccination (p.v., average of 0.06%, or

216   0.05% without or with the B5 epitope, respectively), on day 4 post-challenge (p.c.), than

217   vaccinated mice challenged without boosting (1.57% on day 4 p.c., and 0.5% on day 6 p.c.). The

218   boosting effect does not require the B5 epitope in a significant way, which is not surprising, B5

219   CD4 T cell epitope was included as a way to track specific T cells, not to protect as a single epitope.

220   Heterologous parasite-specific serum antibody was measured by ELISA at day 7 p.c. (**Fig 3C**). As

221   previously reported parasite-specific IgG responses remain stable up to day 200 post-live

222   vaccination [3], ELISA showed that levels of *P. chabaudi* AJ-specific serum IgG in the first week

223   of challenge were not altered by the MCMV booster (**Fig 3C**). The boost was specifically chosen

224   to promote T cell responses to support the ongoing antibody response generated by the live

225   vaccine. In summary, *Plasmodium* vaccination efficacy, but not serum antibody, fades with time,

226   and boosting with MCMV prolonged protection.

10

227

MCMV can boost parasite-specific T cell immunity induced by live malaria vaccine

CMV is a promising CD8 T cell-inducing vaccine vector and generates Tem and inflationary effector T cells [20, 28]. Less is known about CD4 T cell phenotypes in response to MCMV [20], and the potential for continuous generation of phenotypic effector CD4 T cells in the persistent phase is not well understood. In order to determine if MCMV-B5 boosted *Plasmodium*-specific CD4 T cell responses, B5 CD4 TCR Tg cells (Cell Trace Violet (CTV)-labelled) were adoptively transferred into congenic recipients (Thy1.1 BALB/cByJ), which were then immunized with live *P. chabaudi* vaccine, and boosted with MCMV-B5 or MCMV-BAC at day 140 p.v. (**Fig 4A**). B5 T cell effector and effector memory phenotype and cytokine responses were quantified at day 200 p.v.. Using MCMV-B5, as a booster to the live malaria vaccine specifically and significantly increased the numbers of divided B5 TCR Tg T cells ($CD4^+CTV^-Thy1.2^+$) present on day 200 p.v., while MCMV-BAC did not (**Fig 4B**). Therefore, more transferred B5 T cells survived to day 200 after MCMV-B5 boost. B5 TCR Tg cells are not detectable after 200 days in the absence of parasite [8]. The MCMV-B5 boost 60 days earlier specifically increased the numbers of both $CD127^-$ B5 Teff and $CD127^{hi}$ B5 Tmem present at day 200, while MCMV-BAC did not increase B5 T cell numbers (**Fig 4C**). Among Tmem subsets, the number of both $CD27^+$ and $CD27^-$ effector memory T cells ($CD44^{hi}CD127^{hi}CD62L^{lo}$, Tem) were significantly higher in MCMV-B5 boosted mice, while central memory ($CD62L^{hi}$ Tcm) were not increased by MCMV boost (**Fig 4D**). In addition, the MCMV-B5 booster specifically generated or maintained functional B5 T helper type 1-type cells, as shown by their production of IFN-γ (**Fig 4E**), TNF (**Fig 4F**) and IL-2 (**Fig 4G**) at day 200. This data show that the vector generated well-presented epitopes, and

249   could be used as a booster vaccine for T cells. In addition, the vector induced effector T cells for

250   a longer time than reported for acute stimulation, in addition to making Tem.

251

252   **IFN-γ, but not IL-12 and IL-18, induced by MCMV, is associated with protection from *P.***

253   ***chabaudi* infection**

254   As we had observed a protective effect by the MCMV-BAC, we took the opportunity to

255   understand mechanisms explaining the observed benefits of using this virus as a vaccine vector in

256   addition to the ability to present T cell epitopes and generate Teff. To determine if cytokines

257   induced by long-term MCMV infection are mechanistically responsible for promoting protection

258   from heterologous challenge, we used neutralizing antibodies to test the roles of IFN-γ, as well as

259   the combination of IL-12 and IL-18, in the period before challenge after 40 days of MCMV

260   infection. IFN-γ was neutralized starting on day 34 of MCMV-BAC infection, and before *P.*

261   *chabaudi* challenge, as shown schematically in **Fig 5A**. *In vivo* neutralization of IFN-γ after

262   MCMV, but before *P. chabaudi* infection was associated with a substantial loss of protection

263   against *P. chabaudi* challenge. The mice treated with anti-IFN-γ before infection had significantly

264   higher parasitemia compared to isotype-treated controls previously infected with MCMV (**Fig 5B**).

265   The level of parasitemia in mice receiving neutralizing antibody is similar to a first infection

266   suggesting that all or most of the reduction due to MCMV is due to IFN-γ. The effect of

267   neutralizing MCMV-induced IFN-γ on challenge is also reflected in increased pathology, as

268   measured by weight loss (**Fig 5C**), and hypothermia (**Fig 5D**). To determine if there is an effect of

269   MCMV persistence-induced IFN-γ on the T cell response to challenge, the polyclonal CD4^{+} T cell

270   phenotype was examined on day 15 post-challenge with *P. chabaudi.* Surprisingly, there was a

271   significant reduction in responding CD4^{+} Teff after pre-treatment with anti-IFN-γ (**Fig 5E**).

272   Evaluation of serum cytokines in MCMV infected animals showed IL-12p40 production

273   throughout MCMV infection (**Fig 5F)** that is increased upon *P. chabaudi* challenge (**Fig 5G**).

274   Strikingly, IL-12 p40 production on days 7, 8 and 15 p.c. as well as TNF, CXCL-9 and CXCL-10

275   on day 7 p.c. were significantly reduced by neutralization of IFN-γ for six days before challenge

276   while IL-10 level was increased at 7 days p.c. **(Fig 5H)**. We also measured other cytokines

277   including IL-6, IL-15, IL-18, IFN-α and IFN-β, which are known to be produced during MCMV

278   infection to see if they were affected by neutralization of IFN-γ but did not see significant

279   differences at these time points.

280   To investigate potential direct effects of neutralizing IFN-γ on challenge parasitemia due

281   to persisting antibody and determine the role of persistent IFN-γ induced by MCMV in protection,

282   we examined the effect of anti- IFN-γ clone H22 on *P. chabaudi* infection with or without an

283   earlier dose of MCMV (**S4A Fig**). We found that administration of neutralizing antibody up to 2

284   days before infection with *P. chabaudi* does not have a significant measurable effect on peak

285   parasitemia (**S4B Fig**), while it does affect parasitemia significantly, as above, if MCMV is given

286   before infection (**S4C Fig**). To determine if neutralizing levels of antibody remained in the serum

287   during challenge, we developed an assay to test induction of CXCL10, which is induced in

288   macrophages by IFN-γ. CXCL10 mRNA was measured after culture of RAW 264.7 cells with

289   serum from mice before challenge (d40, **S4D Fig**), and on day 7 post *P. chabaudi* challenge with

290   or without prior anti-IFN-γ administration (**S4E Fig**). We found no detectable change for the

291   relative CXCL10 mRNA level with Raw 264.7 cells cultured with d40 or d47 serum, though there

292   was a trend suggesting antibody was there. Adding recombinant IFN-γ (rIFN-γ) to the assay

293   allowed detection of neutralizing antibody. In this assay, a reduced level of CXCL10 mRNA was

294   detectable in the presence of serum from 2 days after anti-IFN-γ (**S4F Fig**) and day 7 *P. chabaudi*

295   challenge (**S4G Fig**). Therefore, though neutralizing antibody does remain in the system during *P.*

296   *chabaudi* challenge, we conclude that the large effect of blocking IFN-γ after MCMV, and before

297   challenge, is mostly due to inhibition of MCMV-induced not *P. chabaudi*-induced IFN-γ.

