

Microgravity-Induced Immune Plasticity and Temporal Modulation of Gene Networks and T Cell Subsets During the MESSAGE Science Mission in Axiom-3 on the ISS

Cihan Tastan

cihantastan.ct@gmail.com

Üsküdar University

Gamze Gulden

Üsküdar University

Ozge Demir

Üsküdar University

Ebru Cam

Üsküdar University

Batuhan Yolver

Üsküdar University

Kursat Kolay

Üsküdar University

Fatmanur Erkek

Üsküdar University

Berranur Sert

Üsküdar University

Tayfun Gozler

Üsküdar University

Ipek Yuksel

Üsküdar University

Busra Tekirdagli

Üsküdar University

Beyza Aydin

Üsküdar University

Keywords: Microgravity, Spaceflight physiology, Immune regulation, T lymphocytes, RNA-Seq, Axiom-3, MESSAGE Science Mission

Posted Date: December 16th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7535063/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Microgravity presents significant challenges to human physiology, particularly the immune system, during spaceflight. T lymphocytes, central regulators of adaptive immunity, are known to be sensitive to gravitational changes; however, the molecular mechanisms underlying immune modulation in space remain incompletely understood. This study, conducted as part of the MESSAGE (Microgravity Associated Genetics) Science Mission during the Axiom-3 mission, investigated how short-term microgravity influences immune regulation (<https://www.nasa.gov/mission/station/research-explorer/investigation/?#id=9100>). Blood samples were collected from three astronauts at Day 4, Day 7, and Day 10 of the mission, as well as from one suborbital astronaut and three healthy controls. Hemogram profiling, flow cytometry-based T cell subpopulation analysis, and RNA sequencing (RNA-Seq) were performed to characterize immune dynamics at cellular and molecular levels. Hemogram analysis showed no statistically significant changes in leukocytes, erythrocytes, haemoglobin, or hematocrit levels, suggesting stable hematological profiles during short-duration spaceflight. Flow cytometry revealed non-significant shifts in effector memory T cell subsets (TemEARLY and TemLATE), with a trend toward increased late effector memory differentiation by Day 10. Transcriptomic profiling identified time-dependent modulation of immune-related genes, including downregulation of CD69, IL2RA, LAT, and IFNG, indicating early suppression of T cell activation pathways. Heatmap analysis revealed coordinated transcriptional changes across 48 immune-associated genes, demonstrating that molecular adaptations occur before detectable alterations in immune cell counts or hematological profiles. These findings provide evidence of early, coordinated transcriptional reprogramming of immune-regulatory pathways under microgravity, highlighting the potential vulnerability of immune function during spaceflight. While cellular and haematological changes remained limited, transcriptomic signatures indicate that molecular responses precede phenotypic shifts. Understanding these mechanisms is critical for developing targeted countermeasures to protect astronaut immune health during long-duration space missions.

1. Introduction

Gravity is a fundamental physical force that has shaped the evolution, structure, and physiology of living organisms on Earth. It plays a pivotal role in cellular behaviour, tissue organization, and systemic biological regulation. Understanding how the absence of gravity—defined as microgravity—affects human biology is crucial for the success of long-duration space missions. Microgravity has been shown to impact various physiological systems, including the cardiovascular, musculoskeletal, and immune systems [1]. Among these, the immune system is of particular concern due to its critical role in host defence and its vulnerability to dysregulation in space.

The immune system is composed of a complex and dynamic network of cells and signalling molecules that coordinate the body's response to pathogens and environmental stressors. T lymphocytes, a central component of adaptive immunity, are responsible for cytotoxic activity, cytokine secretion, and the regulation of immune responses [2]. Numerous studies have demonstrated that spaceflight can alter

immune cell distribution, impair T cell activation and proliferation, and modulate cytokine production, collectively leading to a weakened immune response [3,4]. These alterations are thought to be driven by spaceflight-specific stressors such as microgravity, cosmic radiation, circadian rhythm disruption, and psychological strain [5]. Experimental data from the International Space Station (ISS) have shown that microgravity can disrupt leukocyte trafficking, cytoskeletal architecture, and immune signalling pathways. These disruptions are often associated with reduced interleukin-2 (IL-2) levels and increased tumour necrosis factor-alpha (TNF- α) expression [6]. Furthermore, increased reactivation of latent viruses in astronauts has been reported, suggesting compromised immune surveillance during space missions [7]. Despite the growing body of evidence indicating immune dysregulation in space, the underlying molecular mechanisms remain incompletely understood. There is increasing interest in identifying gravity-sensitive genes and examining how their expression may contribute to immune impairment. Advances in transcriptomic technologies, including RNA sequencing and systems-level analyses, offer new opportunities to explore these questions [8]. In addition to cellular immune profiling, transcriptome-wide gene expression analyses have emerged as a powerful tool to uncover early molecular responses to microgravity. Investigating the expression dynamics of immune-related genes, such as *IL2RA*, *CD69*, and *IFNG*, can help elucidate the transcriptional landscape of immune regulation under altered gravity conditions. Recent studies have shown that long-term exposure to microgravity aboard the International Space Station modulates leukocyte gene expression profiles, suggesting widespread immune adaptation at the transcriptomic level [10]. In this context, integration of transcriptomic data with flow cytometry and hematological analyses enables a multi-dimensional approach to understanding how the immune system responds to spaceflight at both the cellular and molecular levels.

In this study, we investigated the effects of short-term microgravity exposure on immune system parameters in astronauts participating in the Axiom-3 mission, conducted as part of the MESSAGE (Microgravity Associated Genetics) Science Mission. To comprehensively evaluate immune dynamics under spaceflight conditions, blood samples were collected at multiple time points during the mission (Day 4, Day 7, and Day 10 post-launch) and analyzed using a multi-layered approach. Hemogram analyses were performed to assess hematological stability, while flow cytometry was used to characterize T cell subpopulations, including central memory, effector memory, and stem-cell-like memory subsets. In parallel, transcriptomic profiling of peripheral blood mononuclear cells (PBMCs) was conducted using RNA sequencing to identify microgravity-associated gene expression changes in immune-regulatory pathways. This integrative strategy enables the evaluation of immune adaptation at both cellular and molecular levels, providing insights into how short-term spaceflight influences hematological profiles, T cell memory dynamics, and transcriptional immune regulation. Understanding these mechanisms is essential for developing targeted countermeasures to mitigate immune dysregulation in astronauts and ensuring crew health and performance during future long-duration missions.

2. Materials and methods

Ethics Approval and Consent to Participate

Microgravity Associated Genetics (MESSAGE) Science Mission proposal (Study eIRB Number: STUDY00000620) has been reviewed and approved (Date of Approval: 21Sep2023) by the NASA IRB in accordance with ethical standards and the requirements of the Code of Federal Regulations on the Protection of Human Subjects (NASA 14CFR1230 and if applicable, FDA 21CFR50 and 56). MESSAGE Science Mission proposal (No: 61351342/020-275) has also been approved (Date of Approval: 03 July 2023) by Üsküdar University Ethical Commission.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from healthy adult blood samples obtained at the Üsküdar University Transgenic Cell Technologies and Epigenetic Application and Research Center (TRGENMER). Blood samples of 3cc were obtained from three healthy human donors and diluted in a 1:1 ratio with PBS (without Mg/Ca). The blood sample diluted in a 1:2 ratio with Ficoll (Paque PREMIUM 1.073, 17-5446-52) was combined. PBMCs were isolated using density gradient centrifugation. The sample combined with Ficoll was centrifuged at 800 g for 25 minutes with an acceleration of 9 and brake at 0. The Buffy coat formed after centrifugation was collected into a new tube. PBS (without Mg/Ca) was added in a 1:3 ratio (Buffy coat) and centrifuged at 400 g for 10 minutes. This process was repeated until the supernatant became clear. After centrifugation, the pellet was dissolved in 5 ml of PBS (without Mg/Ca) and centrifuged at 400 g for 5 minutes. The pellet was dissolved in RPMI Medium (CAPRICORN SCI, RPMI-A) (10% FBS + 1% penicillin/streptomycin). The 3cc blood sample was analysed for hemogram in NPISTANBUL Brain Hospital, Türkiye.

