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

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Abstract

Plasmodium infection of erythrocytes induces clinical malaria. Parasite-specific $CD4^+$ T cells correlate with reduced parasite burdens and severity of human malaria, and are required 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 *P. falciparum* infection of humans increased expression of an inhibitory receptor (PD-1) associated with T cell dysfunction. *In vivo* blockade of PD-L1 and LAG-3 restored $CD4^+$ T cell function, amplified T follicular helper cell and germinal center B cell and plasmablast numbers, enhanced protective antibodies and rapidly cleared blood-stage malaria in mice. Thus, chronic malaria drives specific T cell dysfunction, which can be rescued to enhance parasite control using inhibitory therapies.

Introduction

Infection of red blood cells by *Plasmodium* species induces clinical malaria, a devastating global health problem that has been exacerbated by emergence of drug resistant parasites[1, 2]. Thus, new approaches to combat malaria, such as efficacious vaccines or other immune interventions, are desperately needed. Given the clear correlation between high parasite density and disease severity in children[3], much effort has gone into developing vaccination

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approaches that target the blood-stage of *Plasmodium* infection with the goal of reducing parasite burden and transmission. However, success has been limited and candidate subunit vaccines in clinical trials have thus far not proven highly efficacious[4, 5], although recent studies with killed blood-stage parasites and specific adjuvant show promise in mouse models[6]. One reason for the limited progress in anti-malarial vaccination likely relates to our incomplete understanding of how the parasite can evade adaptive immunity and the specific characteristics of cellular immune responses that can mediate protection against blood-stage *Plasmodium* infection. While it is well understood from both clinical human correlates[7-9], and experimental rodent models[10-13] that $CD4^+$ T cells are a critical component of protective immune responses that arise following exposure to blood-stage *Plasmodium* parasites, very little is known about how *Plasmodium*-specific $CD4^+$ T cell responses influence the balance between parasite clearance versus persistent blood-stage infection. Additionally, whether or how *Plasmodium* blood-stage infection influences the development of $CD4^+$ T follicular helper cell responses, with subsequent and direct effects on humoral immunity, remains undefined.

In humans that survive *Plasmodium falciparum* infection without treatment, parasites can be detected in the blood for several weeks or months[14] and can also establish a chronic-relapsing blood-stage infection that can persist for years[15-17]. The former scenario is mimicked in mouse models by *P. yoelii*, which establishes patent infections lasting 30 days in immunocompetent hosts, whereas the latter is mimicked by *P. chaubadi*, which can establish persistent, subpatent infections lasting for several months[18]. Importantly, chronic infection of humans with viruses such as HIV or HCV drives the functional 'exhaustion' of anti-viral T cells[19-21], a concept first revealed through studies of $CD8^+$ T cells in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (ref. [22]). In the murine LCMV model, repeated antigen stimulation through the T cell receptor (TCR) drives the sustained expression of T cell inhibitory receptors including programmed death-1 (PD-1) and lymphocyte activation gene-3 (LAG-3) on virus-specific $CD8^+$ T cells. Sustained signaling via these inhibitory receptors directly and indirectly induces transcriptional changes that negatively regulate proliferation and pro-inflammatory cytokine expression by virus-specific $CD8^+$ T cells[23, 24]. Based on these collective observations, we tested the hypothesis that humans exposed to *P. falciparum* would harbor $CD4^+$ T cells that exhibit phenotypic characteristics of T cell exhaustion, and that therapeutic blockade of T cell inhibitory receptor signaling *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 cohort study in Mali where the malaria season is intense and seasonal[25] and occurs during each six-month rainy period from July through December. Study participants consisted of children aged five to eleven years who presented as blood smear negative for *P. falciparum* at the end of the dry season and again seven days after the diagnosis and treatment of symptomatic *P. falciparum* infection (Before Malaria

and After Malaria, respectively, **Fig. 1a**). Consistent with our hypothesis, we observed elevated percentages of PD-1 expressing $CD4^+$ T cells in children after *P. falciparum* infection (**Fig. 1a** and **Supplementary Fig. 1**), suggesting that *P. falciparum* infection is associated with PD-1 T cell inhibitory receptor expression on $CD4^+$ T cells in individuals presenting with clinical malaria.

To address the biological relevance of these data we turned to mouse models of blood-stage malaria. Initially we focused on the prolonged (>30 day) blood-stage infection induced by injection of mice with parasitized red blood cells (pRBC) harboring the normally non-lethal *P. yoelii* (*Py*) strain 17XNL. T cell depletion studies strongly suggested a critical role for $CD4^+$ T cells in survival following *Py* pRBC challenge (**Fig. 1b**). Additionally *Aicda*$^{-/-}$ mice, which harbor an addition deletion of the *Igh* μ secretory domain that contain mature, IgM^+ B cells but cannot undergo isotype switching or secrete antibodies (hereafter called *Aicda*$^{-/-}$ μs$^{-/-}$), also succumbed to *Py* blood-stage infection (**Fig. 1b**). Although not conclusive, these data are consistent with a critical role for secreted antibody in survival following *Py* pRBC challenge. 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. We recently applied a surrogate activation marker approach to evaluate the total $CD8^+$ T cell response to attenuated whole sporozoite vaccines[26]. Importantly, this approach permits tracking of the total $CD8^+$ T cell response to infection or vaccination in the absence of information about MHC restriction, epitopes or antigens[27]. To test our current hypothesis, we applied a modified surrogate activation marker approach, which relies on the coordinate upregulation of CD49d and CD11a on antigen-experienced $CD4^+$ T cells, to directly identify *Plasmodium*-specific $CD4^+$ T cells responding to blood-stage infection. This approach has been extensively validated for virus infections[28] and was confirmed by examining CD49d and CD11a expression patterns on LCMV- or *Listeria monocytogenes* (LM)-induced $CD4^+$ T cell responses of known specificity. Indeed, all LCMV and LM epitope-specific, interferon-γ (IFN-γ) expressing $CD4^+$ T cells exhibited the $CD49d^{hi}CD11a^{hi}$ cell surface phenotype (**Supplementary Fig. 2a,b**). Of note, because not all $CD4^+$ T cells responding to LCMV or LM were specific for the two examined dominant epitopes ($GP_{61\text{-}80}$ and $LLO_{190\text{-}201}$, respectively), and some antigen-specific $CD4^+$ T cells may express cytokines other than IFN-γ, not all $CD49d^{hi}CD11a^{hi}$ T cells expressed IFN-γ in these assays (**Supplementary Fig. 2a,b**).

Naïve C57BL/6 mice exhibited a small fraction (~1%) of $CD49d^{hi}CD11a^{hi}$ circulating $CD4^+$ T cells prior to infection, however infection of C57BL/6 mice with *Py*-parasitized red blood cells (*Py* pRBC) resulted in the appearance of a large population (>20%) of circulating $CD49d^{hi}CD11a^{hi}$ $CD4^+$ T cells by day 7 post-infection (p.i.) (**Fig. 1c**). Importantly, $CD4^+$ T cell expression of the $CD49d^{hi}CD11a^{hi}$ phenotype required TCR crosslinking and was not a result of malaria infection-induced inflammation, because naïve transgenic $CD4^+$ T cells specific for LCMV ($GP_{61\text{-}80}$-specific, SMARTA $CD4^+$ T cells)[29] did not upregulate CD49d or CD11a expression during *P. yoelii* blood-stage infection (**Supplementary Fig. 2c**). To address the specificity of these markers at the memory phase (>60 days post-*Py* infection), we adoptively transferred purified *Py*-specific

CD49d^{hi}CD11a^{hi} memory CD4$^+$ T cells, or CD49d^{lo}CD11a^{lo} naïve CD4$^+$ T cells (both obtained >70 days p.i. from mice that had cleared *Py* infection) into new naïve, allelically marked recipients. Importantly, *Py* infection of recipient mice resulted in secondary T cell expansion only from transferred CD49d^{hi}CD11a^{hi} memory cells, but not CD49d^{lo}CD11a^{lo} naïve CD4$^+$ T cells (**Supplementary Fig. 2d,e**). Thus, the CD49d^{hi}CD11a^{hi} phenotype faithfully and durably identifies *Plasmodium*-specific effector and memory CD4$^+$ T cells.

