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Differential effects of hypergravity on immune dysfunctions induced by simulated microgravity

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Abstract

Microgravity (μg) is among the major stressors in space causing immune cell dysregulations. These are frequently expressed as increased pro-inflammatory states of monocytes and reduced activation capacities in T cells. Hypergravity (as artificial gravity) has shown to have beneficial effects on the musculoskeletal and cardiovascular system both as a countermeasure option for μg-related deconditioning and as "gravitational therapy" on Earth. Since the impact of hypergravity on immune cells is sparsely explored, we investigated if an application of "mild" mechanical loading of $2.8\,g$ is able to avoid or treat μg-mediated immune dysregulations. For this, T cell and monocyte activation states and cytokine pattern were first analyzed after whole blood antigen incubation in simulated μg (s-μg) by using the principle of fast clinorotation or in hypergravity. Subsequent hypergravity countermeasure approaches were run at three different sequences: one preconditioning setting, where $2.8\,g$ was applied before s-μg exposure and two therapeutic approaches in which $2.8\,g$ was set either intermediately or at the end of s-μg. In single g-grade exposure experiments, monocyte pro-inflammatory state was enhanced in s-μg and reduced in hypergravity, whereas T cells displayed reduced activation when antigen incubation was performed in s-μg. Hypergravity application in all three sequences did not alleviate the increased pro-inflammatory potential of monocytes. However, in T cells the preconditioning approach restored antigen-induced CD69 expression and IFNγ secretion to $1\,g$ control values and beyond. This in vitro study demonstrates a proof of concept that mild hypergravity is a gravitational preconditioning option to avoid adaptive immune cell dysfunctions induced by (s-)μg and that it may act as a booster of immune cell functions.

KEYWORDS
artificial gravity, clinostat, cytokines, hypergravity, microgravity, monocytes, preconditioning, T cells

1 | INTRODUCTION

During spaceflight, the human immune system can be severely dysregulated which is predominantly attributed to the condition of microgravity (μg).[1,2] Especially T cells, which are key components of the adaptive immune system, exhibit a reduced responsiveness to antigenic and mitogenic stimulation in space.[3] Insufficient T cell functionality may result in reactivation of latent herpes viruses[4] but also in an enhanced susceptibility to infectious diseases, as it has been already recognized during the Apollo missions.[5] Conversely do monocytes, the sentinels of innate immunity. They show an enhanced pro-inflammatory potential in space, which was demonstrated by increased plasma concentrations of monocyte-specific cytokines such as IL-1β during and shortly after spaceflight.[6,7] The alterations in T cell functions resemble the onset of premature immune aging and together with an enhanced pro-inflammatory state of monocytes, a state of inflammaging may occur,[8,9] causing permanent subclinical inflammation and concurrently higher incidence of viral infection but also autoimmunological reactions as usually seen in the elderly.[6,8,10] During spaceflight, inflammaging-associated symptoms may not only be detrimental for the single crew member but also for mission success.

A detailed understanding of μg-induced effects on the immune system can support the development of adequate countermeasure strategies. However, opportunities to perform investigations in space such as aboard the International Space Station (ISS) are very restricted. Devices to simulate μg (s-μg) allow to explore the effect of reduced gravity in cell culture settings by the for immune cells approved method of fast 2D clinorotation.[11,12] An *ex vivo* whole blood stimulation approach in s-μg provided further evidence for impaired T cell activation and enhanced pro-inflammatory potential of innate immune cells in reduced gravity.[13] To investigate the impact of real μg in Earth-bound spaceflight analog platforms, parabolic flights (PFs) in which μg is repetitively achieved for 22 seconds per each parabola, however, interrupted by hypergravity phases, represent a feasible alternative. Whole blood stimulation with immunostimulatory agents *ex vivo* during PF resulted both in immune cell activation and inhibition as demonstrated by surface activation marker expression and pro-inflammatory cytokine profiles.[14] Yet, it is not known if this observation is attributed to μg, the hypergravity phases of 1.8 g at the pull-up and pull-out of a parabola (each for 22 seconds)[15] or to the interplay of both. However, alternating g-forces were suggested to alleviate μg-induced immune deficiencies.[14]

We aimed to investigate, if an application of hypergravity is capable to counteract immune dysfunctions induced by s-μg. Accordingly, *ex vivo* whole blood stimulation was performed under three different s-μg/hypergravity sequences: preconditioning, where hypergravity of 2.8 g exposure was applied before s-μg, and two therapeutic approaches in which hypergravity was applied intermediately or at the end of s-μg. Results obtained from these investigations will expand the knowledge on the effects of hypergravity on immune cells and provide insights into the effectiveness of gravitational countermeasures.

2 | MATERIALS AND METHODS

2.1 | Blood sampling

All experiments were carried out in accordance with the ethical standards of the appropriate institutional and national committees and with the World Medical Association Helsinki Declaration of 1975.[16] Ethical approval was obtained from the local ethics committee (#20-632) and privacy rights of blood donors were observed at all times. After subject briefing and obtaining informed consent, venous blood was drawn from the forearm of healthy participants ($n = 8$, *age* 40 ± 12 years) into 2×9 mL lithium-heparinized tubes.

2.2 | *Ex vivo* whole blood incubation assay

Lithium-heparin anticoagulated whole blood was diluted with an equal volume of RPMI 1640 (Sigma-Aldrich, Steinheim, Germany) cell culture medium and stimulated with one of the following stimuli: Heat-killed Listeria monocytogenes (HKLM; 10^8 cells/mL; InvivoGen Europe, Toulouse, France), Lipopolysaccharide (LPS; 10 ng/mL; Sigma-Aldrich, Steinheim, Germany), or Pokeweed mitogen (PWM; 5 μg/mL; Sigma-Aldrich, Steinheim, Germany). For negative control, stimulation was performed with cell culture medium only (basal). Samples were incubated for 6 and 24 h at 37°C in 1 g, s-μg or hypergravity of 2.8 g. Following incubation, supernatants were collected and stored at −80°C until cytokine measurement. The remaining sample was conserved with Transfix (Cytomark, Buckingham, UK) and stored at 4°C until flow cytometry analyses.

2.3 | Microgravity simulation and hypergravity induction

For achieving simulated microgravity (s-μg) a pipette-clinostat was used, capable to rotate ten 1 mL pipettes (Sarstedt, Nümbrecht, Germany) at once along their

horizontal axis at 60 rpm. Pipettes were filled with 1 mL of blood/antigen suspension and directly transferred to the clinostat, or incubated under static control conditions at 1 g. Under the chosen clinostat set-up, the residual acceleration acting on the cells was less than ≤0.006 g.[17] All experiments were carried out in an incubator at 37°C. Hypergravity of 2.8 g was achieved by centrifugation with the Multi-Sample Incubation Centrifuge (MuSIC) in 1 mL Nunc cryo tubes (Thermo Fisher Scientific nunc, Waltham, MA, USA). The magnitude of hypergravity corresponds to the gravitational difference between μg and hypergravity in PF (1.8 g) when referring to 1 g as starting point for these experiments. Both the clinostat and the MuSIC platforms were used at the Institute of Aerospace Medicine, DLR Cologne within the scope of an ESA Ground-Based Facility (GBF) project.