Analysis of T Cell Sub-Populations (TN-TCM-TEM-TEF) and Immunodeficiency Profile

T cell sub-populations and Tcm-Tscm profiling studies on days 4, 7, and 10 were analysed using CD3-PC7 (Beckman Coulter, 6607100), CD4-APC-A700 (Beckman Coulter, B10824), CD8-PC5.5 (Beckman Coulter, B21205), CD45RA-ECD (Beckman Coulter, IM2711U), CD45RO-PE (Miltenyi Biotech, 130-113-559), CD27-APC-A750 (Beckman Coulter, B12701) and CD62L-APC (Miltenyi Biotech, 130-113-617) antibodies. Exhaustion profiling studies on days 14 and 21 were analysed using CD3-PC7 (Beckman Coulter, 6607100), CD4-APC-A700 (Beckman Coulter, B10824), CD8-PC5.5 (Beckman Coulter, B21205), CD279 (PD1)-PE (Miltenyi Biotech, 130-117-384), CD366 (TIM3) - APC (Invitrogen, 17-3109-42) and CD223 (LAG-3) - APC-eFluor 780 (Invitrogen, 47-2239-42) antibodies. 100µl samples were taken by pipetting and added to tubes. 1 ml of Staining Buffer was added to the samples (Staining Buffer, 1:20- BSA: Rinsing Solution). After adding buffer, it was centrifuged at 400g for 5 minutes. After centrifugation, the supernatant was discarded, and the final volume was adjusted to 20 µl. Antibodies for the Tcm-Tscm and Exhaustion panels were added according to the manufacturer's instructions and vortexed in the dark. After vortexing, tubes were incubated at +4 °C for 30 minutes. After incubation, 1 ml of Staining Buffer was added to the samples in the dark. It was centrifuged at 400g for 5 minutes. By discarding the supernatant, the pellet was dissolved in 100µl Staining Buffer and analysed by Flow Cytometry. Survival analysis was performed to control the cell viability of PBMC. Trypan blue (Biologica Industries, #03-102-

1B) was applied to identify and count surviving cells. Cell counting and viability analysis were performed with the BIO-RAD TC20 Automated Cell Counter.

Transcriptome Analysis

Transcriptome profiling was conducted in collaboration with SZA OMICS (Genomic Productions, Istanbul), using peripheral blood samples collected from astronauts aboard the International Space Station (ISS), one astronaut who completed a suborbital flight (~100 km), and three healthy volunteers matched for age, height, and weight. Following sample collection, RNA isolation was performed either manually or through automated systems, depending on sample type and content. For automated RNA extraction, the QIAGEN PAXgene QIASYMPHONY Blood RNA Kit (Qiagen, Germany) was utilized, while manual extraction followed the Qiagen RNeasy protocol. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation at Üsküdar University TRGENMER, and the resulting cell pellets were shipped to SZA OMICS for downstream processing, including RNA isolation, library preparation, sequencing, and bioinformatic analysis. The quality and quantity of isolated RNA were assessed spectrophotometrically using a NanoDrop device and confirmed using a Qubit Fluorometer (Thermo Fisher Scientific) and a Victor Nivo (Perkin Elmer) system. Quality control thresholds were defined as an A260/280 ratio close to 2.0 and an A260/230 ratio between 2.0 and 2.2. RNA integrity was further evaluated based on RNA Integrity Number (RIN) values. Although the RNA samples derived from astronaut donors yielded relatively low RIN values (3.9 and 4.2), transcriptome analysis was successfully performed by supplementing with RNA obtained from laboratory-expanded cell cultures of the same donors. Before sequencing, blood samples from healthy individuals were also analyzed to identify stable reference genes, which were subsequently used as normalization factors in comparative gene expression analysis. Library preparation was conducted using the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Kit, automated on a Hamilton NGS Star platform. Library quality was assessed using a Qubit device, and, when necessary, further validated with an Agilent Bioanalyzer or TapeStation. Sequencing was performed on the Illumina platform using a 2×100 bp paired-end protocol, targeting approximately 25 million reads per sample. Raw sequencing data were demultiplexed using Illumina DRAGEN v3.9.5 and delivered in FASTQ format. To support data processing, a dedicated high-performance computing environment was established using a Lenovo ThinkPad P15V workstation configured with an Intel i7-12700H processor, 64 GB RAM, SSD storage, and dual operating systems: Windows 11 Pro and Ubuntu via Windows Subsystem for Linux (WSL). Quality control of raw sequencing reads was carried out using FastQC software, which evaluated key metrics including sequence quality, GC content, base composition balance, duplication levels, and adapter contamination. While general quality parameters were acceptable across all samples, minor declines in sequence quality and elevated GC content were observed in astronaut-derived reads, potentially reflecting transcriptomic instability related to microgravity exposure. Adapter sequences and low-quality bases were trimmed using Trimmomatic. Cleaned reads were mapped to the human reference genome (GRCh38) using the STAR aligner with optimized parameters. Expression levels were calculated in TPM (Transcripts Per Million), and normalization was performed using the housekeeping genes GAPDH, ACTB, and RPL13A. Transcriptome data were generated both from blood samples collected during the mission and from

PBMC-derived cell cultures maintained under laboratory conditions, enabling a more comprehensive analysis of active transcription under spaceflight exposure. To investigate the potential biological roles of expressed transcripts, representative gene sequences were aligned against public databases using the NCBI BLAST (Basic Local Alignment Search Tool) algorithm. These analyses provided insights into the molecular functions and cellular pathways associated with differentially expressed genes identified in the astronaut and control samples.

Statistics Analysis

All statistical analyses were performed using GraphPad Prism v9.5 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics v27 (IBM Corp., Armonk, NY, USA). Data were first evaluated for normality using the Shapiro–Wilk test. For parametric datasets (e.g., hemogram parameters), differences between time points (Day 4, Day 7, and Day 10) were analysed using a repeated-measures one-way ANOVA. When ANOVA indicated significance, Tukey’s post hoc test was applied to determine pairwise differences. For non-parametric datasets or when normality assumptions were not met, the Kruskal–Wallis test was used, followed by Dunn’s multiple comparisons test. T cell subpopulation distributions (TemEARLY, TemLATE, Tcm, Temra, and Tn+Tscm) were analyzed using repeated measures testing across the three spaceflight time points. Where applicable, comparisons between astronaut samples and healthy donor controls were performed using the Mann–Whitney U test for non-parametric data or unpaired two-tailed t-tests for parametric data. For RNA-Seq transcriptomic analysis, raw counts were normalized using Transcripts Per Million (TPM), and differential gene expression was quantified using DESeq2 (v1.40.2) in RStudio (v4.3.1). Genes with a false discovery rate (FDR)-adjusted p-value < 0.05 (Benjamini–Hochberg correction) were considered statistically significant. For genes that did not meet statistical significance but exhibited biologically meaningful modulation, trends were visualized in Figures 6 and 7 to capture their temporal expression profiles. Heatmap clustering of immune-related genes was performed using the pheatmap R package, employing Euclidean distance and complete linkage clustering to represent similarity patterns across time points. All results are presented as mean ± standard deviation (SD) unless otherwise stated. A significance threshold of $p < 0.05$ was applied across all analyses. Where appropriate, effect sizes and confidence intervals (95% CI) were calculated to complement hypothesis testing. No data points were excluded unless identified as technical artifacts following quality control procedures.

3. Results

Conceptual Overview of the MESSAGE Science Mission and Experimental Design

The Microgravity Associated Genetics Research (MESSAGE) Science Mission represents one of Türkiye’s pioneering human space biology initiatives, aiming to explore the effects of microgravity on immune regulation and gene expression in astronauts. The experimental design integrates pre-flight, in-flight, and suborbital investigations to characterize dynamic molecular and cellular responses to altered

gravitational environments. As shown in Figure 1, venous blood samples were collected from astronauts seven days before launch at Earth baseline conditions (Pre-launch, -7 Day) and stored at -80 °C to establish reference control profiles. To assess spaceflight-induced alterations, additional blood samples were obtained onboard the International Space Station (ISS) during the Axiom-3 mission at three distinct time points: Day 4, Day 7, and Day 10 post-launch. These samples were immediately cryopreserved in the MELFI (Minus Eighty-Degree Laboratory Freezer for ISS) at -80 °C to maintain sample integrity and capture in-flight molecular dynamics. To complement the long-duration microgravity data, short-term gravitational effects were also examined using the Galaxy-07 suborbital mission, which reached an altitude of ~100 km at the edge of space. Blood samples were collected immediately before and after suborbital exposure, providing a unique intermediate model between Earth gravity and extended ISS microgravity conditions. Following collection, all biological samples from both orbital and suborbital missions were stored under ultra-low-temperature conditions (-80 °C) and transported for downstream processing. Transcriptomic profiling was subsequently conducted to investigate global changes in gene expression, with a particular focus on immune-related pathways. This integrated experimental framework enabled a multi-dimensional analysis by combining hematological measurements, immune cell phenotyping, and high-resolution transcriptomic data across different gravitational contexts. **Figure 1** illustrates the complete workflow, integrating both spatial (Earth, suborbital, and orbital environments) and temporal (pre-flight and in-flight sampling) dimensions of the MESSAGE mission, highlighting its comprehensive design to evaluate early immune and molecular responses to microgravity.