Using this approach we determined that *Py* infection of mice results in substantial CD4$^+$ T cell responses, and these cells exhibited sustained proliferation over the course of the 30-day period of patent blood-stage infection (**Fig. 1d**). By day 30 p.i, we also observed that a large fraction (>25-40%) of *Py*-specific, CD49d^{hi}CD11a^{hi} CD4$^+$ T cells expressed the T cell inhibitory receptors PD-1 and LAG-3, similar to CD4$^+$ T cells responding to chronic, but not resolved, LCMV infection (**Fig. 1e**). Of note, the fraction of CD4$^+$ T cells expressing PD-1 in mice with clinical malaria (~3.75%) is in line with our observations in *P. falciparum*-infected humans, where we observed ~3.5% of all CD4$^+$ T cells, on average, expressed PD-1 (**Fig. 1a**). Furthermore, and consistent with their exhausted phenotype, *Py*-specific CD4$^+$ T cells also exhibited impaired cytokine (IFN-γ, tumor necrosis factor (TNF) and interleukin 2, IL-2) production in response to phorbol ester (PMA) plus ionomycin stimulation, relative to virus-specific CD4$^+$ T cells from mice either acutely or chronically infected with LCMV on day 31 p.i. (**Fig. 1f**). Additionally, parasite-specific CD8$^+$ T cells (identified by modulation of surface CD8α and CD11a[27]) also exhibited sustained proliferation throughout the infection and both the phenotypic and functional characteristics of T cell exhaustion at day 30 p.i. (**Supplementary Fig. 3**). Collectively, these data show that prolonged *Plasmodium* blood-stage infection elicits dysfunctional parasite-specific T cells including the critical CD4$^+$ T cell subset required for eventual clearance of infection.

Prolonged infection leads to T cell exhaustion

To directly evaluate whether prolonged *Plasmodium* blood-stage infection was responsible for the appearance of dysfunctional populations of T cells in mice with clinical malaria, we treated groups of *P. yoelii* infection-matched mice with injections of chloroquine, or vehicle control (PBS), on days 8 and 9 post-infection. Two successive chloroquine treatments resulted in rapid and substantial reductions in parasite burden without complete parasite clearance (data not shown). We found that ~60% of *ex vivo*-stimulated, parasite-specific CD49d^{hi}CD11a^{hi} CD4$^+$ T cell express IFN-γ at day 8 p.i. (**Fig. 2a, right panel**). The capacity to produce IFN-γ was reduced to ~15% of parasite-specific CD4$^+$ T cells by d 24 p.i. in PBS control mice, however chloroquine treatment partially restored IFN-γ production (**Fig. 2b, right panels**). Importantly, chloroquine treatment also partially reversed the exhausted phenotype at day 24 p.i., with ~40% and ~70% reductions in the fraction of parasite-specific CD4$^+$ T cells expressing PD-1 or LAG-3 (**Fig. 2b, middle panels**). Similar to CD4$^+$ T cells, chloroquine treatment reduced the fraction of CD8$^+$ T cells expressing inhibitory receptors, as well as increased the fraction of CD8$^+$ T cells capable of cytokine production (**Supplementary Fig. 4**). Of note, these data also reveal that distinct phenotypic differences exist between CD4$^+$ and CD8$^+$ T cells undergoing functional exhaustion during chronic infection, as upregulation of the inhibitory receptors CD160 and 2B4 was only observed on exhausted CD8$^+$ T cells (**Supplementary Fig. 4** and data not shown).

Collectively, these data highlight that sustained high parasite burdens in mice with prolonged *P. yoelii* blood-stage infection elicit the phenotypic and functional attributes of parasite-specific T cell exhaustion. These data also show that specific functional attributes (for example, the capacity to express IFN-γ) of parasite-specific T cells progressively deteriorate during prolonged blood-stage *Plasmodium* infection.

Blocking PD-L1 + LAG-3 clears blood-stage malaria

The inhibitory receptor PD-1 interacts with PD-L1 and PD-L2, and in addition, PD-L1 can interact with CD80 (refs 30, 31). LAG-3 mediates negative regulation through interactions with MHC class II[32, 33]. Importantly, synergistic blockade of T cell inhibitory receptor interactions improves $CD8^+$ T cell function and virus control during chronic LCMV cl13 infection[24]. Thus, to address a biological role for inhibitory receptor expression by parasite-specific T cells during prolonged *Plasmodium* blood-stage infection, we administered non-depleting monoclonal antibodies (anti-PD-L1 and anti-LAG-3) that prevent PD-1 and LAG-3 inhibitory receptors from functionally engaging their major ligands (PD-L1 and MHC class II, respectively)[23, 24, 33]. To mimic therapeutic intervention of clinical malaria, mice with matched, high parasite burdens (~25-30% of RBC harbored parasites) at day 14 p.i. were given regular injections of anti-PD-L1 and anti-LAG-3, or control rIgG, and monitored for parasite clearance from the blood. We observed that PD-L1 and LAG-3 blockade resulted in immediate control of parasite burdens and substantially accelerated parasite clearance (**Fig. 3a**), which correlated with substantially increased parasite-specific $CD4^+$ T cell numbers (**Fig. 3b**) and restored parasite-specific $CD4^+$ T cell cytokine responses (**Fig. 3c,d**). PD-L1 and LAG-3 blockade similarly restored both numerical and functional features of parasite-specific $CD8^+$ T cell responses (**Supplementary Fig. 5**). Importantly, therapeutic PD-L1 and LAG-3 blockade failed to improve parasite clearance in $CD4^+$ T cell-depleted or *Aicda*$^{-/-}$ μs$^{-/-}$ mice (data not shown), consistent with a critical role for the $CD4^+$ T cell–B cell axis in PD-L1 and LAG-3 blockade-enhanced parasite control. Of note, anti-PD-L1 blockade alone had a partial effect on parasite clearance, while anti-LAG-3 alone minimally influenced blood-stage infection but rather acted synergistically with anti-PD-L1 to reduce parasite burden and accelerate parasite clearance (**Supplementary Fig. 6**). Collectively, these data show markedly enhanced parasite control during blood-stage *Plasmodium* infection following therapeutic blockade of PD-L1 and LAG-3. Additionally, these data suggest that PD-L1 and LAG-3, and their respective ligand-receptor interactions, act synergistically to inhibit T cell responses during persistent *Plasmodium* blood-stage infection.

