Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection

Noah S Butler¹, Jacqueline Moebius², Lecia L Pewe¹, Boubacar Traore³, Ogobara K Doumbo³, Lorraine T Tygrett⁴, Thomas J Waldschmidt⁴,⁵, Peter D Crompton² & John T Harty¹,⁴,⁵

Infection of erythrocytes with *Plasmodium* species induces clinical malaria. Parasite-specific CD4⁺ T cells correlate with lower parasite burdens and severity of human malaria and are needed to control blood-stage infection in mice. However, the characteristics of CD4⁺ T cells that determine protection or parasite persistence remain unknown. Here we show that infection of humans with *Plasmodium falciparum* resulted in higher expression of the inhibitory receptor PD-1 associated with T cell dysfunction. In vivo blockade of the PD-1 ligand PD-L1 and the inhibitory receptor LAG-3 restored CD4⁺ T cell function, amplified the number of follicular helper T cells and germinal-center B cells and plasmablasts, enhanced protective antibodies and rapidly cleared blood-stage malaria in mice. Thus, chronic malaria drives specific T cell dysfunction, and proper function can be restored by inhibitory therapies to enhance parasite control.

Infection of red blood cells by *Plasmodium* species induces clinical malaria, a devastating global health problem that has been exacerbated by the emergence of drug-resistant parasites¹,². Thus, new approaches to combat malaria, such as effective vaccines or other immune interventions, are much needed. Given the clear correlation between high parasite density and disease severity in children³, much effort has gone into developing vaccines that target the blood stage of *Plasmodium* infection, with the goal of diminishing the morbidity and mortality of malaria. However, success has been limited, and candidate subunit vaccines in clinical trials have not proven highly efficacious thus far⁴-⁵, although studies of killed blood-stage parasites and specific adjuvant show promise in mouse models⁶. One reason for the limited progress in antimalarial vaccination probably relates to incomplete understanding of how the parasite evades adaptive immunity and the specific characteristics of the cellular immune responses that mediate protection against blood-stage *Plasmodium* infection. Whereas it is well understood from clinical human correlates⁷-⁹ and experimental rodent models¹⁰-¹³ that CD4⁺ T cells are a critical component of the protective immune responses that arise after exposure to blood-stage *Plasmodium* parasites, very little is known about how *Plasmodium*-specific CD4⁺ T cell responses influence the balance between parasite clearance and persistent blood-stage infection. Additionally, whether or how blood-stage *Plasmodium* infection influences the development of CD4⁺ follicular helper T cell (Tfh cell) responses, with subsequent and direct effects on humoral immunity, remains undefined.

In humans who survive infection with *Plasmodium falciparum* without treatment, parasites can be detected in the blood for several weeks or months¹⁴ and can also establish a chronic recrudescence of blood-stage infection that can persist for years¹⁵-¹⁷. The former scenario is mimicked in mouse models by *Plasmodium yoelii*, which establishes patent infections that last 30 d in immunocompetent hosts, whereas the latter feature is mimicked by *Plasmodium chaubadi*, which can establish persistent, subpatent infections that last for several months¹⁸. Of note, chronic viral infection in humans, such as infection with human immunodeficiency virus or hepatitis C virus, drives the functional exhaustion of antiviral T cells¹⁹-²¹, a phenomenon first described for CD8⁺ T cells in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (ref. 22). In the mouse LCMV model, repeated antigen stimulation through the T cell antigen receptor drives the sustained expression of T cell inhibitory receptors, including PD-1 and LAG-3, on virus-specific CD8⁺ T cells. Sustained signaling through these inhibitory receptors directly and indirectly induces transcriptional changes that negatively regulate proliferation and the expression of proinflammatory cytokines by virus-specific CD8⁺ T cells²²-²⁴. On the basis of such observations, we tested the hypothesis that humans exposed to *P. falciparum* would have CD4⁺ T cells with phenotypic characteristics of T cell exhaustion and that therapeutic blockade of signaling via T cell inhibitory receptors in vivo would markedly improve clinical outcomes in models of rodent malaria.