Hemogram analysis

To understand the effects of microgravity on cellular aging and the immune system, hemogram analysis, and immune profiling experiments were conducted as part of the ground-based experiments. Blood samples were collected from the astronaut at multiple time points: Day 4, Day 7, and Day 10 during their stay in space. Cell counts were performed, and the samples were cryogenically preserved for further analysis. The hemogram analysis, a laboratory test evaluating the components of blood, particularly erythrocytes (red blood cells), leukocytes (white blood cells), and platelets, was carried out to assess changes in the astronaut's health during spaceflight. These results are detailed in (as shown in **Table 1** and **Fig.2**). The hemogram results provide valuable insights into how space conditions affected the astronaut's haematological profile. The counts of various blood cells at each time point (Day 4, Day 7, and Day 10) show key changes that are indicative of the body's response to microgravity. This data is crucial for understanding how space environments influence overall immune function and cellular aging. The hemogram analysis aimed to assess the astronaut's blood profile during spaceflight at three key points: Day 4, Day 7, and Day 10. Leukocytes were noticeably increased from Day 4 (Mean 1.23 ± 2.05) to Day 10 (Mean 2.80 ± 0.62). While the difference between Day 4 and Day 7 was not statistically significant ($p=0.109$), a higher leukocyte count on Day 10 suggests immune activation. The erythrocyte count remained relatively stable between Day 4 (Mean 4.75 ± 0.75) and Day 7 (Mean 4.77 ± 0.77) but dropped slightly on Day 10 (Mean 4.23 ± 0.50). No significant differences were noted between the time points ($p=0.593$ and $p=0.109$). The hemoglobin levels remained consistent across the three time points, with no statistically significant changes (p -values >0.1). Also, there was a non-significant reduction in hematocrit

on Day 10 compared to Day 4 and Day 7, potentially indicating a change in blood volume or red cell mass during spaceflight. The MCV increased slightly from Day 4 to Day 7 but then dropped on Day 10. However, these changes were not statistically significant ($p=0.285$). MCH and MCHC values exhibited minor fluctuations across the three time points, but no significant changes were observed. Platelet levels increased from Day 4 to Day 7 but then slightly decreased on Day 10, with no statistically significant differences ($p=0.109$, $p=0.593$). RDW parameters, which measure red blood cell distribution width, showed slight variations across the time points, but the differences were not statistically significant. HbA1c levels, a marker of long-term glucose control, remained stable across all time points, indicating no significant changes in glucose metabolism during the space mission. The data suggests that while there were slight fluctuations in some hematological parameters during spaceflight, particularly in leukocyte count and hematocrit levels, most other blood components remained stable. This highlights a relatively controlled response to the space environment in terms of blood cell production and turnover.

Table 1: Hemogram Analysis Results (t-test)

	Day 4 N=3 Mean $\pm$ SD	Day 7 N=3 Mean $\pm$ SD	Day 10 N=3 Mean $\pm$ SD	4-7.day	4-10.day	7-10.day
				p	p	p
Leukocyte	1.23 $\pm$ 2.05	1.69 $\pm$ 2.77	2.80 $\pm$ 0.62	0.109	0.285	0.285
Red blood cell	4.75 $\pm$ 0.75	4.77 $\pm$ 0.77	4.23 $\pm$ 0.50	0.593	0.285	0.109
Hemoglobin	15.20 $\pm$ 0.45	15.56 $\pm$ 0.28	15.16 $\pm$ 0.83	0.109	0.655	0.414
Hematocrit	41.53 $\pm$ 4.84	43.76 $\pm$ 6.35	35.00 $\pm$ 7.07	0.285	0.285	0.109
MCV	87.80 $\pm$ 6.82	92.30 $\pm$ 12.05	82.53 $\pm$ 12.36	0.285	0.285	0.109
MCH	32.53 $\pm$ 5.40	33.20 $\pm$ 5.23	36.30 $\pm$ 6.15	0.102	0.109	0.109
MCHC	37.00 $\pm$ 5.30	36.16 $\pm$ 6.33	44.70 $\pm$ 10.63	0.593	0.109	0.109
Platelet	265.66 $\pm$ 51.03	294.66 $\pm$ 70.51	289.66 $\pm$ 106.95	0.109	0.593	1.000
RDW-SD	79.73 $\pm$ 5.01	76.80 $\pm$ 3.35	87.96 $\pm$ 4.79	0.655	0.109	0.109
RDW-CV	25.86 $\pm$ 0.90	23.96 $\pm$ 3.82	30.30 $\pm$ 3.43	0.109	0.109	0.109
HbA1c	5.40 $\pm$ 0.36	5.50 $\pm$ 0.51	5.33 $\pm$ 0.49	0.414	0.414	0.102

Table 2: Astronauts Time-Dependent Correlation Analysis (correlation analysis)

	Astronaut A	Astronaut B	Astronaut C
	P	P	P
Leukocyte	0,3252	0,8078	0,3248
Red blood cell	0,3508	0,5456	0,2913
Hemoglobin	0,6667	0,0732	>0,9999
Hematocrit	0,4246	0,8965	0,3036
MCV	0,5292	0,9341	0,3223
MCH	0,1919	0,1789	0,2531
MCHC	0,3401	0,9406	0,9421
Platelet	0,4605	0,1603	0,9421
RDW-SD	0,2086	0,7034	0,3333
RDW-CV	0,3534	0,8675	0,3419
HbA1c	0,7877	>0,9999	0,6667

Based on the time-dependent correlation analyses conducted for each astronaut, no statistically significant changes ($p > 0.05$) were observed in the measured parameters between **Day 4 and Day 10**. This finding suggests that the assessed biological processes did not exhibit substantial variation within this timeframe and that the evaluated parameters remained stable in the microgravity environment over the short term.

This study aimed to investigate potential changes in various hematological parameters by analyzing blood samples collected from three different astronauts on days 4, 7, and 10 of their respective missions. The parameters analysed included leukocyte, erythrocyte, hemoglobin, hematocrit, MCV, MCH, MCHC, thrombocyte, RDW-SD, RDW-CV, PDW, MPV, P-LCR, PCT, NEUT, NEUT%, LYMPH, LYMPH%, MONO, MONO%, EO, EO%, BASO, BASO%, IG, and HbA1c. The findings revealed no statistically significant changes in any of the analyzed parameters, suggesting that hematological parameters remain stable during long-duration space missions and indicating a physiological adaptation to the space environment.

Total Cell Viability and Count Analysis

Next, we wanted to analyze the cell viability in astronaut blood samples. **Figure 3** shows live cell counts measured on Day 4, Day 7, and Day 10 for three astronauts (A, B, and C). The average live cell count increased from Day 4 to Day 7, reaching a peak, followed by a decrease on Day 10 across all groups. Astronaut A consistently maintained the highest number of live cells throughout all time points, whereas Astronaut C had the lowest counts, particularly on Day 10, where a sharp decline was observed. Total cell counts also followed a similar trend, with an initial increase from Day 4 to Day 7. Also, by Day 10, a non-significant reduction was observed, particularly in Astronaut C, which had the lowest total cell count. Astronaut A maintained the highest count, while Astronaut B showed intermediate results. The average cell viability percentage remained relatively stable from Day 4 to Day 7 but showed a slight decrease by Day 10. Astronaut A showed the highest viability across all time points, maintaining above 60%, while Astronaut C had the lowest viability, particularly on Day 10, where it dropped below 50%. The data suggest that while live and total cell counts initially increased, possibly due to adaptation to experimental conditions, a decline in both live cell count and viability by Day 10 indicates cellular stress or reduced proliferative capacity over time, especially in Astronaut C. Astronaut A showed a relatively more stable profile across all metrics, suggesting greater resilience under the given microgravity conditions.

T Cell Subpopulation Analysis

We compared various T cell subpopulations between astronauts and healthy donors at three points: Day 4, Day 7, and Day 10. The subpopulations included in the analysis were TemEARLY (Early effector memory T cells), Tcm (Central memory T cells), Tn+Tscm (Naive and stem cell-like memory T cells), Temra (Effector memory T cells re-expressing CD45RA), and TemLATE (Late effector memory T cells), analyzed by flow cytometry (**Fig.4**).

