Immune Cell Dysregulation in Hindlimb Unloading Mouse Model: Implications for Space Applications

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ABSTRACT

Background: Hindlimb unloading (HU) mice is a ground-based model that simulates the effects of microgravity. Since microgravity significantly affects the immune system, understanding immune cell function under these conditions is crucial for developing strategies to protect astronauts from infections and malignancies during long space missions.

Objective: To evaluate how microgravity affects neutrophils and T cells as the key components of innate and adaptive immunity, the activity of these cells in HU mice was compared with untreated control mice.

Material and Methods: In this experimental study, 10 HU male BALB/c mice and 10 untreated control mice were included. Neutrophil-to-lymphocyte ratio (NLR) was evaluated and neutrophil function was assessed using the DHR assay. T cell proliferation was evaluated using the CFSE-dilution assay. IL-4 and IFN-ɣ production by T cell subsets was determined by intracellular cytokine staining with flow cytometry.

Results: The capacity for reactive oxygen species (ROS) production in neutrophils did not differ between HU mice and control mice however, NLR was higher in HU mice. The proliferation of both $CD4^+$ and $CD8^+$ T cells was slightly reduced in HU mice. More notably, IL-4 production by $CD4^+$ T cells and IFN-ɣ production by both $CD4^+$ and $CD8^+$ T cells were significantly decreased in HU mice.

Conclusion: Hindlimb unloading, simulating microgravity, impairs immune cell functions by reducing cytokine production and T cell proliferation. The increased NLR in HU mice could indicate a heightened inflammatory response. These insights are essential for advancing space biology and medicine, ensuring astronaut health during prolonged space travel.

Keywords

Hindlimb Unloading; Immune System; Neutrophils; T-Lymphocytes; Cytokines; Microgravity; Space; Astronauts

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Introduction

Investigating immune system function during space missions presents significant limitations. Such experiments can either be conducted post-mission on Earth or through ground-based analogs simulating microgravity. Due to the potential effects of hyper-gravity during

launch and landing on the immune system, the results of post-flight experiments may not fully represent events occurring during spaceflights. Ground-based experiments that simulate microgravity in a controlled environment are acceptable models that, despite some limitations, have shown results relatively similar to those from spaceflights. The main advantage of these methods is the ability to control confounding factors. The rotating cell culture system (RCCS) is an *in vitro* method that induces microgravity by spinning cultured cells in two or three dimensions [1, 2]. Isolation of cells from their physiological conditions is considered the major limitation of this method [3]. The random positioning machine (RPM) is often used to simulate microgravity by rotating samples or organisms on multiple axes. The RPM operates with two perpendicular frames that rotate independently at different speeds. This random movement negates the effects of gravity, creating an environment that mimics the free-fall conditions experienced in space. Unlike the RCCS, the RPM can typically handle larger samples or whole organisms, although it also has its own limitations [3].

The hindlimb unloading (HU) mouse model, developed in the early 1980s, is one of the most widely used in vivo models for simulating microgravity [4]. In this model, the animal's hindlimbs are elevated, placing all body weight on the forelimbs. This position leads to a cephalic fluid shift and musculoskeletal unloading, similar to what astronauts experience during spaceflights. In addition to its effects on the musculoskeletal system, microgravity also impacts the immune system [2, 5].

The immune system protects the body against infectious agents and tumors through the cellular and humoral components of innate and specific immunity. Innate immunity plays a crucial role in the initial defense against infectious agents. By producing inflammatory substances, it recruits acute inflammatory cells, neutrophils, to the site of infection or tissue injury. These cells, with the help of tissue resident macrophages, clean up microbes and scavenge dead cells. When innate immunity is unable to destroy the infectious agents, cooperates with lymphocytes- key members of the specific immune system- to eradicate these agents.

In this process, foreign antigens are recognized by specific immune cells, leading to the production of antibodies and the activation of T cells. $CD4^+$ helper T (Th) cells coordinate the activity of other immune cells through cytokine production. $CD8^+$ cytotoxic T (Tc) cells migrate to the site of injury to destroy infectious agents and malignant cells. Following the control of infection and the restoration of hemostasis, conditions would be stablished for tissue repair [6].

