Signal Transduction in Primary Human T Lymphocytes in Altered Gravity During Parabolic Flight and Clinostat Experiments

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Key Words
T cell activation • Microgravity • Gravi-sensitivity • Hypergravity • Parabolic flight • Clinorotation

Abstract
Background/Aims: Several limiting factors for human health and performance in microgravity have been clearly identified arising from the immune system, and substantial research activities are required in order to provide the basic information for appropriate integrated risk management. The gravity-sensitive nature of cells of the immune system renders them an ideal biological model in search for general gravity-sensitive mechanisms and to understand how the architecture and function of human cells is related to the gravitational force and therefore adapted to life on Earth. Methods: We investigated the influence of altered gravity in parabolic flight and 2D clinostat experiments on key proteins of activation and signaling in primary T lymphocytes. We quantified components of the signaling cascade 1) in non-activated T lymphocytes to assess the “basal status” of the cascade and 2) in the process of activation to assess the signal transduction. Results: We found a rapid decrease of CD3 and IL-2R surface expression and reduced p-LAT after 20 seconds of altered gravity in non-activated primary T lymphocytes during parabolic flight. Furthermore, we observed decreased CD3 surface expression, reduced ZAP-70 abundance and increased histone H3-acetylation

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in activated T lymphocytes after 5 minutes of clinorotation and a transient downregulation of CD3 and stable downregulation of IL-2R during 60 minutes of clinorotation. **Conclusion:** CD3 and IL-2R are downregulated in primary T lymphocytes in altered gravity. We assume that a gravity condition around 1g is required for the expression of key surface receptors and appropriate regulation of signal molecules in T lymphocytes.

# Introduction

Beginning already in the early days of human spaceflight, an enhanced susceptibility to infections has been observed among astronauts [1–4], while initial evidence of disturbed cellular function appeared during investigations of lymphocytes from astronauts of the Soyuz and Skylab missions [5, 6]. The first clear evidence of cellular sensitivity to gravity was found during the first Spacelab-Mission, where it could be demonstrated that the proliferative response of lymphocytes to mitogenic stimulation in vitro was strongly impaired under space conditions [7]. Consequently, the impairment of lymphocyte activation and the resulting immune deficiency is considered as a serious limitation for manned long-term space flights [8]. Many studies have revealed strong effects of microgravity on immune cell function [9–11], which were performed during manned space flights, on board of orbital, suborbital (sounding rockets) and parabolic flights, and were supported by ground-based facilities for simulation of microgravity such as clinorotation and 3D-random positioning machines [12]. Gravity-sensitivity in T lymphocytes was found for the cell cycle regulation [13], epigenetic regulation [14], chromatin regulation [15], expression profile of microRNA [16], cell motility [17], regulation of apoptosis [18], expression of cytokines [19], and expression of early genes of T lymphocyte activation, which are regulated primarily by the transcription factors NF-κB, CREB, ELK, AP-1, and STAT [13, 20, 21]. Taken together, the phenomenon of reduced activation of T cells and alterations in several cellular processes during microgravity is well described. Nevertheless the exact molecular mechanisms of sensing gravitational changes are not elucidated.

The delivery of the first activation signal, the patching and capping of ConA-binding membrane proteins occurs normally in altered gravity [9]. At the same time, transcriptional regulation relevant in T cell activation is influenced by gravitational change (see above). Therefore the existence of gravity-sensitive cellular targets between TCR/CD3 and gene expression regulation can be assumed. In the current study we investigated the membrane proximal signaling cascade of T lymphocyte activation under the influence of altered gravity to assess whether these early events of T cell activation are responsive to altered gravity conditions. Such responsiveness could be an underlying mechanism for the transcriptional and functional reactions of T lymphocyte activation to altered gravity. We quantified selected components of the signaling cascade 1.) in non-activated T lymphocytes to assess the “basal status” of the cascade and 2.) in the process of activation to assess the signal transduction.

The quantified molecules were CD3, ZAP-70, LAT phosphorylated at tyrosine 171 (LAT (pY171)), acetyl-histone H3 and IL-2R, all indispensable mediators of T cell activation. The T cell antigen receptor (TCR) is a protein complex on the cell surface mediating T lymphocyte activation and consists of variable α and β chains non-covalently linked to the non-polymorphic CD3 proteins, dimers of γε, δε or ζζ chains. CD3 proteins transduce signals from the engaged TCR via phosphorylation of their immunoreceptor tyrosine-based activation motifs (ITAMs) located within their intracellular tails. Then, recruitment of CD3ζ-chain-associated protein kinase ZAP-70, a member of the protein-tyrosine kinase family, is followed by a cascade of phosphorylation events [22]. Among its targets are the transmembrane adapter protein linker for the activation of T cells (LAT), which contains nine tyrosines that are phosphorylated during T cell activation [23, 24]. LAT binds several other signaling molecules, one of them being the C-terminal SH2 domain of phospholipase
Cy1 (PLCγ1) [25], which regulates Ca$^{2+}$ and diacylglycerol (DAG)-induced responses, cytoskeletal rearrangements and integrin activation pathways. Signaling of IL-2 through IL-2R is required to enable lymphocytes to pass the G1 checkpoint when ConA activation of the TCR-CD3 complex and co-stimulation by anti-CD28 cause non-activated T cells to exit G0 phase and enter G1 phase of the cell cycle, a prerequisite for clonal expansion during immune response [26]. Activation of the IL-2R promotes also an activation cascade by recruitment of the adaptor Src homology 2 domain containing transforming protein 1 (SHC1), in which in turn activates the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, the latter including p44/42 MAPK (ERK1/2). T lymphocyte activation finally results in differential gene expression. This is not only mediated by transcription factor induction, but also by chromatin condensation and decondensation. Histones are highly post-translationally modified including the covalent attachment of methyl or acetyl groups to lysine and arginine amino acids and the phosphorylation of serine or threonine. Since recent findings suggest a possible role of histone acetylation in the transduction of gravitational effects [13, 14], we quantified acetyl-histone H3 in response to altered gravity.