2.4 | Immunophenotyping

For monocyte surface activation marker analysis, leukocytes were stained for co-expression of CD14 and CD69, HLA-DR, TLR2, TLR4, CD40, CD80, CD86, or CD11b. For T cells, CD4$^+$ or CD8$^+$ cells were stained for co-expression of CD69, CD28, and PD-1. Except for TLR2 (Cat. No. 130-120-052, Miltenyi Biotec, Bergisch Gladbach, Germany) and TLR4 (FAB6248C, R&D Systems, Minneapolis, MN, USA), all antibodies were obtained from BD Biosciences (Cat. No. CD4: 555346, CD8: 557085, CD69-PE: 555531, HLA-DR: 559866, PD-1: 558694, CD28: 337181, CD14: 345784, CD69-PerCp: 340548, CD40: 555589, CD80: 557227, CD86: 555665, CD11b: 550019, Franklin Lakes, NJ, USA). Samples and antibodies were incubated for 20 min at room temperature, lysed for 10 min (BD FACS lysing solution, BD Biosciences Franklin Lakes, NJ, USA), washed with PBS, and analyzed by flow cytometry (Guava® easyCyte™ 8HT Flow Cytometer, Merck Millipore, Billerica, MA, USA). For each measurement, 10 000 events were recorded. Data analysis was performed with InCyte Software for Guava® easyCyte HT Systems (Merck Millipore, Billerica, MA, USA). Representative dot plots demonstrating the gating strategy of T cells are shown in Supporting Information Figure S1.

2.5 | Cytokine measurements

Cytokine concentrations were quantified from thawed *ex vivo* incubation assay supernatants using the MAGPIX Multiplexing System (Luminex, Austin, TX, USA) and custom-made Multiplex assays (Merck Millipore, Billerica, MA, USA) for detection of IL-1α, IL-1β, IL-6, IL-10, IL-18, TNF, and IFNγ according to the manufacturer's instructions.

2.6 | Statistical analyses

Data analysis was performed with commercially available software (SigmaPlot 12.5, Systat, Erkrath, Germany). Unless otherwise stated, results are expressed as median (IQR). For comparison between two groups, two-tailed unpaired Student's *t* test or Mann–Whitney *U* test were used. A value of $p < .05$ was considered statistically significant.

3 | RESULTS

In this study it was tested, whether exposure to hypergravity is capable to counteract dysregulated immune responses which might be induced by s-μg. Therefore, at first the distinct effects of single s-μg or hypergravity (2.8 g) on antigen-induced monocyte and T cell activation as well as cytokine response were elaborated. Subsequently, the effect of hypergravity on a potential s-μg-induced immune dysregulation was tested in three different exposure sequences.

3.1 | Effects of separate s-μg and hypergravity on monocyte and T cell activation

Activation state of cells was assessed by analysis of cell surface marker expression after *ex vivo* whole blood stimulation with HKLM, LPS and PWM in 1 g, s-μg or hypergravity of 2.8 g for 6 and 24 h. For monocytes, surface expression of CD69 (cell activation), CD40, CD80, CD86 (interaction with adaptive immune cells), HLA-DR (antigen presentation), TLR2/4 (pattern recognition receptors), and CD11b (activation/cell adhesion) was determined as well as CD69 and CD28 (cell activation) and PD-1 (exhaustion marker) on CD4$^+$ and CD8$^+$ T cells.

In monocytes, antigen incubation for 6 h at 1 g induced an increase in surface expression of CD69 and CD40 by LPS and PWM and of CD80 and TLR4 by all three antigens as well as a reduction of CD11b by LPS. These changes in expression intensity were maintained in samples incubated in s-μg and in addition, while a further increase was observed for CD69 and CD40 by HKLM in comparison to the basal s-μg control, HLA-DR expression was reduced by LPS as well as TLR2 by HKLM and LPS and CD11b by PWM under s-μg exposure. Here, no surface markers displayed significant differences in expression between 1 g and s-μg, however, data indicate that the effect of antigens

is more pronounced in s-µg than in 1g. Especially surface expression levels of CD80 were clearly enhanced after antigen incubation in s-µg compared to the respective 1g control. Antigen incubation in hypergravity did not further change surface marker expression (Table 1). These effects were not remarkably different after 24h incubation (Supporting Information Table S1).

On CD4$^+$ and CD8$^+$ T cells, expression of the activation marker CD69 was not altered by s-µg in basal controls (for CD4$^+$ at 1g: 1.2% (0.8–2.5); at µg: 1.0% (0.7–2.7), $p = .841$ and for CD8$^+$ at 1g: 1.8% (1.3–2.4); at µg: 1.7% (1.0–1.9), $p = .340$). However, in comparison to the 1g control, incubation with HKLM and PWM in s-µg resulted in significantly decreased CD69 mean expression levels on CD4$^+$ T cells by 48.6% and

TABLE 1 Cell surface marker expression on CD14$^+$ monocytes after 6h basal control, HKLM, LPS and PWM incubation in 1g, s-µg or hypergravity at 2.8g.

