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Supplemental Information

Sleep Disturbance during Infection Compromises Tfh Differentiation and Impacts Host Immunity

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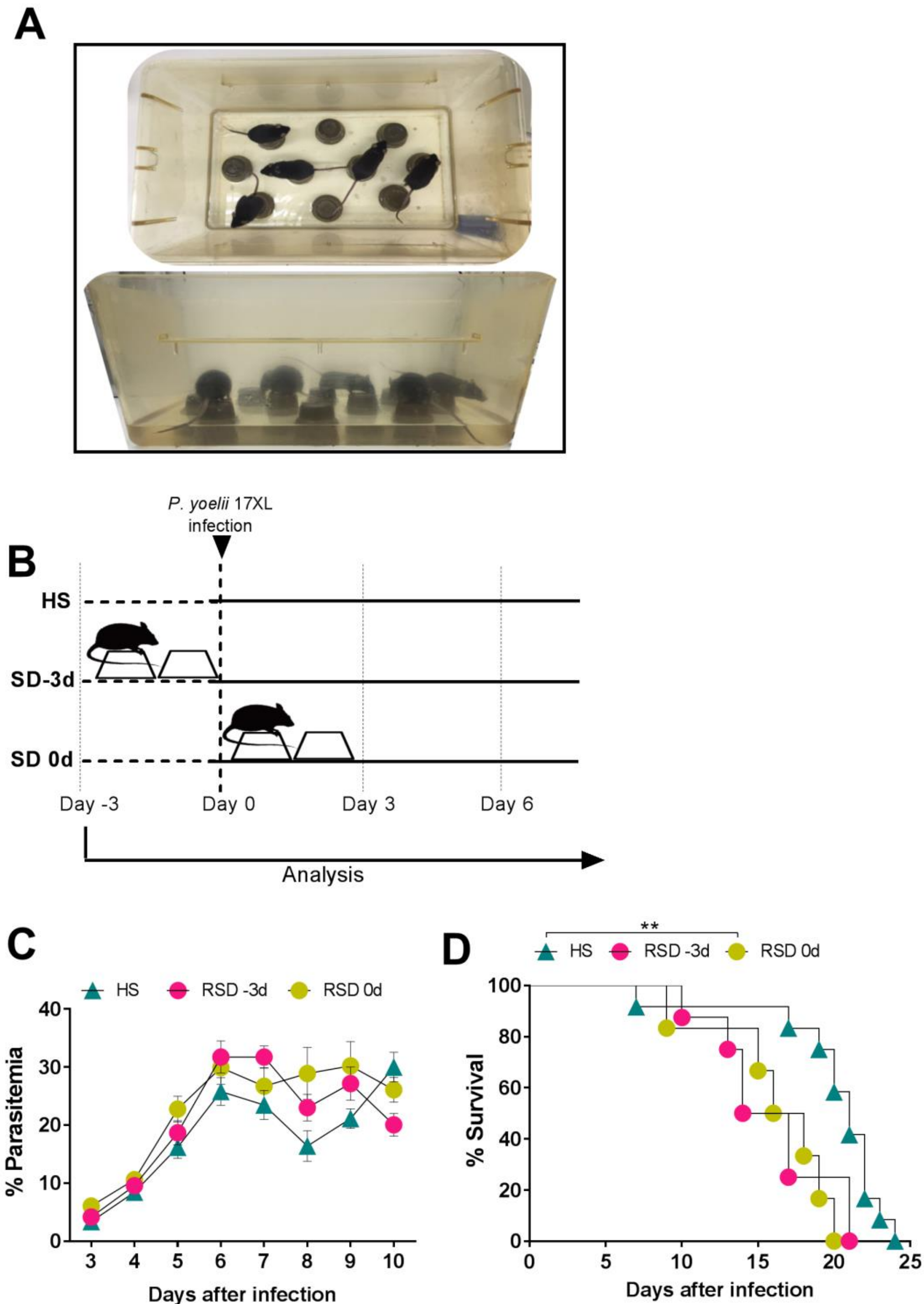

Figure S1. Time-dependent relationship between sleep deprivation and parasite infection drives malaria outcome, Related to Figure 1. Mice were subjected to the RSD protocol at different times post-infection. Here we summarize data obtained when animals were infected with *P. yoelii* at the beginning 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.