We performed experiments on a parabolic flight during the 19th DLR parabolic flight campaign providing 22 s microgravity and used ground-based simulation of microgravity using a 2D-clinorotator providing 5-60 min simulated microgravity. The molecules of interest were analyzed by relative quantification using immunofluorescent staining and flow cytometry. Results were compared to experiments on board of the MASER-12 sounding rocket [27] providing 5 min of microgravity, which were conducted using the same cell type and activation- and fixation regimes and analyzed using the same method.

**Materials and Methods**

*Isolation of CD4⁺ primary human T lymphocytes*

For parabolic flight experiments and for clinorotation experiments CD4⁺ primary human T lymphocytes were used. Cells were isolated fromuffy coats obtained from the blood donation center Zurich ("Blutspende Zürich", Switzerland). Donors were healthy persons of either sex and between 33 and 65 years old. They gave their written consent that their blood or certain components may be used for research purposes. T lymphocytes were not pooled, but taken from single donors for each experimental setup. This was done to avoid uncontrolled lymphocyte activation. First, PBMCs were isolated using Ficoll density gradient centrifugation. Therefore, buffy coats were diluted with an equal volume of PBS (Biochrom, Berlin, Germany), layered on top of Ficoll-Paque PREMIUM (GE Healthcare, Solingen, Germany) and centrifuged for 30 min at 1500g without brake. The PBMC layer was collected from the interphase and washed with PBS. CD4⁺ T lymphocytes were isolated from PBMCs by positive magnetic bead selection using BD IMag Anti-Mouse CD4 Particles (BD Biosciences, Allschwill, Switzerland) as described in the manufacturer’s protocol. Isolated human CD4⁺ T lymphocytes were suspended in RPMI 1640 medium (Biochrom) supplemented with 10% FCS Superior (Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco®/Life Technologies, Zug, Switzerland) and cultured at 37°C in 5% CO₂ atmosphere for 3-4 days before performance of experiments.

*Parabolic flights as microgravity research platform*

During a parabolic maneuver, an aircraft is weightless by flying on a Keplerian trajectory, described as an unpropelled body in ideally frictionless space subjected to a centrally symmetric gravitational field [28]. During this free-fall trajectory, the resultant of all forces acting on the aircraft other than gravity is nulled. During a flight campaign, which normally consists of three individual flights, 31 parabolas are flown on each flight, with 93 parabolas in total. A parabolic maneuver results in a 22 second phase of hypergravity (1.8g), followed by a 22 second phase of reduced gravity (microgravity) and a second phase of 22 seconds hypergravity (Fig. 1a). Special designated flight areas were above the Atlantic Ocean and the Mediterranean Sea. Three researchers executed the experiments on board during each flight.

Experiments were conducted on the second flight day of the 19th Parabolic Flight Campaign of the German Aerospace Center ("Deutsches Zentrum für Luft- und Raumfahrt", DLR) in Bordeaux, France. The campaign used the unique large aircraft that is licensed in Europe to perform parabolic flights for
research purposes, the Airbus A300 ZERO-G. This aircraft is a specially configured test aircraft operated by NOVESPACE (Bordeaux, France) according to the standing orders of NOVESPACE and the CEV (Centre d’essai en vol).

**In-flight hardware for parabolic flight experiments**

A custom-made hardware meeting the requirement for experiments with human cell culture on board the Airbus A300 ZERO-G was developed in collaboration with KEK GmbH, Germany (Fig. 1b). The system has already been used successfully for cell culture experiments during the 8th, 9th, 10th, 13th, 14th and 16th DLR Parabolic Flight Campaigns and during the 45th ESA Parabolic Flight Campaign [13, 15]. The system consists of double-sealed cell containers holding the T lymphocytes and three experimental modules that supply storage of samples before the experiment, half-automated performance of the experiment and storage of the processed samples. The first module holds the cell containers at 36.5°C in a hanging position. From there, containers are transferred into the second module manually. In this module, cells were fixed by addition of fixation reagent upon triggering. Triggering was done manually at defined time intervals (20 seconds) after the onset of the gravitational condition of interest, resulting in the addition of fixation fluid. The third experimental module served as in-flight storage for the fixated samples at 4°C. Three samples could be processed in parallel. Sample exchange required approximately one minute, requiring a defined procedure by three trained persons. Two persons loaded and unloaded the cell containers in the working rack within
60 seconds of the 1g phase between each parabola. A third person was in charge of operating the control unit and monitoring the subsystems. Each was trained to overtake any other position in a case of emergency.

**Experimental setup, sample logistics and protocol of parabolic flight experiments**

Samples were prepared and analyzed in our laboratory at the University of Zurich, Switzerland. T lymphocytes from a single donor were used. Four days before the flight, cells were isolated and cultivated at a concentration of 1x10^6 cells/ml. 32-28 hours before the flight, cells were supplied with fresh medium and added to the cell containers of the hardware under sterile conditions. The samples consisted of 10 ml cell suspension with a concentration of 1x10^6 cells/ml in RPMI 1640, 10% FCS, 1% Glutamine, 0.1% Ciprofloxacin, 1% Amphotericin B. Samples were transported by train to the NOVESPACE facilities at Bordeaux Mérignac airport at 20-25°C in temperature-isolated boxes. After arrival (15 hours before the parabolic flight), cells were incubated at 37°C until they were integrated into the flight hardware 30 min before flight. About one hour after takeoff, the first parabolic maneuver was initiated. Experimental groups on board comprised 1g samples, hypergravity (hyp-g) samples and microgravity (µg) samples, complemented by ground controls. 1g samples were fixed during horizontal flight prior to parabolic maneuvers, hyp-g samples were fixed at the end of a 20 second 1.8g phase of the first parabola, and µg samples were fixed at the end of the microgravity phase of the same parabola, respectively. Each group consisted of three samples. Fixation was achieved by addition of 50 ml of 5% formaldehyde in PBS resulting in a final concentration of 4.2% formaldehyde. For the formaldehyde concentration, the upper tested margin for sample processing and fixation was chosen in order to ensure rapid and efficient fixation even in the case of an unexpected pump function disturbance. Subsequently, cells were stored at 4°C until landing of the airplane. Ground controls were fixed after the flight in the hardware on-board. Cells of all samples were then centrifuged at 300g for 5 min and resuspended in 2 ml Stain Buffer BSA (BD Bioscience). Samples were transported by train back to Zurich where they were stained and analyzed within four weeks after flight. Storage and transport of the flight samples was at 4°C.

