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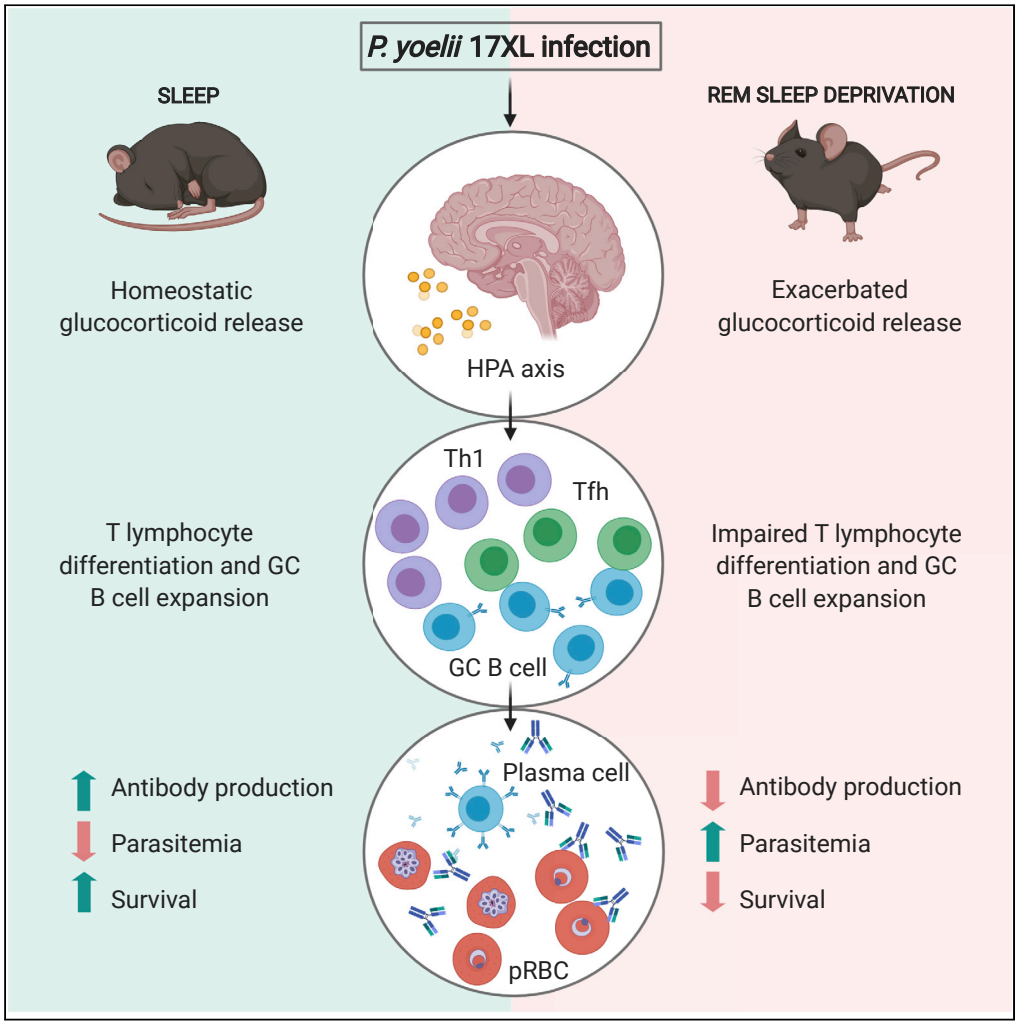

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HIGHLIGHTS

REM sleep deprivation (RSD) worsens malaria induced by *Plasmodium yoelii* infection

RSD decreases germinal center formation and impairs specific antibody production

Exacerbated glucocorticoid production impairs T lymphocyte differentiation

The relationship between sleep and immunity is a target for malaria management

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Sleep Disturbance during Infection Compromises Tfh Differentiation and Impacts Host Immunity

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SUMMARY

Although the influence of sleep quality on the immune system is well documented, the mechanisms behind its impact on natural host immunity remain unclear. Meanwhile, it has been suggested that neuroimmune interactions play an important role in this phenomenon. To evaluate the impact of stress-induced sleep disturbance on host immunity, we used a murine model of rapid eye movement sleep deprivation (RSD) integrated with a model of malaria blood-stage infection. We demonstrate that sleep disturbance compromises the differentiation of T follicular helper cells, increasing host susceptibility to the parasite. Chemical inhibition of glucocorticoid (Glcs) synthesis showed that abnormal Glcs production compromised the transcription of Tfh-associated genes resulting in impaired germinal center formation and humoral immune response. Our data demonstrate that RSD-induced abnormal activation of the hypothalamic-pituitary-adrenal axis drives host susceptibility to infection. Understanding the impact of sleep quality in natural resistance to infection may provide insights for disease management.

INTRODUCTION

Sleep is a behavioral and physiological phenomenon, fundamental for homeostasis. In modern 24-hr society, it is noteworthy a reduction in sleep duration (from nearly 9 hr in the 60s to less than 7 hr nowadays) and in sleep quality, due to extended work routines, stressful situations, and the presence of sleep disorders that alter sleep quality (Chattu et al., 2018). Several studies suggest that inadequate sleep duration is strongly associated with the development of hypertension (Grandner et al., 2018), obesity, cardiovascular diseases (Sabanayagam and Shankar, 2010), diabetes, and cancer (Haus and Smolensky, 2013; Hakim et al., 2014; Ma et al., 2016; Shi et al., 2020). In addition, several studies provided clues regarding the cross talk between sleep homeostasis and the immune system functions (Besedovsky et al., 2019). Thus, the comprehension of the relationship between sleep and the immune system is essential to design new approaches to improve sleep and immune response quality.

Sleep architecture comprises two very distinct phases: the rapid eye movement (REM) sleep (or paradoxical sleep) and non-REM sleep (NREM, which includes three different stages). Under normal circumstances, sleep regulatory substances (SRS), such as melatonin, regulate the circadian rhythm. However, other SRS, such as IL-1β and TNF-α, exert a significant influence in the sleep-awake cycle, acting as somnogenic molecules, modulating NREM sleep. Elevated IL-1β and TNF-α levels, detected during infectious diseases, increase the NREM sleep period (Krueger et al., 2007). Noteworthy, the NREM period is characterized by increased growth hormone (GH) secretion, diminished cortisol release, and a predominant activity of $CD4^+$ T helper-1 (Th1) lymphocytes, which is essential for host immunity against intracellular parasites (Besedovsky et al., 2012; Lange et al., 2006). There is also growing evidence associating longer periods of sleep with a substantial reduction in parasitism levels (Opp, 2009) and reduced sleep quality with increased risk of infection and poor infection outcome (Patel et al., 2012; Prather et al., 2015; Besedovsky et al., 2019).

Considering that the majority of clinical and experimental models to study sleep disturbance involve total or partial sleep deprivation or sleep fragmentation, it is virtually impossible to distinguish between the

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effects of NREM or REM sleep periods. Thus, the relationship between REM sleep and immune system's activity remains poorly understood. Therefore, we used a murine model of REM sleep deprivation (RSD) integrated with a model of malaria blood-stage *Plasmodium yoelii* infection (Sanni et al., 2002) to determine the impact of REM sleep quality on host immunity against a parasitic infection.

Malaria is an infectious disease caused by different *Plasmodium* species, with an estimated 228 million cases and 405,000 deaths worldwide in 2018 (World Health Organization, 2019). In humans, inoculated sporozoites infect hepatocytes and gain circulation as blood-stage parasites (merozoites), invading and replicating in red blood cells (RBCs) (Kurup et al., 2019). The cyclical rupture of the infected erythrocyte is responsible for the clinical manifestations (mainly fever, chills, headache, malaise, and fatigue) of disease, which may lead to life-threatening complications if left untreated (Ashley et al., 2018). In malaria-endemic areas, repeated exposure to the parasite results in a protection against clinical manifestations, mediated by humoral immune response (Tran et al., 2014). Although specific antibodies do not provide sterile immunity, they are essential to control parasitemia levels by limiting parasite replication (Boyle et al., 2015). The key feature for antibody production is the maturation of B cells in the germinal center (GC) and further differentiation into memory or antibody-producing plasma cells (Mesin et al., 2016). This process is closely related to the interaction with a specialized subset of $CD4^+$ T cells, known as T follicular helper (Tfh) cells, that provide differentiation signals to GC B cells (Crotty, 2019). From this perspective, chronic intermittent infection, or even certain types of treatment, has been associated with fatigue, irritability, and sleep disturbances, with loss of up to half of normal sleep (Nevin and Croft, 2016). Therefore, events that influence the development of humoral immunity may contribute to morbidity and mortality associated with mild and severe malaria (Ashley et al., 2018).

Sleep disturbances have been extensively associated with the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Scheiermann et al., 2013), which is characterized by increased endogenous glucocorticoids levels that ultimately may exert immunosuppressive effects (Di Comite et al., 2007). Thus, we hypothesized that the stress induced by RSD during *Plasmodium* infection might impair the development of a protective humoral response. Herein, we show that RSD during the development of the immune response against malaria parasites impairs Tfh differentiation and GC formation, reducing the antibody response and ultimately host resistance to the parasite. The primary mechanism responsible for this phenomenon is the exacerbated production of glucocorticoids (Glcs) due to RSD during the acute phase of *P. yoelii* infection. Thus, our data highlight the impact of sleep quality on the development of protective humoral immunity against malaria. Furthermore, we suggest that Glc levels during the development of the acute phase of malaria infection may represent a useful marker to predict disease severity.

RESULTS

RSD Impairs the Control of Malaria Infection and Increases Host Susceptibility to the Parasite

To determine whether sleep disturbance influences host immunity against malaria parasite, mice were injected with *P. yoelii*-infected erythrocytes before, at the same time, or after being subjected to RSD (Figures 1A and S1A). Our results showed that RSD initiated three days after infection (Figure 1A) exerted a critical effect on parasitemia and survival. Mice from this RSD group failed to control infection and, consequently, presented a lower survival rate. By contrast, the non-sleep deprived control group (Ctrl) controlled parasite growth (Figure 1B) and survived longer (Figure 1C), although blood parasitemia increased exponentially in the first six days post infection. RSD 3 days before, or at the same day of parasite inoculation (Figure S1B), did not impact disease progression to the same extent (Figures S1C and S1D).

