3D microenvironment attenuates simulated microgravity-mediated changes in T cell transcriptome

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Abstract
Human space travel and exploration are of interest to both the industrial and scientific community. However, there are many adverse effects of spaceflight on human physiology. In particular, there is a lack of understanding of the extent to which microgravity affects the immune system. T cells, key players of the adaptive immune system and long-term immunity, are present not only in blood circulation but also reside within the tissue. As of yet, studies investigating the effects of microgravity on T cells are limited to peripheral blood or traditional 2D cell culture that recapitulates circulating blood. To better mimic interstitial tissue, 3D cell culture has been well established for physiologically and pathologically relevant models. In this work, we utilize 2D cell culture and 3D collagen matrices to gain an understanding of how simulated microgravity, using a random positioning machine, affects both circulating and tissue-resident T cells. T cells were studied in both resting and activated stages. We found that 3D cell culture attenuates the effects of simulated microgravity on the T cells transcriptome and nuclear irregularities compared to 2D cell culture. Interestingly, simulated microgravity appears to have less effect on activated T cells compared to those in the resting stage. Overall, our work provides novel insights into the effects of simulated microgravity on circulating and tissue-resident T cells which could provide benefits for the health of space travellers.

Keywords Space biology · Simulated microgravity · 3D cell culture · T cells · Transcriptome

Introduction
Our complex immune system is made up of two arms: the innate immune system and the adaptive immune system that work together to protect our bodies and prevent infection [1]. While cells of the innate immune system are the first responders to pathogens, the slower and more specific adaptive immune system, composed of lymphocytes, plays an essential role in fighting infections and providing long-term immunity. T lymphocytes (T cells) in circulation can infiltrate into the tissue where they are activated by antigen-presenting cells (APCs). They subsequently differentiate into several subtypes that are involved in regulating the long-term immune response and fighting infections. Dysregulation of T cell activation or their functions leads to many adverse effects such as the development of autoimmune diseases or increased risk of infection [2–4].

Currently, an exploration into deep space is of interest to both the industrial and scientific community, and, therefore, space flight duration and distances will be continuously increasing. Unfortunately, a limiting factor is the adverse effects of space flight on human physiology, ranging from...
reduction of bone density to increased risk of cancer, all of which have been documented in astronauts who return from space [5, 6]. Due to the fact that studying the effects of real microgravity (in space) is cost intensive and limited in terms of manual human handling and sample numbers, approaches which simulate microgravity on Earth are established. Methods to study microgravity effects on Earth are becoming more widely used and include drop towers, parabolic flights, and simulated microgravity (μg) platforms, like rotating wall vessels (RWVs) and the random positioning machine (RPM) [7–9]. Using these methods, there have been advances in understanding how microgravity and μg affect physiological and pathological processes [10, 11].

Specifically, immune cells appear to have reduced function under lack of gravity, such as lower inflammatory response and a reduction of leukocyte number [12, 13]. Due to their importance in maintaining efficacy in the immune system, research on the changes that occur in T cells under the effects of microgravity is a primary area of focus. It has been shown that resting Jurkat T cells cultured under μg conditions for up to 24 h have increased proliferation and metabolic rates compared to ground controls [14]. Other studies using μg platforms have shown that exposure of T cells to altered gravitational conditions results in a decrease in DNA repair genes and levels of acetylated histone H3, suggesting an alteration at epigenetic levels [15, 16]. Additionally, microgravity has been shown to increase intra-chromosomal interactions in smaller-sized chromosomes in Jurkat T cells [17]. Interestingly, gene analysis studies have shown that resting Jurkat T cells under different microgravity platforms show changes within 20 s of exposure to microgravity, indicating cells have a rapid response to gravitational changes [18]. These data give insight into how microgravity affects T cells and, in turn, plays a role in attenuating the immune system of astronauts while in orbit and after returning to Earth. However, there is still a great deal to explore in terms of the mechanisms and outcomes of T cell dysregulation as a result of exposure to microgravity.