There are many reports regarding the immune system dysregulation in HU mice. Disability of the immune system to eradicate bacterial infections [7, 8], disorder in the development of B lymphocytes [9], delayed production of protective specific IgG against bacteria [8], reduction in spleen size due to increased catecholamine levels [10], reduced ability of gastrointestinal tract to bacterial products and systemic innate immune activation [11], increased IL-1β in spleen as well as IL-6 and TNF-α in lymph nodes [12] and inhibition of antitumor immunity [13].

Although many studies have examined immune system function in HU mice, a more detailed investigation of immune cell function can lead to a better understanding of astronauts' immune systems. This, in turn, can help develop preventive or alternative strategies for managing infections and malignancies during long spaceflights. In this regard, the current study was designed to analyze neutrophil activity and T cell function in HU mice compared to untreated controls.

Material and Methods

Study design

In this experimental study, male BALB/c

mice aged 8-10 weeks and weighing about 20 g (obtained from Royan Institute, Tehran, Iran) were included. The protocol for this study was designed based on the "Guide for the Care and Use of Laboratory Animals" published by the National Academy Press (https://grants.nih.gov/grants/olaw/ guide-for-the-care-and-use-of-laboratory-animals.pdf) and approved by our University Ethic Committee. The mice were housed individually at 23±1 ºC with 50±5% humidity and a 12-hour light/12-hour dark cycle. They were given a normal regimen of food and water for rodents. After one-week adaptation to the new conditions, half of the mice (n=10) were randomly assigned to establish the HU-mouse model and the rest (n=10) were served as control group.

Hindlimb unloading mouse model induction

The HU-mouse model was established using a previously published method [14, 15]. Briefly, one week after introducing a stainless steel ring between the L5 and L6 vertebrae of the mice, the tail ring was linked to a pulley inside a rail in the cage roof using an S-shaped connector. Each mouse was unloaded by the tail at a 20-30º angle of hind limbs from the horizon, while having free access to water and food. Mice were kept in HU conditions for 21 days, then narcotized with an intraperitoneal injection of 20 µL ketamine (100 mg/mL) and 10 µL xylazine (20 mg/mL) (Alfasan, Woerden, Netherlands). Blood samples were collected from the mice hearts, mixed with heparin, and freshly used for the desired experiments. Samples with insufficient amounts were excluded from the study, and only some tests were performed on samples with low volume.

Neutrophil Activation Test

Dihydrorhodamine (DHR) flow cytometric analysis was used to evaluate neutrophil activation by comparing the amount of reactive oxygen species (ROS) production in phorbol myristate acetate (PMA)-stimulated neutrophils against unstimulated counterparts. For analyzing the results of flow cytometry tests, the granulocyte population was first gated based on forward versus side scatter, and then the mean fluorescent intensity (MFI) of DHR was determined in the gated granulocytes (Figure 1A). The stimulation index (SI) was obtained by dividing the fluorescent

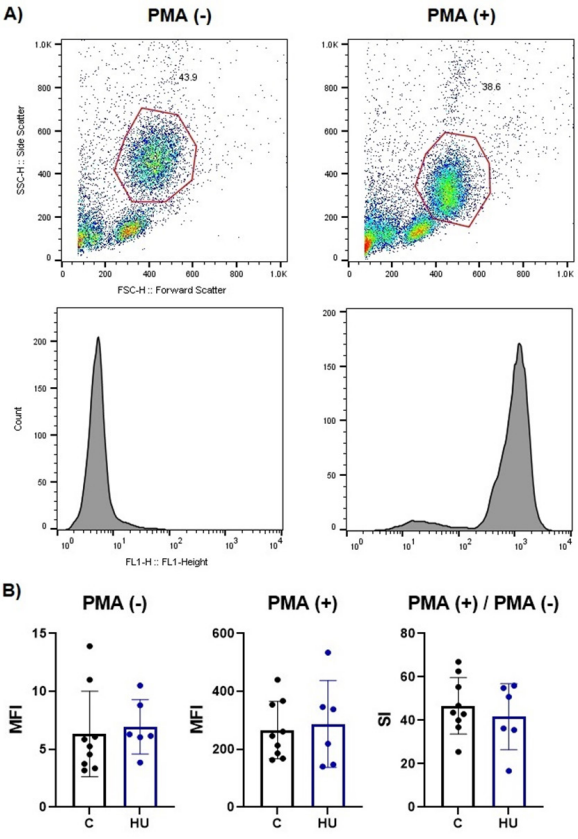

Figure 1: Neutrophil activation test by DHR assay. Neutrophil population was gated based on FSC versus SSC before and after PMA stimulation (**A top**). MFI of DHR is depicted before and after stimulation (**A bottom**). Neutrophil activity in HU mice group compared to the control group (n=10 in each group). Samples with insufficient volume were excluded from the study (**B**).
FSC: Forward Scatter, SSC: Side Scatter, DHR: Dihydrorhodamine, PMA: Phorbol Myristate Acetate, MFI: Mean Fluorescent Intensity, SI: Stimulation Index