PD-L1 + LAG-3 blockade prevents chronic infection

To address whether improved clinical outcomes following therapeutic PD-L1 and LAG-3 blockade are system-specific (*P. yoelii* and C57BL/6 mice) or generalizable, we next evaluated clinical efficacy in outbred Swiss Webster mice, which more closely mimic the genetic complexity of humans. Importantly, therapeutic blockade of PD-L1 and LAG-3 also rapidly reduced parasitemia and accelerated clearance of *P. yoelii* blood-stage infection in outbred Swiss Webster mice (**Fig. 4a**), suggesting that both T cell exhaustion and successful therapeutic PD-L1 and LAG-3 blockade are independent of immunogenetics, background genes and the targeted parasite antigens or epitopes. Additionally, we evaluated the impact

of therapeutic PD-L1 and LAG-3 blockade on the clinical course and persistence of *Plasmodium chabaudi chabaudi* (*Pcc*), 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 naïve mice[34-36]. The rationale for extending our studies to the *P. chabaudi* model was to address the impact of inhibitory receptor blockade on the clearance of persisting malaria parasites that are undetectable by blood-smear, which is an important aspect of human malaria that is not recapitulated in the *P. yoelii* model. For these reasons, *Pcc* blood-stage infection of rodents is widely believed to be the small animal malaria model most relevant for understanding persistent *Plasmodium* blood-stage infection of humans. Therapeutic PDL1 and LAG-3 blockade initiated at day 14 p.i. did not have a discernable effect on clearance of patent *Pcc* blood-stage infection in C57BL/6 mice (**Fig. 4b**). Of note, neither control rIgG-treated nor anti-PD-L1 plus anti-LAG-3 blockade-treated mice showed detectable *Pcc* recrudescence from days 18 through 30, or on day 40 (limit of detection of parasitemia <0.02%). However anti-PD-L1 plus anti-LAG-3 blockade resulted in the complete elimination of persistent, subpatent *Pcc* infection in the majority of mice as determined by the failure of whole blood transfers to initiate blood-stage infection, which was observed for 100% of recipient mice receiving blood from rIgG-treated, *Pcc* infected mice (**Fig. 4c**). Collectively, these data show that the clinical benefits of therapeutic PD-L1 and LAG-3 blockade are generalizable to outbred rodents and occur independently of both host immunogenetics and *Plasmodium* parasite species. Moreover, these data highlight that immunity to even low-grade, subpatent *Plasmodium* infection can be enhanced by PD-L1 and LAG-3 blockade to mediate sterilizing parasite clearance.

PD-L1 + LAG-3 blockade enhances T_{FH} 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,** data not shown), as well as recent data showing modestly enhanced humoral immunity following *in vivo* anti-PD-1 blockade[37, 38], we next evaluated the mechanistic effects of PD-L1 and LAG-3 blockade on T follicular helper (T_{FH}) $CD4^+$ T cells, which regulate germinal center B cell reactions necessary for the generation of high-magnitude and quality antibody responses[39]. Of note, blood-stage *Py* infection resulted in the induction of $CD150^{lo}CXCR5^+$ T_{FH} cells (**Fig. 5a,c**), as was recently reported following chronic LCMV infection of mice[40]. However, we observed a 7-fold enhancement of T_{FH} $CD4^+$ T cell numbers (**Fig. 5a,c**) and a 50-fold enhancement of plasmablast ($CD19^{lo}B220^{lo}CD138^{hi}IgD^-$) B cell numbers (**Fig. 5b,c**) in *Py*-infected mice receiving combined PD-L1 and LAG-3 blockade. Thus, improved parasite control following *in vivo* PD-L1 and LAG-3 blockade is directly associated with enhanced T_{FH} cell numbers and substantial induction of plasma cell differentiation.

Blockade enhances plasmablasts and antibodies

To formally investigate a mechanistic role for B cells and antibody responses during PDL1 and LAG-3 blockade, we next enumerated and characterized total and germinal center B cells as well as the functional production of anti-*Plasmodial* serum immunoglobulins. Relative to rIgG-treated control mice, *P. yoelii*-infection matched mice receiving anti-PD-L1 and anti-LAG-3 *in vivo* blockade therapy exhibited 10-fold greater absolute numbers of

$CD19^+B220^+$ B cells (**Fig. 6a**), 20-fold increases in the absolute number of peanut agglutinin-staining (PNA^{hi}) germinal center B cells (**Fig. 6b,c**) and 30-fold increases in the total number of germinal center B cells that had undergone class switch recombination (**Fig. 6b,c**). Of note, the marked expansion of the $CD19^{lo}B220^{lo}PNA^{int}$ subset of cells observed following PD-L1 and LAG-3 co-blockade (**Fig. 6b, bottom left**) corresponds to B cell plasmablast populations ($CD19^{lo}B220^{lo}CD138^{hi}IgD^-$; **Fig. 5b**). Consistent with the flow cytometric data, immunostaining of cryosectioned spleens from d21 p.i. control rIgG- and blockade-treated mice revealed qualitatively improved splenic architecture marked by preservation of IgM^+ B cell follicles and enhanced PNA^+ germinal center formation (**Supplementary Fig. 7**). Additionally, we directly evaluated titers of serum immunoglobulins against the blood-stage *Plasmodium* parasite antigen and human malaria vaccine candidate, merozoite surface protein 1 ($MSP1_{19}$)[11, 41]. In line with improved T_{FH} and B cell responses, *in vivo* combinatorial blockade of PD-L1 and LAG-3 resulted in the generation of higher-magnitude parasite-specific anti-$MSP1_{19}$ IgG antibody responses (**Fig. 6d**). Importantly, compared to serum from rIgG-treated mice, passive transfer of serum from mice that received anti-PD-L1 and anti-LAG-3 blockade therapy to naïve mice resulted in substantially better control and accelerated parasite clearance following challenge with *Py* blood-stage parasites (**Fig. 6e**). Given that total anti-$MSP1_{19}$ IgG titers were only 2.5 fold different between control and blockade-treated mice, these observations suggest that specific anti-$MSP1_{19}$ antibody subclasses and/or antibodies targeting other blood-stage parasite proteins also significantly contribute to protection following PD-L1 and LAG-3 combinatorial blockade. Collectively, these data show that therapeutic PD-L1 and LAG-3 blockade during established clinical malaria also substantially improves the anti-*Plasmodial* humoral response via potent induction and secretion of protective antibodies.

Discussion

Here, we show that humans with clinical malaria upregulate expression of the inhibitory receptor PD-1 on their $CD4^+$ T cells. We then show that $CD4^+$ T cell dysfunction, which negatively impacts the induction of protective *Plasmodium*-specific antibody responses, is a major underlying factor for prolonged blood-stage *Plasmodium* infections in mice. We also found that administration of PD-L1 + LAG-3 specific blocking antibodies markedly improved effector and T_{FH} $CD4^+$ T cell responses and antibody producing B cell responses, resulting in rapid control of parasite burden and accelerated parasite clearance. Our results are consistent with a previous study showing PD-1 expression on T cells during *P. yoelii* blood-stage infection of mice[42], although the importance of this finding was not experimentally addressed. Of relevance, similar biologics targeting inhibitory pathways are already in clinical trial to improve T cell function during neoplastic disease[32, 43]. Thus, our results reveal new information relevant to the mechanisms of parasite persistence and also have potentially direct relevance for alternative immune-based strategies to combat the malaria global health epidemic.

Chronic or prolonged microbial infection, and subsequent repeated antigenic stimulation, has been associated with functional exhaustion of $CD8^+$ T cells in both humans[20, 21] and mice[22-24]. Although $CD4^+$ T cell exhaustion also likely occurs in humans persistently

infected with HIV[19, 44] or HCV[45], and $CD4^+$ T cell inhibitory receptor expression may contribute to persistent *Mycobacterium tuberculosis* infection of mice[46, 47], direct evidence the relevance of functional T cell exhaustion is limited to $CD8^+$ T cells[48]. On the other hand, *Plasmodium* blood-stage infection provides unique opportunities to evaluate the biological significance of $CD4^+$ T cell exhaustion, as these cells are critical for suppression of parasite replication and full resolution infection[10-13]. Indeed, here we report marked improvement of T cell function when blood-stage infection is truncated by chloroquine treatment, and we see functional improvements in parasite-specific T cells following PD-L1 and LAG-3 co-blockade. This latter point provides conclusive evidence that these inhibitory receptor interactions compromise clearance of blood-stage malaria. Blocking these interactions restores functionality to exhausted $CD4^+$ T cells, resulting in accelerated clearance of infection. Thus, functional exhaustion of parasite-specific $CD4^+$ T cells plays a critical role in the persistence of blood-stage *Plasmodium* infection and provides a unique target to enhance both the control and clearance of malaria parasites.

