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The effects of short-term sleep deprivation on the immune system of *Mus Musculus*

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Abstract

Sleep deprivation has become a prevalent issue, raising concerns about its detrimental effects on immune function and overall health. This study investigates the effect of acute sleep deprivation on the immune response of mice. It was hypothesized that sleep deprivation would significantly alter immune cell distribution and cytokine expression. Using a custom-designed rotor cage that prevented sleep without excessive stress, mice were subjected to 72 hours of continuous sleep deprivation. Results from flow cytometry analysis showed significant reduction in the distribution of Natural Killer (NK) cells and memory T cells in the spleen cells and peripheral blood leukocytes from sleep deprived sacrificed mice, indicating impaired cytotoxic and immune responses. Conversely, an increase in B cell proportions was observed which could reflect that activation of compensatory mechanisms to maintain immune homeostasis. Additionally, the expression of anti-inflammatory genes and the concentration levels of anti-inflammatory cytokines increased, while pro-inflammatory cytokines and related genes were downregulated. These findings reveal that short-term sleep deprivation suppresses the immune system of mice, underscoring the critical role of sleep in maintaining immune balance and suggesting that sleep deprivation could predispose the body to chronic immune diseases.

Keywords

Sleep deprivation, Sleep, Inflammation, Immune suppression, Cytokine expression, Immune homeostasis, Chronic immune disease, Sleep deprivation apparatus, Immune cell distribution, RNA sequencing

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1. Introduction

In modern day society, sleep deprivation has become more common due to individuals sacrificing sleep to meet the pressures of daily life in this fast-paced, high stress environment (1). More than one-third of adults in the United States have reported that they were not getting enough sleep and rest at night (2). This widespread trend of sleep deprivation has led to significant increases in sleep-related disorders, and the deprivation of sleep was also found to cause significant economic consequences. Sleep is essential in maintaining human health and physiological balance, affecting many metabolic functions. Research shows that sleep plays a role in tissue repair, memory consolidation, and hormonal regulation (3). Adequate sleep is especially important for the immune system since it promotes the proper functioning of the body's defense mechanisms, enhancing the body's ability to fight pathogens. Therefore, sleep strengthens immune defenses and results in the production of natural killer (NK) cells as well as T-cells (4). Sleep deprivation refers to the condition of not getting enough sleep, whether voluntarily or involuntarily, over an extended period of time (5). Sleep deprivation can be categorized as either acute or chronic. Acute sleep deprivation refers to a short period of sleep loss, typically lasting from one night to a few days, whereas chronic sleep deprivation occurs when sleep is consistently restricted over a longer duration, often leading to cumulative sleep debt. In healthy adults, the recommended amount of sleep is 7–9 hours per night (6). When an individual sleeps for fewer than these recommended hours, sleep deprivation occurs, which has been shown to be linked to numerous health consequences, including increased inflammation, impaired immune function, and the development of chronic health conditions such as diabetes, heart disease, and obesity (7). Furthermore, when the body is sleep-deprived, the production of pro-inflammatory cytokines such as IL-6 and TNF-α was found to increase (8).

Similarly, another sleep deprivation study in mice demonstrated that prolonged sleep deprivation triggered a cytokine storm by significantly increasing the levels of pro-inflammatory cytokines such as IL-6 and IL-17, which, in turn, led to multiple organ dysfunction syndrome and eventually resulted in the death of the mice (9). In addition, in a past short-term sleep deprivation study, a relationship was found between sleep deprivation and the increased risk of developing a cold. It was found that participants who received insufficient sleep were ~ 3X more likely to develop a cold. Short-term sleep deprivation may hence suppresses the production of cytokines that are used in defending against infections such as the common cold (10). These studies have however focused only on single molecules or specific immune cells, leaving the broader molecular mechanisms largely unexplored. Sleep deprivation models used in previous sleep deprivation studies, such as the flowerpot technique, also introduce unintended physiological and psychological stress on the animals, affecting the validity of the results amid the potential to cause unintended physiological and psychological stress. This study aims to address these gaps in the literature by utilizing a custom-made model, developed previously, designed to assess the effects of sleep deprivation on the gene expression and distribution of immune cells without these confounding factors. By focusing

on both pro-inflammatory and anti-inflammatory pathways, it was hypothesized that short-term sleep deprivation would significantly suppress immune responses by altering gene expression, cytokine concentration levels, and the relative abundance of different types of immune cells.

2. Methods

2.1 Model organism overview

B6 mice (C57BL/6) are commonly used as laboratory animals and are known for their highly purified genetic background. As they inbreed through many generations, B6 mice have become genetically homogeneous, resulting in their low genetic diversity. This uniformity allows for consistent genotypic outcomes across different experiments. In this study, all the mice used were sourced from the National Laboratory Animal Center, Taiwan, and kept and cared for under standard laboratory conditions with a 12-hour light/dark cycle, having access to food and water *ad libitum*.

2.2 Animal handling and care

All mice in the control group were housed in cubed-shaped cages under standard conditions with a 12:12 light/dark cycle (Zeitgeber Time ZT0-12 from 7 am to 7 pm for light; ZT12-24 from 7 pm to 7 am for darkness). On the other hand, all mice in the sleep-deprived group were housed in the homemade rotor cage under the same lighting conditions. For the control group, water was changed every 3-4 days, and food was provided *ad libitum* without feeding restrictions. Similarly, the sleep-deprived group had unrestricted feeding, and the rotor cage environment was maintained with cage changes every 4-5 days. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) under protocol number IACUC-23-106. All procedures followed institutional and ethical guidelines and complied with the principles of the 3Rs. The mice were also housed under pathogen-free conditions in sterile ventilated racks after being originally obtained from the National Laboratory Animal Center.