Based on these results, we sought to explore the effects of RSD 3 days after parasite inoculation. Of note, RSD itself did not impact weight loss in infected mice when compared to Ctrl mice (Figure S2A). However, the increased parasitemia found in the RSD-infected group impacted erythrocyte count (Figure S2B) and hematocrit (Figure S2C), but not hemoglobin levels (Figure S2D). By itself, RSD did not influence the same parameters in uninfected mice (Figures S2E–S2G, respectively).

Analysis of the humoral immune response showed that the susceptibility of the RSD group to *P. yoelii* infection correlated with a significant reduction in parasite-specific IgG titers (Figure 1D) that negatively correlated with parasitemia levels (Figure 1E), thus indicating that RSD impaired the production of protective antibodies. Besides the reduction in anti-parasite-specific IgG titers, the RSD group also presented lower

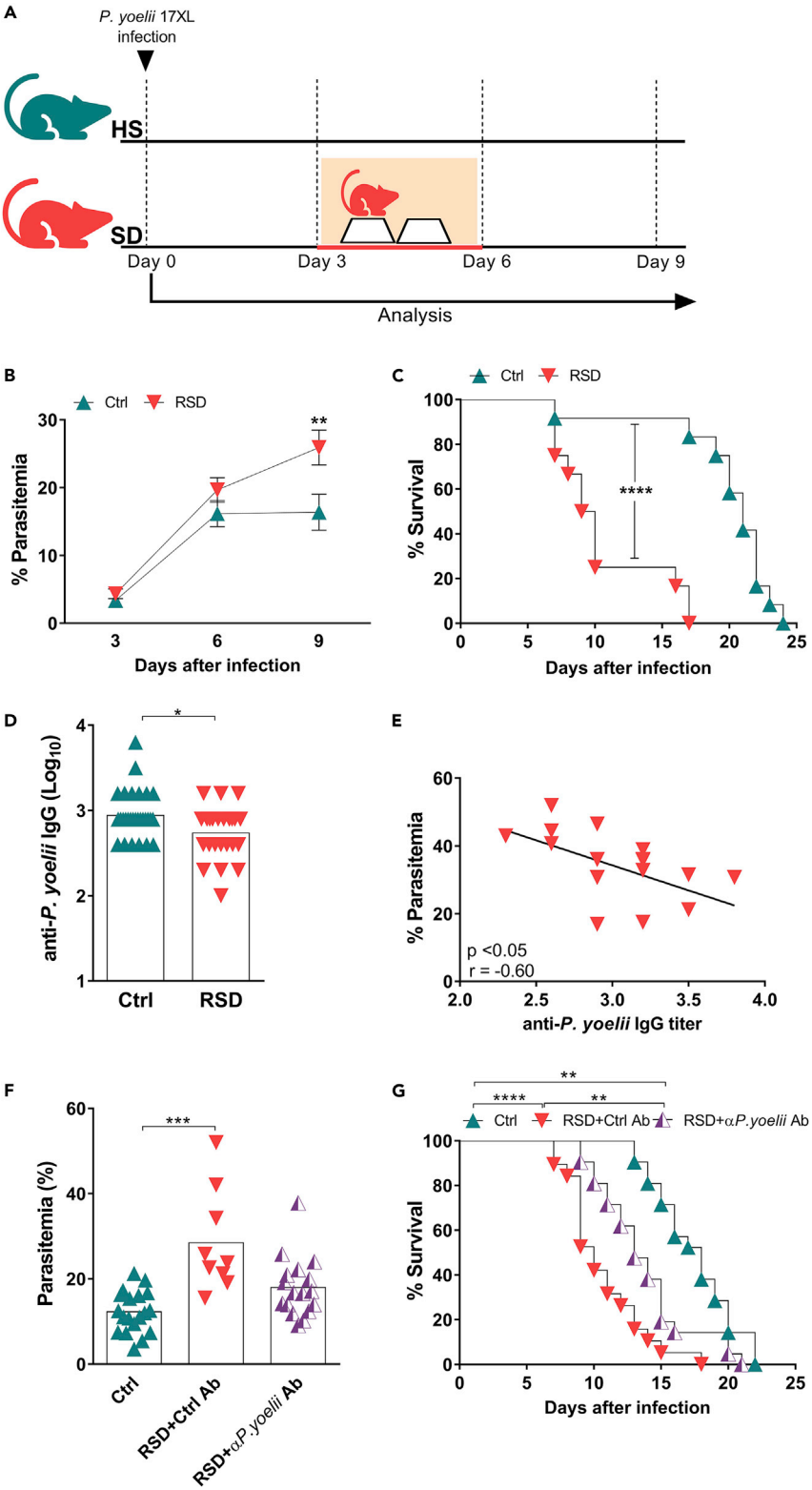
A
P. yoelii 17XL
infection
HS
SD
Day 0
Day 3
Day 6
Day 9
Analysis
B
Ctrl
RSD
% Parasitemia
Days after infection
**
C
Ctrl
RSD
% Survival
Days after infection
****
D
anti-P. yoelii IgG (Log10)
Ctrl
RSD
*
E
% Parasitemia
anti-P. yoelii IgG titer
p <0.05
r = -0.60
F
Parasitemia (%)
***
Ctrl
RSD+Ctrl Ab
RSD+αP.yoelii Ab
G
Ctrl
RSD+Ctrl Ab
RSD+αP.yoelii Ab
% Survival
Days after infection
**
****
**

Figure 1. REM Sleep Deprivation Impairs the Control of Malaria Infection by Reducing Parasite-Specific Antibody Titers

(A) Experimental setup (see also Figure S1). Mice were submitted to REM sleep deprivation (RSD) 3 days after infection with *Plasmodium yoelii*, while the control group (Ctrl) was kept under regular sleep conditions.

(B) Parasitemia in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by two-way ANOVA.

(C) Mice survival in Ctrl and RSD groups. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (****$p < 0.0001$) by log-rank (Mantel-Cox) test.

(D) Anti-*P. yoelii* IgG serum titers in Ctrl and RSD mice at day nine post infection. Data are from six independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

(E) Correlation between parasitemia and anti-*P. yoelii* IgG titers. Data are from three independent experiments with at least five mice in each group.

(F) Six days after infection, RSD mice were transferred with purified IgGs obtained from control or *P. yoelii*-infected HS animals. Three days later, parasitemia levels were evaluated. Data are from two independent experiments with ten mice in each group. Mean ± SEM (***$p < 0.001$) by one-way ANOVA.

(G) Mice survival after passive immunization with purified IgG from control or *P. yoelii*-infected animals. Data are from two independent experiments with ten mice in each group. Mean ± SEM (**$p < 0.01$, ****$p < 0.0001$) by log-rank (Mantel-Cox) test.

levels of circulating IgG titers (Figure S3A) and antibody-secreting cells in the spleen (Figure S3B) against a 19-kDa major *P. yoelii* surface antigen, known as merozoite surface protein 1 ($MSP\text{-}1_{19}$).

To confirm the correlation between IgG titers and protection, we performed passive transfer experiments. Groups of RSD-infected animals received serum-purified IgGs taken from uninfected mice or from *P. yoelii*-infected mice 9–11 days post infection. No protection was conferred by IgGs from control mice, but IgGs from the infected mice reduced parasitemia (Figure 1F) and increased survival (Figure 1G). Thus, RSD impaired the production of protective antibodies.

RSD Alters Antibody Class Switching and Reduces Germinal Center B Cells and Tfh Frequency during Infection

The analysis of parasite-specific antibody production revealed that RSD impaired the production of IgG2b and IgG2c isotypes without altering IgG1 titers (Figures 2A–2C, respectively). This result prompted us to analyze whether the reduction in IgG class switching was due to impaired GC and Tfh differentiation. Our results revealed that RSD impaired the differentiation of GC ($CD3^-B220^+GL7^+CD95^+$) (Figures 2D and 2E) and antibody-producing plasma ($CD3^-B220^{low}CD138^+$) B cells (Figures 2F and 2G). This phenomenon was closely related to a significant reduction in the frequency of Tfh cells ($B220^-CD3^+PD\text{-}1^+CXCR5^+$) (Figures 2H and 2I) since GC expansion and plasma B cell maturation are highly dependent on Tfh help (Victora et al., 2010). Thus, these data indicated that RSD compromised $CD4^+$ T differentiation into Tfh cells and the development of specific B cells.

Sleep Disturbance Impairs T Cell Activity and Inhibits the Transcription of Genes Associated with Tfh Differentiation

In the blood-stage infection with the lethal strain of *P. yoelii* (17XL), IFN-γ production is essential to elicit parasite-specific IgG2a response and restrain parasite growth (Ishih et al., 2013). The *ex vivo* analysis of splenic cells by flow cytometry revealed that RSD mice presented lower numbers of IFN-γ- and TNF-α-producing $CD4^+$ T cells (Figures 3A and 3B, respectively). Further analysis demonstrated that $CD4^+$ T cells from the RSD group were not more susceptible to cell death when compared to those from Ctrl mice (Figures S4A and S4B); instead, these cells expressed lower levels of Ki67, indicating a lower proliferative capacity (Figure S4C). Therefore, these data indicate that RSD impaired T cell function. This idea was strengthened by the fact that splenocytes from RSD mice displayed lower numbers of IFN-γ-producing cells (Figure 3C), produced lower levels of the proinflammatory cytokines IFN-γ (Figure 3D) and TNF-α (Figure 3E), and decreased $CD4^+$ T cell proliferation (Figure 3F) upon specific *in vitro* recall with *P. yoelii* $MSP\text{-}1_{19}$.