It is well established that the $CD4^+$ T cell-antibody secreting B cells axis is critical for resolving non-lethal *Plasmodium* blood-stage infections in mice and is highly associated with protection against severe malaria in humans. While the precise mechanisms remain to be defined, PD-L1 and LAG-3 blockade results in robust numerical and functional enhancement of effector $CD4^+$ T cell, T_{FH} $CD4^+$ T cell and antibody secreting B cell responses. Of interest, a previous report described modestly enhanced humoral immunity following *in vivo* PD-1 blockade in SIV-infected monkeys[37, 38]. However, here we directly demonstrate that therapeutic PD-L1 and LAG-3 blockade during clinical malaria resulted in ~50-fold increases in the number of plasmablasts capable of secreting potent *Plasmodium*-specific antibodies. Of note, therapeutic PD-L1 and LAG-3 blockade does not prevent lethal outcomes following *P. yoelii* infection of mice depleted of $CD4^+$ T cells or in *Aicda*$^{-/-}$ $\mu s^{-/-}$ mice lacking secreted antibodies (data not shown). Thus, the major and relevant functional targets of therapeutic PD-L1 and LAG-3 blockade are likely $CD4^+$ T cells and B cells.

Recent data revealed that chronic LCMV infection directs the differentiation of virus-specific $CD4^+$ T cells into T_{FH} cells[40], although the role these cells play in clearing virus remains unknown. Similarly, we also observe the induction of T_{FH} cell differentiation during prolonged blood-stage *Plasmodium* infection. However, we also show that PD-L1 and LAG-3 blockade results in substantially expanded numbers of the T_{FH} $CD4^+$ T cell subset, which likely drive the attending increases in germinal center B cell numbers, B cell class switching to cytophilic (for example, opsonizing IgG2b) isotypes, and plasmablast differentiation that are not observed in control mice. Thus, the improved clinical outcomes of PD-L1 and LAG-3 blockade in mice with established malaria are directly associated with multifaceted enhancement of both cellular and humoral immunity.

Lastly, our data also highlight the generalizability and potency of therapeutic PDL1 and LAG-3 blockade during clinical malaria. Indeed, our data show improved clinical outcomes in outbred Swiss Webster mice, suggesting that therapeutic PD-L1 and LAG-3 blockade stimulates more potent anti-*Plasmodial* responses independent of MHC alleles or the parasite antigens and epitopes targeted by B cells and $CD4^+$ T cells, respectively. Importantly, due to immunogenetic complexities, studies of phenotypic and functional $CD4^+$

T cell exhaustion in outbred populations are only possible using the surrogate activation marker approach to identify and track *Plasmodium* blood-stage specific $CD4^+$ T cells responses. Furthermore, in mice persistently infected with *Pcc*, which establishes low-grade, subpatent infection of mice, PD-L1 and LAG-3 blockade mediated sterilizing clearance of blood-stage infection, suggesting that even low numbers of parasites or antigen persistence negatively impacts the ability of the host immune response to eliminate *Plasmodium* parasites. This later observation is of particular interest as humans can harbor subpatent *P. falciparum* infections for months or even years in the absence of clinical intervention[14-17], and *Plasmodium ovale* and *Plasmodium vivax* spp. can persist as hypnozoites[49] capable of generating recurring blood-stage infection. Thus, in addition to well-described mechanisms of evolution and antigenic variation during human infection with *Plasmodium* parasites[50], our data reveal a mechanism by which malaria parasites can evade the protective host immune responses through dampening and dysregulating $CD4^+$ T cell and B cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Methods

Mali study site and participants

This study was conducted in Kambila, Mali, a rural village where *P. falciparum* transmission is seasonal and intense from July through December. The present study analyzed PBMCs collected from children aged 5 to 11 years who had a blood smear negative for *P. falciparum* at a cross-sectional visit at the end of the dry season (n = 12; May 2009), and again 7 days after the diagnosis and treatment of acute malaria (n =2 7) during the subsequent transmission season, defined as an axillary temperature ≥37.5°C, asexual parasitemia ≥5000 parasites/μl of blood, and no other cause of fever discernable on physical examination. Ten children had paired PBMC samples available at both time points. The ethics committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, and the Institutional Review Board (IRB) at the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) approved this study (NIAID protocol number 06-I-N147). Written, informed consent was obtained from the parents or guardians of participating children.

US blood donors

PBMCs from nine healthy adult blood bank donors in the U.S. were analyzed. Demographic and travel history data were not available from these anonymous donors but prior *P. falciparum* exposure is unlikely. Blood samples were obtained for research use after written

informed consent was obtained from all study participants enrolled in a protocol approved by the IRB of NIAID, NIH (protocol # 99-CC-0168).

Human T cell staining and flow cytometry

Blood samples were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD, Vacutainer CPT Tubes). PBMCs were isolated according to the manufacturer's instructions and frozen in fetal bovine serum (FBS) containing 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich), kept at −80°C for 24 h, and then stored at −196°C in liquid nitrogen until use. Cryopreserved PBMCs were stained for the expression of CD4 and PD-1 for 30 min at 4°C in the dark, using anti-CD4 (clone RPA-T4, BD Biosciences) and anti-CD279 (clone MIH4, BD Biosciences). Fluorescence was measured on lymphocytes gated by forward and side scatter on the LSRII™ flow cytometer using FACSDiva software. Analysis was performed using FlowJo software (Tree Star).

Mice and parasites

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 *Aicda*$^{-/-}$μs$^{-/-}$ mice[51] were a gift from F. Lund (University of Rochester). Mice were housed at the University of Iowa under the appropriate biosafety level. The University of Iowa Animal Care and Use Committee approved animal experiments. Mice were infected intravenously with ~10,000 to 100,000 cryopreserved *P. yoelii* (*Py*) 17XNL (non-lethal) or freshly passaged *P. chaubaudi chabaudi* (*Pcc*) blood-stage parasites. Parasitized red blood cells in challenged mice were identified by Giemsa stain of thin blood smears at 2 day intervals.

Mouse T cell staining and flow cytometry

Spleens were harvested on the indicated days and cells were stimulated for 3.5 h in the presence of Brefeldin A, 5 ng/ml PMA and 500 ng/ml ionomycin. Cells were subsequently surface stained with antibodies to mouse-Ter119 (Ter119), CD45.2 (104), CD4 (RM4-5), CD49d (R1-2), CD11a (M17/4) for CD4$^+$ T cell analyses, or antibodies to mouse-Ter119, CD45.2, CD8α (53-6.7), and CD11a for CD8$^+$ T cell analyses, followed by permeabilization and intracellular staining with anti-mouse-IFN-γ (XMG1.2) and TNF (XT-22) or IL-2 (JES6-5H4). For T follicular helper cell analyses, splenocytes were incubated for 30 min at 25°C with anti-mouse-CXCR5-biotin (2G8), washed extensively, and subsequently stained for 60 min at 4°C in the presence of fluorochrome conjugated anti-mouse-CD4, -CD49d, -CD11a and -CD150 (TC15-12F12.2). Cells were washed again and incubated in the presence of streptavidin-APC for 20 min at 4°C. For germinal center B cell analyses, splenocytes were incubated with anti-mouse-IgG2b-biotin (LO-MG2b-2), washed and subsequently stained with fluorochrome conjugated antibodies to mouse-Ter119, CD45.2, CD19 (6D5), B220 (6B2), IgM (b76), IgD (11-26c.2a), CD138 (281-2), and FITC-conjugated peanut agglutinin (FITC-PNA). Cells were washed extensively and further incubated in the presence of streptavidin-APC for 20 min at 4°C. For all mouse antibody staining protocols, cells were pre-incubated with anti-CD16/CD32 (2.4G2) to block non-specific Fc receptor binding. All mouse antibodies and reagents were obtained from

Biolegend, BD Pharmingen or Vector Laboratories. Cells were analyzed using BD FACSCanto or LSR II instruments. Data were analyzed using FLOWJO Software (Tree Star, Inc.).

