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# Impact of sleep restriction in B-1 cells activation and differentiation

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ABSTRACT

B-1 lymphocytes are a subtype of B cells with functional and phenotypic features that differ from conventional B lymphocytes. These cells are mainly located in mice's pleural and peritoneal cavities and express unconventional B cell surface markers. B-1 cells participate in immunity by producing antibodies, cytokines, and chemokines and physically interacting with other immune cells. In addition, B-1 cells can differentiate into mononuclear phagocyte-like cells and phagocytize several pathogens. However, the activation and differentiation of B-1 cells are not entirely understood. It is known that several factors can influence B-1 cells, such as pathogens components and the immune response. This work aimed to evaluate the influence of chronic stress on B-1 cell activation and differentiation into phagocytes. The experimental sleep restriction was used as a stress model since the sleep alteration alters several immune cells' functions. Thus, mice were submitted to sleep restriction for 21 consecutive days, and the activation and differentiation of B-1 cells were analyzed. Our results demonstrated that B-1 cells initiated the differentiation process into mononuclear phagocytes after the period of sleep restriction. In addition, we detected a significant decrease in lymphoid lineage commitment factors (*EBF*, *E2A*, *Blnk*) ($^{*}P < 0.05$) and an increase in the *G-CSFR* gene (related to the myeloid lineage commitment factor) ($^{****}P < 0.0001$), as compared to control mice no submitted to sleep restriction. An increase in the co-stimulatory molecules CD80 and CD86 ($^{**}P < 0.01$ and $^{*}P < 0.05$, respectively) and a higher production of nitric oxide (NO) ($^{*}P < 0.05$) and reactive oxygen species (ROS) ($^{*}P < 0.05$) were also observed in B-1 cells from mice submitted to sleep restriction. Nevertheless, B-1 cells from sleep-restricted mice showed a significant reduction in the Toll-like receptors (TLR)-2, −6, and −9, and interleukine-10 (IL-10) cytokine expression ($^{***}P < 0.001$) as compared to control. Sleep-restricted mice intraperitoneally infected with L. amazonensis promastigotes showed a reduction in the average internalized parasites ($^{*}P < 0.05$) by B-1 cells. These findings suggest that sleep restriction interferes with B-1 lymphocyte activation and differentiation. In addition, b-1 cells assumed a more myeloid profile but with a lower phagocytic capacity in this stress condition.

## 1. Introduction

Since discovering B-1 cells in the 1980s, several studies have been performed to understand their origin, function, and involvement in the immune response to infectious diseases. B-1 lymphocytes are a subtype of B cells that differ from other B cells populations by unique surface markers ($CD19^{+}$ $IgM^{hi}$ $IgD^{lo}$ $CD11b^{+}$) and lack the CD23 marker (Hardy and Hayakawa 2001, Berland and Wortis 2002). These cells represent a significant population of B cells in mice's pleural and peritoneal cavities. However, they can also be detected in small quantities in the spleen

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(Baumgarth 2011, Baumgarth 2017). In addition, compared to conventional B cells, also called B-2 lymphocytes, B-1 cells have a less distinct repertoire of antibodies acting mainly on thymus-independent antigens (Whitmore et al., 2004). Also, B-1 cells generate low affinity polyreactive natural IgM antibodies that connect to repetitive epitopes, such as nucleic acids and carbohydrates (Montecino-Rodriguez et al., 2016). The importance of B-1 cells in immunity has been related to the production of immunomodulatory cytokines and; the ability to present antigens to T lymphocytes; they also play a protective or resistant role depending on the experimental infection (revised on (Novaes E Brito et al., 2019)). In addition, B-1 lymphocytes participate in some autoimmune diseases (e Brito et al., 2007, Novaes E Brito et al., 2014).

Murine peritoneal B-1 lymphocytes can differentiate in cells with physiological and phenotypic characteristics of mononuclear phagocytes (Almeida et al., 2001). Besides, it was also established that phagocytes derived from B-1 cells (B-1 cell-derived phagocyte – B-1 CDP) were capable of phagocytizing pathogens, such as Leishmania amazonensis promastigotes (Geraldo et al., 2016), *Escherichia coli* (Novaes e Brito et al., 2010), *Cryptococcus neoformans* (Ghosn et al., 2006), and apoptotic bodies (Novaes e Brito et al., 2010). Furthermore, molecular studies of B-1 cells showed that these cells express both lymphoid and myeloid lineage commitment factors but after phagocyte differentiation, the expression of myeloid commitment factors is increased (Popi et al., 2009). Thus, the demonstration of B-1 cell differentiation in phagocytes in vivo brings a new approach to a better comprehension of the role of this cell population in immunity (Popi et al., 2012, Reis et al., 2020). Moreover, the production of microbicide molecules by B-1 cells, such as reactive oxygen species (ROS) and nitric oxide (NO), supports the hypotheses of their role in innate immune response (Almeida et al., 2001, Popi et al., 2012, Reis et al., 2020). However, some aspects of B-1 cell biology, such as their participation in immunity during chronic stress, are not wholly uncovered.

Some evidence suggests communication between sleep and immune system regulation. For example, neurons and glial cells share standard intercellular signals, such as cytokines, hormones, and neurotransmitters (Besedovsky and del Rey 1996, Ransohoff 2009), which participate in physiological sleep regulation and the alteration of sleep in response to systemic inflammation. The hypothesis that sleep can influence the immune system follows the observation that sleep deprivation can affect various aspects of the immune system, including antibody responses (Spiegel et al., 2002, Lange et al., 2003), antigen uptake (Casey et al., 1974), increase in autoantibody levels (Palma et al., 2006) and phagocytosis (Palmblad et al., 1976). In addition, paradoxical sleep deprivation in mice exhibited a significant increase in systemic levels of glucocorticoids and atrophy of the thymus, a significant decrease in the number of splenic NK cells, and impaired NK cell activity against tumor cells (De Lorenzo et al., 2015, Sousa et al., 2020).

Some studies have demonstrated that B-1 cell activation and differentiation can be affected by the immune response and/or pathogens (Novaes E Brito et al., 2019). However, the effect of chronic stress, such as sleep restriction, is not known on B-1 cell activation and differentiation. Thus, this study aimed to evaluate the effects of sleep restriction on the behavior of B-1 cells. Our data showed sleep restriction-induced murine peritoneal B-1 cells' differentiation into a myeloid profile. Nevertheless, the sleep restriction impaired the production of IL-10 and the phagocytose by B-1 cells. Thus, our work brings a new understanding of the role of sleep on the activation and differentiation of B-1 cells.

## 2. Material and methods

### 2.1. Animals

Male wild type C57BL/6J mice, aged 8–10 weeks were obtained from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia, Universidade Federal de São Paulo, São Paulo, Brazil). Animals were housed in 12 h light/dark cycles under controlled temperature conditions with standard solid food ad libitum and filtered water. All animal practices were presented agreeing to the Federal Law 11.794 (2008), the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA). The local ethical committee approved the study (Comissão de Ética no Uso de Animais – CEUA) (#01/018). All efforts were made to reduce animal suffering and decrease the sample size.