CD14	Basal		HKLM		LPS		PWM	
	1g	s-µg	1g	s-µg	1g	s-µg	1g	s-µg
CD69	4.42 (3.3–5.2)	3.8 (1.7–8.5)	10.0 (4.4–22.5)	**10.8**[#b] (10.1–12.7)	**20.6**[##b] (7.5–35.8)	**18.0**[#a] (6.8–22.6)	**22.8**[##b] (10.8–32.1)	**11.8**[#a] (6.1–23.4)
HLA-DR	93.6 (80.1–95.7)	95.2 (88.9–97.7)	82.4 (73.0–91.1)	94.5 (84.7–94.9)	88.5 (60.7–89.9)	**85.0**[#b] (70.3–90.0)	81.7 (76.1–85.4)	87.1 (71.2–96.3)
TLR2	96.5 (94.0–98.0)	**99.0**[*b] (98.4–99.2)	97.5 (94.6–98.6)	**96.6**[#b] (94.1–98.3)	94.0 (88.1–96.3)	**96.8**[#a] (87.2–98.1)	94.1 (88.3–96.1)	97.3 (91.6–98.7)
TLR4	14.6 (10.5–17.8)	15.1 (7.2–22.2)	**24.6**[#a] (20.4–44.7)	**30.8**[#a] (21.5–35.7)	**31.5**[#a] (21.6–55.1)	**39.5**[##a] (27.5–49.3)	**35.9**[#b] (18.1–43.9)	**29.8**[#a] (21.7–45.4)
CD40	6.7 (5.2–7.6)	4.0 (2.8–10.8)	14.1 (4.9–30.0)	**14.3**[#b] (10.6–29.1)	**15.0**[##b] (10.9–33.0)	**23.7**[#a] (10.1–33.2)	**16.2**[#a] (12.7–35.8)	**19.9**[#a] (7.6–30. 5)
CD80	7.4 (3.8–13.2)	8.1 (3.9–11.6)	**19.6**[#a] (14.7–41.1)	**38.5**[##b] (20.8–53.0)	**44.2**[###a] (32.2–50.2)	**56.7**[###a] (46.5–63.0)	**35.3**[###a] (33.6–51.4)	**47.2**[###a] (41.3–59.1)
CD86	92.7 (82.6–96.0)	90.5 (85.8–96.0)	94.2 (62.1–95.0)	91.5 (87.7–95.2)	81.0 (63.0–91.4)	93.2 (84.7–95.6)	88.8 (72.4–93.4)	92.8 (82.2–96.8)
CD11b	95.2 (90.3–96.8)	98.3 (96.0–98.8)	91.9 (87.7–98.6)	94.7 (92.9–98.7)	**91.1**[#a] (86.3–91.7)	**92.0**[#a] (83.5–95.3)	91.2 (89.5–92.8)	**92.7**[#a] (88.0–97.1)
	1g	**2.8g**	**1g**	**2.8g**	**1g**	**2.8g**	**1g**	**2.8g**
CD69	5.0 (1.3–10.1)	3.0 (2.2–11.2)	8.1 (5.5–14.6)	10.9 (7.2–14.9)	4.1 (3.6–11.6)	6.1 (3.4–13.8)	6.4 (5.9–15.0)	2.9 (2.2–13.1)
HLA-DR	90.7 (90.6–97.1)	97.4 (90.2–98.2)	**88.2**[#b] (65.8–93.2)	92.6 (79.9–94.5)	87.3 (82.5–94.7)	89.2 (81.2–95.6)	86.9 (86.1–91.8)	**89.6**[#a] (88.5–92.5)
TLR2	97.7 (97.1–99.1)	99.0 (96.7–99.3)	94.9 (83.6–98.4)	96.9 (94.5–98.9)	95.9 (90.5–98.3)	97.1 (92.3–99.2)	**92.9**[##a] (91.7–96.2)	98.8 (94.7–99.9)
TLR4	14.5 (8.5–17.6)	15.2 (9.4–25.0)	26.6 (10.8–34.7)	23.3 (9.0–34.0)	**23.4**[#a] (14.0–37.6)	25.9 (15.9–37.9)	**22.4**[#a] (16.7–33.7)	23.4 (14.1–37.5)
CD40	5.9 (0.6–8.8)	4.3 (2.1–10.4)	**11.8**[#a] (6.1–21.0)	**10.9**[#a] (9.2–19.8)	7.4 (5.7–16.3)	9.7 (4.3–15.8)	**9.0**[#b] (8.1–17.4)	3.2 (1.7–15.9)
CD80	5.0 (2.3–9.1)	4.7 (4.2–9.5)	**20.2**[##a] (10.6–22.5)	**17.4**[#b] (9.3–26.1)	**30.4**[###a] (28.8–46.5)	**24.0**[#b] (14.4–47.2)	**26.7**[###a] (24.5–38.6)	**25.6**[##b] (16.0–34.2)
CD86	87.8 (76.8–94.0)	91.6 (89.1–92.4)	87.4 (63.0–90.5)	88.5 (83.0–91.4)	88.3 (64.3–92.7)	90.0 (71.6–90.5)	89.9 (78.3–97.2)	88.1 (82.0–92.8)
CD11b	94.9 (93.5–97.1)	97.3 (94.5–98.7)	95.6 (93.3–96.8)	96.9 (94.2–99.8)	93.4 (88.2–96.7)	94.3 (85.4–96.9)	95.4 (92.1–98.3)	95.5 (94.3–97.1)

Note: Values are given as median (IQR) and represent percentages of surface marker positive cells of total CD14$^+$ monocytes ($n = 5$). Differences between 1g and g-grade (*) or antigen and basal control (#) were calculated using two-tailed unpaired Student's t test ([a]) or Mann–Whitney U test ([b]). Significant differences are indicated in bold font. *$p < .05$. #$p < .05$.

##$p < .01$.

###$p < .001$.

44.3%, respectively. On CD8$^+$ T cells, CD69 increase was downregulated in s-µg by 40.9% with HKLM, 40.0% with LPS, and 40.3% with PWM stimulation (Figure 1).

Expression levels of CD69 were not affected by hypergravity (Table 2). Surface expression analysis of the activation marker CD28 and the exhaustion marker PD-1 revealed almost any changes, neither by antigen incubation nor by altered g-grades (Supporting Information Table S2). Same as for monocytes, the observed effects were not altered after 24 h incubation (data not shown).

3.2 | Cytokine pattern after whole blood stimulation in separate s-µg and hypergravity

To assess the impact of s-µg and mild hypergravity on antigen-induced cytokine secretion, a panel of pro- and anti-inflammatory cytokines reflecting a multifunctional immune response was measured.

After 6 h, basal control incubation in both s-µg and hypergravity did not affect cytokine concentrations (Table 3). Antigen incubation in 1 g resulted in significant increase of IL-1α, IL-1β, IL-6, IL-10, TNF, and IFNγ concentrations. Secretion of IL-18 was not affected by antigens (Table 4). Concentrations of the pro-inflammatory monocyte-specific cytokines IL-1β and IL-18 as well as the innate immune cytokines IL-1α, IL-6, and TNF were clearly increased when antigen stimulation was performed in s-µg, with reaching statistical significance for IL-1β and TNF after PWM stimulation. Levels of antigen-induced concentrations of the anti-inflammatory cytokine IL-10 were unaffected by s-µg (Figure 2A, Table 4). Six hours antigen incubation in hypergravity resulted in reduced cytokine concentrations compared to 1 g with significant differences for IL-18 after PWM and for TNF after stimulation with HKLM and LPS (Figure 2B, Table 4). The striking differences in concentration between sample sets applying s-µg (with respective control) and hypergravity (with respective
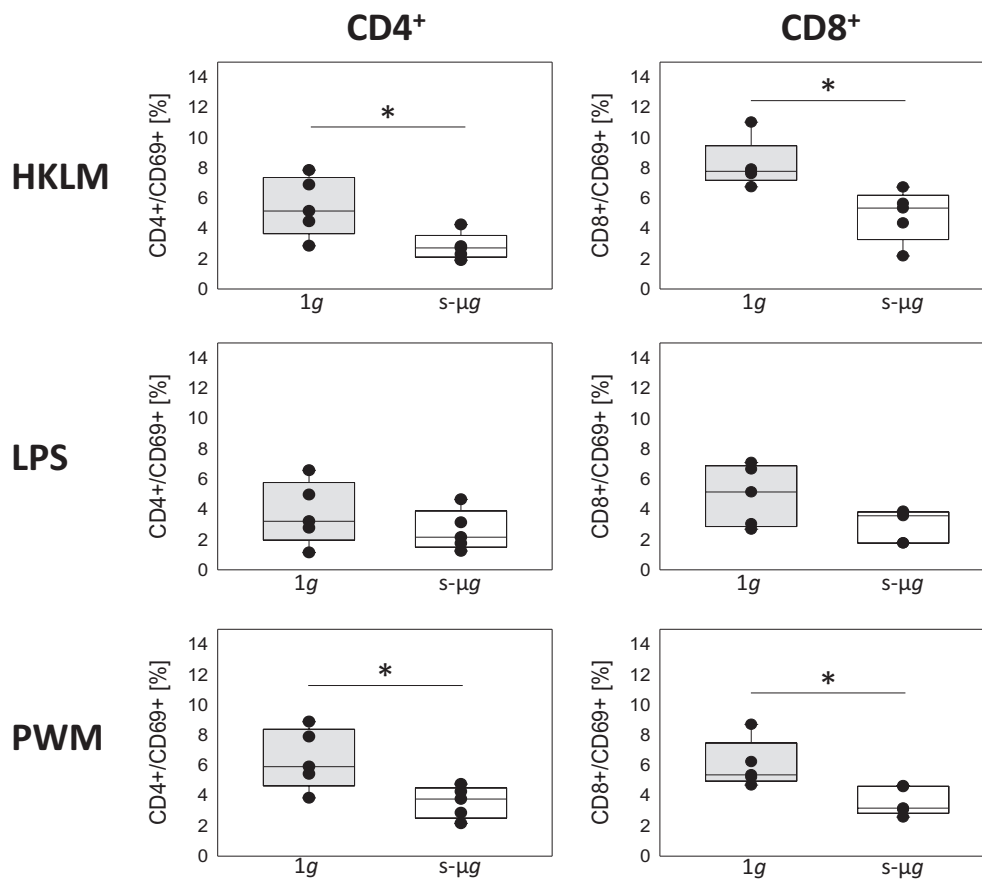
FIGURE 1 CD69 expression on the cell surface of CD4$^+$ and CD8$^+$ T cells after 6 h HKLM, LPS, or PWM incubation in 1 g (co., gray box) and s-µg (white box). Values represent percentages of CD69$^+$ cells of total CD4$^+$ or CD8$^+$ T cells. Boxes indicate median and interquartile range; whiskers represent minimum and maximum, dots represent single values ($n = 5$). Differences between groups were calculated using unpaired two-tailed t test, $*p < .05$.