298   Therefore, we investigated the mechanisms of this strong effect.

299   As IL-12 and IL-18 can induce IFN-γ from NK cells or previously stimulated T cells, we

300   queried their role in induction of IFN-γ by MCMV, but did not find any effect of blocking IL-12

301   and IL-18 together in the time period before challenge on protection from *P. chabaudi* parasitemia

302   or pathology by the MCMV vector (**S5 Fig**).

303   To explore the effect of IFN-γ on persisting epitope-specific T cells and the innate response in the

304   context of late MCMV infection, we adoptively transferred CTV-labelled B5 TCR Tg T cells into

305   Thy1.1 mice and then infected them with MCMV-B5, as illustrated in **Fig 6A**. IFN-γ was

306   neutralized on days 44-48 of MCMV infection. Blocking IFN-γ induced by MCMV-B5 infection

307   did not change the number of CTV⁻ B5 T cells at day 50 p.i. (**Fig 6B**). IFN-γ also did not affect

308   the phenotype, as measured by the fraction of both Teff and Tmem (**Fig 6C**), or representation of

309   Tmem subsets, as defined by CD27 and CD62L expression (**Fig 6D**). B5 T cells induced by

310   MCMV-B5 are mostly memory T cells, with a fraction of effector T cells that is larger than that

311   seen on day 60 of *P. chabaudi* infection [8]. A panel of innate cell markers showed that after IFN-

312   γ neutralization on day 50 post-MCMV infection, the number of CD8α⁻ DCs (CD3⁻

313   CD11c^{hi}MHCII^{hi}) remained constant while the number of CD8α⁺ DCs showed a trend towards a

314   reduction (**Fig 6E**). The level of IL-12p40 in the serum on day 50 post-MCMV infection was

315   significantly reduced in the group that received IFN-γ neutralizing antibody compared to isotype

316   (**Fig 6F**). Splenic CD8α⁺ DCs are the major producers of IL-12p40 in response to systemic stimuli

317   [29].

318

319   **IFN-γ generated by the MCMV booster drives prolonged protection of live malaria vaccine**

320   Given the ability of MCMV to prolong protection from challenge and to promote IL-12 in

321 an IFN-γ dependent manner, we tested mechanisms of the prolonged protection. Mice were

322 vaccinated and then boosted with MCMV at day 140, as in **Fig 4**, with adoptive transfer of B5 T

323 cells to determine the effect on vector-driven T cells. B5 T cell recipients were immunized with

324 live malaria vaccine and boosted with MCMV-B5 at day 140 p.v., as illustrated in **Fig 7A.** Half of

325 these animals received neutralizing antibody to IFN-γ, or isotype control antibody, days 194-198

326 p.v. (days 54-58 after the MCMV-B5 booster). T cell and antigen-presenting cell phenotypes were

327 assessed at day 200 after malaria immunization, in the absence of challenge. Live *P. chabaudi*

328 vaccination plus MCMV-B5 booster tended to increase survival of B5 T cells compared to live

329 vaccine alone, as above, though this effect did not reach significance in this experiment (**Fig 7B**).

330 Neutralization of IFN-γ did not have an effect on specific malaria vaccine-driven, MCMV-B5

331 boosted B5 T cell numbers, or the composition of Tmem subsets (**Fig 7C**). These data indicate that

332 IFN-γ does not promote T cell survival or Tem differentiation directly, after MCMV-B5 boost,

333 supporting the specific effect of MCMV-B5 epitope on the specific T cell responses. Staining for

334 innate cell types, $CD11c^{hi}$ dendritic cells (DC) were found to be increased by MCMV boost (**Fig**

335 **7D**). Furthermore, *in vivo* neutralization of IFN-γ significantly reduced the DC numbers compared

336 to isotype control (**Fig 7D**). This innate effect was seen most strikingly in the $CD8\alpha^+$ DC ($CD3^-$

337 $CD11c^{hi}MHCII^{hi}CD8\alpha^+$) population (**Fig 7E**). We also assessed NK cells, and monocyte derived

338 subsets including monocytes, macrophages and inflammatory monocytes to see if their numbers

339 or activation marker expression were affected by neutralization of IFN-γ, but did not see other

340 significant differences **(S6 Fig)**. Blocking IFN-γ after vaccination and the MCMV booster caused

15

341   a trend towards a reduced serum level of IL-12p40, though this did not reach significance, as it did

342   in MCMV infection alone on day 50 (**Fig 7F**). To test the role of MCMV booster-induced IFN-γ

343   in prolonging vaccine-induced protection, mice were vaccinated and then boosted with MCMV-

344   BAC at day 140, as above in **Fig 3**, and as shown schematically in **Fig 7G**. The control groups

345   either had no live vaccine and no MCMV or no vaccine and then MCMV only. Prior to

346   heterologous challenge with *P. chabaudi* AJ at day 200, vaccinated mice with MCMV boost

347   received IFN-γ neutralizing antibody, or isotype control, at days 194-198 p.v. (54-58 days post-

348   MCMV booster). MCMV boosting live vaccination showed significant protection against weight

349   loss (**Fig 7H)** and parasitemia (**Fig 7I)** compared to the adjuvanting effect of MCMV or

350   unvaccinated control mice. Blocking IFN-γ before challenge significantly reduced the booster

351   effect of the MCMV-BAC on protection at day 200 (**Fig 7J**). This was measured as a significant

352   increase in parasitemia in the IFN-γ neutralized animals (0.4%), compared to the isotype group

353   (0.09%), on day 5 post-challenge. While this is clearly a non-specific effect, as there is no B5

354   epitope here, polyclonal T cell phenotyping on day 15 post-challenge again showed that blocking

355   IFN-γ before reinfection significantly reduced the fraction of Teff generated during *P. chabaudi*

356   challenge, and was associated with higher parasitemia, suggesting a role for antigen presentation

357   during challenge that was not tested here (**Fig 7K**). Serum levels of *P. chabaudi* AJ-specific IgG

358   increased after challenge, but there was no significant difference from neutralization of IFN-γ (**Fig**

359   **7L**). Collectively, these data suggest a novel innate pathway where viral vector-induced IFN-γ

360   drives an increase in CD8α+ DCs, which promote increased protective IL-12 and Teff upon

361   challenge. We have identified two novel mechanisms of adjuvanting supporting the promise of

362   CMV-based vaccines, and defined a promising line of research for prolonging protection from

16

363   *Plasmodium* infection. Testing the full capacity of this strategy would require the identification of

364   more protective T cell epitopes.

365

366   **Discussion**

367   Alternative high-efficacy approaches for all malaria vaccine continue to be developed, but

368   little engineering has been focused on increasing longevity. These data show significant promise

369   for use of chronic vaccination to prolong the current short half-life of vaccines against systemic

370   infections requiring CD4 T cell memory. In summary, we demonstrated an adjuvant effect

371   dependent on the MCMV vector-induced IFN-γ that enhances CD8α$^+$ dendritic cell numbers, and

372   IL-12 production upon challenge. In addition, we demonstrate two basic mechanisms for the

373   extension of protection provided by the chronic MCMV booster to a typical short-lived malaria

374   vaccine. The MCMV vaccine-expressing a malaria epitope prolongs survival of epitope-specific

375   CD4 Teff and Tem. Polyclonal effector T cell numbers also increase when challenge occurs in the

376   presence of MCMV-induced IFN-γ, suggesting that the underlying mechanism of protection from

377   challenge is an increased Th1 response to *Plasmodium* due to increased IL-12 downstream of

378   higher dendritic cell numbers at the time of challenge.

