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參展科別　動物學

作品名稱　短期睡眠剝奪對小鼠免疫系統的影響

得獎獎項　四等獎

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關鍵詞　短期睡眠剝奪、免疫反應

作者簡介

我是顏維萱(Valerie Yen)，目前就讀於台北美國學校的11年級。自然科學一直是我喜歡的科目之一。我也對科學研究感興趣，因為透過實驗我能學到許多新的知識和技能。這次的研究過程雖然艱辛且充滿挑戰，但也讓我獲得了許多的知識和成就感。在此，我特別感謝這次的研究機會以及一直在我身邊支持我的家人和老師、與實驗室的學長姐們。

附件三

研究報告封面

2025年臺灣國際科學展覽會

研究報告

區別:

科別：動物學

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關鍵詞：短期睡眠剝奪、免疫反應、_____（最多三個）

編號:

（編號由國立臺灣科學教育館統一填列）

摘要

現代社會中，睡眠剝奪已成為普遍問題，人們對其對免疫系統及整體健康的負面影響愈加關注。本研究使用特製的旋轉鼠籠讓小鼠連續 72 小時保持清醒，探討急性睡眠剝奪對小鼠免疫反應的影響。研究發現 NK 細胞與脾臟中的記憶 CD8 T 細胞比例明顯減少，顯示細胞毒性功能受損或記憶免疫反應下降。與此同時，抗炎細胞因子的表達增加，而促炎細胞因子和相關基因的表達則有顯著下調。此外，雖然觀察到 B 細胞比例有所增加，這可能是免疫系統在細胞免疫功能受損時，維持免疫穩態的反應。這些發現揭示了睡眠剝奪可能抑制免疫系統造成損害。本研究強調適量睡眠對維持免疫平衡的重要性，並指出睡眠不足可能促進慢性免疫問題的發展。在此基礎上，後續研究可探討短期睡眠剝奪與腫瘤及免疫系統的關聯，並延伸至長期剝奪的影響。

Abstract

Sleep deprivation has now become a prevalent issue in society, raising concerns about its detrimental effects on the immune system and the overall health of human beings. This study aims to investigate acute sleep deprivation's impacts on mice's immune responses. Using a custom-designed sleep deprivation method, the mice were sleep deprived for a continuous 72 hours by a custom-made rotor cage. The research found a notable reduction in the proportions of NK cells and memory CD8 T cells in the spleen after short-term sleep deprivation, suggesting the impairment of cytotoxic functions and diminished immune responses. Although an increase in B cell proportions was observed, it may be the immune system's attempt to compensate for impaired cellular immune functions by enhancing humoral immune responses. In addition, the expression of anti-inflammatory cytokines was found to increase, and expressions of pro-inflammatory cytokines and related genes were significantly downregulated, further supporting

our findings that sleep deprivation may compromise the body's ability to activate an effective immune response when facing pathogens. Thus, the results reveal the critical role of adequate sleep in maintaining immune homeostasis and suggest that sleep deprivation may contribute to the development of chronic immune illnesses. Building on this foundation, future studies could examine the effects of short-term sleep deprivation on the immune response to tumors. Subsequently, future studies could extend these investigations to chronic sleep deprivation conditions, with and without tumor inoculation.

Introduction

I. Research Context and Significance

In modern-day society, sleep deprivation has become more common due to high-stress work environments and the fast pace of life (LaMotte, 2022). Individuals are often sacrificing sleep to meet the pressures of daily life. According to the CDC (Centers for Disease Control and Prevention), more than one-third of adults in the United States reported that they were not getting enough sleep and rest every night. This shift has led to a significant increase in sleep-related disorders and a growing concern about the long-term health implications of chronic sleep deprivation. In addition, deprivation of sleep was also found to lower productivity and increase the absence of workers, adding up to a total loss of about 1.2 million working days annually. Due to these reasons, sleep deprivation also has significant economic consequences. In the United States, this issue costs the economy up to $411 billion per year, which is around 2.28% of the nation's GDP (Hafner, 2016). Other than the economy, health is another critical issue. People who consistently sleep less than six hours each night were found to have a 13% higher mortality risk. Finally, sleep deprivation is also a major contributor to road accidents. Drowsy driving, which is extremely common today causes about 1 million crashes each year, resulting in severe casualties with 500,000 injured and 8000 dead (Sleep Foundation, 2022). These negative consequences of sleep deprivation underscore the significance of this study.

II. Background and Literature Review

Sleep is essential in maintaining human health and physiological balance, affecting many metabolic functions. Research shows that sleep contributes significantly to tissue repairment, memory consolidation, and the regulation of hormonal and metabolic functions, which are

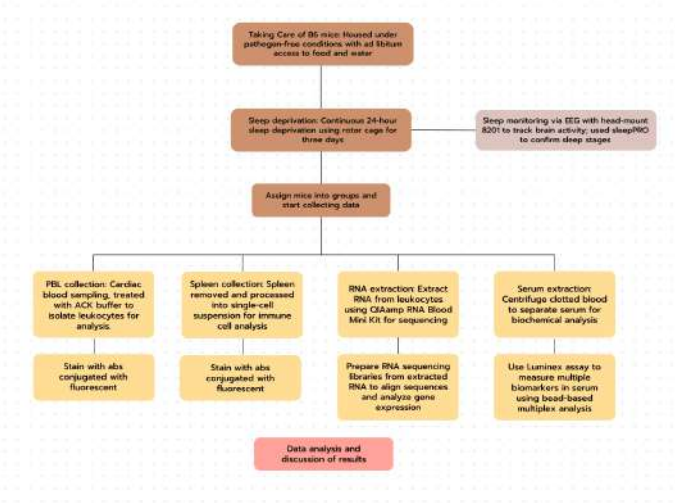
essential for maintaining physiological balance (Hanlon, Erin C *et al*, 2016; "How Sleep Affects Human Health"). In addition, it is found that both short-term and long-term sleep deprivation were highly associated with a greater risk of future cardiovascular events, highlighting the critical role sleep plays in maintaining cardiovascular health (Cappuccio *et al.,* 2011). Adequate sleep is exceptionally important to the immune system, as it promotes the proper functioning of the body's defense mechanisms, enhancing the body's ability to fight off pathogens. Therefore, sleep strengthens immune defenses and produces more natural killer (NK) cells as well as T-cells (Irwin, 2015).

Sleep deprivation refers to the condition of not getting enough sleep, whether voluntarily or involuntarily, over an extended period of time (Basner *et al.*, 2012). 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 (Hirshkowitz *et al.*, 2015). When an individual sleeps for fewer than these recommended hours, sleep deprivation occurs, which previous studies show is 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 (Mullington *et al.,* 2009). Furthermore, when the body is sleep-deprived, the production of pro-inflammatory cytokines like IL-6 and TNF-α was found to increase (Besedovsky *et al*., 2019). 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 like IL-6 and IL-17, which led to multiple organ dysfunction syndrome and eventually resulted in the death of the

mice (Sang, 2023). On the other hand, 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 2.94 times more likely to develop a cold. This highlights that short-term sleep deprivation suppresses the production of cytokines that are used in defending against infections like the common cold (Cohen, 2009).