TABLE 2 CD69 expression on CD4$^+$ and CD8$^+$ T cells after 6 h basal control, HKLM, LPS, and PWM incubation in 1 g or hypergravity at 2.8 g.

	CD4$^+$ T cells		CD8$^+$ T cells	
	1 g	**2.8 g**	**1 g**	**2.8 g**
Basal	0.9 (0.7–2.1)	1.2 (0.7–1.6)	2.1 (1.2–2.5)	1.6 (1.3–2.2)
HKLM	3.0 (1.6–3.6)	2.4 (1.9–3.0)	4.6 (2.6–6.1)	4.7 (3.2–5.6)
LPS	1.3 (1.1–2.4)	2.0 (0.9–2.5)	2.1 (1.8–3.9)	2.5 (2.2–3.2)
PWM	4.2 (2.1–5.1)	2.7 (2.3–4.4)	3.0 (2.4–4.2)	3.6 (3.1–3.9)

Note: Values are given as median (IQR) and represent percentages of surface marker positive cells of total CD4$^+$ or CD8$^+$ T cells ($n = 5$).

TABLE 3 Cytokine concentrations in supernatants after 6 h basal control incubation in 1 g, s-µg or hypergravity at 2.8 g.

	1 g	**s-µg**	**1 g**	**2.8 g**
IL-1α	4.8 (3.4–43.5)	2.5 (1.4–9.0)	6.5 (2.5–9.0)	2.5 (0.8–6.0)
IL-1β	4.2 (0.1–251.7)	2.3 (0.2–5.1)	0.8 (0.1–29.6)	0.1 (0.1–12.4)
IL-6	9.5 (4.0–240.9)	13.4 (2.2–18.1)	1.7 (1.1–19.4)	1.7 (0.2–13.9)
IL-10	11.7 (4.6–52.9)	11.7 (2.8–31.1)	2.1 (1.1–9.5)	3.6 (0.4–3.6)
IL-18	10.1 (0.2–16.7)	0.2 (0.2–9.3)	7.2 (0.2–19.7)	0.2 (0.2–11.1)
TNF	38.1 (17.6–272.5)	29.4 (19.1–126.1)	26.5 (22.3–86.8)	74.8 (35.9–880.9)
IFNγ	7.0 (2.6–11.8)	10.9 (0.6–30.0)	0.2 (0.2–3.6)	0.2 (0.2–4.5)

Note: Concentrations (pg/mL) are given as median (IQR) ($n = 5$).

control) is attributed to the different reaction tubes that had to be used for performing experiments under these two gravitational conditions.

Concentration of the pro-inflammatory T cell-specific cytokine IFNγ was decreased in s-µg, however, without reaching statistical significance (Figure 2A). Six hours incubation in hypergravity did not affect concentration levels (Figure 2B). An incubation time of 24 h did not further change cytokine concentrations (data not shown).

Altogether, data indicate an increase in monocytic pro-inflammatory response in s-µg and a tending decrease in hypergravity at 2.8 g. Moreover, CD4$^+$ and CD8$^+$ T cells displayed reduced activation capacities in s-µg. Because of high inter-individual variabilities within the study subjects, majority of the data failed to reach statistical significance.

3.3 | Effect of hypergravity on s-µg-mediated immune dysregulations

To test, if hypergravity is capable to counteract s-µg-mediated immune dysregulations, whole blood stimulation was performed for 4 h in s-µg with additional 2 h hypergravity sequences at three different phases of antigen incubation. To explore gravitational preconditioning of leukocytes, hypergravity was applied before s-µg (sequence 1 [Seq1]). Moreover, two therapeutic approaches were tested with hypergravity being applied intermediately (sequence 2 [Seq2]) or at the end (sequence 3 [Seq3]) of incubation (Figure 3). Samples that were incubated at 1 g served as control.

3.4 | Monocyte and T cell activation

Expression of CD69 on the cell surface of monocytes was significantly enhanced in basal control samples after all three treatment sequences compared to the 1 g control. Within Seq1 and Seq2, HKLM treatment likewise resulted in significant increase in CD69 expression compared to the 1 g control. PWM had only slight impact on CD69 surface expression with significant differences in Seq3. Surface expression of HLA-DR was significantly decreased after HKLM incubation at Seq1 and Seq2 and TLR2 expression levels were reduced after basal control stimulation within Seq2. TLR4 expression was increased after HKLM incubation at Seq1. CD40 surface expression was increased in basal and HKLM samples at Seq2, indicating an only marginal effect of hypergravity in this setting, especially by Seq2. Surface expression levels of CD80, CD86, and CD11b were not changed by the different gravitational exposure sequences (Table 5).

Same as for single s-µg or hypergravity exposure, expression levels of CD28 and PD-1 on CD4$^+$ and CD8$^+$ T cells were not altered by antigens or gravitational alterations (data not shown). However, compared to the 1 g control, PWM stimulation within Seq2 resulted in significantly reduced mean expression levels of CD69 on CD4$^+$ T cells by 54.1% and within Seq2 (48.6%) and Seq3 (44.4%) in CD8$^+$ T cells, indicating a maintained reduction of activation capacity in these cells by s-µg. Seq1, which encompassed the gravitational preconditioning approach revealed an antigen-induced increase in CD69 expression levels, which were comparable to the 1 g control (Figure 4).

3.5 | Cytokine pattern

Concentration levels of the pro-inflammatory cytokine IL-1α were significantly increased after HKLM incubation

TABLE 4 Cytokine concentrations in supernatants after 6 h HKLM, LPS, and PWM incubation in 1 g, s-µg or hypergravity at 2.8 g.

