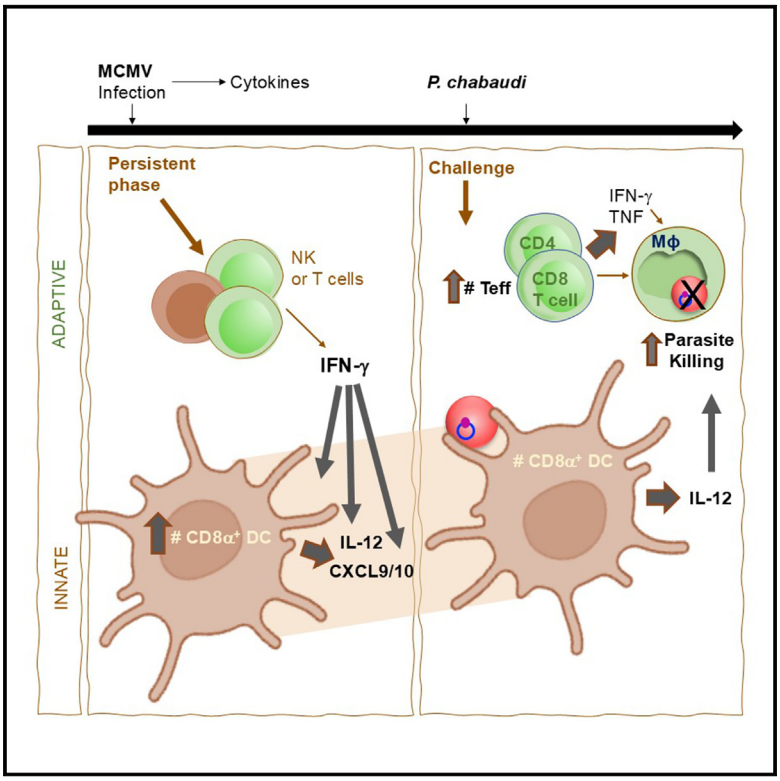
iScience

Article

Heightened innate immune state induced by viral vector leads to enhanced response to challenge and prolongs malaria vaccine protection

Graphical abstract



Highlights

- MCMV-B5 vector maintains specific CD4 T cells and effector memory phenotype
- IFN-γ from persistent MCMV increases CD8α+ DCs, IL-12, and effector T cell response
- Neutralization of IL-12 during challenge limits MCMV-based protection
- MCMV-induced innate immune state improves T cell response to *Plasmodium* challenge

Authors

Komi Gbedande, Samad A. Ibitokou, Mark Joseph D. Endrino, George S. Yap, Michael G. Brown, Robin Stephens

Correspondence

komi.gbedande@rutgers.edu (K.G.), robin.stephens@rutgers.edu (R.S.)

In brief

Natural sciences; Biological sciences; Immunology; Immune response; Microbiology; Viral microbiology

Gbedande et al., 2024, iScience *27*, 111468
December 20, 2024
https://doi.org/10.1016/j.isci.2024.111468

CellPress

iScience

CellPress
OPEN ACCESS

Article

Heightened innate immune state induced by viral vector leads to enhanced response to challenge and prolongs malaria vaccine protection

Komi Gbedande,[1,2,3,7,*] Samad A. Ibitokou,[1,8] Mark Joseph D. Endrino,[2] George S. Yap,[4] Michael G. Brown,[5] and Robin Stephens[1,2,3,6,7,9,*]

[1]Department of Internal Medicine, Division of Infectious Diseases, University of Texas Medical Branch, Galveston, TX 77555-0435, USA
[2]Center for Immunity and Inflammation, Rutgers New Jersey Medical School, Cancer Center, 205 S. Orange Avenue, Newark, NJ 07103, USA
[3]Department of Pharmacology, Physiology and Neuroscience, Rutgers New Jersey Medical School, Cancer Center, 205 S. Orange Avenue, Newark, NJ 07103, USA
[4]Center for Immunity and Inflammation, and Department of Medicine, Rutgers New Jersey Medical School, Cancer Center, 205 S. Orange Avenue, Newark, NJ 07103, USA
[5]Department of Medicine, Division of Nephrology, and the Beirne B. Carter Center for Immunology Research, University of Virginia, Charlottesville, VA, USA
[6]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA
[7]Present address: RS, KG: Center for Immunity and Inflammation, Rutgers New Jersey Medical School, Cancer Center, 205 S. Orange Avenue, G-1230; Newark, NJ 07103, USA
[8]Present address SAI: Fate Therapeutics, 12278 Scripps Summit Drive, San Diego, CA 92131, USA
[9]Lead contact
*Correspondence: komi.gbedande@rutgers.edu (K.G.), robin.stephens@rutgers.edu (R.S.)
https://doi.org/10.1016/j.isci.2024.111468

SUMMARY

Cytomegalovirus is a promising vaccine vector; however, mechanisms promoting CD4 T cell responses to challenge, by CMV as a vector, are unknown. The ability of MCMV to prolong immunity generated by short-lived malaria vaccine was tested. MCMV provided non-specific protection to challenge with *Plasmodium* and increased interleukin-12 (IL-12) and CD8α^+ dendritic cell (DC) numbers through prolonged MCMV-dependent interferon gamma (IFN-γ) production. This late innate response to MCMV increased IL-12 upon challenge and increased the polyclonal CD4 effector T cell response to *Plasmodium*, protecting in an IL-12-dependent manner. Although *Plasmodium*-vaccine-induced protection decayed by d200, MCMV restored protection through IFN-γ. Mechanistically, protection depended on MCMV-induced-IFN-γ increasing CD8α^+ DCs and IL-12p40. MCMV expressing a *Plasmodium* epitope increased parasite-specific CD4 effector and effector memory T cells persisting after malaria vaccination, both phenotypes reported to protect. Overall, enhanced innate cell status, a mechanism of heterologous protection by MCMV, led to a stronger T cell response to challenge.

INTRODUCTION

The development of effective vaccines against human parasitic diseases has been challenging, partially due to the unknown mechanisms required to generate strong effector responses upon challenge.[1,2] Malaria is caused by *Plasmodium* spp. and is one of the most dangerous parasitic diseases in the world, responsible for over 600,000 deaths a year.[3] Overall, approaches tested in humans, including the two most advanced malaria vaccines, RTS,S and R21, show a maximum of 70% efficacy over a year, which is measured as delaying rather than preventing the next infection.[4–6] Importantly, despite impressive progress in malaria vaccine development, including emerging approaches for live and attenuated parasite vaccine strategies, and recombinant protein constructs targeting different stages in the parasite life cycle, none of these vaccine candidates provides long-lived immunity.[4–7] Th1-cytokine-producing CD4 T cells and antibody-producing B cells are required for resolution of the blood stage of *Plasmodium* infection, which leads to the disease.[8–10] Specific CD8$^+$ T cell responses are required to control the liver-stage of malaria infection, which is not studied here. Both natural parasite infection and live malaria vaccine generate immunity to parasitemia on re-infection that wanes quickly, and in mice, this is due to a loss of specific T cells over time.[7,11] Other parasite vaccines similarly do not generate durable immunity, leading to the question of what vaccine vector or adjuvant formulation to use to prolong protection.

Adjuvants can induce trained immunity, a concept for improving innate responses by long-term programming of increased functionality of innate immune cells to improve the immune response to heterologous re-stimulation through epigenetic memory.[12–14] For example, adjuvants targeting Toll-like

receptor 4 (TLR4), including aluminum salts (ASO1, ASO2, ASO4) and glucopyranosyl lipid adjuvant (GLA), enhanced function of myeloid lineage cells through training and have shown promising results in parasite vaccine trials.[14–16] The response to the Bacillus-Calmette Guerin tuberculosis vaccine is a good example of how heterologous infection can induce innate cell expansion and epigenetically modify macrophages, suggesting trained immunity that mediates non-specific protective properties of the vaccine.[13,17] Memory-like innate responses also occur in pregnancy-associated malaria, where immune cells from a cohort of infants with a history of malaria in pregnancy, or during active *Plasmodium falciparum* infection, exhibited increased hyperresponsiveness, secreting more inflammatory cytokines to subsequent TLR stimulation compared to unexposed cells.[18–20] Given that the innate response can exhibit memory-like responses, better understanding of adjuvant mechanisms is a crucial undertaking for parasite vaccine design to uncover how to rapidly induce a strong adaptive response for longer.

Both murine cytomegalovirus (MCMV) and rhesus cytomegalovirus (RhCMV) have been tested as vaccine vectors.[21–25] These studies showed that both species of CMV can promote long-lived CD8 T cell immunity. MCMV vectors expressing specific antigens have been shown to confer protection mediated by CD8 T cells against *L. monocytogenes*, SARS-CoV-2 and influenza,[26,27] as well as humoral protection in influenza.[28] Both MCMV and RhCMV are potent CD8 T-cell-inducing vectors with efficacy against simian immunodeficiency virus (SIV), ebolavirus, tuberculosis, cancer, and liver-stage malaria.[22,29–32] RhCMV-derived vaccine vector (RhCMV/SIVgag) completely protects more than half of all vaccinated rhesus macaques from progressive infection after mucosal challenge with highly pathogenic SIVmac 239 from 1 to 6 years post-initial vaccination.[23,33,34] Additionally, they revealed that CMV can induce non-specific protection against other microbial agents. MCMV infection promotes non-specific protection to *Listeria monocytogenes*, *Yersinia pestis*, and influenza virus, particularly in the latent phase of MCMV infection.[21,35] These studies showed that the innate immune response to MCMV generates Stat1-dependent protection, but detailed mechanism was lacking.

Although CMV has safety issues for immunodeficient patients, viral engineering to improve the virus as a vector is well underway.[36] MCMV and RhCMV infection induce sustained or inflationary CD8 T cell responses with an effector memory phenotype as well as CD4 Th1-type effector function.[37–39] Therefore, we investigated whether MCMV can increase the duration of vaccine-induced immunity to effectively prolong CD4-dependent immune protection to *Plasmodium* infection. We found that interferon gamma (IFN-γ) produced during MCMV infection increased CD8α^+ dendritic cell (DC) numbers and that interleukin-12 (IL-12) promoted protection upon challenge. Mechanistically, at this late time point, MCMV-induced IFN-γ prolonged a heightened innate state including IL-12 and CD8α^+ DCs and increased IL-12 and an enhanced polyclonal T cell response upon challenge. Our findings suggest an interesting paradigm where immunity can be promoted by innate priming due to stimulation by viral vectors, which we found can prolong protection elicited by malaria vaccination and improve the adaptive response to challenge.

RESULTS

MCMV infection elicits immune protection against *Plasmodium chabaudi*

To test MCMV as a potential vector useful for future *Plasmodium* blood-stage vaccine development, we engineered K181 MCMV to express the *P. chabaudi* MSP-1 B5 CD4 T cell epitope (MCMV-B5), enabling evaluation of *Plasmodium*-specific CD4 T cell responses. The B5 epitope was inserted into MCMV intermediate early 1 locus with a linker for processing using bacterial artificial chromosome (BAC) recombineering[40] (Figure S1A). We compared MCMV-B5 with the parental virus to establish its infectivity and immunogenicity. The kinetics and tissue tropism of MCMV-B5 infection were the same as MCMV K181-BAC as shown in the plaque assay (Figure S1B) and also by PCR (Figure S1C). Both viruses were largely cleared from several major organs by day 30, consistent with the establishment of MCMV latency.[41,42] The adaptive immune response was comparable, as polyclonal T cell activation was similar. In order to identify the overall MCMV-driven generation of effector T cells (Teff), CD4 T cells were stained for the IL-7Rα (CD127), which is downregulated on recently activated T cells and re-upregulated in memory T cells (Tmem), which were similar for MCMV-BAC and MCMV-B5 at day 15 post-infection (p.i.; Figure S1D). Viral immunogenicity *in vivo* was confirmed using MCMV-specific MHC tetramers on splenocytes to show specific T cell responses to virus by both CD4 on day 15 p.i. (Figure S1E) and CD8 T cells on day 6 p.i. (Figure S1F).

MCMV has shown early promise as a vaccine vector against liver-stage malaria and other infections where protection relies on CD8 T cells.[22,35] However, the mechanisms of heterologous protective effect of MCMV on infections requiring a Th1-type response are not well defined. Hence, we tested whether MCMV-B5 infection affects *P. chabaudi* immunity (Figure 1A) in mice when active viral replication was no longer detectable (60 dpi). BALB/c mice were used in this study to track *Plasmodium*-specific B5:I-E^d T cell responses and also MCMV induces a more sustained infection and CD4 T cell response in BALB/c than in C57BL/6 mice.[41] Both MCMV-BAC- and MCMV-B5-infected mice had strongly reduced parasite growth (Figure 1B) and reduced weight loss (Figure 1C) upon *P. chabaudi* infection compared to a primary infection in age-matched control mice. Prior MCMV infection thus was sufficient to elicit protective immunity during an initial exposure to *P. chabaudi*.

To identify the basis for this effect, we measured several key cytokines and chemokines present in the serum 2 months post-MCMV infection (Figure 1D). We observed significantly increased levels of CXCL9, IFN-γ, and IL-12p40, whereas tumor necrosis factor (TNF), CCL2, and monocyte chemoattractant protein 1 (MCP-1) were undetectable in MCMV-infected mice. Inflammatory cytokine-producing T cells were also assessed at day 7 post-challenge (p.c.). Prior MCMV infection led to increased proportions and numbers of IFN-γ^+ and TNF$^+$ CD4 and CD8 T cells before *P. chabaudi* challenge (Figure S2) and also during challenge (Figure 1E). These data demonstrate