**2D clinorotation as source of simulated microgravity**

A fast-rotating two-dimensional (2D) clinostat was used to provide simulated microgravity (Fig. 1c). The principle of clinorotation-induced microgravity is rotation of cells in a pipette perpendicular to the Earth’s gravity. During clinorotation the speed of rotation has to be adjusted fast enough to avoid sedimentation, and at the same time slow enough to avoid centrifugation. The cells are then moving on a circular path and should no longer perceive the rapidly turning gravity vector (experience “weightlessness”) because the accelerating forces compensate each other [29]. The clinostat that was used for our experiments was manufactured and certified for cell culture experiments by the German Aerospace Center (DLR, Cologne, Germany). Under our chosen experimental conditions (60 rpm, 4 mm pipette diameter), a maximal residual acceleration of 2x10^-3 g was calculated at the outer radius of the pipette and decreases towards the center. 2D clinorotation with this experimental setup is an established model system for the simulation of microgravity using T lymphocytes and other cell types [13, 30–32]. A recent study (ESA Ground-based facilities / GBF project) compared different ground based microgravity platforms to real microgravity and identified fast 2D clinorotation as a good simulation of microgravity suitable for cell culture studies. In accordance to recommendations from the same study we will refer to clinorotation-induced microgravity as simulated microgravity [33].

**Experimental setup and protocol of clinorotation**

T lymphocytes from a single donor were used, from one donor for 5 min experiments and from a second donor for the 5-60 min experiments. Experimental groups of the clinorotation studies comprised simulated microgravity (s-µg) samples, 1g samples and baseline samples. In some experiments, cell culture samples were included as additional controls. Each group consisted of 3-4 independent samples. Human CD4+ T lymphocytes (5x10^6 cells/ml) were filled into 1 ml serological pipettes (BD Biosciences). The s-µg samples were clinorotated for 5, 15, 30 or 60 minutes. Pipettes with 1g samples were loaded simultaneously and placed inside the clinostat without rotation. To investigate the influence of pipette filling, baseline samples were prepared by fixing cells immediately after transferring them into the pipettes. Culture controls were taken directly from a cell culture vessel and fixed immediately. For T cell activation, Concanavalin A (ConA, Sigma-Aldrich) and anti-CD28 antibody (BD Biosciences) were added to the cells at final concentrations of...
10 μg/ml (ConA) and 4 μg/ml (anti-CD28 antibody) directly before filling of the pipettes. The time interval needed to activate cells prior to the start of altered gravity (mixing and installing on the clinostat) was kept as short as possible and constant over all samples. All experiments were performed in a temperature-controlled incubator at 37°C. After the respective time period (or directly in case of baseline samples and cell culture samples), cells were fixed by adding 800 μl 2.8% formaldehyde (Sigma-Aldrich). For fixation of the s-μg samples the tilting mechanism of the clinostat was used, meaning that the samples were poured into the 800 μl fixation solution while the pipettes were still rotating. Final concentration of formaldehyde was 1.2% and samples were fixed for 2 hours at room temperature before they were centrifuged for 5 min at 300g, resuspended in 250 μl Stain Buffer BSA (BD Biosciences) and stored at 4°C until immunocytochemical staining.

**Immunocytochemical Staining and Flow cytometry analysis**

For immunocytochemical staining, samples were divided in aliquots and each aliquot was stained with one antibody. In the case of samples from parabolic flight (whole sample 2 ml, 10 × 10^6 cells) aliquots of 80 μl, 4 × 10^4 cells were made, and in the case of samples from clinorotation (whole sample 250 μl, 5 × 10^5) aliquots of 10 μl, 20 × 10^4 cells were made. For surface staining of CD3 and IL-2R, the subsequent permeabilization step was omitted. For detection of all other proteins, cells were permeabilized with Perm Buffer III (BD Biosciences) according to the manufacturer’s instructions. Then, cells were centrifuged at 300g for 5 min, supernatant was removed and cells were resuspended in 80 μl of Stain Buffer BSA containing the respective Alexa Fluor® 488-conjugated antibody. The following antibodies and respective dilutions were used: anti-CD3 (PromoCell, Heidelberg, Germany, custom labeled PK-AB913-144), 1:80; anti-IL-2R (PromoCell, custom labeled PK-AB913-104), 1:40; anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, Boston USA, 4374), 1:160; anti-Zap-70 (D1C10E) (Cell Signaling, 9473), 1:80; anti-acyetyl-histone H3 (Lys9) (Cell Signaling, 9683), 1:400; anti-LAT (pY171) (BD Bioscience, 558519), 1:8; Mouse (MOPC-21) mAb IgG1 Isotype Control (Cell Signaling, 2975), 1:80; Rabbit (DA1E) mAb IgG Isotype Control (Cell Signaling, 4878), 1:80. After 30 min of incubation in antibody solution, 250 μl Stain Buffer BSA were added to each reaction. Flow cytometry data were collected on a FACSCanto II flow cytometer (BD Biosciences) using FACSDiva software and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). A gate was set around the T cell population using forward- and sideward-scatter which excluded cell debris and cell doublets. The same gate was used for all analyzed samples. Results are expressed as the relative fluorescent intensity (RFI) which was calculated by dividing the geometric mean of fluorescent intensity (MFI) of the sample incubated with the antibody specific for the antigen of interest by the MFI of the same sample incubated with the isotype- and species-matched unspecific control antibody.