### 2.2. Sleep restriction protocol

The multiple platform sleep restriction (SR) method was used throughout the study (Bergmann et al., 1996). This method abolishes the social isolation, and movement restriction, preserving the social hierarchy established in the home cage. Experimental groups comprised six mice. Mice adaptation to the sleep restriction protocol was performed for 1 h/day three days before the experimental protocol. Mice in the SR group were sleep-restricted for 18 h/day (with a sleep window from 10:00 am to 4:00 pm) for 21 consecutive days. This protocol simulates the chronic condition of sleep restriction (Machado et al., 2004). This technique efficiently leads to SR, reducing the slow-wave sleep by 20 % and 100 % in the paradoxical sleep. Furthermore, an augment in total sleep time was watched in the sleep window, with a slow-wave sleep rebound and paradoxical sleep, indicating that SR occurred. The protocol consisted of a polycarbonate tank (41 × 34 × 17 cm) filled with water. Ten narrow circular platforms (3 cm in diameter) were placed. The water reached 1 cm below the upper border of the platform. A maximum of four animals was placed in each tank, which allowed animals to move and jump from one platform to another. The muscle atony led animals to fall off the platform and wake. This method induced a consistent amount of sleep reduction in mice (Silva et al., 2004). Mice were distributed into two groups: (1) control mice, which remained in their home cages in the SR room, and (2) SR mice placed inside the SR tanks. Animals were euthanized by decapitation after 21 days of SR, and B-1 cells were collected by peritoneal lavage.

### 2.3. Flow cytometry analysis

Total peritoneal cells were collected by washing with sterile PBS and counted in the Neubauer chamber. The cell suspension was distributed in microtubes and diluted up to 100 μL PBS (1 × 106 cell/microtube). The purified rat anti-mouse CD16/CD32 (clone 2.4G2, BD Fc Block™, BD Bioscience, San Jose, CA, USA) was used to block non-antigen-specific binding immunoglobulins to the Fc receptors. Then, total cells were then stained with anti-CD23 coupled with phycoerythrin (PE) (clone B3B4, BD) and anti-CD19 associated with allophycocyanin (APC) (clone 1D3, BD). Lymphocyte population $CD23^{-}$ $CD19^{+}$ was identified as B-1 cells. The monoclonal antibodies fluorescein-isothiocyanate (FITC)-conjugated anti-mouse CD80 (anti-CD80 FITC, clone 16-10A1, BD), anti-CD86 FITC (clone GL1, BD), anti-CD40 FITC (clone 3/23, BD), anti-F4/80 coupled with peridinin-chlorophyll-protein - PerCP (clone BM8, BioLegend, San Diego, CA, USA) were used to evaluate cell surface markers. We used anti-MHC-II coupled with PE to assess the expression of MHC-II. B-1 lymphocytes were identified with anti-CD19 APC (BD) and anti-CD23 FITC (BD). After incubation for 30 min at 4 °C with the respective antibodies diluted at 1:100, cells were washed and resuspended in 500 μL of PBS.

Intracellular NO production was evaluated with DAF2-DA (Sigma, St. Louis, MO, United States), and ROS production was assessed using the fluorescent reagent $H_2$DCFHDA (Sigma). Peritoneal cells were incubated with 5 μM of DAF2-DA or $H_2$DCFHDA for 30 min at 37 °C in the dark. Then, cells were washed with PBS and resuspended with 600 μL of PBS.

Accuri C6 flow cytometer (BD) was used for data acquisition, and the results were analyzed with FlowJo software version 10.6.2 (FlowJo,

LLC, FlowJo™ Software for Mac, Ashland, OR, USA).

### 2.4. Enrichment of peritoneal B-1 cells

Total peritoneal cells were collected from the animals after the sleep restriction protocol was completed. After euthanasia, peritoneal cells were collected by washing and submitted to cells purification protocol as previously described (e Brito et al., 2007, Reis et al., 2020). Briefly, cell suspensions were incubated with Fc block for 60 min at 4 °C. Then, cells were washed with PBS and subjected to negative selection with anti-CD23 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by positive selection with anti-CD19 microbeads (Miltenyi Biotec) (CD23- CD19+), according to the manufacturer's instructions. B-1 cells were considered CD23-CD19+. The purity of the cell suspensions was constantly evaluated by flow cytometry.

### 2.5. Immunofluorescence and confocal laser scanning microscopy

After B-1 cell purification, cells were fixed with 3.7 % formaldehyde for 30 min, washed with PBS, and permeabilized with 0.5 % Triton X-100 for 10 min. Then, B-1 cells were washed three times with PBS and spun down on poly-L-lysine-coated glass slides in a cytological centrifuge (1000 rpm for 2 min). Finally, B-1 cells were incubated with anti-CD11b-FITC (BD Biosciences Pharmingen) diluted at 1:25, and nuclei were stained with DAPI (blue) (Sigma). The cell preparations were analyzed by SP8 confocal laser scanning microscopy (Leica Microsystems) with a 40X objective. Optical sections of 0.5–1 μm were made.

### 2.6. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

A quantitative reverse transcriptase-polymerase chain reaction was performed to analyze the expression of, Toll-like receptors (TLRs), cytokines, and myeloid and lymphoid commitment factors in B-1 cells from wild-type C57BL/6J sleep-restricted and control mice. All qRT-PCR followed the recommendations of the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). First, peritoneal B-1 cells were purified, and total RNA was extracted using PureLinkRNA Mini Kit (Ambion; Thermo Fisher Scientific Brand, Grand Island, NY, United States) following the manufacturer's recommendations. Then, total RNA quantification was performed by UV absorption in a spectrophotometer (Nanodrop 2000c, Thermo Fisher), and RNA integrity was assessed by electrophoresis in 1.5 % agarose gels. After treatment with DNAse (RQ1 RNase-free DNase; Promega, Madison, WI, United States), cDNA synthesis was employed with the Proto Script First Strand cDNA Synthesis Kit (New England Biolabs Inc., MA, United States). SYBR Green Real-Time PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the apparatus StepOnePlus (Applied Biosystems) were used to perform the qRT-PCR. The conditions of qRT-PCR reactions and the following analysis were performed (Reis et al., 2020).

The primers sequences used for each target gene are shown in Supplementary Table 1. The primers sequences were obtained according to PrimerBank (Spandidos et al., 2010) and validated in (Reis et al., 2020).

### 2.7. Parasites

The *L. amazonensis* strain (MHOM/BR/1973/M2269) was kindly provided by Clara Lucia Barbieri (Universidade Federal de São Paulo, São Paulo, Brazil). Parasites were maintained as promastigotes by culturing in 199 medium (Gibco, Life Technologies Brand, Grand Island, NY, United States) supplemented with 4.2 mM sodium bicarbonate, 4.2 mM HEPES, 1 mM adenine, 5 mg/mL hemin (bovine type I) (Sigma, St. Louis, MO, United States) and 10 % fetal calf serum (FCS) (Gibco, Carlsbad, CA, United States). The parasite cultures were maintained at 26 °C until the stationary growth phase.

### 2.8. In vivo phagocytosis of L. amazonensis

Each mouse was intraperitoneally injected on the 21 days of sleep restriction (24 h before euthanasia) with $1x10^7$ L. amazonensis promastigotes in a final volume of 100 μL. Control mice were also injected with L. amazonensis promastigotes. Twenty-four hours after injection, peritoneal cells were recovered by washing and subjected to the purification of the B-1 cells by magnetic cell sorting isolation (as described above) to obtain purified B-1 cells or macrophages. Purified cells were adhered to microscope slides by centrifugation in a cytospin apparatus at 600 rpm for 5 min. The slides containing adherent cells were stained with haematoxylin-eosin (Panótico Rápido LB; Laborclin, Paraná, Brazil). The phagocytic index (PI) was calculated using an average of 100 cells counted in several microscopic fields. First, PI was calculated by multiplying the average number of internalized parasites and the percentage of phagocytic cells that internalized at least one parasite. Five mice were included per group, and each experiment was repeated thrice.

