

PD-1 Dependent Exhaustion of CD8⁺ T Cells Drives Chronic Malaria

Joshua M. Horne-Debets,^{1,9} Rebecca Faleiro,^{1,9} Deshapriya S. Karunaratne,^{1,9} Xue Q. Liu,^{1,2} Katie E. Lineburg,¹ Chek Meng Poh,^{3,4} Gijsbert M. Grotenbreg,^{4,5,6} Geoffrey R. Hill,¹ Kelli P.A. MacDonald,¹ Michael F. Good,^{1,2} Laurent Renia,^{3,4} Rafi Ahmed,⁷ Arlene H. Sharpe,⁸ and Michelle N. Wykes^{1,*}

¹QIMR Berghofer Medical Research Institute, 300 Herston Road, Brisbane, QLD 4029, Australia

²Institute for Glycomics, Griffith University, Gold Coast Campus, QLD 4222, Australia

³Singapore Immunology Network, Agency for Science, Technology and Research (A*STAR), Singapore 136648, Singapore

⁴Department of Microbiology, Life Sciences Institute, National University of Singapore, Singapore 117597, Singapore

⁵Department of Biological Sciences, Faculty of Science, Life Sciences Institute, National University of Singapore, Singapore, Singapore 117597, Singapore

⁶Immunology Programme, Life Sciences Institute, National University of Singapore, Singapore 117597, Singapore

⁷Emory Vaccine Center, 954 Gatewood Road, Atlanta, GA 30329, USA

⁸Harvard Medical School, Department of Microbiology and Immunobiology, Boston, MA 02115, USA

⁹These authors contributed equally to this work and are co-first authors

*Correspondence: michelle.wykes@qimrberghofer.edu.au

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SUMMARY

Malaria is a highly prevalent disease caused by infection by *Plasmodium* spp., which infect hepatocytes and erythrocytes. Blood-stage infections cause devastating symptoms and can persist for years. Antibodies and CD4⁺ T cells are thought to protect against blood-stage infections. However, there has been considerable difficulty in developing an efficacious malaria vaccine, highlighting our incomplete understanding of immunity against this disease. Here, we used an experimental rodent malaria model to show that PD-1 mediates up to a 95% reduction in numbers and functional capacity of parasite-specific CD8⁺ T cells. Furthermore, in contrast to widely held views, parasite-specific CD8⁺ T cells are required to control both acute and chronic blood-stage disease even when parasite-specific antibodies and CD4⁺ T cells are present. Our findings provide a molecular explanation for chronic malaria that will be relevant to future malaria-vaccine design and may need consideration when vaccine development for other infections is problematic.

INTRODUCTION

Malaria is a major cause of global morbidity and mortality. People living in endemic areas can develop partial immunity to this disease, but only after repeated attacks of malaria, over several years. This disease is caused by *Plasmodium* parasites, which after introduction into the host by mosquitoes proceed to infect hepatocytes followed by red blood cells (RBCs). It is the blood-

stage infection that causes the symptoms and lethality associated with malaria. The infection can also become chronic with sustained, low levels of blood-stage parasites. These chronic infections cause profound anemia and splenomegaly with severe symptoms including mental depression and muscular weakness. Drugs used to treat malaria are losing efficacy, and efforts to develop an efficacious vaccine over 30 years have not been successful.

The components of the immune system responsible for killing *Plasmodium* parasites remain unclear, although antibodies are known to have a key role in controlling blood-stage infections (Cohen et al., 1961). In studies investigating the mechanism of protection, mice deficient in mature B cells developed a chronic relapsing parasitemia, confirming the need for antibodies to control chronic malaria (von der Weid et al., 1996). Furthermore, studies in experimental rodent models showed that CD4⁺ Th1-immune responses are also a critical component of protection against chronic blood-stage malaria (Kumar and Miller, 1990; Stephens and Langhorne, 2010; Su and Stevenson, 2002). Finally, depletion of CD8⁺ T cells delayed clearance of *P. chabaudi* malaria, implicating these cells in protection against chronic disease (Podoba and Stevenson, 1991).

Signaling through the programmed death-1 (PD-1) receptor is thought to “exhaust” HIV-specific CD4⁺ and CD8⁺ T cells (Barber et al., 2006; Day et al., 2006; Freeman et al., 2006). T cell exhaustion is characterized by poor effector function, sustained expression of inhibitory receptors, and a transcriptional state distinct from that of functional effector or memory T cells, which prevent optimal control of infections and tumors (Wherry, 2011). Exhaustion can also lead to apoptosis of virus-specific CD8⁺ T cells (Zhang et al., 2008).

Previous research implicated PD-1 in poor immune protection against malaria. PD-1 was shown to be expressed by mouse (Chandele et al., 2011; Hafalla et al., 2012) and human (Butler

et al., 2012; Illingworth et al., 2013) CD4⁺ T cells during malaria, suggesting lymphocyte-exhaustion in the pathogenesis of this disease. Furthermore, blockade of PD-L1 augmented experimental cerebral malaria (Hafalla et al., 2012). In contrast, combined blockade of PD-L1 and lymphocyte-activation gene 3 (LAG-3) accelerated clearance of *P. yoelii* (nonchronic) malaria and correlated with improved CD4⁺ T cell and B cell responses (Butler et al., 2012). However, since PD-L1 can interact specifically with both B7-1 (Butte et al., 2007) and PD-1 (Iwai et al., 2003) to inhibit T cell activation, either or both (B7-1 and PD-1) pathways could contribute to the attrition of malarial immunity. Furthermore, the requirement for combined blockade of LAG-3 with PD-L1 to improve malarial immunity (Butler et al., 2012) adds confusion to our understanding of any role for PD-1 per se in inhibition of protection against malaria.

In the present study, we investigated why malaria persists as a chronic infection. We focused on (1) exhaustion of CD8⁺ T cell, CD4⁺ T cell, and B cell responses; and (2) the direct role of PD-1 in facilitating this process. We used a mouse model of chronic malaria: *Plasmodium chabaudi chabaudi* AS (hereafter referred to as *P. chabaudi*), which produces ongoing cycles of recrudescence or low-grade infections that can persist for up to 3 months (Achtman et al., 2007). We found that parasite-specific CD8⁺ T cells undergo significant PD-1-dependent exhaustion, which exacerbates acute blood-stage malaria and drives chronic disease.

RESULTS

PD-1 Exacerbates Acute Blood-Stage Malaria and Drives Chronic Infection

P. chabaudi parasites are detected in the blood of C57BL/6 (wild-type [WT]) infected mice within 3–4 days postinfection (p.i.). The parasitemia then peaks by day 7–10, rapidly clears by day 10–13, and is followed by a second peak that generally clears around day 20–25 (acute phase). This is followed by the chronic phase with low-level or recrudescence parasitemia, which can occur at different times and levels in individual mice (Figure 1A).