379   We observed a strong adjuvant effect of the MCMV vector itself on *P. chabaudi* infection.

380   The effect of MCMV alone reduced *P. chabaudi* challenge parasitemia by about one half. The

381   adjuvant effect alone also drove a significant fraction of the prolonged protection seen from using

382   the MCMV vector in a boosting strategy, as demonstrated by neutralizing the IFN-γ induced by

383   MCMV-BAC before challenge. MCMV and gamma herpesvirus 68 also have an adjuvant effect

384   against influenza virus and *Listeria monocytogenes*, but not West Nile virus [22, 23], indicating

385   some specificity. This specificity may be due to latency. Gamma Herpesvirus 68 with a point

17

386   mutation in ORF73, a gene required for latency, showed reduced protection from *Listeria*, and also

387   reduced IFN-γ levels and chronic macrophage activation. HSV-1 and Sindbis viruses, which do

388   not establish latency, did not have this protective effect [22]. MCMV-induced cross-protection

389   against influenza also lasts through early and established (5-12 weeks), but not long-standing (9

390   months) MCMV latency. Production of IFN-γ during the latent phase of infection has also been

391   implicated in MCMV-induced protection to *Listeria* and *Yersinia* [22], influenza; which was

392   abrogated in IFN-γ deficient mice [23]. Our work extends these findings mechanistically by

393   showing that neutralizing IFN-γ late in MCMV infection reduced the number of $CD8\alpha^+$ dendritic

394   cells in the spleen, and reduced the amount of some cytokines and chemokines IL-12, CXCL-9

395   and CXCL-10 and the number of CD4 Teff, but increased IL-10 cytokine level on challenge. While

396   we did not specifically test for latency, our vectors showed similar kinetics and outcomes to

397   previous studies suggesting infection with our vectors would be latent in our mouse model by day

398   40 [24]. Mechanisms of this protection had not been specifically established.

399   Decay of immunity, and failure to maintain *Plasmodium*-specific CD4 T cells make the

400   development of an effective and long-lived malaria vaccine challenging. Both natural immunity to

401   the parasite, and vaccines, generate immunity that decays due to a progressive loss of malaria-

402   specific T cells limiting long-lived protection [3, 4, 30, 31]. To our knowledge, there are no current

403   vaccine formulations that drive long-lived CD4 T cell responses [2, 32]. Therefore, the potential

404   of CMV to induce a prolonged T cell response with the desired phenotype was used to extend

405   vaccine protection. In this study, a two-step vaccine strategy that first induces a long-lived

406   protective antibody response, and then boosts with an MCMV strategy was chosen for the potential

407   to extend effector/memory CD4 T cells responses over a longer period to prolong protection. While

408   the protective T cell epitopes are not well defined, using a parasite derived CD4 T cell epitope

409   allowed us to test this strategy in a pre-clinical setting. Boosting with the MCMV vector did

410   prolong the protection generated by the live infection and-drug cure malaria vaccine as evidenced

411   by reduced challenge parasitemia after boost. The data show that animals that received live malaria

412   vaccination became susceptible to heterologous re-infection by day 200 p.v., while the MCMV-

413   B5 boost maintained the protection seen at day 60 until day 200. All malaria vaccines tested in

414   human studies thus far, including the most advanced vaccine RTS,S, show insufficient longevity

415   of protection [25].

416   CMV induces a robust and long-lasting T cell response including both effector and memory

417   components [20, 33]. Hansen et al. showed recently that RhCMV-expressing *Plasmodium*

418   *knowlesi* antigens, generated a parasite antigen-specific immune response that delayed parasite

419   growth upon sporozoite challenge via generation of CD8 T cell responses [16]. However, whether

420   an MCMV vector could be used to boost pre-existing specificities has not been tested, and only a

421   few studies have investigated CD4 T cell responses to MCMV [34]. Here we show that the MCMV

422   vector can be used to specifically boost CD4 epitope-specific effector T cell responses, which is

423   one of the primary gaps in our current malaria vaccine arsenal. Both CD4 Teff and Tmem

424   phenotypes are generated in *P. chabaudi* infection, and both promote protection [8, 10]. Here, we

425   observed that MCMV-B5 increased epitope-specific T cells, and specifically generated GC-Tfh

426   and promoted maintenance of the Tem phenotype and effector functions (IFN-γ, TNF and IL-2)

427   past day 200. CD4 T cells are crucial for protective immunity to *P. chabaudi* as they produce

428   cytokines that both promote phagocytosis and B cell help [35]. Our previous findings revealed the

429   power of Teff in protection from persistent *P. chabaudi* with help from B cells. Adoptive transfer

430   of B5 effector CD4 T cells reduced the peak of *P. chabaudi* parasitemia by half [10]. While

431   memory T cells are less effective, CD27⁻ TemL do reduce parasitemia, and are the most protective

19

432 memory phenotype. In this study, we showed that the MCMV-B5 vector specifically activated and

433 maintained both adoptively transferred epitope-specific T cells and MCMV-specific CD4$^+$ T cells

434 over 2 months. Interestingly, Teff were clearly present among the few m78-Tetramer MCMV-

435 specific CD4 T cells over 2 months, but did not reach significant levels compared to uninfected

436 controls. This may reflect the small number of cells detected, and so we used the B5 TCR Tg to

437 test for MCMV-induced proliferation at late time points, however this assay had high background.

438 Consistent with this, MCMV-B5 maintained both Teff and the potentially protective late effector

439 memory phenotype T cells generated by *P. chabaudi* infection. While we do not present evidence

440 here that the protection provided is due to increased survival of B5 T cells, their numbers are

441 boosted at a timepoint when live vaccine induced T cells and protection would otherwise decay,

442 though antibody levels remain constant. It should be noted that the B5 specificity is not especially

443 potent in protection, as the effect of this one specificity is only seen in immunocompromised

444 animals. CD8 T cells specific for some MCMV loci are given to inflationary activation, even after

445 15 weeks of MCMV infection [36]. The locus where B5 is expressed, IE1, can be a memory

446 inflation inducing locus for CD8 T cells [37]. CD4 T cell responses to MCMV are even less well

447 understood. Therefore, additional T cell epitope discovery in this field will aid in the long-term

448 goal of developing durable vaccine induced protection.

449 The MCMV vector increased CD8α$^+$ DCs and IL-12 both before challenge, and in response

450 to the parasite. Induction of IL-12 upon *P. chabaudi* challenge was reduced in the context of prior

451 neutralization of IFN-γ. Considering that CD8α$^+$ DCs are a key source of IL-12 production in many

452 infections [38], and also present antigen to T cells upon infection, the reduced IL-12 level in

453 response to *P. chabaudi* challenge after IFN-γ neutralization could be directly linked to the change

454 in Teff cell numbers seen. While IL-12 is more commonly associated with stimulating IFN-γ

455   production, IL-12 production by DC and macrophages can also be promoted by IFN-γ present in

456   the milieu, representing a positive feedback mechanism [39, 40]. Therefore, prolonged IFN-γ

457   appears to be maintaining CD8α$^+$ DCs, an IL-12 producing cell type, suggesting a novel

458   mechanism for this feedback. As IL-12 deficient mice have higher *P. chabaudi* parasitemia than

459   WT [41], or Ifng deficient animals [42], the reduced IL-12 response during challenge is likely to

460   explain the reduced control of parasites seen upon neutralization of IFN-γ. While more research

461   will be required to establish if the increase in dendritic cells late in MCMV infection is sufficient

462   to drive the increased IL-12 response or the increase in Teff upon challenge, the increase of CD8α$^+$

463   DCs supports this interpretation.

464   Prior work has shown that giving *P. chabaudi*-infected mice recombinant IFN-γ increased

465   the number of polyclonal CD4 Tem, and also prolonged heterologous immunity. In our system,

466   generation and maintenance of Tem is epitope specific, and not affected by blocking IFN-γ

467   generated during MCMV infection. Our previous work showed that both memory T cell

468   differentiation and prolonged infection contribute to maintenance of Tem numbers in *P. chabaudi*

469   infection [10]. It remains to be proven whether the MCMV-BAC driven increase in DCs, and/or

470   priming of them to respond/present antigen, leads directly to enhanced responsiveness of parasite

471   specific T cells/expansion and clearance of parasite by Th1-promoted macrophage activation and