pg/mL	HKLM		LPS		PWM	
	1 g	s-µg	1 g	s-µg	1 g	s-µg
IL-1α	**780.8**##b (277.2–1653.9)	**1352.1**##b (556.0–2223.7)	**1447.6**##b (572.0–2207.3)	**2835.5**##b (1636.5–3422.2)	**1114.6**##b (543.2–1912.6)	**2090.4**##b (841.0–2888.7)
IL-1β	**3997.8**##b (2639.4–5799.4)	**7521.1**##b (3053.4–11 917.5)	**3346.1**##b (2494.4–6106.3)	**8733.4**##b (3506.6–12 708.6)	**3207.0**###a (2528.2–3589.6)	**7279.8***a, ##b (3998.6–7921.1)
IL-6	**13 017.3**##b (10 378.2–16 651.2)	**14 744.0**##b (9959.7–20 224.2)	**19 219.7**##b (11 125.9–24 232.9)	**24 333.9**##b (13 862.9–32 610.0)	**17 742.3**##b (8232.2–20 126.8)	**22 022.5**##b (11 156.3–24 385.1)
IL-10	**368.3**##a (211.3–642.0)	**262.0**#a (207.8–677.3)	**746.2**##b (414.1–981.4)	**703.6**##b (347.7–942.8)	**483.5**##b (377.9–599.5)	**445.6**##b (234.9–662.5)
IL-18	29.8 (4.1–53.8)	4.8 (3.5–76.2)	6.3 (0.2–30.4)	**11.5**#b (6.1–53.3)	7.2 (0.2–29.2)	**25.8**#b (8.3–53.3)
TNF	**6893.6**#a (1399.2–7925.9)	**3580.1**##b (1626.3–6111.5)	**722.3**#b (367.8–3027.0)	**3068.9**##b (1585.1–5283.8)	**745.5**#a (574.6–1968.6)	**4685.5***a, ##b (2309.2–5303.2)
IFNγ	**148.1**##b (101.5–184.3)	**96.0**##b (58.1–129.0)	**317.2**##b (154.4–517.0)	**148.1**##b (109.7–397.4)	**87.7**##b (54.8–131.7)	**73.0**###a (58.1–92.3)
	1 g	2.8 g	1 g	2.8 g	1 g	2.8 g
IL-1α	**252.1**##b (146.0–653.2)	**217.6**##b (100.0–638.4)	**640.7**##b (168.9–867.3)	**125.8**##b (50.3–500.1)	**608.5**##b (164.0–1121.2)	**194.8**#b (23.8–446.8)
IL-1β	**1386.6**##b (1287.9–2554.3)	**917.9**##b (896.5–1972.1)	**1662.9**##b (700.8–2765.7)	**696.2**#a (216.5–1972.9)	**1419.6**##b (360.5–2799.0)	**620.8**##b (148.3–1752.3)
IL-6	**2609.3**##b (1337.4–7759.3)	**1204.1**##b (767.6–7167.3)	**9411.9**##b (2527.6–15 179.4)	**3297.2**##b (1373.9–5720.5)	**4860.3**##b (1552.3–16 315.4)	**896.8**##b (474.4–7094.3)
IL-10	**90.1**##b (48.4–202.4)	**74.6**##b (36.4–160.2)	**488.2**##a (132.9–505.7)	**302.6**##b (78.7–537.7)	**175.9**##a (140.4–360.7)	**129.0**##a (61.7–156.0)
IL-18	14.9 (7.0–36.1)	0.2 (0.2–10.5)	5.8 (0.2–23.5)	0.2 (0.2–8.5)	20.8 (10.0–32.3)	**0.2***a (0.2–9.4)
TNF	**4350.7**##b (1547.6–4517.3)	**365.4***a (107.7–1246.3)	**2006.4**##b (808.1–3035.3)	**334.1***b (288.2–673.3)	**2975.8**##b (733.0–4234.3)	331.5 (314.6–392.4)
IFNγ	**46.0**##a (40.4–108.8)	**38.5**##b (36.6–183.0)	**106.1**#a (65.1–632.7)	**139.0**#a (29.0–715.7)	**127.1**#a (24.3–174.3)	**71.1**#b (13.7–172.0)

Note: Concentrations (pg/mL) are given as median (IQR) ($n = 5$). Differences between 1 g and g-grade (*) or antigen and basal control (#) were calculated using two-tailed unpaired Student's t test (^{a}) or Mann–Whitney U test (^{b}). Significant differences are indicated in bold font. *$p < .05$. #$p < .05$.
##$p < .01$.
###$p < .001$.

at Seq1 and 3 and after PWM incubation at Seq1, 2, and 3 compared to the 1 g control. For IL-1β, concentrations were higher in basal control and after HKLM and PWM incubation after all three sequences in comparison to the 1 g control, reaching statistical significance for PWM after Seq1 and Seq3 (Supporting Information Table S3), which was comparable to results of the preparatory experiments (Figure 2A). Concentrations of IL-6 were increased after PWM incubation at Seq1 and 3 (Supporting Information Table S3).

Concentration levels of the anti-inflammatory cytokine IL-10 were not affected after antigen incubation in s-µg compared to 1 g controls (Table 4). However, when performing whole blood incubation assays with HKLM and PWM in s-µg with either preconditioning or therapeutic hypergravity exposure, concentrations were significantly increased. One cytokine, for which hypergravity intervention approaches showed a mitigating impact on the s-µg-induced pro-inflammatory state was TNF. Here, values after PWM and HKLM stimulation were comparable in 1 g and within Seq1-3 (Supporting Information Table S3).

Together these data indicate that sequences of alternating s-µg and hypergravity have, except for TNF, no alleviating impact on the enhanced pro-inflammatory potential of monocytes. Moreover, the inflammatory response might be even increased.

The T cell-specific cytokine IFNγ was enhanced after PWM stimulation within all sequences, however, without reaching statistical significance (Figure 5, Supporting Information Table S3).
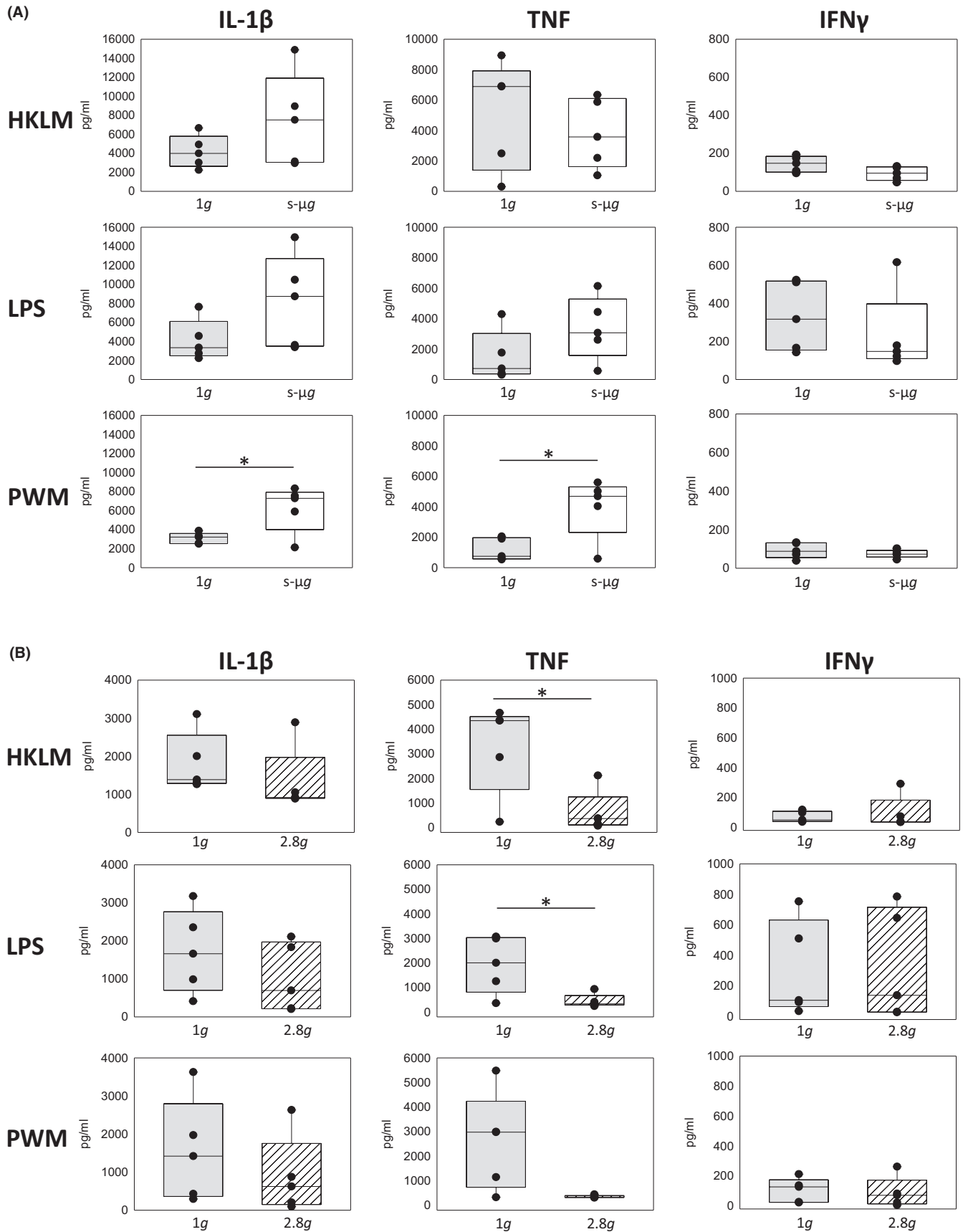
FIGURE 2 Cytokine concentrations (pg/mL) of IL-1β, TNF and IFNγ after 6 h incubation with HKLM, LPS, or PWM in s-μg (A, white box) or hypergravity (2.8 g) (B, dashed box) compared to 1 g control (gray box). Boxes indicate median concentrations and interquartile range; whiskers represent minimum and maximum, dots represent single values (n = 5). Differences between groups were calculated using unpaired two-tailed t test or Mann–Whitney U test, *p < .05.