2.3 Sleep deprivation model

A custom-designed sleep deprivation apparatus, referred to as a rotor cage, was utilized in this experiment (Figure 1). The cage was constructed with acrylic material, using a 3D printer, forming a cylindrical shape with a diameter of 30 cm and a height of 21 cm. The top of the cage was covered with a transparent lid to facilitate observation and video recording. A feeding system was suspended through a 7.5 cm diameter hole in the center of the lid. This system, measuring 7 cm in diameter and 17 cm in height, allowed the mice to access food and water, which were positioned 4-5 cm above the floor, enabling easy access. At the base of the cage, a motor (5 cm in diameter) powered a mechanical cross-shaped arm (12 cm long) that rotated at a speed determined by code 5, which translated to 1 rotation per 13 seconds followed by a 67-second rest (80 seconds total per cycle). This setting was chosen to ensure that the mice could rest but not sleep. The mechanical arm moved in 90-degree intervals, ensuring continuous disturbance without blind spots. Four small barriers (2 mm high) were installed at the base of the cage with a 7 mm gap between the barriers and the arm, requiring the mice to step over the gaps, further disrupting their ability to rest. The rotor cage's operation, including the timing and duration of

movements, was controlled by an integrated microchip and software system. Each cage housed 4-5 mice, and the rotor's speed and activity periods were precisely set based on the experiment's sleep deprivation protocol.

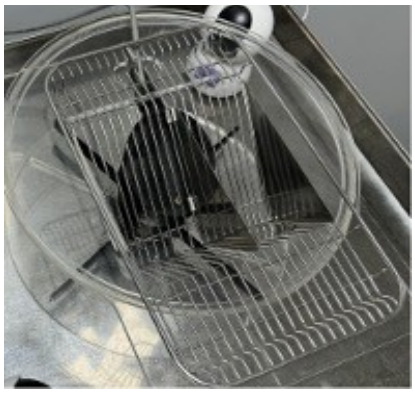

Figure 1. Rotor Cage. A custom-made rotor cage was used to effectively sleep deprive the mice without introducing additional psychological and physical stress that could confound results.

2.4 Sleep monitoring via EEG

An electroencephalogram (EEG) was used to monitor and confirm the wakefulness of mice during the sleep deprivation protocol. To monitor, a wireless head-mount device was surgically implanted to track the brain wave activities in the mice in the sleep deprived group during sleep deprivation. The headmount device used for EEG monitoring was purchased from Pinnacle Technology**,** model 8201 **(**Pinnacle Technology, EEG/EMG Systems Headmount, Cat# 8201). The device was placed over the cortical surface of the mice, and it allowed for continuous monitoring of brain waves, distinguishing wakefulness, rapid eye movement (REM), and non-rapid eye movement (NREM) sleep stages. The raw data collected from the electrode was then transmitted to a monitoring system that recorded the brain activity throughout the experiment. Later, the raw EEG data were processed using Fourier transformation and analyzed with sleepPRO software (Pinnacle Technology Inc.) to classify the sleep stages and verify the effectiveness of the sleep deprivation model.

2.5 Experimental setting

The mice were randomly assigned to one of two groups. Mice assigned to the first group were used for blood and spleen sampling, with 12 mice in the control group and 18 mice in the treatment group. The serum samples for Luminex analysis (Luminex Corp. TX, USA) was also collected from the first group. The second group of mice was used for bulk RNA-sequencing and consisted of 21 mice each for both the control and treatment groups. These mice were then further divided into three subgroups of 7 mice. The blood samples of the 7 mice in each group were pooled to ensure there were enough cell numbers for RNA-Seq analysis. To clarify, the 7 mice with blood pooled together belonged to the same experimental condition. Before the official sleep deprivation, all mice underwent a one-week training period, during which the rotor cage was activated between ZT14 and ZT17.

During training, the rotor operated for 20 seconds every 100 seconds. This training period allowed the mice to acclimate to the mechanical arm's movement. After the training period, the sleep deprivation group underwent continuous sleep deprivation for 24 hours per day over a span of three days, starting at 10 weeks of age. After the sleep deprivation phase, the mice were transferred from the animal center to the laboratory for sample collection. All blood and tissue sampling occurred between ZT 2 and ZT 3 (9:00–10:00 AM), which corresponds to the early light phase of the 12:12 light/dark cycle. In the sleep-deprived group, mice were continuously kept awake throughout the deprivation period using the rotor cage. Since these mice were prevented from entering any sleep stages, the specific sleep stage at the time of sacrifice was not applicable to this group. We did not use EEG monitoring in this study to determine exact sleep stages in the control group at the time of sacrifice. The transfer process between the animal center and laboratory took less than 30 minutes, during which the mice were kept awake and under observation, ensuring experimental consistency and avoiding potential recovery sleep before blood and tissue collection.

2.6 Splenocyte and peripheral blood leukocyte cell collection

After sacrifice, each spleen was carefully excised and transferred into a 50 mL conical tube (GeneDireX, Cat. No. PC150-0500) containing PBS. The spleen was then mechanically disrupted using the plunger of a syringe and passed through a 70 μm cell strainer (Corning, Product No. 352350) to obtain a single-cell suspension. The suspension was centrifuged at 600g for 5 minutes at room temperature. The supernatant was discarded, and 1 mL of ACK lysis buffer (Thermo Fisher Scientific, REF A1049201) was added to remove residual red blood cells. After incubating for 3 minutes at room temperature, the sample was washed with 10 mL of PBS and centrifuged again. The final cell pellet, enriched for splenocytes, was resuspended in PBS for counting and downstream analysis. For the peripheral blood leukocyte collection, peripheral blood was collected via submandibular venipuncture using micro blood collection tubes containing lithium heparin (BD, SKU 365967). To isolate leukocytes, blood samples were carefully layered onto a two-step Percoll gradient (62% and 75%) (Cytiva, Item No. 17089101) and centrifuged twice at 200g for 15 minutes and 400g at 20 minutes at room temperature with no brake. After centrifugation, the leukocyte-enriched layer was collected from the interface between the two Percoll layers. This layer was then washed with PBS, centrifuged, and the resulting cell pellet was resuspended in PBS for cell counting and cell analysis.