472   Tfh-promoted antibody production. It is also possible that antigen presentation and Th1 response

473   are reduced with the presence of detectable anti-IFN-γ auto-antibody [43]. Exploring this

474   hypothesis to determine potential effects of anti-IFN-γ antibody, we found that administration of

475   neutralizing antibody up to 2 days before infection with *P. chabaudi* does not have a significant

476   effect on peak parasitemia, though there may be a trend towards increasing parasitemia. Indeed, in

477   Meding et al, where IFN-γ was neutralized during the infection, the change in parasitemia was

21

478 very small [42], similar to the effects of anti-IFN-γ seen on primary infection in our study. There

479 is a clear reduction of these IFN-γ-inducible chemokines (CXCL9 and CXCL10) in the serum

480 upon infection after anti-IFN-γ. This suggests that anti-IFN-γ directly or indirectly reduces their

481 production in response to the parasite. An indirect example would be that these chemokines can

482 be made by DCs, the reduction of IFN-γ induced chemokines could reflect the changes on DC

483 number that we have documented downstream of MCMV. Our results show that although

484 neutralizing antibody persists into the challenge phase, the largest effect of anti-IFN-γ after

485 boosting, requires MCMV-induced IFN-γ, and is not a direct effect on parasitemia.

486   Neutralization of IFN-γ led to a loss of the extended protection provided by MCMV

487 boosting and protection from *P. chabaudi* infection. Teff were reduced on challenge after IFN-γ

488 neutralization, which is particularly striking given the increase in parasite in anti-IFN-γ pre-treated

489 animals. IFN-γ production has been previously reported in MCMV and HCMV infection during

490 the period coinciding with latency [23]. Data from our laboratory and others establishes that IFN-

491 γ expression during persistent *P. chabaudi* infection, or recombinant IFN-γ also promote

492 protection from heterologous re-infection at day 200 [12, 44]. It was also reported that rIFN-γ

493 increased the number of polyclonal Tem at day 200 after *P. chabaudi* infection [44].

494

495   We have used basic knowledge of T cell differentiation in *P. chabaudi* infection to reverse

496 engineer a novel chronic vaccine strategy. We demonstrate a method and a mechanism to prolong

497 protection by a live malaria vaccine, which alone is short-lived, due to a loss of specific T cells.

498 Our work is novel as it indicates that MCMV works as a booster for CD4 T cell responses both by

499 maintaining specific effector and effector memory T cells and also as an adjuvant driving

500 protective IFN-γ production. The increase in dendritic cell numbers and increased IL-12 induced

22

501   by MCMV, together with and a trend towards increased Th1 cytokines upon challenge, suggest

502   that ultimately, a stronger T cell response to parasite explains the prolonged protection. This

503   strategy has the potential to resolve the major issue of short-lived protection generated by malaria

504   vaccines, as well as help understand how natural immunity could be prolonged. There are safety

505   issues to work out before CMV vectors could be used in humans. However, superinfection, the

506   ability to re-infect CMV carriers, and a varied T cell response are two benefits of this approach

507   [45, 46]. Further understanding of the mechanisms of this exciting new model could lead to more

508   rapidly translatable tools in the quest to prolong immunity to persistent or recurrent pathogens

509   without introducing another pathogen. This could be applicable to malaria, a disease that kills up

510   to half a million people a year; as well as other persistent or recurrent pathogens.

511

512   **Materials and methods**

513   **Ethics statement**

514   All experiments were carried out according to protocol approved by the University of

515   Texas Medical Branch Institutional Animal Care and Use Committee (IACUC).

516

517   **Mice, Parasites and Immunization**

518   All mice used in our study were maintained in the UTMB animal facility under specific

519   pathogen-free conditions with access to food and water *ad libitum*. Recipient Thy1.1 BALB/cByJ

520   were backcrossed to BALB/cJ (N4; The Jackson Laboratory, Bar Harbor, ME). B5 T cell receptor

521   transgenic (TCR Tg), which recognizes a peptide from merozoite surface protein-1 (MSP-1; 1157-

522   1171, ISVLKSRLLKRKKYI/I-E^{d}) was backcrossed to BALB/cJ (N10). B5 TCR Tg mice, and *P.*

523   *chabaudi chabaudi* AS stocks, were a kind gift from J. Langhorne (Francis Crick Institute, London,

524 UK). *P. chabaudi* AJ (MRA-740, a cloned line that is highly related but heterologous to AS [47,

525 48] was purchased from BEI Resources, formerly MR4. B5 TCR Tg mice were typed using

526 primers Vα2, 5′-gaacgttccagattccatgg-3′ and 5′-atggacaagatcctgacagcatcg-3′, and vβ8.1, 5'

527 cagagaccctcaggcggctgctcagg-3′ and 5′-atgggctccaggctgttctttgtggtttttgattc-3′ [8]. Both male and

528 female recipients were used, however no sex differences in infection were noted. All adoptive

529 transfer experiments were done using age- and sex- matched recipient and donors.

530 For infection and drug cure vaccination, the protocol was performed as previously

531 documented in mice and humans [3, 27]. Six- to nine-week old mice were infected (10^6 *P.*

532 *chabaudi)* and treated with two subsequent noncurative doses of chloroquine (10 mg/kg body

533 weight/day; Sigma-Aldrich), when parasitemia reached 1-2% of erythrocytes. Parasites were

534 counted by light microscopy in thin blood smears stained with Giemsa (Sigma-Aldrich, St. Louis,

535 MO). Parasitemia was monitored by microscopic examination of thin blood smears from the tail.

536 Thin blood smears were stained with Giemsa (Ricca chemical company, Arlington, TX), and

537 parasites were counted by light microscopy.

538

539 **Construction of MCMV and viral infection**

540 A vaccine vector, MCMV-K181-B5, was generated as follows. MCMV-K181-Perth was

541 genetically modified to carry the MHC II-E^d-restricted B5 epitope (ISVLKSRLLKRKKYI) from

542 the *Plasmodium chabaudi* Merozoite Surface Protein MSP1 (1157–1171) in frame with the C-

543 terminus end of the MCMV IE1 gene (**Fig S1 A**). The B5 epitope was inserted into the MCMV-

544 K181 genome using bacterial artificial chromosome recombineering, as described previously [49].

545 The resulting virus was denoted MCMV-B5. Mice were infected by injecting 5×10^3 PFU of

24

546   salivary gland-propagated MCMV-B5 virus or the parental virus, MCMV-K181-Perth (MCMV-

547   BAC). Viruses were injected i.p. in 0.2ml of PBS.

548

549   **Virus quantification**

550   MCMV-B5 or MCMV-BAC viral copy number of CMV/β-actin ratios were determined in

551   different organs in infected BALB/cJ mice. DNA was purified from tissue (liver, lung, and salivary

552   gland) using the Gentra Puregene Kit (Qiagen, Valencia, CA) following manufacturer's

553   instructions. All tissues were stored at -80°C in PBS for DNA, or Trizol (Thermo Fisher Scientific,

554   Carlsbad, CA) for RNA purification, until sample processing. qPCR was performed with 50 ng of

555   purified DNA. Specific primers for MCMV immediate early (IE1) gene (NCBI Accession No.