T cell inhibitory receptor blockade

200 μg of control rat IgG (Sigma), anti-PD-L1 (clone 10F.9G2, BioXCell), anti-LAG-3 (prepared from hybridoma clone C9B7W, a gift from D. A. A. Vignali), or a combination of anti-PD-L1 and anti-LAG-3, were injected intravenously into blood-stage parasitized mice every three days beginning on d14 p.i. for approximately two weeks.

T cell depletions

Mice were depleted of $CD4^+$ or $CD8^+$ T cells via two intraperitoneal injections of 400 μg of GK1.5 or 2.43 antibodies, respectively. Depletions were verified by staining peripheral blood mononuclear cells from each individual mouse with anti-CD4 (RM4-5) and anti-CD8 (53-6.7).

BrdU incorporation

Groups of mice were infected with 10^5 *P. yoelii*-parasitized red blood cells. Mice were subsequently injected with 2 mg BrdU (Sigma) and placed on 0.8 mg/ml BrdU in drinking water for a total of four days. Spleens were harvested on the indicated days and parasite-specific ($CD49d^{hi}CD11a^{hi}$) and naïve ($CD49d^{lo}CD11a^{lo}$) $CD4^+$ T cells, and parasite-specific ($CD11a^{hi}CD8\alpha^{lo}$) and naïve ($CD11a^{lo}CD8\alpha^{hi}$) $CD8^+$ T cells were stained intracellularly for BrdU incorporation as described[52].

Immunofluorescence staining of frozen spleen sections

Spleens were excised and incubated for 30 min on ice in phosphate buffer containing 20% (vol/vol) sucrose. Tissues were subsequently flash frozen in isopentane using liquid nitrogen and then cryosectioned. 8 μm sections were air-dried and fixed with acetone, followed by rehydration and blocking for 1 h with PBS containing 10% rat serum and 10% anti-CD16/CD32 (2.4G2). Sections were stained for 1 h with FITC-anti-mouse IgM (prepared and conjugated from clone b76 hybridoma) and biotinylated PNA (Vector Laboratories) washed extensively then incubated with streptavidin conjugated Alexa Fluor 549 (Invitrogen). After additional extensive washing, samples were imaged with an LSM 510 Zeiss laser-scanning confocal microscope.

$MSP1_{19}$ ELISA

Sera from *Py* blood-stage infected mice that were subsequently treated with rIgG or anti-PD-L1 and anti-LAG-3 from days 14 to 32 were collected on day 41 p.i. Dilutions of sera were reacted against purified recombinant $MSP1_{19}$ immobilized in Maxisorb Immunoplates essentially as described[26]. Total $MSP\text{-}1_{19}$-specific IgG antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Jackson

ImmunoResearch) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma). Data are expressed as average endpoint titers with absorbance readings below 0.2 (A_{405}).

Statistical Analyses

Data were analyzed using GraphPad Prism4 software. Specific tests of statistical significance are detailed in figure legends.

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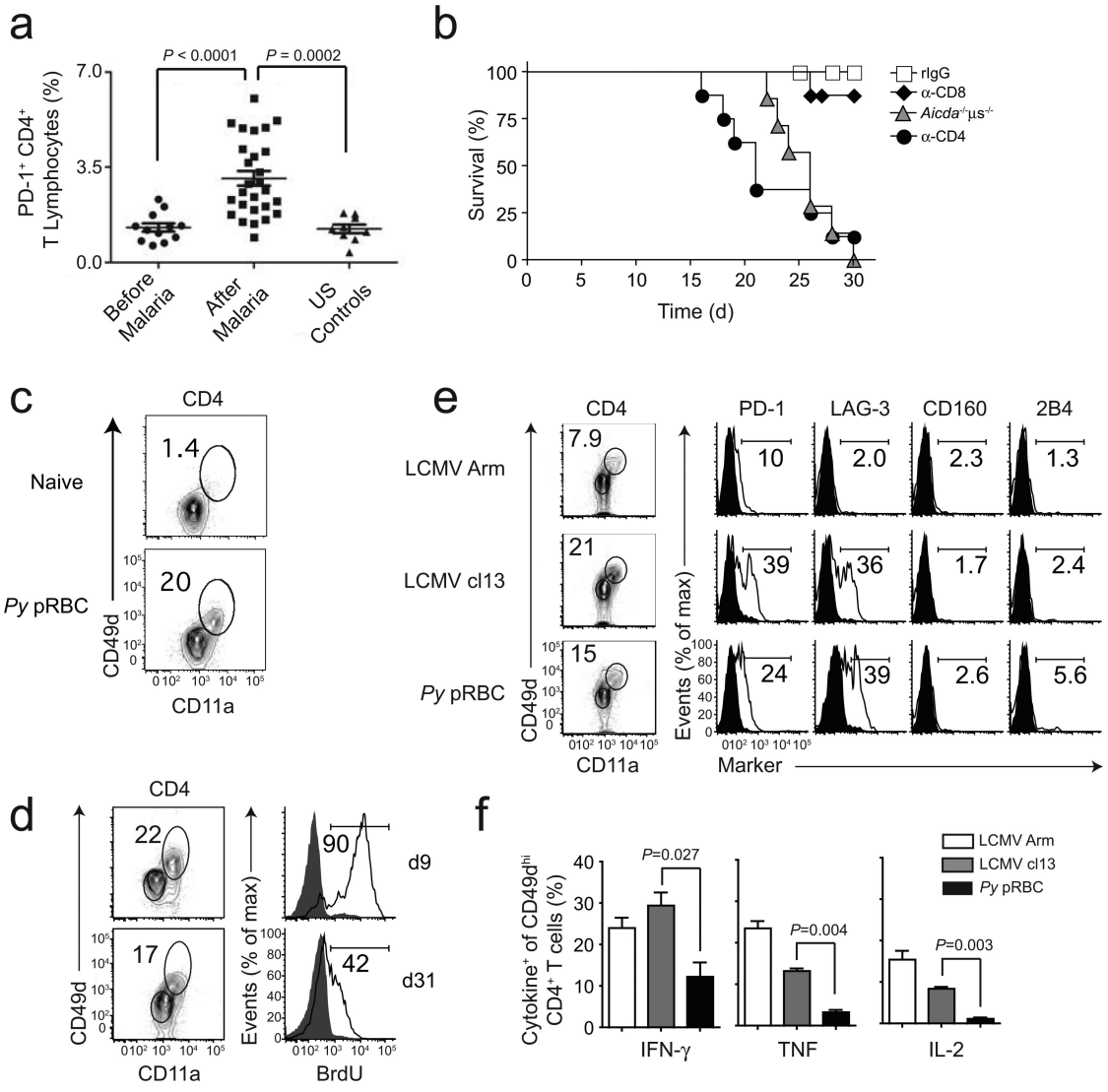

Figure 1. Human and rodent malaria induce specific phenotypic and functional characteristics of CD4$^+$ T cell exhaustion

(**a**) PD-1 expression by CD4$^+$ T cells from Malian children before the malaria season (Before Malaria) and seven days after symptomatic *P. falciparum* infection (After Malaria). The non-parametric Mann-Whitney test was used to compare continuous variables between groups. (**b**) Resolution of *P. yoelii* (*Py*) blood-stage infection in rodents requires CD4$^+$ T cells and antibody secreting B cells. Survival curves of *Py* pRBC infected wild-type C57BL/6 mice, wild-type mice depleted of CD4$^+$ or CD8$^+$ T cells on day 10, or C57BL/6 *Aicda*$^{-/-}$ μs$^{-/-}$ mice. Data represent 2 independent experiments with 5 mice/group. (**c**) Upregulation of CD49d and CD11a identifies *Plasmodium*-specific, infection-induced CD4$^+$ T cells. Longitudinal analyses of PBL before (naïve) and 7 days following (*Py* pRBC) challenge. Data represent 4 independent experiments. (**d**) Prolonged *Py* blood-stage infection results in sustained CD4$^+$ T cell proliferation. Histograms show the fraction of CD49d^{hi}CD11a^{hi} *Plasmodium*-specific (open) or CD49d^{lo}CD11a^{lo} naïve (filled) CD4$^+$ T cells that have incorporated BrdU following a day 4-8 pulse (top panel) or day 27-30 pulse

