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The dual effect of paradoxical sleep deprivation on murine immune functions

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ABSTRACT

We aimed to evaluate the effect of paradoxical sleep deprivation on the cellular migration during inflammation, the peritoneal macrophage phenotype and the infectious stimulus outcomes. A/J mice were inoculated with thioglycollate and exposed to paradoxical sleep deprivation. Sleep-deprived animals presented decreased cell migration compared to controls. Nitric oxide production was reduced in macrophages from sleep-deprived mice compared to controls. Cell surface analysis showed that sleep deprivation reduced F4/80^{+}/CD80^{low} peritoneal cell population induced by thioglycollate injection. Sleep-deprived mice were not more susceptible to infection than control mice. Our findings challenge the general perception that sleep loss always increases infection susceptibility.

1. Introduction

The relationship between sleep and immunity is long recognized (Bryant et al., 2004; Casey et al., 1974; Naitoh, 1976; Palmblad et al., 1979). An increasing body of evidence has demonstrated that changes in the circadian rhythm caused by disruption of sleep integrity affect inflammatory, cellular and humoral immune responses in humans (Faraut et al., 2012). However, the observed phenotype and its intensity may vary depending on: 1) the protocol (sleep restriction or sleep deprivation); 2) the duration of sleep loss; 3) the parameters evaluated (number and/or function); and 4) the moment of the evaluation (during or after sleep loss). Several studies with healthy volunteers agree that sleep restriction and sleep deprivation increase whole leukocyte counts, mainly neutrophils and/or monocytes (Christoffersson et al., 2014; Lasselin et al., 2014; Ruiz et al., 2012; Faraut et al., 2012). The activity of natural killer (NK) cells is shown to be diminished after sleep restriction (Irwin et al., 1996), although protocols of sleep deprivation produce conflicting results regarding these cells (Moldofsky et al., 1989; Faraut et al., 2012; Matzner et al., 2013). On the other hand, the production of inflammatory mediators after sleep deprivation appears increased, decreased or even unchanged depending on the study (Shearer et al., 2001; Zager et al., 2007; Frey et al., 2007; Meier-Ewert et al., 2004; Ruiz et al., 2012). There are several explanations for such divergence including: the procedures used for sample collection, which can represent a confounding factor for these studies; variations in the subjects' behaviors and monitoring throughout the studies; and differences in the genetic backgrounds of the individuals.

A number of factors hamper the understanding of the role of sleep and sleep loss on immune functions, such as nutritional status, underlying diseases, heterogeneous genetic background and availability of an adequate number of individuals in each group for statistical analysis. For all this reasons, the use of a murine model to study the sleep/immunity relationship is desirable, since it allows the control of several variables. Our group has been working with a multiple platform method that inhibits paradoxical sleep, with less impact on slow wave sleep (Zager et al., 2009; Silva et al., 2004). Employing this murine model, we have been able to evaluate several behavioral, neurological, biochemical and physiological parameters over the last few years (Frussa-Filho et al., 2004; Hirotsu et al., 2012; Patti et al., 2010; Zager et al., 2009, 2012. In addition, we have observed a strong reduction of lymphocytes in the circulation (Ruiz

Abbreviations: Con A, Concanavalin A; NK, Natural killer; NO, nitric oxide; PSD, paradoxical sleep deprivation.

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et al., 2007) and in the spleen (Zager et al., 2012) following paradoxical sleep deprivation (PSD). Sleep deprivation induced by the platform technique involves numerous awakenings, which predominantly affect the REM stage of sleep. In this sense, over the three last decades use this procedure in rodents to mimic sleep fragmentation due to repeated awakenings as useful tool to investigate the effects of sleep loss on inflammatory and immune (Tufik et al., 2009). Considering the importance of sleep integrity to immune functions, the present work aimed to expand the understanding of the effects of PSD on the biology of inflammatory and immune responses in vitro, ex vivo and in vivo in this model.

2. Material and methods

2.1. Mice

Male A/J mice (12–20 weeks old) were obtained from the Isogenic Breeding Unit, Department of Immunology, Institute of Biomedical Sciences, Universidade de São Paulo, Brazil. All experiments agree with the ethical principles in animal research adopted by the Brazilian Society of Laboratory Animal Science (SBCAL) and were evaluated and approved by the Institutional Animal Care and Use Committees, under the protocols 146/2010 (Universidade de São Paulo) and 2091/2009 (Universidade Federal de São Paulo).