Given that CD4⁺ T cells were implicated in protection against chronic malaria, we first examined the durability of CD4⁺ T cell-mediated protection during *P. chabaudi* malaria (Figures 1B and 1C). We compared in vitro responses to total parasite antigen extract by CD4⁺ T cells isolated from the spleens of infected WT mice during acute (day 9 p.i.) and chronic (day 31 p.i.) malaria (Figure 1B). Approximately 11% of the CD4⁺ T cells proliferated in response to *P. chabaudi* antigens at day 9 p.i., but this percentage declined to 1% at day 31 (Figure 1B). Similarly, the amount of interferon- γ (IFN- γ) secreted by CD4⁺ T cells declined by 30-fold between days 9 and 31 p.i. (Figure 1C). These results indicated that the numbers and functional capacity of parasite-specific CD4⁺ T cells decline with ongoing infection.

Next, we explored how CD4⁺ T cells might lose their functional capacity over the course of infection. For this, we examined expression of PD-1 and CD11a (which identifies antigen-experienced T cells; Butler et al., 2012) on CD4⁺ and CD8⁺ T cells from *P. chabaudi*-infected mice (Figures 1D and 1E). Compared to CD4⁺ T cells in naive mice, the number of CD4⁺ T cells expressing both CD11a and PD-1 increased 10-fold within 14 days p.i.,

but then declined significantly by day 35 p.i. (Figure 1D). Furthermore, the number of CD8⁺ T cells that expressed both CD11a and PD-1 increased 86-fold within 14 days p.i., but then declined to the levels found in naive mice by day 35 p.i. (Figure 1E). A peptide/major histocompatibility complex (MHC) tetramer (K^b-F4), consisting of parasite-specific peptide F4 (EIYIFTNI) (Lau et al., 2011) in context with MHC class I-K^b, was then used to label and track parasite-specific CD8⁺ T cells in WT and PD-1 knockout (KO) mice. Approximately 4% of CD8⁺CD62L⁻ T cells (which represent effector or effector memory cells) in WT mice were labeled with this tetramer by day 7 p.i., and most of these cells also expressed CD11a (Figure 1F). This result validated that CD11a expression by CD8⁺ T cells did identify parasite-specific CD8⁺ T cells. Furthermore, ~20% of K^b-F4-tetramer⁺CD8⁺ T cells in WT mice (day 7) expressed PD-1 (Figure 1G; 1.17% of 5.36% total tetramer⁺ cells), confirming that parasite-specific CD8⁺ T cells expressed the exhaustion marker.

To explore whether expression of PD-1 affected the clearance of malarial parasitemia, we examined the outcome of *P. chabaudi* infections in PD-1-deficient C57BL/6 mice (*Pdcd1*^{-/-}; hereafter referred to as PD-1 KO mice; Nishimura et al., 2001). As shown in Figure 1H, the first peak in parasitemia at day 7 p.i. was >2-fold lower (note log scale) in PD-1 KO mice (20%) compared to WT animals (45%). PD-1 KO mice also cleared acute parasitemia 7 days earlier than WT mice (arrow in Figure 1H). After clearance of acute parasitemia, 100% of WT mice continued to experience patent parasitemia. In marked contrast, <30% of the PD-1 KO mice developed chronic infections, and parasitemia levels in these mice were >100-fold lower than those in the WT mice. Importantly, by day 40, while all WT mice experienced ongoing parasitemia, all PD-1 KO mice (n = 5) had developed sterile immunity as the transfer of 200–300 μ l of their blood into naive mice did not transfer the infection. Taken together, these results implicated PD-1 in a loss of capacity to protect against malaria infections, thereby exacerbating acute infections and delaying the clearance of parasites.

PD-1 on CD8⁺ and CD4⁺ T Cells Mediates Loss of Capacity to Protect against Malaria

To address whether improved parasite control in PD-1 KO mice was mediated by T cells, several cohorts of WT and PD-1 KO mice were depleted of CD4⁺ or CD8⁺ T cells with specific antibodies or treated with rat immunoglobulin (rlg) 1 day before *P. chabaudi* infection and every 3–4 days until days 14–18 p.i.. WT mice receiving rlg (Figure 2A) had ongoing parasitemia at day 32 unlike PD-1 KO mice (Figure 2B) that cleared acute parasitemia by day 25 (arrow). However, both WT (Figure 2C) and PD-1 KO mice (Figure 2D), depleted of CD4⁺ T cells, were unable to control parasitemia and died or had to be euthanized (Figure S1A) because they developed severe clinical symptoms (Figures S1B and S1C). These observations demonstrated that *P. chabaudi*-infected mice (both WT and PD-1 KO) required CD4⁺ T cells to survive acute malaria.

Depletion of CD8⁺ T cells in WT mice had no significant effect on acute (Figure 2E) or chronic parasitemia (Figure 2F) when compared to rlg-treatment (Figure 2A). Chronic or recrudescence peak parasitemia in individual mice are shown in scatterplots to highlight differences in all mice. However, compared to rlg