Next, we aimed to assess CD4⁺ T cell subpopulation analysis, including central memory T cells (Tcm) and stem-cell-like memory T cells (Tn+Tscm), measured at three time points: Day 4, Day 7, and Day 10 during spaceflight (**Fig. 5**). In day 4, the percentage of TemEARLY and TemLATE cells was high, with approximately 50% of the total CD4⁺ population comprising these effector memory subsets. Tcm and Tn+Tscm populations were minimal, constituting a small fraction of the total CD4⁺ population. Temra cells were present at low levels on Day 4. On the other hand, in day 7, TemEARLY cells slightly decreased in percentage, while TemLATE cells showed a steady increase, indicating a shift toward a more differentiated effector memory T cell population. Tcm and Tn+Tscm populations remained low, but Temra cells exhibited a slight increase compared to Day 4. By Day 10, TemEARLY cells continued to decrease slightly, while TemLATE cells reached their highest levels, with over 60% of the population comprising late effector memory T cells. Tcm and Tn+Tscm populations remained low, like earlier time points, while Temra cells showed a notable increase, reflecting potential ongoing differentiation or immune activation as spaceflight progressed. The data suggests a shift from early effector memory T cells (TemEARLY) to late effector memory T cells (TemLATE) over the course of spaceflight. By Day 10, TemLATE cells constituted the majority of the CD4⁺ T cell population. Central memory T cells (Tcm) and stem-cell-like memory T cells (Tn+Tscm) remained consistently low throughout all time points, suggesting that spaceflight conditions did not favor the expansion of these less differentiated memory subsets. The increase in Temra cells by Day 10 indicates potential ongoing immune activity or response to environmental stressors in space. These results reflect a shift in immune memory and differentiation dynamics during spaceflight, with a predominant focus on effector memory subsets, which could have implications for immune function during long-term space missions.

Immune Transcriptome Dynamics Under Microgravity

To investigate the impact of microgravity on immune regulation, transcriptomic profiling was performed on peripheral blood mononuclear cells (PBMCs) collected from three astronauts aboard the International Space Station (ISS), one suborbital astronaut, and three healthy controls. Blood samples were obtained from ISS astronauts at three time points during the Axiom-3 mission (Day 4, Day 7, and Day 10), with additional samples collected immediately before and after suborbital flight (~100 km). RNA-Seq analysis revealed time-dependent alterations in immune-related gene expression, indicating that microgravity induces early molecular adaptations in immune regulation pathways. Analysis of immune-related transcripts demonstrated coordinated modulation of multiple genes involved in adaptive and innate immune pathways (**Figure 6**). T cell activation markers (CD69, IL2RA, LAT, IFNG) exhibited a progressive decline from Day 4 to Day 10, suggesting impaired T cell activation and signaling. NK cell and macrophage markers (NKG7, PRF1, GZMB) displayed reduced expression, indicating a potential suppression of innate cytotoxic responses. Genes associated with cytokine signaling, including IL6, IL10, and IL18, showed subtle but biologically relevant modulation, consistent with microgravity-induced immune dysregulation. While not all changes reached statistical significance, consistent temporal downregulation trends were observed, suggesting an early and coordinated transcriptional response. These results indicate that microgravity suppresses key immune pathways, potentially lowering immune responsiveness during short-duration missions.

To further characterize the transcriptional landscape, a heatmap of 48 immune-related genes was generated across five sampling stages, including Pre-launch baseline, Post-suborbital exposure, ISS Day 4, Day 7, and Day 10. As shown in **Figure 7**, the heatmap revealed time-resolved immune transcriptional patterns, with noticeable early-phase changes as soon as Day 4 post-launch. Key immune regulatory genes displayed gradual modulation, while later phases (Day 7 and Day 10) exhibited a more pronounced downregulation of cytokine and T cell–associated transcripts. Importantly, these transcriptional changes preceded measurable alterations in haematological parameters or immune cell distributions, suggesting that molecular adaptations occur before phenotypic immune changes become detectable. This figure illustrates the transcriptional alterations observed in immune-related genes following exposure to microgravity. Gene expression differences were statistically assessed using the Kruskal–Wallis test, a non-parametric method suitable for small sample sizes and non-normally distributed data. The threshold for statistical significance was set at $p < 0.05$. The majority of the genes presented in the upper section of the figure exhibited statistically significant differences across time points. However, a subset of genes with $p > 0.05$, which did not reach statistical significance but demonstrated biologically notable expression trends, is displayed in the lower section of the figure. By integrating both statistically significant and biologically relevant, yet non-significant, results into a single visualization, this figure provides a more comprehensive understanding of the immune system’s transcriptional sensitivity to gravitational stress. Integrated analysis of **Figures 6 and 7** demonstrates that short-term spaceflight induces rapid and coordinated transcriptional reprogramming of immune-regulatory pathways. Downregulation of T cell activation genes and cytokine modulators suggests a microgravity-associated immunosuppressive phenotype, while modulation of NK cell and macrophage genes reflects altered innate immune readiness. These findings provide evidence that molecular-level adaptations occur early during exposure to microgravity and highlight potential vulnerabilities in immune surveillance during long-duration space missions.

4. Discussion

The findings of this study highlight the substantial impact of microgravity on the immune system, particularly on T lymphocyte populations. Our observations of altered T cell subpopulations and cellular viability in astronauts during a 10-day space mission provide critical insights into how spaceflight can modulate immune function. Our data shows substantial shifts in T cell subpopulations, blood cell profiles, and cell viability, reflecting the body's adaptive responses to the unique stressors of spaceflight. The hemogram analysis revealed key changes in blood cell populations during the mission. The most notable observation was the gradual increase in leukocytes (white blood cells) from Day 4 to Day 10, suggesting that microgravity may activate immune responses. Leukocytosis, observed by the increase in leukocyte counts on Day 10, might be due to the stress experienced in space, which can trigger immune activation. This increase in leukocyte count has been previously reported in studies that simulated spaceflight conditions, where the body responds to stress by activating innate immune pathways [1]. However, the erythrocyte count showed a slight decline by Day 10, which could indicate a reduction in red blood cell production or increased hemolysis in microgravity. This aligns with findings from other

spaceflight studies that observed alterations in erythropoiesis and red cell mass during prolonged exposure to microgravity [6]. The non-significant reduction in haematocrit on Day 10 further supports the hypothesis of potential blood volume changes or fluid shifts in space. These alterations in haematological parameters, though subtle, highlight the need to monitor astronauts' oxygen-carrying capacity and overall hematologic health during long-term missions [4].

The mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) remained relatively stable, though slight fluctuations in MCV from Day 4 to Day 10 may indicate cellular adjustments to the changing environmental conditions. Importantly, HbA1c levels, a marker of long-term glucose control, remained stable, suggesting that glucose metabolism was not significantly affected during the short duration of the mission. The analysis of cell viability and total cell counts provides additional insights into how immune cells adapt to microgravity. We observed an initial increase in live and total cell counts from Day 4 to Day 7, likely reflecting an initial proliferative response to microgravity. However, by Day 10, there was a notable decline in both live and total cell counts, particularly in astronaut C, suggesting that microgravity imposes cellular stress over time, ultimately reducing proliferative capacity and viability. This decline in cell viability aligns with previous studies that documented reduced cell proliferation and increased apoptosis in microgravity conditions [9]. The sharp decline in viability observed on Day 10 could be indicative of cellular exhaustion or the cumulative effects of stress, which has been reported in both simulated and real microgravity environments. The slight decrease in cell viability percentage from Day 7 to Day 10 further supports the notion that prolonged exposure to microgravity leads to cellular stress and reduced survival [2]. Interestingly, astronaut A maintained the highest live cell count and viability across all time points, suggesting potential individual variability in response to microgravity. This may be due to genetic factors, differences in immune resilience, or varying levels of adaptation to space conditions. Further research is needed to explore these individual differences and identify potential biomarkers of resilience to spaceflight-induced immune stress. Moreover, time-dependent transcriptomic analysis of immune-related genes provided further evidence of immune modulation under microgravity. While most changes were not statistically significant, key immune markers such as *IL2RA*, *CD69*, and *IFNG* exhibited downregulated expression at critical time points, particularly during the ISS phase. These genes are central to T cell activation and cytokine signalling, and their reduction may reflect an immunosuppressive shift in spaceflight conditions. This agrees with prior studies demonstrating altered cytokine profiles and impaired signal transduction during microgravity exposure [3,6].