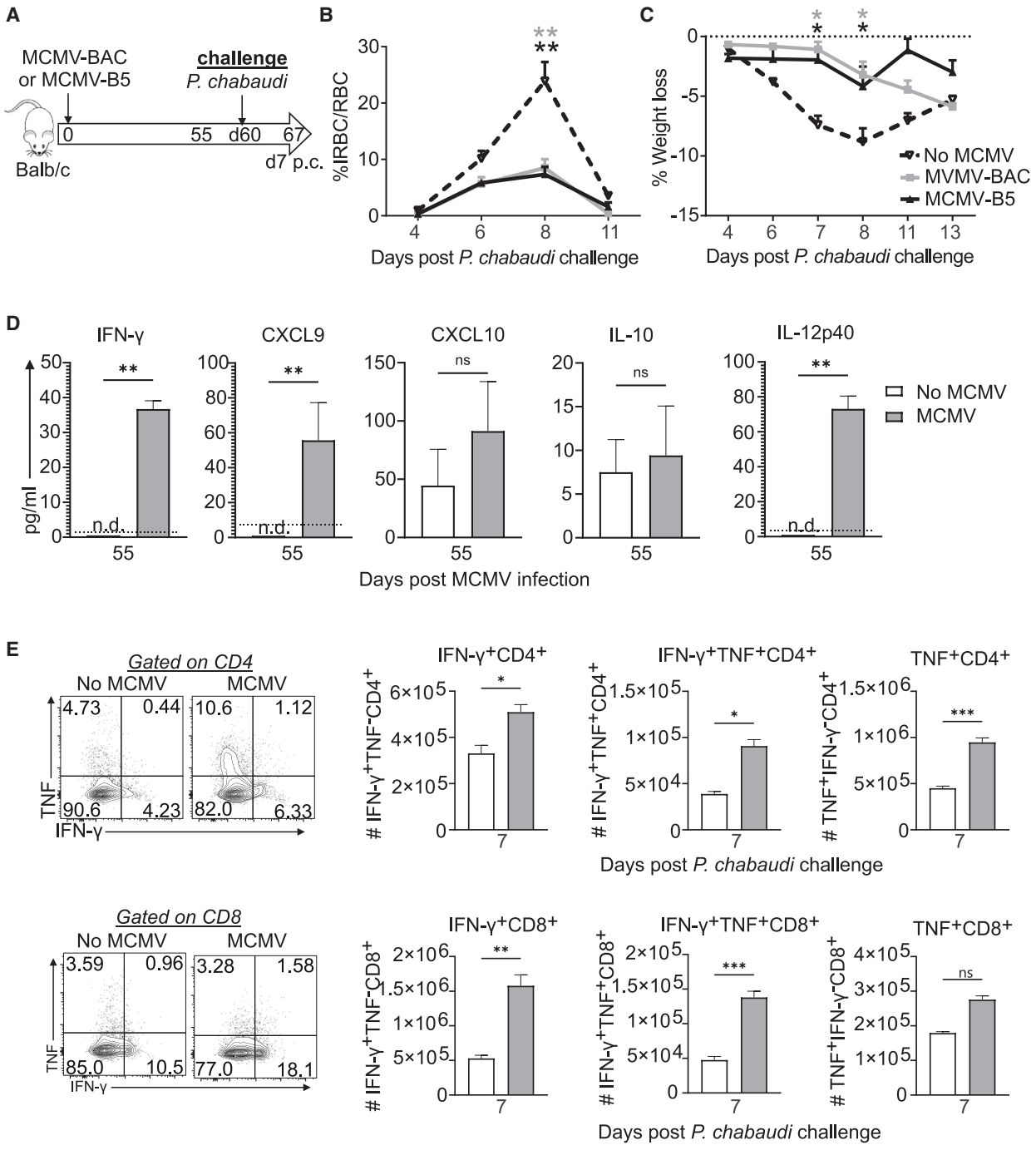


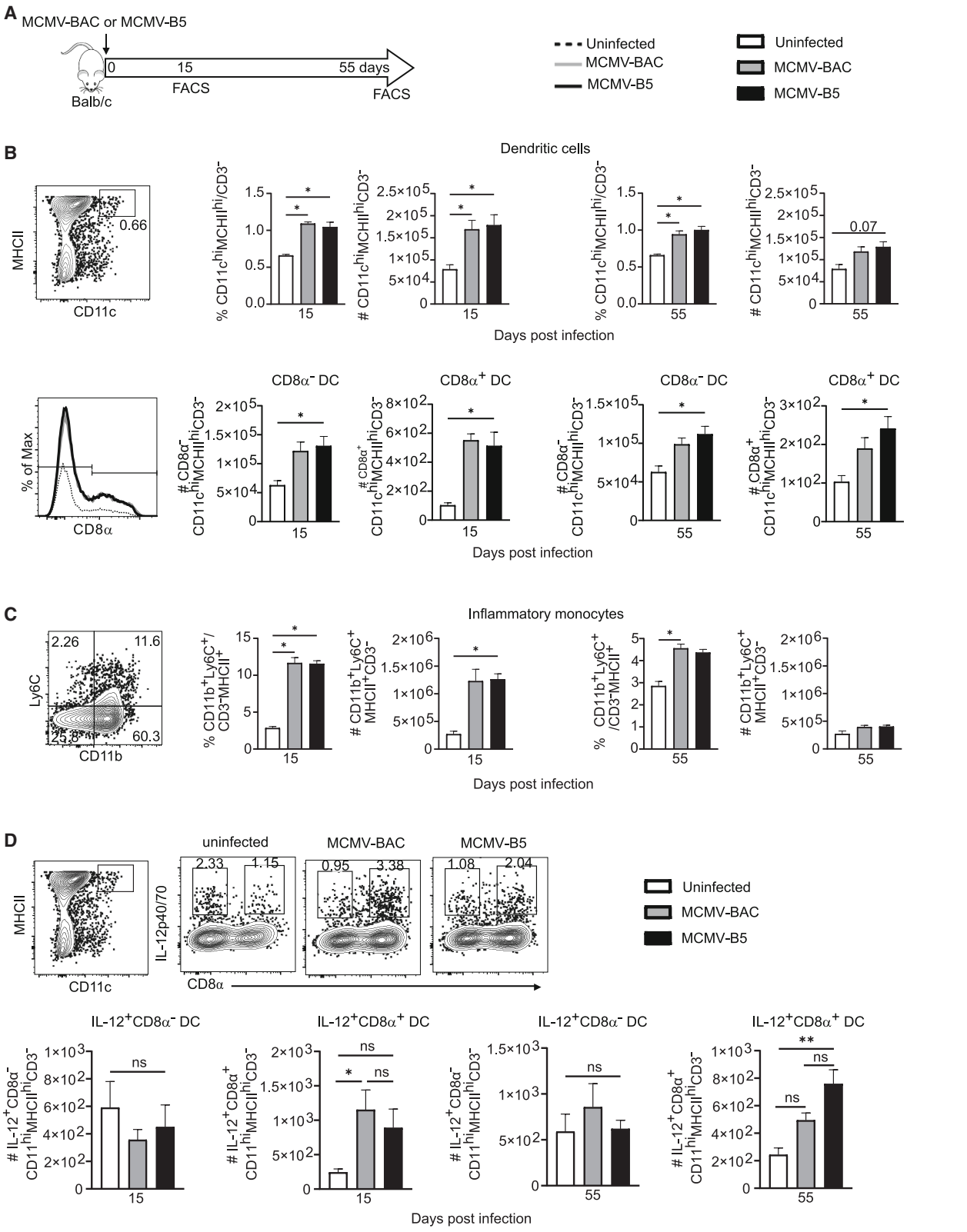
Figure 1. MCMV prime protects against *P. chabaudi* infection

BALB/c mice were infected with MCMV-B5 or -BAC and challenged on day 60 post-MCMV infection with *P. chabaudi* AS, as shown in (A) experimental schematic.

(B) Parasitemia and (C) weight loss after challenge.

(D) Graphs show cytokines and chemokines on day 55 post-MCMV-BAC infection, and dotted line represents the limit of detection (LOD).

(E) Plot and graph show quantification of IFN-γ^+ and TNF$^+$ CD4 (top) and CD8 (bottom) T cells on day 7 post-*P. chabaudi* challenge comparing animals with prior MCMV or not. Data are representative of three independent experiments with five animals per group. Mean shown with error bars representing SEM. Groups were analyzed using ANOVA followed by Tukey's post-test or Mann-Whitney test with $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. See also Figures S1 and S2.



(legend on next page)

that MCMV infection promotes an enhanced Th1 response to challenge infection with *P. chabaudi*.

MCMV enhances DC innate immunity and stimulates *Plasmodium*-specific T cell responses

As both MCMV and MCMV-B5 induced protection to *P. chabaudi*, we tested whether this effect is promoted by an innate response rather than an adaptive response. We evaluated the spleen for changes in innate cell composition during prolonged MCMV infection. BALB/c mice were infected with MCMV-BAC or MCMV-B5, and splenic DCs, macrophages, and inflammatory monocytes were assessed 15 and 55 days post-MCMV infection by flow cytometry (Figure 2A). Phenotypes of splenic innate cells and *Plasmodium*-B5-specific $CD4^+$ T cell subsets were identified with gating strategy as schematically depicted in Figure S3. Both MCMV-BAC and MCMV-B5 promoted an increased proportion and number of DCs on day 15 (Figure 2B). Although this is not reported in acute infection, it is possible that this is a specific effect of latency.[43] The expansion of $CD8\alpha^+$ DC and $CD8\alpha^-$ DC subsets was maintained until day 55 (Figure 2B). The proportion and number of inflammatory monocytes ($CD11b^+Ly6C^+$) were also increased on day 15 in MCMV-infected mice compared to uninfected, with an increased proportion also at day 55 (Figure 2C). Splenic $CD8\alpha^+$ DCs are the major producers of IL-12p40 in response to various infections.[44,45] MCMV-infected DCs also produce IL-12.[46,47] Since an increase of DCs was observed at day 55, we tested if MCMV induced an increase in $IL\text{-}12^+$ DC subsets at day 15 and day 55 post-MCMV infection by intracellular cytokine staining. The number of $CD8\alpha^+$ $IL\text{-}12^+$ DCs also increased in MCMV-infected compared to uninfected mice, whereas $CD8\alpha^-$ $IL\text{-}12^+$ DCs were present but did not increase in infection (Figure 2D). These data suggested that MCMV latent infection established a heightened innate immune state indicated by changed proportions of DCs that may affect adaptive responses.

To assess the quality of the specific T cell response induced to the expressed B5 epitope by measuring their expansion, survival, and phenotype following MCMV exposure, we adoptively transferred B5-specific T cells into congenitally marked BALB/c recipients. The number of donor (Thy1.2+) B5 CD4 T cells that had proliferated (CTV^-) was significantly increased by both MCMV and MCMV-B5 in comparison to uninfected control mice, and this increased response capacity lasted through day 55 (Figure 3B). These data suggest that MCMV infection itself was sufficient to increase CD4 T cell immune responsiveness, although donor-B5-specific CD4 T cell expansion was most pronounced after exposure to MCMV-B5. Phenotypically, the majority of expanded donor-B5-specific T cells on day 15 were effector T cells (Figure 3C, Teff, $CD127^-CD44^+$), whereas by day 55, the fraction of memory T cells ($CD127^{hi}CD44^{hi}$) had increased (Figure 3D). A significant number of B5 Teff were also present at day 55 post-MCMV-infection supporting continuous priming, as suggested by the MCMV-driven increase in DCs. Together these data demonstrate that MCMV-B5 can enhance durable *Plasmodium*-specific CD4 T cells.

MCMV-induced IFN-γ drives protection against *P. chabaudi* infection

In subsequent experiments, we sought to understand the basis for MCMV-induced protection. Given the substantial amount of IFN-γ still present at day 55 of MCMV infection, we first tested the role of MCMV-induced IFN-γ by neutralizing this cytokine after 40 days of MCMV infection, in the period before *P. chabaudi* challenge (Figure 4A). *In vivo* neutralization of MCMV-induced IFN-γ was associated with a substantial loss of protection against *P. chabaudi* challenge (Figure 4B). The anti-IFN-γ-treated mice had significantly higher parasitemia compared to isotype-treated controls previously infected with MCMV. The effect of neutralizing MCMV-induced IFN-γ on challenge was also reflected in increased pathology, as measured by weight loss (Figure 4C) and hypothermia (Figure 4D). Mice receiving neutralizing antibody had a peak level of parasitemia similar to a first infection, suggesting that the protection from parasitemia during MCMV was mostly due to IFN-γ.[9] Evaluation of serum IL-12p40 production throughout MCMV infection and upon *P. chabaudi* challenge showed that IL-12 increased on challenge in an IFN-γ-dependent manner, as shown on days 7, 8, and 15 p.c. (Figure 4E). The chemokines CXCL9 and CXCL10 were also significantly reduced on day 7 p.c. due to IFN-γ neutralization, whereas the level of IL-10 level was increased (Figure 4F). We also measured other cytokines including IL-6, IL-15, IL-18, IFN-α, and IFN-β, which are produced during MCMV infection.[46,47] However, IFN-γ neutralization had no significant impact on their expression (data not shown). To better understand the protective effect of MCMV-induced IFN-γ, we next evaluated its effect on the $CD4^+$ T cell response induced by the *P. chabaudi* challenge. There was a significant reduction in the fraction of $CD4^+$ Teff responding to challenge after pre-treatment with anti-IFN-γ (Figure 4G). These data suggest that MCMV infection promotes protection against *P. chabaudi* in an IFN-γ-dependent manner including increased serum IL-12 and CXCR9/10 and an enhanced CD4 T cell response to challenge infection.

We next investigated the potential caveat that would arise if the effects of neutralizing IFN-γ at this time point on parasitemia were due to persistence of the IFN-γ neutralizing antibody. Anti-IFN-γ was administered to MCMV-infected or uninfected mice, which were then challenged with *P. chabaudi* (Figure S4A). Control animals were given anti-IFN-γ before a first *P. chabaudi* infection to test the effect of a full neutralization of the cytokine on infection. The results clearly show that administration of

Figure 2. MCMV prime induces and maintains myeloid cell numbers

BALB/c mice were infected with MCMV-BAC or MCMV-B5, as shown in (A) experimental schematic. Splenocytes were phenotyped at days 15 and 55 post-MCMV prime. Plots and graphs show percent and number of (B) dendritic cells and subsets ($CD3^-MHCII^{hi}CD11c^{hi}$ and $CD8\alpha^{+/-}$), (C) inflammatory monocytes ($CD3^-MHCII^+CD11b^+Ly6C^+$).

(D) Plots and graphs show gating and quantification of IL-12-secreting $CD8\alpha^{+/-}$ DCs. Data are representative of 3–7 animals per group. Mean shown with error bars representing SEM and analyzed using one-way ANOVA followed by Dunn's multiple comparisons test. ns, not significant; $^*p < 0.05$; $^{**}p < 0.01$. See also Figure S3.

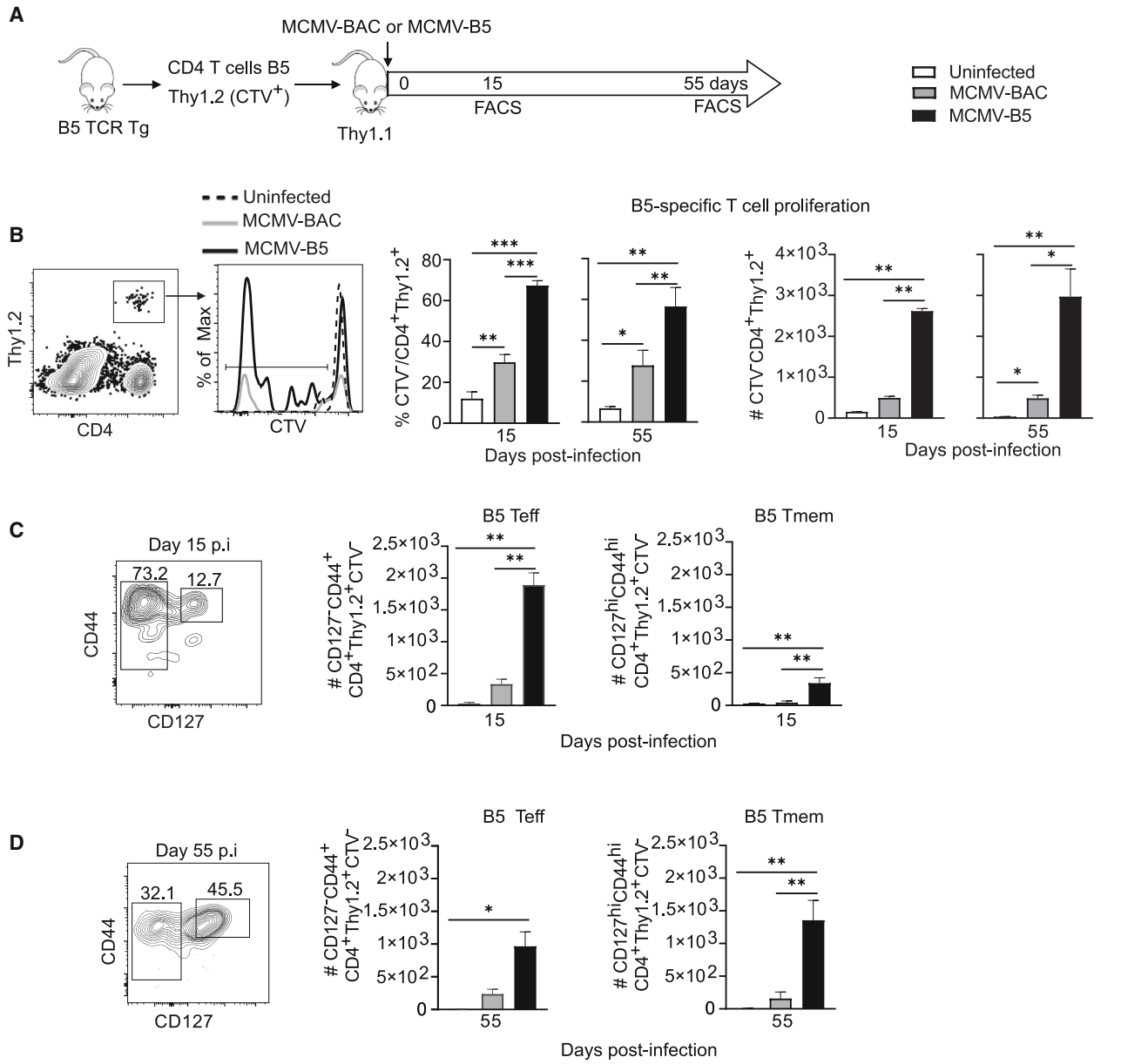


Figure 3. MCMV-B5 can stimulate and maintain epitope-specific T cell responses

MSP-1-specific B5 TCR Tg CD4 T cells ($CTV^+Thy1.2^+$) were adoptively transferred into Thy1.1 mice that were then infected with MCMV-BAC or MCMV-B5, as shown in (A) experimental schematic. Splenic T cells were gated on B5 TCR Tg ($CD4^+CTV^-Thy1.2^+$) recovered at days 15 and 55 post-MCMV infection.