**RESULTS**

*Plasmodium* infection induces T cell exhaustion

To identify potential relationships between *P. falciparum* infection and exhaustion of circulating CD4⁺ T cells, we focused on a
Human and rodent malaria induce specific phenotypic and functional characteristics of CD4+ T cell exhaustion. (a) PD-1 expression by CD4+ T cells from Malian children before the malaria season (Before malaria) and 7 d after symptomatic Plasmodium falciparum infection (After malaria) and from malaria-naive US control subjects. Each symbol represents an individual subject; small horizontal lines indicate the mean. *P = 0.0002 and **P = 0.0001 (nonparametric Mann-Whitney test). (b) Survival of P. yoelii-infected wild-type C57BL/6 mice treated with rat IgG (rIgG), wild-type mice depleted of CD4+ T cells (α-CD4) or CD8+ T cells (α-CD8) on day 10, or C57BL/6 Aicda−/− mice lacking the immunoglobulin heavy-chain μ-chain secretory domain (Aicda−/−μs-KO; n = 5 mice per group). (c) Flow cytometry of peripheral blood leukocytes before (Naive) and 7 d after (PY pRBC) challenge with P. yoelii pRBCs, assessing upregulation of CD49d and CD11a to identify Plasmodium-specific, infection-induced CD4+ T cells. (d) Flow cytometry (left) of CD4+ T cells during prolonged blood-stage P. yoelii infection (n = 3 mice per group), and incorporation of the thymidine analog BrdU (right) by CD49dhiCD11ahi Plasmodium-specific CD4+ T cells (open curves) or CD49dloCD11alo naive CD4+ T cells (filled curves) after a BrdU pulse on days 4–8 (top) or days 27–30 (bottom). Numbers below bracketed lines (right) indicate percent cells that incorporated BrdU. (e) Flow cytometry analysis of PD-1 and LAG-3 and other markers at day 31 on splenic, pathogen-specific CD4+ T cells (open curves) and naive CD4+ T cells (filled curves) during acute LCMV infection (Armstrong (Arm) strain), chronic LCMV infection (strain c13) or prolonged blood-stage Plasmodium infection (PY pRBC; n = 3–5 mice per group). Numbers below bracketed lines indicate percent cells with marker expression. (f) Production of IFN-γ, tumor necrosis factor (TNF) and interleukin 2 (IL-2) in response to stimulation with PMA and ionomycin in pathogen-specific CD49dhiCD11ahi CD4+ T cells from mice (n = 4–5 per group) infected as in e. *P = 0.027, **P = 0.004 and ***P = 0.003 (two-tailed, unpaired Student’s t-test). Numbers adjacent to outlined areas (c–e) indicate percent CD49dhiCD11ahi cells. Data are representative of two (a,b), four (c) or three (d–f) experiments (error bars, s.d.).

A cohort study in Mali, where P. falciparum transmission is intense and seasonal, and occurs during the 6-month rainy period from July through December. Study participants were children 5–11 years of age who were analyzed at two time points: at the end of the dry season when they had a blood smear negative for P. falciparum (before malaria) and again 7 d after the diagnosis and treatment of the first symptomatic P. falciparum infection of the malaria season (after malaria; Fig. 1a). Consistent with our hypothesis, we observed higher frequencies of PD-1-expressing CD4+ T cells in children after infection with P. falciparum (Fig. 1a and Supplementary Fig. 1), which suggested that P. falciparum infection is associated with expression of the T cell inhibitory receptor PD-1 on CD4+ T cells in people with clinical malaria.