intensity of DHR in PMA-stimulated neutrophils by that in unstimulated neutrophils. The neutrophil to lymphocyte ratio (NLR) was also determined in freshly isolated blood samples based on forward versus side scatter by flow cytometry (Figure 2 Left).

T Cell Proliferation and Functional Assay

Carboxyfluorescein succinimidyl ester (CFSE) dilution assay was applied to assess the proliferation index (PI) of two main T cell subsets. Intracellular cytokine staining with flow cytometry was also used to determine T cell function. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Inno-Train Diagnostik GmbH, Kronberg, Germany). After staining with CFSE (Biolegend, San Diego, CA, USA), PBMCs were stimulated with concanavalin A (Con-A, Sigma-Aldrich) for 96 h; unstimulated cells were cultured in parallel as controls. The harvested cells were then stained with PerCP-Cy5.5-conjugated anti-CD4 or anti-CD8 antibodies (Biolegend). A cocktail of PMA/ionomycin/brefeldin-A (Sigma-Aldrich) was added to equivalent cultures and incubated for an additional 4 h. The cells were permeabilized using Perm/Wash buffer (Biolegend), and intracellular staining was done using PE-conjugated anti-IL-4 and APC-conjugated anti-IFN-γ (Biolegend). All tubes were finally evaluated using a 4-color flow cytometer, and the data were analyzed using FlowJo software version 10.

For analyzing the results of flow cytometry assays, lymphocytes were first gated based on forward versus side scatter. T cell subsets were determined based on the expression of CD4 or CD8 markers. The median fluorescent intensity of CFSE was measured in the gated $CD4^+$ or $CD8^+$ T cells. The rate of T cell proliferation in each subset was then compared between the stimulated and their corresponding unstimulated controls. The median fluorescent intensity of IFN-γ in $CD4^+$ or $CD8^+$ T cells, as well as the median fluorescent intensity of IL-4 in $CD4^+$ lymphocytes, was determined (Figure 3).

Statistical Analysis

To compare the SI of neutrophils, PI in $CD4^+$ and $CD8^+$ T cells, and the amount of cytokines produced by each T cell subset, pair-wise analyses were conducted between the HU mice group and the untreated control group using

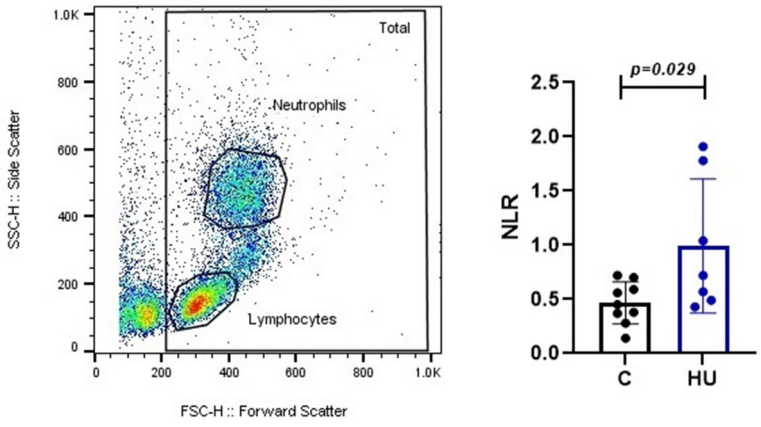

Figure 2: NLR was calculated after gating of neutrophils and lymphocytes based on FSC versus SSC (**left**). Comparison of NLR between HU mice and controls (**right**).
(NLR: Neutrophil To Lymphocyte Ratio, HU: Hindlimb Unloading, SSC: Side Scatter, FSC: Forward Scatter)