Although most gene expression changes were subtle or statistically non-significant, biologically relevant trends were observed across different phases of the mission. The observed patterns suggest that microgravity initiates an early transcriptional response in immune cells, potentially as part of a stress adaptation mechanism. These trends were further supported by a heatmap analysis of immune-related gene expression, showing dynamic but coherent modulation over time. The non-significant elevation of effector memory T cells (TemEARLY and TemLATE) during spaceflight underscores a shift towards an activated immune state. This shift may be an adaptive response to the unique stressors encountered in the microgravity environment, including altered cellular signalling and reduced mechanical stress on

cells. Previous studies have suggested that microgravity induces a hyper-responsive state in immune cells, which could predispose astronauts to inflammatory conditions or other immune-related health issues [3,6]. The persistent low levels of central memory T cells (T_{cm}) and stem cell-like memory T cells (T_n+T_{scm}) observed in this study may indicate a potential vulnerability in the immune system's ability to respond to new antigens or fight off recurring infections. The reduction in these cell populations could lead to a compromised ability to form long-term immunological memory, which is essential for protecting against diseases over a lifetime [4]. The increase in late effector memory T cells (T_{em}LATE) by Day 10 suggests a more differentiated state, potentially leading to an enhanced immediate defensive response but at the expense of proliferative capacity and longevity of the immune response. This observation aligns with findings from [1], who noted that spaceflight conditions could trigger early senescence of naive T cells, thereby accelerating the aging of the immune system. Despite the biological signals observed, the findings of this study indicate that, within the timeframe of the Axiom-3 mission, microgravity did not induce statistically significant changes in the haematological parameters and T cell subpopulations of the participating astronauts. The MESSAGE mission lasted only a limited number of days on the ISS, with blood samples collected on post-launch Day 4, Day 7, and Day 10. Previous studies suggest that immune dysregulation and haematological adaptations in microgravity may require prolonged exposure to space conditions. The duration of our study may not have been sufficient for significant physiological alterations to manifest, as previous research has shown more pronounced immune shifts during long-duration missions (>30 days). Astronauts undergo extensive pre-flight conditioning, including exercise regimens, nutritional optimization, and immune health monitoring, which could contribute to maintaining physiological stability in space. Additionally, onboard countermeasures such as exercise routines and controlled diets may have helped mitigate immune fluctuations and haematological shifts, preventing significant changes from occurring.

Our findings from Figure 6 and Figure 7 demonstrate that short-term exposure to microgravity induces rapid and coordinated transcriptional reprogramming of immune-regulatory pathways. The observed downregulation of T cell activation markers such as CD69, IL2RA, LAT, and IFNG suggests an early impairment of adaptive immune responses during spaceflight. This pattern is consistent with previous studies reporting reduced T cell activation and cytokine secretion in astronauts during and after space missions, indicating that microgravity alters critical signaling cascades required for immune surveillance. Similarly, the decreased expression of innate immune regulators, including NKG7, PRF1, and GZMB, highlights a potential suppression of natural killer (NK) cell cytotoxic activity, which may increase susceptibility to viral reactivation and opportunistic infections under spaceflight conditions. The heatmap analysis (Figure 7) further supports these findings by revealing time-resolved transcriptional modulation across 48 immune-related genes, with distinct shifts evident as early as Day 4 post-launch and progressively amplified through Day 10. Notably, these transcriptional alterations emerged before measurable changes in hematological profiles or T cell subpopulation distributions, suggesting that molecular adaptations precede phenotypic immune alterations in microgravity. This temporal dissociation highlights the importance of transcriptomic profiling as a sensitive early biomarker for detecting immune dysregulation in astronauts. Together, these results provide new insights into the

early-phase immune adaptations to microgravity, revealing that both adaptive and innate immune pathways undergo coordinated suppression during short-duration spaceflight. These findings underscore the necessity of monitoring immune function at the molecular level during missions and may inform the development of targeted countermeasures to mitigate immune dysfunction in long-duration deep-space exploration. While microgravity is known to influence immune function, other spaceflight-associated stressors such as radiation exposure, circadian rhythm disruption, and psychological stress may play a more significant role in immune changes over time. The absence of significant findings in this study suggests that short-term microgravity exposure alone might not be sufficient to induce acute immune shifts without the compounded effects of other stressors present in long-duration missions. With a cohort of only three astronauts, statistical power is inherently limited. The small sample size increases variability in individual responses and reduces the ability to detect significant trends. Future studies with a larger number of participants across multiple missions would provide a more comprehensive understanding of immune adaptations in space.

The findings of this study provide valuable insights into the early immune adaptations that occur during short-term spaceflight, with a particular focus on T cell dynamics and transcriptional regulation under microgravity. While no statistically significant changes were observed in haematological parameters or T cell subpopulations, the transcriptomic analyses revealed time-dependent modulation of immune-related genes, suggesting that molecular responses precede phenotypic alterations. These results highlight the importance of early and sensitive transcriptomic profiling to understand how the immune system adapts to the unique stressors of spaceflight. The implications of these findings are particularly relevant for long-duration space missions, where astronauts are subjected to prolonged microgravity exposure. Understanding the molecular mechanisms that drive these immune alterations will be critical for developing countermeasures aimed at preserving astronaut health and maintaining operational performance. Enhancements in spacecraft design, including facilities to simulate partial gravity, may help maintain a balanced immune response, as suggested by studies in simulated microgravity environments [9]. Future research will focus on identifying microgravity-associated candidate genes through advanced transcriptomic analyses, followed by CRISPR-based knock-out studies to define their functional roles in immune modulation. These approaches will enable a more precise understanding of gravitationally sensitive pathways and their contribution to immune stability during spaceflight. Beyond space exploration, these findings have broad translational relevance on Earth. Insights into the regulation of immune resilience under extreme environmental stressors could inform the development of novel therapeutic strategies for immunosuppression, infectious diseases, and cancer immunotherapy. To strengthen these findings, future studies should incorporate longer-duration missions, larger astronaut cohorts, and multi-omic profiling approaches to comprehensively characterize immune adaptations. Additionally, developing on-ground analog systems to better simulate microgravity conditions will be essential for pre-mission training, in-flight countermeasure development, and post-mission recovery planning [2]. Collectively, this study establishes a foundation for understanding the molecular basis of immune regulation in space, paving the way for targeted countermeasures that safeguard astronaut health during deep-space exploration while simultaneously advancing immunological research on Earth.

Abbreviations

MESSAGE (Microgravity Associated Genetics) Science Mission, TemLATE(Transcriptional Enhancer-Like Activator), International Space Station (ISS), Peripheral blood mononuclear cells (PBMC), (TCM) memory T cells, Mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), stem-cell-like memory T cells (TSCM), interleukin-2 (IL-2), increased tumour necrosis factor-alpha (TNF- α), Transgenic Cell Technologies and Epigenetic Application and Research Center (TRGENMER).

Declarations

Acknowledgements

This manuscript was presented at the International Astronautical Congress (IAC) 2024, Milan, Italy, 14–18 October 2024. This research process initially began with two TÜBİTAK 2209-A Projects (Application No: 1919B012112510 and 1919B012208729), followed by a Scientific Research Project at Üsküdar University (Project No: ÜÜBAP-YP-2021-016), which focused on the establishment of a device simulating microgravity conditions. The experience and infrastructure gained through these projects laid the foundation for the Turkish Astronaut and Science Mission (TABM) Project (Project No: 1007-KAMAG-121L002 / Contract No: TABM-HZT-23-25), supported by the Turkish Space Agency (TUA) and the Scientific and Technological Research Council of Turkey (TÜBİTAK). We sincerely thank TUA, TÜBİTAK, and Üsküdar University for their valuable contributions. The opinions and findings presented in this publication are solely the responsibility of the authors and do not necessarily reflect the views of the official institutions. Finally, we extend our gratitude to all colleagues, data analysts, and support staff who contributed to the MESSAGE Science Mission.

Funding Acknowledgement: This study was supported by the Scientific and Technological Research Council of Türkiye (TÜBİTAK) and the Turkish Space Agency (TUA) through the Turkish Astronaut and Scientific Mission (TABM) Project (1007-KAMAG-121L002 / Agreement No: TABM-HZT-23-25. We would like to thank TÜBİTAK UZAY and TUA for their support and declare that none of the opinions and findings contained in this publication are the official views of TÜBİTAK and TUA.

Author Contributions: **C.T.** conceived and supervised the study, coordinated the MESSAGE Science Mission project, and contributed to manuscript writing. **G.G.** performed experimental procedures and processed biological samples. **O.D.** conducted data analysis, interpreted transcriptomic results, and contributed to manuscript preparation. **E.Ç.** analyzed data, reviewed figures, interpreted results, and contributed to manuscript writing. **B.Y.** established the bioinformatics pipeline and performed computational analyses. **K.K.** assisted with transcriptomic data processing and software optimization. **F.E.** performed experimental implementation and sample handling. **B.S.** provided experimental support and managed project documentation. **T.G.** contributed to the laboratory setup and provided technical assistance. **I.Y.** assisted with experimental procedures and bench work. **B.T.** prepared cell samples and

coordinated experimental workflows. **B.A.** designed the MESSAGE mission schematic and contributed to manuscript preparation.

Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

References

1. Crucian, B. E., Stowe, R. P., Mehta, S. K., Yetman, D. L., Leal, M. J., Quiriarte, H. D., et al. (2009). Immune status, latent viral reactivation, and stress during long-duration head-down bed rest. *Aviat Space Environ Med*, 80 [2] Mann, V., Okoro, E., Sodipe, A., Williams, C., Ngantcha, P., & Sundaresan, A. (2018). Lymphocyte signaling and function in altered physiological environments. In *Lymphocytes*. IntechOpen [3] Borchers, A. T., Keen, C. L., & Gershwin, M. E. (2002). Microgravity and immune responsiveness: implications for space travel. *Nutrition*, 18(10), 889–898.
2. Cooper, D., & Pellis, N. R. (1998). Suppressed PHA activation of T lymphocytes in simulated microgravity is restored by direct activation of protein kinase C. *Journal of leukocyte biology*, 63(5), 550–562.
3. Crucian, B. E., Stowe, R. P., Mehta, S. K., Yetman, D. L., Leal, M. J., Quiriarte, H. D., et al. (2009). Immune status, latent viral reactivation, and stress during long-duration head-down bed rest. *Aviat Space Environ Med*, 80 [6] Hughes, J. H., & James P. Long. (2001). Simulated Microgravity Impairs Respiratory Burst Activity in Human Promyelocytic Cells. *In Vitro Cellular & Developmental Biology. Animal*, 37(4), 209–215.
4. Rucian, B., Stowe, R., Mehta, S., Uchakin, P., Quiriarte, H., Pierson, D., et al. (2012). Immune system dysregulation occurs during short duration spaceflight on board the space shuttle. *Journal of Clinical Immunology*, 33:456–65.
5. Wainberg, M., Sinnott-Armstrong, N., Mancuso, N., Barbeira, A. N., Knowles, D. A., Golan, D., et al. (2019). Opportunities and challenges for transcriptome-wide association studies. *Nature Genetics*, 51(4), 592–599.
6. Li, L., Gu, N., Dong, H., Li, B., & Kenneth, T. V. G. (2020). Analysis of the effects of acoustic levitation to simulate the microgravity environment on the development of early zebrafish embryos. *RSC Advances*, 10(72), 44593–44600.
7. Stratis, D., Trudel, G., Rocheleau, L., Pelchat, M., & Laneuville, O. (2023). The transcriptome response of astronaut leukocytes to long missions aboard the International Space Station reveals immune modulation. *Frontiers in Immunology*, 14, 1171103.

Figures

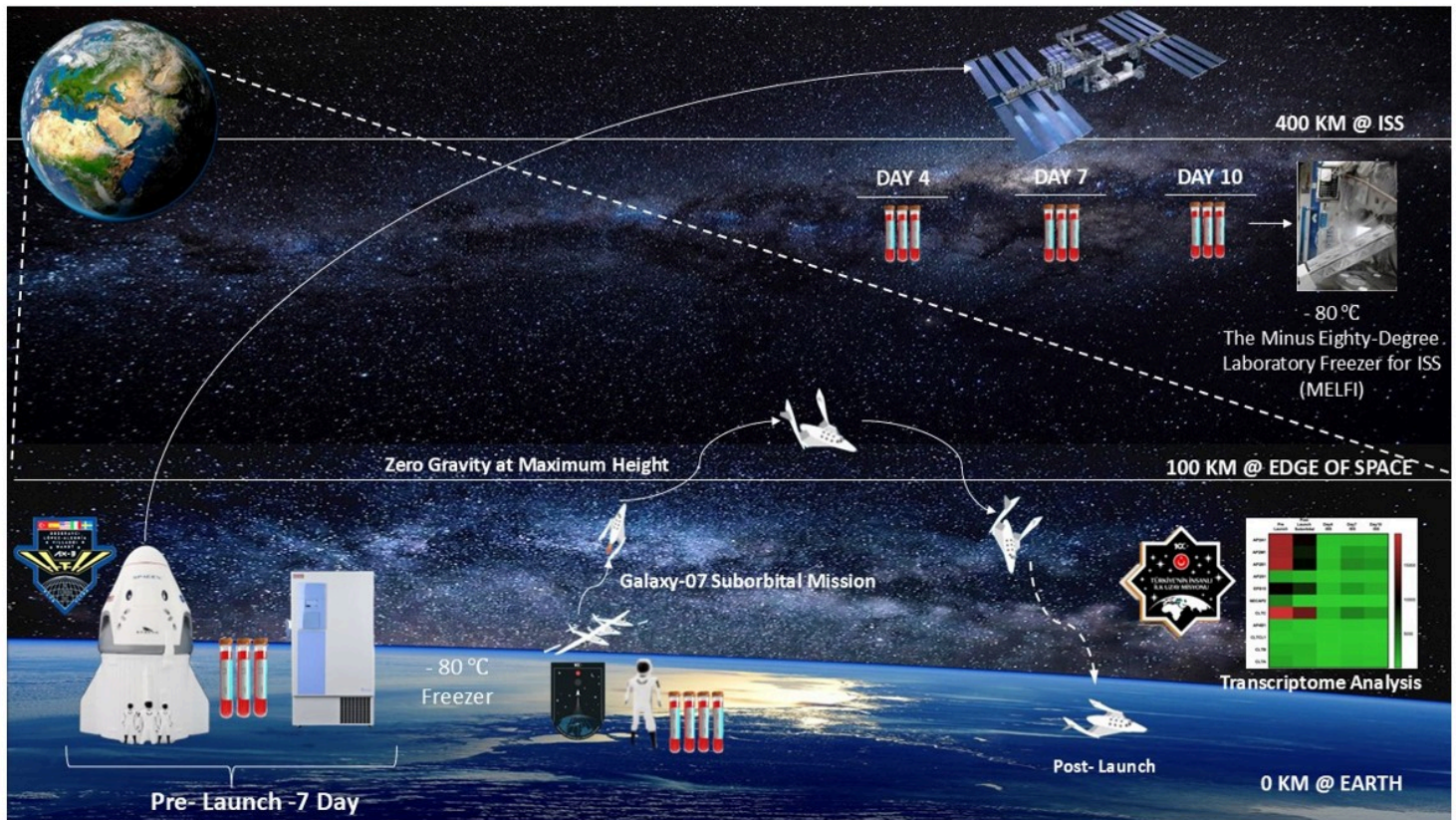


Figure 1

Experimental design of the MESSAGE Science Mission. Schematic overview of the MESSAGE (Microgravity Associated Genetics) Science Mission workflow. Venous blood samples were collected from astronauts seven days pre-launch (Earth baseline), during the Axiom-3 mission on the ISS at Day 4, Day 7, and Day 10, and cryopreserved at -80°C in the MELFI system. Short-term microgravity effects were additionally assessed using the Galaxy-07 suborbital mission (~ 100 km altitude), with samples collected before and after flight. All samples were stored at -80°C and later analyzed through hematological profiling, immune cell phenotyping, and transcriptomic sequencing to evaluate microgravity-induced immune and molecular responses.

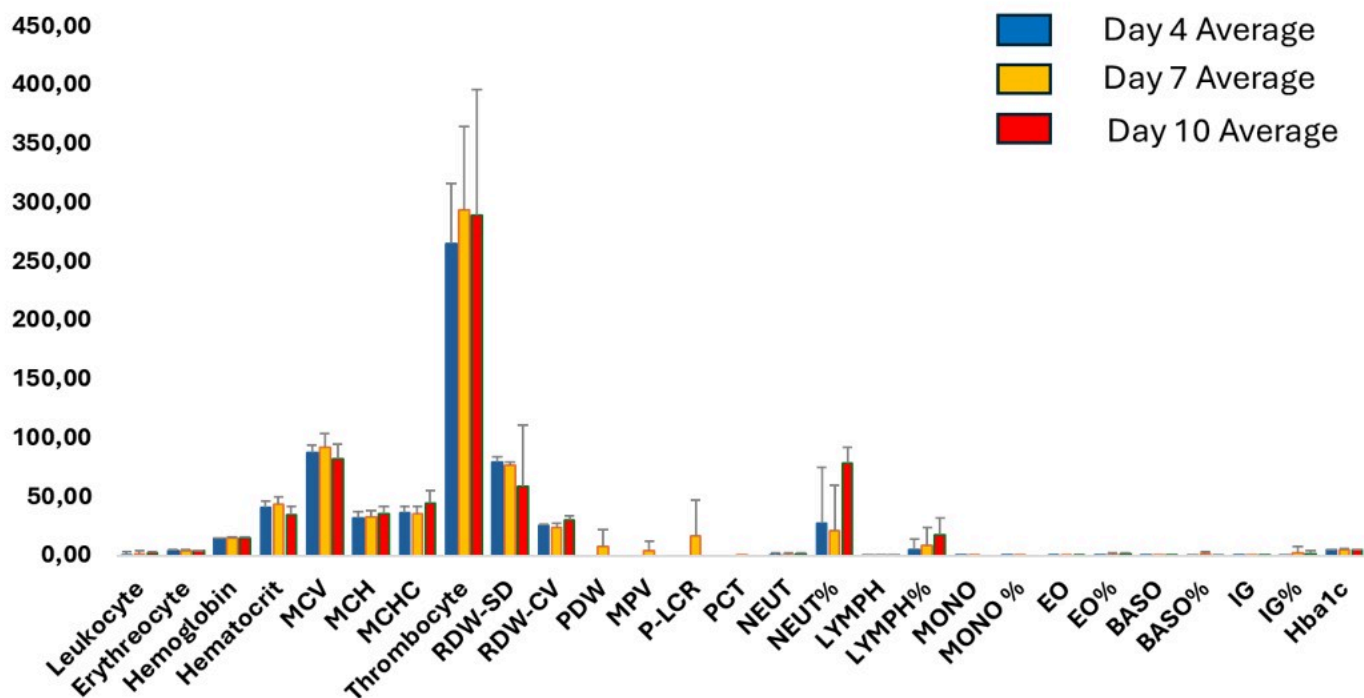


Figure 2

Hemogram Analysis Results of astronauts whose blood samples were withdrawn in space on Day 4, 7, and 10.