2.7 Flow cytometry

Cells were stained with fluorochrome-conjugated antibodies targeting surface markers to identify immune cell subsets. The antibodies used were as follows: CD11b (Brilliant Violet 421™, Clone M1/70, BioLegend, Cat. No. 101236), CD4 (BV480, Clone RM4-5, BD Biosciences, Cat. No. 565634), CD62L (BB515, Clone MEL-14, BD Biosciences, Cat. No. 565261), CD8a (PerCP/Cyanine5.5, Clone 53-6.7, BioLegend, Cat. No. 100734), NK-1.1 (PE, Clone PK136, BioLegend, Cat. No. 108708), Ly-6C (PE-CF594, Clone AL-21, BD Biosciences, Cat. No. 562728), CD11c (PE/Cyanine7, Clone

N418, BioLegend, Cat. No. 117318), CD44 (APC, Clone IM7, BD Biosciences, Cat. No. 559250), and B220 (APC-Cy7, Clone RA3-6B2, BD Biosciences, Cat. No. 552094). Zombie NIR™ Fixable Viability Kit (BioLegend, Cat. No. 423105) was used to discriminate live from dead cells according to the manufacturer's instructions. Staining was performed by incubating cells with the antibody cocktail at 4°C for 30 minutes, followed by two washes with staining buffer. After final resuspension in phosphate-buffered saline (PBS), samples were analyzed using a Thermo Fisher Attune NxT V6 flow cytometer. The instrument was equipped with four lasers (405 nm, 488 nm, 561 nm, and 637 nm) and configured with the following fluorescence filter and voltage settings: VL2 (450/40, BV421, 290 V), VL3 (525/50, BV480, 340 V), BL1 (530/30, BB515, 380 V), BL2 (695/40, PerCP5.5, 420 V), YL1 (585/16, PE, 380 V), YL2 (620/15, PE-CF594, 390 V), YL3 (780/60, PE/Cy7, 430 V), RL1 (670/14, APC, 370 V), and RL3 (780/60, APC-Cy7, 350 V). Forward scatter (FSC) and side scatter (SSC) voltages were set to 180 V and 390 V, respectively, and VL1 (used for thresholding) was set to 300 V. Flow cytometry data were analyzed using FlowJo software (version 10.8.1, BD Biosciences)

2.8 Leukocyte bulk RNA sequencing

Peripheral blood leukocytes (PBLs) were isolated using a two-step Percoll gradient (62% and 75%, Cytiva, density 1.130 g/mL). Whole blood was carefully layered onto the gradient and centrifuged at 1200g for 30 minutes at room temperature with no brake. The leukocyte-rich interphase was collected, washed with PBS, and used for RNA extraction. Blood samples from seven mice of the same experimental group were pooled to ensure sufficient RNA yield. RNA was extracted using the QIAamp RNA Blood Mini Kit (Qiagen, Cat. No. 52304), following the manufacturer's instructions. This step was performed by the student author. All subsequent steps, including RNA quality assessment, cDNA synthesis, library preparation (Illumina TruSeq Library Prep Kit v2), and high-throughput sequencing, were performed by trained personnel at the institutional core facility. Sequencing reads were processed by removing low-quality raw data and adaptors using Trimmomatic. Reads were aligned to the prebuilt mouse reference genome (GRCm38/mm10) using STAR. Gene-level counts were generated with FeatureCounts from the Subread package, and expression levels were normalized as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Visualization of differential gene expression was performed using RStudio, including the generation of heat maps and volcano plots.

2.9 Serum extraction and Luminex analysis

Following peripheral blood collection, samples were transferred into serum collection tubes without anticoagulant (BD Microtainer®, Cat. No. 365956) and left undisturbed at room temperature for 30–45 minutes to allow clotting to occur. After confirming full coagulation, samples were centrifuged at 1000 g for 5 minutes at room temperature to separate serum from blood cells. The clear supernatant (serum) was carefully extracted using a pipette, avoiding contamination with cells or clots, and stored at −80°C until analysis. Serum cytokine levels were quantified using the ProcartaPlex™ Mouse Cytokine and Chemokine Panel 80-Plex (Thermo Fisher Scientific, Cat. No. EPX800-

26088-901), a preconfigured multiplex panel designed for mouse samples. Each well of the assay plate received 50 µL of capture beads followed by 25 µL of serum sample, standard, or control. Plates were incubated at room temperature for 2 hours on a shaker, washed to remove unbound materials, and then incubated with 25 µL of biotinylated detection antibodies for 30 minutes. After an additional wash, 50 µL of Streptavidin-PE was added, followed by a final wash and the addition of 120 µL of Reading Buffer. Fluorescence was measured on a Luminex 200™ System (Luminex Corp., Austin, TX) according to the manufacturer's protocol. Cytokine concentrations were calculated using a standard curve generated from known concentrations provided in the kit.

2.10 Data analysis

Statistical analyses were performed using Prism 10. T-test was used to determine the statistical significance of the results for comparisons between control and sleep-deprived (SD) groups. A p-value threshold of < 0.05 was considered statistically significant. The RNA sequencing data and data visualization were performed using external core facility resources.

3. Results

3.1 Electroencephalography *(EEG)* data analysis

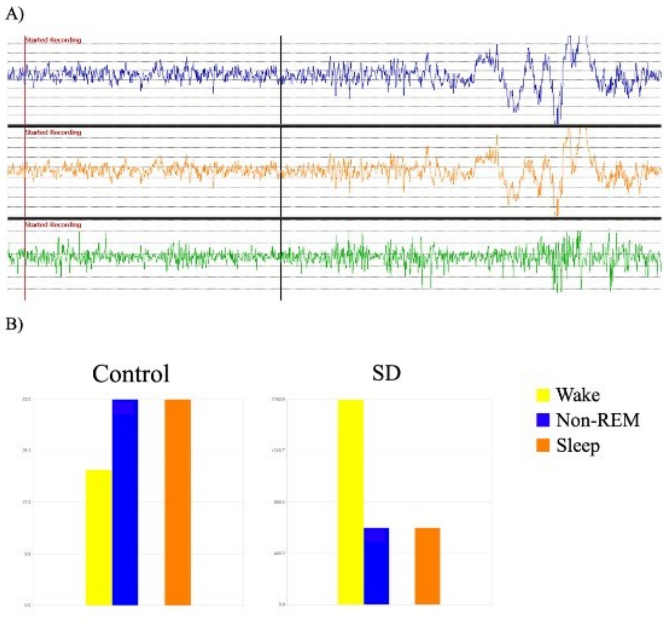

Figure 2. Customized sleep deprivation model and its effectiveness. (A) EEG and EMG data from two recording sites (blue and yellow traces for EEG, green trace for EMG) to differentiate sleep stages and muscle activity during wakefulness and sleep. Recorded under controlled laboratory conditions to analyze sleep patterns. **(B)** Two bar graphs comparing the duration of wakefulness, REM, and non-REM sleep between control and sleep-deprived groups. The results show an increase in wakefulness in the sleep-deprived group, proving the effectiveness of the customized sleep deprivation device.