To further characterize these $CD4^+$ T cells, we analyzed the expression of the Th1 key transcription factor T-bet (*Tbx21*) (Szabo et al., 2000). Indeed, sorted $CD4^+$ T cells from RSD mice displayed lower expression of the *Tbx21* gene when compared to Ctrl mice (Figure 3G). Taken together, these data demonstrated that RSD impaired the development of fully functional $CD4^+$ T cells.

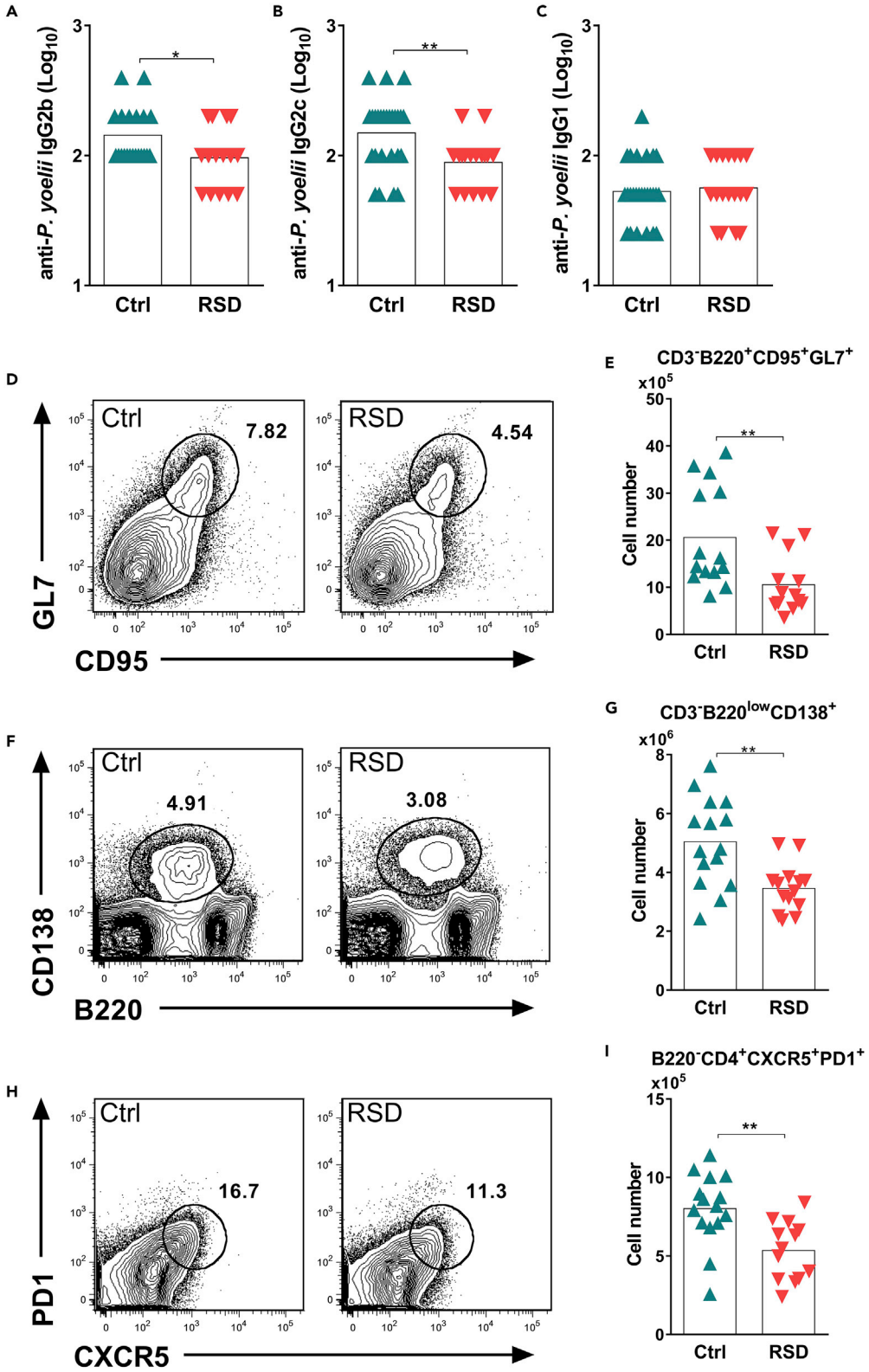
A
anti-P. yoelii IgG2b (Log10)
Ctrl
RSD
B
anti-P. yoelii IgG2c (Log10)
C
anti-P. yoelii IgG1 (Log10)
D
Ctrl
7.82
RSD
4.54
GL7
CD95
E
CD3-B220+CD95+GL7+
x10^5
Cell number
F
4.91
3.08
CD138
B220
G
CD3-B220lowCD138+
x10^6
H
16.7
11.3
PD1
CXCR5
I
B220-CD4+CXCR5+PD1+

Figure 2. REM Sleep Deprivation Impairs Antibody Production and Differentiation of Germinal Center B Cells, Plasma B Cells, and Tfh Cells

Infected Ctrl and RSD mice were analyzed at day nine post infection.

(A) Anti-*P. yoelii* IgG2b serum titers in Ctrl and RSD mice. Data are from six independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

(B) Anti-*P. yoelii* IgG2c serum titers in Ctrl and RSD mice. Data are from six independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by two-tailed unpaired t test.

(C) Anti-*P. yoelii* IgG1 serum titers in Ctrl and RSD mice. Data are from six independent experiments with at least five mice in each group. Mean ± SEM.

(D) Representative flow cytometry analysis of splenic GC B cells ($CD3^-B220^+GL7^+CD95^+$) in Ctrl and RSD mice.

(E) Absolute numbers of splenic GC B cells ($CD3^-B220^+GL7^+CD95^+$) in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by two-tailed unpaired t test.

(F) Representative flow cytometry analysis of splenic plasma B cells ($CD3^-B220^{low}CD138^+$).

(G) Absolute numbers of splenic plasma B cells ($CD3^-B220^{low}CD138^+$) in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by two-tailed unpaired t test.

(H) Representative flow cytometry analysis of splenic Tfh cells ($B220^-CD3^+PD\text{-}1^+CXCR5^+$).

(I) Absolute numbers of splenic Tfh cells ($B220^-CD3^+PD\text{-}1^+CXCR5^+$) in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by two-tailed unpaired t test.

We next evaluated the impact of RSD on $CD4^+$ T cell differentiation into Tfh, a critical cellular population for GC formation. The differentiation of naive $CD4^+$ T cells into the Tfh subpopulation is an intricate event coordinated by cytokines, such as IL-6 and IL-21 (Choi et al., 2013; Nurieva et al., 2008). $CD4^+$ T cells, activated in the IL-6/21 context, start to express transcription factors, such as *Bcl-6* and *Maf* (Nurieva et al., 2012; Kroenke et al., 2012). As a consequence, these cells increase the expression of cell surface molecules such as ICOS, PD-1, and CXCR5, which define the Tfh population (Vinuesa and Cyster, 2011). Although we did not detect a significant production of IL-21 by splenocytes upon *in vitro* specific recall (data not shown), IL-6 levels were lower in the RSD than in the Ctrl group (Figure 3H). Accordingly, flow cytometry analysis showed a reduction in the number of ICOS-expressing $CD4^+$ T cells in RSD versus Ctrl splenocytes (Figure 3I). As a proof of concept, we used sorted $CD4^+$ T cells to determine the expression of genes linked to Tfh differentiation such as *Bcl-6*, and *Maf*. Expression analysis showed that RSD downregulated *Maf* expression (Figure 3J), while we did not detect a significant difference in the levels of *Bcl-6* transcripts between the Ctrl and RSD groups (Figure 3K). Overall, these data support the idea that RSD impaired factors associated with Tfh differentiation.

Exacerbated Synthesis of Glucocorticoids Triggered by RSD and *P. yoelii* Infection Inhibits Tfh Differentiation, GC Formation, and Protective Humoral Responses

The data outlined above indicated that sleep disturbance during the development of the immune response against malaria triggered an immunosuppressive response that impaired host resistance to infection. As we did not find a significant increase of suppressive cytokines, such as IL-10 (data not shown), we sought to explore other potential immunosuppressive mechanisms. A previous study demonstrated that the modified multiple platform method used for RSD activates the HPA axis, increasing Glc synthesis (Suchecki et al., 1998). In addition, infection itself is a Glc inducer. Physiological production of Glcs is required to contain immune-mediated tissue injury; however, it also compromises host immunity against pathogens (Jamieson et al., 2010).