(bottom panel). Data represent 3 independent experiments with 3 mice/group. (**e**) Chronic virus (LCMV cl13) and prolonged *Plasmodium* blood-stage infection (*Py* pRBC), but not acute virus infection (LCMV Arm) induce T cell inhibitory receptors PD-1 and LAG-3 at day 31 on splenic, pathogen-specific (open) but not naïve (filled), $CD4^+$ T cells. Data represent 3 independent experiments with 3-5 mice/group. (**f**) Pathogen-specific $CD49d^{hi}CD11a^{hi}$ $CD4^+$ T cells from *Py* infected mice exhibit dysfunctional IFN-γ, TNF and IL-2 production in response to PMA/ionomycin stimulation. Data (mean±s.d.) are from 4-5 mice/group and are representative of 3 independent experiments. Statistics in (**f**) were determined by two-tailed, unpaired student's *t*-test.

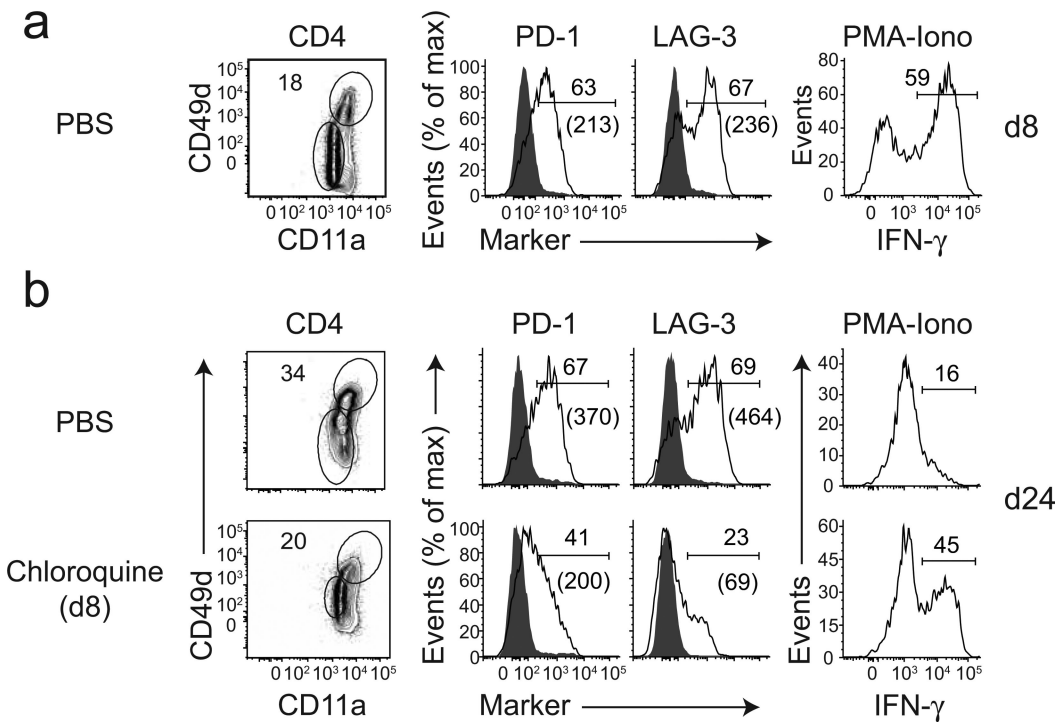

Figure 2. Truncation of *P. yoelii* blood-stage infection with chloroquine reverses CD4$^+$ T cell exhaustion

Groups of C57BL/6 mice were infected with 10^5 *P. yoelii* pRBC and subsequently treated with PBS or 80 mg/kg chloroquine/PBS on day 8 and 9 p.i. Spleens were harvested from mice on day 8 (**a**), or day 24 p.i. (**b**) and cells were examined for the expression of the indicated cell surface markers or for the functional production of IFN-γ following *ex vivo* stimulation with PMA-ionomycin (PMA–iono). Numbers in histograms refer to frequency of CD49d^{hi}CD11a^{hi} cells expressing the indicated marker or IFN-γ. Numbers in parentheses show mean fluorescence intensity of T cell inhibitory receptor staining. Data in **a,b** are representative of 2 independent experiments with 5 mice/group.

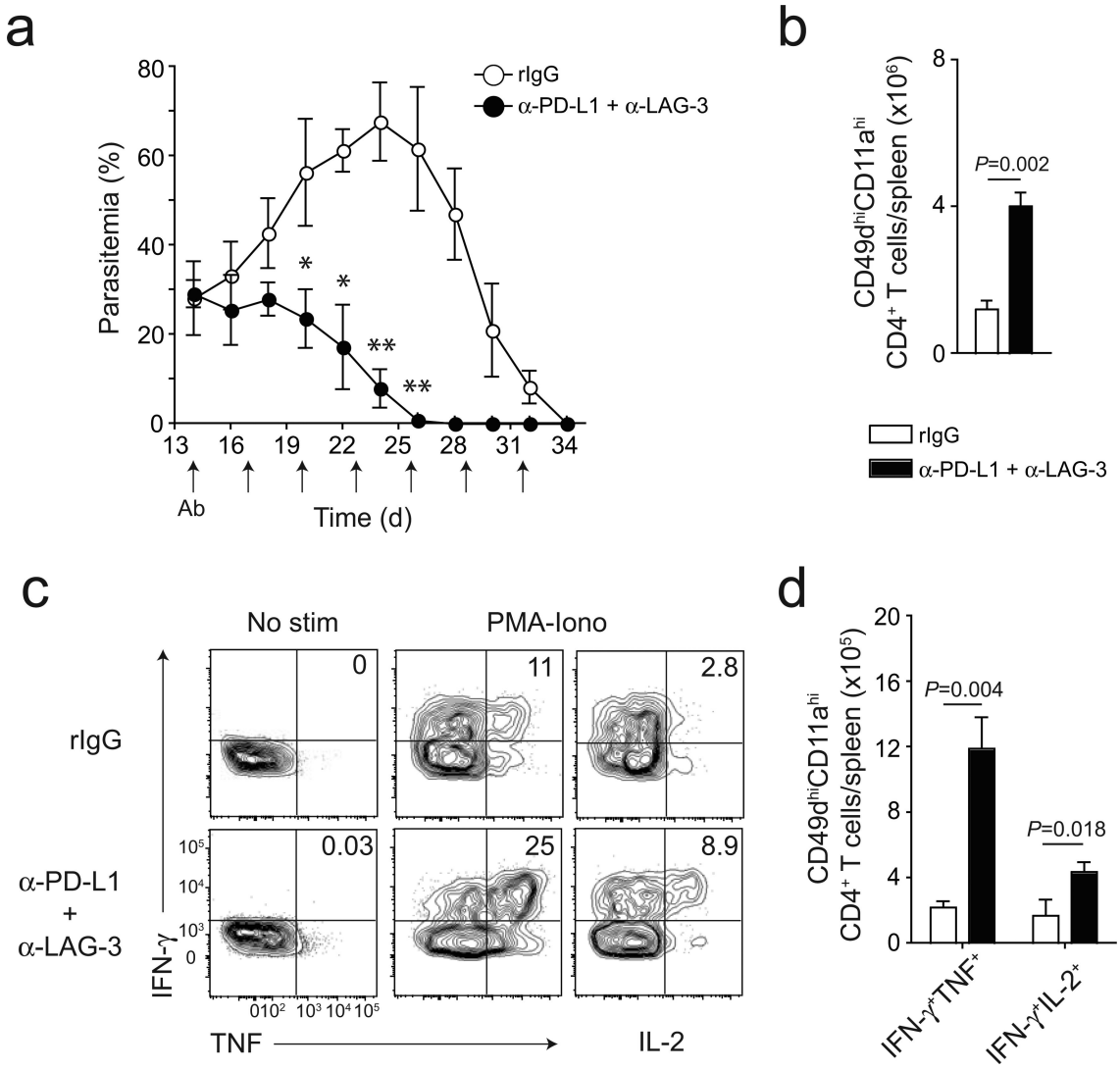

Figure 3. Therapeutic *in vivo* blockade of PD-1 and LAG-3 in mice improves the anti-*Plasmodial* CD4$^+$ T cell response and accelerates parasite clearance