**Statistical Analysis**

Statistical analysis was performed using the Student’s t-test by PRISM software (GraphPad software Inc., San Diego, CA). A p-value of < 0.05 was considered statistically significant. RFI values were given with one decimal, whereas statistical tests were performed with the original three decimal values. Values are expressed as dot plots with the mean according to recommendations of Cumming et al. [34].

**Results**

**Rapid decrease of CD3 and IL-2R surface expression and reduced p-LAT in T lymphocytes upon 20 seconds of altered gravity during parabolic flight**

Human CD4+ T lymphocytes were exposed to inflight 1g, hypergravity and microgravity during parabolic flight. Additionally ground control samples (flight-identical hardware but no flight) were taken to observe possible effects of the flight maneuvers before parabolas. Components of T cell signaling were quantified by flow cytometry. Results are shown in Fig. 2. An overview of significant up- and downregulations of the investigated molecules is given in Table 1. Comparison of the inflight 1g samples with the ground controls showed no significant difference in abundance of all 6 investigated markers (CD3, ZAP-70, LAT (pY171), P-p44/42 MAPK, acetyl-histone H3, IL-2R) indicating that the flight phase of the aircraft before onset of the parabolic flight maneuvers had no effect. Surface staining of CD3 was
significantly reduced in hyp-g samples (4.9+/−0.2, n=3) and in μg samples (5.4+/−0.3, n=3) compared to inflight 1g samples (p=0.004 and p=0.015, respectively). Hyp-g and μg samples did not differ significantly from each other (p=0.245).

The level of LAT phosphorylated at tyrosine 171 (LAT (pY171)) in the hyp-g samples (2.7+/−0.1, n=3) and in the μg samples (2.8+/−0.1, n=3), LAT (pY171) was significantly downregulated compared to the inflight 1g samples (p=0.0346 and p=0.0266, respectively). Hyp-g samples and μg samples did not differ from each other (p=0.767).

The level of ZAP-70 staining in the T lymphocytes was not significantly different among all four experimental groups. No difference was detected in the level of phosphorylated p44/42 MAPK in response to the different gravitational conditions. Also the level of acetylated histone H3 did not change among the experimental groups.

The level of IL-2R in the hyp-g samples (15.4+/−0.8, n=3) and in the μg samples (15.5+/−1.2, n=3) were significantly decreased compared to the inflight 1g samples (p=0.007 and p=0.010, respectively).

Enhanced histone H3 acetylation in T lymphocytes after 5 minutes of clinorotation
Having seen effects of short-term altered gravity on the key proteins of T lymphocyte signaling induced by 20 seconds hyp-g and μg phases of the parabolic flight, we then investigated the influence of 5 minutes of simulated microgravity in 2D clinorotation experiments. In the first set of clinorotation experiments, the effects of microgravity on non-activated cells was investigated. Results are expressed as RFI and shown in Fig. 3. An overview of significant up- and downregulations of the investigated molecules is given in Table 1.
Table 1. Summary of the qualitative changes of selected proteins of T lymphocyte signal transduction in non-activated or activated cells induced by altered gravitational conditions during parabolic flight, suborbital flight and 2D clinorotation. Cells were either not stimulated or stimulated with Concanavalin A (ConA)/CD28 and exposed to parabolic flight, suborbital flight or clinorotation. GC, ground control (cells that have been exposed to the flight hardware of parabolic flight or suborbital flight experiments); BL, baseline samples (cells have been shortly in the experimental hardware of clinorotation experiments before fixation) CC, cell culture control (optimal culture conditions before fixation); 1g, normal gravitational conditions (cell were clinostat but not rotated); inflight 1g (cells were fixed before parabolas during parabolic flight or were on an onboard centrifuge providing 1g during suborbital flight); hyp-g, hypergravity (fixation after the 1.8g phase during parabolic flight or after the launch phase of suborbital flight); μg, microgravity during parabolic flight or suborbital flight; s-μg, simulated microgravity provided by clinorotation. Cells were stained by immunocytochemistry, staining was quantified by flow cytometry analysis and the relative fluorescent intensity was calculated. ↓ indicates a downregulation, ↑ indicates an upregulation, - indicates no significant difference within the compared groups; nd, not determined; * p<0.05, ** p<0.01

| CD3 | ZAP-70 | LAT (pΥ117) | P-p44/42 MAPK | Acetyl-Histone H3 | IL-2R |
|-----|--------|-------------|--------------|-----------------|-------|
| 19th DLR parabolic flight campaign / non-activated T lymphocytes |
| Flight controls (flight compared to ground, inflight 1g vs. GC) | - | - | - | - | - |
| Hypergravity compared to normal gravity (hyp-g vs. inflight 1g) | ↑** | ↓* | - | - | ↓** |
| Microgravity compared to normal gravity (μg vs. inflight 1g) | ↑* | ↓* | - | - | ↑* |
| Microgravity compared to hypergravity (μg vs. hyp-g) | - | - | - | - | - |
| 2D clinorotation (5 min) / non-activated T lymphocytes |
| Hardware control (BL vs. CC) | - | - | - | - | - |
| 5 min hardware control (s-μg and 1g vs. BL) | - | - | - | - | ↑* |
| 5 min clinorotation (s-μg vs. 1g) | - | - | - | - | ↑* |
| 2D clinorotation (5-60 min), ConA/CD28-activated T lymphocytes |
| Hardware control (s-μg and 1g vs. CC) | ↓* | - | ↑* | ↑** | - |
| 5 min clinorotation (s-μg vs. 1g) | ↑** | ↓* | - | - | ↑* |
| 15 min clinorotation (s-μg vs. 1g) | ↓* | nd | nd | nd | ↓* |
| 30 min clinorotation (s-μg vs. 1g) | - | nd | nd | nd | ↓* |
| 60 min clinorotation (s-μg vs. 1g) | - | nd | nd | nd | ↓* |
| MASER-12 suborbital space flight / controls (GC vs. CC) |
| Launch phase / hypergravity compared to normal gravity on ground (hyp-g vs. GC) | ↑** | ↓* | - | - | ↓** |
| Microgravity compared to normogravity (μg vs. inflight 1g) | - | - | - | - | - |
| MASER-12 suborbital space flight / non-activated T lymphocytes (6 min) |
| Microgravity compared to normogravity (μg vs. inflight 1g) | - | - | - | - | - |