To address the biological relevance of those data, we turned to mouse models of blood-stage malaria. Initially we focused on the prolonged blood-stage infection (>30 d) induced by injection of mice with parasitized red blood cells (pRBCs) containing the normally nonlethal P. yoelii strain 17XLN. Studies of mice depleted of T cells suggested a critical role for CD4+ T cells in survival after challenge with red blood cells infected with P. yoelii (P. yoelii pRBCs; Fig. 1b). Additionally, mice deficient in the cytidine deaminase AID (Aicda−/−) with additional deletion of the immunoglobulin heavy-chain μ-secretory domain, which have mature B cells positive for immunoglobulin M (IgM) but cannot undergo isotype switching or secrete antibodies, also succumbed to blood-stage P. yoelii infection (Fig. 1b). Although not conclusive, these data were consistent with a critical role for secreted antibody in survival after challenge with P. yoelii pRBCs. Of note, the paucity of identified epitopes has hampered efforts to define the precise characteristics of CD4+ T cells that either determine protection or correlate with persistent blood-stage Plasmodium infection. A surrogate-activation-marker approach has been used to evaluate the total CD8+ T cell response to attenuated whole-Plasmodium sporozoite vaccines26. This approach allows monitoring of the total CD8+ T cell response to infection or vaccination in the absence of information about major histocompatibility complex (MHC) restriction, epitopes or antigens27. To test our hypothesis, we applied a modified surrogate-activation-marker approach that relies on coordinated upregulation of the integrins CD49d and CD11a on antigen-experienced CD4+ T cells to directly identify Plasmodium-specific CD4+ T cells responding to blood-stage infection. The utility of this approach has been extensively confirmed for viral infection28, and we further confirmed it by examining the expression patterns of CD49d and CD11a on CD4+ T cells of known specificity induced by infection with LCMV or Listeria monocytogenes. Indeed, all LCMV epitope–specific or
L. monocytogenes epitope–specific CD4+ T cells expressing interferon-γ (IFN-γ) had a CD49dhiCD11ahi cell surface phenotype (Supplementary Fig. 2a,b). Of note, because not all CD4+ T cells responding to LCMV or L. monocytogenes were specific for the two dominant epitopes examined (glycoprotein amino acids 61–80 (LCMV) or listeriolysin O amino acids 190–201 (L. monocytogenes)), and some antigen-specific CD4+ T cells may express cytokines other than IFN-γ, not all CD49dhiCD11ahi T cells expressed IFN-γ in these assays (Supplementary Fig. 2a,b).

Naïve C57BL/6 mice had a small fraction (~1%) of CD49dhiCD11ahi circulating CD4+ T cells before infection; however, infection of C57BL/6 mice with P. yoelii PBMCs resulted in the appearance of a large population (>20%) of circulating CD49dhiCD11ahi CD4+ T cells by day 7 after infection (Fig. 1c). Notably, expression of the CD49dhiCD11ahi phenotype by CD4+ T cells required crosslinking of T cell antigen receptors and was not a result of Plasmodium-induced inflammation because naïve transgenic CD4+ T cells specific for LCMV (SMARTA CD4+ T cells (with a T cell antigen receptor specific for LCMV glycoprotein amino acids 61–80)) did not upregulate CD49d or CD11a expression during blood-stage P. yoelii infection (Supplementary Fig. 2c). To address the specificity of these markers at the memory phase, we adoptively transferred purified P. yoelii–specific CD49dhiCD11ahi memory CD4+ T cells or CD49dloCD11alow naïve CD4+ T cells (both obtained >70 d after infection from mice that had cleared P. yoelii infection) into naive, allelically marked mice (~3.75%) and treated them with injections of chloroquine or vehicle control (PBS) on days 8 and 9 after infection. Two successive treatments with chloroquine resulted in a rapid and substantial decrease in parasite burden without complete parasite clearance (data not shown). We found that ~60% of ex vivo–stimulated, parasite-specific CD49dhiCD11ahi CD4+ T cells expressed IFN-γ at day 8 after infection (Fig. 2a). The capacity to produce IFN-γ was lowered to ~15% of parasite-specific CD4+ T cells by day 24 after infection in PBS control mice; however, chloroquine treatment to diminish parasitemia partially restored IFN-γ production (Fig. 2b). It also partially reversed the exhausted phenotype at day 24 after infection, with ~40% or ~70% lower fractions of parasite-specific CD4+ T cells expressing PD-1 or LAG-3, respectively (Fig. 2b). Similarly, chloroquine treatment resulted in a lower fraction of CD8+ T cells expressing inhibitory receptors and a greater fraction of CD8+ T cells able to produce cytokines (Supplementary Fig. 4). Notably, these data also demonstrated distinct phenotypic differences between CD4+ T cells and CD8+ T cells undergoing functional exhaustion during chronic infection, as we observed upregulation of the inhibitory receptors CD160 and 2B4 only on exhausted CD8+ T cells (Supplementary Fig. 4 and data not shown). Collectively, these data indicated that prolonged blood-stage P. yoelii infection elicited the phenotypic and functional