Seq1: Preconditioning with hypergravity

Seq2: Intermediate hypergravity

Seq3: Hypergravity at the end
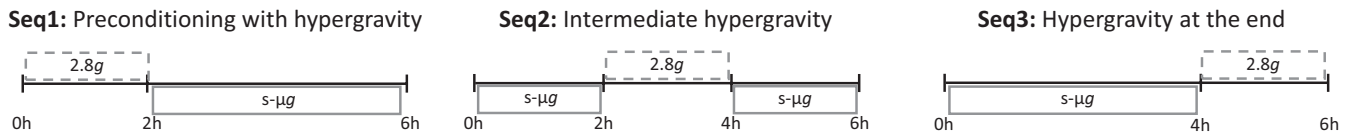
FIGURE 3 Scheme of different sequences to test counteracting functions of hypergravity (2.8 g) for s-μg-induced immune dysregulations. (Seq1) preconditioning by application of hypergravity prior to s-μg; Therapeutic action by application of hypergravity intermediately (Seq2) or at the end (Seq3) of s-μg.

In summary data from surface marker expression staining on monocytes and T cells as well as cytokine expression patterns indicate that a preconditioning or therapeutic application with hypergravity at 2.8 g does not influence the increased pro-inflammatory potential of monocytes which is induced by s-μg. However, T cell deficiencies regarding activation and cytokine secretion can be mitigated by preconditioning with hypergravity.

4 | DISCUSSION

Microgravity (μg) is a unique condition space crews are exposed to and regarded as the key stressor for alterations of immune system functional capacities.[1,2] Hereby T cells display reduced responsiveness to antigenic and mitogenic stimulation[3] whereas monocytes show an increased pro-inflammatory potential.[6] In view of envisaged future long-term explorations, the elaboration of suitable countermeasure strategies against deconditioning in space is urgently needed to ensure the crews′ health. One approach that has been extensively tested within the *Multiple Artificial-gravity Research System* (*MARS*) located in the Japanese Kibo module on the ISS is artificial gravity (AG). AG can be achieved by specialized centrifuges and encompasses both the restoration of Earth-like conditions of 1 g and the induction of hypergravity.[18] The restoration of 1 g on the ISS was reported to alleviate μg-induced detrimental effects on several physiological structures in mouse models, such as apoptosis of retina vascular endothelial cells[19] and thymic atrophy.[20] The application of hypergravity, that means mechanical loading, has shown promising effects on various physiological systems in μg and in Earth-bound approaches. In rodent models, mild hypergravity (2 g-4 g) reversed μg-induced bone and muscle loss and even increased bone density and muscle volume in normogravity (1 g),[21–23] indicating a therapeutic applicability of hypergravity in rehabilitation programs and age-related diseases such as osteoporosis and sarcopenia. These effects are mainly attributed to mechanical stimulation, which is required for osteogenesis[24] and the upregulation of bone formation- and myogenesis-related genes as well as downregulation of protein-degrading genes.[23] The implementation of hypergravity was also

discussed to have a potential relevance for the therapy of diabetes and neurodegenerative diseases.[25,26] In Uruguay, gravitational therapy with mild hypergravity (up to ~2.5 g) is applied for more than forty years to alleviate symptoms in patients with vascular-based pathologies such as coronary artery disease, lymphedema, and secondary Raynaud's phenomenon.[27] Despite these positive effects of mild hypergravity both on μg-mediated deconditioning and human diseases on Earth, the impact on the immune system is less explored. By now, the findings derive either from cell culture models or from long-term hypergravity exposure of rodents and the observed effects strongly depend on the experimental model and immune cell subset. Adrian et al. demonstrated in vitro in rat alveolar macrophages that hypergravity gradually enhances the production of zymosan-induced reactive oxygen species (ROS).[17] Conversely, in mouse lymphocytes a long-term exposure to mild hypergravity resulted in reduced responsiveness to LPS and Concanavalin A as well as decreased IFNγ and IL-2 secretion.[28] Another study using an ovalbumin (OVA)-induced asthma mouse model revealed reduced serum total and OVA-specific IgE levels, less immune cell infiltration and pro-inflammatory T$_h$2-cytokine production as well as less eosinophils in lung parenchyma, when allergy was induced under 5 g compared to 1 g.[29,30] These findings demonstrate the susceptibility of immune cells toward changes in g-grade in cell culture and mouse models.

Studies on primary human immune cells were conducted in PF in which μg and hypergravity alternate. Thereby, modulated signal transduction and activation capacities were identified in T cells[31] and polymorphonuclear leukocytes were shown to display enhanced stimulation-induced oxidative burst capacities *ex vivo* which was considered as immune cell priming.[32] In a whole blood stimulation approach, alternating g-forces in PF enhanced CD69 expression on monocytes and reinforced innate and adaptive pro-inflammatory cytokine secretion after antigen stimulation. Concomitantly characteristics of immunosuppression such as reduced HLA-DR or TLR2 on monocytes and increased anti-inflammatory cytokine levels were observed.[14] Together with findings of dysregulated immune responses in (simulated) μg[1,6,13] these various effects in PF corroborate the assumption

TABLE 5 Cell surface marker expression on CD14$^+$ monocytes after basal control, HKLM, and PWM incubation at 1 g, with preconditioning hypergravity (Seq1), therapeutical hypergravity intermediately (Seq2) or at the end of s-µg exposure (Seq3) (in total 4 h s-µg and 2 h hypergravity at 2.8 g).