2.2. Paradoxical sleep deprivation (PSD)

The method of PSD was adapted from the multiple platform method, originally developed for rats (Zager et al., 2009, 2012; Frussa-Filho et al., 2004). Groups of 5–8 mice (PSD group) were placed in water tanks (41 cm × 34 cm × 16.5 cm), containing 12 platforms (each 3 cm in diameter), equally distributed across the area, surrounded by water up to 1 cm beneath the platforms. In this method, the animals are able to move inside the tank, jumping from one platform to the other. When mice reach the paradoxical phase of sleep, they fall into the water, due to muscle atonia, and wake up. The animals were sleep deprived for 72 h, starting immediately after the procedures described below, and then they were either returned to their cages or used for experimental assays. Control mice were maintained in their home-cages in the same room. All animals were kept in the same room during the entire protocol.

2.3. Thioglycollate-induced peritonitis

Cell recruitment to the peritoneal cavity was induced by i.p. inoculation of 0.5 mL of 3% aged thioglycollate broth (Difco Laboratories, Detroit, MI). Following the inoculation, a group of mice was returned to their cage (control group) and another group was submitted to the sleep deprivation protocol (PSD group). After 72 h, mice were euthanized and peritoneal cavity lavage was performed with 3 mL of cold medium (RPMI 1640, GIBCO-Life Technologies, Grand Island, NY). Cells were collected, washed twice and a total count was performed in a Neubauer's chamber. Differential counts were performed in cytocentrifuge preparations (Sa-Nunes et al., 2004).

2.4. Nitric oxide production

A suspension containing 1.5×10^6 peritoneal cells/mL, prepared in RPMI 1640 medium was distributed in 96-well flat bottom plates at 100 μL aliquots/well. After 2 h, non-adherent cells were removed by 3 washes with warm sterile PBS. Cells were then incubated with complete medium only (RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol) or stimulated with IFN-γ (10 ng/mL — Sigma-Aldrich, St. Louis MO) plus ultrapure LPS (10 ng/mL — InvivoGen, San Diego, CA). Cell-free supernatant was removed after 48 h culture and nitric oxide (NO) production was estimated by determination of nitrite (NO_2^-), the stable product of NO oxidation, by Griess reaction as previously described (Medeiros et al., 2004; Sá-Nunes et al., 2007).

2.5. Flow cytometry

Cells collected from the peritoneal cavity of control and PSD animals were centrifuged, resuspended in flow cytometer buffer (PBS containing 1% fetal bovine serum) and counted. Suspensions from each individual mouse containing the same number of cells were incubated for 15 min at 4 °C with anti-CD16/CD32 antibody to block unspecific binding to Fc receptors. Fluorochrome-conjugated antibodies were added to the cells followed by further incubation for 30 min at 4 °C protected from light. After washing, cells were acquired in FACSCalibur (BD Biosciences, San Jose, CA) and analysis was performed using FlowJo software, version 7.5.5 (Tree Star, Ashland, OR).

2.6. Blood samples and corticosterone evaluation

Following PSD procedures, the animals were euthanized by decapitation. Blood was collected in sterile tubes containing EDTA, centrifuged (1000 *g* at 4 °C) to obtain samples of plasma and stored at −80 °C. Concentration of the stress-related hormone, corticosterone, was assayed by a double antibody radioimmunoassay method using a commercial kit specific for mice (MP Biomedicals, NY, USA). The sensitivity of the assay was 0.25 ng/mL.

2.7. Trypanosoma cruzi infection

Mice were intraperitoneally (i.p.) infected with 1000 blood trypomastigote forms of *T. cruzi* (Y strain) and then were either kept in their cage or subjected to PSD for 3 consecutive days as described above and their survival was monitored for 30 days.

2.8. Histoplasma capsulatum infection

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and intratracheally infected with 2×10^6 *H. capsulatum* yeasts. Then, mice were either kept in their cage or subjected to PSD for three consecutive days as described above and their survival was monitored for 60 days.

2.9. Lymphocyte proliferation and IFN-γ determination

Following euthanasia, spleens and inguinal lymph nodes of control and PSD mice were aseptically removed and a cell suspension containing 10^6 cells/mL was prepared. Cells were then distributed in 96-well plates (100 μL/well), incubated with medium only or stimulated with Concanavalin A (Con A), a polyclonal mitogen that induces proliferation of T lymphocytes (0.5 μg/mL final concentration — Sigma-Aldrich), and incubated at 37 C and 5% CO_2. After 48 h incubation, 25 μL of 0.01% resazurin were added to all wells, followed by additional 24 h incubation. Cell proliferation was evaluated by reading the culture absorbance at 570 and 600 nm, as previously described (Sá-Nunes et al., 2009; Bizzarro et al., 2013).

IFN-γ production was evaluated in cell-free culture supernatants by OptEIA ELISA set according to the manufacturer's instructions (BD Biosciences, San Diego, CA). Sensitivity was ≥10 pg/mL.

2.10. Statistical analysis

Statistical analysis of differences between means of experimental groups was performed using Student's *t* test or analysis of variance (ANOVA) followed by Tukey as a post-test. The log-rank test was used for statistical analysis of mortality. In all cases, a value of $p \leq 0.05$ was considered statistically significant.