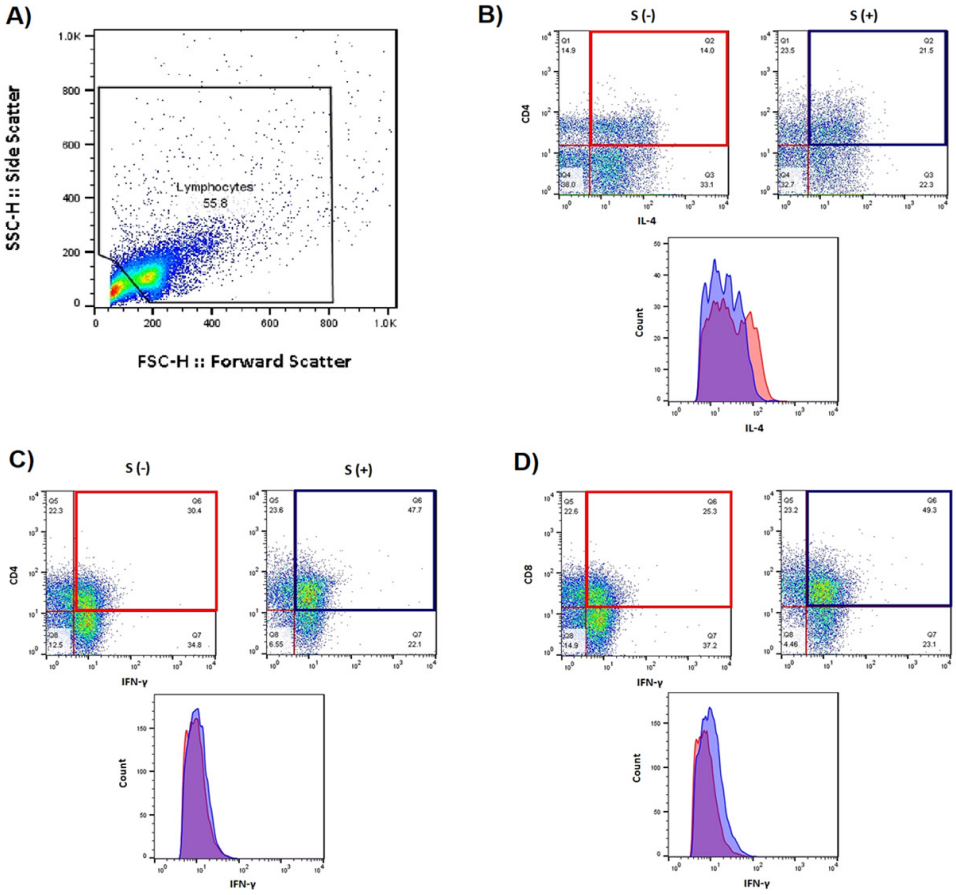

Figure 3: Gating strategy for analysis of cytokine production by different T cell subsets. Lymphocyte population was gated based on FSC versus SSC (**A**). Red and blue quadrants show cytokine producing lymphocytes before stimulation S(-) and after Con-A stimulation S(+), respectively (**B**/**C**/**D**). (SSC: Side Scatter, FSC: Forward Scatter)

t-tests. A *P*-value less than 0.05 was considered significant. Statistical analyses and graphical presentations were performed using GraphPad Prism 9.

Results

In this study, the effects of microgravity on neutrophils and T cells were evaluated. NLR was assessed by flow cytometry, DHR assay was used to measure the capacity of ROS production by neutrophils, CFSE dilution assay was used to determine the capacity of T cell proliferation, and intracellular cytokine staining with flow cytometry was used to investigate cytokine production by different T cell subsets.

The results showed that HU mice lost 1-4 g of weight after 21 days, while control mice gained 1-2 g during this period. The results of DHR assay indicated a non-significant increase in ROS production by PMA-stimulated neutrophils in both HU and control mice compared to their corresponding unstimulated neutrophils. However, SI showed no change in either group (Figure 1B). There was no difference in ROS production capacity by neutrophils between the two groups, but an elevated NLR was observed in HU mice compared to controls (P=0.029) (Figure 2 Right).