(B) Plot and histogram show gating of divided B5 T cells, and graphs show percent and number of divided B5 T cells.

(C and D) Plots and graphs show B5 Teff ($CD4^+Thy1.2^+CTV^-$, $CD127^-CD44^+$) and Tmem ($CD4^+Thy1.2^+CTV^-$, $CD127^{hi}CD44^{hi}$) (C) on day 15, or (D) day 55. Plots are concatenated, whereas graphs represent 3–5 animals per group. Data are representative of two experiments. Mean shown with error bars representing SEM and analyzed using one-way ANOVA followed by Tukey's post-test; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. See also Figure S3.

neutralizing antibody up to 2 days before infection with *P. chabaudi* only affects parasitemia significantly, and dramatically, as shown earlier in Figure 4B, if MCMV is given before infection, and not in the absence of prior MCMV infection (Figures S4B and S4C), supporting the interpretation that MCMV-driven IFN-γ promotes protection from *P. chabaudi* better than the IFN-γ produced in response to parasite alone. The IFN-γ-secreting cell types at this late time point of MCMV infection were quantified by intracellular cytokine staining on day 60 post-MCMV. There is an increased fraction of IFN-γ^+ from CD4 and CD8 T cells and natural killer (NK) and NKT cells, but not gamma-delta (gd) T cells, in MCMV-infected mice compared to uninfected mice even at this late time point (Figure S4D). The largest fraction of IFN-γ^+ cells are CD8 T cells (Figure S4E). We conclude that the large effect on parasitemia of blocking IFN-γ after MCMV, and before challenge, is mostly due to inhibition

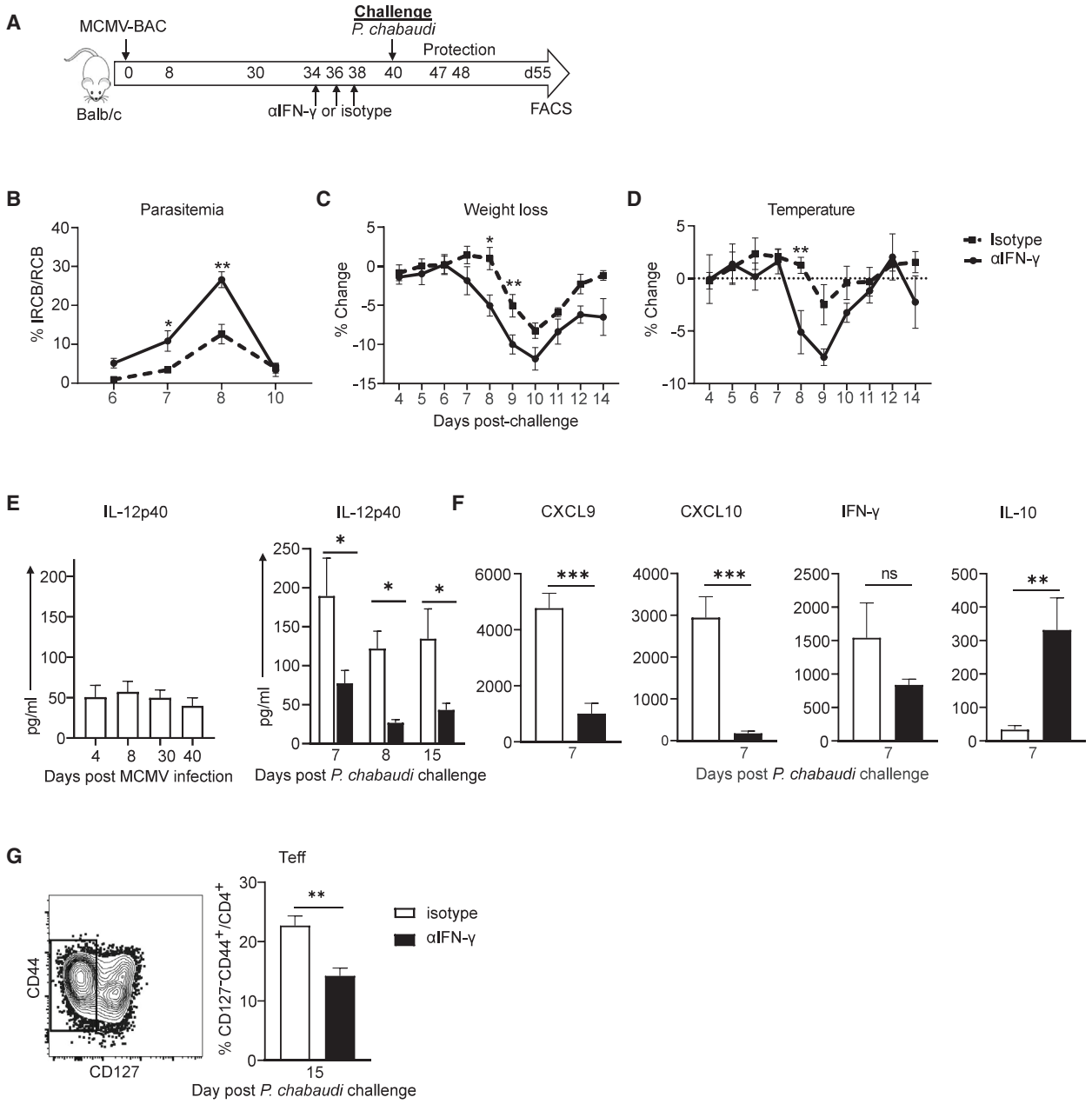


Figure 4. IFN-γ induced by persistent MCMV promotes IL-12 production and protection from *P. chabaudi* infection

BALB/c mice were infected with MCMV-BAC, and anti-IFN-γ or isotype control was administered days 34, 36, and 38 p.i., prior to challenge with *P. chabaudi* at day 40, as shown in (A) experimental schematic. Graphs show (B) percentage of parasitemia, (C) weight loss represented as percent change in weight and (D) hypothermia represented as percent change in body temperature upon *P. chabaudi* challenge. Graphs show (E) plasma IL-12p40 levels throughout MCMV infection and upon challenge (on days 7, 8, and 15 p.c.) and (F) CXCL9, CXCL10, IFN-γ, and IL-10 on day 7 of *P. chabaudi* challenge after MCMV-BAC and antibody treatment; isotype-treated in white and anti-IFN-γ in black bars.

(G) Plot and graph show fraction of polyclonal effector T cells at day 15 p.c. Graphs include 5 animals per group and show mean with error bars representing SEM. Multiple comparisons Student's t test, One-way ANOVA and unpaired or Mann Whitney tests were used. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant. See also Figure S4.

of MCMV-induced, not *P. chabaudi*-induced, IFN-γ, from multiple cellular sources.

As IL-12 and IL-18 can induce IFN-γ from NK cells or previously stimulated T cells, we queried the role of these cytokines in protection from challenge after MCMV. There was no significant effect of blocking both IL-12 and IL-18 together, in the time period before challenge, on protection from *P. chabaudi* by MCMV infection (Figure 5A). As shown earlier, MCMV

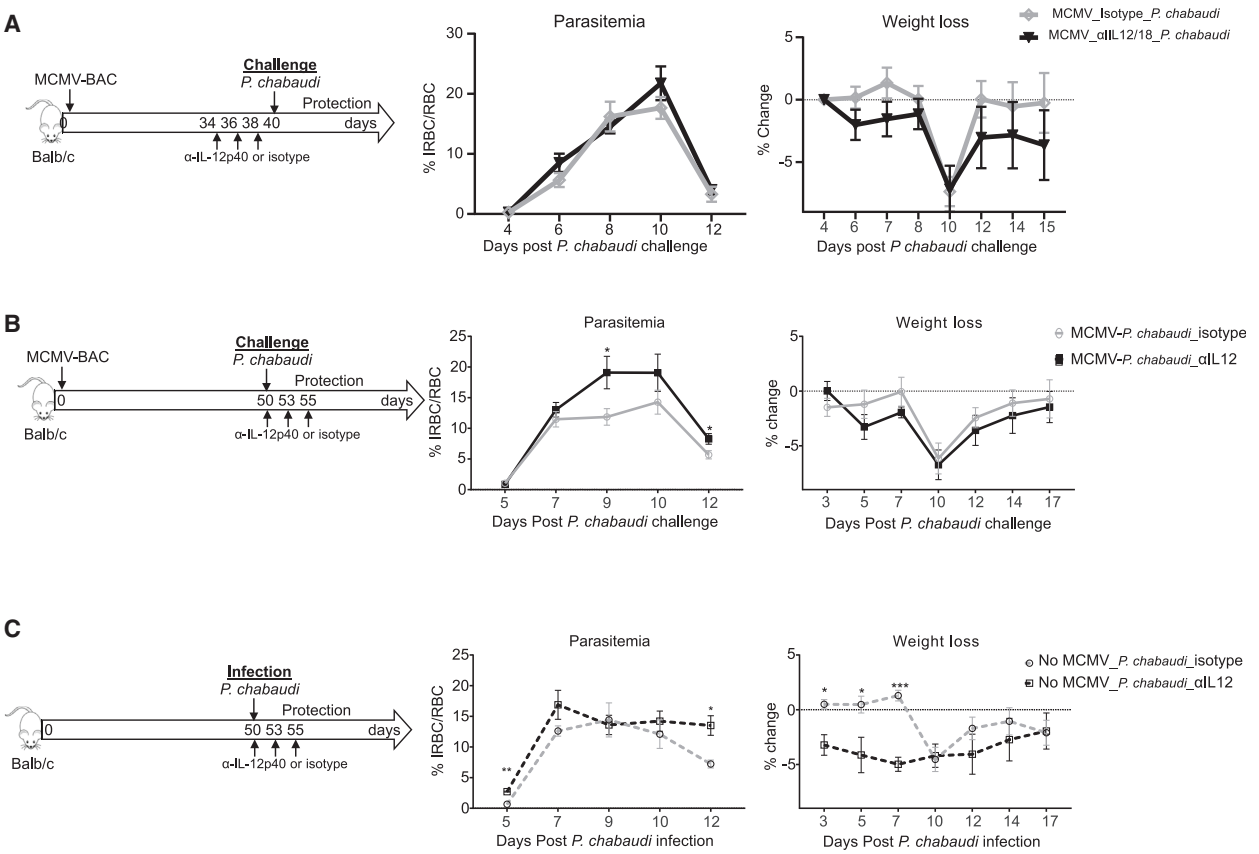


Figure 5. Combined IL-12 and IL-18 neutralization did not affect protection induced by MCMV

BALB/c mice infected (or not) with MCMV-BAC and neutralizing antibody or isotypes were given (A) pre- (d34–38 p.i.) or (B and C) post- (d50–55)-MCMV infection and then infected with *P. chabaudi*, as shown in experimental schematics. Line graphs show percentage of parasitemia and weight loss (A) upon combined neutralization of IL-12p40 and IL-18 before *P. chabaudi* challenge; (B) IL-12p40 neutralization during *P. chabaudi* challenge in MCMV-infected mice; and (C) IL-12p40 neutralization during *P. chabaudi* infection in the absence of MCMV vaccination. Data shown represent 5 animals per group and mean shown with error bars representing SEM and analyzed using multiple t test; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

infection promotes protection from challenge through an IFN-γ-dependent effect before challenge. Since MCMV also leads to more IL-12 production during *P. chabaudi* challenge compared to *P. chabaudi* alone, as shown in previous studies,[48–50] we also evaluated the importance of this increased IL-12 after challenge. Here, IL-12p40 was neutralized in MCMV-infected animals starting on the day of *P. chabaudi* challenge (Figure 5B). *In vivo* neutralization of IL-12 during challenge in MCMV-experienced animals resulted in a significant reduction of control of *P. chabaudi* parasite growth, of a similar magnitude to neutralization of IFN-γ before challenge. As a control, we also tested the effects of neutralizing IL-12 during *P. chabaudi* infection in the absence of MCMV and found a much smaller and earlier effect (Figure 5C). This demonstrates that the MCMV-induced increase in IL-12 during challenge plays an important role in the protective potential of MCMV.

MCMV-induced IFN-γ promotes IL-12 from DCs but does not regulate the number of MCMV-driven T cells

To this point, the data support the interpretation that the increased number of CD8α$^+$ DCs induced by MCMV-induced IFN-γ make IL-12 on challenge, which drives improved Th1 responses and protection. To explore this hypothesis further, adaptive and innate cell phenotypes including T cells, DCs, monocytes, macrophages, and NK cells were quantified after 2 months of infection in the presence or absence of anti-IFN-γ. B5-epitope-specific T cells (CTV-labeled B5 TCR Tg T cells) were adoptively transferred into congenic (Thy1.1) mice that were then infected with MCMV-B5. IFN-γ was neutralized days 44–48 of MCMV infection (Figure 6A). Blocking MCMV-B5-induced IFN-γ did not change the number of B5 T cells (CD4$^+$Thy1.2$^+$CTV$^-$) at day 50 p.i. (Figure 6B). B5 T cells induced by MCMV-B5 include both memory (CD127^{hi}) and effector (CD127$^-$) T cells (Figure 6C), with a fraction of effector T cells that is larger than that seen on day 60 of *P. chabaudi* infection.[10] However, persistent IFN-γ was not the mechanism driving either the B5 CD127$^-$ effector or memory phenotypes (Figure 6D).

A panel of innate cell markers showed that after IFN-γ neutralization on day 50 post-MCMV infection, the number of CD8α$^+$ DCs were reduced, whereas the number of CD8α$^-$ DCs did not (Figure 6E). Consistent with this finding, the level of IL-12p40 in the serum on day 50 post-MCMV infection was

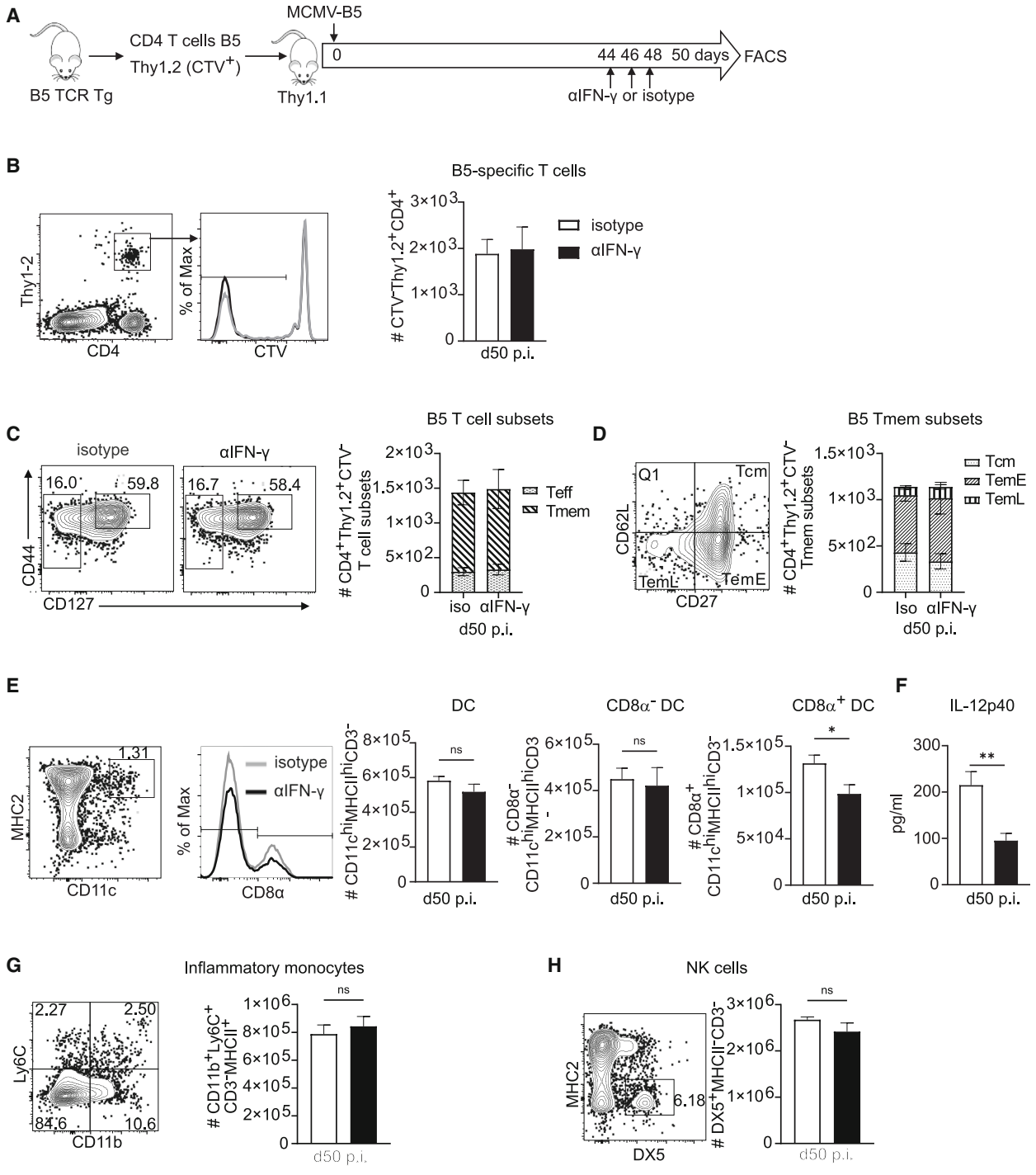


Figure 6. IFN-γ induced by MCMV does not affect T cell phenotype but does increase DC number and IL-12p40

CTV-labeled B5 TCR Tg T cells were adoptively transferred into Thy1.1 (BALB/c) mice that were then infected with MCMV-B5 and received anti-IFN-γ antibody or isotype days 44–48 p.i., as shown in (A) experimental schematic. Splenocytes were phenotyped for (B–D) B5 TCR Tg (Thy1.2+) CD4+ T cells and (E–G) innate cells (dendritic cells, and inflammatory monocytes) and quantification of serum IL-12p40 level 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 bar graph show MCMV-B5-specific effector and memory T cell numbers.