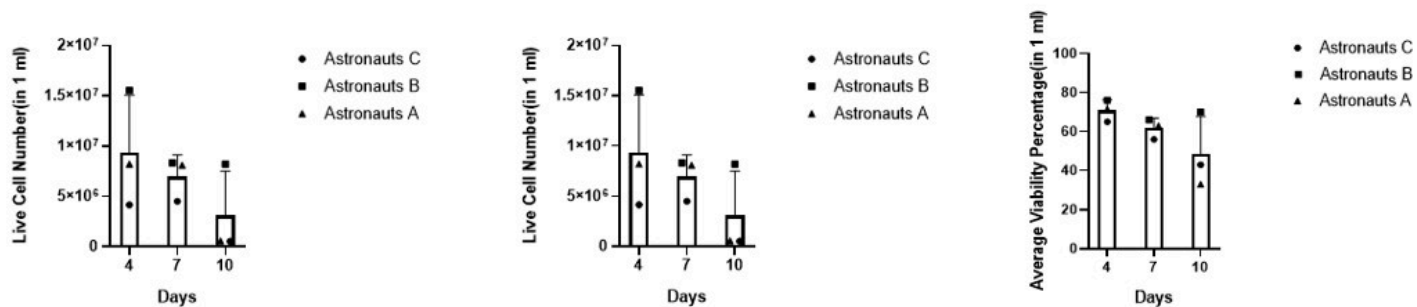


Figure 3

Viability, number of viable cells, and total cell count graphs obtained because of cell count readings.

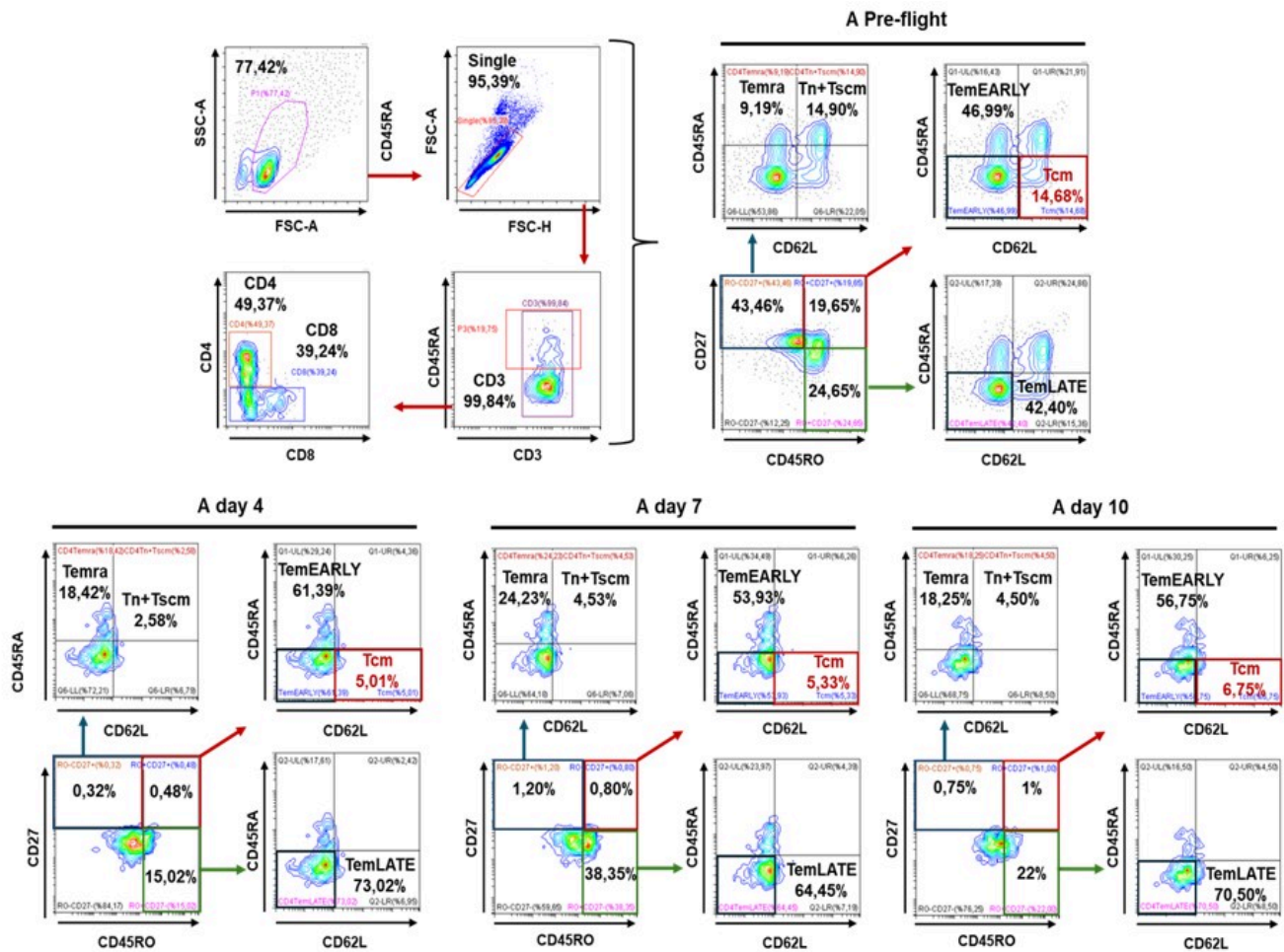


Figure 4

T cell subpopulations between astronauts and healthy donors at different times (Pre-flight, Day 4, Day 7, and Day 10).

Astronaut Total CD4+ TCM-TSCM

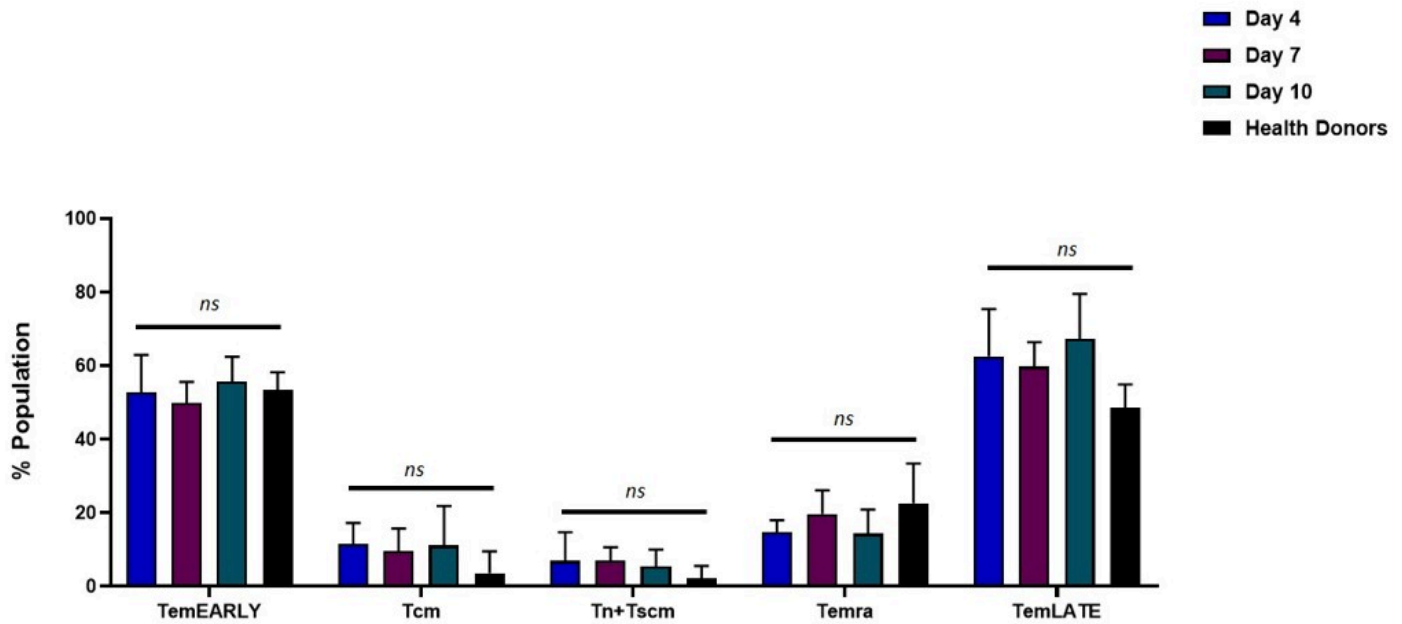


Figure 5

CD4+ T cell subpopulations, including central memory T cells (TCM) and stem-cell like memory T cells (TSCM), measured at three time points: Day 4, Day 7, and Day 10 during spaceflight