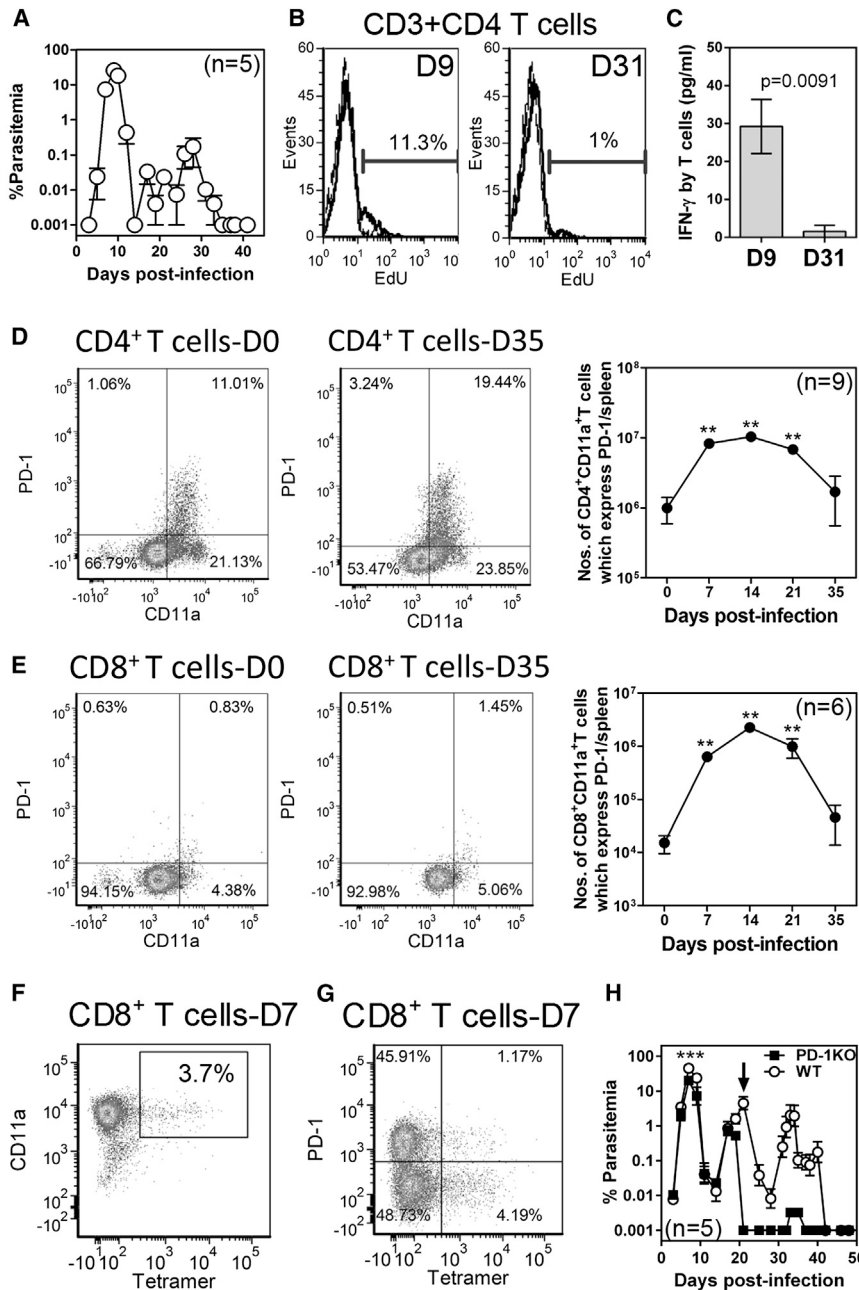


Figure 1. *P. chabaudi* AS Malaria Induces PD-1 Expression on Parasite-Specific CD4⁺ and CD8⁺ T Cells and PD-1 Dependent Loss of Protection

(A) Mean percent parasitemia for a typical course of infection in a cohort of mice infected with *P. chabaudi* and monitored for 40 days.

(B) In vitro proliferation of CD3⁺CD4⁺ T cells in response to total parasite antigen, as shown by the uptake of EdU on days 9 (D9) and 31 (D31) p.i.

(C) IFN- γ secretion by CD3⁺CD4⁺ T cells in response to total parasite antigen on D9 and D31 p.i.

(D) Flow cytometric profiles of *Plasmodium* infection-induced expression of PD-1 and CD11a on CD4⁺ T cells at days 0 and 35 (D0 and D35). (The line chart summarizes pooled data from 3 independent experiments (total n = 9 mice). Error bars represent SEM (**p < 0.005).

(E) Flow cytometric profiles of *Plasmodium* infection-induced expression of PD-1 and CD11a on CD8⁺ T cells at D0 and D35. The line chart summarizes pooled data from two independent experiments (total n = 6 mice). Error bars represent SEM (**p < 0.005).

(F) Flow cytometric profiles of CD11a and parasite-specific tetramer labeling of CD8⁺CD62L⁻ T cells from WT mice on day 7 p.i.

(G) Flow cytometric profiles of CD8⁺CD62L⁻ T cells labeled with PD-1 and parasite-specific tetramers.

(H) Mean percent parasitemia for a typical course of infection in cohorts of WT and PD-1 KO mice infected with *P. chabaudi*. The arrows represent end of acute malaria PD-1 KO mice. Data represent five mice for this experiment with a total of 26 mice for all experiments (**p < 0.0001 between WT and PD-1 KO groups, during peak parasitemia). Error bars represent SEM. Data represent one of five independent experiments, using groups of three, five, or six mice, for which similar results were obtained.

treatment (Figure 2B), depletion of CD8⁺ T cells in PD-1 KO mice, increased peak parasitemia by 2-fold (p < 0.01) during the acute phase of infection (Figure 2G). Furthermore, 100% of the PD-1 KO mice developed chronic malaria when depleted of CD8⁺ T cells (Figure 2H), compared to only 33% of rlg-treated PD-1 KO mice (Figure 2H). Critically, parasitemia levels in CD8⁺ T-cell-depleted PD-1 KO mice were significantly higher than rlg-treated PD-1 KO mice (Figure 2H), similar to those of WT animals (Figure 2F). Taken together, these findings showed that PD-1 mediated a loss of CD8⁺ T cells in WT mice, which were required to control acute and chronic malaria.

taken from infected WT mice showed minor protection on the course of recrudescence parasitemia in the recipient mice, for 6 days only (Figure 2I). By contrast, the CD8⁺ T cells derived from infected-PD-1 KO mice profoundly controlled recrudescence infections for 16 days (Figure 2J). The donor CD8⁺ T cells were also assessed for parasite specificity by measuring IFN- γ production in response to parasite-specific (F4) peptide (Figure S2A). CD8⁺ T cells from PD-1 KO mice had nearly 3-fold more IFN- γ -secreting, parasite-peptide-specific CD8⁺ T cells than WT mice, which explained the better protection seen in recipients. Taken together, these data provide further support

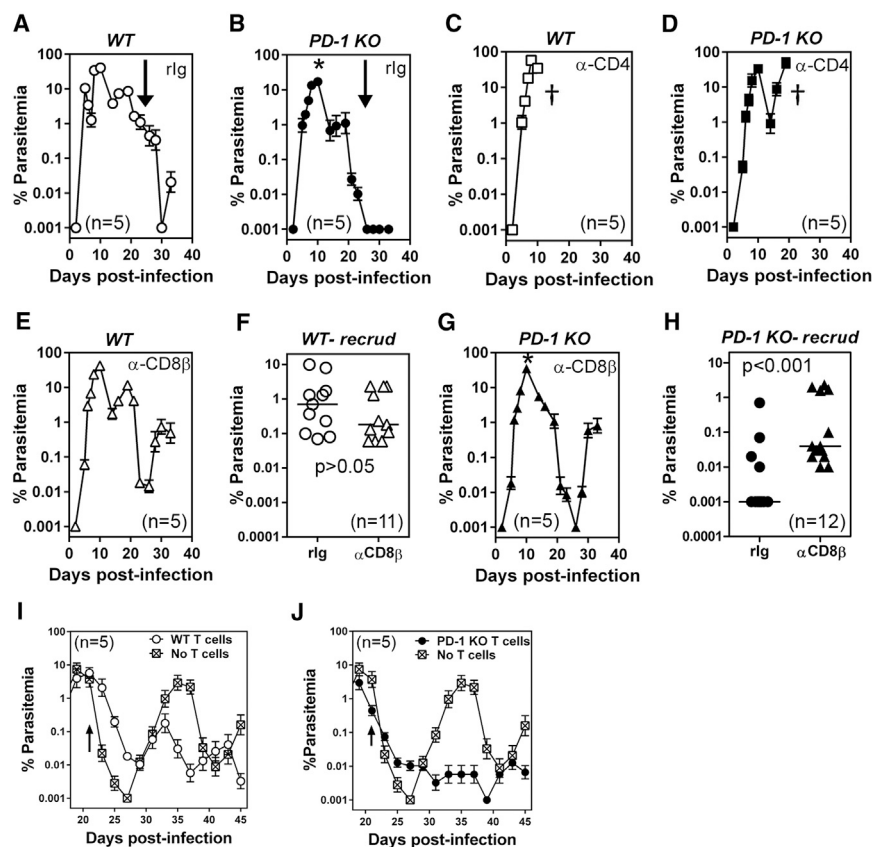


Figure 2. CD4⁺ and CD8⁺ T Cell Depletion and CD8⁺ T Cell Transfer Studies Show the Role of These Cells in Protection Against Acute and Chronic Malaria