There was no difference between cell culture controls and baseline samples in any of the investigated markers, indicating that the filling of the clinostat pipettes had no effect
Fig. 3. Analysis of selected molecules involved in T lymphocyte signal transduction in non-activated T lymphocytes during 2D clinorotation (s-μg) or 1g for 5 min. CD4+ T lymphocytes were exposed to simulated microgravity or to 1g for 5 min using a 2D-clinostat. Experimental groups comprise control cells from standard cell culture (CC), baseline samples that were exposed to the experimental hardware (BL), cells that were clinorotated (s-μg) and cells that were placed on a non-rotating part inside the clinostat in parallel to the s-μg samples (1g). Samples were immunofluorescently stained and analyzed by flow cytometry; data are expressed as relative fluorescence intensity (RFI). Single data points and means are shown for each experimental group (* p<0.05, ** p<0.01).

Fig. 4. Analysis of selected molecules involved in T lymphocyte signal transduction in ConA/CD28-activated T lymphocytes during clinorotation (s-μg) or 1g for 5 min. Concanavalin A (ConA)/CD28-activated CD4+ T lymphocytes were exposed to simulated microgravity for 5 min using a 2D-clinostat. Experimental groups comprised baseline samples that were exposed to the experimental hardware (BL), cells that were clinorotated (s-μg), and cells that were placed on a non-rotating part inside the clinostat in parallel to the s-μg samples (1g). Samples were immunofluorescently stained and analyzed by flow cytometry; data are expressed as relative fluorescence intensity (RFI). Single data points and means are shown for each experimental group (* p<0.05, ** p<0.01).

on the abundance of the molecules. Therefore in the following, s-μg and 1g samples will be compared only to the baseline samples and not additionally to the CC controls.
Surface staining of CD3 in 1g samples (14.6+/−0.6, n=3) and s-μg samples (13.6+/−0.6, n=3) did not differ from each other significantly (p=0.313) giving no evidence for sensitivity to gravity. However, CD3 staining was decreased in 1g samples and in s-μg samples compared to baseline samples (16.7+/−0.4, n=3; p=0.040 and p=0.014, respectively) showing an influence of culturing the cells in the clinostat for 5 min.

The intensities of ZAP-70 staining, and staining of LAT phosphorylated at tyrosine 171 and phosphorylated p44/42 MAPK did not differ between any of the experimental groups baseline samples.

The level of stained acetyl-histone H3 was significantly higher in s-μg samples (51.7+/−1.5, n=3) compared to 1g samples (38.7+/−2.8, n=3; p=0.016). 1g samples did not differ from baseline samples (43.5+/−1.3, n=3; p=0.197), whereas in s-μg samples the level was significantly higher than in baseline samples (p=0.015) indicating an s-μg-induced upregulation of acetylated histone H3.

The only significant change of surface IL-2R expression among the experimental groups was measured between the baseline samples (8.3+/−0.2, n=3) and the 1g samples (9.2+/−0.3, n=3), the latter having a significantly higher level (p=0.045). In contrast, in the s-μg samples (8.7+/−0.3, n=3) the level of IL-2R surface staining was neither different from the baseline samples, nor from the 1g samples (p=0.251 and p=0.267, respectively).

Taken together, among the investigated molecules, only for acetylated histone H3 a microgravity-induced change (an upregulation) was detected after 5 min of clinorotation.
Decreased CD3 surface expression, reduced ZAP-70 abundance and increased histone H3-acetylation in Concanavalin A/CD28-stimulated T lymphocytes after 5 minutes of clinorotation

In a second set of clinorotation experiments, cells were activated by Concanavalin A (ConA) and CD28 to evaluate if and how microgravity changes the composition of the key components of T lymphocyte signaling during activation. Except for the ConA/CD28 activation, experiments were performed analogous to the previous ones. Results are depicted in Fig. 4. An overview of significant up- and downregulations of the investigated molecules is given in Table 1.

Activated T lymphocytes exposed to simulated microgravity showed a significant reduction of CD3 surface staining (14.2±0.6, n=3) in comparison to the 1g samples (16.2±0.3, n=3) and to the baseline samples (16.2±0.3; p=0.045 and p=0.047, respectively) demonstrating a sensitivity to gravity. CD3 surface staining of the 1g samples was not significantly different compared to baseline samples (p=0.920).

ZAP-70 staining of s-µg samples (1.8±0.0, n=3) was significantly reduced in comparison to that of the 1g samples (2.0±0.1, n=3) and of the baseline samples (2.3±0.1, n=3; p=0.037 and p=0.003, respectively). Furthermore, the ZAP-70 staining of 1g samples was significantly reduced compared to baseline samples (p=0.028). These results demonstrated a sensitivity of ZAP-70 to cultivation in the clinostat and to simulated microgravity.

Staining for LAT phosphorylated at tyrosine 171 (pY171) was found to be significantly stronger in s-µg samples (2.0±0.0, n=3) than in baseline samples (1.9±0.0; p=0.019). The LAT (pY171) staining of 1g samples (2.3±0.2, n=3) seems to be more intense than that of the baseline samples and that of µg samples, however, these differences are not significant (p=0.075 and p=0.176, respectively), probably due to the rather big standard deviation of the 1g samples.

The staining for phosphorylated p44/42 MAPK in s-µg samples (1.6±0.0, n=3) is significantly higher than that of baseline samples (1.4±0.0, n=3; p=0.003), but not different from that of 1g samples (1.5±0.1, n=3; p=0.442). Phosphorylated p44/42 MAPK staining of baseline samples and 1g samples did not differ (p=0.108).

Staining of acetylated histone H3 revealed that the level of acetyl-histone H3 is significantly higher in s-µg samples (53.1±1.6, n=3) than in 1g samples (47.1±1.3, n=3; p=0.044). No difference could be observed between s-µg samples and baseline samples (48.9±1.4, n=3; p=0.121) or between 1g samples and baseline samples (p=0.384).