The results of CFSE dilution assay showed decreased T cell proliferation capability in both $CD4^+$ and $CD8^+$ T cells in HU mice, although these differences were not significant

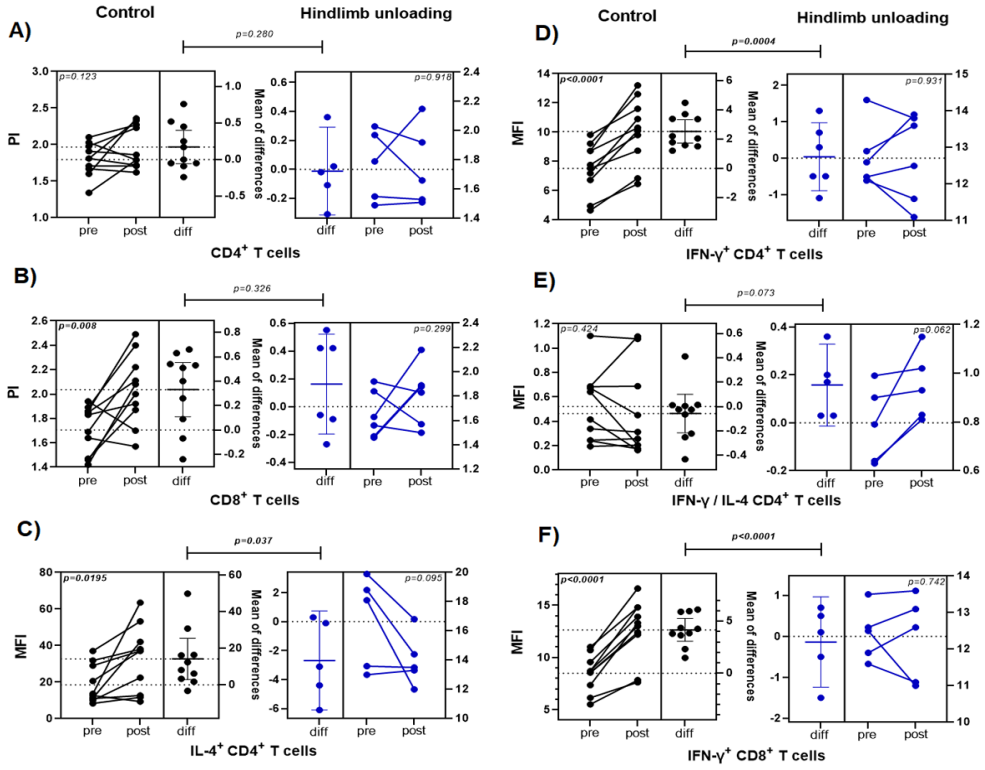

Figure 4: Proliferation capacity of $CD4^+$ and $CD8^+$ T cells (**A & B**). MFI of IL-4 (**C**), IFN-γ (**D**) and IFN-γ/IL-4 ratio (**E**) produced by $CD4^+$ T cells from HU mice compared to the controls. MFI of IFN-γ produced by $CD8^+$ T cells from HU mice compared to the control group (**F**). n=10 in each group and samples with insufficient volume were excluded from the study.
(HU: Hindlimb Unloading, PI: Proliferation Index, MFI: Median Fluorescent Intensity, Pre: Before Con-A Stimulation, Post: After Con-A Stimulation)

compared to untreated controls (Figure 4A/B). No uniform trend in T cell proliferation was observed in HU mice after Con-A stimulation, whereas T cell proliferation in the control group mainly increased. The PI showed no significant difference between HU and normal mice.

The production of both IL-4 and IFN-γ by $CD4^+$ T cells was significantly reduced in HU mice compared to controls (P=0.037 and P=0.0004, respectively), although the IFN-γ/IL-4 ratio showed no remarkable difference with controls (Figure 4C/D/E). IFN-γ production by $CD8^+$ T cells also showed a significant reduction in HU mice (P<0.0001) (Figure 4F).

Discussion

To evaluate the effects of microgravity on neutrophils and T cells as the most effective cells of innate and adaptive immunity, the activity of these cells was compared between HU mice and untreated control mice.

Our results showed despite free access to food and water, HU mice had weight loss. In addition to overall stress, nutritional change and muscle disuse, microgravity-induced muscle atrophy is a major complication of spaceflights [16]. Weight loss resulting from muscle atrophy was previously reported in HU mice [17, 18].

Acute increase of neutrophil count in HU mice and astronauts' blood after a 140-day spaceflight was previously reported [19, 20]. Myotubular injury and decreased myotubular tension lead to the release of neutrophil chemotactic factors. Then neutrophil infiltra-

tion promotes muscle atrophy by releasing lysosomal enzymes and reactive nitrogen and oxygen intermediates [21].

Furthermore, the role of neutrophils in progressive muscle destruction following age-related sarcopenia [22], ischemia-reperfusion muscle injury [23], sepsis-induced muscle atrophy [24], and motor-neuron muscular dystrophy [25] has earlier been documented.