(A–H) Mean percent parasitemia in WT (open symbols) and PD-1 KO (closed symbol) mice following treatment with rlg or the depletion of CD4⁺ T cells or CD8⁺ T cells († denotes the median point of death). Data represent one of three independent experiments in WT (total n = 11 mice) or PD-1 KO (total n = 12) mice. Arrow shows when PD-1 KO mice given rlg cleared acute infection (*p < 0.01). (F and H) The scatterplots show peak parasitemia during recrudescence or chronic malaria in individual mice. The scatterplots represent data from three pooled experiments and the bar represents the median of peak parasitemia.

(I and J) Mean percent parasitemia in *P. chabaudi*-infected WT mice following transfusion at day 21 p.i. with 3×10^5 CD8⁺ T cells from WT or PD-1 KO mice at day 21 p.i. The arrows indicate the day on which CD8⁺ T cells were transferred. The data represent one of three independent experiments (n = 4 or 5), twice with 3×10^5 and once with 3×10^6 CD8⁺ T cells per mouse. Data are presented as the mean % parasitemia \pm SEM.

for the above findings that CD8⁺ T cells protect against the chronic phase of malaria and that PD-1 reduced the capacity of these cells to protect against chronic disease.

PD-1 Mediates Loss of Parasite-Specific CD8⁺ T Cell Numbers and Function during Malaria

To determine the extent to which PD-1 mediated exhaustion of CD8⁺ T cells, WT and PD-1 KO mice were infected with *P. chabaudi* and their CD8⁺ T cells were analyzed for numbers and immune function, over 35 days. Total numbers of CD3⁺CD8⁺ T cells were similar in WT and PD-1 KO mice and did not change significantly over the course of infection (Figure 3A). Furthermore, the number of tetramer⁺CD8⁺CD62L⁻ T cells at day 7 p.i. in WT and PD-1 KO mice was also similar (Figure 3B). However, by day 35, PD-1 KO mice had 5-fold more parasite-specific CD8⁺ effector or effector memory T cells than WT mice (Figure 3B). We next examined CD8⁺CD62L^{+/−} T cells for expression of Ki-67, a marker of recent cell proliferation (Figure S2B). On the basis of Ki-67 expression, the proliferation of CD8⁺CD62L⁻ T cells (Figure 3C) and CD8⁺CD62L⁺Ki-67⁺ T cells (Figure S2C) was similar in WT and PD-1 KO mice from days 0 to 21. However, between days 21 and 35, WT mice lost CD8⁺CD62L⁻Ki-67⁺ T cells, whereas these were maintained in the PD-1 KO mice (Figure 3C). In agreement with these data, CD8⁺ T cells from WT and PD-1 KO mice showed similar *in vitro* proliferation activity in response to parasite proteins on day 14 (Figure 3D), but by day 35 p.i., CD8⁺ T cells from PD-1

KO mice had a significantly greater capacity to proliferate (Figure 3D). Taken together, the data showed that PD-1-mediated 80% reduction in numbers of tetramer⁺CD8⁺CD62L⁻ T cells and 95% reduction in capacity of CD8⁺ cells to proliferate in response to parasites during the chronic phase of malaria.

Next, we assessed cytokine responses. WT and PD-1 KO mice had similar numbers of IFN- γ -secreting CD8⁺CD62L⁻ T cells at day 7 p.i.; however, the WT mice then showed a steady decline in the number of these cells, whereas there was no obvious change in PD-1 KO mice (Figure 3E). Furthermore, the number of parasite-specific IFN- γ -secreting cells at 35 days p.i., as determined by CD8⁺ T cells responding to parasite-specific peptide F4 in an enzyme-linked immunosorbent spot (ELISPOT) assay, confirmed this finding (Figure 3F). In addition, T-bet, a transcription factor required for CD8⁺ T cell effector function (including IFN- γ production), was present in similar proportions of CD8⁺CD62L⁻ T cells in WT as in PD-1 KO mice at day 7 (Figure S2D) but in a significantly greater proportion in PD-1 KO mice at day 35 (Figure S2E). In contrast, the number of interleukin-10 (IL-10)-secreting CD8⁺CD62L⁻ T cells, which counteract protective Th1 responses, was similar in both groups of mice throughout the course of infection (Figure S2F). Taken together, the data showed that based on numbers of tetramer⁺CD8⁺ T cells/spleen (Figure 3B), 18% of parasite-specific CD8⁺ T cells from PD-1 KO mice could respond to peptide F4 in culture, compared to only 11% from WT mice at day 35 (Figure 3F), indicating functional exhaustion of parasite-specific CD8⁺ T cells in WT mice.

Finally, to determine if CD8⁺ T cells had the ability to mediate protection by cytotoxicity, (activated) CD69⁺CD8⁺ T cells were

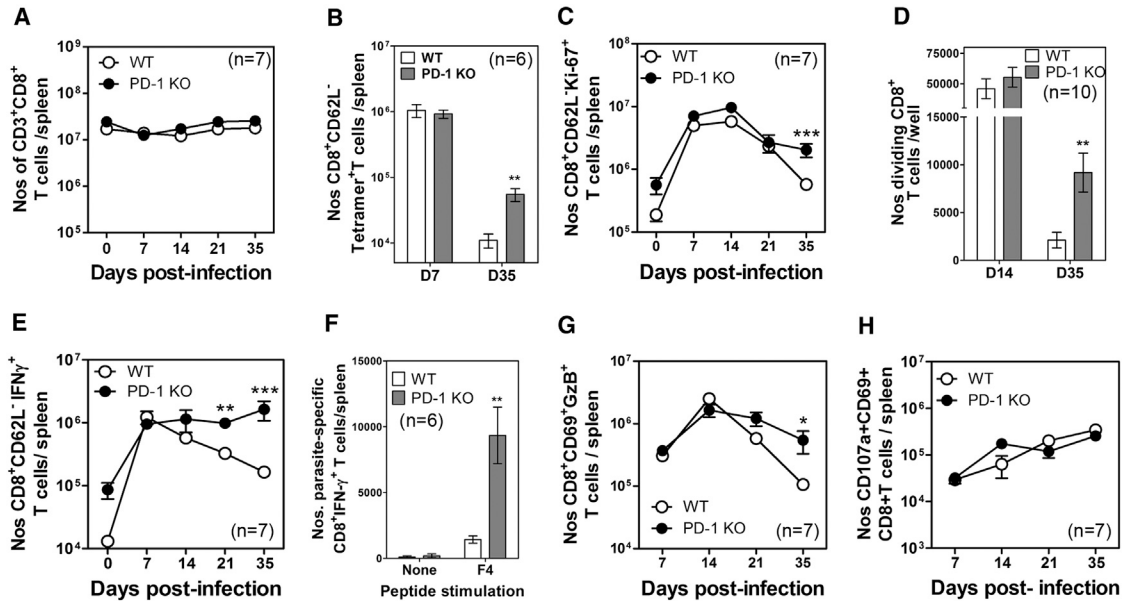


Figure 3. A Phenotypic and Functional Analysis of CD8⁺ T Cells from *P. chabaudi*-Infected WT and PD-1 KO Mice over 35 Days