Although RSD and *Plasmodium* infection themselves resulted in Glc production, sleep disturbance during the early onset of infection exerts an additive effect, resulting in a systemic peak of Glc production (Figure 4A). Moreover, we observed that uninfected mice submitted to RSD restore normal levels of corticosterone in a few days; however, the higher corticosterone concentration in infected RSD mice persists even after 3 days of recovery (Figure 4A), suggesting a prolonged systemic exposure to Glcs. To explore the influence of the Glc peak on the development of host immunity against *Plasmodium*, we treated mice with metyrapone, an inhibitor of Glc synthesis (Igarashi et al., 2005; Macphee et al., 1989; Besedovsky et al., 2014). Metyrapone treatment reduced both infection and RSD-induced Glc levels (Figure 4B). Despite reducing infection-induced Glc synthesis in Ctrl mice, metyrapone treatment did not improve parasite burden control, indicating that the production of Glcs in response to infection did not impact immunity against malaria (Figure 4C). By contrast, in the infected-RSD group, inhibition of Glc synthesis increased resistance against infection, reducing parasitemia, and mice death (Figures 4C and 4D, respectively). This phenomenon was associated with an increase in specific antibody titers (Figure 4E) and a recovery in GC (Figure 4F) and plasma B cell numbers (Figure 4G). Also, following metyrapone treatment, the

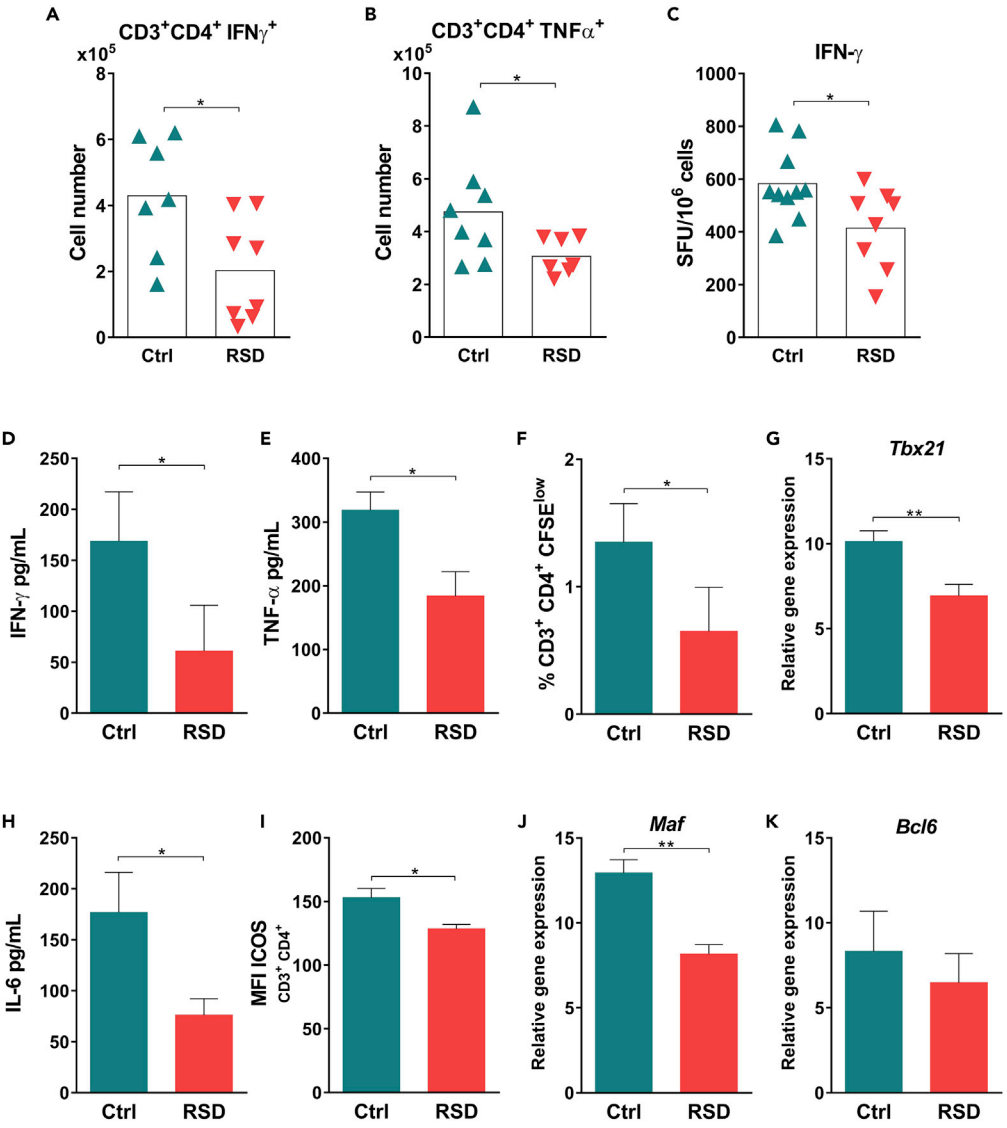

Figure 3. REM Sleep Deprivation Impairs T Cell Activity and Inhibits the Transcription of Genes Associated with Tfh Differentiation

Infected Ctrl and RSD mice were analyzed at day nine post infection.

(A) *Ex vivo* analysis of splenic IFN-γ-producing $CD3^+CD4^+$ T cells from Ctrl and RSD mice. Data represent one experiment with at least 7 mice per group. Mean ± SD (*$p < 0.05$) by two-tailed unpaired t test.

(B) *Ex vivo* analysis of splenic TNF-α-producing $CD3^+CD4^+$ T cells from Ctrl and RSD mice. Data represent one experiment with at least 7 mice per group. Mean ± SD (*$p < 0.05$) by two-tailed unpaired t test.

(C) ELISPOT analysis of IFN-γ-producing cells from Ctrl and RSD mice, after culture with recombinant *P. yoelii* $MSP1_{19}$. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

(D) Levels of IFN-γ in culture supernatants from Ctrl and RSD splenocytes after culture with recombinant *P. yoelii* $MSP\text{-}1_{19}$ for 48h. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

(E) Levels of TNF-α in culture supernatants from Ctrl and RSD splenocytes after culture with recombinant *P. yoelii* $MSP\text{-}1_{19}$ for 48 hr. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

(F) Frequency of specific proliferating ($CFSE^{low}$) $CD3^+CD4^+$ T cells after culture in the presence of recombinant *P. yoelii* $MSP\text{-}1_{19}$. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by two-tailed unpaired t test.

Figure 3. *Continued*

(G) *Tbx21* mRNA expression by sorted splenic $CD4^+$ T cells from Ctrl and RSD mice. Data are from two independent experiments with at least four mice in each group. Mean ± SEM (**p < 0.01) by two-tailed unpaired t test.

(H) Levels of IL-6 in culture supernatants from Ctrl and RSD splenocytes after culture with recombinant *P. yoelii* $MSP\text{-}1_{19}$ for 48hr. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*p < 0.05) by two-tailed unpaired t test.

(I) Median fluorescence intensity (MFI) of ICOS on splenic $CD3^+CD4^+$ T cells from Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*p < 0.05) by two-tailed unpaired t test.

(J) *Maf* mRNA expression by sorted $CD4^+$ T cells from Ctrl and RSD mice. Data are from two independent experiments with at least four mice in each group. Mean ± SEM (**p < 0.01) by two-tailed unpaired t test.

(K) *Bcl6* mRNA expression by sorted $CD4^+$ T cells from Ctrl and RSD mice. Data are from two independent experiments with at least four mice in each group. Mean ± SEM.

number of Tfh cells was restored (Figure 5A) and $CD4^+$ T cell recovered their unspecific (Figure 5B) and parasite-specific (Figure 5C) proliferative responses. In line with these results, splenocytes from metyrapone-infected RSD (RSD + M) group produced higher levels of IFN-γ (Figure 5D) and TNF-α (Figure 5E). The Glc decay was associated with the recovery of *Tbx21* and *Maf* transcript expression in sorted $CD4^+$ T cells (Figures 5F and 5G, respectively). Overall, we conclude that inhibition of Glc synthesis recovered host functional immune response.

DISCUSSION

The notion that sleep disturbance reduces natural resistance against pathogens is part of folk wisdom, and a fact well documented by several studies (Besedovsky et al., 2019). However, the underlying mechanisms of these events remain poorly understood, primarily due to the difficulty of addressing the influence of a specific sleep stage in physiological processes as immune responses. Thus, it is a considerable challenge to depict the REM sleep influence on the immune system's activity.

To assess the active influence of REM sleep on the development of immunity against *P. yoelii*, we used the modified multiple platform method to selectively deprive mice of REM sleep at pre-determined periods. Although RSD has been linked to increased susceptibility to another species of murine malaria parasite, *Plasmodium chabaudi* (Lungato et al., 2015), the mechanisms underlying this phenomenon were not evaluated.

First, we observed that a time window between RSD and parasite inoculation constituted a turning point for host susceptibility. Starting RSD three days after infection resulted in the worst disease outcome, indicating that the immune context at this time point was the most affected by sleep disturbance. Previous studies demonstrated that protection against clinical malaria is dependent on the humoral responses (Cohen et al., 1961; Egan et al., 1996). In agreement with this notion, specific IgG titers inversely correlated with parasitemia levels in RSD mice. The key role of IgG in protection against the parasite was further confirmed by transferring total IgG from previously infected mice to RSD-infected mice. Therefore, our data indicated that in this specific time window, RSD impaired immunological events involved in the development of IgG-producing B cells.

In germinal centers, mature B cells differentiate into memory and plasma cells in a T cell-dependent reaction (Victora et al., 2010; Schwickert et al., 2011). Although experimental *Plasmodium* infection has been described to induce specific $CD4^+$ T cell depletion (Xu et al., 2002), our *ex vivo* analysis did not reveal any increase in $CD4^+$ T cell death. Instead, we found that $CD4^+$ T cells displayed a reduced proliferative capacity both *ex vivo* and *in vitro,* to unspecific and specific stimuli. In parallel, T cells also exhibited impaired effector activity, characterized by a reduced production of proinflammatory cytokines, such as IFN-γ and TNF-α. As a consequence, B cells exhibited a reduced capacity to differentiate into plasma B cells, as demonstrated by the reduced numbers of GC B cells, IgG-producing B cells, and lower titers of IgG2b/c in RSD mice. Also, we did not find a significant difference in the number of $Ki67^+$ B cells between the Ctrl and RSD groups (Figure S4D), suggesting that the problem within the B cell compartment was associated with the maturation process.