### 2.9. Statistical analysis

Data are shown as the mean and standard deviation (SD) and are illustrative of a minimum of two independent experiments. The student's *t*-test was used to compare two groups. Comparisons between multiple groups were performed with analysis of variance (ANOVA) followed by Tukey's post-test. Two-way ANOVA followed by the Bonferroni post-test was used for multiple comparisons. $P$ values $< 0.05$ were considered significant. Statistical tests were performed in PRISM version 6.00 for Mac (GraphPad Software, La Jolla, CA, USA; www.graphpad.com).

## 3. Results

### 3.1. Sleep restriction for 21 days (SR21) leads to a decrease in the peritoneal B-1 cell population and alters NO and ROS production

Finding evidence of the relationship between sleep disorders and the immune system is a task with many obstacles. Thus, this work used an experimental model to uncover the modulation of peritoneal B-1 cells in mice submitted to sleep restriction protocol. After sleep restriction for 21 days (SR21), peritoneal B-1 cells were evaluated for activation markers and their differentiation into phagocytes. All experiments described in the results session are presented in the workflow in Fig. 1.

Several works have demonstrated that sleep restriction interferes with the quantity and activation of immune cells (Mullington, Simpson et al., 2010). Thus, we first assessed the influence of sleep restriction in the B-1 lymphocytes population present in the peritoneal cavity in the C57BL/6J mice. Total cells from peritoneal cavities were labeled with anti-CD19, anti-CD23, and anti-F4/80 to identify B-1 cells (CD19+CD23−F4/80−) and B-1 CDP (CD19+CD23−F4/80+). Mice submitted to sleep restriction (SR) protocol showed a significant reduction in the percentage of peritoneal B-1 cells compared to control animals (not submitted to sleep restriction) (Fig. 2A, 2B, and 2C; $^{\#\#}P < 0.01$ [blue bars]). On the other hand, there was a statistically significant increase in the percentage of pre-B-1 CDP cells (Fig. 2A, 2B, and 2C; $^{****}P < 0.001$ [red bars]) in SR mice. It is worth mentioning that pre-B-1 CDP are cells undergoing a differentiation process into phagocytes derived from B-1 cells. These cells still express the CD19 in their membrane and simultaneously display the F4/80 marker (Mussalem et al., 2012).

After initial flow cytometry analysis of the B-1 cell population, we examined the expression of cell surface molecules CD40, CD80, CD86, CD45R, CD11b, IgM, and MHC II molecules in peritoneal B-1 cells from control and SR mice. No statistical differences were detected in the expression of these molecules comparing B-1 cells from the control and sleep-restricted groups (data not shown). However, the NO (Fig. 3A) and

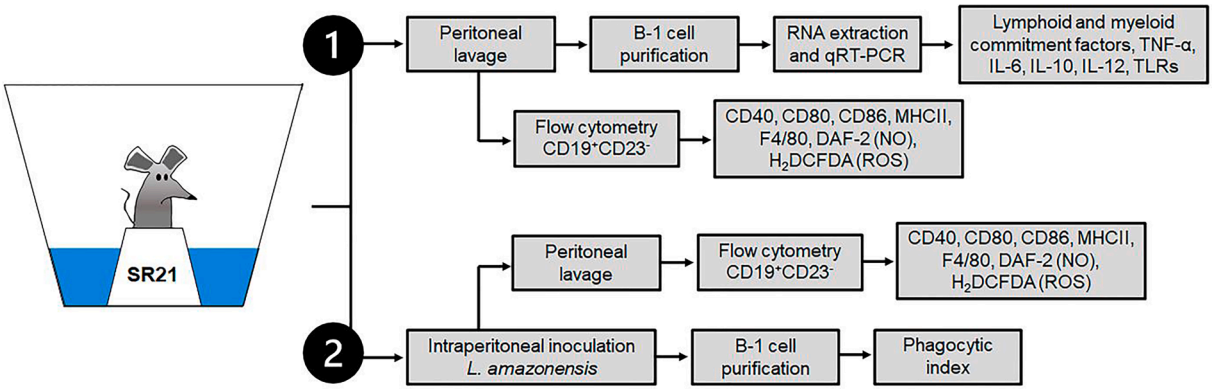


**Fig. 1.** Workflow is used to analyze B-1 cells. (1) After sleep restriction for 21 consecutive days (SR21), total peritoneal cells were collected, and the B-1 population (CD19 + CD23-) was analyzed by flow cytometry for the presence of CD40, CD80, CD86, MCHII, NO, and ROS. In addition, B-1 cells were purified using negative selection with anti-CD23 microbeads and positive selection with anti-CD19 microbeads. Total RNAs from purified B-1 cells were used to evaluate the gene expression of lymphoid and myeloid commitment factors (EBF - Early B-cell factor; E2A – E box protein; BLNK - B-cell Linker; M-csfr – Macrophage colony-stimulating factor; Spi-1 - transcription factor PU.1; G-csfr – Granulocyte colony-stimulating factor), cytokines (TNF-α, IL-6, IL-10, and IL-12), and Toll-like receptors (TLR2, -6, and -9). (2) On the 20th day after the start of the sleep restriction protocol, mice were intraperitoneally infected with L. amazonensis promastigotes. After 24 h, B-1 cells were evaluated for the presence of CD40, CD80, CD86, MCHII, NO, and ROS. Additionally, the phagocytose of L. amazonensis by B-1 cells was evaluated using a light microscope in purified B-1 cells stained with hematoxylin and eosin.

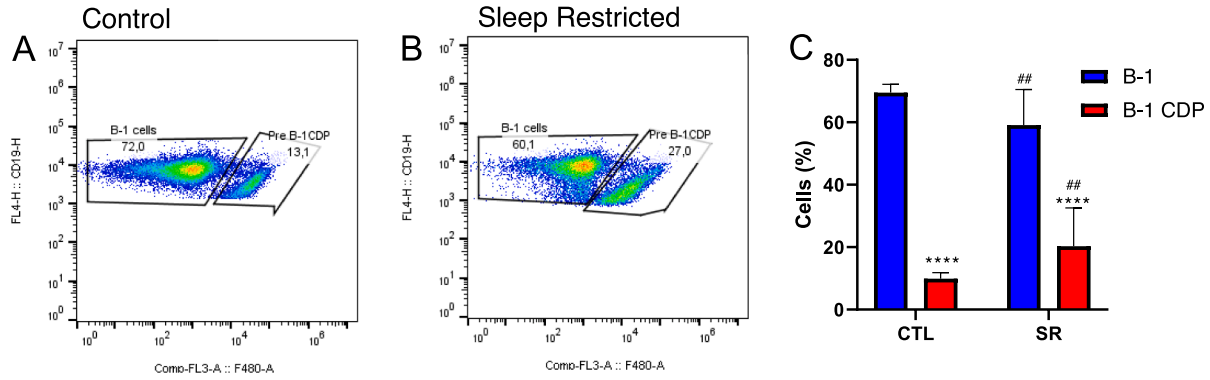


**Fig. 2.** Percentages of B-1 cells and B-1 CDP in the peritoneal cavity of animals submitted to sleep restriction for 21 days. Peritoneal washing was performed on control animals (CTL) and subjected to sleep restriction for 21 days (SR). The cells were labeled with appropriate fluorescent antibodies, and the population was selected according to their surface molecules. (A) and (B) dot plot representative of B-1 cells and B-1 CDP, identified in $CD19^+$ population by F4/80 expression. (C) B-1 cells $CD19^+F4/80^-$ (blue bars), and B-1-CDP $CD19^+F4/80^+$ (red bars), assessed by flow cytometry. The data are presented as mean ± standard error of the mean. Two-away ANOVA followed Bonferroni test. $^{****}P < 0.001$, comparing B-1 and B-1CDP in each group (CTL or SR); $^{\#\#}P < 0.01$, comparing B-1 CDP of CTL and SR group. N = 5 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