(A) Mean numbers of CD3⁺CD8⁺ T cells per spleen. (B) Mean numbers of parasite-specific F4-tetramer⁺CD8⁺CD62L⁻ T cells per spleen at days 7 and 35 p.i. (C) Mean numbers of CD8⁺CD62L⁻ T cells expressing Ki-67 per spleen. (D) Mean numbers of CD8⁺ T cells (days 14 and 35 p.i.) that proliferated in culture in response to parasite antigen, in the presence of naive DCs, as measured by EdU uptake. (E) Mean numbers of ex vivo CD8⁺CD62L⁻ T cells per spleen that secreted IFN- γ , as measured by a flow cytometry-based assay. (F) Mean numbers of parasite-specific CD8⁺ T cells per spleen at 35 days p.i. that secreted IFN- γ in response to parasite-specific peptide F4 compared to cultures without peptide, as measured by ELISPOT assay. (G and H) Mean numbers of CD8⁺CD69⁺ T cells per spleen that expressed (G) GzB or (H) CD107a. Error bars represent SEM (**p* < 0.05; ***p* < 0.005; ****p* < 0.0005 between WT and PD-1 KO groups).

assessed for granzyme B (GzB) expression. WT and PD-1 KO mice had similar numbers of GzB-expressing cells for 14 days p.i.; however, over the following 21 days, the WT mice showed a greater decline in numbers of these cells than PD-1 KO mice (Figure 3G). In contrast, surface acquisition of CD107a, which indicates recent degranulation or secretion of cytokines (e.g., IFN- γ or IL-10), increased similarly in both groups of mice over the course of infection (Figure 3H). Taken together, these findings showed that the pool of functional CD62L⁻CD8⁺ T cells capable of secreting IFN- γ , expressing GzB, and proliferating in response to parasite antigens was depleted in WT mice during the chronic phase of blood-stage malaria and that this depletion was mediated by PD-1.

PD-1 Mediates a Reduction of CD4⁺ T Cell Numbers and Function during Chronic Malaria

To determine if CD4⁺ T cells experienced PD-1-mediated exhaustion during chronic malaria, WT and PD-1 KO mice were infected with *P. chabaudi* and their CD4⁺ T cells were analyzed over the course of 35 days. Total numbers of CD3⁺CD4⁺ T cells in the spleen were similar in WT and PD-1 KO mice over the course of infection (Figure 4A). Furthermore, in both groups of mice, T-bet was predominantly expressed in CD4⁺CD62L⁻ cells in similar proportions (Figure 4B), and both groups had similar numbers of these cells per spleen up to day

21 (Figure 4C). However, by day 35 p.i., PD-1 KO mice had significantly more CD4⁺CD62L⁻T-bet⁺ T cells per spleen (Figure 4C). In addition, ex vivo proliferation (as determined by Ki-67 expression) of CD4⁺CD62L⁻ (Figure 4D) and CD4⁺CD62L⁺ T cells (Figure S3A) was similar over the first 21 days of infection in both groups. However, by day 35, WT mice had fewer proliferating CD4⁺CD62L⁻Ki-67⁺ T cells than PD-1 KO animals (Figure 4D). These data were then confirmed using an in vitro EdU-uptake assay to measure proliferation in response to parasite antigens. On day 35, PD-1 KO CD4⁺ T cells had significantly more parasite-specific proliferative capacity than did WT CD4⁺ T cells (Figures 4E).

Analysis of CD4⁺ T cell function found that while numbers of IFN- γ -secreting CD4⁺CD62L⁻ T cells were significantly different between WT and PD-1 KO mice on day 0, both groups had similar numbers on day 14 p.i. and these numbers declined by day 21 (Figure 4F). By day 35, only the PD-1 KO mice responded to chronic malaria with an increase in numbers of IFN- γ -secreting CD4⁺CD62L⁻ T cells (Figure 4F). Furthermore, using CD11a as a surrogate marker of antigen-specific CD4⁺ T cells (Butler et al., 2012), we found similar numbers at day 14 in WT and PD-1 KO mice but 2-fold higher numbers in PD-1 KO mice by day 35 (Figure 4G). Further, similar percentages of antigen-experienced CD4⁺ T cells secreted IFN- γ in WT and PD-1 KO mice at day 14, but