The entirety of studies performed to determine the effects of microgravity on the human immune system have been conducted from the peripheral circulating blood of astronauts during or after spaceflight or in vitro in 2D cell culture models. In addition, due to the difficulty in obtaining tissue samples from astronauts, there have been no human studies investigating the effects on tissue-resident immune cells after exposure to microgravity. To address this aspect, 3D cell culture models have been established as a well-defined physiologically and pathologically relevant in vitro system, allowing for the study of cells in their native microenvironment [19]. Despite the fact that there have been extensive studies on T cells exposed to microgravity, the examination of microgravity effects on T cells cultured in 3D culture systems, which allows us to mimic in vivo tissue-resident T cells, has not been explored. The work here focuses on the phenotypic changes, activation levels, as well as transcriptome level changes in T cells cultured in 2D and 3D cell culture systems on the ground (1 g) and under μg conditions using the RPM.

Methods and materials

Cell culture and activation of T cells

Jurkat cells, a human T Lymphocyte cell line, were maintained in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum (FBS), 1% HEPES, 1% sodium pyruvate, 0.01% beta-mercaptoethanol, and 1% penicillin/streptomycin at 37 °C, 95% humidity, and 5% CO2 (standard cell culture conditions). Cell culture media and supplements were purchased from Gibco, InVitrogen, Thermo Fisher Scientific Inc., Dreieich, Germany.

For all 2D cell culture experiments, cells were seeded at 1 × 10^5 cells/well in 4-well plates (Thermo Fisher Scientific Inc, Dreieich, Germany). For 3D cell culture experiments, 3D collagen matrices were prepared by mixing type I collagen from rat tail (Advanced BioMatrix, Inc. San Diego, CA, USA), 0.1% acetic acid (Sigma-Aldrich, St. Louis, MO, USA), and 500 mM phosphate buffer (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 2 mg/mL, as previously published [20]. To allow for covalent binding of the collagen matrix via a lysine side chain, the collagen solution transfer was added onto a glutaraldehyde-coated coverslip (13 mm in diameter; VWR, Darmstadt, Germany) [21]. Fibrillogenesis of the collagen matrix occurred by placing the coverslips at 37 °C, 5% CO2 and 95% humidity. Afterwards, the 3D collagen matrices were washed 3 times with phosphate buffer saline (PBS; Thermo Fisher Scientific Inc, Leicestershire, UK). Subsequently, the 3D collagen matrices were placed in 4 well plates (Thermo Fisher Scientific Inc, Dreieich, Germany), seeded with 1 × 10^5 cells/well, and placed in the incubator overnight to allow for cell infiltration.

For activation of T cell in 2D and 3D culture, cell culture medium was supplemented with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Merck KGaA, Germany) and 1 μg/ml ionomycin (Merck KGaA, Germany), as established [22]. Afterwards, a custom-built microvessel was placed in both 2D and 3D cell culture settings, as previously described [23]. The well plates were placed on the RPM or on the ground (1 g) and cultured at standard cell culture conditions for 24 h prior to further analysis.

Settings of random positioning machine

All experiments using simulated microgravity (μg) were performed on the desktop RPM (Airbus Defence and Space
Netherlands B.V., Leiden, The Netherlands). The RPM was placed inside an incubator with standard cell culture conditions (37 °C 5% CO2 and 95% humidity). Afterwards, cell plates were placed at the center of rotation of the RPM to maintain the quality of μg as suggested by the manufacturer of the RPM. The operational setting of the RPM was adjusted for a 3D random mode with random motion, random direction while maintaining an average velocity of 60 deg/s, as established [24]. Control samples at 1 g were placed in the same incubator next to the RPM.

Cell imaging and analysis of nucleus morphology

For cell staining, cells were fixed with 4% paraformaldehyde (Biolegend, San Diego, CA, USA) for 10 min and subsequently permeabilized with 0.1% Triton X100 (Merck KGaA, Darmstadt, Germany) for 10 min. After each step, cells were washed three times with PBS. Afterwards, cells were stained with Hoechst-33342 (dilution 1:10,000 in PBS; Invitrogen, Carlsbad, CA, USA). The RPM solution, which was placed inside an incubator with standard cell culture conditions (37 °C 5% CO2 and 95% humidity). Afterwards, cell plates were placed at the center of rotation of the RPM to maintain the quality of μg as suggested by the manufacturer of the RPM. The operational setting of the RPM was adjusted for a 3D random mode with random motion, random direction while maintaining an average velocity of 60 deg/s, as established [24]. Control samples at 1 g were placed in the same incubator next to the RPM.

