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Regulatory T cells impede acute and long-term immunity to blood-stage malaria through CTLA-4

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Abstract

Malaria, caused by the protozoan *Plasmodium* is a devastating mosquito-borne disease, that puts nearly half the world's population at risk[1]. Despite mounting substantial T and B cell responses, humans fail to efficiently control blood-stage malaria or develop sterilizing immunity to reinfections[2]. Though Foxp3$^+$ regulatory T cells (Tregs) form a part of these responses[3–5], their influence remains disputed, and mode of action unknown. Here we show that Tregs, which expand in both humans and rodents during blood-stage malaria, interfere with conventional T helper (Th) cell responses and the Follicular T helper (Tfh) cell:B cell partnership in germinal centers, in a critical temporal window to impede protective immunity, through the Cytotoxic T-lymphocyte-Associated protein (CTLA)-4. Targeting Tregs or CTLA-4 with precisely timed depletion or blocking enhanced immune responses, accelerated clearance, and generated species-transcending immunity to blood-stage malaria in mice. Our study uncovers a critical mechanism of immunosuppression associated with blood-stage malaria that delays parasite clearance and prevents development of potent adaptive immunity to reinfection. These data also reveal a temporally discrete and therapeutically amenable functional role for Tregs in limiting anti-malarial immunity.

CD4 T helper (Th) cells are vital to the control of malaria in humans and the rodent models of disease[6]. Frequencies of activated Th cells increased during the course of malaria in mice and humans (Supplementary figures 1C, 2) and their depletion after established infection resulted in uncontrolled parasitemia and death in mice infected with the normally non-lethal

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Author contributions

S.P.K. designed, performed, analyzed and interpreted experiments and wrote the paper. N.O-A, S.M.A designed, performed, analyzed and interpreted experiments. N.S.B designed, performed, analyzed and interpreted experiments and wrote the paper. B.T. and O.K.D. supervised the human studies, designed, analyzed and interpreted experiments. P.D.C supervised the human studies, designed, analyzed and interpreted experiments and wrote the paper. J.T.H supervised the project, designed and interpreted experiments and wrote the paper.

rodent parasite, *Plasmodium yoelii* 17XNL (*Py*) (Supplementary figure 1A,B). However, unlike in most other infections (eg. *L. monocytogenes*), the expansion of pathogen-specific Th cells (defined as $CD49d^{+}CD11a^{hi}CD4^{+}$)[7] in *Py* infection is distinctly and inexplicably biphasic. Specifically, the frequency and total numbers of pathogen-specific Th cells temporarily fall or plateau before rising again prior to clearance of *Py* infection (Supplementary figure 1D–F). Though the mechanism behind this hiatus in Th cell expansion has remained unknown, we perceived it as a tangible sign of the general immunosuppression associated with blood-stage malaria. This assumption was underscored by the consistently observed increase in the key immunosuppressive cell population: $Foxp3^{+}$ Tregs, during blood-stage malaria in humans (Supplementary table 1) and mice (Figure 1A–D), as well as the numerical and functional correlation between Tregs and disease susceptibility[5,8,9]. Higher parasite densities in blood were associated with higher Treg frequencies in humans; and chloroquine treatment to decrease parasitemia reduced Treg frequencies in mice (Supplementary figure 3A–B), also tempering the drop in Th frequencies (Supplementary figure 3C). The impact and function of Tregs in malaria has remained controversial[5,10,11], with independent studies suggesting that Tregs suppress[8,12,13], or enhance[14,15] protection in this infection. While some concluded that depleting Tregs helped control parasitemia, disease severity or morality[8,12,16], others saw no impact[14,17,18] or even increased parasitemia and disease severity in some cases[15]. Importantly, these studies invariably manipulated the Treg response prior to or shortly after *Plasmodium* infection, mostly using anti-CD25 depleting antibodies[8,12,14,15,18] with some studies using the more precise Foxp3-DTR system[16,17]. Although variations in model systems may have contributed to some of these inconsistencies, the timing of interventions that target Tregs may also be a critical consideration in malaria. Here, we observed that the expansion of Tregs in *Py* infected mice preceded or matched in time the hiatus in Th cell responses, suggesting a causal relationship that manifests ~10 days post infection. To test this, we depleted circulating and lymphoid[19–21] Tregs in *Py* infected Foxp3-DTR (Supplementary figure 4A–B) or C57BL/6 (Supplementary figure 5A) mice with diphtheria toxin[22] or anti-CD25 antibody[8] respectively, beginning at day 9 just prior to the Th cell hiatus. Treg depletion at this time with both treatments interrupted the hiatus, restored *Plasmodium*-specific Th cell expansion and substantially accelerated control of *Py* infection (Figures1E–F, Supplementary figure 5B–C). In contrast, Treg depletion in Foxp3-DTR mice at the onset of *Py* infection resulted in death of infected mice (Supplementary Figure 4C).