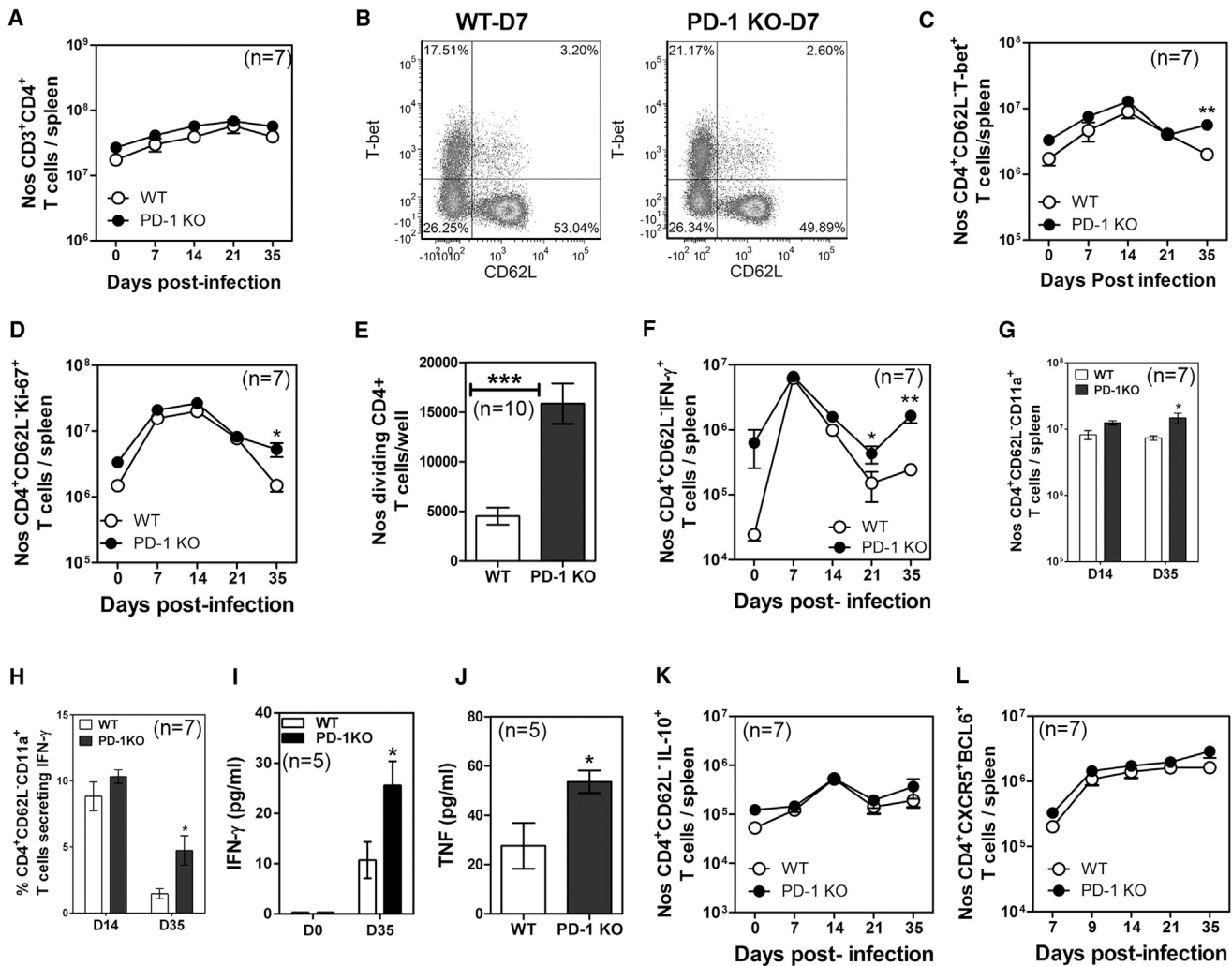


Figure 4. A Phenotypic and Functional Analysis of CD4⁺ T Cells from *P. chabaudi*-Infected WT and PD-1 KO Mice over 35 Days

(A) Mean numbers of CD3⁺CD4⁺ T cells per spleen.
 (B) Flow cytometric profiles of CD3⁺CD4⁺ T cells expressing CD62L and T-bet.
 (C) Mean numbers of T-bet-expressing CD4⁺CD62L⁻ T cells per spleen.
 (D) Mean numbers of CD4⁺CD62L⁻ T cells expressing Ki-67 per spleen.
 (E) Mean numbers of CD4⁺ T cells (day 35 p.i.) that proliferated in culture in response to parasite antigen in the presence of naive DCs, as measured by EdU uptake.
 (F) Mean numbers of ex vivo CD4⁺CD62L⁻ T cells per spleen that secreted IFN- γ , as measured by a flow cytometry-based assay.
 (G) Mean numbers of CD4⁺CD62L⁻ T cells expressing CD11a per spleen at days 14 and 35 p.i.
 (H) Percentage of CD4⁺CD62L⁻CD11a⁺ T cells that secreted IFN- γ , as measured by a flow cytometry-based assay on days 14 and 35 p.i.
 (I) Ex vivo secretion of IFN- γ by CD4⁺ T cells in culture, in response to parasite antigen in the presence of naive DCs, at days 0 and 35 p.i.
 (J) Ex vivo secretion of TNF- α by CD4⁺ T in culture, in response to parasite antigen in the presence of naive DCs, at day 35 p.i.
 (K) Mean numbers of ex vivo CD4⁺CD62L⁻ T cells per spleen that secreted IL-10, as measured by a flow-cytometry-based assay.
 (L) Mean numbers of CD4⁺CXCR5⁺BCL6⁺ T_{FH} cells per spleen. Error bars represent SEM. The data represent one of two or three independent experiments that had similar results (* $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$ between WT and PD-1 KO groups).

with 3.2-fold higher percentages in PD-1 KO mice at day 35 (Figure 4H).

CD4⁺ T cells were then taken from naive and *P. chabaudi*-infected WT and PD-1 KO mice (at day 35) and cultured with naive dendritic cells (DCs), with and without *P. chabaudi* antigens to measure parasite-specific cytokine responses. At day 0, in both PD1 KO and WT mice, antigen-specific IFN- γ responses

were negligible and equivalent (Figure 4I). In contrast, by day 35, CD4⁺ T cells taken from WT mice secreted 2.4-fold less IFN- γ (Figure 4I) in response to parasite antigens than those from PD-1 KO mice. Similarly, at day 35, CD4⁺ T cells taken from WT mice also secreted 2-fold less tumor necrosis factor- α (TNF- α) in response to parasite antigens than those from PD-1 KO mice (Figure 4J). However, there were no differences