---

**Figure 2** Truncation of blood-stage P. yoelii infection with chloroquine reverses CD4+ T cell exhaustion. Expression of cell surface markers (left) and functional production of IFN-γ (far right) in spleen cells after *ex vivo* stimulation with PMA and ionomycin (PMA-ions) on day 8 (a) or day 24 (b) after infection of C57BL/6 mice (*n* = 5 per group) with 1 × 10⁹ P. yoelii PBMCs and treatment with PBS alone or chloroquine in PBS (80 mg per kg body weight) on days 8 and 9 after infection. Numbers adjacent to outlined events (%) indicate mean fluorescence intensity of staining. Data are representative of two experiments.
attenuates parasite-specific T cell exhaustion. They also showed that specific functional attributes of parasite-specific T cells (such as the ability to express IFN-γ) progressively deteriorated during prolonged blood-stage Plasmodium infection.

Blockade of PD-L1 and LAG-3 clears blood-stage malaria
PD-1 interacts with its ligands PD-L1 and PD-L2 and, in addition, PD-L1 can interact with the costimulatory molecule CD80 (refs. 30,31). LAG-3 mediates negative regulation through interactions with MHC class II (refs. 32,33). Notably, synergistic blockade of PD-L1 and LAG-3. Additionally, these data suggested that PD-L1 and LAG-3 acted synergistically to inhibit T cell responses during persistent blood-stage Plasmodium infection, we administered nondepleting monoclonal antibodies (anti-PD-L1 and anti-LAG-3) that prevent the inhibitory receptors PD-1 and LAG-3 from functionally engaging their ligands (PD-L1 and MHC class II, respectively) 23, 24, 33. To mimic therapeutic intervention of clinical malaria, we gave mice with matched large parasite burdens (~25-30% of RBCs contained parasites) at day 14 after infection regular injections of anti-PD-L1 and anti-LAG-3, or rat IgG as a control, and monitored clearance of parasites from the blood. We observed that blockade of PD-L1 and LAG-3 resulted in immediate control of parasite burdens and substantially accelerated parasite clearance (Fig. 3a), which correlated with many more parasite-specific CD4+ T cells (Fig. 3b) and restoration of parasite-specific CD4+ T cell cytokine responses (Supplementary Fig. 5). However, therapeutic blockade of PD-L1 and LAG-3 failed to improve parasite clearance in mice depleted of CD4+ T cells or Aicda−/− mice lacking the immunoglobulin heavy-chain μ-chain secretory domain (data not shown), consistent with a critical role for the CD4+ T cell–B cell axis in parasite control. Of note, blockade with anti-PD-L1 alone had a partial effect on parasite clearance, whereas anti-LAG-3 alone minimally influenced blood-stage infection but instead acted synergistically with anti-PD-L1 to accelerate parasite clearance (Supplementary Fig. 6). Collectively, these data showed markedly enhanced parasite control during blood-stage infection with Plasmodium after therapeutic blockade of PD-L1 and LAG-3. Additionally, these data suggested that PD-L1 and LAG-3, as well as their respective ligand-receptor interactions, acted synergistically to inhibit T cell responses during persistent blood-stage Plasmodium infection.
Blockade of PD-L1 and LAG-3 prevents chronic infection