Despite these findings, much of the current research focuses on single molecules or specific immune cells, leaving the broader molecular mechanisms largely unexplored. There remains a lack of comprehensive understanding of how short-term sleep deprivation impacts the immune system at the genomic level. Existing studies often look at the effects of chronic sleep deprivation, but short-term sleep deprivation and its effects on pro-inflammatory and anti-inflammatory pathways have limited research. Additionally, previous sleep deprivation models, such as the flowerpot technique, have introduced unintended physiological and psychological stress on the animals, affecting the validity of the results (Youngblood, B. D., *et al*, 1997). To address these limitations, a specialized model developed in my mentor's lab will be used. This new custom-made model allows for more accurate assessments of the effects of sleep deprivation on immune function without confounding stress responses

The experimental framework of this study is shown below:



III. Research Objectives

This study hypothesizes that short-term sleep deprivation affects the gene expression and distribution of immune cells. The research objectives are as follows:

1. To investigate how short-term sleep deprivation influences the expression of pro-inflammatory and anti-inflammatory genes.
2. To analyze the changes in immune cell distribution before and after sleep deprivation.
3. To utilize a custom-made sleep deprivation model to minimize the psychological stress and other confounding factors introduced by previous models, ensuring more accurate results in assessing immune responses.

Materials and Methods

I. Research Instruments and Equipment (Table 1)

Table 1: Experimental Devices and Materials

Number	Name	Brand/Specifications	Notes
1	10 mL Serological Pipet	Simply Biologics	
2	2 mL Serological Stripette	Simply Biologics	
3	Pipet Controller	Falcon	
4	15 mL Centrifuge Tube	Simply Biologics	Flat cap, conical bottom
5	50 mL Centrifuge Tube	Simply Biologics	
6	14 mL Polypropylene Round-Bottom Tube	Falcon	17 x 100 mm style
7	5 mL Polypropylene Round-Bottom Tube	Falcon	12 x 75 mm style
8	Cell Strainer	GeneDireX	40 μm mesh pore size
9	25G Single-Use Needle	Terumo	25mm
10	Disposable Syringe	Terumo	1 cc/mL
11	Automated Cell Counter	Invirotgen: Countess 3	
12	ACK Lysing Buffer	Thermo Fisher Scientific	A10492-01 100 mL
13	Micro Blood Collection Tube	Shanghai TEAMSTAND CORPORATION	0.5 mL
14	Microfuge 22R Centrifuge	Beckman Coulter	
15	1000 mL Percoll	Cytiva	1.130 g/mL
16	Dulbecco's Phosphate Buffered Saline	Gibco	21600-069 1 x 10 L
17	Luminex Kit: Mouse ProcartaPlex Mix&Match 30-plex	Invitrogen	PPX-30-MX2XA33
18	Hemacytometer	Reichert Bright-Line	
19	Illumina Stranded Total RNA Prep	Illumina	

20	Direct-zol RNA Miniprep Plus Kits	Zymo research	

II. 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 experiment, all the mice used were sourced from the National Laboratory Animal Center and kept and cared for under standard laboratory conditions with a 12-hour light/dark cycle, having access to food and water ad libitum. The experimental procedures were also carried out in compliance with the institutional guidelines for animal care.

III. 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 (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 experiments were approved by the Institutional Animal Care & Use Committee (IACUC) and performed in accordance with the Taiwan Animal Protection Act. The mice were also housed under pathogen-free conditions in sterile ventilated racks after being originally obtained from the National Laboratory Animal Center.

IV. Sleep Deprivation Model

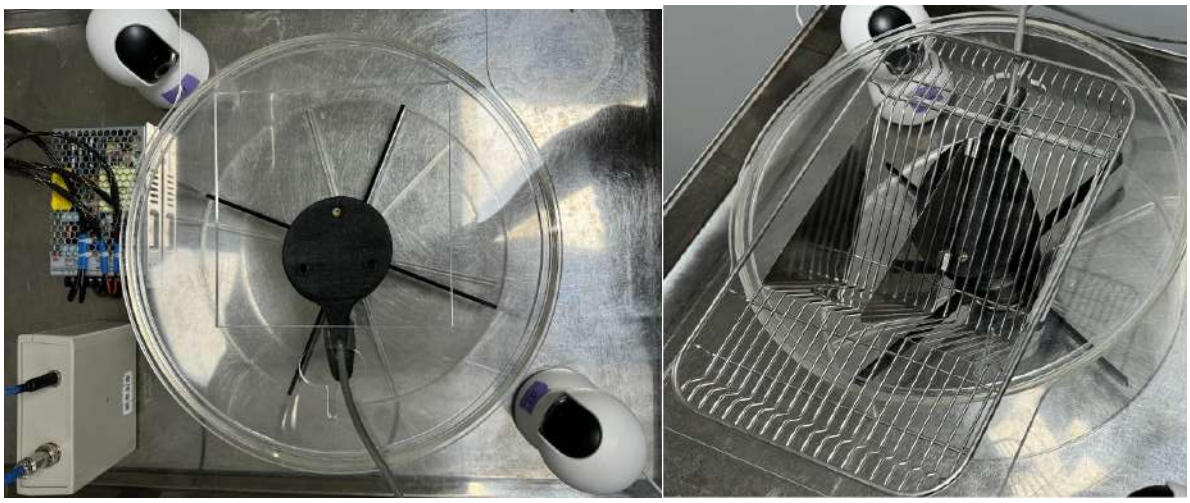


Figure 1. Rotor Cage (picture taken by the first author)

A custom-designed sleep deprivation apparatus, referred to as a rotor cage, was utilized in this experiment (Figure 1). The cage was constructed through 3D printer with acrylic material, 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 translates 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.

V. 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 implanted through surgery to track the brain wave activities in the mice 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.

VI. 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 second group of mice was used for bulk RNA-sequencing and had 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. Following the sleep deprivation phase, the mice were transferred from the animal center to the laboratory for sample collection.

VII. Peripheral Blood Leukocyte Separation

Each mouse was anesthetized and cardiac blood was collected in an EDTA anticoagulant tube using a 25G needle. 1cc of Ammonium-Chloride-Potassium (ACK) lysis buffer was then added to the blood sample, disintegrating the red blood cells within. After lysing, the sample was then incubated at room temperature for 3 minutes, during which the sample was gently turned upside down 2 times. This allows the buffer to coat and penetrate the cells more effectively. After this incubation period, 10cc of phosphate-buffered saline (PBS) was added. The sample was then centrifuged at 600g for 5 minutes at room temperature. Following centrifugation, the supernatant was removed, and the remaining cell pellet was resuspended in 10cc of PBS and counted. Lastly, the cells were collected in a 15cc tube.

To collect the PBL (peripheral blood leukocyte) cells for RNA sequencing, the blood was layered onto a percoll gradient using 62% and 75% and centrifuged to separate the layers. After

careful handling to avoid disturbing the layers, the cloud-like layer containing PBLs was collected. The combined blood samples from seven mice in the same experimental group were processed to extract total RNA using the QIAamp RNA Blood Mini Kit (52304, Qiagen), adhering strictly to the guidelines provided by the manufacturer. Sequencing libraries were then prepared using the Illumina TruSeq Library Prep Kit v2.