556   M11788) 5-tca gcc atc aac tct gct acc aac-3 and 5-gtg cta gat tgt atc tgg tgc tcc tc-3 and for murine

557   β-actin (Accession No. M12481) 5-gct gta ttc ccc tcc atc gtg -3 and 5-cac ggt tgg cct tag ggt tca-

558   3. Serial dilutions of a control plasmid were used to generate a standard curve for both MCMV-

559   IE1 and β-actin, as described previously, and qPCR of viral DNA has been shown to correlate

560   quantitatively with the plaque assay throughout the viral infection [50]. All sample measurements

561   were performed in triplicate. Real time PCR (VIIA, AB Biosystems, Life Technologies, Carlsbad,

562   CA).

563

564   **Cell culture with serum, RNA isolation and real-time PCR analysis**

565   RAW264.7 macrophage-like cell line was cultured in RPMI medium (Gibco)

566   supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 U/ml penicillin and 100

567   lg/ml streptomycin), Sodium Pyruvate (100x, Gibco), 45% D-Glucose (100x, Sigma), L-glutamine

568   (100x, Corning) at atmosphere of 5% CO_2. RAW264.7 cells were incubated with sera form IFN-

569   γ neutralizing mice with or without recombinant IFN-γ for 24h. Total RNA was extracted with

570   Direct-Zol RNA reagents (Zymo research, R2025) as described by the manufacturer. cDNA was

571   synthesized from total RNA using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was

572   performed with cDNA using the SYBR Green Supermix (Bio-Rad). The β-actin gene was used for

573   normalization. Relative changes in gene expression were calculated using the comparative

574   threshold cycle (Ct) method. The following primers were used: CXCL-10 5'-

575   cctgctgggtctgagtggga-3' and 5'-gataggctcgcagggatgat-3'; β-actin 5-gctgtattcccctccatcgtg-3 and 5-

576   cacggttggccttagggttca-3.

577

578   **Adoptive transfers and *in vivo* studies**

579   MACS-purified CD4$^+$ T cells from young, uninfected B5 TCR-Tg mouse spleens (Miltenyi

580   San Diego, CA) were labelled with cell trace violet (CTV) and adoptively transferred (2×10^6) into

581   Thy1.1 congenic mice. For cytokine neutralization, three doses of anti-IFN-γ antibody (H22, 0.5

582   mg) or isotype control antibody (PIP, 0.5 mg); or a combination of anti-IL-12p40 (C17.8, rat

583   IgG2a, 300 μg) and anti-IL-18 antibody (YIGIF74–1G7, control Rat IgG2a, 200 μg) were given,

584   every other day. All *in vivo* antibodies were from BioXCell (Lebanon, NH).

585

586   **Flow cytometry**

587   Single-cell suspensions from spleens were prepared at the indicated time points after infection in

588   HBSS) with HEPES (Thermo Fisher Scientific, Waltham, MA) using gentleMACS (Miltenyi, San

589   Diego, CA) and incubated in RBC lysis buffer (eBioscience, San Diego, CA). Single cells were

590   stained extracellular in PBS, 2% FBS, and 0.1% sodium azide with antibodies. For T cells: anti-

591   CD127 (A7R34), anti-PD-1 (RMP1-30), anti-CD62L (MEL-14), CD4 (RM4-5), (anti-CD90.2

592 [30-H12], anti-CD44 [IM7], and anti-CD27 [LG-7F9]) (all from eBioscience/Invitrogen and

593 Biolegend, San Diego, CA). Phenotype of polyclonal CD4^{+} T cell subsets and *Plasmodium* B5-

594 specific CD4^{+} T cell subsets were identified with gating strategy as schematically depicted in **S7**

595 **Fig**. For follicular T cell staining with CXCR5, cells were stained in PBS + 0.5% BSA + 0.1%

596 sodium azide + 2% Normal Mouse Serum (NMS) and 2% FBS (Sigma, St. Louis, MO). Rat anti-

597 mouse purified CXCR5 (2G8, BDbioscience, San Jose, CA, 1 hr., 4°C) was followed by biotin-

598 conjugated AffiniPure Goat anti-rat (H+L, Jackson ImmunoResearch, West Grove, PA, 30 min,

599 4°C) followed by Streptavidin-eFluor 450. For antigen presenting cells were stained with anti-

600 Ly6C, anti-Ly6G (1A8), anti CD11b (M1/70), anti-CD11c (N418), DX5, MHCII/I-A/Eb

601 (M5/114.15.2). For intracellular cytokine staining, cells were cultured for 5 hours at 37°C, 5%

602 CO_2 in the presence of GolgiPlug (BD Bioscience) before extracellular staining and then fixed

603 with 2% paraformaldehyde overnight. Cells were then permeabilized using BD 10X Permeability

604 buffer diluted with water, and stained with anti-IFN-γ (XMG1.2), anti-TNF (MP6-XT22), anti-IL-

605 2 (JES6-5H4). Virus-specific CD4^{+} T cells were identified using m78/I-A[d] tetramers, with peptide

606 (SQQKMTSLPMSVFYS) and CLIP/I-A[d] as tetramer control (PVSKMRMATPLLMQA) and

607 CD8^{+} T cells with H2D[d] restricted m45-tetramer peptide 507-VGPALGRGL-515 [20, 21] (both

608 from NIH Tetramer Core Facility, Emory, Atlanta, GA). Tetramer staining was performed within

609 6 months of receiving the reagents at 4C for 1 hour before/after surface and intracellular staining.

610 Cells were acquired on a LSR II Fortessa using FACSDiva software (BD Biosciences, San Jose,

611 CA). Compensation was performed in FlowJo using single-stained splenocytes (with CD4 in all

612 colors). All data analysis was performed using the FlowJo software package (version 10, FlowJo,

613 BD San Jose, CA). Cell numbers were calculated as the % of recovered Tg cells/lymphocytes x

614 the number of lymphocytes, except for **Fig 6** and **Fig 7** which the recovered cells were counted by

27

615 inclusion of counting beads (Accu-Check, Molecular Probes, Thermo Fisher Scientific) in the

616 FACS sample according to manufacturer's instructions and calculated using the equation: Total

617 cell number = ((all cell event count / bead event count) x bead conc. (per µl) x (bead volume / cell

618 volume)) x volume of original sample. This formula only works if the whole volume of FACS

619 tube is collected.

620

621 **Cytokines and antibody measurements**

622 Cytokines and chemokines (CXCL10 (IP-10), IFN-γ, IFN-β, IFN-α, IL-10, IL-12p40, IL-

623 15, IL-18, IL-6, and TNF-α) were measured in the plasma by Legendplex according to

624 manufacturer's protocol (Biolegend, San Diego, CA). Plasma samples were collected at the

625 indicated days and were stored in -80°C until analysis. All data were acquired on an LSR II

626 Fortessa flow cytometer using FACSDiva software, version 8 (BD Biosciences, San Jose, CA).

627 The concentration of each analyte is determined with cytometric data based on a standard curve

628 using the LEGENDplex™ data analysis software (Biolegend, San Diego, CA). *P. chabaudi* AJ-

629 specific IgG was evaluated in plasma at indicated days by ELISA, as described previously [5].

630 Plates were coated with *P. chabaudi* AJ parasite lysate overnight at 4°C in PBS with 0.05%

631 Sodium Azide. Plates were blocked with 1% BSA, 0.3% Tween 20, 0.05% Sodium Azide and then

632 sera were incubated. Bound antibody was detected using Alkaline Phosphatase-conjugated goat

633 anti-mouse IgG (Southern Biotech, Birmingham, AL). The reaction was revealed with a p-

634 Nitrophenyl phosphate disodium salt (Sigma, USA) solution (1mg/ml) in diethanolamine buffer.

635 Plates were analyzed with an Omega plate reader (FLUOstar Omega BMG LABTECH Inc,

636 Durham, NC, USA).

637

638 **Statistical analysis**

639   All data were analyzed by ANOVA, followed by Tukey's or two-tailed Mann-Whitney test

640 or two-tailed unpaired Student t test, where indicated. All data are presented as mean ± SEM.