RNA Isolation and purification

RNA was extracted using TRIzol (Invitrogen, Thermo Fisher Scientific, Inc., Dreieich, Germany), followed by chloroform extraction (Sigma-Aldrich, Schnelldorf, Germany) using the manufacturer’s protocol. Afterwards, a purification step using the RNeasy mini kit (Qiagen, Hilden, Germany) was performed according to the manufacturer’s protocol. The obtained RNA concentration and purity (the ratio of absorbance at 260 nm and 280 nm) were quantified using NanoDrop (Thermo Fisher Scientific, Inc., Dreieich, Germany) and confirmed by Qi RNA kit using Qubit 4 fluorometer (Thermo Fisher Scientific, Inc., Dreieich, Germany) prior to performing RNA sequencing.

RNA sequencing and analysis

Purified RNA samples were prepared with NEB Ultra II RNA kit (New England Biolabs, Ipswich, MA, USA) as per protocol instructions using NEBNext Poly(A) mRNA Magnetic Isolation module (New England Biolabs, Ipswich, MA, USA), and uniquely dual indexed. The resulting libraries concentration, size distribution, and quality were assessed on a Qubit 4 fluorometer (Thermo Fisher Scientific, Inc., Dreieich, Germany) with a dsDNA high sensitivity kit (Invitrogen, Carlsbad, CA, USA) and on a 4200 TapeStation using a High Sensitivity D5000 kit (Agilent, Santa Clara, CA, USA). Based on these results, libraries were normalized according to their molarity and pooled, then quantified with a library quantification kit for Illumina platforms (Roche, Basel, Switzerland) on a StepOnePlus qPCR machine (Thermo Fisher Scientific, Inc., Dreieich, Germany). Finally, pooled libraries were loaded at 350 pM with 1% PhiX on S2 FlowCell, and paired-end sequenced (2 × 150 bp) on a
NovaSeq 6000 next-generation sequencer (Illumina, San Diego, CA, USA). RNA-Seq was performed in triplicate.

Raw FASTQ sequenced reads were first assessed for quality using FastQC v0.11.5 (available online at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) [27]. The reads were then passed through Trimmomatic v0.36 [28] for quality trimming and adapter sequence removal with the parameters (ILLUMINA_CLIP: trimmomatic_adapter. fa:2:30:10 TRAILING:3 LEADING:3 SLIDINGWINDOW:4:15 MINLEN:36). The surviving trimmed read pairs were then processed with Fastp [29] to remove poly-G tails and NovaSeq/Nextseq specific artifacts. Following the quality trimming, the reads were assessed again using FastQC. Post QC and QT, the reads were aligned to the human reference genome GRCh38.p4 using HISAT2 [30] with the default parameters, and additionally by providing the –dta flag. The resulting SAM alignments were then converted to BAM format and coordinate sorted using SAMTools v1.3.1 [31]. The sorted alignment files were then passed through HTSeq-count v0.6.1p1 [32] using the following options (-s no -t exon -i gene_id) for raw count generation. Concurrently, the sorted alignments were processed through Stringtie v1.3.0 [33] for transcriptome quantification. Briefly, the process was: stringtie—>stringtie merge (to create a merged transcriptome GTF file of all the samples) —> stringtie (this time using the GTF generated by the previous merging step). Finally, Qualimap v2.2.2 [34] was used to generate RNA-Seq specific QC metrics per sample.

RNA-Seq data were merged using the NASQAR tool- box (publicly accessible at http://nasqar.abudhabi.nyu.edu/; accessed on: 20th March 2022)) [35] and the analysis was performed using iDEP 0.95 (http://bioinformatics.sdstate.edu/idep95/ (accessed on: 20th March 2022); publicly accessible by South Dakota State University) [36]. Raw counts of genes were first converted into TPM [37]. These data were then used with the JMP Genomics (JMP®, Version < 10.1 >, SAS Institute Inc., Cary, NC, 1989–2021) software to determine differentially expressed genes (DEGS; FC ≥ 2, FDR ≤ 0.05). DEGs were then analyzed using the Ingenuity Pathway Analysis (IPA) software from QIAGEN [38] to determine the statistically significant enriched pathways based on gene expression (Padj ≤ 0.05).