The EEG data showed a notable increase in wakefulness, with a corresponding reduction in both REM and non-REM sleep stages for the sleep deprived mice (Figure 2). The significant difference between the proportions of wakefulness compared to the other sleep stages in length provided evidence that the mice were sleep-deprived in the experiment.

3.2 Comparison of immune cell distributors and proportions in mice spleens

To investigate the effects of short-term sleep deprivation on immune cell distributions, we performed flow cytometric analysis on spleen samples from the control and sleep-deprived groups. To examine the changes in proportions and distributions of the various immune cells, specific surface markers were used to differentiate between each cell (Figure 3A). After converting numerical data into a bar chart, changes in the immune cell distributions between the control and sleep-deprived groups in the spleen were noticed (Figure 3B). There was a statistically significant reduction in the proportion of NK cells in sleep-deprived mice ($p < 0.01$, two-sample t-test), suggesting a compromised innate immune response. In contrast, the proportion of B cells increased significantly ($p < 0.05$, two-sample t-test), which indicated a potential compensatory mechanism to maintain immune homeostasis.

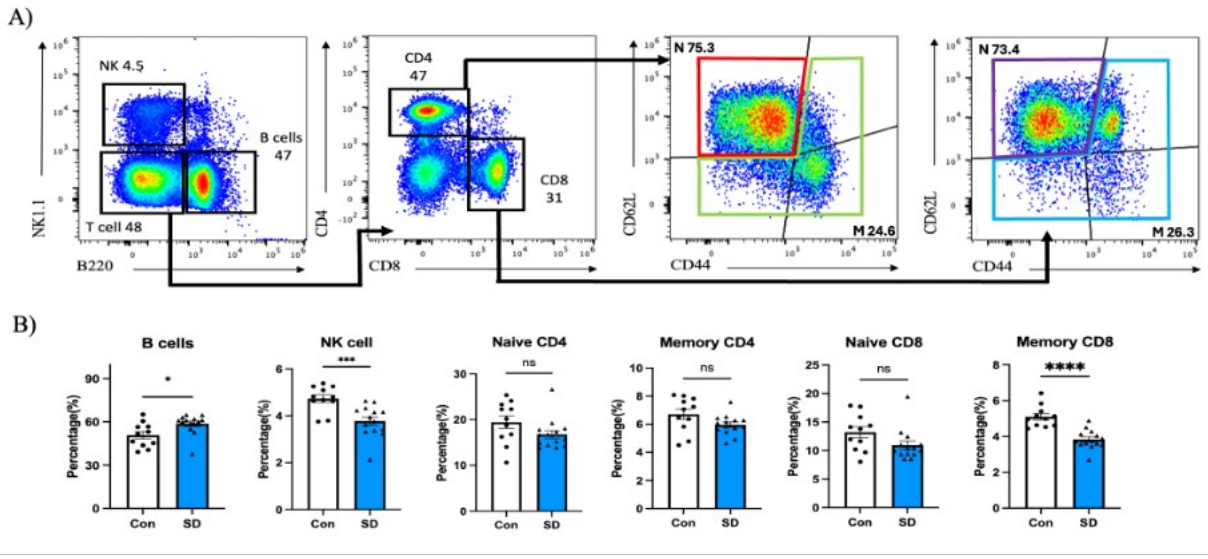

Figure 3. Effects of short term sleep deprivation on spleen immune cells. (A) Identifying the distribution of NK cells, B cells, $CD4^+$ T cells, and $CD8^+$ T cells in the spleen after sleep deprivation based on specific surface markers. Left panel: NK cells ($NK1.1^{hi}$, $B220^{lo}$) and B cells ($NK1.1^{lo}$, $B220^{hi}$); middle panels: $CD4^+$ T cells gated as $CD4^{hi}$ and $CD8^{lo}$, further subdivided into naive (high CD62L, low CD44) and memory (low CD62L or high CD44) cells; right panels: $CD8^+$ T cells gated as $CD4^{lo}$ and $CD8^{hi}$, similarly subdivided (n=18). *p<0.05, **p<0.01, ***p<0.001, ****p=0.0001. **(B)** Bar graph comparing immune cell proportions in the control and sleep-deprived groups. Statistical analysis was conducted using a two-sample t-test, visualized using Prism software (n=30). *p<0.05, **p<0.01, ***p<0.001, ****p=0.0001.

Additionally, although both the naive and memory subsets of $CD4^+$ and $CD8^+$ T cells were observed to be similar, a statistically significant decrease in the memory $CD8^+$ T cell population ($p < 0.001$, two-sample t-test) was noted. This decrease is indicative of potential vulnerabilities of the immune system after sleep deprivation. It could also represent a regulatory adaptation to mitigate excessive inflammatory responses and prevent over-inflammation under sleep-deprived conditions.

3.3 Comparison of immune cell distributors and proportions in the peripheral blood leukocytes of mice

Flow cytometry was also employed to analyze immune cell distributions in peripheral blood leukocytes between mice subjected to short-term sleep deprivation and mice from the control group (Figure 4A). A significant decrease in memory CD4 T cells and NK cells proportions in sleep-deprived mice was observed, which is similar to the results found in the spleen cells, indicating impaired immune capabilities ($p < 0.05$, two-sample t-test, Figure 4B).