B cell maturation is strictly linked with the help of Tfh cells (Crotty, 2014). In this sense, we found that in parallel with the decrease in the T cell effector activity, RSD mice presented a reduced number of Tfh cells. A recent study reported that during *Plasmodium* infection, a dominant Tfh population emerges as soon as four days after infection (Arroyo and Pepper, 2019). In our model, this phase represents the beginning of

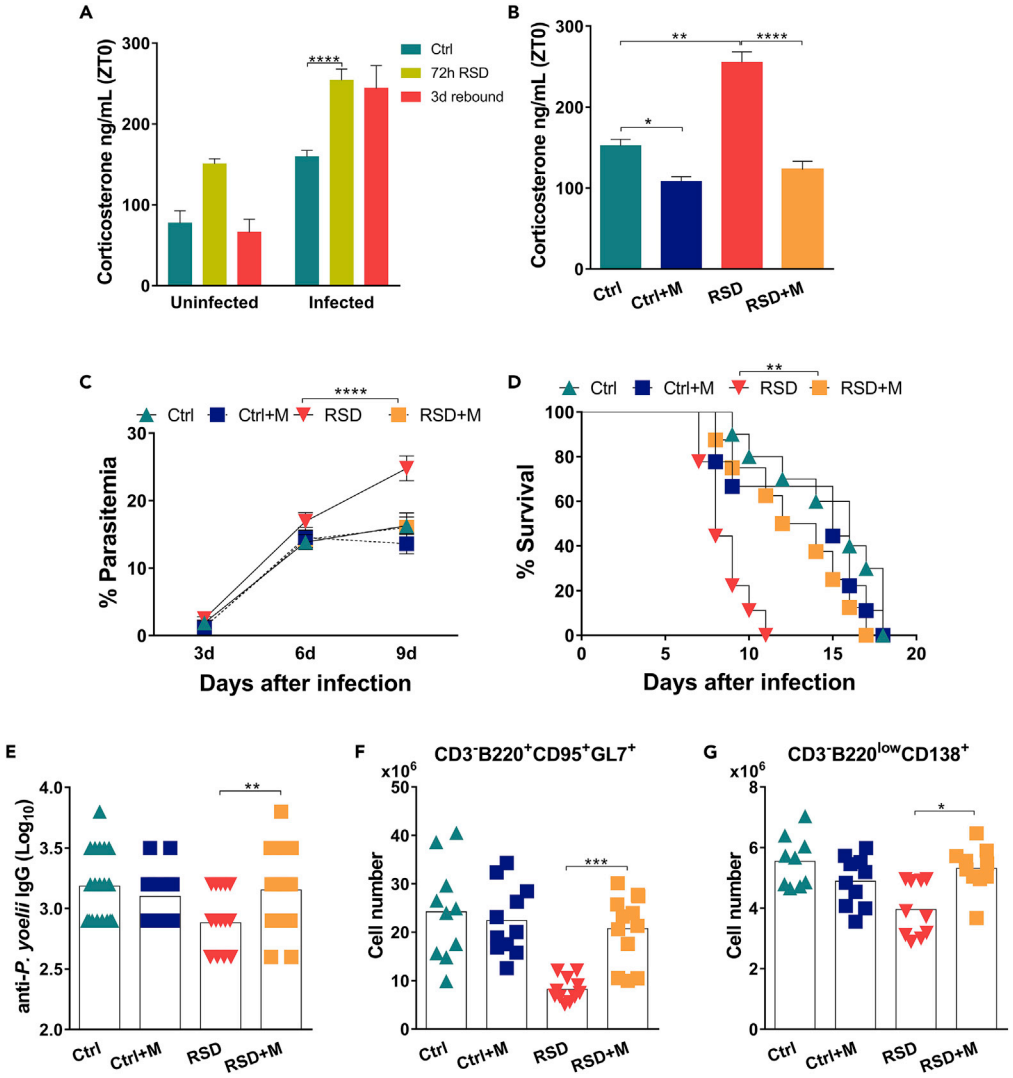

Figure 4. Exacerbated Synthesis of Glucocorticoids Triggered by REM Sleep Deprivation and *P. Yoelii* Infection Inhibits GC Formation and Host Humoral Immunity

Inhibition of corticosterone synthesis was accomplished by treating mice with metyrapone during the RSD period (RSD + M). As an internal control of the drug effect on disease outcome, infected Ctrl received the same treatment (Ctrl + M).

(A) Corticosterone concentration in uninfected and infected mice immediately after RSD and after 3 days of sleep recovery (3d rebound). Data of uninfected Ctrl and uninfected RSD mice represent one experiment with at least 5 mice in each group. Mean ± SD; data from Ctrl and RSD infected mice are from two independent experiments with at least five mice in each group. Mean ± SEM (****$p < 0.0001$) by two-way ANOVA.

(B) Corticosterone plasma levels in infected Ctrl and RSD mice after metyrapone treatment. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$) by one-way ANOVA.

(C) Parasitemia levels in Ctrl and RSD mice treated or not with metyrapone. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (****$p < 0.0001$) by two-way ANOVA.

(D) Mice survival following treatment with metyrapone. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by log-rank (Mantel-Cox) test.

(E) Anti-*P. yoelii* IgG1 serum titers in Ctrl and RSD mice treated or not with metyrapone. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by one-way ANOVA.

(F) Absolute numbers of splenic GC B cells ($CD3^-B220^+GL7^+CD95^+$) in Ctrl and RSD mice treated or not with metyrapone. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (***$p < 0.001$) by one-way ANOVA.

Figure 4. *Continued*
(G) Absolute numbers of splenic plasma B cells ($CD3^{-}B220^{low}CD138^{+}$) in Ctrl and RSD mice treated or not with metyrapone. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by one-way ANOVA.

the sleep disturbance period; therefore, we hypothesized that RSD during the acute phase of the immune response to malaria compromised both Tfh differentiation and Th1 polarization. This idea was reinforced by the fact that at day seven post infection, both Maf, a Tfh differentiation factor (Andris et al., 2017; Nurieva and Chung, 2010), and T-bet, a pro-Th1 transcription factor (Szabo et al., 2000; Saravia et al., 2019), were reduced in the RSD group.

As we did not find a significant production of the immunosuppressive cytokine IL-10 in RSD mice (data not shown), we explored other potential mechanisms, and Glcs (cortisol in humans and corticosterone in rodents) appeared as candidates based on their known immunosuppressive effects (Di Comite et al., 2007). Sleep loss has multiple effects on the homeostasis of multiple hypothalamic areas (Fifel et al., 2018). Stress is inherent to sleep disorders, leading to a potent increase in HPA axis activation and subsequently Glc release by the adrenal gland cortex (Balbo et al., 2010). The Glc release is the primary and major response to stressful events, and several studies have demonstrated that RSD potently increases corticosterone production (Galvao Mde et al., 2009; Andersen et al., 2005; Nunes et al., 2018; Zager et al., 2009). Stressful events may also include infectious processes. Malaria patients infected with *P. vivax* and *P. falciparum* have been found to present increased levels of blood cortisol (van Zon et al., 1982; Dekker et al., 1997; Davis et al., 1997; van Thien et al., 2001; Wilson et al., 2001; Blumer et al., 2005; Muehlenbein et al., 2005). Moreover, elevated cortisol levels were also reported in *P. falciparum*-infected patients with cerebral malaria (Blumer et al., 2005), while other studies correlated cortisol levels with increased parasitemia in pregnant women (Vleugels et al., 1989; Bouyou-Akotet et al., 2004, 2005; Adam et al., 2007). Studies in murine models of malaria showed that an elevation in corticosterone levels upon infection correlated with increased parasitemia (Barthelemy et al., 2004) and disease severity (Van Zon et al., 1983).

In agreement with these findings, we observed that *P. yoelii* infection, as well as RSD, increased the release of endogenous Glcs, albeit to a lesser extent. However, the combination of RSD and *P. yoelii* infection resulted in an additive effect, leading to the exacerbated production of Glcs. The exacerbated Glc production induced higher parasitemia, a lower survival rate, and inhibition of Tfh, GC, and plasma B cell differentiation. These results are in line with those of previous studies using influenza infection and stress-inducing models that demonstrated that, together, these stimuli exert an additive effect on Glc production and impair specific IgG production and immune cell distribution in the periphery (Hermann et al., 1994). Therefore, our data indicated the HPA axis hyperactivation is responsible for impairing host immune response to the parasite.

Endogenous physiological concentration of Glcs is an important mechanism for immune regulation during *Plasmodium* infection (Vandermosten et al., 2018) and also for antibody production (Shimba et al., 2018). However, the long-term continuous exposure to exacerbated levels of Glcs may potentially suppress T lymphocyte-mediated immune responses (Van Laethem et al., 2001a, 2001b; Ashwell et al., 2000; Kovacs, 2014).

The inhibition of Glcs synthesis by metyrapone (Besedovsky et al., 2014; Machado et al., 2013) did not influence the outcome of malaria infection in Ctrl mice, corroborating the idea that induction of Glcs synthesis by is a physiological event, insufficient to hamper host immunity. By contrast, in the RSD-infected group, metyrapone treatment restored parasite control and survival, which was associated with higher specific antibody titers when compared to non-treated RSD mice. Additionally, metyrapone-treated RSD mice displayed higher numbers of Tfh, GC, and plasma B cells. *In vitro* assays revealed that T cells from these mice also produced higher levels of IFN-γ and TNF-α and restored proliferative capacity upon stimulation with cognate antigens. Furthermore, Glcs have been described as important modulators for transcription factors, such as T-bet (Liberman et al., 2007) and Maf (Mao et al., 2007). We found that metyrapone treatment restored the expression of *Tbx21* and *Maf*, indicating that high levels of endogenous Glcs hindered host immunity through the inhibition of these transcription factors.