To address whether the improved clinical outcomes after therapeutic blockade of PD-L1 and LAG-3 were system specific (P. yoelii and C57BL/6 mice) or could be generalized, we next evaluated clinical efficacy in outbred Swiss Webster mice, which more closely mimic the genetic complexity of humans. Therapeutic blockade of PD-L1 and LAG-3 also resulted in a rapid decrease in parasitemia and accelerated clearance of P. yoelii blood-stage infection in outbred Swiss Webster mice (Fig. 4a), which suggested that both T cell exhaustion and successful therapeutic blockade of blockade of PD-L1 and LAG-3 were independent of host genetic background and the targeted parasite antigens or epitopes. Additionally, we evaluated the effect of blockade of PD-L1 and LAG-3 on the clinical course of infection with Plasmodium chabaudi chabaudi, a malaria parasite known to establish long-term, subpatent infections in mice, as determined by the ability of transferred blood to establish new infections in naive mice34-36. The rationale for extending our studies to the P. chabaudi model was to address the effect of the blockade of inhibitory receptors on the clearance of persisting malaria parasites that are undetectable by blood smear, an important aspect of human malaria that is not recapitulated in the P. yoelii model. For those reasons, blood-stage infection of rodents with P. chabaudi is considered to be the small-animal model that most closely resembles persistent blood-stage Plasmodium infection in humans. Therapeutic blockade of PD-L1 and LAG-3 initiated at day 14 after infection did not have a discernable effect on clearance of patent blood-stage P. chabaudi infection in C57BL/6 mice (Fig. 4b). Of note, neither control mice treated with rat IgG nor mice treated by blockade of PD-L1 and LAG-3 showed detectable recrudescence of P. chabaudi on days 18-30 or on day 40 (limit of detection of parasitemia, <0.02%). However, blockade of PD-L1 and LAG-3 resulted in the complete elimination of persistent, subpatent P. chabaudi infection in most mice, as determined by the failure of whole-blood transfer to initiate blood-stage infection, which we observed for 100% of recipient mice that received blood from P. chabaudi–infected mice treated with rat IgG (Fig. 4c). Collectively, these data showed that the clinical benefits of therapeutic blockade of PD-L1 and LAG-3 could be generalized to outbred rodents and occurred independently of host immunogenetics and Plasmodium parasite species. Moreover, these data indicated that blockade of PD-L1 and LAG-3 induced sterilizing immunity even to low-grade, subpatent Plasmodium infection.

Blockade of PD-L1 and LAG-3 enhances Tfh and plasma cells

Given our results suggesting a critical role for the CD4+ T cell–B cell axis in clearance of blood-stage Plasmodium infection (Fig. 1b and data not shown), as well as published data showing modestly enhanced humoral immunity after in vivo PD-1 blockade37,38, we next evaluated the mechanistic effects of blockade of PD-L1 and LAG-3 on CD4+ Tfh cells, which regulate germinal-center B cell reactions necessary for the generation of high-affinity antibody responses39. Of note, blood-stage infection with P. yoelii resulted in the induction of CD150+CXCR5+ Tfh cells (Fig. 5a), as has been reported after chronic LCMV infection of mice40. However, blockade of PD-L1 and LAG-3 in P. yoelii–infected mice resulted in a doubling of the frequency of Tfh CD4+ T cells (Fig. 5a), which correlated with an increase of over 15-fold in the frequency of plasmablasts (CD19+B220+CD138+IgD−; Fig. 5b). In all, blockade of PD-L1 and LAG-3 resulted in sevenfold more total Tfh CD4+ T cells and 50-fold more plasmablasts in P. yoelii–infected mice (Fig. 5c). Thus, improved parasitic control after in vivo blockade of PD-L1 and LAG-3 was directly associated with enhanced Tfh cell numbers and plasma cell differentiation.