(**a**) Starting on day 14 after infection with 10^5 *Py* pRBC, C57BL/6 mice were treated with 200 μg each of anti-PD-L1 and anti-LAG-3, or control rIgG, every 3 days and monitored for parasite burden every two days. Data (mean±s.d.) are from 5 mice/group and are representative of 4 independent experiments. (**b**) Mice were infected and treated as in (**a**) and *Plasmodium*-specific (CD49d^{hi}CD11a^{hi}) splenic CD4$^+$ T cells were enumerated on day 21 p.i. (**c**) Representative plots showing PMA–iono-induced cytokine expression by CD49d^{hi}CD11a^{hi} CD4$^+$ T cells from rIgG- and inhibitory receptor blockade-treated mice analyzed on day 21p.i. (**d**) Summary cytokine expression by CD49d^{hi}CD11a^{hi} CD4$^+$ T cells from rIgG- and inhibitory receptor blockade-treated mice. Data (Mean±s.e.m.) in (**b**) and (**d**) derive from 2 independent experiments with 3-5 mice/group. Statistics in (**a**), (**d**) and (**d**) were determined by two-tailed, unpaired student's *t*-test. (*=P<0.05, **P<0.01).

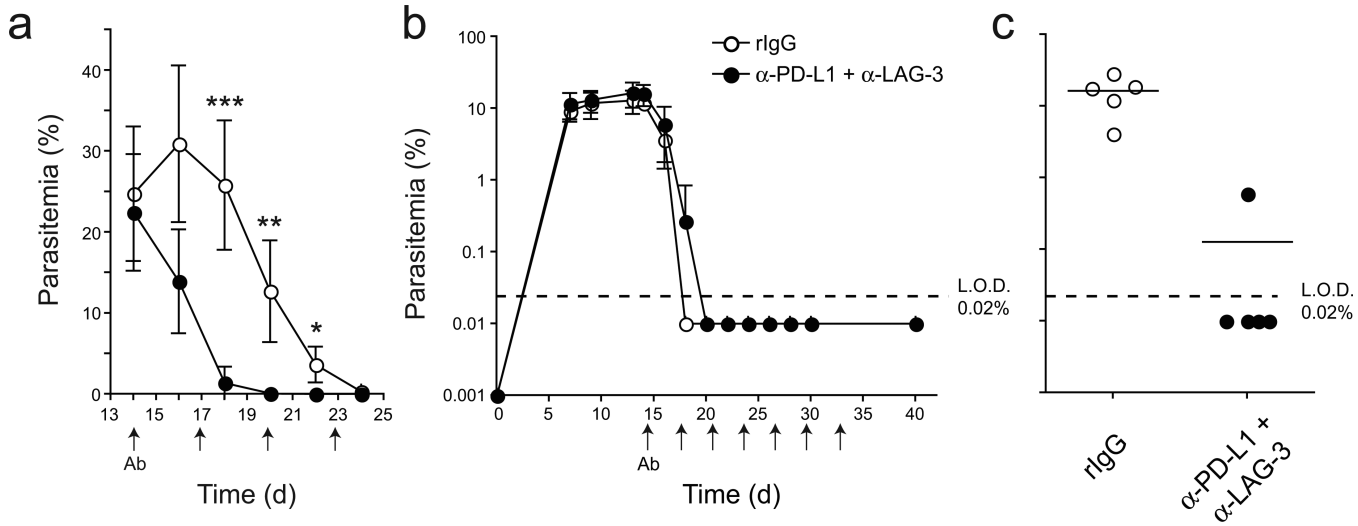

Figure 4. Therapeutic T cell inhibitory blockade accelerates parasite clearance in genetically diverse backgrounds and prevents chronic *Plasmodium* infection

(**a**) Female Swiss Webster mice (n=10/group) were infected with 10^5 *P. yoelii* parasitized red blood cells and given 200 μg of anti-PD-L1 and anti-LAG-3, or control rIgG, every three days starting on day 14 p.i. Parasitemia was monitored in individual mice until day 24. Statistics were determined by two-tailed, unpaired student's *t*-test. Data (Mean±s.d.) are representative of 2 independent experiments. Asterisks indicate $P < 0.005$. (**b-c**) Therapeutic *in vivo* blockade of PD-1 and LAG-3 signaling results in sterilizing clearance of persistent, subpatent *P. chabaudi chabaudi* (*Pcc*) infection in the majority of mice. (**b**) C57BL/6 mice were infected with 10^4 *Pcc* blood-stage parasites and given 200 μg of anti-PD-L1 and anti-LAG-3 every three days from days 14 to 32. Parasitemia was monitored on the indicated days. Data (Mean±s.d.) are from 5 mice/group. On day 40 post-challenge, 100 μl of whole blood was collected via cardiac puncture from each donor mouse, diluted 1:2 in saline and injected intravenously into new naïve recipient mice. Recipient mice were monitored for the development of patent *Pcc* parasitemia from day 2 to day 20 post-transfer. (**c**) Frequency of patent infection and quantification of parasite burden on day 9 post-transfer in recipient mice are shown. For **b-c**, the parasitemia limit of detection (L.O.D.) was 0.02%. Data in **b-c** are representative of 2 independent experiments.

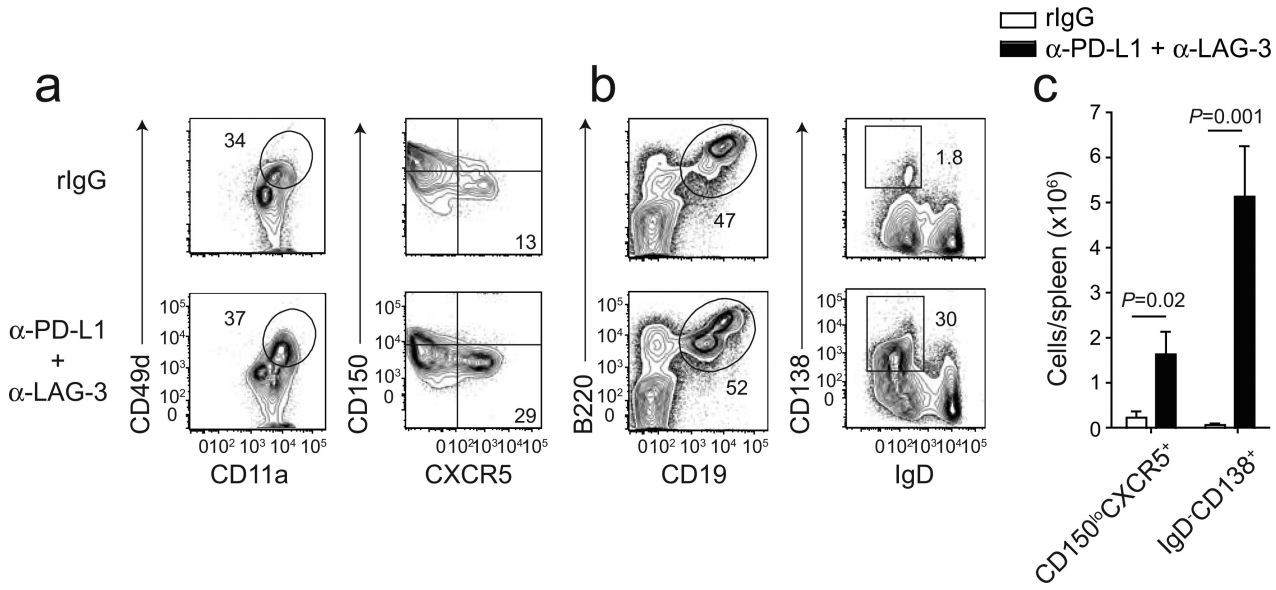

Figure 5. Therapeutic *in vivo* blockade of PD-L1 and LAG-3 in mice enhances T_{FH} $CD4^+$ T cell and plasmablast differentiation during clinical malaria