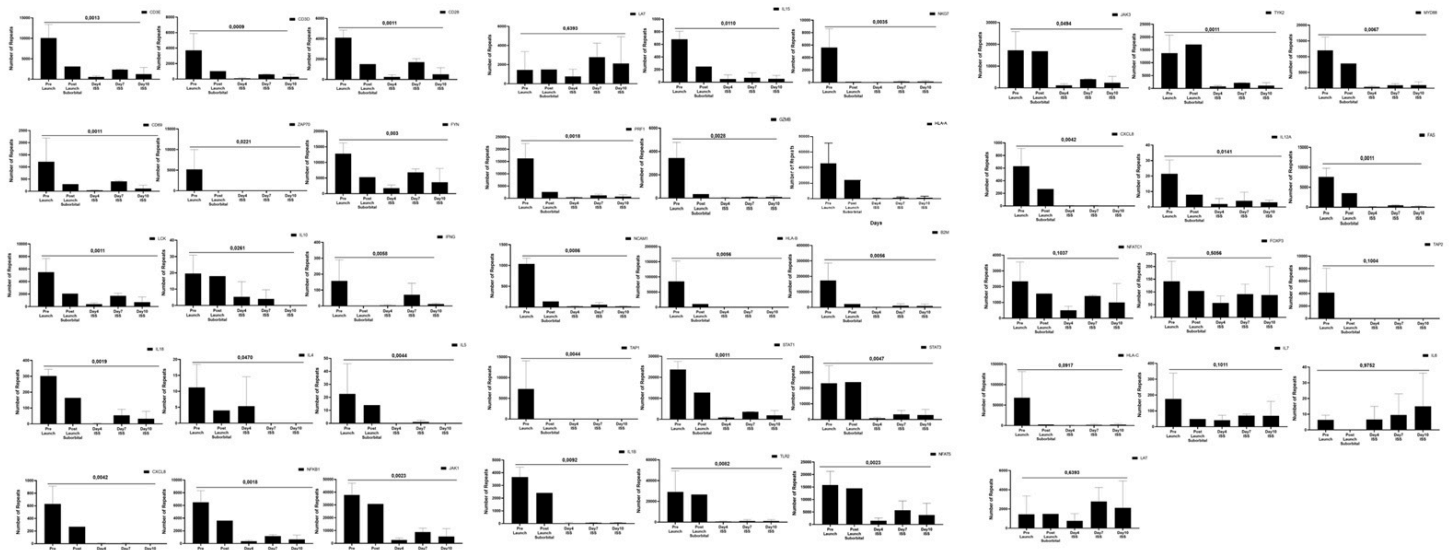


Figure 6

Differential expression of immune-related genes under microgravity conditions. Bar plots illustrate the expression patterns of selected immune-regulatory genes obtained from RNA-Seq analysis of astronaut PBMCs collected during the Axiom-3 mission at Day 4, Day 7, and Day 10 compared with pre-flight baseline levels. Genes associated with T cell activation (CD69, IL2RA, LAT, IFNG), NK cell cytotoxicity

(NKG7, PRF1, GZMB), and cytokine signalling (IL6, IL10, IL18) demonstrated progressive downregulation across in-flight time points, suggesting reduced immune responsiveness in microgravity. Although some changes did not reach statistical significance, the consistent temporal patterns highlight an early and coordinated transcriptional adaptation of the immune system during spaceflight.

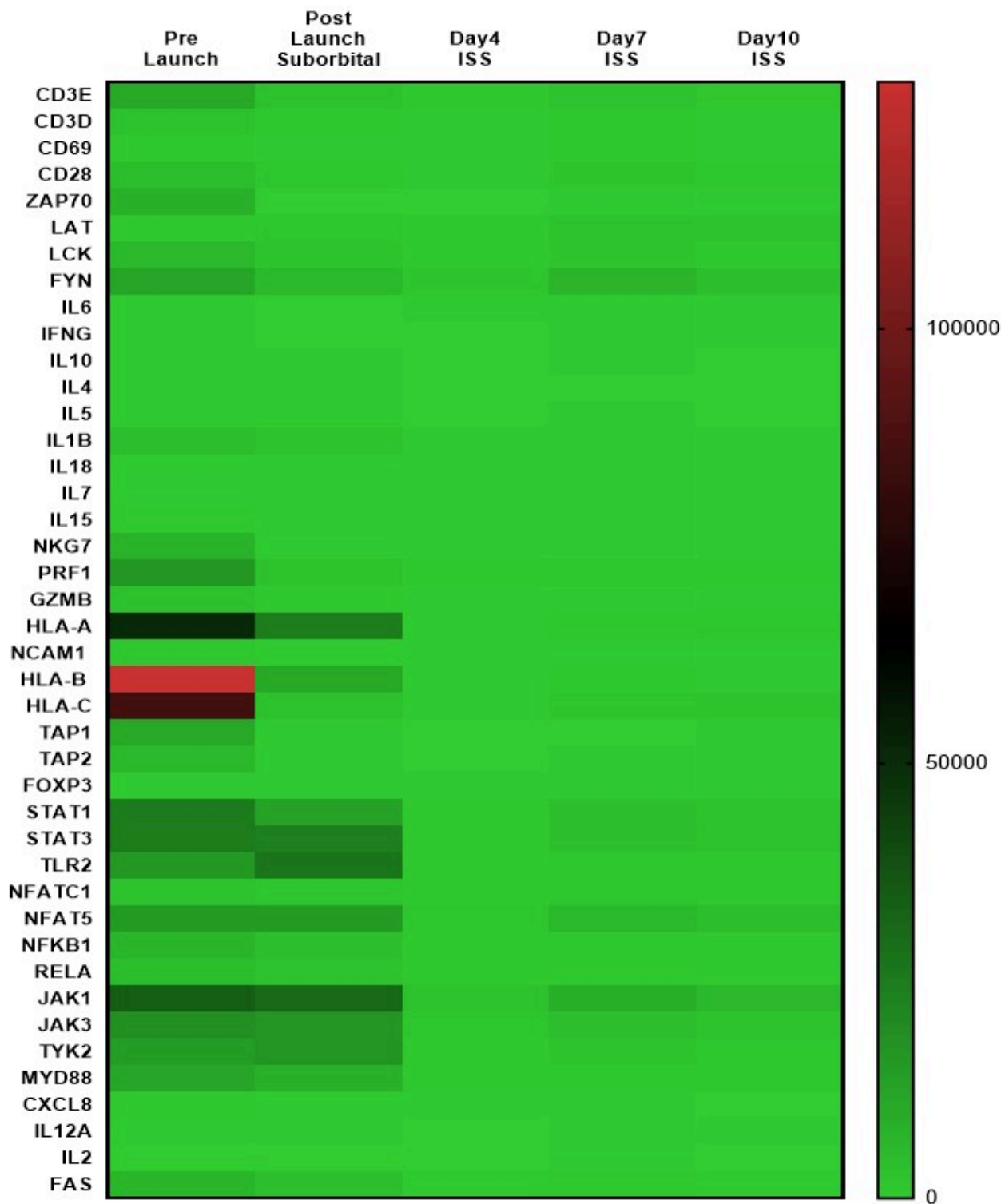


Figure 7

Heatmap visualization of immune gene expression across pre-flight, suborbital, and ISS time points.

Heatmap depicting the temporal expression profiles of 48 immune-related genes across five experimental stages: pre-launch baseline, post-suborbital exposure (~100 km), and ISS Days 4, 7, and 10. The color scale represents normalized expression levels (TPM), with red indicating higher expression and green representing lower expression. The heatmap reveals dynamic but coordinated transcriptional shifts, including the downregulation of T cell activation markers and cytokine-associated genes during ISS exposure. These molecular alterations emerged before observable changes in immune cell counts, suggesting that transcriptional responses precede phenotypic immune modulation under microgravity conditions.