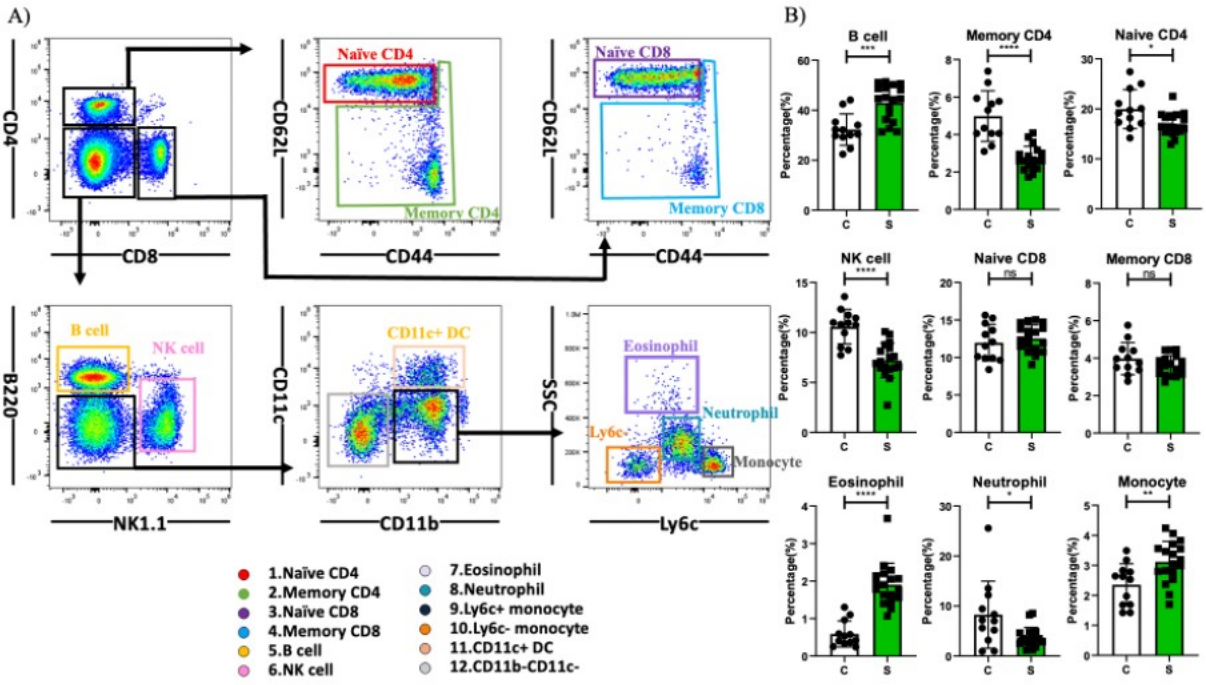

Figure 4. Effects of short-term sleep deprivation on peripheral blood leukocytes. (A) Flow cytometry results and identification of naive and memory CD4 and CD8 T cells, B cells, NK cells, neutrophils, eosinophils, $Ly6C^+$ monocytes, $Ly6C^-$ monocytes, and dendritic cell populations ($CD11c^+$ and $CD11b^-CD11c^-$) (n=18). **(B)** Bar charts showing the comparison of immune cell proportions in the control and sleep-deprived groups. Data points represent individual mice with mean ± SD shown (n=30). Statistical significance determined by two-sample t-tests is indicated: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant (n=30).

This suggests a potential weakening of both adaptive and innate immune defenses. In contrast, naive and memory $CD8^+$ T cells did not differ, highlighting the different impact of sleep deprivation on various components of the immune system. Similar to what was observed in the spleen, increases in B cells was found ($p < 0.01$, two-sample t-test, Figure 4B). Additionally, an significant increase in eosinophils was found, further suggesting a compensatory response to maintain immune functionality despite the reduction in other cell types.

3.4 Comparison of levels of gene expression

To emphasize the differences between the results of bulk RNA sequencing for the control and sleep-deprived groups, heat maps and volcano plots were generated to better visualize the data. This allowed a clear comparison between the differences in gene expression levels between the control and treatment groups. A significant difference in the gene expression levels between the control CD4 T cell group and the sleep-deprived CD4 T cell group was found (Figure 5A). Specifically, within group, all three sleep-deprived groups and three control groups behaved similarly. They all showed an upregulation in specific gene clusters, which is represented by warmer colors (red), whereas all three control groups displayed cooler tones (blue) in the corresponding regions. This contrast suggested that upregulation of certain genes in one group was accompanied by the downregulation of the same set of genes in the other group and vice versa, emphasizing the difference in gene expression between the two groups. When examining a more specific list of immune-related genes, the levels of expression for genes with anti-inflammatory properties such as Foxp3 and Tnfrsf21 increased after sleep deprivation (Figure 5A). The upregulation of Foxp3 suggested that short-term sleep deprivation may activate mechanisms that mitigate inflammation. Similarly, the increase in expression of Tnfrsf21, which is significant as it plays a crucial role in disposing of overly activated immune cells and preventing chronic inflammation, could potentially contribute to the suppression and termination of inflammatory responses. On the other hand, genes such as Gzma and Gzmb showed a decrease in expression. Both granzymes are proteases released primarily by cytotoxic T cells ($CD8^+$) and natural killer (NK) cells. The decrease in the two gene expressions suggests a potential downregulation of cytotoxic T cell and NK cell activity, which might alter how the immune system responds to infected or malignant cells, leading to a decreased ability to eliminate harmful cells effectively. This means that immune homeostasis is skewed toward a state of increased susceptibility to infections or diseases after sleep deprivation.

Examining the changes in gene expression levels in CD8 T cells, Smad6 and Pdcd7 genes were upregulated in the sleep-deprived group (Figure 5B). Smad6 serves as an inhibitory SMAD protein that moderates the signaling pathways of transforming growth factor-beta (TGF-β) and bone morphogenetic protein (BMP). These signaling pathways are pivotal in regulating immune responses and maintaining cellular homeostasis. Thus, the upregulation of Smad6 could also act as a suppressor of excessive immune responses and potentially reduce the risk of inflammation and autoimmune diseases. On the other hand, the increase in Pdcd7 expression shows the immune system's response of clearing out cells

that are dysfunctional or potentially dangerous to maintain a level of immune surveillance. A downregulation in key genes like Granzyme B (Gzmb), Interferon-gamma (Ifng), and T-bet (Tbx21) was also observed. Similar to the changes in gene expressions in CD4 T cells, the reduction in Gzmb, Ifng, and Tbx21 in $CD8^+$ T cells suggested that defenses against infections might be compromised, leading to a weakened immune response, making it less effective at coordinating attacks against pathogens. The overall downregulation of these genes that play critical roles in the immune system points to a significant dampening of both the cytotoxic abilities and the general immune functions of $CD8^+$ T cells under sleep deprivation, emphasizing the importance of adequate sleep for maintaining immune health.