Blockade enhances germinal-center B cells and antibodies

To formally investigate the mechanistic role of B cells and antibody responses during blockade of PD-L1 and LAG-3, we next counted and characterized total and germinal-center B cells, as well as the functional production of serum immunoglobulins to Plasmodium. For mice with matched P. yoelii infection, those that received blockade therapy with anti-PD-L1 and anti-LAG-3 had tenfold more CD19+B220+ B cells (Fig. 6a), 20-fold more germinal-center B cells that stained with peanut agglutinin (PNAhi; Fig. 6b,c) and 30-fold more germinal-center B cells that had undergone class-switch recombination (Fig. 6b,c) than did control mice treated with rat IgG. Of note, the considerable expansion of the CD19+B220+PNAhi subset of cells observed after combined blockade of PD-L1 and LAG-3 (Fig. 6b) corresponded to B cell plasmablast populations (CD19+B220+CD138+IgD−; Fig. 5b). Consistent with the flow cytometry data, immunostaining of cryosectioned spleens 21 d after infection showed that mice treated with combined blockade of PD-L1 and LAG-3 had qualitatively improved splenic architecture marked by preservation of IgM+ B cell follicles and enhanced PNAhi germinal center formation relative to that of control mice treated with rat IgG (Supplementary Fig. 7). Additionally, we measured serum titers of immunoglobulins specific for the blood-stage Plasmodium antigen and human malaria vaccine.
candidate merozoite surface protein 1 (MSP119)11,41. In line with the improved Tfh cell and B cell responses, in vivo blockade of PD-L1 and LAG-3 resulted in much better control and accelerated parasite clearance after challenge with blood-stage P. yoelii parasites than did transfer of serum from naive mice treated with rat IgG (Fig. 6d). Notably, transfer of serum from mice that received therapy with anti-PD-L1 and anti-LAG-3 into naive mice resulted in much better control and accelerated parasite clearance after challenge with blood-stage P. yoelii parasites than did transfer of serum from mice treated with rat IgG (Fig. 6e). The finding that MSP119-specific IgG titers were only 2.5-fold higher in blockade-treated mice than in control mice suggested that MSP119-specific antibodies of other subclasses and/or antibodies specific for other blood-stage Plasmodium antigens contributed substantially to protection. Collectively, these data showed that therapeutic blockade of PD-L1 and LAG-3 during established Plasmodium blood-stage infection substantially improved the antiplasmodial humoral response through potent induction and secretion of protective antibodies.

**DISCUSSION**

Here we have shown that P. falciparum infection in humans was associated with more PD-1-expressing CD4+ T cells. We then showed that CD4+ T cell dysfunction, which negatively affects the induction of protective Plasmodium-specific antibody responses, was a major underlying factor for prolonged blood-stage Plasmodium infection in mice. We also found that the administration of PD-L1- and LAG-3-specific blocking antibodies markedly improved effector T cell and CD4+ Tfh cell responses, as well as antibody-producing B cell responses, which resulted in a rapid decrease in parasitemia and accelerated parasite clearance. Our results are consistent with a published study showing PD-1 expression on T cells during blood-stage P. yoelii infection in mice42, although the functional importance of this finding was not experimentally addressed. Of relevance, biological agents targeting similar inhibitory pathways that have been designed to improve T cell responses to neoplastic disease are now in clinical trials32,43. Thus, our results provide new insights into the mechanisms of parasite persistence and support the idea that alternative immune-based strategies may have clinically relevant applications in parasitic infections such as malaria.

Chronic or prolonged microbial infection and repeated antigenic stimulation are associated with functional exhaustion of CD8+ T cells in both humans30,31 and mice22-24. Although exhaustion of CD4+ T cells may be associated with infection of humans with human immunodeficiency virus19,44 or hepatitis C virus45, as well as persistent infection of mice with Mycobacterium tuberculosis46,47, direct evidence for the functional exhaustion of CD4+ T cells is lacking48. Blood-stage Plasmodium infection provided an opportunity for evaluating the biological importance of CD4+ T cell exhaustion, as these cells are critical for the suppression of parasite replication and full resolution of infection10-13. Indeed, we observed considerable improvement in T cell function after blood-stage infection that was truncated by chloroquine treatment, and we noted functional improvement in parasite-specific T cells after combined blockade of PD-L1 and LAG-3. The latter point provided conclusive evidence that engagement of PD-L1 with its receptors (PD-1 or CD80) and LAG-3 with its ligand (MHC class II) compromised the clearance of blood-stage malaria. Blocking these interactions restored functionality to exhausted CD4+ T cells, which resulted in accelerated clearance of infection. Thus, functional exhaustion of parasite-specific CD4+ T cells had a critical role in the persistence of blood-stage Plasmodium...
infection and provided a unique target for enhancing both the control and clearance of malaria parasites.