(**a**) Starting on day 14 after *Py* pRBC infection, groups of C57BL/6 mice were treated with 200 μg each of anti-PD-L1 and anti-LAG-3, or control rIgG, every 3 days. On day 21 p.i., splenic $CD49d^{hi}CD11a^{hi}CXCR5^{hi}CD150^{lo}$ $CD4^+$ T follicular helper (Tfh) cells were enumerated. (**b**) Mice were infected and treated as in (**a**) and $CD19^{hi/int}B220^{hi/int}$ B cells were analyzed for early plasma cell differentiation (evidenced by coordinate downregulation of IgD and upregulation of CD138 prior to loss of CD19 and B220 expression). (**c**) Summary data (Mean±s.d.) showing total numbers of T_{FH} and pre-plasma cells in rIgG- and inhibitory receptor blockade-treated mice 21 days p.i. Statistics in (**c**) were determined by two-tailed, unpaired student's *t*-test. Data in **a-c** are representative of 3 independent experiments with 5 mice/group.

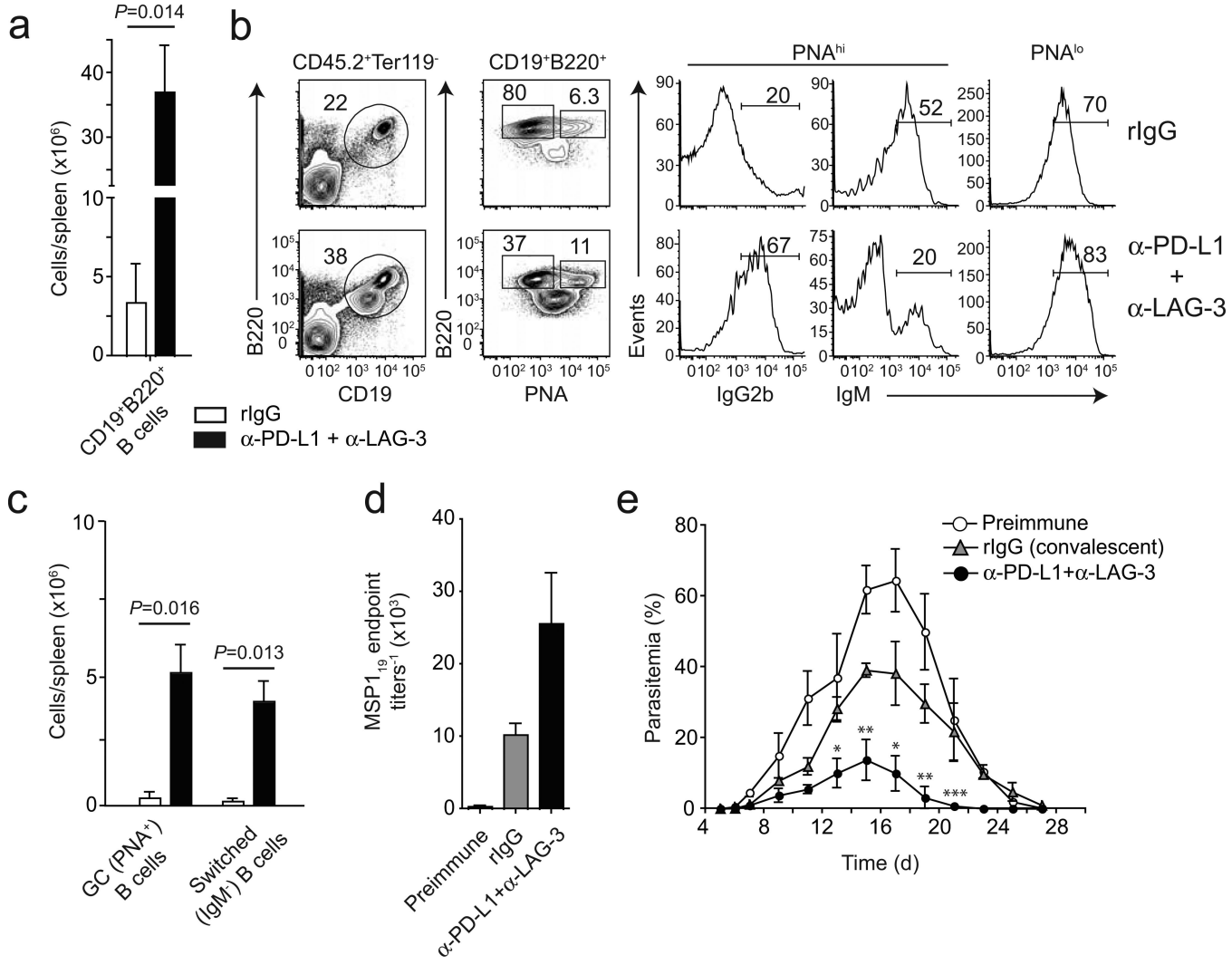

Figure 6. Enhanced germinal center B cell reaction, class switch recombination and functionally protective anti-*Plasmodial* antibody secretion following therapeutic PDL1 and LAG-3 blockade during clinical malaria

(**a**) C57BL/6 mice were infected with *Py* pRBC and subsequently treated with blocking antibodies as described for Figure 2. On day 21 p.i., total $CD19^+B220^+$ B cells were enumerated. Data (Mean±s.d.) represent 3 independent experiments with 3 mice/group. (**b**) Representative dot plots showing enhanced splenic germinal center ($B220^{hi/int}PNA^{hi}$) B cell responses on day 21 p.i. in mice that received anti-PD-L1 and anti-LAG-3 treatment from days 14 to 20 p.i. Histograms show the frequency of class-switching (IgM^{lo} and $IgG2b^{hi}$) in germinal center (PNA^{hi}) and non-germinal center (PNA^{lo}) B cells. (**c**) Summary data (Mean ±s.d.) show total numbers of germinal center and class-switched B cells in rIgG- versus anti-PD-L1 and anti-LAG-3 treated mice and are representative of 3 independent experiments with 3 mice/group. Statistics in (**a**) and (**c**) were determined by 2-tailed unpaired student's t-test. (**d**) Sera from *Py* infected mice that were subsequently treated with rIgG or anti-PDL1 and anti-LAG-3 from days 14 to 32 were collected on day 41 p.i. Total $MSP\text{-}1_{19}$-specific IgG antibodies were detected as described in Methods. Data (Mean±s.d.) are expressed as average endpoint titers with absorbance readings below 0.2 (A_{405}) and are representative of

2 experiments with 4 mice/group. (**e**) Parasite burdens and clearance kinetics following *Py* pRBC challenge of naïve mice receiving passive transfer of 150 μl of serum from donor mice subjected to the indicated treatments during *Py* malaria. Donor serum was obtained 41 days after initial *Py* pRBC challenge (2 weeks after cessation of blockade therapy). Data (Mean±s.d.) in (**e**) were analyzed by One-way ANOVA with Tukey's post-test of multiple comparisons and are representative of 2 independent experiments with 4 mice/group (*P<0.05; **P<0.01; ***P<0.001 for rIgG versus α-PD-L1+α-LAG-3).