Staining of surface IL-2R revealed no differences in the surface abundance between any of the experimental groups.

Microgravity-induced transient downregulation of CD3 and stable downregulation of IL-2R during 60 minutes of clinorotation

To evaluate the development of simulated microgravity-induced effects on T lymphocyte activation over time, kinetic clinorotation experiments were performed with measurements after 5, 15, 30, and 60 min. CD3 and IL-2R were chosen as markers for analysis, as both of these surface receptors were altered in their surface abundance upon altered gravity during the parabolic flight and clinorotation experiments. This choice covers one very early component of the T lymphocyte activation cascade (CD3) and one component far more downstream (IL-2R), enabling to assess possible differences in the effects of altered gravity on early and late components of T lymphocyte activation cascade. Results are shown in Fig. 5. An overview of significant up- and downregulations of the investigated molecules is given in Table 1.

As already seen in the previous experiment, activated T lymphocytes exposed to clinorotation for 5 minutes (s-µg samples) were stained significantly less for CD3 (19.5±0.2, n=3) than 1g samples (21.6±0.5, n=3; p=0.010). Likewise, after 15 minutes of clinorotation the s-µg samples were still stained less (18.2±0.2, n=3) than 1g samples (20.1±0.4, n=3; p=0.014). In contrast, after 30 and 60 minutes, no significant difference was detected anymore between s-µg samples and 1g samples (30 minutes: s-µg samples 18.2±0.2, n=3,
1g samples 18.6+/−0.2, n=3; p=0.293; 60 minutes: s-μg samples 18.9+/−0.6, n=3, 1g samples 18.7+/−0.3, n=3; p=0.707), suggesting an adaptation of CD3 expression to the microgravity environment.

The IL-2R staining was less intensive in s-μg samples than in 1g samples throughout all investigated time intervals of clinorotation. However, this difference was significant only after 30 and 60 minutes (30 minutes: s-μg samples 13.1+/−0.2, n=3, 1g samples 15.1+/−0.4, n=3; p=0.014; 60 minutes: μg samples 13.3+/−0.2, n=3, 1g samples 14.4+/−0.3, n=3; p=0.023). In contrast, after 5 and 10 minutes, the difference was not significant (5 minutes: s-μg samples 14.9+/−1.0, n=3, 1g samples 17.2+/−1.1, n=3; p=0.204; 15 minutes: s-μg samples 13.4+/−0.5, n=3, 1g samples 14.6+/−0.9, n=3; p=0.305), coherent with the previous experiment of 5 min clinorotation, which also revealed no significant difference between 1g and s-μg samples (Fig. 4). Taken together these results suggest that surface IL-2R expression is down regulated upon simulated microgravity either within 30 min and lasts at least for 60 min, or (assuming that the differences between 1g and s-μg samples would be significant with a larger sample number) is present already after 5 min and gets more pronounced over 60 min.

Discussion

To investigate the influence of microgravity on signal transduction in CD4+ T lymphocytes, selected molecules that are involved in signal transduction were analyzed 1.) after 20 sec real microgravity provided during the 19th DLR parabolic flight campaign and 2.) after 5-60 min simulated microgravity provided by a fast rotating 2D clinostat. Due to the technical limitation of the used DLR clinostat device (experiment stop by activation of a tilting mechanisms pouring the cell suspension into the fixative), ultra-short clinorotation experiments (less than 1 min incubation time) were not possible. Using these microgravity platforms primary human CD4+ T lymphocytes were exposed to altered gravity conditions, either non-stimulated or stimulated with ConA/CD28. Due to the technical limitation (cell numbers and culture volumes) and logistical constraints (experiment preparation, execution and sample recovery), flow cytometry analysis was chosen as the most appropriate method for quantification of a wide range of signal molecules in a small sample size. The number of experimental groups, the group size and the number of different analysis parameters were dictated by the technical conditions and were strictly limited by the maximal cell number.

Results were compared to a MASER-12 sounding rocket experiment that was conducted previously using the same cell type and activation- and fixation regimes [27] allowing to compare our findings between 3 different research platforms and microgravity time periods. The results that we got for the individual markers partly differ among the experiments from the different microgravity platforms. An example is the reducing effect of altered gravity on CD3 surface abundance after 20 seconds observed during parabolic flight, and the lack of gravi-sensitivity of CD3 during 5 min clinorotation (both non-activated T lymphocytes). The same situation was found for IL-2R. Besides the obvious assumption that different time duration of microgravity can lead to different cellular reactions, it must be kept in mind that such differences are not necessarily solely due to the different time duration, but can also be influenced by the different characteristics of the used microgravity platforms with respect to the quality of the provided gravitational changes. Concerning the simulated microgravity during clinorotation the quality of the “weightlessness”, (meaning that the system does not sense any accelerating forces), depends on the rotation speed, the radius of the samples, but also on the systems threshold for sensing acceleration forces [29]. The latter is basically unknown, meaning that is not sure if and to what degree the cells in our system sense the remaining 2x10^−3 g acceleration forces in the clinostat. Therefore clinorotation-induced microgravity is called “simulated” microgravity, and this limitation must be kept in mind comparing the results to “real microgravity” platforms. On the other hand, clinorotation has the clear advantage that it does not require a phase of hypergravity prior to microgravity, as it is unavoidable in parabolic flight and sounding rockets experiments. Such hypergravity
phases can make it difficult to distinguish between hypergravity effects and effects of altered gravity in general, and effects of microgravity might be masked by hypergravity effects that extend into the μg phase. In our experiments, we had to face this restriction for the down regulation of CD3 in hyp-g and in μg samples during parabolic flight, which must therefore be understood as sensitivity to altered gravity instead of sensitivity to a gravity below or above 1g. In the suborbital flight experiment, we also observed a very strong downregulation of CD3 upon the hyp-g phase (Table 1), which might mask an effect of microgravity in the following 6 min phase. Additionally, the hypergravity phase of a sounding rocket flight has other physical characteristics than the hypergravity phase of a parabolic flight.