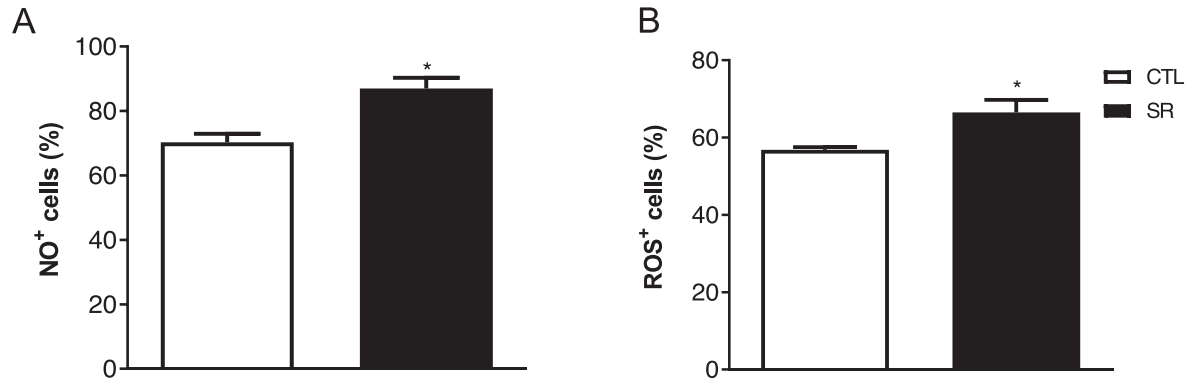


**Fig. 3.** NO and ROS production by B-1 cells from SR and control animals. Cells from the peritoneal lavage of control animals (white bars) and SR mice (black bars) were labeled as described in the material and methods. Analysis of NO (A) production and ROS (B) was performed by flow cytometry. The data are presented as mean ± standard error. Student *t*-test, * $P < 0.05$, n = 5 per group. Graphs are representative of three independent experiments.

ROS production (Fig. 3B) were significantly increased in B-1 cells from sleep-restricted mice compared to the control group (*$P < 0.05$).

### 3.2. Sleep restriction alters lymphoid and myeloid commitment factors and changes the expression of cytokines and TLR of B-1 cells from C57BL/6J mice

All data from the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis were obtained from total RNA of