To further address the opposing contributions of Tregs and Th cells in the control of parasitemia during the Th hiatus in *Py* infected mice, we selectively expanded Tregs (with IL-2/JES6 Ab complexes) or pathogen-specific Th cells (with IL-2/S4B6 Ab complexes)[23] starting at day 9 post infection. Increasing the frequencies of Tregs further dampened the pathogen-specific Th cell response and resulted in higher parasitemia and death, while increasing the frequencies of pathogen-specific Th cells resulted in better control of the infection (Supplementary figure 5D–F). Together, these data suggested that Tregs suppress Th cell responses during a critical window of time during blood-stage malaria, compromising control of acute infection.

There are two prominent mechanisms by which Tregs counter Th cell responses in the context of infection: through contact-independent, IL-10 mediated inhibition, or through

contact-dependent, CTLA-4 mediated repression of co-stimulation by antigen presenting cells (APCs)[24]. Tregs in mouse malaria transcriptionally upregulate both IL-10 and CTLA-4[16]. Consistent with another study[16], blocking IL-10 after established infection altered neither the kinetics of Th cell response nor the course of parasitemia in *Py* infected mice (Supplementary figure 6). However, Tregs in *Py* infected mice exhibited enhanced upregulation of CTLA-4, compared to Tregs in acute infections with influenza or vaccinia viruses (Figure 2A,B). Additionally, CTLA-4 was detectable in serum and spleen lysates in *Py* infected mice (Figure 2C), where the fraction of Tregs expressing CTLA-4 remained higher than the small percentages of Th cells with detectable CTLA-4 expression (Figure 2D)[16]. Of note, longitudinal analyses in humans showed that blood-stage (febrile) malaria increased the frequencies of circulating Th cells and Tregs expressing CTLA-4 (Figure 2E–F, Supplementary figure 7A). Febrile malaria in humans was also associated with higher frequencies of circulating, Helios (a marker of superior suppressive function[25,26]) expressing Tregs, Helios$^+$ Tregs expressing CTLA-4, as well as CTLA-4 expressing Tregs of lymphoid follicular origin (Tfr)[27,28] (Supplementary figure 7B–D). Taken together, these data suggested the hypothesis that Tregs may modulate Th and possibly humoral immunity to blood-stage malaria through CTLA-4.

Humoral immunity, built on efficient follicular T helper (Tfh) cell: B cell cooperation in the secondary lymphoid organs, is perhaps the most important component of acquired immunity that controls blood-stage malaria[29,30]. To examine if either Tregs or CTLA-4 interfered with humoral immunity against malaria, we examined their roles in the Tfh:B cell partnership in the germinal centers (GCs). Within the GCs in secondary lymphoid organs, (CD4$^+$) Th cells and (GL-7$^+$B220$^+$) B cells appeared to form discrete clusters of interaction (Video 1) after *Py* infection. These clusters were composed of CTLA-4-expressing follicular Treg (Tfr) and Th (Tfh) cells[31], in close apposition with the GC plasmablasts or B cells (Figure 2G, Video 2). Expression of Neuropilin (Nrp)-1 or Foxp3 distinguished Tfr from Tfh cells[32].

In the context of infection, CTLA-4 expressed on T cells bind to B7 ligands on APCs and limits immune responses by 1) competitive inhibition of B7:CD28 co-stimulatory interactions, 2) inducing inhibitory indoleamine 2,3-dioxygenase (IDO) in the APCs or 3) transendocytosing the B7 molecules from the surface of APCs [33]. Since B cells (and not follicular dendritic cells) are the primary APCs that sustain Tfh cell responses[34] and dictate protective antibody responses in malaria, we investigated if B cells might broker the CTLA-4-mediated immunomodulation. After *Py* infection, the CTLA-4$^+$ Tfr cells appeared to directly associate with GC B cells (Video 3, Figure 2G), with CTLA-4 detectable on the B cells at their interface with Tfr cells (Video 4). Further, individual Tfr cells appeared to transiently interact with multiple B cells in GCs (Video 5), indicating how the relatively few Tfr cells could effectively modulate the GC reaction. We failed to observe any induced IDO (*in vitro*) in, or discernable transendocytosis of B7 molecules (*in vitro* or *in vivo*) from, Th or B cells after *Py* infection (data not shown). These observations suggested that CTLA-4 expressed or secreted by the Tfr cells might be directly binding to B7 ligands on the B cell surface, restricting productive co-stimulation of Tfh cells in the B cell follicles. Additionally, it also implied that blocking the CTLA-4: B7 interactions (checkpoint blockade) might augment immunity and clearance of blood-stage malaria.