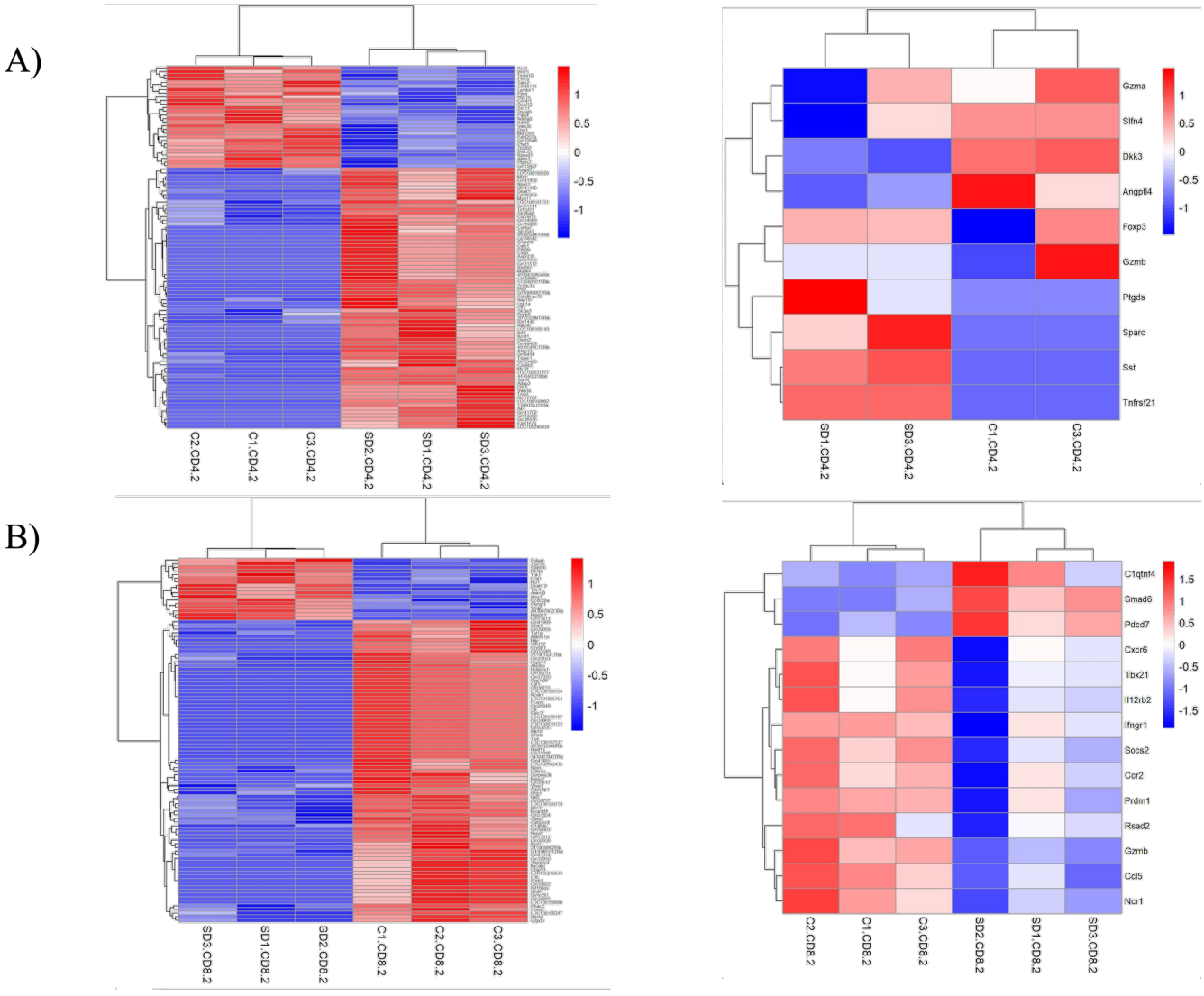

Figure 5. Effects of short-term sleep deprivation on gene expression. (A) Heat maps of gene expression in CD4 T cells. Bulk RNA sequencing analysis was done to examine changes in expression levels (n=42). **(B)** Heat maps of gene expression in CD8 T cells. Bulk RNA sequencing used to observe transcriptional changes. (n=42)

In addition, the gene expression data after sleep deprivation for CD4 and CD8 T cells were expressed in the format of a volcano plot (Figure 6). For CD4 T cells after sleep deprivation, the volcano plots show a more balanced mix of upregulated and downregulated genes. Meanwhile, the volcano plot for CD8 T cells after sleep deprivation exhibited a greater skew towards upregulation, suggesting that sleep deprivation impacted a wider range of genes in CD4 T cells but triggered a larger increase in anti-inflammatory genes in CD8 T cells, thereby contributing to the suppression of the immune system.