Initial down-regulation and adaptation of CD3 surface expression in microgravity

In our study, non-activated T cells showed a rapid downregulation of CD3 surface expression during the hyp-g phase of the parabolic flight, which was not reconstituted during the following 20 seconds of μg phase. After 5 min clinorotation, the expression of CD3 on the cell surface of non-activated T cells was reduced in both, the 1g and the μg samples, thus no simulated microgravity-induced effect could be detected. In contrast, in activated T lymphocytes a clear difference between the 1g and the μg sample was detected, an effect obviously due to the gravitational change. In accordance with this, exposure of CD4+ T lymphocytes to different durations of simulated microgravity also demonstrated a reduced surface expression of CD3 after 5 min and 15 min microgravity in comparison to the 1g samples in activated T lymphocytes. However, this difference in the CD3 surface expression disappeared within 30 min. These experiments suggest, that CD3 surface expression is sensitive for altered gravity and adapts to altered gravity with time.

In our preceding study on board of the sounding rocket MASER-12 providing 6 min real microgravity, we found a reduced CD3 surface expression after the hyp-g phase supporting the suggested gravisensitivity. Furthermore, CD3 surface expression reconstituted after T cell activation during 1g conditions but not in microgravity [27]. Investigations of TCR internalization in stimulated human PBMCs using rotating wall vessel system revealed that CD3 internalization was delayed in simulated microgravity [30] an effect in accordance with our findings of gravity-dependence of CD3 surface expression.

ZAP-70 is reduced in activated T cells in simulated microgravity

Upon T lymphocyte activation, ZAP-70 is recruited to the doubly phosphorylated ζ-chain of the TCR at the plasma membrane [35], where it phosphorylates LAT protein, which leads to interleukin production, T cell proliferation and differentiation [36, 37]. Furthermore, ZAP-70 participates in regulation of cytoskeleton modifications, adhesion and mobility of T-lymphocytes. ZAP-70 is also required for TCR-CD3 complex internalization [38].

ZAP-70 was not influenced during the parabolic flight experiment in non-activated T cells, which could be confirmed during 5 min clinorotation. However, exposure of activated T cells to simulated microgravity led to a significant decrease of the ZAP-70 compared to the 1g sample. Similarly to these results, it has been reported that more than 2-fold downregulation of ZAP-70 occurred after 96 hours simulated microgravity using rotating wall vessels [39, 40].

Since ZAP-70 is degraded in parallel with TCR internalization upon T cell activation [41], it can be assumed that degradation is enhanced by microgravity and thus, transduction of TCR mediated signaling leading to formation of the immunological synapse, T cell polarization, proliferation and differentiation is disturbed.

6 min real microgravity on board of MASER-12 did not reveal any effect of microgravity on ZAP-70. However, the preceding hypergravity phase led to a strong decrease of ZAP-70, thus ZAP-70 degradation might already have occurred during the hyp-g phase, overlaying any microgravity-specific effect [27].

Tyr171-phosphorylated LAT is reduced in hypergravity

In the parabolic flight experiment, the phosphorylation of LAT at Tyr171 in non-activated T cells was reduced during the hyp-g phase and was not reconstituted during
the following 20 sec µg phase. In contrast phosphorylated LAT in non-activated T cells was not influenced by 5 min simulated microgravity during clinorotation. Phosphorylated LAT in ConA/CD28 activated cells was not statistically shown to be different between 1g and s-µg samples (this might be due to a rather high deviation in the 1g samples), but it seems that in s-µg samples the level of phosphorylated LAT is higher. Thus, a sensitivity of LAT phosphorylation at Tyr171 was shown in parabolic flight but could not be proven during simulated microgravity.

Reduced IL-2R surface expression after 30 minutes clinorotation

Non-activated T cells exhibited rapid downregulation of IL-2R surface expression during the hyp-g phase of the parabolic flight, which was not reconstituted during the following 20 sec µg phase.

The exposure of non-activated and activated T lymphocytes to 5 minutes simulated microgravity during 2D clinorotation showed no difference between IL-2R surface level of the then µg group and the 1g group. This results suggest that IL-2R is sensitive to hypergravity but not to simulated microgravity after 5 min. However, after 30 minutes simulated microgravity, a significantly reduced IL-2R surface expression was observed, which continued over one hour of clinorotation. This effect of microgravity had its onset either quite late compared to the other investigated parameters, or it was just under the detection level and became more pronounced and significant with time. A late onset of an effect of clinorotation would be unique among the here investigated molecules. Nevertheless these findings support the results of previous gene expression studies: Both IL-2 and IL-2R mRNA expression were downregulated in microgravity experiments [26, 42]. Since IL-2 and IL-2 receptor interaction transduces a crucial signal for full T cell activation and proliferation, we suppose that reduced IL-2R stimulation could contribute to reduced proliferative response of activated T cells in microgravity [7].

Different effects of microgravity on MAPK phosphorylation

Alterations of gravity during the parabolic flight experiment as well as during clinorotation did not affect phosphorylation and therefore activation of p44/42 MAPK. These results suggest that p44/42 MAPK is not influenced by altered gravity.

However, in the MASER-12 experiment, we found that phosphorylation of p44/42 MAPK was reduced in non-activated T cells after 6 min microgravity, but not in activated T cells. P-p44/42 MAPK was also reduced during the hyp-g phase [27]. In a previous study with Jurkat T cells, we detected an enhanced phosphorylation level of p44/42 MAPK in CD3/CD28 stimulated as well as non-stimulated Jurkat T cells after 5 minutes clinorotation and an enhanced phosphorylation level of MEK [15]. During a parabolic flight experiment with 20 seconds real microgravity, we detected an enhanced MEK phosphorylation in stimulated, but not in non-stimulated Jurkat cells [15]. The MAPK cascade is a central signal system which is triggered and inhibited by a multitude of intra- and extracellular events and which comprises various negative and positive feedback loops. Thus, the MAPK cascade is known as an oscillating signaling system [43, 44] and thus, only kinetic studies with various time points would probably allow to evaluate the effect of gravity on the MAPK signaling cascade.