VIII. Splenocyte Separation

In terms of splenocyte collection, the spleen was removed, crushed, and filtered through a strainer into a 50 ml conical tube. The spleen was rinsed with PBS, and the splenocytes were collected by further crushing and rinsing until all the red parts were processed. After centrifugation, RBC lysis buffer was used to remove red blood cells, leaving splenocytes for analysis.

IX. Flow Cytometry

Cells were stained with the following antibodies: Brilliant Violet 421™ anti-mouse/human CD11b, BD Horizon™ BV480 Rat Anti-Mouse CD4, BD Horizon™ BB515 Rat Anti-Mouse CD62L, PerCP/Cyanine5.5 anti-mouse CD8a, PE anti-mouse NK-1.1, BD Horizon™ PE-CF594 Rat Anti-Mouse Ly-6C, PE/Cyanine7 anti-mouse CD11c, BD Pharmingen™ APC Rat Anti-Mouse CD44, and BD Pharmingen™ APC-Cy™7 Rat Anti-Mouse CD45R. Zombie NIR Fixable Viability staining was performed for live/dead discrimination according to the manufacturer's instructions. Samples were incubated under 4°C for 30 minutes with the antibody mixture in the staining buffer, then washed twice with the staining buffer. Finally, samples were resuspended in phosphate-buffered saline (PBS) and analyzed by flow cytometry. Flow cytometry data analysis was performed using Flowjo 10.

X. Leukocyte Bulk RNA Sequencing

Low quality-raw data and adaptors were removed by Trimmomatics. STAR was used to align readings to the prebuilt mouse reference GRCm38/mm10. Mapped readings were then assigned to genes based on the annotation file using FeatureCounts from Subread package and normalize expression levels for each sample to obtain Fragments Per Kilobase Million (FPKM). To visualize and organize data, heat maps and volcano plots showing gene expression data were generated.

XI. Serum Extraction

Following the cardiac blood sampling from the mice, the collected blood samples were transferred into collection tubes and were left undisturbed for a short period of time to promote clotting. After ensuring the blood had clotted, the samples were then centrifuged at 1000 g for 5 minutes at room temperature. Centrifugation allows the blood to into different components based on their different densities, effectively isolating the blood cells from the serum. Once centrifugation was complete, the upper clear layer of serum was carefully extracted using a pipette. During this process, no blood cells or clots were accidentally included in the sample. After extraction, the serum was divided and stored at the appropriate temperature for future analysis, which enables the measurement of various biomarkers relevant to the study.

XII. Luminex

Serum samples were analyzed using the ProcartaPlex™ Human Immune Response Panel 80-Plex to simultaneously measure multiple cytokines. Initially, 50 µL of capture beads were added to each assay plate well to bind cytokines. Then, 25 µL of serum samples, standards, and controls were introduced. The plate was incubated at room temperature for two hours with gentle

shaking for mixing. After incubation, wells were washed to clear unbound substances. Next, 25 µL of biotinylated detection antibodies were added to mark the cytokines, incubating for 30 minutes before another wash. Then, 50 µL of Streptavidin-PE was applied to generate a fluorescent signal, incubating for another 30 minutes followed by a final wash. Finally, 120 µL of Reading Buffer was added before analyzing the plate with the Luminex 200™ instrument, which measures the fluorescence to quantify cytokines in the samples.