Although checkpoint blockade regimens applied without an understanding of the underlying immunology or disease progression could be ineffective or even detrimental to the host, they can be intuitively tailored to generate safe, clinically approved therapeutic choices[35,36]. For example, therapeutic blockade of CTLA-4 at the onset of blood-stage malaria or the absence of PD-L1 in a non-lethal model of LCMV infection, resulted in severe immunopathology and death in mice[37–39]. The precise definition of Treg kinetics, CTLA-4 expression dynamics, and timing of GC reactions uncovered here suggested that CTLA-4: B7 interactions may be meaningfully targeted post-establishment of *Py* infection. Hence, *Py* infected C57BL/6 mice were treated with CTLA-4 blocking (anti-CTLA-4) or IgG control (IgG ctrl) antibodies at the onset of the hiatus in the expansion of pathogen-specific Th cells (Figure 3a). Similar to Treg depletion, therapeutic blockade of CTLA-4 truncated the hiatus and enhanced the total numbers of CD4 T cells, pathogen-specific Th, follicular CD4 T cells, and Tfh cells in the spleen (and peripheral lymph nodes, data not shown) compared to IgG ctrl treated mice (Figure 3B–E). Hypothetically, CTLA-4 blockade could target Tregs, Th cells or both (Figure 2D). However, CTLA-4 blockade failed to further improve the Th cell response in Treg-depleted mice, suggesting a minor role for CTLA-4 expressed on Th cells in shaping their kinetics during this interval (Supplementary figure 8). Also, unlike in tumor models[40], anti-CTLA-4 treatment during malaria did not deplete Tregs or Tfr cells in spleen (Supplementary figure 9, Figure 3F). We also observed a corresponding increase in the total numbers of splenic B cells, plasmablasts, GC B cells, GC plasmablasts, and *Py*-specific, protective serum-antibodies[41] in anti-CTLA-4 treated mice (Figure 3G–K). Depleting GC B cells with anti-CD40L treatment[34] prevented the revival of Th cell responses after CTLA-4 blockade, indicating that Treg interaction with GC B cells likely mediated the repression of Th cell responses in *Py* infection (Supplementary figure 10), although effects on other APC cannot be ruled out. *Plasmodium* infection notoriously causes severe splenomegaly and obliterates the splenic architecture in its hosts. In *Py* infected mice, CTLA-4 blockade resulted in considerably improved resolution of the spleen (Figure 3L) and its architecture, with distinct T cell zones, B cell follicles and GC reactions visible shortly after treatment (Figure 3M). Strikingly, therapeutic blockade of CTLA-4 also dramatically accelerated control of *Py* infection in C57BL/6 mice and the relatively more susceptible BALB/c mice (Figure 4A–B). Additionally, CTLA-4 blockade controlled parasitemia better and partially rescued (40% survival) BALB/c mice from lethal *P. berghei* ANKA infection (Figure 4C). However, CTLA-4 blockade before or after the critical window of expansion of Tregs was unable to productively alter the Th cell responses or accelerate control of *Py* infection (Supplementary figure 11), consistent with some previous results[17,37]. We previously showed that blockade of PD-1 and LAG-3 signaling during the post-hiatus revival of Th cell responses resulted in accelerated clearance of *Py* infection in mice[7]. In contrast, blocking PD-1 and LAG-3 signaling during the Treg-mediated hiatus in Th cell responses after *Py* infection provided no tangible improvement in immunity or parasite clearance, indicating a minimal contribution of these pathways to dampening immune responses during this critical interval (Supplementary figure 12). Of note, a suitably timed stimulation of OX40 signaling can also improve immunity against blood-stage malaria[42]. Together, these results reinforce the notion that immunomodulation during blood-stage malaria is complex and based on multiple molecular pathways that may be dominant during discrete time-windows during infection.