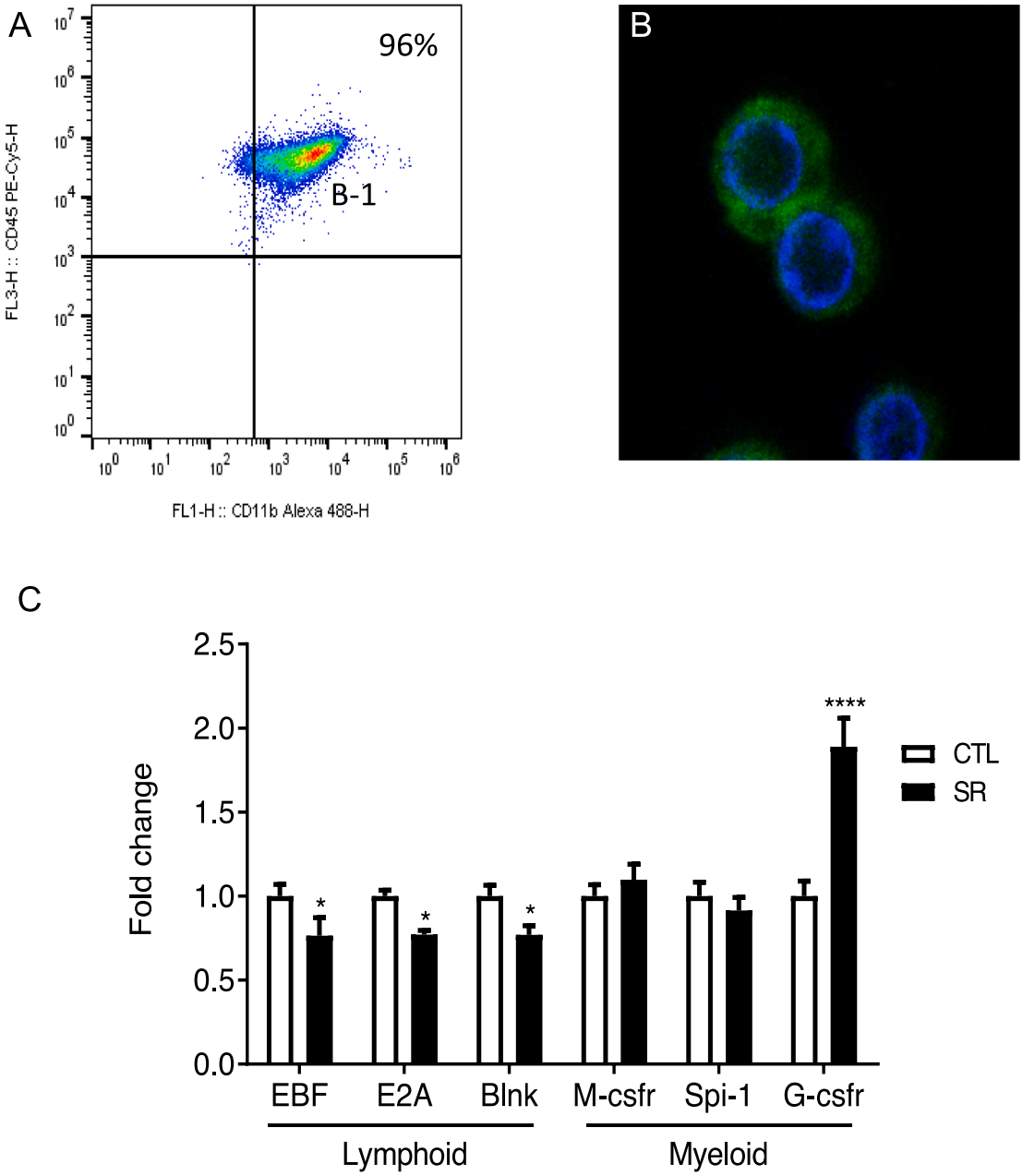


**Fig. 4.** Immunofluorescence, confocal laser scanning microscopy, and relative expression of lymphoid and myeloid commitment factors in enriched B-1 cells from sleep-restricted mice (SR) and control group (CTL). (A) B-1 cells were isolated from peritoneal cavity cell suspensions by magnetic cell sorting. The cells first underwent negative selection with anti-CD23 microbeads, according to the manufacturer's instructions. Following the first selection, CD23 − cells were positively selected with anti-CD19 microbeads. Then, these cells were labeled with appropriate fluorescent antibodies, and the population was selected according to their surface molecules. Dot plot (A) representative of B-1 cells, identified by double labeling with CD45R + and CD11b. A 96 % pure B-1 cell suspension was obtained, as verified by flow cytometry. (B) Confocal microscopy images of enriched B-1 cells identified by immunofluorescence with anti-CD11b-ALEXA 488 (green) and nuclei were stained by DAPI (blue). (C) Relative expression of lymphoid and myeloid commitment factors in peritoneal B-1 cells from sleep-restricted mice (SR) and control group (CTL). After SR protocol, B-1 cells were purified, total RNA extracted, and used to perform the cDNA. Real-time PCR (qPCR) was used to detect changes in the expression of the lymphoid or myeloid genes. The amount of cDNA input was normalized by analyzing the ARBP control transcripts. Results are representative of three independent experiments. EBF (early B-cell factor); E2A (E box protein); BLNK (B cell linker); M-CSFR (macrophage colony-stimulating factor); SPI1 (transcription factor PU.1) and G-CSFR (granulocyte colony-stimulating factor receptor). Student *t*-test, * $P < 0.05$, **** $P < 0.0001$, comparing each gene between SR and CTL. N = 5 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purified B-1 cells. First, peritoneal B-1 cells were purified on magnetic columns based on the expression of CD19 and the absence of CD23. Then, RNA was extracted, and the qRT-PCRs were performed. Fig. 4A shows a flow cytometry analysis of B-1 cells achieved after the magnetic cell sorting. The 96 % purity of B-1 cells was obtained. In addition, a small sample of B-1 cells was prepared for immunofluorescence. Fig. 4B shows a representative confocal microscopy image of purified B-1 cells identified by anti-CD11b-FITC (green) and nuclei stained by DAPI (blue). Although the increase in mRNA expression (qRT-PCR) may not reflect the production of proteins, our studies showed statistically significant changes in the mRNA of lymphoid and myeloid involvement factors, cytokines, and TLR of B-1 cells from SR mice as compared to CTL group. As seen in Fig. 4C, lymphoid commitment factors EBF (early B-cell factor), E2A (E box protein), and BLNK (B cell linker) were negatively modulated after 21 days of sleep restriction ($^{*}P < 0.05$). However, the expression of the G-CSFR gene in B-1 cells from the restricted sleep group was significantly higher in B-1 cells than in control animals ($^{***}P < 0.01$) (Fig. 4). No differences were detected in the expression of M-CSFR and SPI1 genes of B-1 cells from sleep-restricted animals compared to the control group. Altogether these data suggest that the SR21 protocol induced the differentiation of B-1 cells to a myeloid profile.

B-1 cells can produce pro and anti-inflammatory cytokines, contributing to immune response activation (Novaes e Brito et al., 2019). Thus, we investigated the expression of IL-6, IL-10, IL-12, and TNF-α cytokines in B-1 cells from mice submitted to the SR protocol. The results showed no statistically significant differences for IL-6, IL-12, and TNF-α between the control and experimental groups (SR) (Fig. 5A). However, the IL-10 expression was significantly lower in the SR group ($^{***}P < 0.001$) compared to the control group (Fig. 5A).

TLRs participate in the innate immune response by interacting with pathogen-associated molecular patterns (PAMPs) and stimulating the immune cells' activation and cytokine production (Newton and Dixit 2012). Therefore, we decided to verify the influence of SR on the TLRs expression of B-1 cells. As a result, sleep-restricted mice significantly decreased TLR-2, TLR-6, and TLR-9 expression in B-1 cells ($^{***}$ P < 0.001), compared to B-1 cells from the control group (Fig. 5B). Based on these data, B-1 cells from sleep-restricted mice showed altered microbicidal capacity by increasing NO and ROS production, impaired IL-10 expression, an important anti-inflammatory cytokine, and decreased TLRs expression.

### 3.3. *In vivo phagocytosis of L. amazonensis promastigotes by B-1 cells from animals subjected to sleep restriction for 21 days*

B-1 cells can produce microbicidal molecules and internalize pathogens like L. amazonenses (Geraldo et al., 2016). Thus, we evaluated whether sleep restriction could interfere with the activation and phagocytosis of *L. amazonensis* promastigotes by B-1 cells. First, we verified the activation of B-1 cells after intraperitoneal stimulation with *L. amazonensis* promastigotes. B-1 cells from sleep-restricted mice showed a significant increase in CD80 and CD86 co-stimulatory molecules (P < 0.05) after intraperitoneal infection with the parasite (Fig. 6). No changes were observed to CD40 and MHC II.

The NO production showed a significant increase in sleep-restricted mice infected with *L. amazonensis* promastigotes compared to the control group (Fig. 7). No differences in ROS production were detected in B-1 cells from infected mice submitted or not to sleep restriction protocol (Fig. 7).

By analyzing the phagocytic function of B-1 cells, the results demonstrated that cells from sleep-restricted animals exhibited no differences in the percentage of infected cells (Fig. 8A) but showed a significantly lower number of internalized parasite rates than control

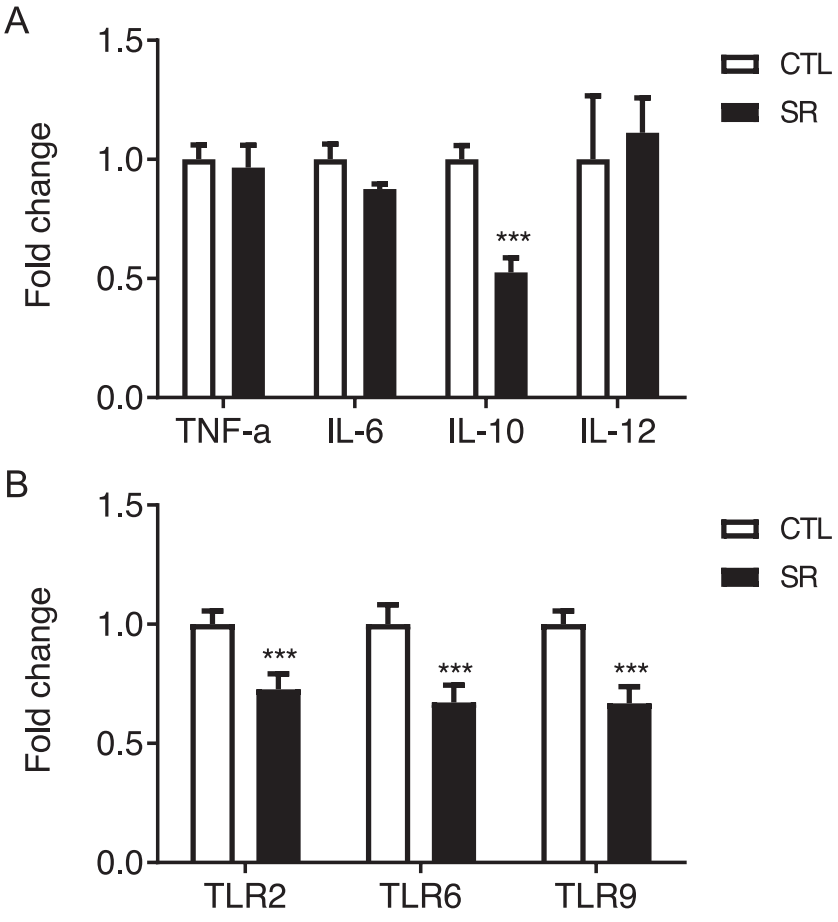


**Fig. 5.** Cytokine production and toll-like receptors (TLR) expression in peritoneal B-1 cells from SR and control mice. After SR protocol, B-1 cells were purified, total RNA extracted, and used to perform the cDNA. Real-time PCR (qPCR) was used to detect changes in the expression of the (A) TNF-a, IL-6, IL-10, and IL-12 cytokines genes and (B) TLR-2, TLR-6, and TLR-9 genes. The amount of cDNA input was normalized by analyzing the ARBP control transcripts. Results are representative of three independent experiments. Student t-test, $^{***}$ P < 0.001, comparing each gene between SR and CTL. N = 5 per group.