Collectively, our findings provide evidence that exacerbated endogenous Glcs production, during the development phase of the B cell response due to REM sleep disturbance and infection, was the mechanism

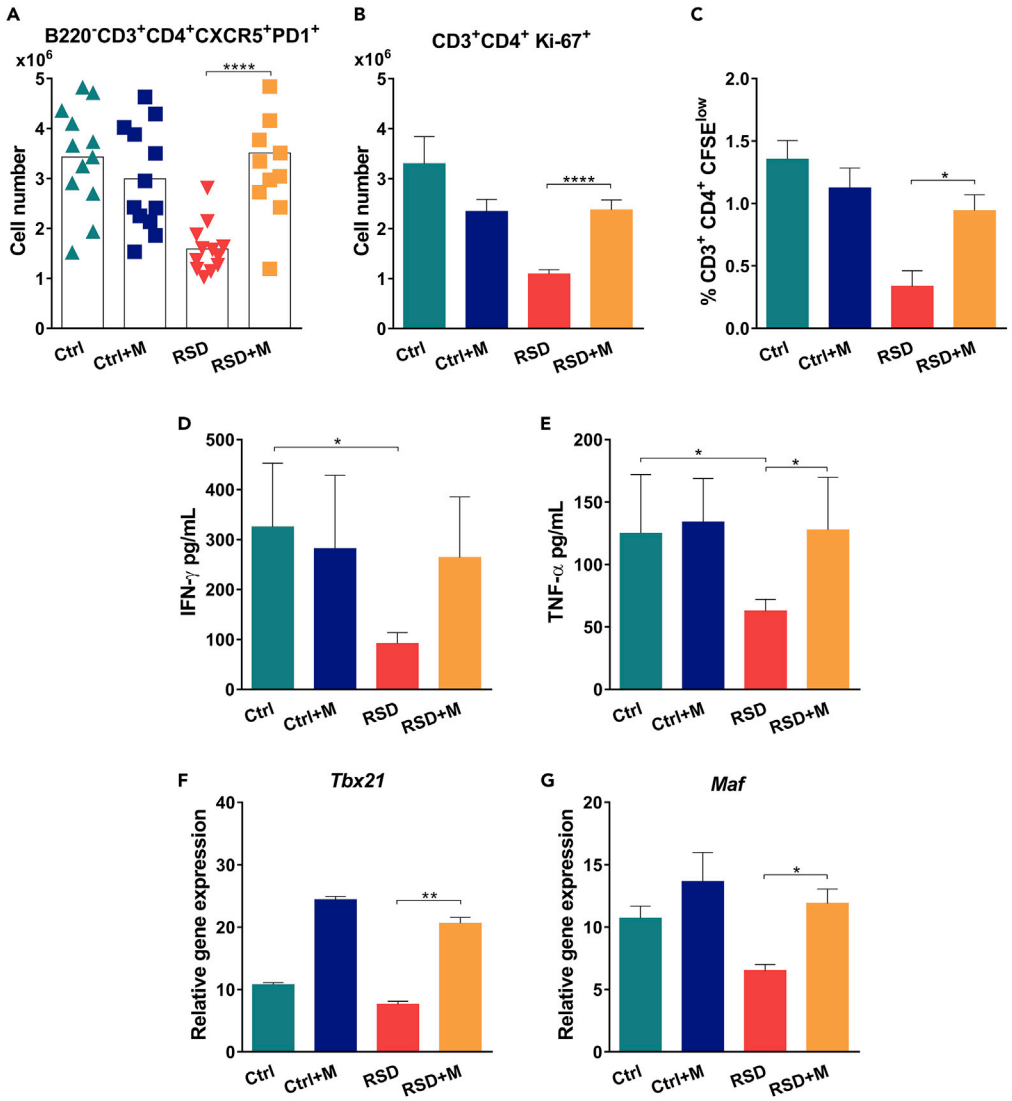

Figure 5. Exacerbated Synthesis of Glucocorticoids Triggered by REM Sleep Deprivation and *P. Yoelii* Infection Inhibits Tfh Cell Differentiation

Inhibition of corticosterone synthesis was accomplished by treating mice with metyrapone during the RSD period (RSD + M). Analyses were performed at day nine post infection. As an internal control of the drug effect on malaria outcome, infected Ctrl received the same treatment (Ctrl + M).

(A) Absolute numbers of splenic Tfh cells ($B220^{-}CD3^{+}PD\text{-}1^{+}CXCR5^{+}$) in Ctrl and RSD mice treated or not with metyrapone. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (****$p < 0.0001$) by one-way ANOVA.

(B) *Ex vivo* analysis of proliferating $CD4^{+}$ T cells stained with $Ki67^{+}$. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (****$p < 0.0001$).

(C) Frequency of specific proliferating ($CFSE^{low}$) $CD3^{+}CD4^{+}$ T cells after culture in the presence of recombinant *P. yoelii* MSP-1_{19} for five days. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by one-way ANOVA.

(D) Levels of IFN-γ in cell supernatants from Ctrl and RSD splenocytes after culture with recombinant *P. yoelii* MSP-1_{19} for 48 hr. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by one-way ANOVA.

(E) Levels of TNF-α in cell supernatants from Ctrl and RSD splenocytes after culture with recombinant *P. yoelii* MSP-1_{19} for 48 hr. Data are from three independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by one-way ANOVA.

Figure 5. *Continued*
(F) *Tbx21* mRNA expression by sorted CD4$^+$ T cells from Ctrl and RSD mice. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (**$p < 0.01$) by one-way ANOVA.
(G) *Maf* mRNA expression by sorted CD4$^+$ T cells from Ctrl and RSD mice. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (*$p < 0.05$) by one-way ANOVA.

responsible for hampering host resistance to the parasite. Thus, Glc levels may be a potential target for predicting disease severity or developing new therapeutic strategies for disease management in endemic areas.

Limitations of the Study

The comprehension of the bidirectional relationship between the nervous and immune systems is essential for the development of new strategies to potentiate the immune response and mitigate its effects on sleep homeostasis and *vice versa*. Herein we showed the effect of RSD on the host response against the blood stage of the malaria parasite. Thus, further experiments are important to extend these findings to the *Plasmodium* complex life cycle and determine the impact of host immunity on sleep homeostasis and how to manipulate this cycle to improve disease management.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniela Santoro Rosa (dsrosa@unifesp.br).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

There are no data sets and/or code associated with the paper.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101599.

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AUTHOR CONTRIBUTIONS

E.R.F., M.L.B., M.P.A., F.B.S., and J.S.A. performed experiments; E.R.F., S.B.B., A.C.K., and D.S.R. designed the experiments; E.R.F., A.C.K., and D.S.R. analyzed the data; A.C.K. and D.S.R. wrote the paper; S.B.B., S.T., and M.L.A. edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Sleep Disturbance during Infection Compromises Tfh Differentiation and Impacts Host Immunity

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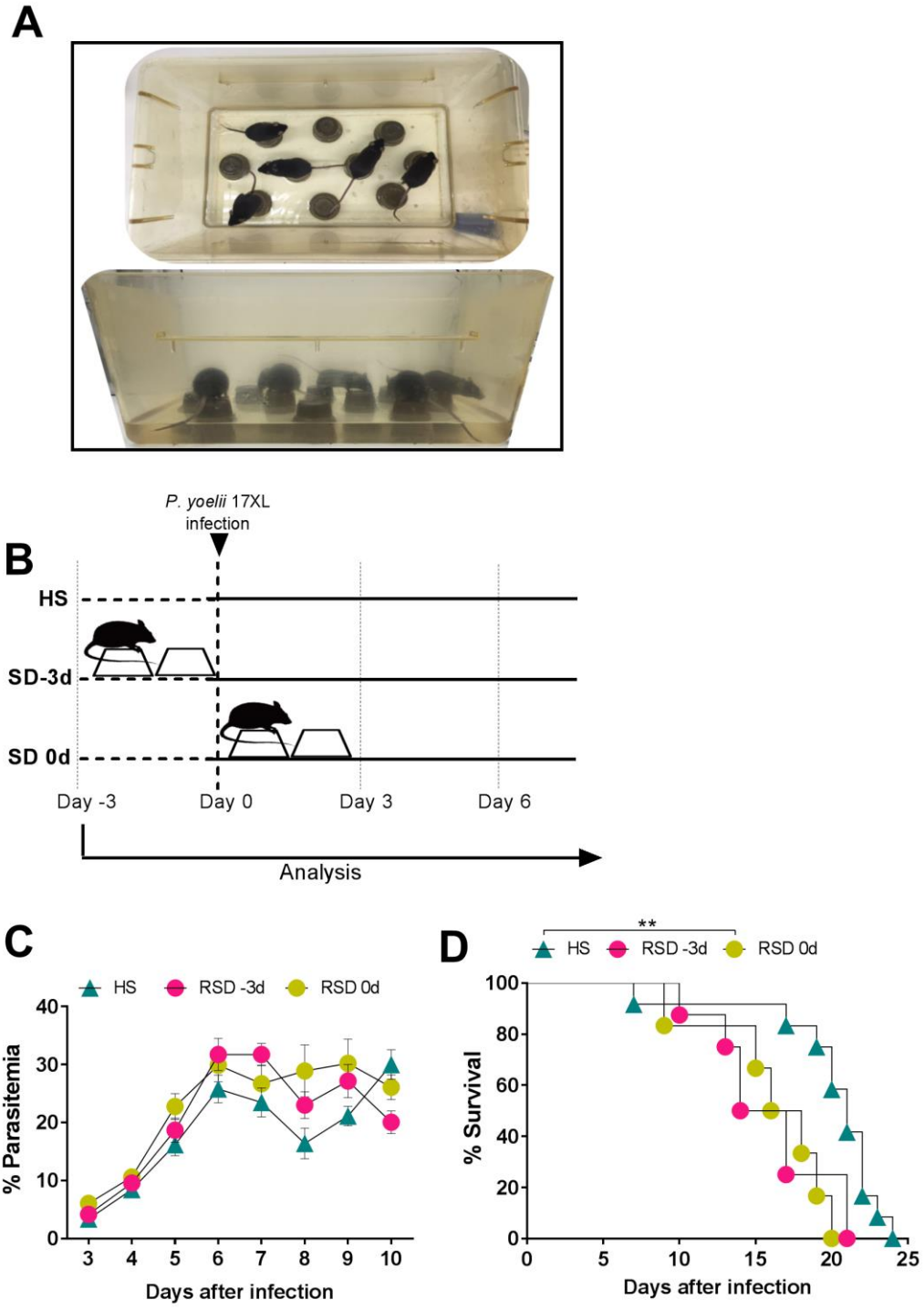

Figure S1. Time-dependent relationship between sleep deprivation and parasite infection drives malaria outcome, Related to Figure 1. Mice were subjected to the RSD protocol at different times post-infection. Here we summarize data obtained when animals were infected with *P. yoelii* at the beginning of the RSD protocol or just after the period of 72h of RSD. **(A)** Modified multiple platform method. **(B)** Experimental set up. **(C)** Parasitemia in Ctrl and RSD mice. Data were pooled from two independent experiments with at least five mice in each group. Mean ± SEM. **(D)** Survival in Ctrl and RSD mice. Data are from two independent experiments with at least five mice in each group. Mean ± SEM (** $p<0.005$) by log-rank (Mantel-Cox) test.