(D) Plot and bar graph show the number within each MCMV-B5-specific Tmem ($CD127^{hi}CD44^{hi}$) subset (Tcm, TemE, and TemL), as defined by CD27 and CD62L.

(E) Plot and histogram show $CD8\alpha^{+/-}$ DC population gating and quantification.

(legend continued on next page)

significantly reduced in the group that received IFN-γ neutralizing antibody compared to isotype (Figure 6F). We did not observe significant changes in other innate cell types including inflammatory monocytes (Figure 6G) or NK cells with IFN-γ neutralization (Figure 6H). Together these data demonstrate that MCMV-induced IFN-γ is indispensable for promoting IL-12 post-parasite challenge, which coincides with higher DC numbers but not CD4 T cell number or phenotype.

MCMV enhances long-term protection of live malaria vaccine through prolonged IFN-γ

Malaria vaccines so far tested in mice or humans lose their protective capacity after only 1 to 2 years.[51,52] In mouse models, this short-lived effect has been correlated with the loss of malaria-specific T cells, not antibodies.[7,11] CMV is an excellent T-cell-inducing vaccine vector, which was previously interpreted to mean that it promotes the Tem phenotype.[22,29–32,38,39] We next tested whether MCMV-induced IFN-γ can prolong immunity, potentially provided by an enhanced T cell response, as suggested by Figure 4G. A live malaria vaccine, which is *Plasmodium* spp. infection followed by drug treatment, is an effective vaccination protocol in C57BL/6 (B6) mice and also in human studies.[11] Although this protocol induces a long-lived B cell response, the protection shows measurable decay by day 200 in mice accompanied by a decay of T cell responsiveness.[11,53] To determine if MCMV after *P. chabaudi* vaccination could prolong protectiveness, we set up to utilize this vaccination protocol in BALB/c. Firstly, challenge parasitemia was tested at day 200 after heterologous challenge of animals first infected with *P. chabaudi* strain AS with strain AJ (diagrammed in Figure S5A). *P. chabaudi* infection induced sterilizing immunity on day 60 p.i., followed by a measurable decay in protection that was evident on day 200 (6.6 months) p.i. in BALB/c (Figure S5B). Secondly, no measurable decay in antibody concentration was observed between 2 and 6.6 months p.i., as shown previously in B6 mice (Figure S5C). To test whether MCMV can supplement the malaria immunity provided by long-lived plasma cells induced by live malaria vaccine, mice were given the live malaria vaccine (*P. chabaudi* AS quickly treated with chloroquine; Figure 7A, top) followed or not by infection with MCMV-BAC or MCMV-B5 140 days later (Figure 7A, bottom). The mice were challenged by re-infection with the heterologous strain, *P. chabaudi* AJ, at day 200 p.v., which was 60 days after MCMV infection. *Plasmodium*-vaccinated mice, with or without MCMV exposure, were challenged with heterologous *Plasmodium* infection. Mice that experienced MCMV infection had a lower parasitemia (p.v., average of 0.06% or 0.05% without or with the B5 epitope, respectively) on day 4 p.c. in comparison to vaccinated mice that had not experienced MCMV infection (1.57% on day 4 p.c. and 0.5% on day 6 p.c.; Figure 7B). Parasite-specific serum antibody to the vaccine and challenge strains was measured by enzyme-linked immunosorbent assay (ELISA) at day 7 p.c. and found to be equivalent in all groups (Figure 7C). We conclude that *Plasmodium* vaccination efficacy, but not the antibody response to challenge, fade with time and that MCMV infection prolonged protection.challenge.

We next tested if MCMV-induced IFN-γ is the factor prolonging protection in this malaria vaccination protocol, as in MCMV infection alone seen earlier. Mice were vaccinated against malaria with *P. chabaudi* infection followed by drug treatment and then infected with MCMV-BAC at day 140. Prior to heterologous challenge with *P. chabaudi* AJ at day 200, MCMV-infected mice received IFN-γ neutralizing antibody, or isotype control, at days 194–198 p.v. (54–58 days post-MCMV booster) (Figure 8A). The control groups either had no live vaccine and no MCMV or only MCMV with no malaria vaccine. Although MCMV alone reduced parasitemia by about half compared to no MCMV, MCMV infection after *P. chabaudi* live vaccination reduced parasitemia by a full order of magnitude (Figure 8B) and also protected against weight loss (Figure 8C). Blocking IFN-γ before challenge significantly reduced the stimulatory effect of the MCMV-BAC on protection at day 200. This was measured as a 4-fold increase in parasitemia in the IFN-γ neutralized animals (0.4%), compared to the isotype group (0.09%), on day 5 p.c. Serum levels of *P. chabaudi* AJ-specific immunoglobulin G (IgG) increased after challenge with that parasite, but there was no significant difference in antibody after neutralization of IFN-γ (Figure 8D). T cell phenotyping on day 15 p.c. showed that blocking IFN-γ before reinfection significantly reduced the fraction of polyclonal T cells with a Teff phenotype generated during *P. chabaudi* challenge, corresponding to less control of parasitemia (Figure 8E). These data demonstrate that MCMV infection after malaria live vaccination can prolong protection to challenge in IFN-γ-dependent manner.

Given the power of MCMV infection to enhance durable protection during malaria challenge by promoting an IFN-γ, we further probed the mechanistic basis by investigating the effect of MCMV on DCs and T cell immunity. MCMV infection generates both $CD62L^{lo}$ antigen-specific CD4 and CD8 effector and effector memory T cells. For some CD8 epitopes, this has been shown to be due to memory inflation in MCMV infection models.[38,39,54–56] To identify parasite-specific CD4 T cell phenotypes present after MCMV stimulation of the live vaccine, $Thy1.2^{+}$ CD4 donor B5 TCR Tg cells (CellTrace Violet [CTV]-labeled) were adoptively transferred into $Thy1.1^{+}$ congenic recipients, which had been immunized with live vaccine and then infected with MCMV-B5 at day 140 p.v. (Figure S6A). *P. chabaudi* live vaccine induces proliferation of B5 T cells, which remain at a low level through day 200 in the absence of MCMV infection. MCMV-B5 specifically and significantly increased the numbers of donor B5 CD4 T cells (CTV^{-}) remaining on day 200 p.v., whereas MCMV-BAC did not (Figure S6B), indicating an effect specific to MCMV expression of the B5 epitope. The phenotypes present due to the MCMV-B5 boost 60 days earlier include both B5 Teff ($CD127^{-}$) and B5 Tmem ($CD127^{hi}$), as well as $CD27^{+}$ and $CD27^{-}$ effector memory T cells ($CD44^{hi}CD127^{hi}CD62L^{lo}$, Tem; Figure S6C). We have previously defined these subsets as Tem^{Early} or Tem^{Late}. Central memory T cells (Tcm) were not

(F) Graph shows serum IL-12p40 levels.

(G) Plots and graphs show gating and quantification of inflammatory monocytes $CD3^{-}MHCII^{+}CD11b^{+}Ly6C^{+}$.

(H) Plots and graph show gating and quantification of $CD3^{-}DX5^{+}MHCII^{-}$ NK cells. Data shown are representative 5 mice per group and mean shown with error bars representing SEM. Student's t test was used; ns, not significant $^{*}p < 0.05$; $^{**}p < 0.01$. See also Figure S3.

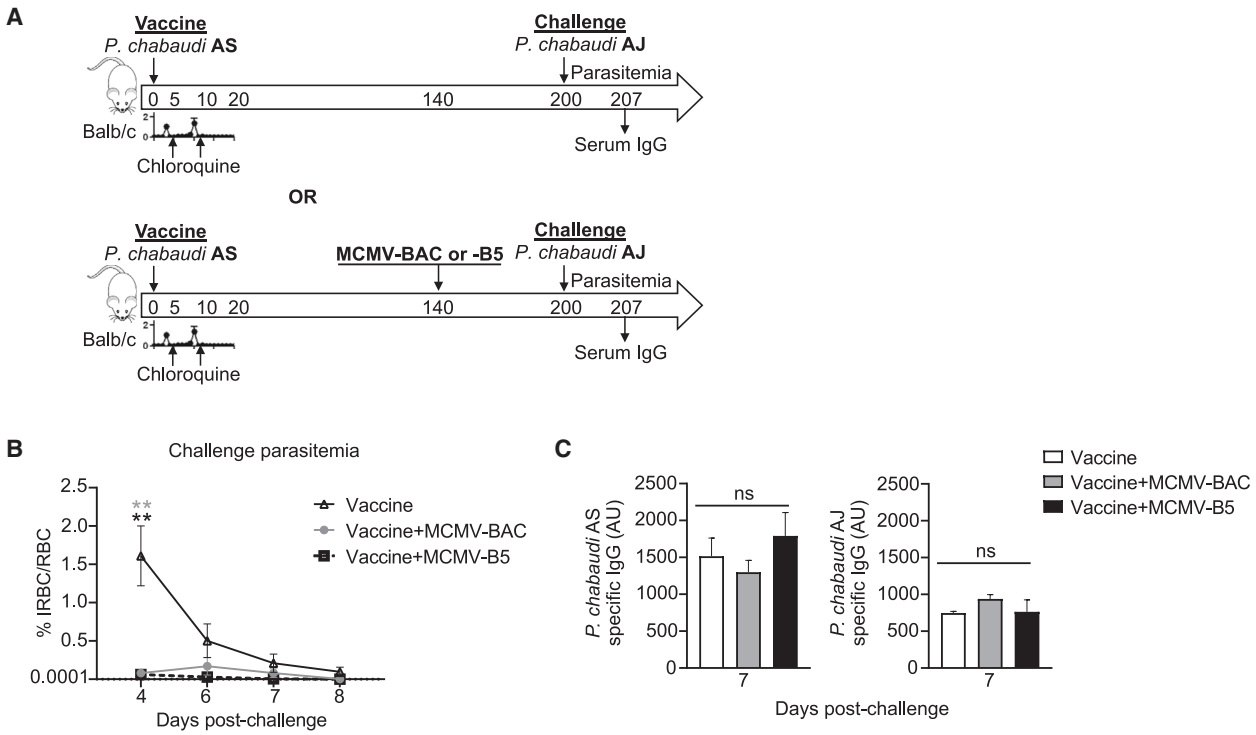


Figure 7. MCMV vector prolongs protection of live malaria vaccine

Infection and drug-cure live vaccine combined with MCMV boosting strategy for protection to heterologous rechallenge. Mice were infected with *P. chabaudi* AS and treated with two subsequent non-curative doses of chloroquine (CQ). Some groups received MCMV-BAC or MCMV-B5 booster at day 140 (bottom timeline). All mice were challenged with heterologous *P. chabaudi* AJ strain 200 days after live vaccination, as shown in (A) experimental schematic. Graph showing (B) parasitemia of *P. chabaudi* AJ challenge and (C) serum level of *P. chabaudi* AS- and *P. chabaudi* AJ-specific IgG antibody at day 7 post-challenge. Data are representative of two independent experiments with five mice per group showing mean, with error bars representing SEM, and analyzed using ANOVA followed by Tukey's post-test with $^{**}p < 0.01$; ns, not significant. See also Figure S5.

increased. Neither IFN-γ nor MCMV-BAC increased B5 T cell numbers (Figures S6D).

To determine what MHCII+ cells present at day 200 of this protocol were dependent on MCMV-induced IFN-γ, we used flow cytometry on splenocytes before the challenge. Vaccinated and MCMV-infected animals received IFN-γ blocking antibody, or isotype control antibody, days 194–198 p.v. (days 54–58 after the MCMV booster) (Figure 8F). Antigen-presenting cell phenotypes were assessed at day 200 after malaria immunization, in the absence of *P. chabaudi* challenge. DCs were still increased by the MCMV boost at day 200, and *in vivo* neutralization of IFN-γ significantly reduced DC numbers compared to isotype control (Figure 8G). This innate effect was seen again most strikingly in the CD8α$^+$ DC population (Figure 8H). Blocking MCMV-B5-induced IFN-γ after vaccination also reduced the serum level of IL-12p40, although this did not reach significance in this experiment (Figure 8I). Numbers of NK cells and inflammatory monocytes were not significantly changed by IFN-γ blockade (Figure 8J). As MCMV-driven IFN-γ affected innate cells and polyclonal effector T cells upon challenge, our data showed that MCMV infection significantly enhanced host defense mechanisms upon malaria challenge via their enduring effect on innate immunity. Collectively, these data suggest an innate pathway for viral vector function through prolonged IFN-γ driving an increase in CD8α$^+$ DCs, which promote both increased protective IL-12 and an increased effector T cell response to challenge.

DISCUSSION

Alternative high-efficacy approaches for malaria vaccination continue to be developed, but little engineering has been focused on increasing the longevity of protection. Here, we show that a basic immune stimulatory mechanism can be applied to extend the protection provided by a typical short-lived malaria vaccine. These data show significant promise to prolong vaccine protection against systemic infections through effects on the innate immune system. In summary, we demonstrated a protective effect dependent on persistent MCMV-induced IFN-γ that enhances IL-12 and CD8α$^+$ DC numbers and promotes a Th1 response upon challenge.