XIII. 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 was outsourced to a core facility.

Results

I. EEG Data Analysis

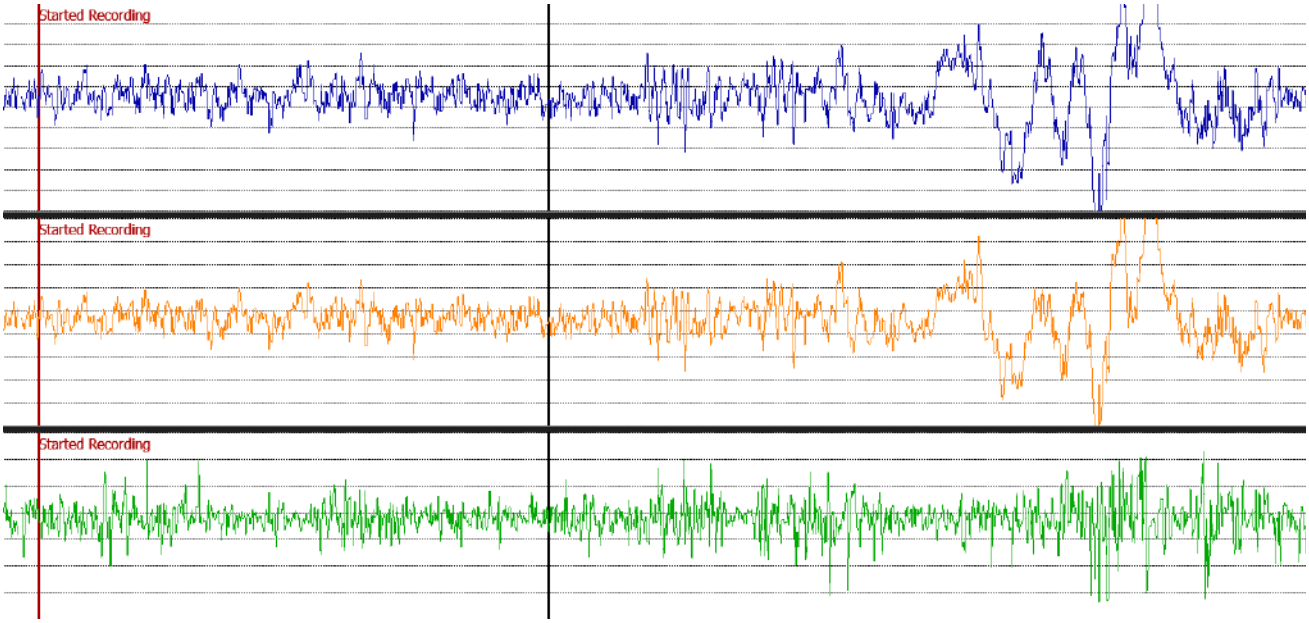


Figure 2: Raw EEG Data

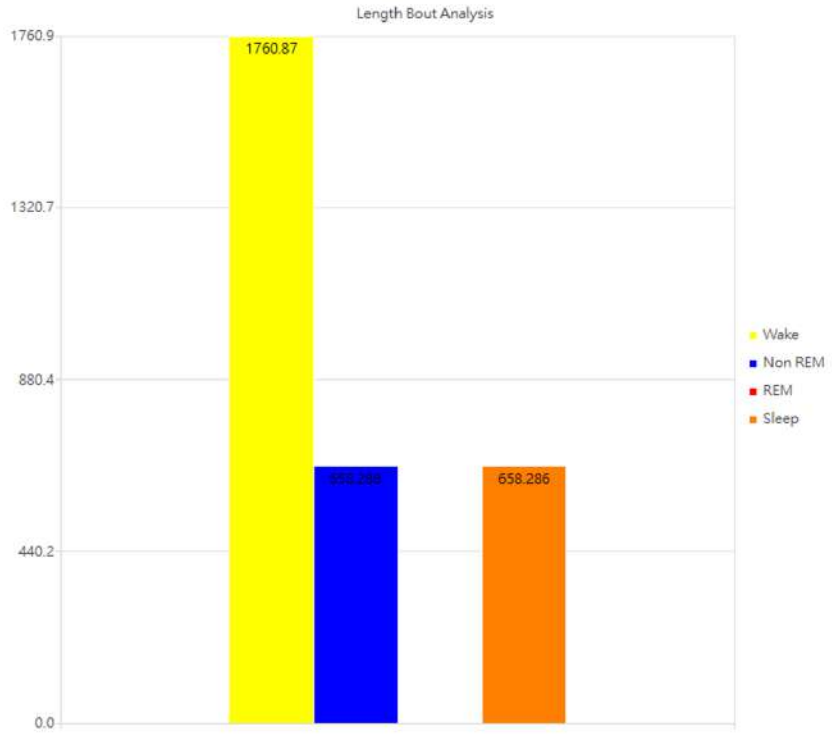


Figure 3: EEG Sleep Stage Distribution Under Sleep Deprivation

Raw EEG data was recorded with the blue and yellow traces representing two independent detecting sites, which differentiate different sleep stages based on the frequency and patterns of brain waves, and the green trace representing the electromyography (EMG) data (Figure 2). EMG was used to distinguish between wakefulness and sleep, as it reflects muscle activity, which is typically reduced during sleep states. The results were transformed into a statistic bar chart, highlighting the duration of wakefulness, REM, and non-REM sleep (Figure 3). The significant difference between the proportions of wakefulness compared to sleep stages in length is evident that the mouse was truly sleep-deprived in the experiment.

II. Comparison of Immune Cell Distributors and Proportions in Mice spleens

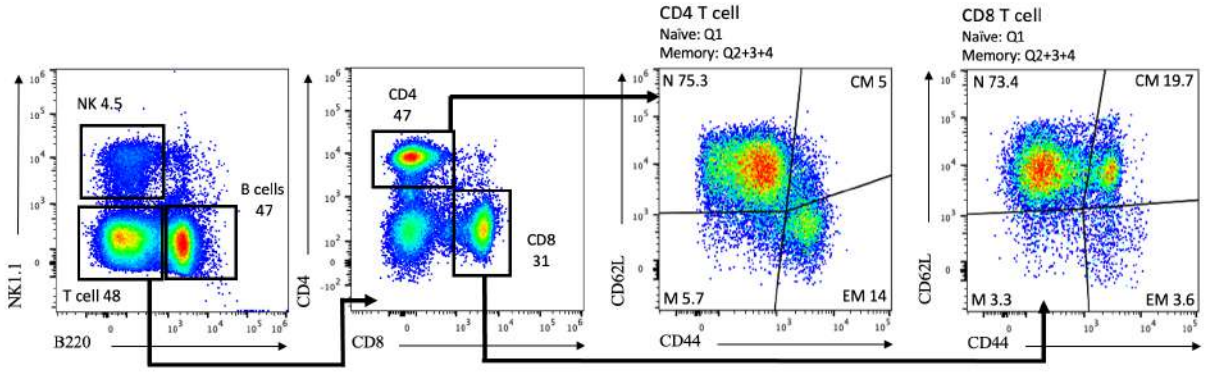


Figure 4: Flow cytometry results of immune cell distributions after short-term sleep deprivation

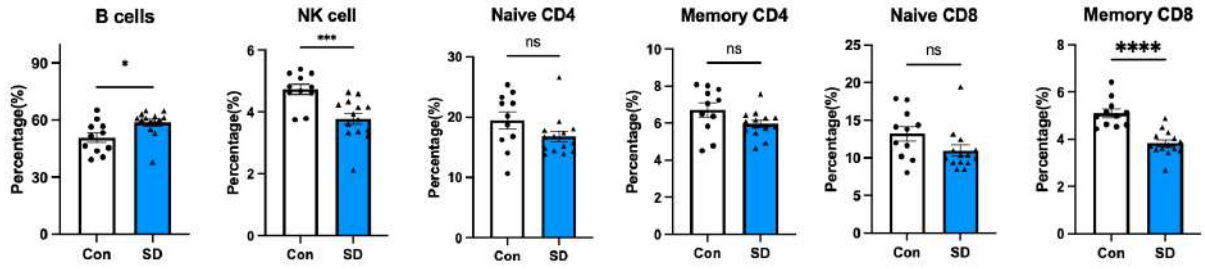


Figure 5: Comparison of immune cell proportions in control and sleep-deprived mice

To investigate the impacts of short-term sleep deprivation on the distributions of immune cells in the spleens of mice, analysis was done on the flow cytometry data. The different immune cell populations were identified and gated based on their different combinations of surface marker expression levels (Figure 4). Natural Killer cells were defined as $NK1.1^{hi}$ and $B220^{lo}$. On the other hand, B cells were recognized based on $NK1.1^{lo}$ and $B220^{hi}$. CD4+ T cells were isolated by gating on cells that expressed $CD4^{hi}$ and $CD8^{lo}$. They were then further categorized into naive and memory groups. In contrast, CD8+ T cells were identified through $CD4^{lo}$ and $CD8^{hi}$. Within the four quadrants of the two axes: CD62L and CD44, the first quadrant

represents the naïve CD4 T cell; meanwhile, the rest of the three quadrants are memory cells (Figure 4). Naïve cells were identified through their high expression of CD62L and low expression of CD44. On the other hand, the memory population was located with their low expression of CD62L or high expressions of CD44. At the end, the analysis of immune cell proportions was visualized through a bar chart. Significant alterations in the proportions of immune cells between the control (Con) and sleep-deprived (SD) groups were revealed (Figure 5). Statistical analysis using t-test highlighted the differences, with significance levels shown directly above each bar graph through asterisks. The proportion of NK cells was shown to have a statistically significant decrease, indicating the body's increased susceptibility to infections after sleep deprivation. This suggests that after sleep deprivation, the body's immune system is compromised and weakened. Both naive and memory subsets of CD4 and CD8 T cells displayed no statistically significant changes, suggesting their stability under short-term sleep deprivation conditions. However, from the chart, slight reductions in proportions were still shown. Memory CD8 T cells, though, showed a significant decrease in proportion in the sleep-deprived group, which may be indicative of the presence of a regulatory mechanism aimed at preventing the overreaction of inflammatory responses. On the contrary, the proportion of B cells showed a significant increase in sleep-deprived mice compared to controls, suggesting an increase in antibody production. This may be a result of immune homeostasis, where the immune system constantly seeks to maintain a balance between different types of immune responses. When other immune cells such as NK cells and T cells are decreased, the body upregulates other aspects of the immune system, in this case, B cells, to ensure that pathogens are still under surveillance.