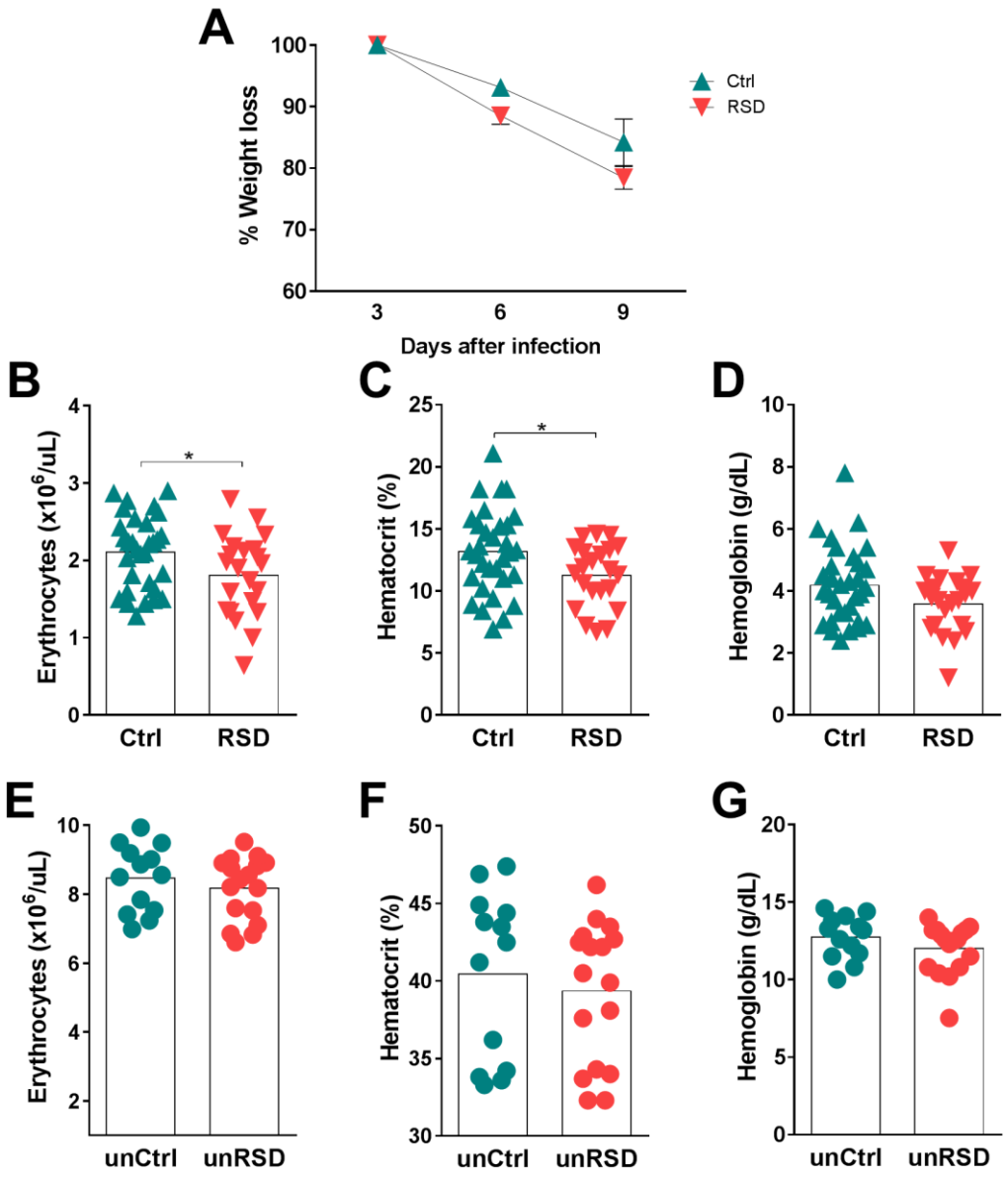

Figure S2. REM sleep deprivation during acute phase of malaria infection reduces erythrocytes and hematocrit counts, Related to Figure 1.
(A) Weight loss during the experimental period in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group Mean ± SEM. **(B, C)** Erythrocytes and hematocrits counts. **(D)** Hemoglobin blood levels in infected Ctrl and RSD mice. Data are from six independent experiments with at least five mice in each group. Mean ± SEM (* $p<0.05$) two-tailed unpaired t test. **(E, F)** Erythrocytes and hematocrit counts. **(G)** Hemoglobin blood levels in uninfected Ctrl and RSD mice. Data are from six independent experiments with at least two mice in each group. Mean ± SEM.

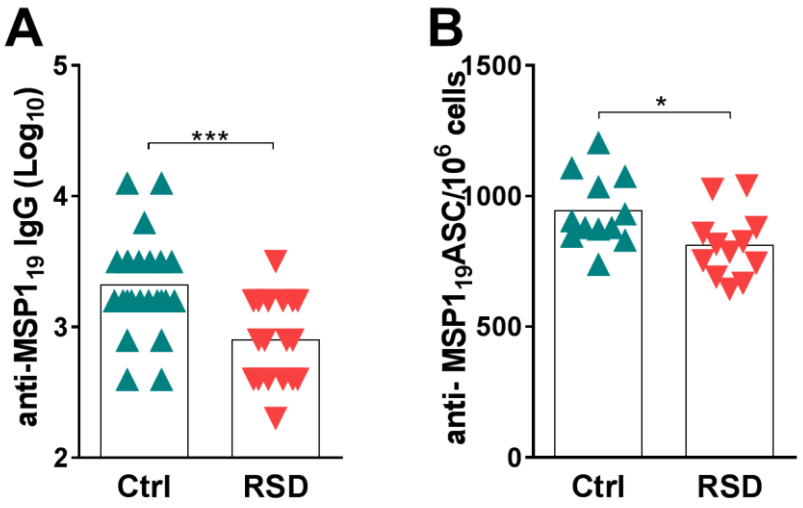

Figure S3. REM sleep deprivation impairs the production of anti-MSP1$_{19}$ antibodies and reduces the number of specific antibody-secreting cells, Related to Figure 2. Infected Ctrl and RSD mice were analyzed at day nine post-infection. **(A)** Anti-MSP1$_{19}$ IgG serum titers in Ctrl and RSD mice. Data are from six independent experiments with at least five mice in each group. Mean ± SEM (*** p<0.001) two-tailed unpaired t test. **(B)** ELISpot analysis of splenic anti-MSP1$_{19}$ antibody-secreting cells (ASC). Data are from two independent experiments with at least five mice in each group. Mean ± SEM (* p<0.05) two-tailed unpaired t test.

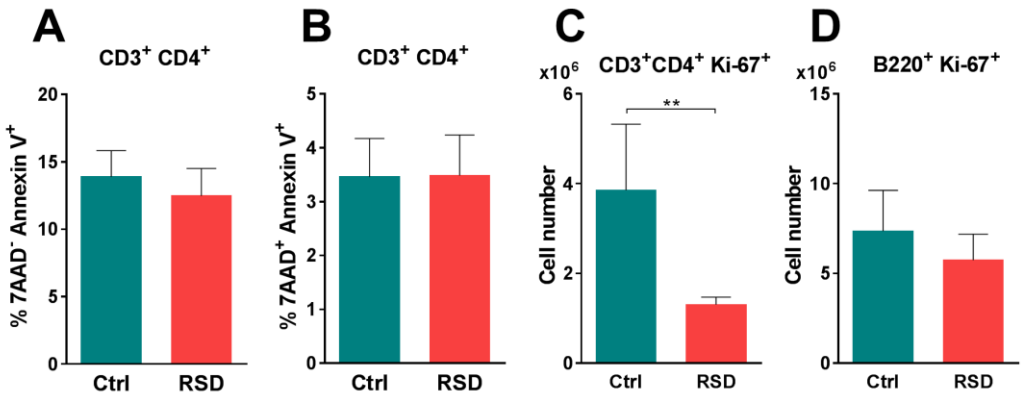

Figure S4. REM sleep deprivation impairs CD4$^+$ T cell proliferation, Related to Figure 3. Infected Ctrl and RSD mice were analyzed at day nine post-infection. **(A)** *Ex vivo* flow cytometry analysis of apoptotic splenic CD4$^+$ T cells (7AAD$^-$AnexinV$^+$). Data are from two independent experiments with at least five mice in each group. Mean ± SEM. **(B)** *Ex vivo* flow cytometry analysis of necrotic splenic CD4$^+$ T cells (7AAD$^+$AnexinV$^+$). Data are from two independent experiments with at least five mice in each group. Mean ± SEM. **(C)** *Ex vivo* analysis of proliferating CD4$^+$ T cells stained with Ki67$^+$. Data represent one experiment with at least five mice in each group. Mean ± SD (** $p<0.005$) two-tailed unpaired t test. **(D)** *Ex vivo* analysis of proliferating B220$^+$ cells stained with Ki67$^+$. Data represent one experiment with at least five mice in each group. Mean ± SD.

Transparent methods

Mice and Plasmodium yoelii infection

Seven-week-old C57BL/6 WT male mice were obtained from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia) and kept under specific pathogen-free conditions, in a controlled 12:12 light-dark cycle, with water and food *ad libitum* at Department of Psychobiology animal facility, Federal University of São Paulo. Mice were inoculated intraperitoneally (i.p.) with $5x10^4$ *Plasmodium yoelii* 17XL-GFP infected red blood cells (iRBCs) (kindly provided by Dr. Claudio Romero Farias Marinho, University of São Paulo) and evaluated daily to ensure mice welfare.