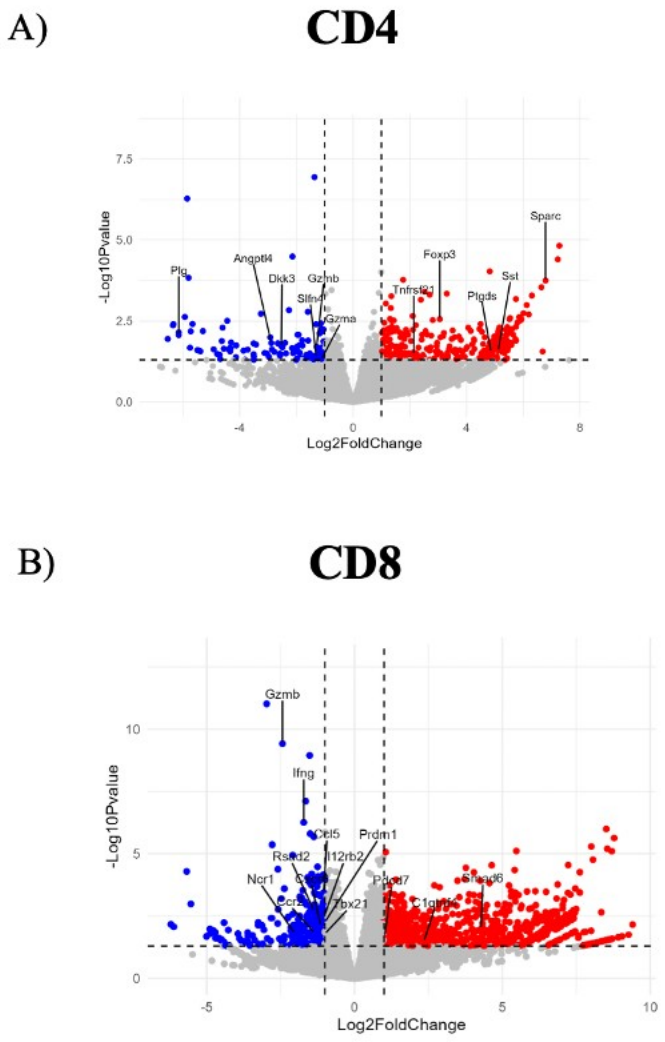

Figure 6. Gene expression changes in T cells after sleep deprivation. **(A)** Volcano plot showing the changes in gene expression in CD4 T cells. **(B)** Volcano plot showing the changes in gene expression in CD8 T cells. Panel A displays the differential gene expression in CD4 T cells, and Panel B shows the changes in CD8 T cells following short-term sleep deprivation. Each point represents a gene; blue points indicate significantly downregulated genes, red points denote significantly upregulated genes, and gray points are genes that did not meet the significance threshold. The x-axis represents the log2 fold change, and the y-axis denotes the -log10 p-value from a two-sample t-test (n=42).

3.5 Luminex assay analysis

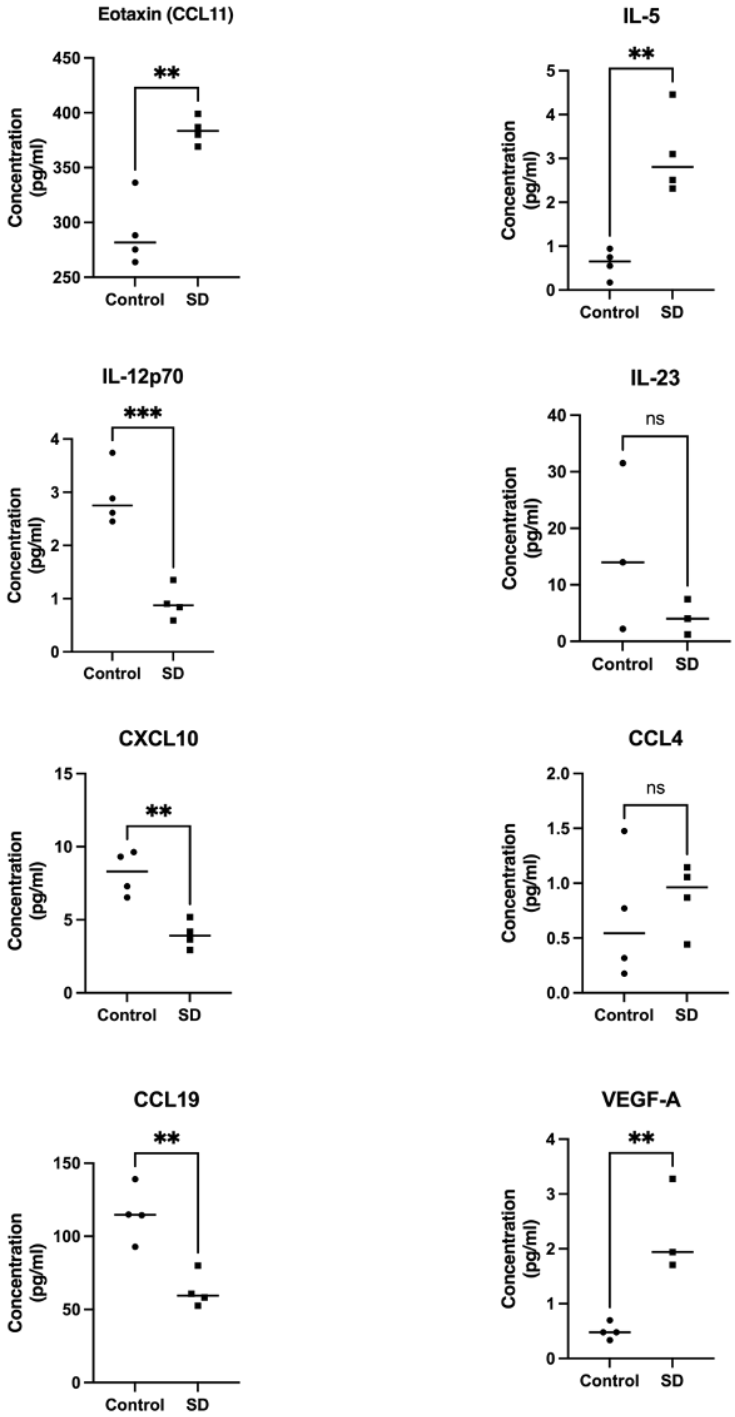

Figure 7. Effects of short-term sleep deprivation on the concentration levels of chemokines and cytokines. Comparison of cytokine and chemokine concentrations in control versus short-term sleep deprivation groups. Serum cytokine and chemokine levels were measured using the Luminex system (n=30). *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