Growing resistance to anti-malarial drugs is a major therapeutic concern that would not arise with approaches such as CTLA-4 blockade that target host molecules[43]. Yet, CTLA-4 blockade might be a less realistic independent treatment option for malaria in endemic areas, with its frequent dosages, parenteral administration, precise timing requirements, and currently prohibitive costs. Nevertheless, a major unresolved issue in malaria is why humans fail to generate potent adaptive immunity to subsequent *Plasmodium* infections, which may also involve multiple species of the parasite in endemic areas[4,44]. To address a potential role of Tregs/CTLA-4 in limiting generation of long-term immunity, we transferred (56 days post infection (dpi)) sera from C57BL/6 mice that cleared *Py* infection with or without CTLA-4 blockade, into C57BL/6 recipients with fresh (0 dpi) or established (10 dpi) *Py* infections. Recipients of sera from anti-CTLA-4 treated mice (that contain no detectable residual anti-CTLA-4 antibodies, Supplementary figure 13A) exhibited significantly lower parasitemia (Figure 4D, Supplementary figure 14). To test if anti-CTLA-4 therapy aided long-term and perhaps species-transcending immunity, we re-challenged C57BL/6 mice originally infected with *Py*, and cured with or without CTLA-4 blockade, using the lethal, cerebral malaria-causing *P. berghei* ANKA strain at 56 or 100 dpi after initial *Py* infection. CTLA-4 blockade during *Py* infection resulted in durable, CD4 T cell driven immunity against *P. berghei* ANKA, and dramatically improved long-term survival of the mice (Figure 