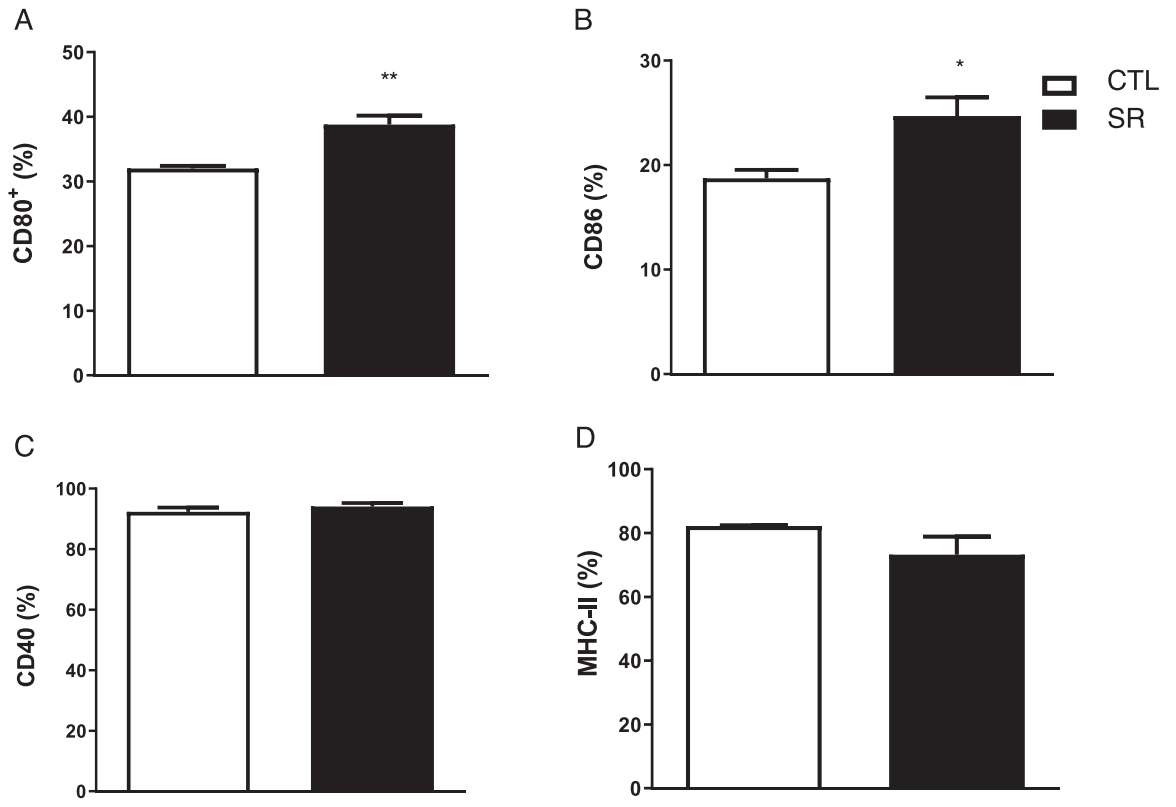


**Fig. 6.** CD80, CD86, CD40, and MHC II expression on the cell surface of B-1 cells from sleep-restricted animals (SR) intraperitoneally infected with L. amazonensis promastigotes. B-1 cells from the peritoneal cavity of control animals (white bars) and RS21 (black bars) were identified as CD19 + CD23-. The presence of the (A) CD80, (B) CD86, (C) CD40, and (D) MHC II was evaluated by flow cytometry in SR and control, both infected with the parasite. Data are presented as mean ± standard error of the mean. Results are representative of three independent experiments. Student t-test** $P < 0.01$, * $P < 0.05$, n = 5 per group.

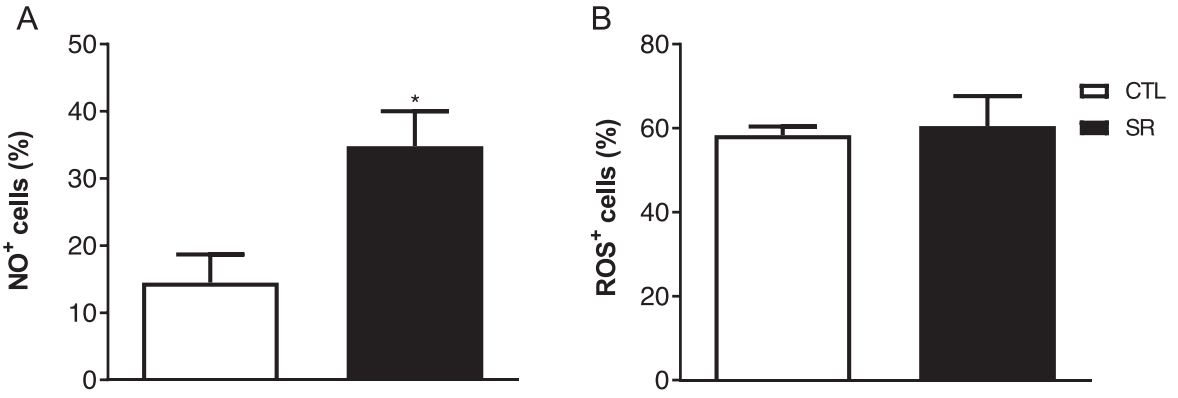


**Fig. 7.** NO and ROS production by B-1 cells from SR21 and control mice infected with *L. amazonensis* promastigotes. Cells from the peritoneal lavage of control animals (white bars) and SR mice (black bars) were labeled as described in the material and methods. Analysis of (A) NO production and (B) ROS was performed by flow cytometry. The data are presented as mean ± standard error. Student *t*-test *$P < 0.05$, n = 5 per group. Graphs are representative of three independent experiments.

animals (Fig. 8B). In addition, macrophages from sleep-restricted animals exhibited a significantly lower percentage of infection than control animals (Fig. 8A) but no differences in the number of internalized parasites (Fig. 8B). Fig. 8C and 8D represent images of infected B-1 cells and macrophages, respectively.

## 4. Discussion

Sleep quality is an essential mediator in the homeostasis and physiology of mammals. Sleep disruption acts as a stressor that harms the health and the function of the immune system (Mullington et al., 2010). Although several studies have demonstrated the development, functions, and activation of B-1 cells under physiological conditions and some diseases, the impact of sleep restriction on B-1 lymphocyte biology remains unclear. Thus, this work addressed the effects of stress-induced sleep restriction on B-1 cells in mice submitted to sleep restriction for 21 days.

In previous studies, Almeida et al. (2001) and other researchers established that B-1 lymphocytes could differentiate into macrophage-like phagocytes (Almeida et al., 2001, Bogsan et al., 2005, Ghosn