We observed a strong protective effect of MCMV itself on *P. chabaudi* infection. The effect of MCMV alone reduced *P. chabaudi* challenge parasitemia by about one-half, a strong effect. However, it should be noted that specific immunity can reduce challenge parasitemia by orders of magnitude in addition, likely the result of long-lived plasma cells and serum antibody. Both MCMV and gamma herpesvirus 68 (gHV68) also have a protective effect against influenza virus and *Listeria*

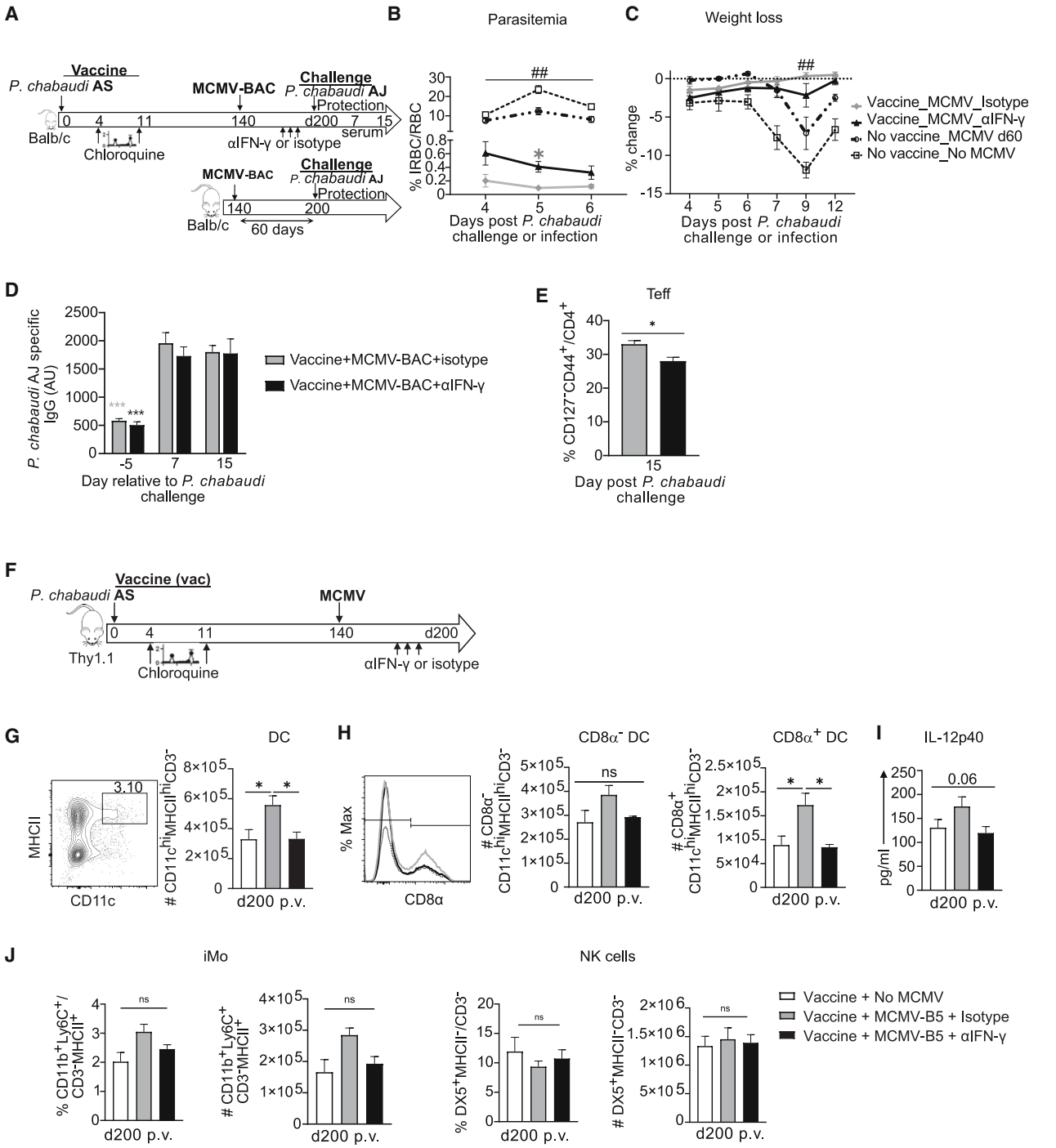


Figure 8. IFN-γ induced by MCMV boosts and drives increase in CD8α$^+$ dendritic cells for long-term protection by live malaria vaccine

Infection and drug-cure live vaccine combined with MCMV boosting strategy for protection to heterologous rechallenge testing effect of neutralizing IFN-γ on innate cells and challenge. BALB/c mice were given infection and drug-cure vaccine combined with MCMV boosting strategy for protection to heterologous rechallenge. Age-matched mice were infected with *P. chabaudi* AS and then treated with two non-curative doses of chloroquine (CQ). Then they received MCMV booster at day 140, and some received anti-IFN-γ antibody or isotype at indicated days (194, 196, 198) after *P. chabaudi* vaccination (p.v.). All mice were re-infected with *P. chabaudi* AJ strain at 200 days p.v., as shown in (A) experimental schematic. Graphs showing (B) parasitemia curves and (C) weight loss after *P. chabaudi* AJ challenge or infection.

(B and C) ## represents significance between groups by ANOVA. Comparisons of parasitemia and weight loss in vaccinated (vaccine_MCMV_isotype and vaccine_MCMV_αIFN-γ) and non-vaccinated groups (No vaccine_MCMV and No vaccine_No MCMV) show a significant difference using ANOVA and Tukey's

(legend continued on next page)

monocytogenes, but not West Nile virus, HSV-1, or Sindbis viruses, indicating some specificity in terms of the viruses that can do this.[21,35] This specificity may be due to viral latency, as gHV68 with a point mutation in ORF73, a gene required for latency, showed reduced protection from *Listeria* and also reduced IFN-γ levels and chronic macrophage activation.[57] MCMV-induced cross-protection against influenza lasted through early and established (5–12 weeks), but not long-standing (9 months), MCMV latency. In terms of mechanism, production of IFN-γ during the latent phase of infection was implicated in MCMV-induced protection from influenza,[21] and STAT1 expression was further implicated in protection against *Listeria* and *Yersinia*.[35] Although we did not specifically test for latency, MCMV-BAC and MCMV-B5 showed similar kinetics and outcomes to previous studies, suggesting viral latency was established by both MCMV strains in our mouse model by day 40.[41,42] Our work extends these findings mechanistically by showing that persistent MCMV-induced IFN-γ increased IL-12 and the number of CD8α$^+$ DCs in the spleen. We also showed that these changes led to differences in the immune response to challenge that are responsible for immunity, namely that there are more CD4 Teff in the response, and a stronger induction of IL-12, which is required for control of the challenge. Both changes during the challenge were linked to the presence of IFN-γ before challenge. Additionally, we applied this insight to a highly significant problem, that of extending protection to malaria.

Neutralization of IFN-γ led to a loss of the extended protection provided by MCMV stimulatory effect and protection from *P. chabaudi* infection. Numbers of Teff after challenge were reduced after neutralization of persistent IFN-γ, which is particularly striking given the reduced protection from parasite growth in anti-IFN-γ pre-treated animals. IFN-γ production has been previously reported in MCMV and HCMV infection during the period coinciding with latency.[21] We show here that several cell types are involved in IFN-γ production. Persistent infection protects in general by generating short-lived effector T cells and promoting their cytokine production with innate cytokines.[10,23,58] This study demonstrates that there are also innate mechanisms, regulated by IFN-γ, that can strongly contribute to the protective effect of persistent stimulation. These mechanisms may be at play in parasitic infections as well. We recently showed that neutralizing IFN-γ also reduces resistance to reinfection with *P. chabaudi* in a phagocyte-dependent manner.[59] Data from our laboratory and others establish that either IFN-γ expression during persistent *P. chabaudi* infection or recombinant IFN-γ also promote protection from heterologous re-infection at day 200 and increase the fraction of polyclonal Tem.[59,60] Here, Tem numbers are B5 epitope specific and not affected by blocking IFN-γ. This may depend on the expression pattern in the two systems; for example, IE1 is reported to be sporadically expressed throughout the latent phase and to drive CD8 T cell memory inflation.[61] Our previous work in *P. chabaudi* infection showed that both memory T cell differentiation from Tcm and prolonged infection contribute to the maintenance of CD4 Tem numbers.[62]

Induction of IL-12 upon *P. chabaudi* challenge was increased by prior MCMV infection and did not occur in the context of prior neutralization of IFN-γ during the persistent phase of MCMV before challenge. The role of the enhanced IL-12 production after challenge in MCMV-infected animals in controlling parasitemia during challenge was confirmed by neutralization. This is consistent with studies showing both that recombinant IL-12 reduces *P. chabaudi* parasitemia and that IL-12-deficient mice have higher parasitemia than wild type (WT).[50,63] Classically, Th1 differentiation depends on the induction of IFN-γ in T cells by IL-12 from DCs. However, in the context of prolonged antigen presentation, such as in a tumor, IL-12 production by DCs can also be reduced by anti-IFN-γ, suggesting a positive feedback loop.[64–67] Neutralization or deficiency of IFN-γ also reduced recruitment of IL-12$^+$ DCs to the cornea upon abrasion.[68] Our data suggest a positive feedback mechanism between IFN-γ, CXCL9/10, and IL-12 to enhance DC function in MCMV infection, although it is not clear if this is due to migration to the spleen or differentiation or proliferation of newly generated DCs. IFN-γ has been shown to promote monocyte differentiation into an IL-12-producing cell type dependent on CCL2 and also to promote DC production of IL-12 in *Toxoplasma* infection,[69] whereas IFN-γ has been implicated in monocyte differentiation in *P. chabaudi*.[70,71] As each infection appears to be distinct, the increased DC population and their increased production of IL-12 may stem from a combination of different factors and mechanisms that need to be further investigated. The possibilities include enhanced differentiation from common myeloid precursors in the bone marrow, or enhanced recruitment of monocytes or DC1 precursors, and maturation within the spleen.

All malaria vaccines tested in human studies thus far, including the most advanced vaccines RTS,S and R21/matrix, show insufficient longevity of protection.[51] Natural immunity to both the parasite and vaccines decays over time, due to a progressive loss of malaria-specific T cells, limiting long-lived protection.[5–7,11] The effector memory T cell population generated in malaria does not re-expand upon rechallenge.[72,73] Memory T cells do produce cytokines in the presence of inflammatory cytokines, due to epigenetic memory.[72,74] To our knowledge, there are no current vaccine formulations that drive long-lived CD4 T cell responses.[2,75] Therefore, a crucial result here is showing

multiple comparisons ($p < 0.01$, for all time points of parasitemia (B) and day 9 p.c. for weight loss (C)). There is also a significant difference ($p < 0.05$) in parasitemia (B) between vaccine_MCMV_isotype and vaccine_MCMV_αIFN-γ at day 5 post-challenge.

(D) Graph of serum level of IgG specific to *P. chabaudi* AJ at indicated days (5, 7, and 15, relative to challenge).

(E) Graph of proportion of polyclonal effector T cells (CD127$^-$) after challenge (d15 p.c.) out of CD4$^+$.

(F–J) As above, mice were vaccinated and boosted with MCMV at day 140 followed by anti-IFN-γ neutralization at indicated days (194, 196, 198), but not challenged, before spleen cell recovery for phenotyping at day 200 p.v., as shown in (F) experimental schematic. Plots and graphs show (G) gating and number of total DC population (CD3$^-$CD11c^{hi}MHCII^{hi}) and (H) CD8α$^-$ or CD8α$^+$ DCs. (I) Graph of serum IL-12p40 level at day 200 p.v. (J) Plots and graphs show gating and quantification of inflammatory monocytes (iMo, CD3$^-$CD11b$^+$Ly6C$^+$) and NK cells (CD3$^-$DX5$^+$MHCII$^-$). Data shown are representative of 5 animals per group. Mean shown with error bars representing SEM. Groups were analyzed using ANOVA followed by Tukey's post-test or with Student's t test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant. See also Figure S6.

the potential of CMV to both enhance innate immunity and promote an appropriate T cell response upon challenge, extending vaccine protection. CMV induces a robust and long-lasting T cell response including both effector and memory components.[38,76] Hansen et al. showed that RhCMV-expressing *Plasmodium knowlesi* antigens generated a parasite-antigen-specific immune response that delayed parasite growth upon sporozoite challenge via generation of CD8 T cell responses.[22] However, whether an MCMV could be used to restimulate pre-existing CD4 T cell specificities through innate immune effects has not been tested previously. Here, we show that the MCMV vector can be used to specifically increase CD4-epitope-specific effector T cell responses, which is one of the primary gaps in our current malaria vaccine arsenal. Both CD4 Teff and Tmem phenotypes are generated in *P. chabaudi* infection, and both can promote protection.[10,62] Here, we observed that MCMV-B5 promoted maintenance of the Tem phenotype beyond day 200. CD4 T cells are crucial for protective immunity to *P. chabaudi*, as they produce cytokines that promote both phagocytosis and B cell help.[8] Our previous findings revealed the power of Teff in protection from persistent *P. chabaudi* with help from B cells. Although *P. chabaudi* MSP-1-specific B5 $CD4^+$ T cells prevented lymphocyte-deficient animals from dying, they were insufficient to control parasitemia in WT mice.[9] Adoptive transfer of B5 effector CD4 T cells with immune B cells, or *in vivo* activation of naive T cells before infection, reduced the peak of *P. chabaudi* parasitemia by half.[62] Although memory T cells are less effective, $CD27^-$ Tem^{Late} do reduce parasitemia and are the most protective memory phenotype. In this study, we showed that the MCMV-B5 vector specifically activated and maintained both adoptively transferred epitope-specific T cells and MCMV-specific $CD4^+$ T cells over 2 months, in addition to enhancing the effector response to challenge. This suggests that a more potent set of CD4 epitopes, which need to be identified for a future malaria vaccine, expressed in a CMV vector could prolong adaptive immunity along with the significant innate effect seen here, possibly in a synergistic fashion. The study suggests that a two-step vaccine strategy that first induces a long-lived protective antibody response and then enhances protection through innate effects on T cells is a reasonable approach.

Several exciting vaccine studies have used attenuated MCMV and RhCMV-derived recombinants to study the effects of various attenuation strategies on vector-induced T cell immunity.[27,37,77,78] These studies address potential safety issues of potentially virulent CMV vectors to be rectified before this strategy could be used in humans. However, superinfection, the ability to re-infect HCMV carriers, and a varied T cell response are two benefits of this approach in addition to the innate and adaptive responses studied here.[77,79] Further understanding of the mechanisms of this model could also lead to new adjuvants to prolong immunity to persistent or recurrent pathogens without introducing another pathogen. In summary, we have identified mechanisms of adjuvanting by MCMV, supporting the promise of CMV-based vaccines and defining a promising line of research for prolonging protection from *Plasmodium* infection. In addition, as MCMV is a chronic viral infection and both *P. chabaudi* and the human malaria pathogen *P. falciparum* can also be both persistent and recurrent, this work informs our basic understanding of the immunology and vaccinology of persistent infections.

Limitations of the study

MCMV infection was used here as a surrogate for the CMV vaccine vectors being engineered for human trials. Using attenuated MCMV-derived recombinant viruses instead of MCMV K181-derived viral recombinants with intact immune evasion mechanisms as this research progresses will enhance the potential for translation. The goal was to study the effects of long-lasting antigen and immunity induced by MCMV. Their induction of prolonged responses including enhanced innate and adaptive functionality and the ability of HCMV to super-infect and to continuously express antigen makes them an exciting platform. While we started the study to define the T cell response to an epitope when testing MCMV-B5 as a vector, the finding that MCMV alone reduced challenge led to the exciting finding that enhanced innate priming by virus could explain improvements in the adaptive response to parasite challenge that has not been previously observed.

RESOURCE AVAILABILITY

Lead contact

All data generated or analyzed during this study are included in this published article and its supplementary information files. Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Robin Stephens (Robin.Stephens@Rutgers.edu) upon request.

Materials availability

This study did not generate any new materials or reagents except MCMV-B5, which is available upon request.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

We would like to thank Mariapia A. Degli-Esposti for scientific input and good advice as well as Monique L. Ong and Peter Fleming in their generation and validation of the MCMV-BAC and B5 viruses. We thank Florentin Aussenac, Victor H. Carpio, Nadia D. Domingo, and Lucinda Puebla-Clark for feedback and discussion. Thanks to Drs. Maria I. Giraldo Giraldo, Gregg Milligan, and Jojo Reyes for technical advice. We thank the NIH Tetramer Core Facility at Emory University, Atlanta, GA for generating the MCMV M78-Tetramer and IE1-Tetramer used in this study. This work was supported by the James W. McLaughlin Fellowship Fund (K.G.) at UTMB, the American Association of Immunologists Careers in Immunology fellowship (S.A.I.), and NIH R01AI135061, R01AI089953 (R.S., S.A.I., K.G.).

AUTHOR CONTRIBUTIONS

Conceptualization: K.G., M.G.B., G.S.Y., and R.S.; investigation: K.G. and S.A.I.; data analysis and interpretation: K.G., S.A.I., R.S., G.S.Y., and M.G.B.; methodology and resources: K.G., M.J.D.E., S.A.I., G.S.Y., and M.G.B.; funding acquisition: K.G. and R.S.; project administration, supervision: R.S. and M.G.B.; writing—original draft preparation: K.G.; writing—review & editing: R.S., M.G.B., G.S.Y., and K.G. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Mice and parasites
  - Construction of MCMV and viral infection
- METHOD DETAILS
  - Immunization
  - Virus quantification
  - Adoptive transfers and *in vivo* studies
  - Flow cytometry
  - Cytokines and antibody measurements
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.111468.