Acetylation of histone H3 is regulated by gravity

Histone H3 acetylation was increased in non-activated as well as in activated T lymphocytes after 5 minutes simulated microgravity in the 2D clinostat in comparison to the corresponding 1g control. However, we detected no changes after 20 seconds microgravity during the parabolic flight experiment. In a rotary cell culture system, reduced histone H3 acetylation in T lymphocytes was described after 72 hours and 7 days rotation, associated with a reduced HDAC1 level [14]. During the MASER-12 experiment, acetylation of histone H3 was reduced strongly during the hypergravity phase of the launch, but did not change during the 6 min microgravity phase [27]. Thus, acetylation of histone H3 is regulated by gravity, possibly downregulated by hypergravity and upregulated by microgravity.
Conclusion

T lymphocytes are one of the most important regulatory cells of the human immune system. During space flights and in microgravity analogues, dysregulation of T cell function has been observed in a multitude of studies [7, 8, 13–20, 45, 46]. We found a rapid decrease of CD3 and IL-2R surface expression and reduced p-LAT after 20 seconds of altered gravity during parabolic flight, decreased CD3 surface expression, reduced ZAP-70 abundance and increased histone H3-acetylation in activated T lymphocytes after 5 minutes of clinorotation and a transient downregulation of CD3 and stable downregulation of IL-2R during 60 minutes of clinorotation. Thus, we assume that a gravity condition around 1g is required for the expression of key surface receptors. During the MASER-12 experiment, many key molecules of T cell signal transduction were not altered, which is probably the consequence of the preceding hypergravity launch phase, masking the microgravity effects afterwards. Indeed, in the clinostat system, where a hypergravity phase is lacking, downregulation of CD3 and IL-2R could be confirmed.

Taken together in this study we have identified single proteins that react sensitive to altered gravity. This indicates that altered gravity can influence key processes of T lymphocyte signal transduction including receptor dynamics, phosphorylation and histone modification. Nevertheless, as other components of the signaling cascade did not react to altered gravity conditions, we assume that the signaling process during T cell activation as a whole is not disturbed severely by altered gravity. Therefore, the question for the system or components that react first to altered gravity and lead to the phenotype of inhibited T lymphocyte function, remains to be elucidated.

Despite the fact that there is broad knowledge about the consequences of altered gravity on the cellular and molecular level in immune cells, it is still unknown by which mechanism cells sense gravity. Comparing the weight of a single cell with the other physical forces within a cell, it is unlikely that the cell can detect the vector of gravity. Searching for the initial gravity sensor; research has been focusing mainly on the cytoskeleton [47]. A popular model for how cells can sense mechanical forces (and possibly gravitational changes) is the idea that the cell cytoskeleton is built in tensegrity architecture, which provides the cell’s stability. According to this model the cytoskeleton is in a state of tension, induced by the actin-myosin network, focal adhesions and cell-cell adhesions [48]. Cytoskeleton-associated proteins which are bound to the actin-network of the cytoskeleton can exist in different folding states dependent on the strain in the actin-network, and these folding states can have different affinities to bind other proteins as a part of signal transduction processes [49]. Therefore, by changing the mechanical forces inside a cell, an alteration of gravity could influence the state of cytoskeleton-associated proteins and thereby influence subsequent signal transduction - a conversion of a mechanical into a chemical signal.

T cell activation and the cytoskeleton are strongly interdependent. The formation of the immunological synapse leads to tightly coordinated remodeling of the actin and microtubule cytoskeleton in the context of the T-cell polarization process [50]. Subsequently the cytoskeleton supports T cell activation by maintaining cell-to-cell contact, facilitating T cell receptor triggering and providing a scaffold for protein clustering, translocation and spatial segregation [51, 52]. Additionally to this physical support, the cytoskeleton has also impact on the signal transduction of T cell activation by regulating molecular components of the signaling cascade: It was shown that altered polymerization of tubulin and actin is accompanied by modulation of IL-2 signaling at the receptor level [53]. Activation of p44/42 MAPK has been shown to be inducible by mechanical strain transduced through the cytoskeleton [54]. Interestingly the T cell receptor itself (with CD3 as a component) has not only been described to be functionally influenced by the cytoskeletal environment, but it was also described as a mechanosensor [55]. This relationship between the cytoskeleton (and the mechanical forces it can transduce) and the components of T cell signaling could support the assumption that T cell signaling can be influenced by different states of tension within the cytoskeleton.
Several limiting factors for human health and performance in microgravity have been clearly identified for the musculoskeletal system, the immune system and the cardiovascular system during spaceflight conditions. Considering these constraints, substantial research activities are required in order to provide the basic information for appropriate integrated risk management. In particular, bone loss during long stays in microgravity still remains an unacceptable risk for long-term and interplanetary flights. Recently, there is emerging evidence that the immune and skeletal system are tightly linked by cytokine and chemokine networks and direct cell-cell interactions: It has been demonstrated that the immune system and T cells influence metabolic, structural and functional changes in bones directly [56, 57]. Therefore, knowing the cellular and molecular mechanisms of how gravity influences T cells is an invaluable requirement for the provision of therapeutic or preventive targets to keep the bone and immune systems of astronauts fully functional during long-term space missions.

**Abbreviations**

CC (cell culture control); ConA (Concanavalin A); ESA (European Space Agency); ESRANGE (European Space and Sounding Rocket Range); GC (ground control); H/W (Hardware); IL-2 (Interleukin-2); IL-2R (Interleukin-2 receptor); LAT (Linker of activation of T cells); MAPK (Mitogen-activated protein kinase); PBMC (Peripheral blood mononuclear cells); RFI (Relative fluorescence intensity); SSC (Swedish Space Corporation); ZAP (Zeta-chain-associated protein kinase 70).

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**Disclosure Statement**

The authors declare that they have no competing interests.

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