III. Comparison of Immune Cell Distributors and Proportions in the Peripheral Blood Leukocyte of Mice

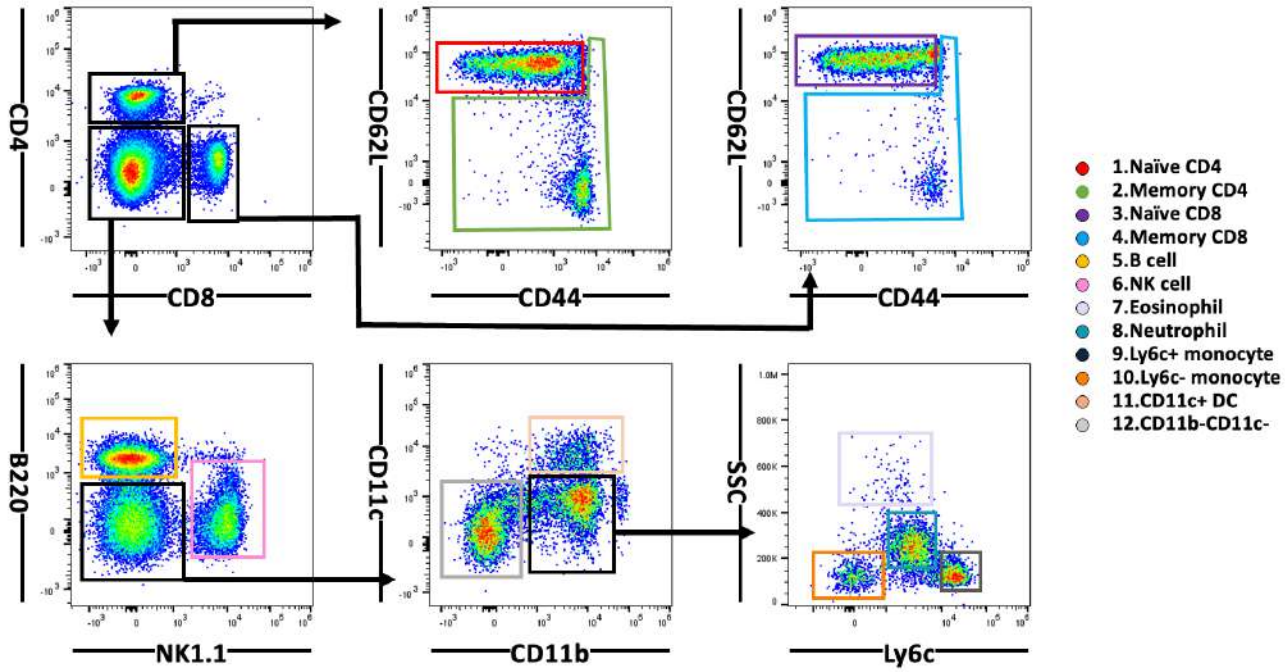


Figure 6: Flow cytometry results of immune cell distributions after short-term sleep deprivation

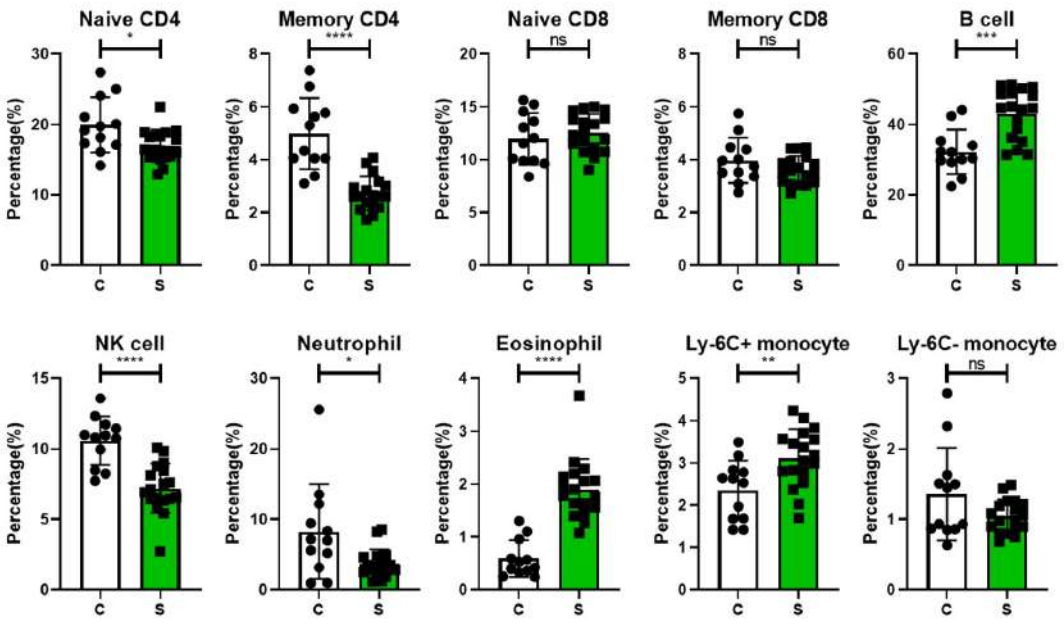


Figure 7: Comparison of immune cell proportions in control and sleep-deprived mice

After flow cytometry was conducted on peripheral blood leukocytes (PBLs) from mice subjected to short-term sleep deprivation, various immune cell populations were categorized through specific gating strategies (Figure 6). T cells were initially gated based on CD3 positivity. It was then further separated into CD4+ and CD8+ cells identified by their respective markers. Like the immune cells in the spleen, the naive and memory statuses within these groups were distinguished by the expression levels of CD62L and CD44. NK cells were isolated based on high NK1.1 and low B220 expression. Monocytes were classified by their expression of CD11b and later categorized into classical and non-classical types based on the intensity of Ly6C. Neutrophils were identified through high expressions of CD11b and Ly6G, while eosinophils were differentiated by their high side scatter characteristics combined with low to moderate expression of CD11b (Figure 6). After locating the different types of immune cells, comparisons between the proportion of each immune cell between the control and sleep-deprived groups were

made and shown in a bar chart that illustrates the shifts in cell populations (Figure 7). In sleep-deprived mice, the proportion of memory CD4 T cells showed a notable decrease as well as for NK cells. This finding implies a potential weakening of both adaptive and innate immunity. This suggests that short-term sleep deprivation may not only affect the body's readiness to respond to known pathogens but can also compromise its initial line of defense, increasing susceptibility to infections and potentially impairing immune regulation. Both naive and memory CD8+ T cells, which greatly decreased in proportion in the spleen, displayed no notable changes, though. This could imply the relative stability in these populations under the condition of sleep deprivation. This also hints at how short-term sleep deprivation can have different impacts on the immune cell population in different tissues. On the other hand, the proportions of B cells were again significantly increased as well as eosinophils and Ly-6C+ monocytes. Ly-6C+ monocytes are pro-inflammatory cells; their decrease suggests a reduction in the body's ability to mount an inflammatory response. The increase in cell proportions for B cells and eosinophils underscores how the immune system is highly dynamic and capable of initiating compensatory responses to maintain homeostasis. Overall, when pro-inflammatory responses are suppressed, the increase in B cells and eosinophils may be a way the body uses to maintain some of its defense mechanisms.