Received: June 13, 2024
Revised: October 1, 2024
Accepted: November 21, 2024
Published: November 26, 2024

REFERENCES

1. Kennedy, R.B., Ovsyannikova, I.G., Palese, P., and Poland, G.A. (2020). Current Challenges in Vaccinology. Front. Immunol. *11*, 1181. https://doi.org/10.3389/fimmu.2020.01181.
2. Pollard, A.J., and Bijker, E.M. (2021). A guide to vaccinology: from basic principles to new developments. Nat. Rev. Immunol. *21*, 83–100. https://doi.org/10.1038/s41577-020-00479-7.
3. WHO (2022). World Malaria Report 2022.
4. Datoo, M.S., Natama, M.H., Somé, A., Traoré, O., Rouamba, T., Bellamy, D., Yameogo, P., Valia, D., Tegneri, M., Ouedraogo, F., et al. (2021). Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. Lancet *397*, 1809–1818. https://doi.org/10.1016/S0140-6736(21)00943-0.
5. RTSS Clinical Trials Partnership (2014). Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med. *11*, e1001685. https://doi.org/10.1371/journal.pmed.1001685.
6. RTSS Clinical Trials Partnership (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet *386*, 31–45. https://doi.org/10.1016/S0140-6736(15)60721-8.
7. Palacpac, N.M.Q., and Horii, T. (2020). Malaria vaccines: facing unknowns. F1000Res. *9*, F1000 Faculty Rev-296. https://doi.org/10.12688/f1000research.22143.1.
8. Meding, S.J., and Langhorne, J. (1991). CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi*. Eur. J. Immunol. *21*, 1433–1438. https://doi.org/10.1002/eji.1830210616.
9. Stephens, R., Albano, F.R., Quin, S., Pascal, B.J., Harrison, V., Stockinger, B., Kioussis, D., Weltzien, H.U., and Langhorne, J. (2005). Malaria-specific transgenic CD4(+) T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. Blood *106*, 1676–1684. https://doi.org/10.1182/blood-2004-10-4047.
10. Stephens, R., and Langhorne, J. (2010). Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria. PLoS Pathog. *6*, e1001208. https://doi.org/10.1371/journal.ppat.1001208.
11. Freitas do Rosário, A.P., Muxel, S.M., Rodriguez-Malaga, S.M., Sardinha, L.R., Zago, C.A., Castillo-Mendez, S.I., Alvarez, J.M., and D'Imperio Lima, M.R. (2008). Gradual decline in malaria-specific memory T cell responses leads to failure to maintain long-term protective immunity to *Plasmodium chabaudi* AS despite persistence of B cell memory and circulating antibody. J. Immunol. *181*, 8344–8355. https://doi.org/10.4049/jimmunol.181.12.8344.
12. Netea, M.G., Domínguez-Andrés, J., Barreiro, L.B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L.A.B., van der Meer, J.W.M., Mhlanga, M.M., Mulder, W.J.M., et al. (2020). Defining trained immunity and its role in health and disease. Nat. Rev. Immunol. *20*, 375–388. https://doi.org/10.1038/s41577-020-0285-6.
13. Saeed, S., Quintin, J., Kerstens, H.H.D., Rao, N.A., Aghajanirefah, A., Matarese, F., Cheng, S.C., Ratter, J., Berentsen, K., van der Ent, M.A., et al. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science *345*, 1251086. https://doi.org/10.1126/science.1251086.
14. de Laval, B., Maurizio, J., Kandalla, P.K., Brisou, G., Simonnet, L., Huber, C., Gimenez, G., Matcovitch-Natan, O., Reinhardt, S., David, E., et al. (2020). C/EBPbeta-Dependent Epigenetic Memory Induces Trained Immunity in Hematopoietic Stem Cells. Cell Stem Cell *26*, 657–674.e8. https://doi.org/10.1016/j.stem.2020.01.017.
15. McKee, A.S., and Marrack, P. (2017). Old and new adjuvants. Curr. Opin. Immunol. *47*, 44–51. https://doi.org/10.1016/j.coi.2017.06.005.
16. Zhu, J., Liu, J., Yan, C., Wang, D., and Pan, W. (2023). Trained immunity: a cutting edge approach for designing novel vaccines against parasitic diseases? Front. Immunol. *14*, 1252554. https://doi.org/10.3389/fimmu.2023.1252554.
17. Kaufmann, E., Sanz, J., Dunn, J.L., Khan, N., Mendonça, L.E., Pacis, A., Tzelepis, F., Pernet, E., Dumaine, A., Grenier, J.C., et al. (2018). BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell *172*, 176–190.e19. https://doi.org/10.1016/j.cell.2017.12.031.
18. Gbédandé, K., Varani, S., Ibitokou, S., Houngbegnon, P., Borgella, S., Nouatin, O., Ezinmegnon, S., Adeothy, A.L., Cottrell, G., Massougbodji, A., et al. (2013). Malaria modifies neonatal and early-life toll-like receptor cytokine responses. Infect. Immun. *81*, 2686–2696. https://doi.org/10.1128/IAI.00237-13.
19. Schrum, J.E., Crabtree, J.N., Dobbs, K.R., Kiritsy, M.C., Reed, G.W., Gazzinelli, R.T., Netea, M.G., Kazura, J.W., Dent, A.E., Fitzgerald, K.A., and Golenbock, D.T. (2018). Cutting Edge: Plasmodium falciparum Induces Trained Innate Immunity. J. Immunol. *200*, 1243–1248. https://doi.org/10.4049/jimmunol.1701010.
20. Feeney, M.E. (2020). The immune response to malaria in utero. Immunol. Rev. *293*, 216–229. https://doi.org/10.1111/imr.12806.
21. Furman, D., Jojic, V., Sharma, S., Shen-Orr, S.S., Angel, C.J.L., Onengut-Gumuscu, S., Kidd, B.A., Maecker, H.T., Concannon, P., Dekker, C.L., et al. (2015). Cytomegalovirus infection enhances the immune response to influenza. Sci. Transl. Med. *7*, 281ra43. https://doi.org/10.1126/scitranslmed.aaa2293.
22. Hansen, S.G., Womack, J., Scholz, I., Renner, A., Edgel, K.A., Xu, G., Ford, J.C., Grey, M., St Laurent, B., Turner, J.M., et al. (2019). Cytomegalovirus vectors expressing Plasmodium knowlesi antigens induce immune responses that delay parasitemia upon sporozoite challenge. PLoS One *14*, e0210252. https://doi.org/10.1371/journal.pone.0210252.
23. Picker, L.J., Lifson, J.D., Gale, M., Jr., Hansen, S.G., and Früh, K. (2023). Programming cytomegalovirus as an HIV vaccine. Trends Immunol. *44*, 287–304. https://doi.org/10.1016/j.it.2023.02.001.

24. Šustić, M., Cokaric Brdovcak, M., Krmpotic, A., and Jonjic, S. (2021). CD8 T Cell Vaccines and a Cytomegalovirus-Based Vector Approach. Life *11*, 1097. https://doi.org/10.3390/life11101097.

25. Ynga-Durand, M.A., Dekhtiarenko, I., and Cicin-Sain, L. (2019). Vaccine Vectors Harnessing the Power of Cytomegaloviruses. Vaccines *7*, 152. https://doi.org/10.3390/vaccines7040152.

26. Kavazović, I., Dimitropoulos, C., Gasparini, D., Roncevic Filipovic, M., Barkovic, I., Koster, J., Lemmermann, N.A., Babic, M., Cekinovic Grbesa, D., and Wensveen, F.M. (2023). Vaccination provides superior in vivo recall capacity of SARS-CoV-2-specific memory CD8 T cells. Cell Rep. *42*, 112395. https://doi.org/10.1016/j.celrep.2023.112395.

27. Trsan, T., Busche, A., Abram, M., Wensveen, F.M., Lemmermann, N.A., Arapovic, M., Babic, M., Tomic, A., Golemac, M., Brinkmann, M.M., et al. (2013). Superior induction and maintenance of protective CD8 T cells in mice infected with mouse cytomegalovirus vector expressing RAE-1gamma. Proc. Natl. Acad. Sci. USA *110*, 16550–16555. https://doi.org/10.1073/pnas.1310215110.

28. Kim, Y., Zheng, X., Eschke, K., Chaudhry, M.Z., Bertoglio, F., Tomić, A., Krmpotić, A., Hoffmann, M., Bar-On, Y., Boehme, J., et al. (2022). MCMV-based vaccine vectors expressing full-length viral proteins provide long-term humoral immune protection upon a single-shot vaccination. Cell. Mol. Immunol. *19*, 234–244. https://doi.org/10.1038/s41423-021-00814-5.

29. Beverley, P.C.L., Ruzsics, Z., Hey, A., Hutchings, C., Boos, S., Bolinger, B., Marchi, E., O'Hara, G., Klenerman, P., Koszinowski, U.H., and Tchilian, E.Z. (2014). A novel murine cytomegalovirus vaccine vector protects against Mycobacterium tuberculosis. J. Immunol. *193*, 2306–2316. https://doi.org/10.4049/jimmunol.1302523.

30. Hansen, S.G., Ford, J.C., Lewis, M.S., Ventura, A.B., Hughes, C.M., Coyne-Johnson, L., Whizin, N., Oswald, K., Shoemaker, R., Swanson, T., et al. (2011). Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature *473*, 523–527. https://doi.org/10.1038/nature10003.

31. Tsuda, Y., Parkins, C.J., Caposio, P., Feldmann, F., Botto, S., Ball, S., Messaoudi, I., Cicin-Sain, L., Feldmann, H., and Jarvis, M.A. (2015). A cytomegalovirus-based vaccine provides long-lasting protection against lethal Ebola virus challenge after a single dose. Vaccine *33*, 2261–2266. https://doi.org/10.1016/j.vaccine.2015.03.029.

32. Wilski, N.A., and Snyder, C.M. (2019). From Vaccine Vector to Oncomodulation: Understanding the Complex Interplay between CMV and Cancer. Vaccines *7*, 62. https://doi.org/10.3390/vaccines7030062.

33. Hansen, S.G., Vieville, C., Whizin, N., Coyne-Johnson, L., Siess, D.C., Drummond, D.D., Legasse, A.W., Axthelm, M.K., Oswald, K., Trubey, C.M., et al. (2009). Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat. Med. *15*, 293–299. https://doi.org/10.1038/nm.1935.

34. Malouli, D., Hansen, S.G., Hancock, M.H., Hughes, C.M., Ford, J.C., Gilbride, R.M., Ventura, A.B., Morrow, D., Randall, K.T., Taher, H., et al. (2021). Cytomegaloviral determinants of CD8(+) T cell programming and RhCMV/SIV vaccine efficacy. Sci. Immunol. *6*, eabg5413. https://doi.org/10.1126/sciimmunol.abg5413.

35. Barton, E.S., White, D.W., Cathelyn, J.S., Brett-McClellan, K.A., Engle, M., Diamond, M.S., Miller, V.L., and Virgin, H.W., 4th (2007). Herpesvirus latency confers symbiotic protection from bacterial infection. Nature *447*, 326–329. https://doi.org/10.1038/nature05762.

36. Marshall, E.E., Malouli, D., Hansen, S.G., Gilbride, R.M., Hughes, C.M., Ventura, A.B., Ainslie, E., Selseth, A.N., Ford, J.C., Burke, D., et al. (2019). Enhancing safety of cytomegalovirus-based vaccine vectors by engaging host intrinsic immunity. Sci. Transl. Med. *11*, eaaw2603. https://doi.org/10.1126/scitranslmed.aaw2603.

37. Snyder, C.M., Cho, K.S., Bonnett, E.L., Allan, J.E., and Hill, A.B. (2011). Sustained CD8+ T cell memory inflation after infection with a single-cycle cytomegalovirus. PLoS Pathog. *7*, e1002295. https://doi.org/10.1371/journal.ppat.1002295.

38. Snyder, C.M., Cho, K.S., Bonnett, E.L., van Dommelen, S., Shellam, G.R., and Hill, A.B. (2008). Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity *29*, 650–659. https://doi.org/10.1016/j.immuni.2008.07.017.

39. Verma, S., Weiskopf, D., Gupta, A., McDonald, B., Peters, B., Sette, A., and Benedict, C.A. (2016). Cytomegalovirus-Specific CD4 T Cells Are Cytolytic and Mediate Vaccine Protection. J. Virol. *90*, 650–658. https://doi.org/10.1128/JVI.02123-15.

40. Corbett, A.J., Coudert, J.D., Forbes, C.A., and Scalzo, A.A. (2011). Functional consequences of natural sequence variation of murine cytomegalovirus m157 for Ly49 receptor specificity and NK cell activation. J. Immunol. *186*, 1713–1722. https://doi.org/10.4049/jimmunol.1003308.

41. Andrews, D.M., Estcourt, M.J., Andoniou, C.E., Wikstrom, M.E., Khong, A., Voigt, V., Fleming, P., Tabarias, H., Hill, G.R., van der Most, R.G., et al. (2010). Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J. Exp. Med. *207*, 1333–1343. https://doi.org/10.1084/jem.20091193.

42. Carroll, V.A., Lundgren, A., Wei, H., Sainz, S., Tung, K.S., and Brown, M.G. (2012). Natural killer cells regulate murine cytomegalovirus-induced sialadenitis and salivary gland disease. J. Virol. *86*, 2132–2142. https://doi.org/10.1128/JVI.06898-11.

43. Wikstrom, M.E., Khong, A., Fleming, P., Kuns, R., Hertzog, P.J., Frazer, I.H., Andoniou, C.E., Hill, G.R., and Degli-Esposti, M.A. (2014). The early monocytic response to cytomegalovirus infection is MyD88 dependent but occurs independently of common inflammatory cytokine signals. Eur. J. Immunol. *44*, 409–419. https://doi.org/10.1002/eji.201243109.

44. Farrand, K.J., Dickgreber, N., Stoitzner, P., Ronchese, F., Petersen, T.R., and Hermans, I.F. (2009). Langerin+ CD8alpha+ dendritic cells are critical for cross-priming and IL-12 production in response to systemic antigens. J. Immunol. *183*, 7732–7742. https://doi.org/10.4049/jimmunol.0902707.

45. Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R.N., and Sher, A. (1997). In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J. Exp. Med. *186*, 1819–1829. https://doi.org/10.1084/jem.186.11.1819.

46. Andoniou, C.E., van Dommelen, S.L.H., Voigt, V., Andrews, D.M., Brizard, G., Asselin-Paturel, C., Delale, T., Stacey, K.J., Trinchieri, G., and Degli-Esposti, M.A. (2005). Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. Nat. Immunol. *6*, 1011–1019. https://doi.org/10.1038/ni1244.

47. Dalod, M., Hamilton, T., Salomon, R., Salazar-Mather, T.P., Henry, S.C., Hamilton, J.D., and Biron, C.A. (2003). Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. J. Exp. Med. *197*, 885–898. https://doi.org/10.1084/jem.20021522.

48. Seixas, E., Cross, C., Quin, S., and Langhorne, J. (2001). Direct activation of dendritic cells by the malaria parasite, *Plasmodium chabaudi chabaudi*. Eur. J. Immunol. *31*, 2970–2978. https://doi.org/10.1002/1521-4141(2001010)31:10<2970::aid-immu2970>3.0.co;2-s.

49. Stevenson, M.M., Tam, M.F., Wolf, S.F., and Sher, A. (1995). IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J. Immunol. *155*, 2545–2556.

50. Su, Z., and Stevenson, M.M. (2002). IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice. J. Immunol. *168*, 1348–1355. https://doi.org/10.4049/jimmunol.168.3.1348.

51. Campo, J.J., Sacarlal, J., Aponte, J.J., Aide, P., Nhabomba, A.J., Dobaño, C., and Alonso, P.L. (2014). Duration of vaccine efficacy against malaria: 5th year of follow-up in children vaccinated with RTS,S/AS02 in Mozambique. Vaccine *32*, 2209–2216. https://doi.org/10.1016/j.vaccine.2014.02.042.