4E–F, Supplementary Figure 13B–D) with no incidence of cerebral malaria. Taken together, these findings revealed that Treg expression of CTLA-4 is a major mechanism that limits acquired, cross-species immunity to malaria. Direct evidence of a role for Tregs and CTLA-4 in limiting protection from *Plasmodium* reinfection in humans can only be obtained through clinical trials and, like the translation of checkpoint blockade from animal models to human cancer immunotherapy[45–47], the precise pathway forward for malaria must be carefully defined. However, in the face of rapidly developing drug resistance by *Plasmodium*, our findings provide important mechanistic insights to consider while designing and evaluating evidence-based interventions to target host-immunity for improved control of malaria.

Malaria is a global health threat, with close to 200 million clinical cases and many deaths reported annually. It is critically important to understand how *Plasmodia* circumvent effective immune responses in humans[2]. Here, we build on high-resolution studies of immune cell dynamics during blood-stage malaria of humans and mice, to show how Tregs can act in a discrete temporal window through CTLA-4 to suppress the Th and humoral immune responses. Thus, Tregs may function as an essential component of the immunoregulation observed in blood-stage malaria to inhibit clearance of acute infection and development of long-term sterilizing immunity to future infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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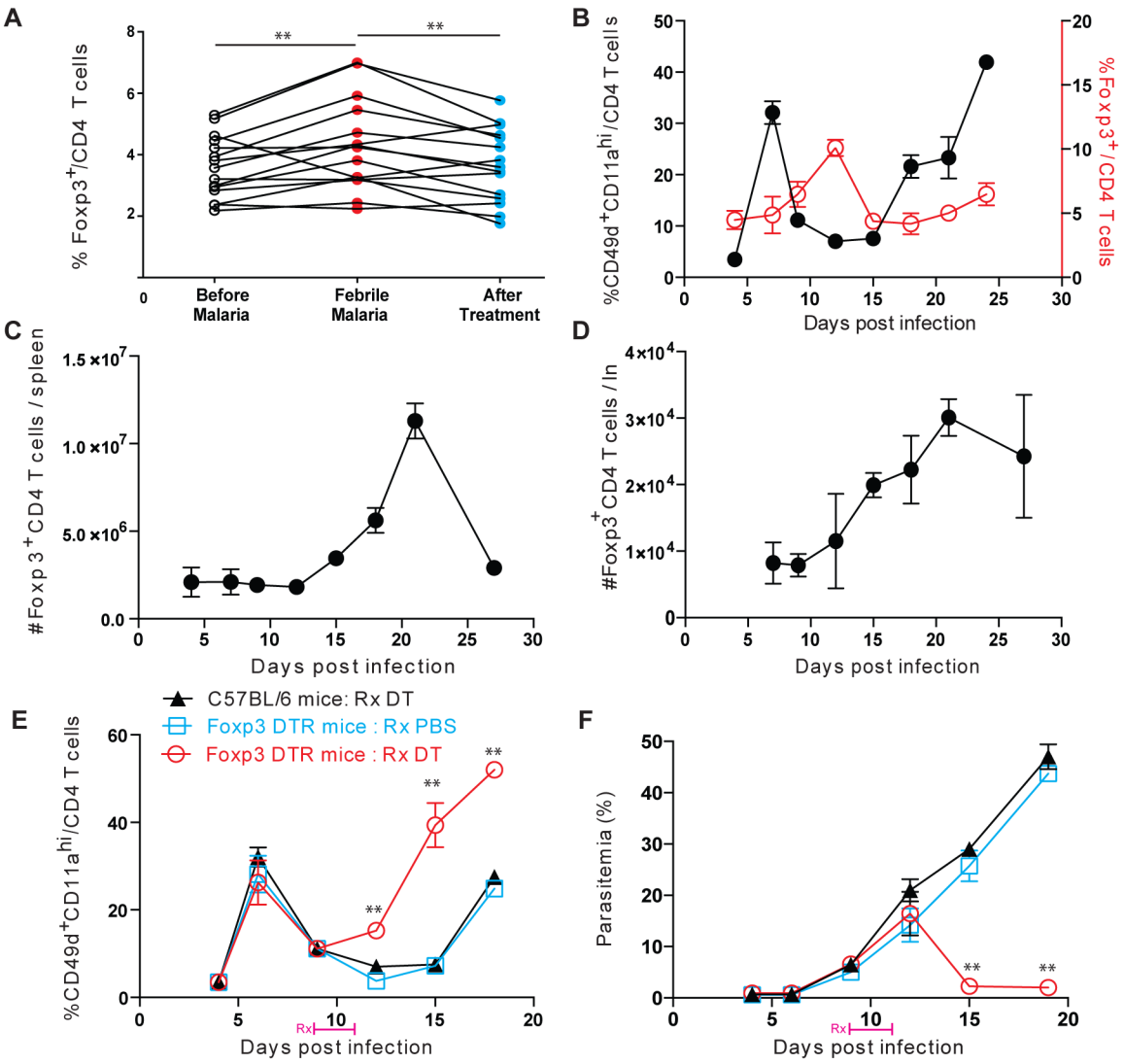