IV. Comparison of Levels of Gene Expression

A) B)

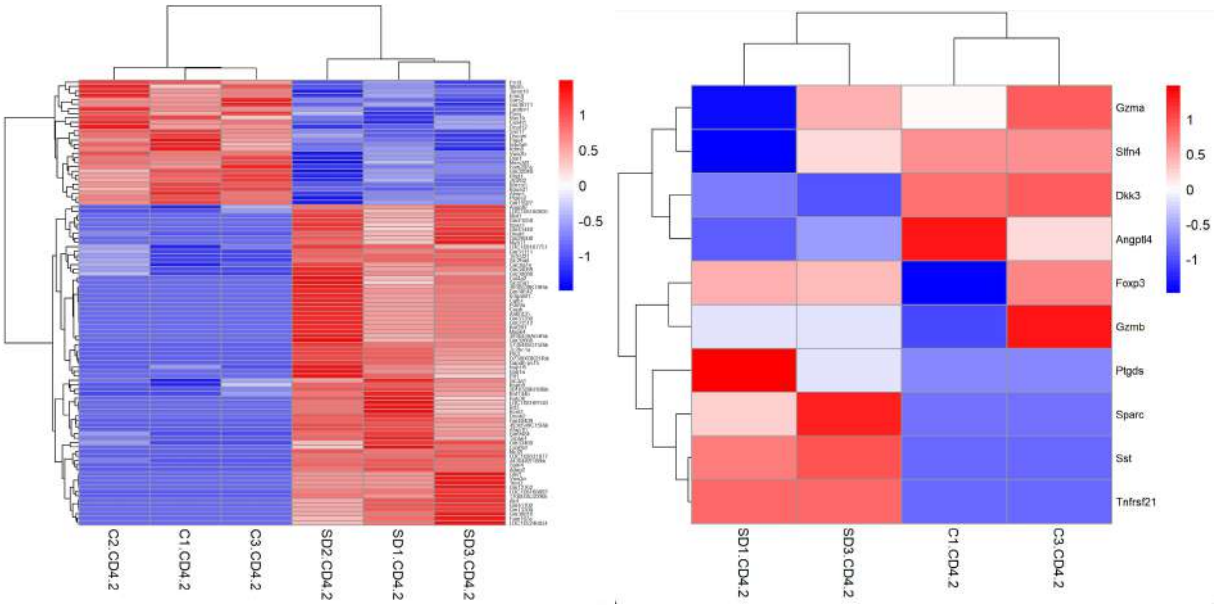


Figure 8: Heat maps of gene expression in CD4 T cells from RNA sequencing data

To emphasize the differences between the results of bulk RNA sequencing for the control and sleep-deprived groups, heat maps were generated. This allows a clear comparison between the differences in gene expression levels between the control and treatment groups. A significant difference between the control CD4 T cell group and the sleep-deprived CD4 T cell group was indicated on the graph (Figure 8A). Specifically, all three sleep-deprived groups behaved similarly, all exhibiting upregulation in specific gene clusters, represented by warmer colors (red), whereas all three control groups displayed cooler tones (blue) in the corresponding regions. This contrast suggests that when a gene was upregulated in one group, it was downregulated in the other, and vice versa, emphasizing the difference in gene expression between the two groups. When looking at a more specific list of immune-related genes, allowing

for analysis of the changes in immune response, it is found that the levels of expression for genes such as Foxp3 and Tnfrsf21 increased after sleep deprivation (Figure 8B). Foxp3 is a transcription factor that is known as the regulator of regulatory T cells (Tregs), which suppress immune responses and prevent autoimmune diseases. The upregulation of Foxp3 suggests that short-term sleep deprivation may activate mechanisms that mitigate inflammation. Similarly, the increase in expression of Tnfrsf21, also known as Death Receptor 6 (DR6), is significant as it is involved in the induction of apoptosis in immune cells. It plays a crucial role in getting rid of overly activated immune cells and preventing chronic inflammation. The elevation in Tnfrsf21 expression could potentially contribute to the termination of inflammatory responses. On the other hand, genes like 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 Gzma expression suggests a potential downregulation of cytotoxic T cell and NK cell activity in initiating apoptosis, which might alter how the immune system responds to infected or malignant cells, leading to a decreased ability to eliminate harmful cells effectively, possibly increasing susceptibility to infections or diseases in a state of sleep deprivation. Similarly, a reduction in Granzyme B may imply a compromised ability of the immune system to control and eliminate cells that pose a threat. Therefore, the reduction in granzyme levels may contribute to an overall diminished immune response, underscoring the importance of adequate sleep for maintaining optimal immune function and health.

A) B)

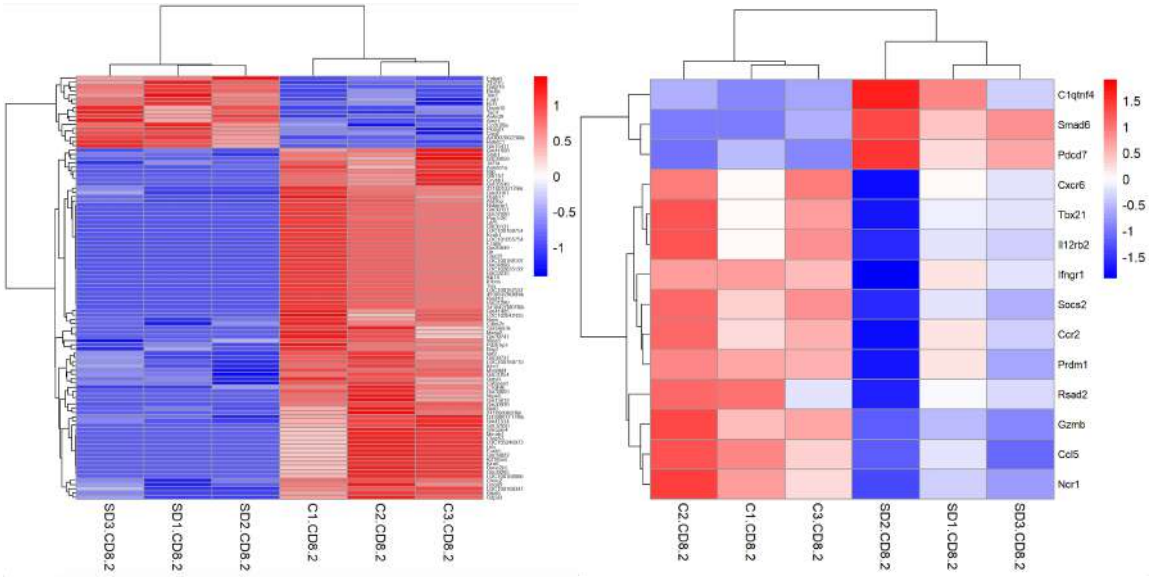
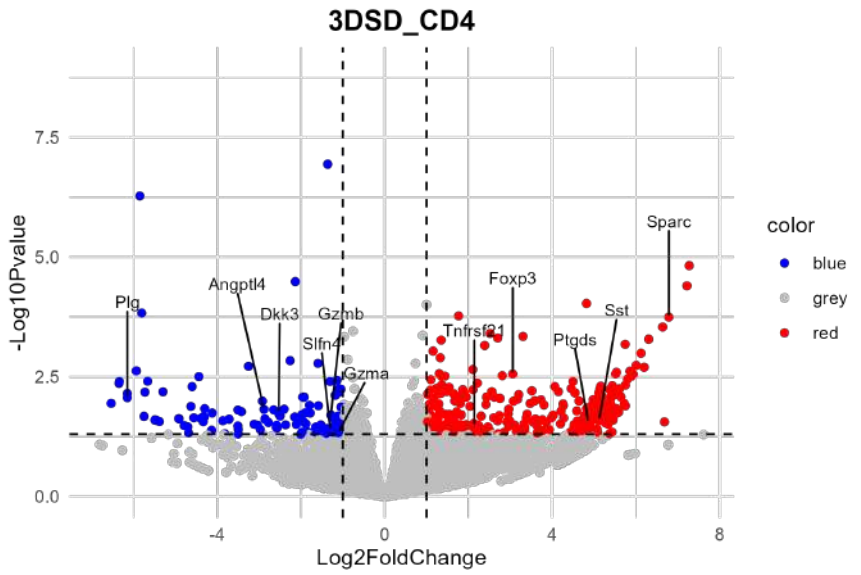