52. Raja, A.I., Cai, Y., Reiman, J.M., Groves, P., Chakravarty, S., McPhun, V., Doolan, D.L., Cockburn, I., Hoffman, S.L., Stanisic, D.I., and Good, M.F. (2016). Chemically Attenuated Blood-Stage Plasmodium yoelii Parasites Induce Long-Lived and Strain-Transcending Protection. Infect. Immun. *84*, 2274–2288. https://doi.org/10.1128/IAI.00157-16.

53. Pombo, D.J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K., Mahakunkijcharoen, Y., Martin, L.B., Wilson, D., et al. (2002). Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. Lancet *360*, 610–617. https://doi.org/10.1016/S0140-6736(02)09784-2.

54. Snyder, C.M., Loewendorf, A., Bonnett, E.L., Croft, M., Benedict, C.A., and Hill, A.B. (2009). CD4+ T cell help has an epitope-dependent impact on CD8+ T cell memory inflation during murine cytomegalovirus infection. J. Immunol. *183*, 3932–3941. https://doi.org/10.4049/jimmunol.0900227.

55. Zangger, N., Oderbolz, J., and Oxenius, A. (2021). CD4 T Cell-Mediated Immune Control of Cytomegalovirus Infection in Murine Salivary Glands. Pathogens *10*, 1531. https://doi.org/10.3390/pathogens10121531.

56. Holtappels, R., Freitag, K., Renzaho, A., Becker, S., Lemmermann, N.A.W., and Reddehase, M.J. (2020). Revisiting CD8 T-cell 'Memory Inflation': New Insights with Implications for Cytomegaloviruses as Vaccine Vectors. Vaccines *8*, 402. https://doi.org/10.3390/vaccines8030402.

57. Xie, W., Bruce, K., Belz, G.T., Farrell, H.E., and Stevenson, P.G. (2024). Indirect CD4(+) T cell protection against mouse gamma-herpesvirus infection via interferon gamma. J. Virol. *98*, e0049324. https://doi.org/10.1128/jvi.00493-24.

58. Yap, G., Pesin, M., and Sher, A. (2000). Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. J. Immunol. *165*, 628–631. https://doi.org/10.4049/jimmunol.165.2.628.

59. Ibitokou, S.A., Gbedande, K., Opata, M.M., Carpio, V.H., Marshall, K.M., and Stephens, R. (2023). Effects of Low-Level Persistent Infection on Maintenance of Immunity by CD4 T Cell Subsets and Th1 Cytokines. Infect. Immun. *91*, e0053122. https://doi.org/10.1128/iai.00531-22.

60. da Silva, H.B., de Salles, E.M., Panatieri, R.H., Boscardin, S.B., Rodríguez-Málaga, S.M., Alvarez, J.M., and D'Império Lima, M.R. (2013). IFN-gamma-induced priming maintains long-term strain-transcending immunity against blood-stage *Plasmodium chabaudi* malaria. J. Immunol. *191*, 5160–5169. https://doi.org/10.4049/jimmunol.1300462.

61. Simon, C.O., Holtappels, R., Tervo, H.M., Böhm, V., Däubner, T., Oehrlein-Karpi, S.A., Kühnapfel, B., Renzaho, A., Strand, D., Podlech, J., et al. (2006). CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. J. Virol. *80*, 10436–10456. https://doi.org/10.1128/JVI.01248-06.

62. Opata, M.M., Ibitokou, S.A., Carpio, V.H., Marshall, K.M., Dillon, B.E., Carl, J.C., Wilson, K.D., Arcari, C.M., and Stephens, R. (2018). Protection by and maintenance of CD4 effector memory and effector T cell subsets in persistent malaria infection. PLoS Pathog. *14*, e1006960. https://doi.org/10.1371/journal.ppat.1006960.

63. Meding, S.J., Cheng, S.C., Simon-Haarhaus, B., and Langhorne, J. (1990). Role of gamma interferon during infection with *Plasmodium chabaudi chabaudi*. Infect. Immun. *58*, 3671–3678. https://doi.org/10.1128/iai.58.11.3671-3678.1990.

64. Alizadeh, D., Wong, R.A., Gholamin, S., Maker, M., Aftabizadeh, M., Yang, X., Pecoraro, J.R., Jeppson, J.D., Wang, D., Aguilar, B., et al. (2021). IFN-gamma Is Critical for CAR T Cell-Mediated Myeloid Activation and Induction of Endogenous Immunity. Cancer Discov. *11*, 2248–2265. https://doi.org/10.1158/2159-8290.CD-20-1661.

65. Garris, C.S., Arlauckas, S.P., Kohler, R.H., Trefny, M.P., Garren, S., Piot, C., Engblom, C., Pfirschke, C., Siwicki, M., Gungabeesoon, J., et al. (2018). Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-gamma and IL-12. Immunity *49*, 1148–1161.e7. https://doi.org/10.1016/j.immuni.2018.09.024.

66. Grohmann, U., Belladonna, M.L., Vacca, C., Bianchi, R., Fallarino, F., Orabona, C., Fioretti, M.C., and Puccetti, P. (2001). Positive regulatory role of IL-12 in macrophages and modulation by IFN-gamma. J. Immunol. *167*, 221–227. https://doi.org/10.4049/jimmunol.167.1.221.

67. Boulch, M., Cazaux, M., Loe-Mie, Y., Thibaut, R., Corre, B., Lemaître, F., Grandjean, C.L., Garcia, Z., and Bousso, P. (2021). A cross-talk between CAR T cell subsets and the tumor microenvironment is essential for sustained cytotoxic activity. Sci. Immunol. *6*, eabd4344. https://doi.org/10.1126/sciimmunol.abd4344.

68. Gao, Y., Li, Z., Hassan, N., Mehta, P., Burns, A.R., Tang, X., and Smith, C.W. (2013). NK cells are necessary for recovery of corneal CD11c+ dendritic cells after epithelial abrasion injury. J. Leukoc. Biol. *94*, 343–351. https://doi.org/10.1189/jlb.1212633.

69. Goldszmid, R.S., Caspar, P., Rivollier, A., White, S., Dzutsev, A., Hieny, S., Kelsall, B., Trinchieri, G., and Sher, A. (2012). NK cell-derived interferon-gamma orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. Immunity *36*, 1047–1059. https://doi.org/10.1016/j.immuni.2012.03.026.

70. Belyaev, N.N., Biró, J., Langhorne, J., and Potocnik, A.J. (2013). Extramedullary myelopoiesis in malaria depends on mobilization of myeloid-restricted progenitors by IFN-gamma induced chemokines. PLoS Pathog. *9*, e1003406. https://doi.org/10.1371/journal.ppat.1003406.

71. Belyaev, N.N., Brown, D.E., Diaz, A.I.G., Rae, A., Jarra, W., Thompson, J., Langhorne, J., and Potocnik, A.J. (2010). Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. Nat. Immunol. *11*, 477–485. https://doi.org/10.1038/ni.1869.

72. Opata, M.M., and Stephens, R. (2017). Chronic *Plasmodium chabaudi* Infection Generates CD4 Memory T Cells with Increased T Cell Receptor Sensitivity but Poor Secondary Expansion and Increased Apoptosis. Infect. Immun. *85*, e00744-16. https://doi.org/10.1128/IAI.00744-16.

73. Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101–105. https://doi.org/10.1038/35065111.

74. Peters, N.C., Pagán, A.J., Lawyer, P.G., Hand, T.W., Henrique Roma, E., Stamper, L.W., Romano, A., and Sacks, D.L. (2014). Chronic parasitic infection maintains high frequencies of short-lived Ly6C+CD4+ effector T cells that are required for protection against re-infection. PLoS Pathog. *10*, e1004538. https://doi.org/10.1371/journal.ppat.1004538.

75. Draper, S.J., Sack, B.K., King, C.R., Nielsen, C.M., Rayner, J.C., Higgins, M.K., Long, C.A., and Seder, R.A. (2018). Malaria Vaccines: Recent Advances and New Horizons. Cell Host Microbe *24*, 43–56. https://doi.org/10.1016/j.chom.2018.06.008.

76. Cicin-Sain, L., Sylwester, A.W., Hagen, S.I., Siess, D.C., Currier, N., Legasse, A.W., Fischer, M.B., Koudelka, C.W., Axthelm, M.K., Nikolich-Zugich, J., and Picker, L.J. (2011). Cytomegalovirus-specific T cell immunity is maintained in immunosenescent rhesus macaques. J. Immunol. *187*, 1722–1732. https://doi.org/10.4049/jimmunol.1100560.

77. Caposio, P., van den Worm, S., Crawford, L., Perez, W., Kreklywich, C., Gilbride, R.M., Hughes, C.M., Ventura, A.B., Ratts, R., Marshall, E.E., et al. (2019). Characterization of a live-attenuated HCMV-based vaccine platform. Sci. Rep. *9*, 19236. https://doi.org/10.1038/s41598-019-55508-w.

78. Krauter, S., Büscher, N., Brauchle, E., Ortega Iannazzo, S., Penner, I., Kramer, N., Gogesch, P., Thomas, S., Kreutz, M., Dejung, M., et al. (2022). An Attenuated Strain of Human Cytomegalovirus for the Establishment of a Subviral Particle Vaccine. Vaccines *10*, 1326. https://doi.org/10.3390/vaccines10081326.

79. Liu, J., Jaijyan, D.K., Tang, Q., and Zhu, H. (2019). Promising Cytomegalovirus-Based Vaccine Vector Induces Robust CD8(+) T-Cell Response. Int. J. Mol. Sci. *20*, 4457. https://doi.org/10.3390/ijms20184457.

80. Landau, I., and Killick-Kendrick, R. (1966). Rodent plasmodia of the Republique Centrafricaine: the sporogony and tissue stages of *Plasmodium chabaudi* and *P. berghei yoelii*. Trans. R. Soc. Trop. Med. Hyg. *60*, 633–649. https://doi.org/10.1016/0035-9203(66)90010-1.

81. Li, C., Corraliza, I., and Langhorne, J. (1999). A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice. Infect. Immun. *67*, 4435–4442. https://doi.org/10.1128/IAI.67.9.4435-4442.1999.

82. Wheat, R.L., Clark, P.Y., and Brown, M.G. (2003). Quantitative measurement of infectious murine cytomegalovirus genomes in real-time PCR. J. Virol. Methods *112*, 107–113. https://doi.org/10.1016/s0166-0934(03)00197-6.

83. Redwood, A.J., Messerle, M., Harvey, N.L., Hardy, C.M., Koszinowski, U.H., Lawson, M.A., and Shellam, G.R. (2005). Use of a murine cytomegalovirus K181-derived bacterial artificial chromosome as a vaccine vector for immunocontraception. J. Virol. *79*, 2998–3008. https://doi.org/10.1128/JVI.79.5.2998-3008.2005.

84. Achtman, A.H., Stephens, R., Cadman, E.T., Harrison, V., and Langhorne, J. (2007). Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi chabaudi*. Parasite Immunol. *29*, 435–444. https://doi.org/10.1111/j.1365-3024.2007.00960.x.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD127 (A7R34) anti-mouse	Biolegend	Cat#135016; RRID: AB_1937261
Brilliant Violet 785™ anti-mouse/human CD44	BioLegend	Cat No#103059, RRID: AB_2571953
anti-mouse/human CD44	BioLegend	Cat#103057; RRID: AB_2564214
anti-CD62L (MEL-14) anti-mouse	BD Biosciences	Cat#563252; RRID: AB_2738098
CD4 (RM4-5) anti-mouse	BD Biosciences	Cat#612844; Gene ID: 12504
Rat Anti-Mouse CD90.2-AF647 (30-H12)	Southern Biotech	Cat#1750-31; RRID: AB_2795242
CD90.2 (Thy-1.2) (30-H12), FITC	eBioscience	Cat# 11-0903-82; RRID: AB_2735033
PE/Cyanine7 anti-mouse CD90.2 (Thy1.2) (30-H12)	BioLegend	Cat#105326; RRID: AB_2201290
CD90.2 (Thy-1.2) (30-H12), PerCP-eFluor 710	eBioscience	Cat#46-0903-82; RRID:AB_10670882
anti-CD44 [IM7] anti-mouse, APC-eFluor 780	eBioscience	Cat#47-0441-82; RRID: AB_1272244
anti-CD27 [LG-7F9], PE/Cyanine7	BioLegend	Cat# 124216; RRID: AB_10639726
Ly6C (HK1.4) anti-mouse FITC	Biolegend	Cat#128006; RRID: AB_1186135
Ly6G (1A8) anti-mouse PE	Biolegend	Cat# 127607; RRID: AB_1186104
anti CD11b (M1/70) anti-mouse PE-Cy5	ebioscience	Cat#15011283; RRID: AB_468714
PE/Cyanine7 anti-mouse CD11c (N418)	BioLegend	Cat#117318; RRID: AB_493568
anti-CD11c (N418) anti-mouse PE	Bio Legend	Cat#117307; RRID: AB_313776
BV421 Rat Anti-Mouse CD49b (DX5)	BD Biosciences	Cat#563063; RRID: AB_2737983
MHCII/I-A/Eb (M5/114.15.2), APC	eBioscience	Cat#17-5321-82, RRID: AB_469455
anti-IFN-γ (XMG1.2) anti-mouse FITC	eBioscience	Cat# 11-7311-41; RRID: AB_10718840
anti-IFN-γ (XMG1.2) anti-mouse PerCp-Cy5.5	Invitrogen	Cat#45731180; RRID: AB_1107020
PE/Cyanine7 anti-mouse TNF-α (MP6-XT22)	Biolegend	Cat#506324; RRID: AB_2256076
PE Rat Anti-Mouse IL-12 p40/p70 (C15.6)	BD Biosciences	Cat#554479; RRID: AB_395420
H-2L(d) MCMV IE1 168–176 YPHFMPTNL	NIH Tetramer Core Facility	Pubmed ID: 29867968
M78/I-A^d tetramer (SQQKMTSLPMSVFYS)	NIH Tetramer Core Facility	GenBank ID: CCE57242.1
CLIP/I-A^d as tetramer control (PVSKMRMATPLLMQA)	NIH Tetramer Core Facility	GenBank ID: NP_001020329.1
*InVivo*MAb anti-mouse IFN-γ	BioXCell	Cat#BE0312; RRID: AB_2736992
*InVivo*MAb polyclonal Armenian hamster IgG	BioXCell	Cat#BE0091; RRID: AB_1107773
*InVivo*MAb anti-mouse IL-12 p40	BioXCell	Cat#BE0051; RRID: AB_1107698
*InVivo*MAb rat IgG2a isotype control, anti-trinitrophenol	BioXCell	Cat#BE0089; RRID: AB_1107769
*InVivo*MAb anti-mouse IL-18	BioXCell	Cat#BE0237; RRID: AB_2687719
Bacterial and virus strains		
MCMV-K181-B5 (MHC II-E^d-restricted B5 epitope (ISVLKSRLLKRKKYI)	This paper	N/A
MCMV-K181-Perth	Corbett et al., 2011[40]	N/A
Chemicals, peptides, and recombinant proteins		
Phorbol Myristate Acetate (PMA)	Sigma	Cat #P1585
Ionomycin	Sigma	Cat No. I0634
CellTrace™ Violet Cell Proliferation Kit	Invitrogen	Cat #C34571
Chloroquine diphosphate salt	Sigma	Cat #C6628
BD GolgiPlug (Containing Brefeldin A)	BD Bioscience	Cat #BDB555029
Ricca Chemical Giemsa stain	Fisher Scientific	Cat #3250-4
Cell Stimulation Cocktail (500X)	eBioscience	Cat #00-4970-93
Custom cytokines/chemokines Legendplex	Biolegend	Cat #900001181
Fugene 6 Transfection Reagent	Promega	Cat#E2691