Fig. 1. Regulatory T cells expand; modulate helper T cell responses and immunity to malaria

(A) Longitudinal frequencies of Foxp3$^+$ regulatory T cells in PBMCs in a cohort of children in Mali (Supplementary table 1) before, during and after acute febrile malaria. Each connected line indicates a separate subject. (B) Kinetics of activated CD4 helper or Foxp3$^+$ regulatory T cell frequencies in circulation in *Py* infected C57BL/6 mice. (C–D) Absolute numbers of Foxp3$^+$ Treg cells in spleen (C) or lymph nodes (D) at the indicated time points in *Py* infected C57BL/6 mice. (E–F) Frequencies of activated CD4 helper T cells in circulation (E) or parasitemia (F) at various time points post infection with *Py* in C57BL/6 or Foxp3 DTR mice treated with DT (Rx DT) or PBS (Rx PBS) on 9 and 11dpi. All experimental data represent 1 of at least 3 separate experiments with 5 mice/group and are presented as mean ± s.e.m. **indicates P≤ 0.01 comparing the indicated groups using one-way ANOVA with Bonferroni's correction (A) or Foxp3 DTR:Rx DT or Foxp3 DTR:Rx PBS groups at the indicated time points using two-way ANOVA with Tukey's correction (B)

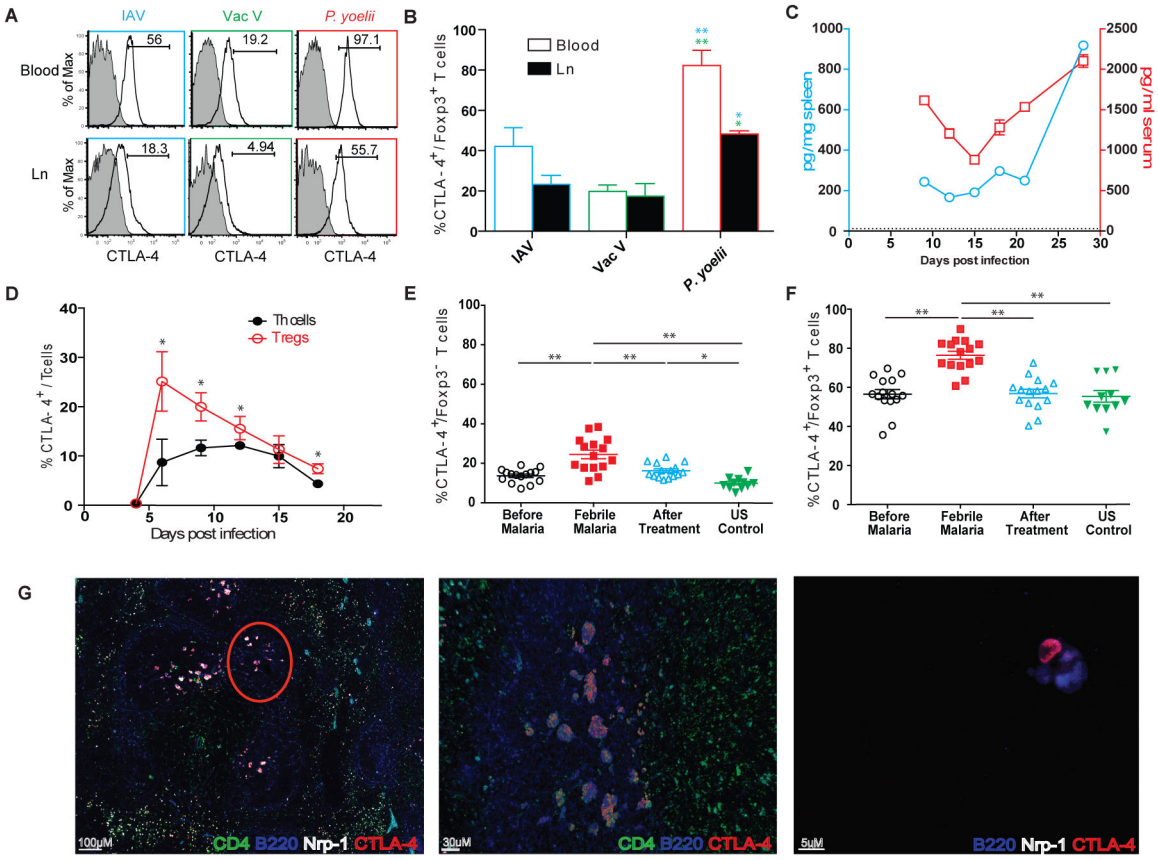

Fig. 2. CTLA-4 expression enhanced in malaria, integral to Tfh:B:Tfr cell interactions in the germinal centers

(A–B) Representative histograms showing CTLA-4 expression in Foxp3$^+$ Tregs in Influenza A virus (IAV), vaccinia virus (VacV) or *Py* infected C57BL/6 mice, 9dpi (A). Gates and numbers inset indicate the proportions of CTLA4$^+$ Foxp3$^+$ Tregs, summarized in (B). (C–D) Kinetics of CTLA-4 expressed in spleen or serum (C) or in splenic Foxp3$^+$ Tregs or Th cells (D) after *Py* infection in C57BL/6 mice. Dotted line in (C) is threshold of detection. (E–F) Proportions of CTLA-4 expressing Th cells (E) or Tregs (F) in a cohort of children in Mali before, during and after acute febrile malaria compared to healthy controls. (G) Representative pseudocolored images of fluorescently labeled sections from a C57BL/6 mouse spleen (left, middle panels) or lymph node (right panel) with a resolving (21–27dpi) *Py* infection, indicating Tfh:B:Tfr clusters in the GCs. Middle panel represents the encircled portion in the left panel. The right panel shows a single B cell and CTLA-4 expressing Tfr cell in close apposition. All experimental data represent 1 of at least 3 experiments with 5 mice per group, presented as mean ± s.e.m. *or **indicate P≤ 0.05 or 0.01 respectively, comparing the indicated groups using two-way ANOVA with Bonferroni correction (B), two-tailed student t-test (D) or one-way ANOVA with Tukey's correction (E–F).