**Computational simulation of fluid dynamics**

To model the flow field of the medium within a microvessel with and without 3D collagen matrices, we computationally solved governing equations of conservation of mass and conservation of linear momentum. Our 3D collagen matrix is modeled as a porous media and represented as a sink term. In addition, because of the uncertainty in the characteristic flows within the collagen matrix, we support our governing equations through turbulence modeling. In this study, we used a transition turbulence model since it is best suited for the Low-Reynolds phenomenon studied here while maintaining flexibility and accuracy.

As the cell culture media is of a constant density, the conservation of mass and conservation of momentum equations are represented by Navier Stokes equation with a source term as follows,

\[
\frac{\partial u}{\partial t} + u \cdot \nabla u = -\nabla p + (v + v_t)\Delta u + \left( \mu \frac{\partial u}{\partial x} + \frac{1}{2} \rho F u^2 \right),
\]

where \( u \) is the velocity field, \( p \) is the normalized pressure field, \( v \) is the kinematic viscosity of media, \( t \) is time, \( \mu \) is turbulence viscosity, \( D \) is the Darcy contribution, \( F \) is the Forchheimer contribution.

The above governing equations are generally used for modeling fluid flow in biological tissues [39].

The rotation of the RPM causes the microvessel and its contents to rotate about two perpendicular axes. In this study, we model the RPM to rotate at a constant speed, and in analogous transposes, the rotating direction at arbitrary positions with time. Assuming rigid body dynamics, the governing equation of the center of mass of the RPM can be expressed as [40].

\[
\vec{P}(t) = R_x \cdot R_y \cdot \vec{P}(t_0),
\]

where, \( \vec{P}(t) \) is the position vector, \( R_x \) is the rotation about the x-axis (60 deg/s), \( R_y \) is the rotation about the y-axis (60 deg/s).

All the mentioned models were available and implemented on our microvessel model in OpenFOAM® (CFD Direct Ltd., Reading, UK), which was also used to mesh, and solve for the variables presented in this study. Particularly, the model was generated in Gmsh [41] and analyzed in OpenFOAM® using pimpleFoam solver. Prior to solving, we performed a mesh refinement exercise, ensuring that the results obtained are independent of the number of elements that contribute to the mesh (Supplementary Fig. S2).

**Data and statistical analysis**

Statistical significance was determined by a one-way unpaired ANOVA test followed by a Kruskal–Wallis post-hoc test and uncorrected Dunn’s test for multiple comparisons using Prism 9 (GraphPad Software Inc., San Diego, USA) and the level of significance was set to \( p < 0.05 \) [42]. Unless otherwise stated, all experiments were performed in at least 3 replicates. Data are represented as mean ± standard deviation (SD).
Results and discussion

T cells are key players in the adaptive immune response and act by killing infected cells, producing cytokines, and regulating other aspects of the immune response. Generally, T cells are circulating in blood vessels or reside in tissues to serve their specific functions. Changes in the phenotypic state, activation states, and transcriptome profile of T cells can have a consequence on their immunological functions. Specifically, exposure to microgravity has shown an alteration in circulating T cells both in vitro cell culture [43], in vivo models [44], and from the peripheral blood of astronauts [45]. However, sufficient invasive tissue biopsies performed on consenting astronauts to obtain ample tissue-resident T cells for research is a limitation, if at all possible. To address this underlying problem, we utilized 3D collagen matrices to mimic interstitial tissue, as it has been well-established for many physiological models [1, 19, 46]. By doing this, it allows for the study of tissue-resident T cells in their cellular microenvironment.

In this work, we investigated T cell behavior in 2D and 3D cell culture models, mimicking circulating and tissue-resident T cells, respectively. Jurkat T cell line was used in this study due to its establishment in many biomedical studies [47]. T cells were studied on the ground (1 g) and under μg for 24 h in two different stages, namely the resting stage or the activation stage using PMA and ionomycin. Cell surface markers associated with T cell activation, nucleus irregularity, and transcriptome profile were then investigated. A schematic illustration of the experimental design and setup is depicted in Fig. 1.