641 Statistical tests were performed using the statistical software package in Prism (version 9.2,

642 GraphPad Software, USA): $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and n.s., not significant.

643

644 **Supporting information**

645

646 **Acknowledgments**

653 **Author Contributions**

654 **Conceptualization:** KG, MGB, MAD, RS

655 **Investigation:** KG, SAI

656 **Formal Analysis:** KG, SAI, RS

657 **Methodology and Resources:** KG, SAI, MLO, MAD, MGB

658 **Funding acquisition:** KG, RS, MGB, MAD

659 **Project Administration, Supervision:** RS, MAD

660 **Writing – Original Draft Preparation:** KG

661   **Writing – Review & Editing:** RS, MGB, MAD

662

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865

866   **Figure Legends**

867

868   **Figure 1. Prior MCMV infection protects against *P. chabaudi* infection.**

869   **(A)** Experimental schematic shows mice infected with MCMV-B5 or -BAC and challenged on

870   day 60 post MCMV infection with *P. chabaudi* AS. **(B)** Parasitemia and **(C)** weight loss were

871   determined after challenge. Data are representative of three independent experiments with 5

872   animals per group. Groups were compared using ANOVA followed by Tukey's post-test with

873   *, $P < 0.05$; **, $P < 0.01$. Mean shown with error bars representing SEM.

874

875   **Figure 2. MCMV-B5 stimulates and maintains parasite-specific effector T cell responses.**

876   **(A)** Experimental schematic shows MSP-1 specific B5 TCR Tg CD4 T cells (CTV⁻Thy1.2⁺) were

877   adoptively transferred into Thy1.1 mice that were then infected with MCMV-BAC or MCMV-B5.

878   Splenic T cells were gated on B5 TCR Tg (CD4⁺CTV⁻Thy1.2⁺) recovered at days 15 and 55 post-

879   MCMV infection. **(B)** Plot, histogram and graphs show gating of proliferated B5 T cells. **(C)** Plots

880   and graphs show B5 Teff (CD4⁺Thy1.2⁺CTV⁻, CD127⁻CD44ʰⁱ) and Tmem (CD4⁺Thy1.2⁺CTV⁻,

881   CD127ʰⁱCD44ʰⁱ) **(D)** on day 15, or **(E)** day 55. Plots and graphs show B5 GC Tfh days 15 and 55

882   p.i.. Plots are concatenated from 3-5 animals per group. Data are representative of two experiments

883   with 3-5 mice in each group. Mean shown with error bars representing SEM and analyzed using

884   One-Way ANOVA followed by Tukey's post-test; *, $P < 0.05$; **, $P < 0.01$.

885

886   **Fig 3. MCMV boosts long-term protection of live malaria vaccine.**

887   **(A)** Experimental schematic shows infection and drug-cure live vaccine combined with MCMV

888   boosting strategy for protection to heterologous rechallenge. Mice were infected with *P. chabaudi*

40

889   AS and treated with two subsequent non-curative doses of chloroquine (CQ). Some groups

890   received MCMV-BAC or MCMV-B5 booster at day 140 (bottom timeline). All mice were

891   challenged with heterologous *P. chabaudi* AJ strain 200 days after live vaccination. Graph

892   showing **(B)** parasitemia of *P. chabaudi* AJ challengeand **(C)** serum level of *P. chabaudi* AJ-

893   specific IgG antibody at day 7 post-challenge. Data are representative of two independent

894   experiments with five mice per group showing mean, with error bars representing SEM and

895   analyzed using ANOVA followed by Tukey's post-test with **, $P < 0.01$; n.s., not significant.

896

897   **Fig 4. MCMV-B5 promotes maintenance of *Plasmodium*-specific effector T cells. (A)**

898   Experimental schematic shows live vaccine combined with MCMV boosting strategy with

899   adoptively transferred $CD4^+$ *Plasmodium* MSP1-specific T cells (B5 TCR Tg). $CD4^+$ $Thy1.2^+$

900   CTV^+ B5 T cells were adoptively transferred into Thy1.1 mice that were then vaccinated. Recipient

901   mice received MCMV-BAC or MCMV–B5 boosters at day 140, with T cell phenotyping at day

902   200 post-vaccination (p.v.). **(B)** Plot, histogram and graph show gating and quantification of

903   parasite-specific CTV^- B5 T cells recovered on d200. **(C)** Plots and graphs showing number of

904   Teff ($CD127^-$) and Tmem ($CD127^{hi}CD44^{hi}$) out of CTV^- B5 TCR Tg cells on day 200. **(D)** Plots

905   and graphs showing percentage and number of B5 memory T cell subsets gated on $CD44^{hi}CD127^{hi}$,

906   Tcm ($CD62L^+CD27^+$), Tem^{Early} (TemE, $CD62L^{lo}CD27^+$) or Tem^{Late} (TemL, $CD62L^{lo}CD27^-$).

907   Cytokines in B5 T cells were quantified and shown as histograms and graphs quantifying **(E)** IFN-

908   γ, **(F)** IL-2- and **(G)** TNF- positive CTV^- B5 T cells. Data are representative of two independent

909   experiments. Plots are concatenated from 5 animals per group, while graphs include individual

910   animals and show mean with error bars representing SEM. Groups were analyzed using One-Way

911   ANOVA followed by Tukey's post-test with *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.

41

912

Fig 5. IFN-γ induced by MCMV leads to increased cytokines and protection from *P. chabaudi* infection.

(A) Experimental schematic shows mice infected with MCMV-BAC and anti-IFN-γ or isotype control was administered at indicated days (34, 36 and 38 p.i.) prior to challenge with *P. chabaudi* at day 40. Graphs show **(B)** percentage of parasitemia, **(C)** weight loss and **(D)** hypothermia upon *P. chabaudi* challenge. **(E)** Plot and graph show fraction of polyclonal effector T cells at day 15 p.c., isotype in white and anti-IFN-γ in black bars. Graphs show plasma IL-12 p40 levels **(F)** throughout MCMV infection and **(G)** after *P. chabaudi* challenge. **(H)** Graphs show chemokines and cytokines on day 7 of *P. chabaudi* challenge after MCMV-BAC and antibody treatment. Graphs include 5 animals per group and show mean with error bars representing SEM. Multiple comparisons Student *t* test and Unpaired or Mann Whitney were used for all except (F) which was analyzed with One-Way ANOVA. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$; n.s., not significant.

925

Fig 6: IFN-γ induced by MCMV does not regulate MCMV driven T cell phenotype, but does induce IL-12 p40.

(A) Experimental schematic shows CTV-labelled B5 TCR Tg T cells were adoptively transfer into Thy1.1 (Balb/c) mice which were then infected with MCMV-B5 and received anti-IFN-γ antibody or isotype at indicated time points and spleen phenotyped for dendritic cells and B5 TCR Tg (Thy1.2$^+$) CD4$^+$ T cell at day 50 p.i.. **(B)** Plot, histogram and graph show gating and quantification of CD4$^+$Thy1.2$^+$CTV$^-$ B5 T cells. **(C)** Plots and summary bar graph show MCMV-B5-specific effector and memory T cell numbers. **(D)** Plot and summary bar graph show the number within each MCMV-B5-specific Tmem subset (Tcm, TemE and TemL) as defined by CD27 and CD62L

935   **(E)** Plot and histogram show DC and CD8α DC population gating and quantification forCD8α⁻

936   DC, CD8α⁺ DC and **(F)** serum IL-12p40 levels. Data shown are representative 5 mice per group

937   and mean shown with error bars representing SEM. The Student *t* test was used; *, $P < 0.05$.

938

939   **Fig 7. IFN-γ induced by MCMV vector drives increase in CD8α⁺ dendritic cells, and boost of**

940   **long-term protection by live malaria vaccine.**

941   **(A)** Experimental schematic shows B5 TCR Tg CD4 T cells labelled with cell trace violet (CTV)

942   were adoptively transferred into Thy1.1 mice that were then vaccinated with live malaria vaccine.