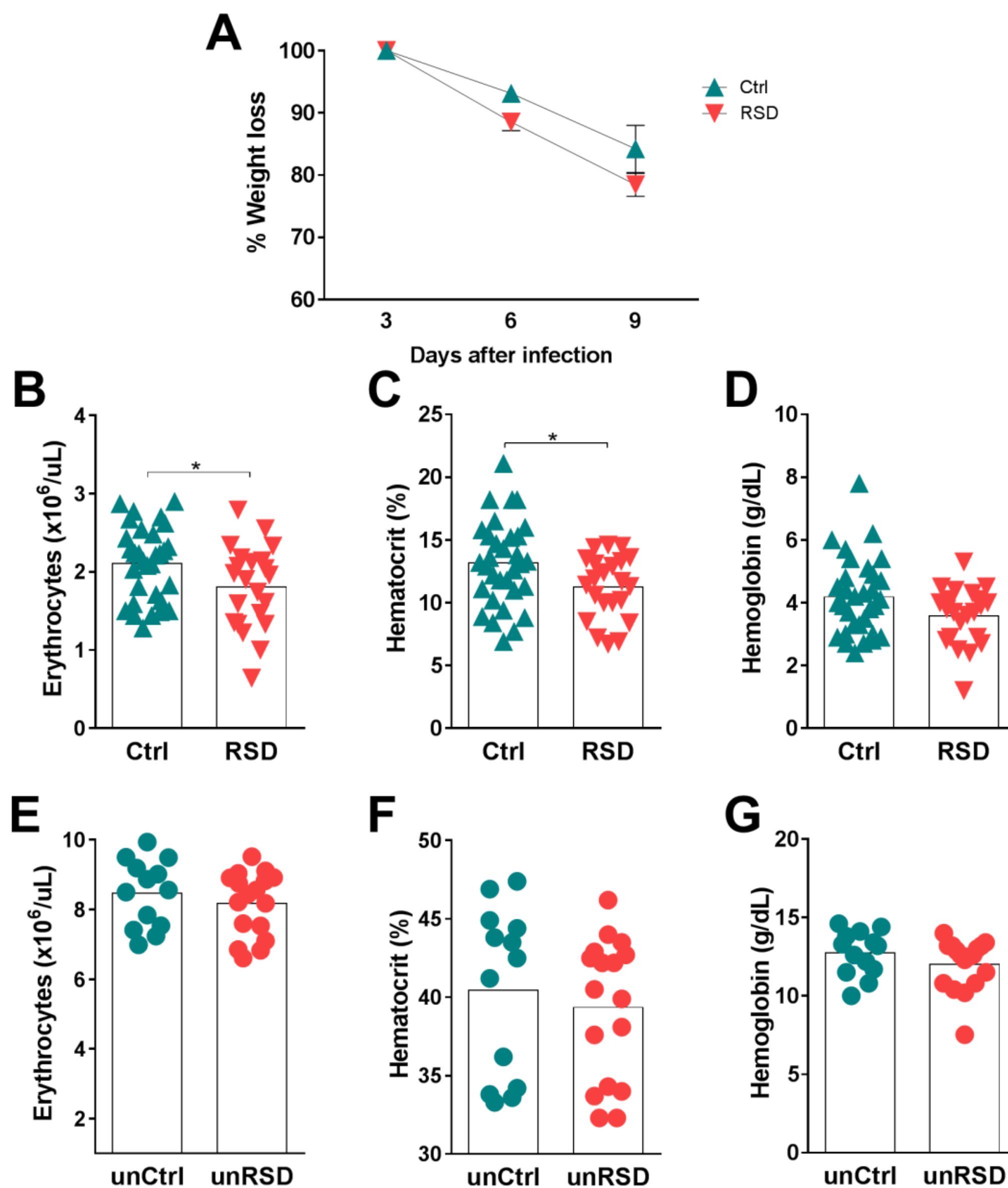

Figure S2. REM sleep deprivation during acute phase of malaria infection reduces erythrocytes and hematocrit counts, Related to Figure 1.
(A) Weight loss during the experimental period in Ctrl and RSD mice. Data are from three independent experiments with at least five mice in each group Mean ± SEM. **(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.