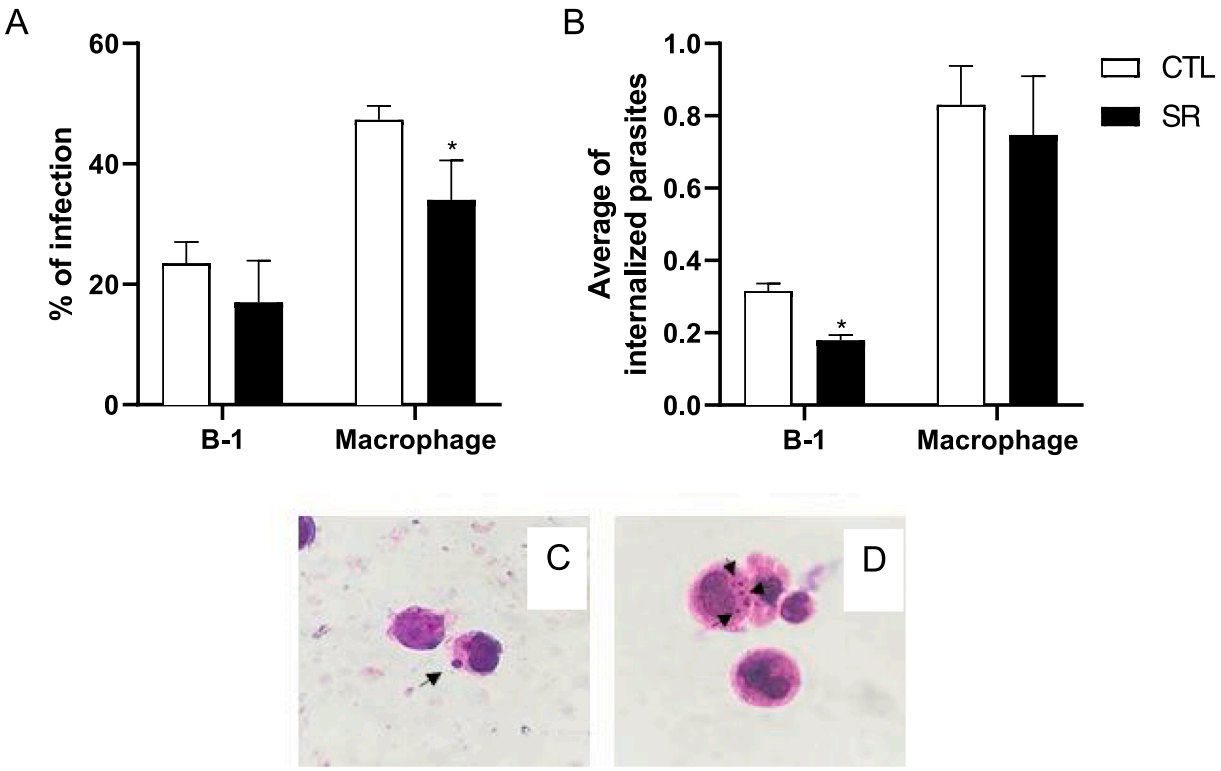


**Fig. 8.** In vivo phagocytosis of L. amazonensis promastigotes by B-1 cells and macrophages from animals subjected to sleep restriction for 21 days. Peritoneal B-1 cells were purified on magnetic columns using anti-CD23-conjugated magnetic beads for negative selection and anti-CD19-conjugated magnetic beads for positive selection. Macrophages were purified using negative selection with anti-CD23-conjugated magnetic beads and anti-CD19-conjugated magnetic beads; and positive selection with anti-F4/80 microbeads. (A) Percentage of in vivo infection of L. amazonensis promastigotes by B-1 cells and peritoneal macrophages. (B) Average of internalized parasites. Control animals (white bars) and SR21 (black bars). Photomicrographs of (C) purified B-1 cells and (D) macrophages stained with hematoxylin and eosin. Arrows show the parasites within B-1 cells or macrophages. Both images show 1000x magnification. Data are presented as mean ± standard error of the mean. Values of p are indicated in the figure. Student *t*-test, comparing SR and control, *P < 0.05. N = 5 per group.

et al., 2006, Popi et al., 2009, Novaes e Brito et al., 2010, Geraldo et al., 2016). Such cells can efficiently perform phagocytosis of different microorganisms and apoptotic cells (Novaes e Brito et al., 2010, Mussalem et al., 2012, Geraldo et al., 2016). Our results demonstrated that mice that underwent the sleep restriction protocol for 21 consecutive days had significant changes in B-1 cell populations compared to the control group that remained with regular sleep/vigilance. As seen in Fig. 2, there was a discrete decrease in the population of B-1 lymphocytes found in the peritoneum of SR21 mice. In contrast, there was an increase in B-1 CDP cells. Some studies have shown the differentiation of B-1 cells into phagocytes after stimulation with pathogens or their components (Novaes e Brito et al., 2010, Popi et al., 2012, Geraldo et al., 2016). However, this is the first work that brings evidence of possible differentiation of B-1 cells under stress conditions. Understanding which factors led to this differentiation may help better understand the role of B-1 cells in the immune system.

Interestingly, an early experimental study, conducted by Kuhn et al., (Kuhn et al., 1969), involving up to five days and nights of the vigil, reported an increase in leukocytes, mainly neutrophils, in response to sleep deprivation. In other studies, it was found that continuous sleep deprivation above 40 h led to a considerable increase in leukocytes (Dinges et al., 1994, Bøyum et al., 1996, Born et al., 1997, Heiser et al., 2000, Boudjeltia et al., 2008). The increase in leukocytes in these sleep deprivation studies was recognized as a signal of the host's immune system activation. Thus, analyzing and comparing our results with data from the literature, we observed that the period of sleep restriction that the animals suffered for 21 consecutive days was sufficient to increase the percentage of B-1 CDP. Furthermore, our model detected a discrete decrease in the percentage of peritoneal B-1 cells in SR mice, suggesting differentiating of B-1 cells into B-1 CDP. Although B-1 cells can migrate to an inflammatory milieu (Bogsan et al., 2005) and leave the peritoneal cavity, the in vivo differentiation of these cells into phagocytes was already verified (Novaes e Brito et al., 2010, Geraldo et al., 2016) and both hypotheses are under investigation by our group.

Analyzes of purified B-1 cells showed an increased expression of myeloid factors in B-1 cells from SR animals compared to B-1 cells from control animals. It is essential to highlight that with the purification protocol performed, it is impossible to separate B-1 cells from B-1 cells that differentiate into phagocytes. However, as shown in Popi et al. (2009) (Popi et al., 2009), fully differentiated B-1 cells into phagocytes lose CD19 expression. Thus, it is essential to note that B-1 cells were obtained from total peritoneal cells and subjected to negative selection with anti-CD23 microbeads, followed by positive selection with anti-CD19 microbeads. Therefore, b-1 cells were considered CD23-CD19+. In this way, our sample does not contain or contains small, B-1 cells differentiated into phagocytes. Some studies showed that during the myeloid differentiation of B-1 cells, there is an alteration in the expression of cell surface markers and some factors involved with the myeloid/lymphoid development of these cells (Popi et al., 2009). Popi et al. (2009) followed the expression of surface markers and transcription factors during this differentiation. It has been shown that B-1 cells spontaneously express both transcription factors restricted to lymphoid and myeloid cell involvement (Popi et al., 2009). Therefore, as the physiology of B-1 cells is still not completely understood, we investigated the expression of commitment factors involved in determining the hematopoietic lineages of B-1 cells from sleep-restricted animals. After the sleep restriction period, the relative expression of lymphoid genes EBF, E2A, and BLNK were negatively modulated. In contrast, the expression of genes related to myeloid involvement (SPI-1 and M-CSFR) showed no significant changes compared to control animals.

Interestingly, the relative expression of the G-CSFR gene (myeloid involvement) in B-1 cells from the restricted sleep group was higher than in B-1 cells from control animals. These data suggest that B-1 cells from sleep-restricted mice tend to compromise the myeloid profile. This fact is corroborated by the increase of B-1 CDP cells observed in Fig. 2. The clarification of mechanisms that determine lineage involvement is considered for understanding cell differentiation and elucidating mechanisms involved in possible neoplastic transformations. Thus, the promiscuity of gene expression by B-1 cells is a model for studying possible commitment factors that induce nuclear alterations related to the permissiveness of the coexistence of lymphoid and myeloid programs in these cells after the sleep restriction protocol.