943   Recipient vaccinated mice received MCMV-B5 boosters at day 140 and then received anti-IFN-γ

944   antibody or isotype at indicated days (194, 196, 198) before spleen cell recovery for phenotyping

945   at day 200 p.v. **(B)** Plot, histogram and graph show gating and quantification of B5-epitope-

946   specific T cells (CTV⁻Thy1.2⁺) recovered on day 200. **(C)** Plot and summary bar graph show the

947   number within each vaccine and booster-B5-specific Tmem subset (Tcm, TemE and TemL) as

948   defined by CD27 and CD62L. * indicates significant difference between TemL subsets recovered

949   between groups. **(D)** Plots and graphs show gating and number of total DC population (CD3⁻

950   CD11cʰⁱMHCIIʰⁱ), and **(E)** CD8α⁻ or CD8α⁺ DC. Plots are concatenated from 5 animals per group.

951   **(F)** Graph of serum IL-12p40 level at day 200 p.v. before and after boosting. **(G)** Experimental

952   schematic for infection and drug-cure vaccine combined with MCMV boosting strategy for

953   protection to heterologous rechallenge. Age-matched mice were infected with *P. chabaudi* AS and

954   treated with two subsequent non-curative doses of chloroquine (CQ). Then they received MCMV

955   booster at day 140. Some mice received anti-IFN-γ antibody or isotype at indicated days (194,

956   196, 198) after *P. chabaudi* vaccination. All mice were reinfected with *P. chabaudi* AJ strain at

957   200 days p.v. Graphs showing **(H)** weight loss and **(I)** parasitemia after *P. chabaudi* AJ challenge

43

958   or infection. Comparisons of vaccinated or non-vaccinated groups are significantly different by

959   ANOVA and Tukey's multiple comparisons significance is not shown for the sake of simplicity

960   ($p < 0.01$, for all time points for parasitemia and at day 9 p.c. for weight loss). **(J)** Graphs showing

961   parasitemia on day 5 *P. chabaudi* AJ challenge after IFN-γ neutralization (black bar) compared to

962   isotype (grey bar). **(K)** Graph of fraction of polyclonal effector T cells (CD127⁻) responding to

963   challenge (d15 p.c.). **(L) graph of** serum level of *P. chabaudi* AJ-specific IgG antibody at

964   indicated days (-5, 7 and 15) relative to challenge. Data shown represent 5 animals per group.

965   Mean shown with error bars representing SEM. Groups were analyzed using ANOVA followed

966   by Tukey's post-test for all except (J and K) which were analyzed with Student t test. *, $P < 0.05$;

967   **, $P < 0.01$, ***, $P < 0.001$; n.s., not significant.

968

969   **S1 Fig. *Plasmodium chabaudi* MSP-1 B5 antigen-expressing MCMV vaccine vector**.

970   **(A)** *P. chabaudi* merozoite surface protein MSP-1 (B5:1157–1171) B5 epitope is expressed as an

971   MCMV immediate early gene. B5 epitope was introduced into an MCMV-K181 bacterial artificial

972   chromosome (BAC) using recombineering. **(B)** The indicated organs were collected from MCMV

973   infected mice at different times post- MCMV infection, homogenized, and MCMV titres were

974   determined by plaque assay. The dashed line represents the limit of detection (L.O.D.) for each

975   organ. Mean ± SEM are plotted with three mice for each time point. **(C)** Time course of qPCR

976   assessment of MCMV infection in multiple organs. Mice were infected with MCMV-B5 or

977   MCMV-BAC and organs were collected at indicated times for vector quantification with n=6 per

978   time point. Copy number of CMV/β-actin (±SEM) ratio and L.O.D. of the assay are shown. L.O.D

979   was calculated using data from uninfected animal. (**D**) Mice were infected with MCMV-BAC or

980   MCMV-B5 and splenocytes were recovered 15 days later for MCMV-induced polyclonal T cell

981   phenotype. Plots show gating and graphs show numbers of polyclonal CD4⁺ T cells effector

44

982   (CD127$^-$) and memory (CD44^{hi}CD127^{hi}) phenotype cells recovered after MCMV-B5 compared to

983   MCMV-BAC. **(E)** Enumeration of MCMV-specific m78-Tetramer-positive CD4 T cells upon

984   MCMV-B5 compared to MCMV-BAC from day 15 MCMV-infected mice. Plots represents

985   control CLIP tetramer or m-78-tetramer staining after MCMV-B5. Graph shows number of

986   MCMV-specific m78 Tet+ T cells (±SEM). **(F)** Enumeration of MCMV-specific m45-Tetramer-

987   positive CD8 T cells. Mice were infected with MCMV-B5 and splenocytes were recovered 11 and

988   60 days later for CD8 T cell quantification by staining with m45 tetramers. Plots and graph show

989   m45-Tet$^+$ CD8$^+$ T cells from uninfected and MCMV-B5 infected mice gating on vector specific T

990   cells (+/- SEM). Data shown are representative of two experiments with 3 or 5 mice each. Groups

991   were analyzed using One-Way ANOVA followed by Tukey's post-test with *, $P < 0.05$;

992   **, $P < 0.01$.

993

994   **S2 Fig. Persistent MCMV-B5 infection induces vector-specific T cell response and may**

995   **promote continued B5 TCR Tg T cell proliferation.**

996   **(A)** Experimental schematic shows mice were infected with MCMV-B5, and splenocytes were

997   recovered 11 or 60 days later to quantify MCMV m-78-specific Tetramer$^+$ CD4 T cells. **(B)** Plots

998   and graph show uninfected and m78 tetramer-specific T cell numbers. Virus-specific CD4$^+$ T cells

999   were identified using m78/I-A^d tetramers, with peptide (SQQKMTSLPMSVFYS) and CLIP/I-A^d

1000 as tetramer control (PVSKMRMATPLLMQA). **(C)** Plots and graphs show the number of MCMV-

1001 specific-Teff (CD44$^+$CD127$^-$) and Tmem (CD44^{hi}CD127^{hi}) in the spleen gated on MCMV m78-

1002 tetramer CD4$^+$ T cell at day 11 and 60 p.i.. Data shown are representative of two experiments with

1003 5 mice each and error bars representing SEM. **(D)** Experimental Schematic shows MCMV-BAC

1004 or MCMV-B5 infection of different groups of age-matched Thy1.1 mice at indicated times (0, 30

1005 and 50 days) followed by adoptive transfer of CTV[+] B5 TCR Tg CD4 T cells to all groups 5 days

1006 before phenotyping, and 55 days after first infection. **(E)** Plots and graphs showing gating and

1007 frequency of CTV-B5 T cells out of CD4 T cells, or number of proliferated B5 epitope-specific

1008 CD4 T cells in response to MCMV-BAC or MCMV-B5 infection at different time points. Data are

1009 representative of three independent experiments with 5 animals per group. Mean shown with error

1010 bars representing SEM. Groups were analyzed using One-Way ANOVA followed by Tukey's

1011 post-test with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1012

1013 **S3 Fig. Decay of *P. chabaudi* protection by days 200**

1014 **(A)** Experimental schematic shows age-matched mice were infected with *P. chabaudi* AS for

1015 assessment of protection to heterologous challenge with *P. chabaudi* AJ 60 or 200 days later.

1016 Graphs show **(B)** the percentage of parasitemia (iRBC/RBC) after heterologous challenge, and **(C)**

1017 *P. chabaudi* AJ-specific IgG antibody serum level at day 7 post-challenge. Data are representative

1018 of two independent experiments with five mice per group showing mean, with error bars

1019 representing SEM. Groups were analyzed using One-Way ANOVA followed by Tukey's post-test

1020 with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1021

1022 **S4 Fig: Evaluation of neutralizing effect of anti-IFN-γ antibody**

1023 **(A)** Experimental schematic shows mice were infected or not infected with MCMV-BAC to assess

1024 the neutralizing effect of anti-IFN-γ naïve mice or in MCMV-induced protection to *P. chabaudi*.