Cell surface markers associated with T cell activation are altered by μg

We first visualized cell morphology using a bright field microscope. As shown in Fig. 2A, no differences in cell shape or size for T cells cultured in 2D or 3D settings could be visually observed at 1 g or μg conditions. It is also important to note that, while there may seem to be less cells in the 3D cell culture conditions compared to 2D, it is due to the fact that the cells are located at different positions within the collagen matrices and only one z-position is shown in Fig. 2A. As expected, the activated samples cultured with the addition of PMA and ionomycin showed an increase in the number of cell clusters, indicating proliferation that is characteristic of activated T cells [47].

T cell activation occurs when T cells interact with surface receptors on APCs. To gain insight into the cell surface markers associated with T cell-APC mediated activation, the expression level of CD3 and CD28 were quantified using flow cytometry. A representative gating strategy is shown in Supplementary Figure S3. Both CD3 and CD28 are necessary for the resting T cells to be activated by binding to the surface receptor of APCs. CD3 (part of the T cell receptor) binds to an MHC receptor, while CD28 is involved in the interaction of co-stimulatory markers and binds to CD80 or CD86 on the surface of APCs [48]. Dysregulation of either receptor can lead to improper activation and decreased differentiation in T cells. As shown in Fig. 2B(i), CD3 expression levels were significantly lower in 3D cell cultured T cells when compared to those cultured in 2D at 1 g (Fig. 2B(i)). By exposing T cells to μg, a slight reduction of CD3 expression can be observed in cells cultured in 2D conditions when compared to the 1 g, whereas no
A difference could be observed in 3D counterparts (Fig. 2B(ii)). On the other hand, CD28 expression levels remain relatively unchanged regardless of cell culture models or condition of culture (1 g or sμg) in the resting and activated stages. Our results are in line with a previous study showing a decrease in CD3 levels on resting primary human T cells after exposure to microgravity in parabolic flight and clinostat [49].

We next investigated whether the T cell activation stage is affected by the sμg. In our case, we used PMA and ionomycin (PMA/iono) to activate T cells by triggering intracellular signaling and bypassing CD3/CD28-mediated activation [22, 50]. T cell activation using PMA/iono overcomes receptor-mediated activation using APCs, soluble antibodies, or dynabeads, which are CD3/CD28-dependent and the expression of these markers is sμg sensitive (Fig. 2Bi). PMA activates protein kinase C, while ionomycin is a calcium ionophore facilitating the transfer of calcium ions into and out of cells. In conjunction, they activate the NFKB and NFAT transcription factors that result in the activation of T cells [50–52]. In our study, PMA and ionomycin were added to cells and cultured under the previously mentioned conditions for 24 h. This time frame was chosen because it has been reported that PMA/iono can effectively activate Jurkat T cells within 6 h [22]. In addition, the prolonged culture of T cells in the presence of activators can lead to an increase in cell death which in turn could release a myriad of factors that could additively be artefacts to the effects of sμg [53]. Coincidentally, intravital imaging revealed that the dwelling time of murine T cells within LN, a 3D environment, range from 4 to 25 h before egress [54]. Following this, cell surface markers of T cell activation, namely membrane-bound type II C-lectin receptor (CD69), programmed cell death protein 1 (PD-1) and lymphocyte activation gene-3 (LAG-3) [55–57], were analyzed using flow cytometry. As shown in Fig. 2Ci, an increase in CD69 expression levels in all activated cell conditions can be observed, when compared to resting controls. In addition, we found a decrease in CD69 expression in activated T cells in both 2D and 3D culture.
under su g when compared to the activated T cells under 1 g condition (Fig. 2Ci). We next analyzed the expression level of PD-1 which is commonly known as an exhaustion marker of T cells but is also rapidly induced on T cells following activation [58]. As shown in Fig. 2Cii, we found an overall increase in PD-1 expression in 3D cell culture conditions compared to 2D cell culture. Additionally, in both 2D and 3D cell culture, PD-1 was slightly reduced in activated T cells cultured under su g when compared to 1 g condition (Fig. 2Cii). Interestingly, previous work has shown a decrease in PD-1 levels in mouse T cells activated with PMA/ino after exposure to microgravity [44]. Another T cell marker that is important in T cell exhaustion and synergistically impacts T cell function with PD-1 is LAG-3 [59]. As shown in Fig. 2Ciii, Lag-3 shows similar results to PD-1 in that it is significantly decreased in activated T cells in 3D cell culture under su g conditions when compared to 1 g condition (Fig. 2Ciii). In activated T cells at 1 g the expression level of LAG-3 is significantly higher in 3D cell culture than in 2D cell culture (Fig. 2Ciii). However, under su g conditions, resting and activated T cells express lower levels of LAG-3 in 3D cell culture than those in 2D culture (Fig. 2Ciii).