REM sleep deprivation protocol

REM sleep deprivation protocol was performed according to the modified multiple platform method adapted to mice (Asakura et al., 1992, Suchecki and Tufik, 2000, Machado et al., 2004). The muscle relaxation is a common phenomenon of the REM sleep phase. Taking advantage of this feature, mice are housed in a ventilated home cage (38 x 31 x 17 cm) containing many platforms (frequently 2 platforms per mouse, with 3.5 cm in diameter) surrounded by water up to 1 cm beneath the surface of the platform. Mice were able to move inside the cage jumping across the platforms. To reduce stress, mice were exposed to this protocol for 1 hour during three consecutive days. After training, mice were submitted to this protocol for 72 hours with access to water and food *ad libitum* and a controlled 12:12 light-dark cycle. This protocol resulted in total REM sleep deprivation and a partial (~30%) NREM sleep deprivation. It is important to note that the protocol is carried out with socially stable animals in the same cage and

several platforms to avoid social isolation and movement restriction (Machado et al., 2004). Mice were submitted to REM sleep deprivation 3 days before, at the same day or 3 days after infection.

Blood cell count and parasitemia

Blood was collected in the presence of anticoagulant (0.1M EDTA) by cardiac puncture after anesthesia. Hematological analysis was performed using BC-2800Vet® (*Mindray*). Parasitemia was monitored daily after collecting 2µL of blood from the tip of the tail. Parasitemia was quantified by flow cytometry after selection of GFP^+ cells on the red blood cells gate, and confirmed by counting infected red blood cells in blood smears.

Flow cytometry

Cellular profile in the blood and spleen were evaluated by flow cytometry. Blood leukocyte suspension was obtained after erythrocyte lysis with ACK solution (0.15 mol/L NH_4Cl, 1mmol/L $KHCO_3$, 0.1 mmol/L Na_2 EDTA). Splenocytes were obtained after tissue dissociation with a cell strainer followed by erythrocyte lysis with ACK. After one wash with RPMI 1640 (Gibco), cells were labeled with fluorochrome-conjugated antibodies: CD45 Pacific Blue or peridinin-chlorophyll-protein complex (PerCP), clone 3D-F11; CD3 phycoerythrin (PE) or allophycocyanin-Cy7 (APC-Cy7), clone 145-2C1; CD4 PerCP or PE-Cy7, clone RM4-5; CD8 APC or Pacific Blue, clone 53-6.7; CD45R (B220) PerCP or Pacific Blue, clone RA3-6B2; CD95 PE, clone Jo2; GL7 fluorescein isothiocyanate (FITC), clone GL7; CXCR5 APC, clone 2G8; CD279 (PD1) PE, clone J43; CD69 FITC, clone H1.2F3-m; CD278 (ICOS) FITC, clone C398.4A; CD138 APC, clone 281-2, CD19 APC, clone 1D3. For *ex vivo* intracellular staining, cells were surface

stained, treated with Cytofix/Cytoperm kit (BD Biosciences) followed by staining with fluorochrome-conjugated antibodies: IFNγ APC or PerCP, clone XMG1.2; TNFα PE-Cy7, clone MP6-XT22. Intranuclear staining was performed after Transcription Factor Staining Buffer Set kit (Ebioscience) protocol according to the manufacturer's instructions followed by staining with fluorochrome-conjugated antibodies: Ki67 Pacific Blue, clone B56. Cellular acquisition was performed by FACSCanto II or LSR Fortessa flow cytometer (BD Biosciences) followed by FlowJo software analysis (v.10; BD Biosciences). All flow cytometry experiments were performed using unstained and all single-color controls to ensure proper compensation and analysis.

Gene expression

Total RNA from sorted $CD4^+$ T lymphocytes was obtained with the RNeasy plus micro kit (Qiagen) following the manufacturer's instructions. RNA was then reverse transcribed into cDNA using the SuperScript™ II Reverse Transcriptase kit (Invitrogen). Gene expression was analyzed by quantitative real-time PCR (PowerUp™ SYBR Green™ Master Mix, Applied Biosystems) and the reaction was performed in a 7500 Real-Time PCR System (Applied Biosystems). Gene expression was calculated by the ΔCt or ΔΔCt method. The primers used in the analyses are described:

Gapdh-F: 5'-AAATGGTGAAGGTCGGTGTG-3'

Gapdh-R: 5'- TGAGGGGTCGTTGATGG-3'

Tbx21-F: 5'- TCAACCAGCACCAGACAGAC-3'

Tbx21-R: 5'- ATCCTGTAATGGCTTGTGGG-3'

Bcl6-F: 5'- CAGAGATGTGCCTCCATACTGC-3'

Bcl6-R: 5'- CTCCTCAGAGAAACGGCAGTCA-3'

Maf-F: 5'- AGCAGTTGGTGACCATGTCG-3'

Maf-R: 5'- TGGAGATCTCCTGCTTGAGG-3'

Cell culture and proliferation assay

Splenocytes were isolated and stained with 1.25 µM of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) diluted in warm PBS 1X ($50x10^6$ cells/mL) for 10 minutes at 37°C. Cells were then washed with RPMI 1640 supplemented with 10% of FBS (R10) and cultured ($5x10^5$/200µL in triplicates) in 96-well plates (Costar) for 5 days at 37°C and 5% CO_2 in the presence of recombinant *P. yoelii* MSP-1_{19} (10 µg/mL) (kindly provided by Dr. Silvia Beatriz Boscardin, University of São Paulo). After the incubation period, culture supernatant was collected. Cells were then stained with fluorochrome-conjugated antibodies: CD3 PE or APC-Cy7, clone 145-2C1; CD4 PerCP or PE-Cy7, clone RM4-5; CD8 APC or Pacific Blue, clone 53-6.7. The frequency of proliferating $CD3^+CD4^+$ T cells was calculated by subtracting background values. For each experiment performed, unstained and all single-color controls were processed to allow proper compensation.

Cytokine quantification

Cytokine production in culture supernatants was quantified by Cytometric Bead Array (mouse Th1/Th2/Th17 kit, BD Pharmingen) according to the manufacturer's instructions.

ELISpot

ELISpot assay was performed using IFN-γ ELISpot Ready-SET-Go! kit (eBiosciences) following the manufacturer's instructions. Splenocytes

($3x10^5$/well) were stimulated for 18 hours with recombinant *P. yoelii* MSP-1_{19} (10µg/ mL). For B cell ELISpot, 96-well plates (MAIPS 4510, Millipore) were coated with recombinant *P. yoelii* $MSP1_{19}$ protein (5µg/ mL) in PBS and incubated overnight at room temperature. After three washes with PBS, wells were blocked with R10 for one hour at 37ºC and 5% CO_2. Five hundred thousand splenocytes were added and incubated for 16 hours at 37 ºC and 5% CO_2. The plates were washed with PBS and incubated with horseradish peroxidase labeled goat anti-mouse IgG (1:1,000; KPL) for 2 hours at room temperature. After extensive washes with PBS, the reaction was developed by 3-amino-9-ethylcarbazole (AEC; BD Biosciences). Spots were counted with an AID ELISpot Reader System (Autoimmun Diagnostika GmbH).

ELISA

Specific antibodies were quantified by coating 96-well high binding flat bottom plates with recombinant *P. yoelii* MSP-1_{19} (200ng/ well) or total parasite protein extract (500ng/ well) in carbonate buffered solution overnight at 4°C. Parasite extract was obtained from an infected C57BL/6 mice whole blood (parasitemia > 50%) as previously described (65). Plates were then blocked with 200 µL/well of PBS 1X containing 1% BSA for 2h at RT. After incubation, mice sera were added in serial dilutions and incubated for 1h. Then, plates were incubated with horseradish peroxidase anti-mouse IgG, IgG1, IgG2b and IgG2c (SouthernBiotech, Birmingham, Ala) for 1h at RT. After incubation, the reaction for detection of conjugated antibodies was performed with the addition of phosphate-citrate buffer (pH 5,0) containing 1 mg/mL o-phenylenediamine (Sigma) and 0,03% (vol/vol) hydrogen peroxide for 15 minutes. The reaction was

quenched with 4N H_2SO_4 solution. Colorimetric reaction was analyzed at 492 nm (Thermofisher Scientific).

Adoptive antibody transfer

Antibodies were purified by affinity chromatography with immobilized protein G columns (GE healthcare) from infected donor mice sera collected 9-11 days after infection. Antibody concentration was determined by spectrophotometry (NanoDrop, Thermofisher). REM sleep deprived mice were injected intravenously with 200 µg of purified antibodies 6 days after infection (immediately after REM sleep deprivation protocol). A control group received antibodies purified from uninfected mice.

Metyrapone treatment

Twice a day with 12h intervals, mice were injected intraperitoneally with 100 mg/kg of 2-Methyl-1,2-di-3-pyridyl-1-propanone (Metyrapone, Sigma) during REM sleep deprivation protocol started 3 days after infection. The non-sleep deprived control group also received the same dose during this period. In addition, control non-sleep deprived and REM sleep deprived groups were treated only with vehicle.

Corticosterone measurement

Corticosterone levels were measured in plasma samples by ultrafast liquid chromatography-tandem mass spectrometry on a Xevo TQ-S Micro triple quadrupole mass spectrometer (Waters, Milford, Mass) using a Acquity UPLC® BEH C18 1,7µm (2,1x50mm) column at flow rate of 0,2mL/minute, controlled by MassLynx 4.1 software (Waters).

Data Analysis

Statistical analyses were performed using GraphPad Prism 7 software (San Diego, CA). For comparison between two groups, two-tailed unpaired t test was performed. For three or more groups, one-way ANOVA followed by Kruskal-Wallis test or two-way ANOVA followed by Bonferroni's test were used. For survival rate, the log-rank (Mantel-Cox) test was used. Summary data from two or more experiments were represented by mean ± SEM and representative data were represented by mean ± SD as detailed in figure legends.

Study approval

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) (#9067160615) and carried out in compliance with the recommendations of Federal Law 11.794 (2008), the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA) and the ARRIVE guidelines.

References

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