Although microgravity-induced muscle atrophy in astronauts was previously explained as a result of increased neutrophil activity during space travel [21], Domont et al. ruled out the role of neutrophils in muscle atrophy by depleting neutrophils in HU mice [26] which remains to be more proven by further experiments.

While we observed no difference in ROS production capacity by neutrophils between two groups, elevated NLR was seen in HU mice. Increased NLR was previously reported in both HU mice and astronauts [27].

Our data revealed a slight decrease in the proliferation of $CD4^+$ and $CD8^+$ T cells in HU mice. These results are in the line of the findings reported by Gaignier et al. who observed decreased lymphocyte proliferation following mitogen challenge in HU mice [28], and Nash et al. who also showed reduced ^{3}H-thymidine incorporation in mitogen-stimulated PBMCs of in rats suspended for one week [29]. Inhibitory effect of unloading on proliferation of other cells such as osteogenic precursors, preosteoblasts and bone marrow cells has also been reported [30, 31]. As it seems, unloading condition down-regulates cell cycle related genes while up-regulates cell cycle arrest genes that leads to significant inhibition of lymphocyte proliferation [32], although further studies are needed on the cell cycle-related proteins in immune cells under suspending condition.

The results of current study showed that, the trend of IL-4 production by $CD4^+$ T cells in HU mice was non-significantly decreasing after Con-A stimulation, while IFN-γ production in both $CD4^+$ and $CD8^+$ T cells showed no remarkable change.

Contrary to our results, a transient increase in IFN-γ production was reported after Con-A challenge of lymphocytes in suspended rats by Berry et al. [33]. Gaignier et al. showed no difference in IL-4 and IFN-γ production in culture supernatant of HU mice splenic lymphocytes 72 h after Con-A stimulation compared to the controls [28]. Aghajari et al. also reported no change in the serum concentration of IL-4 and IFN-γ between HU mice and controls [14].

Regarding to the results of this study, it seems that, unloading may interfere with the immune cell function by affecting T cell proliferation and cytokine production. Several mechanisms have been suggested to be involved in decreasing protein synthesis in HU rodents. Altered hormonal balances in HU animals such as dysregulations in growth hormone [34, 35], insulin-like growth factor 1 [36, 37] and testosterone levels [38, 39] can affect protein synthesis process. Increased oxidative stress resulting from suspending may contribute to protein synthesis impairment [40], and increased catabolic pathways are also reported under suspending conditions [32]. These mechanisms have been widely studied in skeletal muscles but further studies are needed to generalize these mechanisms to immune cells.

Due to financial limitations, we did not able to investigate the humoral factors of the innate and specific immune system e.g., checking the serum levels of complement components and antibodies.

Examining the humoral and cellular components of the immune system following the challenge of mice with a certain antigen or infectious agent in future studies can clear many obscure points about the immune system status of HU mice.

Conclusion

According to the results of this study, HU conditions may interfere with immune cell

functions by affecting cytokine production and the proliferation of T cells. Additionally, the elevated NLR may indicate an increased potential for inflammation in HU mice. These findings are crucial for space biology and medicine, suggesting that long-term exposure to microgravity could impair astronauts' immune responses, making them more susceptible to infections and inflammatory conditions.

To mitigate these risks, future research should focus on the use of compensatory techniques, such as specific exercise regimens, pharmacological interventions, or dietary supplements, to support immune function during space missions. Investigating the underlying mechanisms of immune dysregulation in microgravity can also provide insights into novel therapeutic targets. Long-term studies involving actual spaceflight conditions and more comprehensive analyses of other immune cell types will be essential to validate these findings and enhance the overall health and safety of astronauts during extended space travel.

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Authors' Contribution

S. Masoudi deigned the animal cages, established the HU-mice model and carried out the experiments. M. Kalani and A. Alavianmehr planned and helped carrying out the experiments and data extraction. A. Alavianmehr did statistical analyses and helped in figure design and writing the manuscript draft. A. Mosleh Shirazi involved in experimental design. S. Farjadian and SMJ. Mortazavi supervised the project, designed the experiments, rechecked all analyses and figures, corrected the manuscript draft, and reviewed the final version. All authors read and approved the final manuscript.

Ethical Approval

The protocol of this study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1398.649).

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Conflict of Interest

SMJ. Mortazavi as one of the Editorial Board Members was not involved in the peer-review and decision-making processes for this manuscript.

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