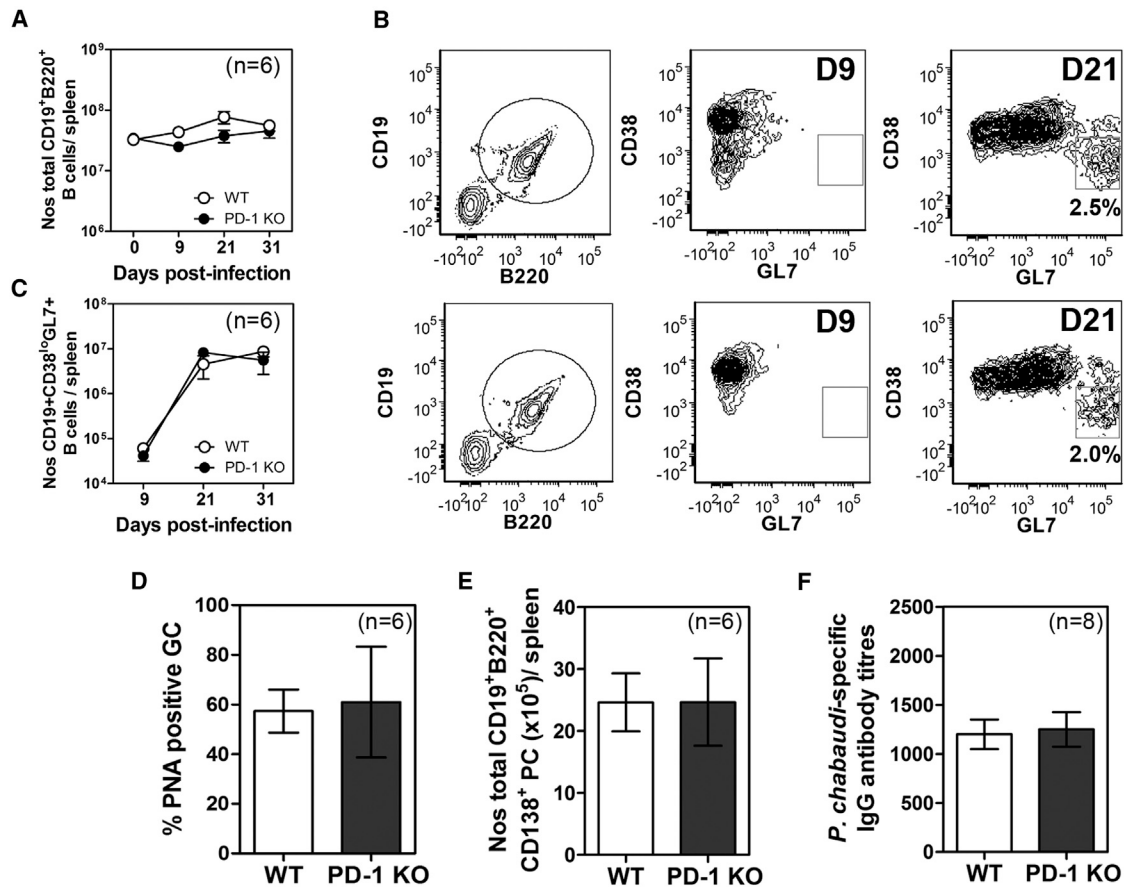


Figure 5. A Phenotypic and Functional Analysis of B Cells from *P. chabaudi*-Infected WT and PD-1 KO Mice

(A) Mean numbers of CD19⁺B220⁺ B cells per spleen.

(B) Flow cytometric profiles of CD19⁺B220⁺ B cells expressing CD38^{lo}GL7⁺ GC B cells at days 9 and 21 p.i.

(C) Mean numbers of CD19⁺B220⁺CD38^{lo}GL7⁺ GC B cells per spleen on days 9, 21, and 31 p.i.

(D) Mean percentages of IgM⁺ B cell follicles with PNA⁺ GCs as measured by microscopy on day 21 p.i.

(E) Mean numbers of CD19⁺B220⁺CD138⁺ plasma cells per spleen at day 21 p.i.

(F) Mean titers of *P. chabaudi*-specific antibody in infected mice at days 21 p.i. Error bars represent SEM. The data represent pool of two independent experiments that yielded similar results.

in IL-10 production by CD4⁺CD62L⁻ T cells from the two groups of mice (Figure 4K). Taken together, the data showed that PD-1 mediated a reduction in the capacity of parasite-specific CD4⁺ T cells to proliferate and to secrete IFN- γ and TNF- α during the chronic phase of malaria (day 35), indicating exhaustion of these cells.

Finally, to determine if other T cell populations experienced PD-1-mediated exhaustion during chronic malaria, we assessed CD3, CD4, CXCR5, and BCL-6 expression to count follicular T helper (T_{FH}) cells. T_{FH} are required to support the development of germinal centers and memory B cells in the spleen and their numbers were similar in PD-1 KO and WT mice during the course of infection (Figure 4L). However, an examination of regulatory T cells in both experimental groups found that infected PD-1 KO mice had higher proportions of both CD4⁺CD25⁺FoxP3⁺ regulatory T (T_{reg}) cells (Figure S3B) and T_{FR} cells (FoxP3⁺ T_{FH}; Sage et al., 2013) than WT mice (Figure S3C). Overall, compared with WT animals, PD-1 KO mice maintained superior effector

and regulatory CD4⁺ T cell function during the chronic phase of malaria.

PD-1 Does Not Affect B Cell Responses during Malaria

Previous research has shown that mice deficient in mature B cells can reduce a primary acute infection with *P. chabaudi* to low levels but cannot eliminate the parasites (von der Weid et al., 1996). Thus, we examined whether PD-1 mediated a loss of B cell responses over the course of 31 days. Following *P. chabaudi* infection, WT and PD-1 KO mice showed no significant differences in the number of CD19⁺B220⁺ B cells (Figure 5A) or CD19⁺B220⁺CD38^{lo}GL7⁺ germinal center B cells (Figures 5B and 5C), as assessed by flow cytometry, or in the percentage of germinal center cells in tissue sections expressing PNA (a germinal center marker; Figure 5D). The number of CD19⁺CD138⁺ plasma cells (Figure 5E) and the antibody titers (Figure 5F) were also equivalent at 21 days p.i. Overall, PD-1 did not affect B cell responses against malaria.

DISCUSSION

Taken as a whole, our findings show PD-1-mediated losses in numbers and functional capacity of parasite-specific CD8⁺ T cells during the acute phase of malaria, which exacerbates the infection and drives chronic disease. Furthermore, PD-1 also modestly affects CD4⁺ T cell function during malaria but does not affect primary B cell responses.

As described in the [Introduction](#), previous research had shown indirect links between PD-1 and CD4⁺ T cell exhaustion in the pathogenesis of malaria. Furthermore, combined blockade of PD-L1 and LAG-3 in *P. yoelii*-infected mice correlated with increases in the numbers of T_{FH} cells, germinal center (GC) B cells, and titers of antibodies with accelerated parasite clearance ([Butler et al., 2012](#)). However, given that PD-L1 can interact specifically with both PD-1 and B7-1, the role of PD-1 remained unclear. Furthermore, the role of CD8⁺ T cells in protection against malaria was not investigated. In our studies using PD-1 KO mice, we found that deletion of PD-1 substantially accelerated parasite clearance and improved CD4⁺ T cell numbers and function. However, even though PD-1 KO mice had more functional CD4⁺ T cells than WT mice and similar titers of parasite-specific antibodies, they still developed chronic malaria if CD8⁺ T cells were depleted. These observations highlight the crucial role of CD8⁺ T cells in protection against chronic malaria.

A role for CD8⁺ T cells in the clearance of chronic, blood-stage malaria is not generally acknowledged. Naive mice transfused with CD8⁺ T cells derived from mice that survived two successive bouts of *P. yoelii* infections (not a chronic form of malaria) survived lethal malaria ([Imai et al., 2010](#)), similar to protection seen previously ([Mogil et al., 1987](#)). Furthermore, depletion of CD8⁺ T cells in WT mice during *P. chabaudi* infections delayed the overall clearance of the infection, compared to nondepleted animals ([Podoba and Stevenson, 1991](#)). Critically, we found that WT mice experienced a significant loss in the numbers and capacity of parasite-specific CD8⁺ T cells to protect against chronic malaria. The PD-1-mediated loss of these cells is probably the result of several factors, including an inability of exhausted cells to respond to interleukin-7 and interleukin-15, which regulate T cell homeostasis ([Yi et al., 2010](#)).

These studies have conclusively shown that CD8⁺ T cells contribute to parasite clearance during acute malaria and control chronic disease. Parasite-specific CD8⁺ T cells were shown to secrete IFN- γ and express GzB. Secretion of IFN- γ by CD8⁺ T cells indicates that protection is mediated by support to innate cells (e.g., macrophages) to clear parasites. Furthermore, the increase in GzB⁺CD69⁺CD8⁺ T cells during infection indicates cytotoxic-mediated killing of parasite-infected cells. This would only be applicable to clearance of infected reticulocytes, but not mature red cells, which express MHC class I molecules required by CD8⁺ T cells for recognition. This possibility is supported by a previous study that found that while *P. chabaudi* parasites had a preference for mature red cells early in the infection, parasites could infect reticulocytes during periods of severe immune pressure on the parasites such as peak parasitemia and ensuing “crisis” phase ([Taylor-Robinson and Phillips, 1994](#)).