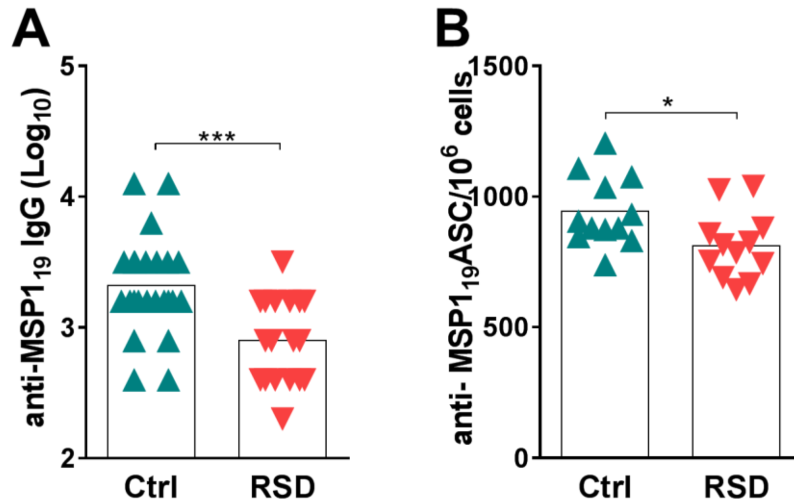

Figure S3. REM sleep deprivation impairs the production of anti-MSP1$_{19}$ antibodies and reduces the number of specific antibody-secreting cells, Related to Figure 2. Infected Ctrl and RSD mice were analyzed at day nine post-infection. **(A)** Anti-MSP1$_{19}$ IgG serum titers in Ctrl and RSD mice. 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.

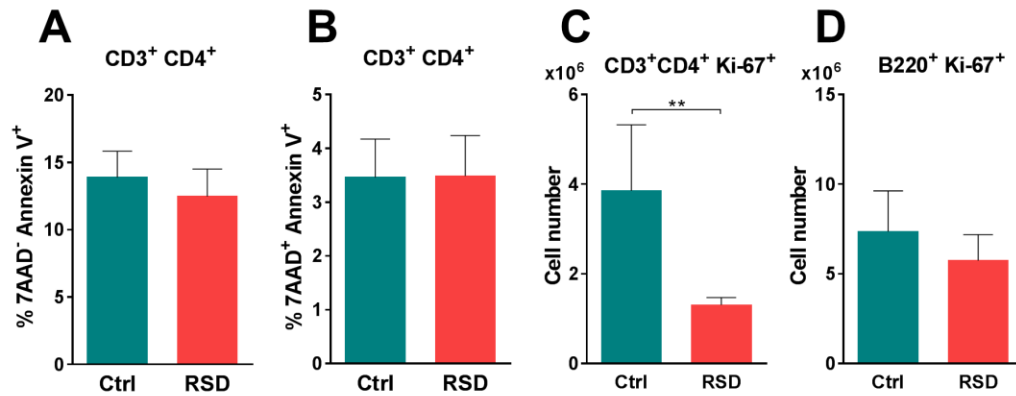

Figure S4. REM sleep deprivation impairs $CD4^{+}$ T cell proliferation, Related to Figure 3. Infected Ctrl and RSD mice were analyzed at day nine post-infection. **(A)** *Ex vivo* flow cytometry analysis of apoptotic splenic $CD4^{+}$ T cells ($7AAD^{-}AnexinV^{+}$). Data are from two independent experiments 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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