Remarkably, chronic sleep restriction can lead to a differential state of immune cells, even in the absence of infection (De Lorenzo et al., 2011, De Lorenzo et al., 2018). Therefore, given that B-1 cells produce microbicidal molecules (Geraldo et al., 2016, Novaes E Brito et al., 2019), we evaluated the production of NO and ROS by B-1 cells from sleep-restricted mice. When phagocytic cells encounter foreign pathogens, they can produce large amounts of ROS and NO, which contribute to the death of invading microorganisms. ROS is essential in several physiological processes, including hormone biosynthesis, host defense, and apoptosis (Donkó et al., 2005). NO is a gas with diverse physiological activities produced from arginine by NO synthases. It can interact with several molecules, mainly superoxide, forming peroxynitrite, which mediates microbicidal reactions (Robbins and Grisham 1997). Our results showed a significant increase in NO and ROS production by B-1 cells from sleep-restricted mice. Pandey and Kar (2018) showed an increase in the levels of ROS and NO in hepatocytes (184.33 % and 404.40 %, respectively) from rats subjected to REM sleep deprivation (Rapid Eye Movement) compared to the control group (Pandey and Kar 2018). A similar phenomenon was also observed by Mullington et al., 2010 who showed that only acute sleep deprivation and chronic sleep restriction could lead to an initial state of inflammation in the absence of pathogenic microorganisms or overt injury (Mullington et al., 2010). Besides, chronic sleep restriction activates microglia and promotes phagocytosis of synaptic elements even in the absence of evident signs of neuroinflammation (Bellesi et al., 2017). Thus, it seems reasonable to speculate that sleep restriction increases the microbicidal capacity of B-1 cells since we find high levels of NO and ROS in these cells. However, after intraperitoneal stimulus with *L. amazonensis* promastigotes, no differences in ROS production were observed. Therefore, we hypothesize that the parasite can modulate ROS production even after sleep restriction. Furthermore, a previous study by Almeida et al. (2012) indicated that ROS production in macrophages was inhibited by the presence of *L. amazonensis* for its survival inside these cells (Almeida et al., 2012). So, it is likely that the parasite has controlled ROS production in B-1 cells by a similar mechanism observed in macrophages.

B-1 cells were initially known to play an essential role as a source of natural antibodies (Baumgarth et al., 2005). However, their functions go far beyond the production of antibodies. B-1 cells are producers of a variety of molecules, such as IL-10, IL-3, and GM-CSF (Chousterman and Swirski 2015), which are capable of exerting influence on other cell populations and, therefore, can influence the susceptibility or resistance in infection models (Popi et al., 2009, Popi et al., 2012, Arcanjo et al., 2017). In the present work, we showed that B-1 cells from C57BL/6 mice submitted to the RS21 protocol had lower IL-10 expression gene than the control group. Several studies have reported that B-1 cells play a role in IL-10 production (De-Gennaro et al., 2009, Geraldo et al., 2016, Liu et al., 2016). IL-10 is a cytokine that can act on different cell types and inhibit several molecules that play an important role in phagocyte activation and NO production (Liu et al., 2016). da Rocha et al. (2019) showed that the reduction in NO production by macrophages co-cultured with B-1 cells might result from the action of IL-10 on macrophages. Their results revealed that NO production was significantly reduced in high concentrations of IL-10 (da Rocha et al., 2019).

Furthermore, Arcanjo et al. (2017) showed increased intracellular macrophage parasitism by *L. major* in the presence of IL-10 from B-1 cells (Arcanjo et al., 2017). These data corroborate our results, which demonstrated that B-1 cells from SR21 mice showed an increase in the production of intracellular NO concomitant with a marked reduction in the relative expression of the IL-10 gene. Therefore, we can suggest that sleep restriction leads to a decrease in IL-10, increasing the intracellular nitric oxide concentration in B-1 cells of mice submitted to a sleep restriction protocol for 21 days.

Costimulatory molecules CD80 and CD86 are mainly expressed on antigen-presenting cells (APC), including dendritic cells, macrophages, and conventional B cells (B-2). They are absent or expressed at low levels in resting antigen-presenting cells (APCs). Various stimuli, including microbial products, induce CD80 and CD86. For example, Mussalem et al. (2012) demonstrated that BALB/c mice intraperitoneally stimulated with Propionibacterium acnes increased peritoneal B-1 cells and anticipated differentiation of B-1 cells into phagocytes (B-1 CDP). Additionally, an increase in the expression of costimulatory molecules CD80 and CD86 was also observed (Mussalem et al., 2012) in B-1 cells from these animals. Similarly, our results clearly showed that after RS21 protocol and intraperitoneal injection of L. amazonensis promastigotes, B-1 cells increased CD80 and CD86 expressions, suggesting that these cells may have assumed the antigen-presenting function.

Notwithstanding being classified as lymphocytes, due to the expression of BCR and production of immunoglobulins, B-1 cells differentiate into "B-1 cell-derived phagocytes" (B-1 CDPs) both *in vitro* and in vivo (Almeida et al., 2001, Popi et al., 2009, Mussalem et al., 2012, Popi et al., 2012). In addition, some studies have shown that the activation of these cells is mediated by Toll-like receptors (TLR), stimulation with LPS (Takahama et al., 1990), or CpG sequences (unmethylated DNA sequence) (Bamba et al., 2005). As B-1 cells exhibit characteristics of both macrophages and lymphocytes, we sought to investigate the effect of sleep restriction on the phagocytic potential of these cells. The results showed that, despite the increase in intracellular NO and ROS in sleep-restricted mice, there was a reduction in the average internalized parasites by B-1 cells from these animals compared to the control group. This data may be related to the decrease in the relative expression of Toll-like receptor genes (TLR-2, TLR-6, and TLR-9) in B-1 cells from sleep-restricted mice. Thus, it seems plausible to infer that sleep restriction can modulate the phagocytic potential of B-1 cells and the expression of Toll-like receptors.

The results suggest that sleep restriction for 21 days can promote the beginning differentiation of peritoneal B-1 cells into phagocytes. In addition, there was an increase in their microbicidal capacity by higher NO and ROS production. Although the sleep restriction alone did not interfere with the expression of costimulatory molecules CD80 and CD86 (data not shown), in the presence of L. amazonensis promastigotes and sleep restriction, there was an increase in these markers, suggesting that B-1 cells could be acting as antigen-presenting cells. On the other hand, B-1 cells from sleep-restricted animals reduced the expression of Toll-like receptors and the phagocytose ability. Together, our results showed that sleep restriction could influence the B-1 cells activation and differentiation, probably increasing the composition of the phagocyte population of these animals. Additional studies may contribute to uncovering the relationship of B-1 cells from sleep-restricted animals with other immune cells. Furthermore, understanding how sleep restriction can affect the response of B-1 cells to different pathogens and cancer may help to unravel better the role of sleep and B-1 cells in immunity.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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*Author statement*

**Andrey Sladkevicius Vidal:** Participated in all experiments and contributed to sample preparation. **Natasha Ferraz de Campos Reis:** Contributed to sample preparation. Enrichment of peritoneal B-1 cells. **Beatriz Helena Pizarro De Lorenzo:** Contributed to sample preparation. Acquisition and analysis of flow cytometry data. Supervised the findings of this work. **Anuska Marcelino Alvares-Saraiva:** Contributed to sample preparation. Acquisition and analysis of flow cytometry data. **Patricia Xander:** Participated in the experiments. Conceived of the presented idea. Conceived and planned the experiments. Supervised the findings of this work. Took the lead in writing the manuscript. **Ronni Rômulo Novaes e Brito:** Participated in the experiments. Conceived of the presented idea. Conceived and planned the experiments. Supervised the findings of this work. Took the lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript. In addition, all authors provided critical feedback and helped shape the research, analysis, and manuscript.

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