Our results indicate that exposure to su g affected CD3-expression in resting T cells in 2D culture, while expression of CD69 is lower in activated T cells under su g regardless of cell culture models. However, we could not make a conclusion about PD-1 and LAG-3, since they are known as both activation and exhaustion markers of T cells.

Irregular nuclear morphology on T cells cultured in 2D setting, but not 3D, upon exposure to su g

Microgravity has been shown to induce DNA damage and impair DNA damage repair in a variety of cells and conditions [15, 60–63]. We investigated whether this phenomenon can be observed in T cells in both 2D and 3D cell culture settings. To do that, we stained resting and activated T cells using 4',6-diamidino-2-phenylindole (DAPI) and visualized the cells using epifluorescence microscopy. As shown in Fig. 3A, upon exposure to su g, both resting and activated T cells cultured in 2D settings demonstrated an increased number of cells with irregular nuclear morphology (yellow arrow) compared to cells cultured in 2D settings at 1 g. It is interesting to note that in 3D cell culture conditions, there appears to be little to no irregularly shaped nuclei compared to 2D cell culture conditions. We analyzed the obtained images to quantitatively determine the irregularity of the nuclei based on the solidity calculated as shown in Supplementary Figure S1. By calculating this morphological parameter, we were able to determine that in 2D cell culture at 1 g there is no difference in number of irregular nuclei between resting and activated cells (Fig. 3B, C). In 3D cell culture, there were no significant differences between resting and activated cells cultured at both 1 g or under su g (Fig. 3B, C). However, cells cultured in 3D have a higher percentage of irregular nuclei than cells cultured in 2D settings at 1 g (Fig. 3B, C). This is due to the cells migration through the collagen matrix pores. Interestingly, we found a significant increase in cells with irregular nuclei when cultured in 2D under su g conditions regardless of their activation stages compared to their relative controls (Fig. 3B, C). In addition, activated cells cultured in 2D under su g exhibit a higher number of cells with irregular nuclei when compared to resting counterparts (Fig. 3B, C). In sum, our analyses suggest that su g has the greatest effect on resting and activated cells cultured in 2D but not in 3D.

To determine if these irregularities in nuclear morphology were affecting the viability of the cells, we treated T cells with calcein-AM, a cell-permeant dye that can be used to determine cell viability. The percentage of live cells was analyzed using flow cytometry. As shown in Fig. 3D, the viability of the cells is not affected in any condition after 24 h of culture. This data indicates that, although the nucleus of the cells cultured in 2D cell culture appears to be more irregular, it is not necessarily affecting the viability of the cells after 24 h of culture. It may be possible that these nuclear irregularities could have more of an effect on cell function and at later time points, on cell viability.

Overall, our results demonstrate that the irregular nuclear morphology could be found in both resting and activated T cells upon exposure to su g in 2D cell culture, suggesting that the 3D cellular microenvironment is able to diminish the effect of su g on cell nuclear irregularity. The irregular nuclear morphology of T cells in 2D culture might be associated with an increase in DNA damage, which has been found in many reports [64, 65].

3D microenvironment maintains transcriptome of both resting and activated T cells exposed to su g

As demonstrated in the previous sections, we found significant changes in T cell behavior in both resting and activated stages upon exposure to su g. To map the changes in T cell function through its transcriptome, we performed RNA sequencing on resting and activated T cells cultured for 24 h in 2D and 3D cell culture conditions at 1 g and under su g.