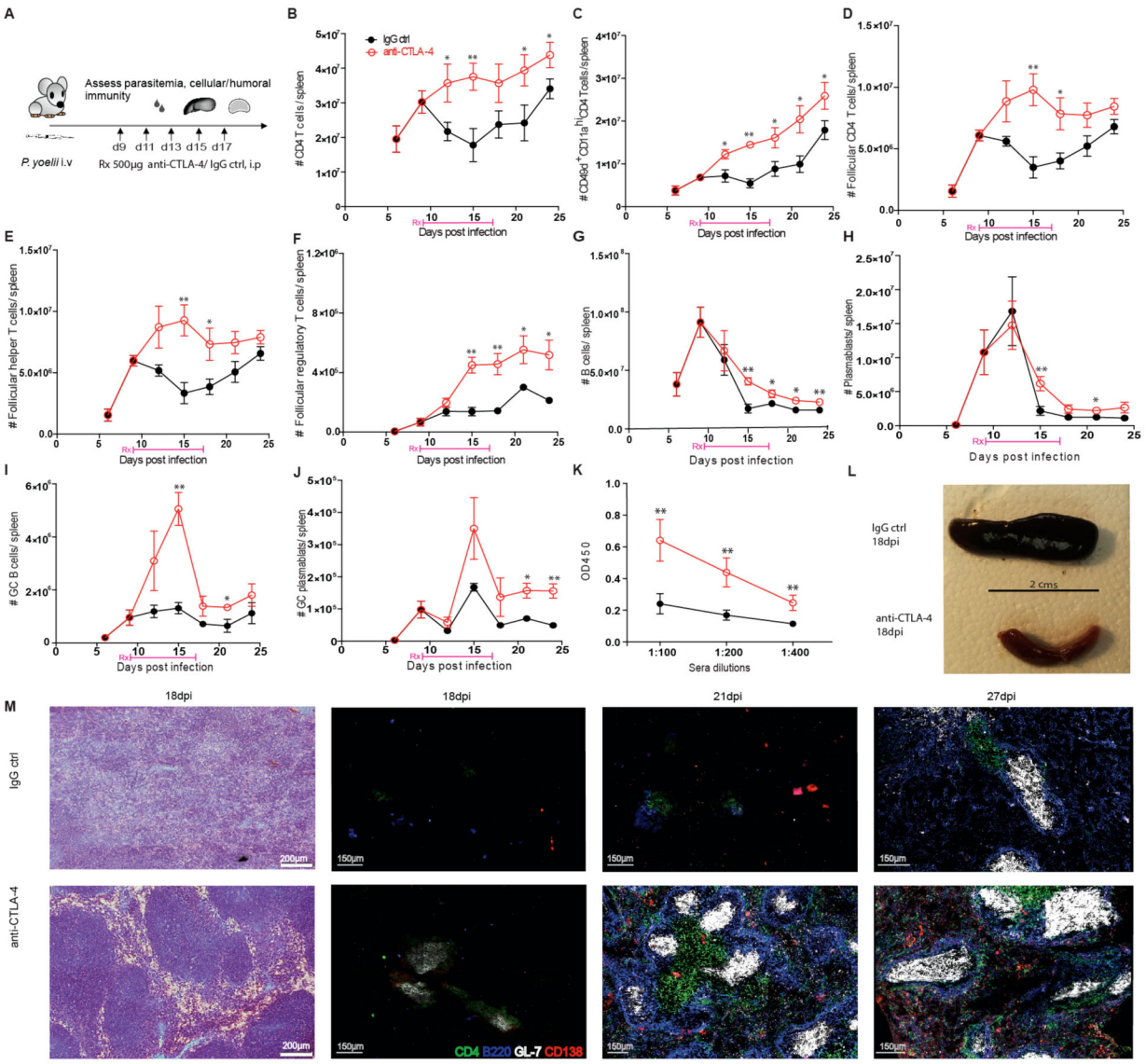

Fig. 3. CTLA-4 blockade enhances CD4 T and B cell responses, GC reaction and antibody titers after *Py* infection

(A) A schematic of therapeutic blockade in *Py* infected C57BL/6 mice. (B–J) Total numbers of CD4 Th (B), *Plasmodium*-specific $CD49d^{+}CD11a^{hi}CD4^{+}$Th (C), $CXCR5^{+}ICOS^{+}PD\text{-}1^{+}CD4^{+}$T follicular cells (D), $CXCR5^{+}ICOS^{+}PD\text{-}1^{+}Foxp3^{-}CD4^{+}$Tfh (E) $CXCR5^{+}ICOS^{+}PD\text{-}1^{+}Foxp3^{+}CD4^{+}$Tfr (F) cells, $CD19^{+}B220^{+}$B cells (G), $CD138^{+}IgD^{-}CD19^{+}B220^{+}$plasmablasts (H), $CD95^{+}GL7^{+}CD19^{+}B220^{+}$GC B cells (I) and $CD95^{+}GL7^{+}CD138^{+}IgD^{-}CD19^{+}B220^{+}$GC plasmablasts (J) in spleen at various time points and *Py* MSP_{1-19} specific relative serum antibody titers at 18 days (K), post *Py* infection in C57BL/6 mice with or without CTLA-4 blockade as in Figure 3(A). Data presented as mean ± s.e.m at each time point or sera dilution and represent 1 of 3 separate experiments, each with at least 5 mice per group. * or ** indicate P≤ 0.05 or 0.01 respectively, comparing the treatment and control groups at the indicated time points or sera dilutions with two-tailed student t-tests. Representative image of the gross appearance (18dpi, L), H&E stained or

pseudocolored fluorescently labeled sections (M) of spleen from *Py* infected C57BL/6 mice at the indicated time points with or without CTLA-4 blockade. Markers and colors are indicated in the figure.