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lipopolysaccharides (LPS)	Sigma	Cat#L4391
TAPI2	Biosynth	Cat #INH-3852-PI
Experimental models: Cell lines		
3T3 cells	NIH	N/A
Experimental models: Organisms/strains		
Plasmodium chabaudi chabaudi AS	Laudau et al., 1966[80]; Li et al., 1999[81] Jean Langhorne, Crick Institute	N/A
P. chabaudi AJ (BEI/MR4, MRA-740)	Laudau et al., 1966[80]; Li et al., 1999[81]	N/A
Oligonucleotides (See Table S1)		
IE1-Galk Fwd: AGATCCAAGTCTTC TGAGAATCAACAGCAGCCCAAGA AGAAGAGCAAGAAGCCTGTTGAC AATTAATCATCGGCA.		N/A
IE1-Galk Rev: TAGTAAAAAAACATTG TCCATGGTGATGGGTGGGGAGCTG GGCTTGTGGATATCAGCACTGTCCT GCTCCTT		N/A
IE1 Exon 4 Fwd: AGCTGGAAGGATCACAGC		N/A
IE1 Exon 4 Rev: GTGTCTAAGTGGGCAGGTG		N/A
B5(*leader*): (*GCGAACCAGAAAGTGAAAGAA*) ATTAGCGTGCTGAAAAGCCGCCTGCTGA AACGCAAAAAATATATT		N/A
M06 Fwd: TTGGAGCGATACGTTGACAATG	Corbett et al., 2011[40]	N/A
6530 Rev: TCCGTTAGCCTTAGATTCCACCG	Corbett et al., 2011[40]	N/A
6147 Fwd: GCCAGCTCCTACTCGTCATC		N/A
6631 Rev: TATCGTTCTTGACGCCCTTT		N/A
IE1 MG Fwd: AGATCCAAGTCTTCTGAGAA		N/A
IE1 MG Rev: TAGTAAAAAAACATTGTCCA		N/A
MSP-1; 1157–1171, ISVLKSRLLKRKKYI/I-E^d	Stephens and Langhorne, 2010[10]	N/A
Vα2 Fwd: GAACGTTCCAGATTCCATGG	Stephens and Langhorne, 2010[10]	N/A
Vα2 Rev: ATGGACAAGATCCTGACAGCATCG	Stephens and Langhorne, 2010[10]	N/A
vβ8.1 Fwd: CAGAGACCCTCAGGCGGCTGCTCAGG	Stephens and Langhorne, 2010[10]	N/A
vβ8.1 Rev: ATGGGCTCCAGGCTGTTCTTTGTGGTTTTGATTC	Stephens and Langhorne, 2010[10]	N/A
MCMV (IE1) Fwd: TCA GCC ATC AAC TCT GCT ACC AAC	Wheat et al., 2003[82]	N/A
MCMV (IE1) Rev: GTG CTA GAT TGT ATC TGG TGC TCC TC	Wheat et al., 2003[82]	N/A
β-actin Fwd: GCT GTA TTC CCC TCC ATC GTG	Wheat et al., 2003[82]	N/A
β-actin Rev: CAC GGT TGG CCT TAG GGT TCA	Wheat et al., 2003[82]	N/A
Software and algorithms		
FlowJo software version 10 package	FlowJo LLC	https://cloud.flowjo.com/
Qognit LEGENDplex software	Biolegend	https://legendplex.qognit.com/user/login?next=workflow.workflow
FACSDiva software	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software
GraphPad Prism (version 9 and version 10) Software	GraphPad	https://www.graphpad.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice and parasites

All mice used in our study were maintained in the University of Texas Medical Branch (UTMB) or Rutgers New Jersey Medical School (NJMS) animal facility under specific pathogen-free conditions with access to food and water *ad libitum*. Recipient Thy1.1 BALB/cByJ were backcrossed to BALB/cJ (N4; The Jackson Laboratory, Bar Harbor, ME). B5 T cell receptor transgenic (TCR Tg), which recognizes a peptide from merozoite surface protein-1 (MSP-1; 1157–1171, ISVLKSRLLKRKKYI/I-E^d) was backcrossed to BALB/cJ (N10). B5 TCR Tg mice, and *P. chabaudi chabaudi* AS stocks, were a kind gift from J. Langhorne (Francis Crick Institute, London, UK). *P. chabaudi* AJ (MRA-740, a cloned line that is highly related but heterologous to AS[80,81] was purchased from BEI Resources, formerly MR4. B5 TCR Tg mice were typed using primers Vα2, 5′-gaacgttccagattccatgg-3′ and 5′-atggacaagatcctga cagcatcg-3′, and vβ8.1, 5′ cagagaccctcaggcggctgctcagg-3′ and 5′-atgggctccaggctgttctttgtggttttgattc-3′.[10] Both male and female recipients were used, however no sex differences in infection were noted. All adoptive transfer experiments were done using age- and sex-matched recipient and donors. All experiments were carried out according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) of either the UTMB or Rutgers NJMS.

Construction of MCMV and viral infection

To follow *Plasmodium*-specific CD4 T cells, MCMV-K181-B5 was generated using B5 epitope (ISVLKSRLLKRKKYI) from the *P. chabaudi* Merozoite Surface Protein MSP1 cloned into the MCMV IE1 gene as follows (Figure S1A). MCMV-K181-Perth was genetically modified using bacterial artificial chromosome (BAC) recombineering[40] to carry the MHC II-E^d-restricted B5 epitope (**ISVLKSRLLKRKKYI**) from the *P. chabaudi* Merozoite Surface Protein MSP1 (1157–1171) in frame with the C-terminus end of the MCMV IE1 gene (Figure S1A).[41] Here the p*GalK* plasmid was used as a template with primers 1 and 2 (Table S1) to generate a PCR product containing the *Galk* gene flanked by 50 bp of target sequence homologous to the insertion site located at the end of IE1 exon 4. The resulting IE1-*GalK* PCR product was transformed into SW102 *E.coli* containing the MCMV pARK25-K181 BAC.[83]Single colonies of recombinants containing the *GalK* gene insert were selected by growth on minimal media containing galactose and confirmed by PCR using primers 3, 4 (Table S1). The resulting construct was denoted pARK25-IE1-GalK. Next, a PCR product was amplified using a 171b IE1-B5 minigene (IDT) consisting of a 7 amino acid leader sequence, the B5 epitope[9] (Table S1) and the same 50bp target sequences used above, as template with primers 10 and 11 (Table S1).The resulting IE1-B5 PCR product was then transformed into SW102 containing the pARK25-IE1-GalK.Recombinants containing the IE1-B5 sequence in place of IE1-*Galk* were obtained by negative selection on minimal media with glycerol and 2-deoxy-galactose and confirmed by PCR using primers 4 and 5 (Table S1). These BAC recombinants, designated pARK25-IE1-B5, were streaked to give single colonies and the IE1 exon 4 region was sequenced to ensure that they contained the correct IE1-B5 sequence. Next, pARK25-IE1-B5 BAC DNA was prepared and used to transfect NIH3T3cells with the Fugene 6 Transfection Reagent (Promega) to generate infectious Tissue Culture Virus (TCV). Excision of the BAC from the viral genome of the reconstituted virus was observed by the absence of GFP expression in infected cells and confirmed by PCR using primers flanking the region where the BAC was inserted into the viral genome (Table S1, primers 6–9). RFLP was performed to confirm the absence of gross mutations and sequencing confirmed the insertion of the B5 gene at the 3′ end of IE1 exon 4 without disruption to the region (Table S1, primers 10, 11). The resulting virus was denoted MCMV-B5. 3weeks old BALB/c mice were infected with TCV to generate primary and secondary Salivary Gland Virus (SGV) stocks. A secondary SGV stock was used to infect mice by injecting 5×10^3 PFU i.p. in 0.2mL of PBS.

METHOD DETAILS

Immunization

For infection and drug cure vaccination, the protocol was performed as previously documented in mice and humans.[11,53] Six-to nine-week old (Age- and sex-matched) mice were infected (10^6 *P chabaudi* AS) and treated with two subsequent noncurative doses of chloroquine (10 mg/kg body weight/day; Sigma-Aldrich), when parasitemia reached 1–2% of erythrocytes. Parasites were counted by light microscopy in thin blood smears stained with Giemsa (Sigma-Aldrich, St. Louis, MO). The mice were challenged by re-infection with the heterologous strain, *P. chabaudi* AJ, at day 200 p.v., which is 60 days after MCMV during the chronic phase. This timing was chosen on the basis that neither immunity nor the T cell response to live vaccination has completely decayed by day 120[11,59], and that the MCMV infection is below detectable by day 60,[41] though we expected it to show prolonged stimulatory capacity at this timepoint.[35] Parasitemia was monitored by microscopic examination of thin blood smears from the tail. Thin blood smears were stained with Giemsa (Ricca chemical company, Arlington, TX), and parasites were counted by light microscopy.

Virus quantification

MCMV-B5 or MCMV-BAC viral copy number of CMV/β-actin ratios were determined in different organs in infected BALB/cJ mice. DNA was purified from tissue (liver, lung, and salivary gland) using the Gentra Puregene Kit (Qiagen, Valencia, CA) following manufacturer's instructions. All tissues were stored at −80°C in PBS for DNA, or Trizol (Thermo Fisher Scientific,

Carlsbad, CA) for RNA purification, until sample processing. qPCR was performed with 50 ng of purified DNA. Specific primers for MCMV immediate-early (IE1) gene (GenBank: M11788,1) 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 β-actin (GenBank: M12481.1) 5-gct gta ttc ccc tcc atc gtg −3 and 5-cac ggt tgg cct tag ggt tca-3. Serial dilutions of a control plasmid were used to generate a standard curve for both MCMV-IE1 and β-actin, as described previously, and qPCR of viral DNA has been shown to correlate quantitatively with the plaque assay throughout the viral infection.[82] All sample measurements were performed in triplicate. Real-time PCR (VIIA, AB Biosystems, Life Technologies, Carlsbad, CA).

Adoptive transfers and *in vivo* studies

MACS-purified $CD4^+$ T cells from young, uninfected B5 TCR-Tg mouse spleens (Miltenyi San Diego, CA) were labeled with cell trace violet (CTV) and adoptively transferred (2×10^6) into Thy1.1 congenic mice. For cytokine neutralization, three doses of anti-IFN-γ antibody (H22, 0.5 mg) or isotype control antibody (PIP, 0.5 mg); or a combination of anti-IL-12p40 (C17.8, rat IgG2a, 300 μg) and anti-IL-18 antibody (YIGIF74–1G7, control Rat IgG2a, 200 μg) were given, every other day. All *in vivo* antibodies were from BioXCell (Lebanon, NH).

Flow cytometry

Single-cell suspensions from spleens were prepared at the indicated time points after infection in HBSS) with HEPES (Thermo Fisher Scientific, Waltham, MA) using gentleMACS (Miltenyi, San Diego, CA) and incubated in RBC lysis buffer (eBioscience, San Diego, CA). Single cells were stained extracellular in PBS, 2% FBS, and 0.1% sodium azide with antibodies. For T cells: anti-CD127 (A7R34), anti-PD-1 (RMP1-30), anti-CD62L (MEL-14), CD4 (RM4-5), (anti-CD90.2 [30-H12], anti-CD44 [IM7], and anti-CD27 [LG-7F9]) (all from BD Biosciences, eBioscience/Invitrogen and Biolegend, San Diego, CA). For antigen-presenting cells were stained with anti-Ly6C, anti-Ly6G (1A8), anti CD11b (M1/70), anti-CD11c (N418), DX5, MHCII/I-A/Eb (M5/114.15.2). Phenotype of phagocytes, polyclonal $CD4^+$ T cell subsets and *Plasmodium* B5-specific $CD4^+$ T cell subsets were identified with gating strategy as schematically depicted in Figure S3 as polyclonal $CD4^+$ T cell subsets including Teff ($CD4^+CD127^-CD44^+$) and Tmem $CD4^+CD44^{hi}CD127^{hi}$); *P. chabaudi* B5-specific cells including proliferated ($CD^+Thy1.2^+CTV^-B5$); Teff ($CD4^+Thy1.2^+CTV^-$, $CD127^-CD44^+$); Tmem and subsets (Tmem, $CD4^+Thy1.2^+CTV^-CD44^{hi}CD127^{hi}$; Tcm, $CD4^+Thy1.2^+CTV^-CD44^{hi}CD127^{hi}$, $CD62L^+CD27^+$); Tem^{Early} (TemE, $CD4^+Thy1.2^+CTV^-CD44^{hi}CD127^{hi}$, $CD62L^{lo}CD27^+$); or Tem^{Late} (TemL, $CD4^+Thy1.2^+CTV^-CD44^{hi}CD127^{hi}$, $CD62L^{lo}CD27^-$) and innate cells including $CD8\alpha^+$ DCs ($CD3^-CD11c^{hi}MHCII^{hi}CD8\alpha^+$); $CD8\alpha^-$ DCs ($CD3^-CD11c^{hi}MHCII^{hi}CD8\alpha^-$), inflammatory monocytes ($CD3^-CD11b^+Ly6C^+$), NKT ($DX5^+CD3^+$) and NK cells ($DX5^+MHCII^-CD3^-$).For intracellular cytokine staining, cells were cultured with PMA and ionomycin (IFN-γ, TNF) or LPS for IL-12 for 5 h at 37°C, 5% CO_2 in the presence of GolgiPlug (BD Bioscience) before extracellular staining and then fixed with 2% paraformaldehyde overnight. Cells were then permeabilized using BD 10X Permeability buffer diluted with water and stained with anti-IFN-γ (XMG1.2), anti-TNF (MP6-XT22), anti-IL-2 (JES6-5H4) and IL-12 p40/p70 (C15.6). Virus-specific $CD4^+$ T cells were identified using M78/$I\text{-}A^d$ tetramers, with peptide (SQQKMTSLPMSVFYS) and CLIP/$I\text{-}A^d$ as tetramer control (PVSKMRMATPLLMQA) and $CD8^+$ T cells with H-2L(d) MCMV IE1 168–176 YPHFMPTNL (both from NIH Tetramer Core Facility, Emory, Atlanta, GA). Tetramer staining was performed within 6 months of receiving the reagents at 4C for 1 h before/after surface and intracellular staining. Cells were acquired on an LSR II Fortessa or Symphony A5 using FACSDiva software (BD Biosciences, San Jose, CA). Compensation was performed in FlowJo using single-stained splenocytes (with CD4 in all colors). All data analysis was performed using the FlowJo software package (version 10, FlowJo, BD San Jose, CA). Cell numbers were calculated as the % of recovered Tg cells/lymphocytes x the number of lymphocytes using countless 3 (Invitrogen), except for Figures 6 and 8 which the recovered cells were counted by inclusion of counting beads (Accu-Check, Molecular Probes, Thermo Fisher Scientific) in the FACS sample according to manufacturer's instructions and calculated using the equation: Total cell number = ((all cell event count/bead event count) x bead conc. (per μl) x (bead volume/cell volume)) x volume of original sample. This formula only works if the whole volume of FACS tube is collected.

Cytokines and antibody measurements

Cytokines and chemokines (CXCL9, CXCL10 (IP-10), CCL2, MCP1, IFN-γ, IFN-β, IFN-α, IL-10, IL-12p40, IL-15, IL-18, IL-6, and TNF-α) were measured in the plasma by Legendplex according to manufacturer's protocol (Biolegend, San Diego, CA). Plasma samples were collected at the indicated days and were stored at −80°C until analysis. All data were acquired on an LSR II Fortessa flow cytometer using FACSDiva software, version 8 (BD Biosciences, San Jose, CA). The concentration of each analyte is determined with cytometric data based on a standard curve using the LEGENDplex data analysis software (Biolegend, San Diego, CA). *P. chabaudi* AS and *P. chabaudi* AJ-specific IgG were evaluated in plasma at indicated days by ELISA, as described previously.[84] Plates were coated with *P. chabaudi* AS or *P. chabaudi* AJ parasite lysate overnight at 4°C in PBS with 0.05% Sodium Azide. Plates were blocked with 1% BSA, 0.3% Tween 20, 0.05% Sodium Azide and then sera were incubated. Bound antibody was detected using Alkaline Phosphatase-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL). The reaction was revealed with a *p*-Nitrophenyl phosphate disodium salt (Sigma, USA) solution (1 mg/ml) in diethanolamine buffer. Plates were analyzed with an Omega plate reader (FLUOstar Omega BMG LABTECH Inc, Durham, NC, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed by ANOVA, followed by Tukey's or two-tailed Mann-Whitney test or two-tailed unpaired Student's t test, where indicated. All data are presented as mean ± SEM. Statistical tests were performed using the statistical software package in Prism (version 9.2, GraphPad Software, USA): $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and n.s., not significant.