		1 g control	Seq1	Seq2	Seq3
Basal	CD69	1.7 (1.3–2.1)	**3.4**[*b] (2.0–4.2)	**5.0**[*b] (1.9–10.7)	**3.4**[**b] (2.7–6.7)
	HLA-DR	84.7 (77.1–88.0)	81.1 (69.0–88.9)	77.7 (69.4–93.0)	76.4 (73.7–86.2)
	TLR2	97.3 (95.8–98.3)	95.6 (88.3–97.9)	**89.6**[**a] (86.0–94.0)	94.2 (89.8–97.0)
	TLR4	14.3 (12.3–17.2)	17.0 (12.6–21.2)	21.3 (15.4–26.7)	19.0 (10.6–24.7)
	CD40	2.8 (1.8–3.7)	4.9 (1.7–8.0)	**8.5**[**b] (4.5–18.8)	5.0 (2.9–9.9)
	CD80	7.0 (5.2–11.9)	6.9 (3.9–13.2)	6.5 (4.0–15.2)	9.0 (6.6–14.9)
	CD86	74.1 (70.5–78.1)	75.2 (64.6–81.7)	84.6 (61.0–90.0)	74.0 (66.2–80.4)
	CD11b	91.7 (85.0–94.5)	88.6 (78.8–94.4)	92.2 (86.6–96.9)	89.2 (78.1–92.6)
HKLM	CD69	13.0 (9.1–14.3)	**24.3**[**a] (19.5–32.6)	**17.4**[*a] (15.5–28.1)	20.0 (11.4–22.9)
	HLA-DR	58.7 (46.7–65.8)	**42.6**[*a] (36.9–46.6)	**32.9**[*a] (27.0–55.4)	44.5 (36.7–55.5)
	TLR2	91.7 (86.0–93.7)	87.0 (83.2–92.3)	88.8 (84.4–93.8)	89.9 (87.7–95.3)
	TLR4	33.2 (29.7–44.3)	**50.6**[*a] (45.8–57.2)	44.6 (32.1–57.3)	38.3 (31.5–45.9)
	CD40	18.0 (13.0–25.3)	33.3 (15.5–58.5)	**33.6**[*a] (25.0–53.3)	27.0 (21.9–31.1)
	CD80	36.6 (17.1–42.8)	40.7 (17.4–57.9)	36.2 (27.0–42.0)	31.1 (27.0–34.6)
	CD86	59.5 (49.5–71.9)	55.7 (31.4–71.9)	39.7 (30.4–56.9)	54.9 (49.0–63.5)
	CD11b	88.2 (83.9–93.5)	86.4 (78.3–89.7)	85.1 (65.5–92.3)	86.3 (76.0–90.1)
PWM	CD69	6.0 (4.5–7.6)	9.5 (6.2–10.7)	7.2 (5.5–11.5)	**10.7**[*a] (8.2–18.2)
	HLA-DR	69.3 (62.3–76.4)	64.8 (46.3–71.5)	56.1 (43.3–76.4)	59.1 (53.2–75.6)
	TLR2	94.1 (87.6–97.0)	91.0 (81.3–92.4)	83.8 (79.8–93.1)	93.9 (77.9–96.8)
	TLR4	34.0 (23.1–37.0)	31.5 (27.2–48.6)	30.2 (21.5–39.2)	31.7 (22.9–37.8)
	CD40	10.3 (6.6–17.4)	19.0 (8.3–24.8)	13.1 (9.6–18.3)	14.4 (13.2–28.1)
	CD80	50.7 (37.8–57.9)	36.5 (24.3–54.4)	36.1 (28.0–43.5)	43.1 (38.7–48.6)
	CD86	69.0 (60.9–74.7)	60.1 (33.2–74.7)	44.7 (41.2–61.7)	60.0 (54.2–69.2)
	CD11b	84.4 (79.9–90.9)	84.9 (75.4–91.4)	85.0 (68.1–88.9)	84.6 (72.5–86.8)

Note: Values are given as median (IQR) and represent percentages of surface marker positive cells of total CD14$^+$ monocytes ($n = 6$). Differences between 1 g and sequences were calculated using two-tailed unpaired Student's t test ([a]) or Mann–Whitney U test ([b]). Significant differences are indicated in bold font.
*$p < .05$;; **$p < .01$.
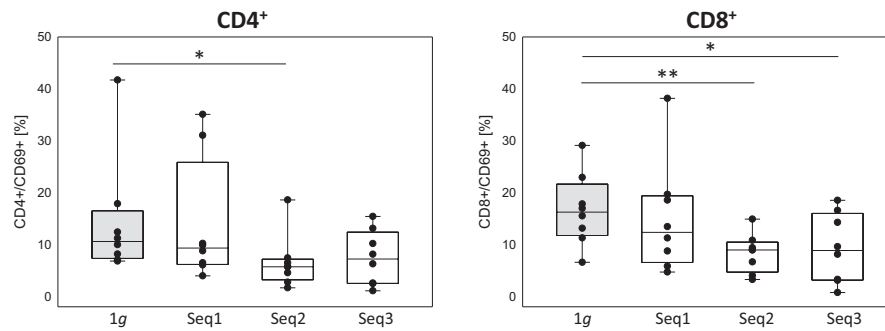
FIGURE 4 CD69 expression on the cell surface of CD4$^+$ and CD8$^+$ T cells after PWM incubation in s-µg (white boxes) and preconditioning with hypergravity (Seq1), therapeutic intermediate hypergravity (Seq2) or at the end of s-µg (Seq3) and 1g control (gray box) (in total 4 h s-µg and 2 h hypergravity at 2.8 g). Values represent percentages of CD69$^+$ cells among total CD4$^+$ or CD8$^+$ T cells. Boxes indicate median and interquartile range; whiskers represent minimum and maximum, dots represent single values ($n = 8$). Differences between groups were calculated using unpaired two-tailed t test or Mann–Whitney U test, $*p < .05$, $**p < .01$.
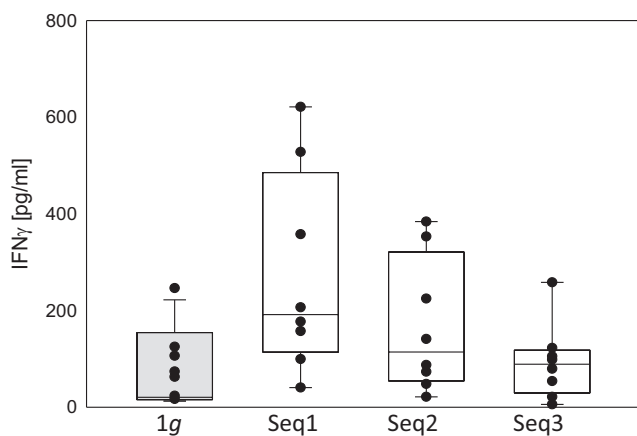
FIGURE 5 Cytokine concentrations (pg/mL) of IFNγ after incubation with PWM at Seq1 (hypergravity preconditioning), Seq2 (intermediate hypergravity) or Seq 3 (hypergravity at the end) (white boxes) compared to 1g control (gray box) (in total 4 h s-µg and 2 h hypergravity at 2.8 g). Boxes indicate median concentrations and interquartile range; whiskers represent minimum and maximum, dots represent single values ($n = 8$).

that an application of mild hypergravity is capable to counteract immune dysfunctions which may occur in µg.