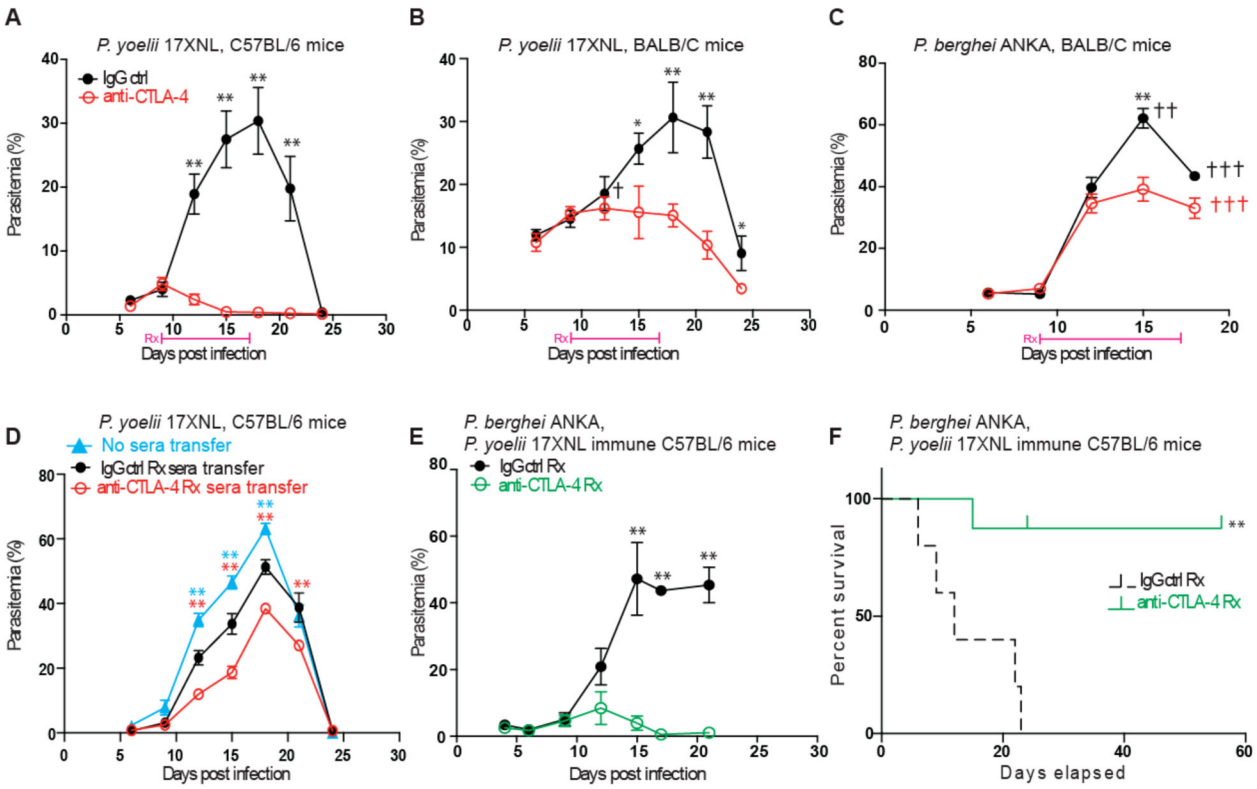

Fig. 4. Therapeutic blockade of CTLA-4 enhances immunity to malaria

(A–C) Percentages of parasitemia at the indicated time points in C57BL/6 (A) or BALB/C (B–C) mice infected with *Py* (A–B) or *P. berghei* (C) with or without CTLA-4 blockade as in Figure 3(A). (D) Parasitemia at the indicated time points in *Py* infected C57BL/6 mice, that received sera (100μl) from 56 dpi donor mice as in panel (A), at 0 dpi. (E–F) Parasitemia at the indicated time points (E) or survival (F) in mice from panel (A) heterologously challenged with *P. berghei* at 56 dpi. All data represent 1 of at least 3 separate experiments, each started with 5 mice per group. Error bars represent s.e.m and color coded *or **indicate P≤ 0.05 or 0.01 respectively, comparing the corresponding treatment and control groups at the indicated time points with two-tailed student t-tests (A–E) or chi-square test (F). †indicates death of one mouse in the corresponding group.