The Luminex Assay was utilized in order to detect the concentrations of the cytokines and chemokines in serum. The results from the Luminex Assay showed a significant decrease in serum protein concentrations of several inflammatory cytokines and chemokines such as Interleukin-12p70 (IL-12p70), CXCL10 (IP-10), and CCL19 (MIP-3β) (Figure 7). In addition, there were non-significant decreases observed in Interleukin-23 (IL-23) and CCL4 (MIP-1β). IL-12p70 is a pro-inflammatory cytokine that is crucial for the activation of NK cells and the differentiation of CD4 T cells into TH1 cells. Likewise, IL-23 also plays a pivotal role in cell activation and inflammation. CXCL10, CCL4, and CCL19 are chemokines that attract immune cells to sites of inflammation. The reduction in concentration of these pro-inflammatory cytokines and chemokines suggests a suppression of inflammation. The increase in the concentrations of Eotaxin (CCL11) and Interleukin-5 (IL-5), though, contradicts this finding of a decreasing trend in expression of pro-inflammatory proteins, as even though they are pro-inflammatory cytokines, they were not downregulated. However, their increase in concentration can be explained by their association with eosinophils. Eosinophils were found to increase in proportion when analyzing the proportions of immune cells in the peripheral blood leukocytes, possibly to maintain immune homeostasis. Therefore, this upregulation of Eotaxin (CCL11) and Interleukin-5 (IL-5) in the serum aligned with and supported our previous findings in the PBL cells. Finally, the concentration of Vascular Endothelial Growth Factor A (VEGF-A), an anti-inflammatory cytokine, was found to be increased. VEGF-A possesses anti-inflammatory properties and is typically involved in the healing process by promoting the repair of damaged tissues and restoring normal tissue function after inflammation. The increase in its levels could indicate a trigger in regulatory responses as a priming mechanism to react to and reduce inflammation that may occur after sleep deprivation. Taken together, the general decrease in pro-inflammatory cytokine concentrations and the increase in the anti-inflammatory protein concentrations suggest that short-term sleep deprivation leads to immune suppression.

4. Discussion

The findings of this study demonstrate that acute sleep deprivation exerts a suppressive effect on the immune system, rather than inducing inflammation. Consistent with our findings of a suppression of pro-inflammatory cytokines and reduced immune cell proportions following short-term sleep deprivation, previous studies have also demonstrated reduced natural killer cell activity and alterations in cytokine production, suggesting a compromised innate immune response (4,7). Moreover, the increase in B cells and eosinophil proportions found in this study is consistent with past findings that also observed elevated inflammatory markers under conditions of sleep deprivation (11). The increase of certain immune cells observed indicates that despite the impairment of specific immune functions, the body actively attempts to restore and maintain immune homeostasis. While our findings show partial overlap with those of previous studies, direct comparisons should be interpreted cautiously. Notably, many past studies used sleep deprivation methods such as the flowerpot technique, which specifically targets REM sleep. Our rotor-based method induces general

sleep disruption. Mice in the sleep deprived group were kept continuously awake using the rotor cage throughout the deprivation period with sleep deprivation monitored by EEG. However, in the control group, EEG monitoring was not performed.

Contrary to the conventional belief that sleep deprivation predominantly increases inflammation (8), our research's findings show that sleep deprivation appears to affect the immune system differently. The suppression of key cytotoxic genes, a generalized downregulation of pro-inflammatory genes, and the decrease in the proportion of immune cells, implies that short-term sleep loss might induce a less responsive and less active immune state overall. This challenges the association often made between sleep deprivation and increased inflammation in past studies and suggests instead that sleep loss might reduce immune activity, thereby leading to a higher susceptibility to infections, since necessary inflammatory responses are not triggered. Our findings indicate that acute sleep deprivation may undermine the immune system, as shown by the decrease in the number of immune cells such as NK cells and memory CD8 T cells, which are essential for defending against pathogens and protecting the body. Our further analysis on gene expression levels also showed a reduction in key cytotoxic gene expression, underscoring the negative effects of sleep loss on cellular immune functions. These results highlight the critical importance of sufficient sleep for maintaining immune health, especially during situations that demand robust immune responses, such as during pandemics.

However, there are several limitations that should be considered and could be addressed in future studies. To begin with, although our custom-designed rotor cage reduced psychological and physical stress compared to the traditional methods, this research could be improved by incorporating direct measurement of stress-related indicators. For example, researchers could assess plasma corticosterone levels, which are well-established biomarkers used to quantify the physiological stress response in mice. This would allow researchers to further understand how sleep deprivation influences stress pathways and how these stress responses might lead to immune changes. Incorporating direct measurement of stress indicators may allow us to clarify whether changes in the immune function are mediated by sleep deprivation itself, stress, or a combination of both. Next, the subjects of the experiment were male B6 mice, indicating that the results may not be fully generalizable. Immune responses and immune function changes caused by sleep deprivation may differ between sexes. Including both sexes would enhance the generalizability of the results. Finally, while this study focused on the immediate effects of acute sleep deprivation on the immune system of mice, it did not assess whether the observed immune changes would persist or reverse following a period of recovery sleep. Investigating the immune system's response to recovery sleep could provide additional insights into the role of sleep in regulating immune function. Additionally, EEG monitoring was performed only in the sleep-deprived group to confirm successful wakefulness, but not in the control group. As immune responses can vary between NREM and REM sleep, the absence of sleep-stage confirmation in controls at the time of blood

draw presents a limitation that may introduce variability in immune readouts.

Looking ahead, future research could extend this study in several directions. First, blood samples could be collected from individuals with varying sleep patterns, such as healthcare workers, firefighters, night shift workers or military personnel, to validate the findings from mice in a human context. Second, comparing the effects of long-term sleep deprivation with short-term deprivation could also provide deeper insights into how chronic sleep loss impacts the immune system. Additionally, introducing tumors into the mouse model and examining the interplay between sleep, tumor growth, and immune system activity would help elucidate the role of sleep in cancer progression and immune surveillance.

5. Conclusion

Short-term sleep deprivation suppresses the immune system by reducing the distribution of key immune cells, downregulating pro-inflammatory genes, and upregulating anti-inflammatory genes. This research confirms the vital role of sleep in regulating the immune system and maintaining an effective immune defense. It also paves the way for further investigations into how optimizing sleep can enhance overall health and well-being as it provides insights into how lifestyle choices in terms of sleep can significantly impact health.

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Author contributions

Valerie Yen was responsible for conceptualization and experimental design (with guidance from Dr. Bo-Yi Sung), mouse monitoring, sample collection, PBL RNA extraction, flow cytometry data acquisition and analysis, data interpretation, and manuscript writing. Hong-Ru Chang was responsible for EEG implantation and setup, rotor cage design, mouse handling, data analysis support, and coding assistance. Lin-Keng Lee was responsible for animal care and monitoring, assistance with sample collection, EEG setup, and general experimental support.

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