In the present study incubation assays using human whole blood were performed in s-µg, hypergravity of 2.8 g, as well as under alternating s-µg and hypergravity and the resulting immune cell activation state and cytokine response was analyzed. In monocytes, s-µg slightly enhanced antigen-mediated alterations in activation cell surface marker expression levels. Cytokine levels revealed an increase in the monocyte-specific IL-1β as well as of IL-1α and TNF in s-µg compared to antigen incubation in 1g, which is in accordance with previous reports of increased innate immune cell cytokine secretion when antigen incubation is performed in s-µg[13] and confirms an overall increased pro-inflammatory potential of monocytes in µg.

Hypergravity on the other side resulted in decreased concentrations of IL-1β and TNF, indicating less pro-inflammatory potential of monocytes under this condition. The exact mechanisms leading to this divergence in cytokine secretion under stimulation in s-µg or hypergravity are yet unknown, and definitely worth being studied. For adhesion-dependent cytokine production, the actin cytoskeleton was shown to be crucial.[33,34] Gravitational alterations were shown to induce structural changes of the cytoskeleton in adherent cells,[35–37] however, analyses of such effects in suspension cells were, to the best of our knowledge, neglected thus far. The restructuring of the cytoskeleton in s-µg and hypergravity, as well as effects on signal transduction pathways and cytokine production shall be investigated in distinct immune cell subsets in future studies.

Both CD4$^+$ and CD8$^+$ T cells displayed reduced antigen-induced CD69 expression in s-µg, demonstrating an impaired activation of this immune cell subset. Concentration levels of the T cell cytokine IFNγ were reduced after antigen incubation in s-µg compared to 1g. Main reasons for reduced activation capacities may constitute remodeling of cytoskeletal components by s-µg[38] leading to an inhibited formation of immune synapses with antigen-presenting cells[39] and consequently reduced TCR activation.[38] In contrast to findings in mouse models[28–30] single hypergravity exposure had no effect on T cells with regard to the parameters analyzed.

4.1 | Mitigation of s-µg-induced immune changes by hypergravity

To investigate an alleviating effect of hypergravity on s-µg-mediated immune dysfunctions, three different sequences of alternating s-µg and hypergravity were tested. The first sequence included preconditioning which allows cells/

organs to adapt to subsequent more severe cellular stress (here gravitational stress) by an initial (gravitational, mechanical loading) insult and to confer tolerance, similarly to hypoxic, pharmacological, or ischemic preconditioning.[40] The other two approaches were therapeutic approaches with an intermediate hypergravity period or exposure to hypergravity at the end of s-μg.

Expression of CD69 on monocytes was significantly increased within all three sequences in basal controls. This is most probably due to the acute gravitational alterations which are known to enhance CD69 surface expression,[41] rather than to short-term exposure to either s-μg or hypergravity. Moreover, after HKLM incubation, expression of CD69 was higher than in 1g, whereas HLA-DR expression was reduced. This indicates that immune enhancing and dampening effects as seen prior in PF[14] are also rather caused by g-grade alterations than by one condition alone. In basal controls changes in g-grade induced an increase in IL-1α, IL-1β, IL-6, and TNF concentrations which reached due to high inter-individual variabilities for only few cytokines statistical significance. However, since this increase in concentration did not occur after single s-μg exposure in basal control samples, a pro-inflammatory reaction of innate immune cells caused by alternating g-forces is strongly assumed. After antigen incubation cytokine levels were mostly increased in all three sequences compared to incubation at 1g. Altogether these observations in a short-term in vitro model of 4 h s-μg and 2 h hypergravity of 2.8g lead to the conclusion that an application of mild hypergravity, irrespective at which phase of s-μg exposure has no mitigating effect on the enhanced pro-inflammatory potential of monocytes in s-μg. In addition, this intervention might even enhance monocyte responses. It cannot be ruled out that prolonged exposure times and stronger hypergravity intensities might show different effects. However, this shall be addressed in future investigations. In this context, levels of applied hypergravity should thereby not exceed mild hypergravity and stay in a range which is well tolerable for humans.

Analyses on T cell activation after PWM incubation revealed a different pattern. The two therapeutic approaches comprising hypergravity application intermittently or at the end of s-μg exposure did not have beneficial effects on T cell activation and CD69 expression levels were still significantly reduced compared to the 1g control. This indicates that the dampening impact of s-μg on T cell activation is pronounced to such a degree that it cannot be easily reversed by subsequent exposure to hypergravity. This is corroborated by the observation of only low activation capacities of resting T cells which were pre-exposed to s-μg before stimulation.[2] The implementation of the preconditioning approach (Seq1), however, resulted in a restoration of CD69 expression after antigen stimulation up to levels which were comparable to 1g controls. Moreover, concentration of IFNγ which was tendentially reduced in single s-μg reached remarkably enhanced concentration levels by Seq1. Altogether these results show that preconditioning with hypergravity has the capability to protect CD4$^+$ and CD8$^+$ T cell from μg-induced functional loss and consequently detrimental effects on health.

A potential human application of AG of 1g or hypergravity to counteract spaceflight-associated deconditioning onboard a spacecraft was discussed for at least two decades.[42] In 2011, the NASA engineers Edward Henderson and Mark Holderman conceptualized the rotating wheel space station NAUTILUS-X (Non-Atmospheric Universal Transport Intended for Lengthy United States eXploration) which was intended to be equipped with a centrifuge to counteract spaceflight-associated deconditioning.[43] However, this concept did not further advance beyond proposal. But still the idea of producing AG aboard a spacecraft has not yet been rejected and studies regarding efficiency, magnitude of g-load, and duration are ongoing. The present investigation has shown that hypergravity preconditioning prevents s-μg-induced loss of T cell activation. Thus, in view of the upcoming space tourism, hypergravity might be implemented as potential preventive approach. The high g-forces which act on the body during launch are in view of proportional duration obviously insufficient to achieve protection. In this context, beneficial effects of hypergravity preconditioning on other systems such as the cardiovascular or the musculoskeletal systems shall represent a topic in future studies. On Earth, gravitational therapy as it is already applied for vascular-based pathologies[27] might strengthen the adaptive immune system which might be advantageous for immune-depressed patients.

5 | LIMITATIONS

We acknowledge the limitations of these investigations targeting primary human cell culture, which does not allow drawing conclusions on long-term effects related to the short duration of the experiments. Investigations on a "second window of protection," which occurs 24–78 h after preconditioning was therefore also not possible, but could represent an in vitro cell culture research topic in the future. Moreover, to investigate the relationship of a cell-specific transcriptional and/or cytokine secretory effect transcriptomic alterations in separated leukocyte subsets would be needed to be assessed as a function of time, stimuli, and g-forces. These analyses might be then complemented with an in vitro approach including, for example, THP-1 and Jurkat cells to achieve more standardized conditions, to reduce deviations by inter-individual

variability and to exclude possible effects by other blood components. However, this study represents a promising proof of concept showing the potential of hypergravity as an immunological countermeasure option for (s-)µg-induced T cell dysregulations. A more detailed resolution of duration of hypergravity required for preconditioning has to be further elaborated in future studies.

AUTHOR CONTRIBUTIONS

Dominique Moser and Alexander Choukér designed the study. Katharina Biere, Dominique Moser, and Marina Tuschen performed experiments and data analysis, and collected the data. The article was drafted by Dominique Moser and Alexander Choukér, and critical revision for important intellectual content was performed by all authors (Dominique Moser, Katharina Biere, Christian Liemersdorf, Marina Tuschen, Ruth Hemmersbach, and Alexander Choukér). All authors contributed to the article and approved the submitted version.

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DISCLOSURES

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Supplementary information accompanies this manuscript and is attached as single files.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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