It is well established that the axis of CD4+ T cells and antibody-secreting B cells is critical for the resolution of nonlethal blood-stage Plasmodium infection in mice and is associated with protection against severe malaria in humans. Although the precise mechanisms remain to be defined, blockade of PD-L1 and LAG-3 resulted in greater numbers and enhanced function of effector CD4+ T cells, CD4+ TFH cells and antibody-secreting B cells. Of note, modestly enhanced antibody responses have been noted after in vivo PD-1 blockade in monkeys infected with simian immunodeficiency virus. We directly demonstrated that therapeutic blockade of PD-L1 and LAG-3 during an established blood-stage Plasmodium infection resulted in a 50-fold more plasmablasts secreting Plasmodium-specific antibodies. Notably, therapeutic blockade of PD-L1 and LAG-3 did not prevent lethal outcomes after Plasmodium yoelii infection in mice depleted of CD4+ T cells or Aicte+ mice in which the deletion of the immunoglobulin heavy-chain μ-chain secretory domain prevents antibody secretion (data not shown), which indicated that the likely targets of such blockade were CD4+ T cells and B cells.

Chronic LCMV infection drives the differentiation of virus-specific CD4+ T cells into Tfh cells, although the role of these cells in clearing viral infection remains unknown. Similarly, we observed the induction of Tfh1 cell differentiation during prolonged blood-stage Plasmodium infection. We found that blockade of PD-L1 and LAG-3 further increased the frequency of Tfh1 CD4+ T cells, which may have led to the observed enhancement of germinal-center B cell numbers, class switching to isotypic isotypes (such as opsonizing IgG2b) in B cells, and plasmablast differentiation. Thus, the improved clinical outcome after blockade of PD-L1 and LAG-3 in mice with established malaria was directly associated with the enhancement of both cellular and humoral immunity.

Finally, we showed that enhanced control of Plasmodium infection through blockade of PD-L1 and LAG-3 could be generalized to outbred Swiss Webster mice, which suggested that therapeutic blockade of PD-L1 and LAG-3 works independently of MHC alleles or the parasite antigens or epitopes targeted by B cells and CD4+ T cells. Of note, studies of phenotypic and functional CD4+ T cell exhaustion in outbred populations are possible only with the surrogate-activation-marker approach, which allows the identification and tracking of Plasmodium-specific CD4+ T cells. Furthermore, in mice persistently infected with Plasmodium chabaudi, blockade of PD-L1 and LAG-3 mediated sterilizing clearance of blood-stage infection, which suggested that even low, subpatent parasitemia negatively affected the host’s ability to clear the infection. This is of particular interest, as untreated humans can harbor subpatent Plasmodium falciparum infection for months or even years, and Plasmodium ovale and Plasmodium vivax parasites can persist as hypnozoites in the liver for years, putting people at risk for relapses of blood-stage infection. Although antigenic variation by Plasmodium contributes to immune evasion, we found that dysregulation of CD4+ T cell and B cell function was another mechanism by which Plasmodium parasites evaded the host immune response.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/reprints/index.html.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank the residents of Kambila, Mali, for their participation; F. Lund (University of Rochester) for C57BL/6 Aicte+ mice lacking the immunoglobulin heavy-chain μ-chain secretory domain; D.A.A. Vignali (St. Jude Children’s Research Hospital) for hybridoma clone C987W; and S. Perlman, N. Schmidt and V. Badovinac for comments. Supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases, the US National Institutes of Health (for work in Mali; and AI39551 and AI42767 for work in the J.T.H. laboratory) and the Department of Microbiology, University of Iowa (for work in the J.T.H. laboratory).

AUTHOR CONTRIBUTIONS

N.S.B. and J.M. designed the experiments, did the work, analyzed the data and wrote the manuscript; T.J.W., P.D.C. and J.T.H. designed the experiments, analyzed the data and wrote the manuscript; B.T. and O.K.D. coordinated the field studies and study site participants; and L.L.P. and J.T.T. did the histological studies and analyzed the data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/natureimmunology/reprints/index.html.
25. Weiss, G.E. et al. The Plasmodium falciparum-specific human memory B cell compartment expands gradually with repeated malaria infections. *PLoS Pathog.* 6, e1000912 (2010).