1025 Blocking of IFN-γ *in vivo* with anti-IFN-γ antibody or isotype control antibody was performed at

1026 days 34, 36 and 38 prior to the challenge or infection with *P. chabaudi* at day 40 in both MCMV

1027 infected and naïve mice. Graphs show percentage of parasitemia in **(B)** no MCMV mice and **(C)**

46

1028   MCMV vaccinated mice. Relative CXCL-10 mRNA level normalized with β-actin from Raw

1029   264.7 cells cultured for 24h with sera from: (**D**) 2 days after anti-IFN-γ administration, (**E**) day 7

1030   post *P. chabaudi* challenge, (**F**) 2 days after anti-IFN-γ administration in presence of rIFN-γ

1031   (25ng/ml), and (**G**) day 7 post *P. chabaudi* challenge administration in presence of rIFN-γ

1032   (25ng/ml). Data shown represent 5 animals per group and mean shown with error bars representing

1033   SEM. Groups were analyzed using Student *t* test (C, D and F) or One-Way ANOVA (B, E and G)

1034   with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1035

1036   **S5 Fig. Combined IL-12 and IL-18 neutralization did not affect protection induced by**

1037   **MCMV.**

1038   (**A**) Experimental schematic shows mice infected with MCMV-BAC and the combination of anti-

1039   IL-12p40 and anti-IL-18 antibody or isotypes were given post MCMV infection. Graphs show

1040   percentage of parasitemia (**B**), weight loss (**C**), and hypothermia (**D**) upon combined IL-12 and

1041   IL-18 neutralization. Data shown represent 5 animals per group and mean shown with error bars

1042   representing SEM and analyzed using multiple comparisons Student *t* test; n.s., not significant.

1043

1044   **S6 Fig. IFN-γ induced by MCMV vector does not change most antigen-presenting cells types**

1045   **and their activation significantly.**

1046   (**A**) Experimental schematic shows mice infected with MCMV-B5 and received anti-IFN-γ

1047   antibody or isotype at indicated time points. Splenic antigen-presenting cells types (monocytes,

1048   macrophages and inflammatory monocytes) and NK cells were phenotyped at day 50 p.i.. (**B**)

1049   Plots and graphs show gating and quantification of CD3$^-$CD11b$^-$Ly6C$^+$, CD3$^-$ CD11b$^+$Ly6C$^+$,

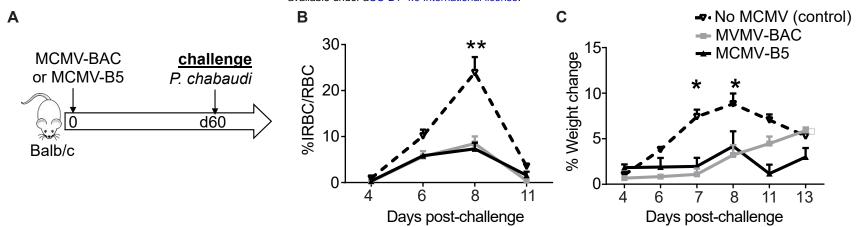1050   CD3$^-$ CD11b$^+$Ly6C$^-$ cells. (C) Histograms and graphs show MFI of CD3- CD11b+Ly6C-

1051   expressing activation marker MHCII. (D) Histograms and graphs show MFI of CD3-

1052   CD11b+Ly6C- expressing activation marker CD86. (**E**) Plots and graphs show gating and

1053   quantification of CD3$^-$ DX5$^+$MHCII$^-$ NK cells.

1054   (**F**) Experimental schematic shows Thy1.1 mice vaccinated with live malaria vaccine. Then

1055   vaccinated mice received MCMV-B5 boosters at day 140 and then received anti-IFN-γ antibody

1056   or isotype at indicated days (194, 196, 198) before spleen cell recovery for cells phenotyping at

1057   day 200 p.v. **(G)** Plots and graphs show gating and quantification of CD3$^-$ CD11b$^-$Ly6C$^+$, CD3$^-$

1058   CD11b$^+$Ly6C$^+$, CD3$^-$ CD11b$^+$Ly6C$^-$ cells. **(H)** Histograms and graphs show MFI of macrophages

1059   (CD3$^-$CD11b$^+$Ly6C$^-$) expressing activation marker MHCII. **(I)** Histograms and graphs show MFI

1060   of inflammatory monocytes (CD3$^-$ CD11b$^+$Ly6C$^+$) expressing activation marker MHCII. (**J**) Plots

1061   and graphs show gating and quantification of CD3$^-$DX5$^+$MHCII$^-$ NK cells. Data shown are

1062   representative 5 mice per group. Plots concatenated per group and mean shown with error bars

1063   representing SEM. The Student *t* test or One-Way ANOVA were used.

1064

1065   **S7 Fig. An example of a gating strategy used to identify polyclonal CD4$^+$ T cells subsets and**

1066   **B5-specific CD4$^+$ T cells subsets**

1067   **(Top row)** Plots show gating strategy for polyclonal CD4$^+$ T cell subsets Teff (CD4$^+$CD127$^-$

1068   CD44^{hi}), Tmem (CD4$^+$CD127^{hi}CD44^{hi}); and memory T cell subsets gated on CD44^{hi}CD127^{hi} as

1069   Tcm (CD62L$^+$CD27$^+$), Tem^{Early} (TemE, CD62L^{lo}CD27$^+$) or Tem^{Late} (TemL, CD62L^{lo}CD27$^-$).

1070   **(Bottom row)** Plots show gating strategy for B5-specific T cells: Teff (CD4$^+$Thy1.2$^+$CTV$^-$,

1071   CD127$^-$CD44^{hi}), Tmem (CD4$^+$Thy1.2$^+$CTV$^-$, CD127^{hi}CD44^{hi}) and B5 memory T cell subsets

1072   gated on CD44^{hi}CD127^{hi}, Tcm (CD62L$^+$CD27$^+$), Tem^{Early} (TemE, CD62L^{lo}CD27$^+$) or Tem^{Late}

1073   (TemL, CD62L^{lo}CD27$^-$).

A

MCMV-BAC
or MCMV-B5

challenge
P. chabaudi

Balb/c

0   d60

B

C

- ▿· No MCMV (control)
- ━ MVMV-BAC
- ━ MCMV-B5

Figure 1

A

MCMV-BAC or MCMV-B5

B5 TCR Tg → CD4 T cells B5 Thy1.2 (CTV⁺) → Thy1.1

| 0 | 15 | 55 days |
| | FACS | FACS |

☐ Uninfected
▨ MCMV-BAC
■ MCMV-B5

B

- - - Uninfected
── MCMV-BAC (grey)
── MCMV-B5 (black)

C

Day 15 p.i

D

Day 55 p.i

E

Figure 2

A

Vaccine
P. chabaudi **AS**

Challenge
P. chabaudi **AJ**

Balb/c

0 5 10 20   140   200 207

↓Parasitemia

↓Serum IgG

Chloroquine

OR

Vaccine
P. chabaudi **AS**

Boost
MCMV-BAC or -B5

Challenge
P. chabaudi **AJ**

Balb/c

0 5 10 20   140   200 207

↓Parasitemia

↓Serum IgG

Chloroquine

B Challenge parasitemia

- △ Vaccine
- ● Vaccine+MCMV-BAC
- ■ Vaccine+MCMV-B5

% IRBC/RBC

**

Days post-challenge

C

□ Vaccine
▨ Vaccine+MCMV-BAC
■ Vaccine+MCMV-B5

P. chabaudi AJ specific IgG (AU)

ns

7

Day post-challenge

Figure 3

Figure 4

A

MCMV-BAC

Challenge
P. chabaudi

Balb/c

0 8   30   34 36 38   40   48   55 days

Protection

αIFN-γ or isotype

FACS

B Parasitemia

C Weight loss

D Temperature

Isotype
αIFN-γ

E Teff

F IL-12p40

G IL-12p40

isotype
αIFN-γ

H

MCMV-BAC

Challenge
P. chabaudi

Balb/c

0 8   30   34 36 38   40   47 days

Cytokines and chemokines

αIFN-γ or isotype

I CXCL9   CXCL-10   IFN-γ   IL-10

isotype
αIFN-γ

Days post challenge

Figure 5

Figure 6

Figure 7