While PD-1 KO mice had significantly better T cell function than WT mice, their B cell function was not improved, unlike

the studies where combined blockade of PD-L1 and LAG-3 improved antibody responses ([Butler et al., 2012](#)). One likely explanation for this apparent contradiction is that PD-1 KO mice compared with WT mice had a significantly higher proportion of T_{FR} cells ([Figure S3C](#)). Regulatory T_{FR} cells are known to be suppressive in vitro and to limit the numbers of T_{FH} cells and GC B cells in vivo ([Linterman et al., 2011](#)). In addition, the improvement in B cell immunity produced by the coblockade of PD-L1 and LAG-3 ([Butler et al., 2012](#)), not observed in the PD-1 KO mice, could be explained by the interruption of inhibitory signals to LAG-3 on B cells ([Kisielow et al., 2005](#)) or PD-L1 signals to B7-1 on B cells.

In conclusion, we show that during chronic malaria, PD-1 mediates the loss and exhaustion of parasite-specific CD8⁺ T cells with some loss in the capacity of CD4⁺ T cells to function. T cell exhaustion affects protection against the parasite, and an improvement in T cell function alone is sufficient to control chronic malaria. Given the difficulties in developing antibody-based malaria vaccines, the role of CD8⁺ T cells requires consideration. Significantly, our data also provide rationale for considering CD8⁺ T cells as targets of future malaria vaccines and may need consideration for other diseases where vaccine development for chronic or prolonged infections is problematic.

EXPERIMENTAL PROCEDURES

Further details are provided in the [Supplemental Experimental Procedures](#).

Mice

Specific pathogen-free C57BL/6 (WT) mice were obtained from the Animal Resources Centre. PD-1 KO (*Pdcd1*^{-/-}) mice on a C57BL/6 background were kindly provided by T. Honjo through the Riken BRC ([Nishimura et al., 2001](#)). Mice were housed in the QIMR animal research facility, and all procedures were approved and monitored by the QIMR Animal Ethics Committee. Work was conducted under QIMR animal ethics approval number A0209-622M in accordance with the “Australian code of practice for the care and use of animals for scientific purposes” (Australian National Health and Medical Research Council).

Parasitic Infection and Monitoring

Cohorts of WT or PD-1 KO mice were inoculated intravenously with 10⁵ parasitized red blood cells (pRBCs) freshly obtained from C57BL/6 mice previously infected with *P. chabaudi chabaudi* AS (*P. chabaudi*). Tail-tip blood films were made every 1–2 days, stained using the Quick Dip modified Wright-Giemsa stain (Thermo Fisher Scientific), and examined for parasitemia, for up to 60 days. The percentage of pRBCs was assessed by counting at least 300 RBCs during parasitemia >1% or ~10,000 cells at other times. Mice were monitored daily for anemia and physical symptoms of disease, including posture (hunching), lack of activity, and fur texture. Mice were euthanized if they showed signs of significant distress.

In Vivo Depletion of CD4⁺ and CD8⁺ T Cells

For the depletion of CD4⁺ or CD8⁺ T cells, intraperitoneal injections of anti-CD4 (500 μ g; GK-1.5, Bio X Cell), anti-CD8 β (500 μ g; 53.5.8, Bio X Cell) or control rat IgG (500 μ g; Bio X Cell) were administered on day -1 and then every 3–4 days for 14–18 days. Mice were infected on day 0.

Tetramer Labeling

Parasite-specific F4 epitopes (H-2K^b restricted) previously described for *P. berghei* ANKA ([Lau et al., 2011](#)) and also present in *P. chabaudi* were used to produce phycoerythrin-labeled K^b-F4-tetramers ([Grotenbreg et al., 2008](#)). CD8⁺ T cells were labeled with these tetramers and a range of standard

CD8⁺ T-cell-related molecules. Refer to the [Supplemental Experimental Procedures](#) for expanded details.

In Vitro Analysis of T Cell Proliferation and Cytokine Secretion

CD4⁺ or CD8⁺ T cells were isolated from *P. chabaudi*-infected mice, and dendritic cells were isolated from naive mice (for use as antigen-presenting cells), using a Dynabeads Untouched Mouse T cells or DC kit (Life Technologies) and then immunomagnetically isolated using anti-mouse CD4, CD8, or CD11c Micro Beads (Miltenyi Biotec). Approximately 2×10^5 T cells, from individual mice, were cocultured with 2×10^4 DCs, with or without *P. chabaudi* antigen, in 8–12 replicate wells, for 4 days. On day 3, 20 μ M EdU (5-ethynyl-2'-deoxyuridine; a nucleoside analog that is incorporated into replicating DNA) (Life Technologies) was added to cultures. After 24 hr, EdU uptake was measured with a Click-it EdU Kit (Life Technologies). In addition, the supernatants were collected from parallel cultures of 10^6 T cells after 36 hr of culture (without EdU) and analyzed for cytokine secretion using the BD Cytometric Bead Array kit (BD Biosciences). Antigen-specific responses (proliferation and cytokine secretion) were calculated by subtracting nonspecific responses (no antigen) from responses in cultures with antigen. Assays to measure ex vivo secretion of IFN- γ and IL-10 by flow cytometry were purchased from Miltenyi Biotec.

ELISPOT Assay to Measure Peptide-Specific IFN- γ Production

PVDF micro plates (Millipore) were coated according to the manufacturer's directions using the Mouse IFN- γ ELISPOT Ready-Set-Go kit (eBioscience). Spleen cells from infected mice were cultured in 8–12 wells per treatment per sample in these plates with 20 μ g/ml peptide F4 or no peptide as previously described (Howland et al., 2013). After 36 hr, the plates were processed according to the manufacturer's instructions.

Isolation and Transfer of CD8⁺ T Cells

Cohorts of WT mice and PD-1 KO mice were infected with *P. chabaudi*. At day 21 p.i., CD8⁺ T cells were isolated from these mice (see above) and 3×10^5 or 3×10^6 cells were transferred intravenously into WT mice that had been infected 21 days prior. These recipient mice were then monitored for the development of recrudescence parasitemia and compared to control infected mice that had not been administered CD8⁺ T cells. For this isolation, CD8⁺ T cells were enriched from red-blood-cell-depleted spleen cells using a Dynabeads Untouched Mouse T Cells kit (Life Technologies) and then immunomagnetically isolated using anti-mouse CD8a (Ly-2) Micro Beads (Miltenyi Biotec). The samples were rerun on the AutoMacs (Miltenyi Biotec) until a purity of >98% was obtained.

Statistics

Except where otherwise noted, the data are shown as the mean \pm SEM. p values were determined using the nonparametric Mann-Whitney U test on pooled data from replicate experiments, analyzed using GraphPad Prism 5.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.11.002>.

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