Figure 9: Heat maps of gene expression in CD8 T Cells from RNA sequencing data

For gene expression levels in CD8 T cells, Smad6 and Pdcd7 genes were upregulated in the sleep-deprived group (Figure 9AB). Smad6 serves as an inhibitory SMAD protein that moderates the signaling pathways of TGF-β (Transforming Growth Factor-beta) and BMP (Bone Morphogenetic Protein). These pathways are pivotal in regulating immune responses and maintaining cellular homeostasis. The upregulation of Smad6 could thus act as a feedback mechanism, tempering excessive immune responses and potentially reducing the risk of inflammation and autoimmune diseases. On the other hand, the rise in Pdcd7 expression, which is associated with programmed cell death, suggests a bolstered pro-apoptotic response. This response might be the immune system's strategy to clear out cells that are dysfunctional or potentially dangerous, thereby preserving the integrity of the immune system and averting abnormal cellular activities. The observed increases in Smad6 and Pdcd7 hint at distinct modulatory actions within the immune system’s signaling pathways, demonstrating their critical

roles in immune regulation. In contrast, we observed a downregulation in key genes like Gzmb, Ifng, and Tbx21. This reduction in Granzyme B (Gzmb), Interferon-gamma (Ifng), and T-bet (Tbx21) in $CD8^+$ T cells shed light on how the immune system's functionality alters after short-term sleep deprivation. Similarly, in CD4 T cells, the decrease in Granzyme B suggests that the body's defenses against infections and cancer might be compromised. Interferon-gamma, a crucial cytokine for boosting the immune response through activation of macrophages and enhancing antigen presentation, was also reduced. This decline could lead to a weakened immune response, making it less effective at coordinating attacks against pathogens and tumor cells. T-bet, vital for transforming $CD8^+$ T cells into effective effector cells, also showed decreased levels. This drop indicates a reduced capacity to produce these crucial effector cells, further weakening the immune response to threats. The overall downregulation of these critical genes 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. This adjustment in CD8 T cell functions, indicative of a broader shift under stress conditions like short-term sleep deprivation, might be recalibrating the balance between immune activation and regulation to prevent overreaction and preserve systemic health. Consequently, the analysis reveals that both CD8 and CD4 T cells show a suppression of inflammatory genes and a rise in anti-inflammatory markers, suggesting a widespread modulation of the immune response that compromises the system's efficiency in targeting and eliminating dangerous cells during periods of sleep loss.

A)



B)

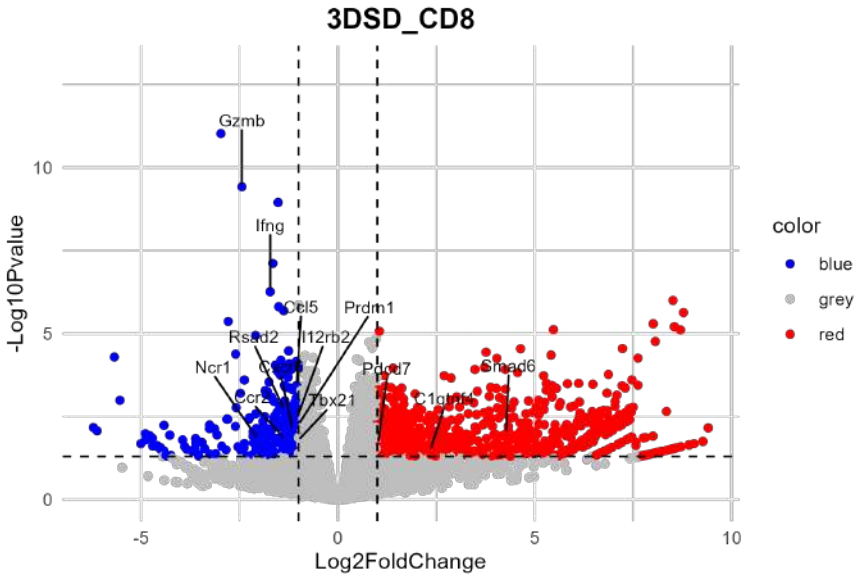


Figure 10: Volcano plots of differential gene expressions in CD4 and CD8 T cells after short-term sleep deprivation

In addition, the gene expression data after sleep deprivation for CD4 and CD8 T cells were expressed in the format of a volcano plot for comparisons to be easier to make (Figure 10). The volcano plot shows both the magnitude of gene expression changes and the statistical significance. The x-axis shows the log2 fold change and the y-axis indicates the negative logarithm of the p-value to better visualize the data. The data points were also color-coded with red meaning having a significant upregulation, blue for significant downregulation, and grey for when a gene had non-significant changes in expression levels. Key immune-related genes, such as cytokines and transcription factors, are identified within this plot. For CD4 T cells after sleep deprivation, the volcano plots show a balanced mix of upregulated and downregulated genes. Meanwhile, the volcano plot for CD8 T cells after sleep deprivation exhibits a more pronounced skew towards upregulation, suggesting that sleep deprivation impacts a broader spectrum of genes in CD4 T cells and triggers more activation and upregulatory processes in CD8 T cells, possibly reflecting the stress-responsive mechanisms that are specific to CD8 T cells.