Resting T cells

We first analyzed differentially expressed genes (DEGs) in resting T cells in both 2D and 3D culture settings with and without exposure to su g. As shown in Fig. 4A, RNA sequencing revealed that there were 597 upregulated and 930 downregulated DEGs in resting T cells cultured in 2D settings under su g when compared to 1 g conditions.
Interestingly, resting T cells cultured on 3D settings demonstrated much lower upregulated (34 genes) and downregulated (28 genes) DEGs. We found 13 upregulated and 9 downregulated DEGs that were solely affected by sμ g independent of cell culture dimensionality as reflected in Fig. 4B, revealing common microgravity-sensitive genes in resting T cells. Some upregulated genes include FLT1, which is involved in the suppression of T cell activation, and TP53BP1, which plays a role in DNA damage response and is recruited to sites of double-strand breaks [66]. CXCR3, which has high expression in T-helper 1 (Th-1) cells, is downregulated under sμ g conditions compared to 1 g [67]. Additionally, RBM3, which protects T cells from apoptosis and is involved in cell proliferation was found to be downregulated in both 2D and 3D cell culture environments under sμ g compared to 1 g controls [68]. These genes point towards the notion that T cell activation and differentiation could be impaired under sμ g conditions.

A deeper look at the DEGs in resting T cells cultured in 2D cell culture shows a variety of biological pathways that are enriched under sμ g conditions compared to 1 g controls (Fig. 4C). Using IPA software analysis of the DEGs in our samples, we were able to predict if pathways would be inhibited or activated under certain conditions. We first investigate the pathways involved in T cell activation. As shown in Fig. 4C, T cell receptor signaling is predicted to be downregulated, which is corroborated by the reduction in CD3 expression, as previously demonstrated in Fig. 2Ai. In addition, the pathways of ICOS-ICOSL, NFAT, and NFκB signaling, which are required for T cell activation, were predicted to be downregulated. These data again hint that T cell activation and differentiation might be diminished by sμ g. Besides these, three signaling pathways, namely senescence, sumoylation, and RHOGDI signaling, were predicted to be upregulated under sμ g. RHOGDI has been reported to be involved in mammalian cell adaptation to microgravity [69].
Interestingly, we found enrichment in pathways associated with DNA repair pathways that are in line with and could explain the irregularly shaped nuclei exhibiting damage seen in Fig. 3. Current literature suggests that the DNA double-strand break and increase in sumoylation can drive and balance the senescence process [71, 72]. This might explain the increase in \( \mu g \)-induced cellular senescence which has been reported in many mammalian cells [73–75]. On the other hand, there were much fewer enriched pathways in resting T cells in 3D cell culture under \( \mu g \) conditions compared to \( 1g \) controls. As Fig. 4D shows, these pathways could not be predicted to be inhibited or activated except for the HIF1\( \alpha \) pathway which is predicted to be inhibited under these conditions.

Overall, the transcriptome analysis data from resting T cells under \( \mu g \) conditions compared to \( 1g \) controls implies that T cell function, especially the pathways associated with T cell activation, is impaired in 2D cell culture conditions under \( \mu g \). In addition, there is an upregulation of DNA damage pathways under these same conditions. This data also indicates that there are fewer changes occurring at a transcriptome level in resting T cells cultured in 3D cell culture under \( \mu g \) compared to \( 1g \) controls.

**Activated T cells**

Prior to studying the impact of \( \mu g \) on activated T cells, we first confirmed that our T cells are successfully activated in...
both 2D and 3D cell culture settings. As shown in Fig. 2C, CD69 confirmed that T cells in both 2D and 3D were activated by the PMA and ionomycin treatment. To gain in-depth knowledge of the T cell transcriptome in the early activation stage, we performed a transcriptome analysis of T cells cultured on 2D and 3D at 1 g. The analysis showed 113 upregulated and 43 downregulated DEGs in a 2D culture setting, while 77 upregulated DEGs and 118 downregulated DEGs were found in 3D counterparts (Supplementary Figure S4A). Results also revealed 38 upregulated and 15 downregulated DEGs upon T cell activation, which are independent of cell culture dimensionality (Supplementary Figure S4B). These overlapped genes in 2D and 3D cell culture settings, e.g. NFkBIA, NFKB2, CD70, and IFIT3, which are found to be induced in activated T cells (Supplementary Fig. S5A–D). By analyzing biological pathways, we found an enrichment in T cell receptor signaling, NFkB signaling, CD27 signaling, and regulation of IL-2 expression in both 2D and 3D cell culture (Supplementary Fig. S4C, D). Although PMA and ionomycin bypassed the TCR receptor signaling, they activated the same downstream signaling of the TCR-mediated T cell activation [22]. Our data support a successful activation of T cells in both cell culture settings.