26. Butler, N.S.S. et al. Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. *Cell Host Microbe* 9, 451-462 (2011).

27. Rai, D., Pham, N.L., Harty, J.T. & Badovinac, V.P. Tracking the total CD8 T cell response to infection reveals substantial discordance in magnitude and kinetics between inbred and outbred hosts. *J. Immunol.* 183, 7672-7681 (2009).

28. McDermott, D. & Varga, S.M. Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. *J. Immunol.* 183, 390-400 (1999).

29. Oxenius, A., Bachmann, M.F., Zinkernagel, R.M. & Hengartner, H. Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur. J. Immunol.* 28, 397-401 (2007).

30. Brown, K.E., Freeman, G.J., Wherry, E.J. & Sharpe, A.H. Role of PD-1 in regulating acute infections. *Curr. Opin. Immunol.* 22, 397-401 (2010).

31. Sharpe, A.H., Wherry, E.J., Ahmed, R. & Freeman, G.J. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* 8, 239-245 (2007).

32. Goldberg, M.V. & Drake, C.G. LAG-3 in Cancer Immunotherapy. *Curr. Top. Microbiol. Immunol.* 344, 269-278 (2011).

33. Huang, C.T. et al. Role of LAG-3 in regulatory T cells. *Immunity* 21, 503-513 (2004).

34. Achtman, A.H., Stephens, R., Catman, E.T., Harrison, V. & Langhorne, J. Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi* chabaudi. *Parasite Immunol.* 29, 435-444 (2007).

35. Langhorne, J. The role of CD4+ T-cells in the immune response to *Plasmodium chabaudi*. *Parasitol. Today* 5, 362-364 (1989).

36. Meding, S.J. & Langhorne, J. CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi* chabaudi. *Eur. J. Immunol.* 21, 1433-1438 (1991).

37. Titanji, K. et al. Acute depletion of activated memory B cells involves the PD-1 pathway in rapidly progressing SIV-infected macaques. *J. Clin. Invest.* 120, 3878-3890 (2010).

38. Velu, V. et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 458, 206-210 (2009).

39. Orthy, S. Follicular helper CD4 T cells (Tfh). *Annu. Rev. Immunol.* 29, 621-663 (2011).

40. Fahey, L.M. et al. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J. Exp. Med.* 208, 987-999 (2011).

41. Wipasa, J. et al. Effect of *Plasmodium yoelii* exposure on vaccination with the 19-kidolation carboxyl terminus of merozoite surface protein 1 and vice versa and implications for the application of a human malaria vaccine. *Infect. Immun.* 77, 817-824 (2009).

42. Chandele, A., Mukerjee, P., Das, G., Ahmed, R. & Chauhan, V.S. Phenotypic and functional profiling of malaria-induced CD8 and CD4 T cells during blood-stage infection with *Plasmodium yoelii*. *Immunology* 132, 273-286 (2011).

43. Klene, J. & Gajewski, T.F. Distinct functions of antigen-specific CD4 T cells during murine *Plasmodium yoelii* infection. *Cell Host Microbe* 11, 1354-1359 (2010).

44. Porichis, F. et al. Responsiveness of HIV-specific CD4 T cells to PD-1 blockade. *Blood* 118, 965-974 (2011).

45. Golden-Mason, L. et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J. Virol.* 83, 9122-9130 (2009).

46. Barber, D.L., Mayer-Barber, K.D., Feng, C.G., Sharpe, A.H. & Sher, A. CD4 T cells promote rather than control tuberculosis in the absence of PD-1-mediated inhibition. *J. Immunol.* 186, 1598-1607 (2011).

47. Reiley, W.W. et al. Distinct functions of antigen-specific CD4 T cells during murine *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* 107, 19408-19413 (2010).

48. Wherry, E.J. T cell exhaustion. *Nat. Immunol.* 12, 492-499 (2011).

49. White, N.J. The treatment of malaria. *N. Engl. J. Med.* 335, 800-806 (1996).

50. Barford, L. et al. Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PFEMP1. *Proc. Natl. Acad. Sci. USA* 108, 12485-12490 (2011).