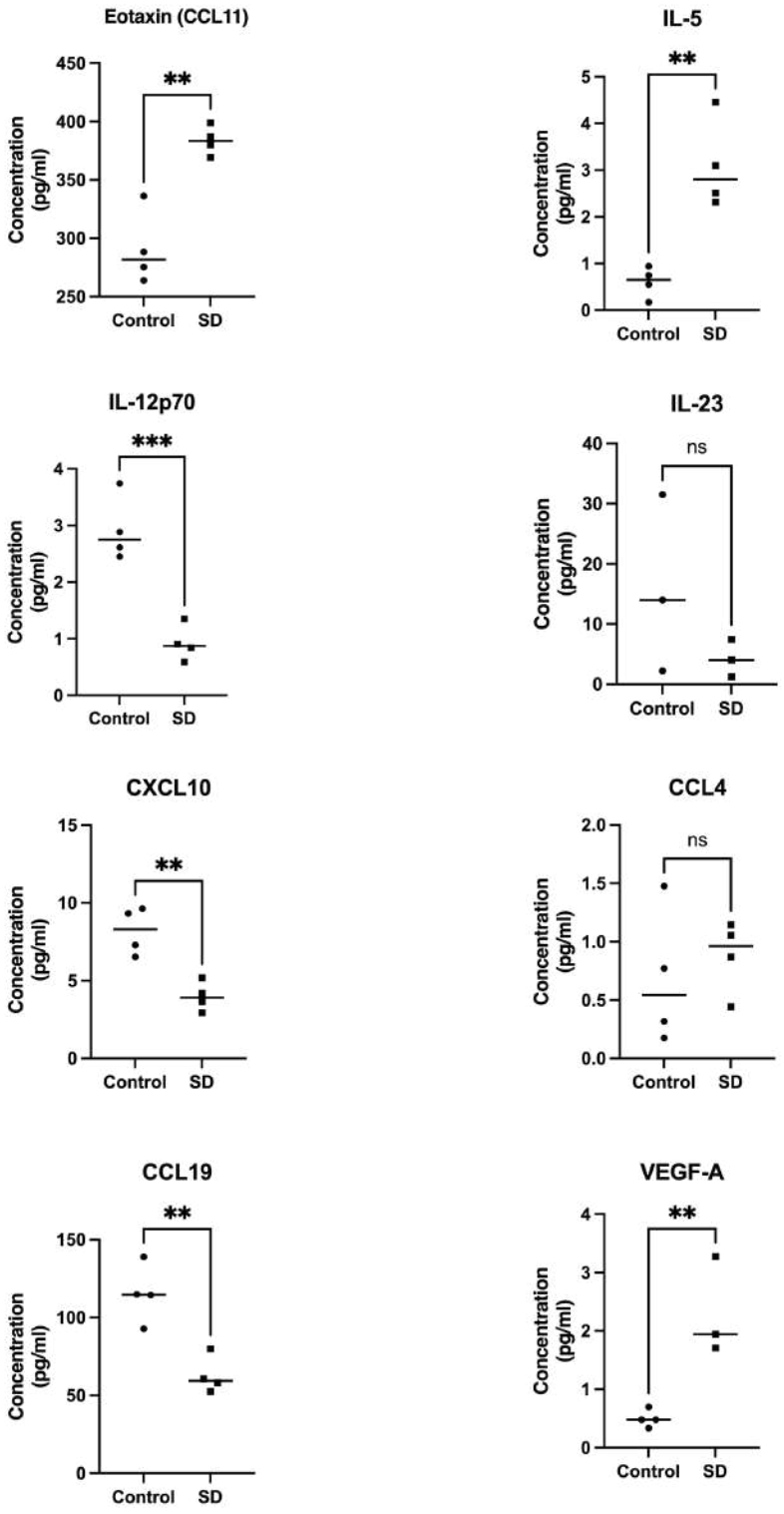


Figure 11: Comparison of cytokine and chemokine concentrations in control versus short-term sleep deprivation Groups by Luminex Assay

V. Luminex Assay Analysis

In order to detect serum protein and the concentrations of these proteins, Luminex Assay was used. The results from the Luminex Assay, analyzed through a median plot, indicate a notable decrease in serum protein concentrations of several inflammatory cytokines and chemokines such as Interleukin-12p70 (IL-12p70), Interleukin-23 (IL-23), CXCL10 (IP-10), CCL4 (MIP-1β), and CCL19 (MIP-3β) (Figure 11). IL-12p70 is a pro-inflammatory cytokine that is crucial for the activation of natural killer (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 for 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 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) aligns with our previous findings. Finally, the concentration of Vascular Endothelial Growth Factor A (VEGF-A), an anti-inflammatory cytokine, was found to be increased. VEGF-A is known for its anti-inflammatory properties and is typically involved in the healing process by promoting angiogenesis, repairing damaged tissues, and restoring normal tissue function after inflammation has subsided. The rise in its levels could indicate a regulatory response by the body to counteract or reduce ongoing inflammation. Ultimately, the general decrease in pro-inflammatory cytokine concentrations and the increase in the only anti-

inflammatory cytokine measured indicates that short-term sleep deprivation leads to immune suppression.

Discussion

The findings of this investigation demonstrate that acute sleep deprivation exerts a suppressive effect on the immune system, rather than inducing inflammation. Consistent with our findings of suppressed pro-inflammatory cytokines following short-term sleep deprivation, studies such as those by Irwin et al. (1996) have demonstrated reduced natural killer cell activity and alterations in cytokine production, suggesting a compromised innate immune response. Moreover, Mullington et al. (2009) have explained how acute sleep deprivation can alter cytokine profiles, possibly toning down pro-inflammatory responses while increasing anti-inflammatory cytokines. An increase in B cells and eosinophils proportions corresponds with findings by Spiegel et al. (1999), who observed elevated inflammatory markers under conditions of sleep deprivation. This indicates that despite the impairment of specific immune functions, the body actively attempts to restore and maintain immune homeostasis. Contrary to the conventional belief that sleep deprivation predominantly catalyzes inflammation (Besedovsky et al., 2019), this research's findings show that sleep deprivation appears to affect the immune system in more complex ways. The suppression of key cytotoxic genes, such as Gzmb and Ifng, implies that short-term sleep loss might overall induce a less responsive and active immune state, rather than simply causing inflammation. This challenges the association often made between sleep deprivation and increased inflammation and suggests instead that sleep loss might reduce immune reactivity, thereby enhancing susceptibility to infections without necessarily triggering pronounced inflammatory responses. These insights underscore the delicate equilibrium the

immune system strives to maintain even under sleep deprivation and emphasize the essential role of adequate sleep in sustaining immune efficacy and overall health resilience.

Conclusion

In conclusion, our findings indicate that acute sleep deprivation may undermine the immune system, as shown by the decreased numbers of NK cells and memory CD8 T cells—both essential for defending against pathogens and maintaining immune memory. Further analysis of gene expression also shows a reduction in key cytotoxic genes, underscoring the negative effects of sleep loss on cellular immune functions. These results highlight the critical importance of sufficient sleep for preserving immune health, especially given the increased risks to both individual and public health in situations that demand strong immune responses, such as pandemics or during vaccination campaigns.

Looking ahead, future research could extend this study in several directions. First, we could collect blood samples from individuals with varying sleep patterns, such as healthcare workers, firefighters, 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 would 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. Ultimately, this research not only confirms the vital role of sleep in regulating the immune system but also paves the way for further investigations into how optimizing sleep can enhance overall health and well-being.

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【評語】050005

本研究使用旋轉鼠籠進行 72 小時的短期睡眠剝奪，探討其對小鼠免疫系統的影響。結果顯示，NK 細胞和記憶型 CD8 T 細胞比例顯著下降，抗炎細胞因數(如 VEGF-A)濃度升高，促炎細胞因數(如 IL-1β）濃度下降。此外，B 細胞比例增加，可能作為免疫平衡的補償機制。

缺點與改進建議：

1. 未研究慢性影響：

   僅針對短期剝奪進行分析，需探討慢性睡眠剝奪對免疫系統的長期影響。

2. 樣本與組別單一：

   擴展樣本量與實驗組別（如不同剝奪時間段）將有助於提高數據普適性。

3. 壓力源影響：

   旋轉鼠籠的使用可能引入額外的壓力源，需驗證其是否影響免疫反應。

4. 機制描述不足：

   促炎和抗炎因數之間的互作機制不清晰，建議增加分子層面的機制探討。