We next analyzed the effects on the transcriptome of μg on activated T cells. In 2D cell culture conditions, 18 DEGs were up- and 228 were down-regulated under μg conditions when compared to 1 g, whereas in 3D collagen cell culture, we found 13 up- and 46 down-regulated DEGs in μg compared to 1 g conditions (Fig. 5A). As shown in Fig. 5B, KDM5B is the only gene that is up-regulated independent of cell culture settings, while 14 DEGs are found down-regulated (Fig. 5B). KDM5B is
a key regulator of genome stability [76] and represses immune response [77]. Interestingly, three down-regulated DEGs in activated T cells under sμ g, namely RBM3, CXCR3, APOBEC3C, were also found to be downregulated in resting T cells (Fig. 4B). RBM3 and APOBEC3C are involved in DNA damage and repair [78, 79]. CXCR3 is a chemokine receptor and is induced during early T cell differentiation [80]. Along with CXCR3, down-regulation of T cell activation and differentiation-associated genes, namely, HES1, ARID5A, LANCL2, RNASEH1, supports the reduction of T cell activation under sμ g conditions [81–84].

We next analyzed the biological pathways of activated T cells under sμ g conditions. Overall pathway analysis shows that under sμ g, activated T cells cultured in 2D cell culture predominantly have pathways involved in T cell signaling, such as IL-2 signaling, T cell receptor signaling, and JAK/STAT signaling, that are predicted to be significantly inhibited (Fig. 5C). On the other hand, in 3D cell cultured activated T cells, there are fewer significantly enriched pathways (Fig. 5D). The prediction of biological pathways associated with T cell activation, e.g. NFAT signaling, T cell exhaustion, IL-2 expression, and Th1/Th2 differentiation, could not be determined to be inhibited or activated (Fig. 5D).

Overall, our data alludes to the 3D cellular microenvironment maintaining the transcriptome profile of T cells regardless of their activation stages, as there were fewer changes in the transcriptome in cells cultured on 3D culture setting compared to 2D counterparts.

sμ g enhances activity on specific genome loci in resting and activated T cells in 2D culture

Aside from the functional transcriptome analysis, we mapped the DEGs to their genome location using the PREDA software package [85], predicting enrichment of genome location and local structural organization of the genome. By mapping the DEGs to their genome loci, we found no enriched genome loci in resting and activated T cells cultured at 1 g and under sμ g in a 3D cell culture setting, which is correlated with the low change in DEGs in 3D culture (Figs. 4A, and 5A). On the other hand, we found 69 enriched genome loci (FDR ≤ 0.05), especially on chromosomes 1 and 19 in resting T cells in 2D culture (Fig. 6A). From the enriched genome loci, it could be observed that most genes on chromosome 1 were upregulated, whereas most genes on chromosome 19 were downregulated. Our findings are in line with a previous report, demonstrating the strong downregulation of genes on chromosome 19 of resting Jurkat T cells in parabolic flight and suborbital ballistic rocket flight experiments [17]. In activated T cells in 2D culture, we found 12 enriched genome regions, especially on chromosome 11 and 19, while there are no significant enriched genome regions in activated T cells cultured in a 3D setting under sμ g (Fig. 6). In both resting and activated T cells, we found that chromosomes 11q12, 11q13, 19p13, and 21p11 are persistently downregulated under sμ g. Our results pinpoint that resting T cells are more prone to be affected by sμ g when compared to activated cells, which might be explained by the stiffening of cell and nucleus upon T cell activation [86]. It has been shown that nuclear deformability
can influence gene expression [87]. In addition, our results suggest that the 3D cellular environment can attenuate the effects of suG on T cells at a gene level.

Computational fluid dynamic simulations confirm the observed effects on T cells are due to su g, not fluid shear stress

As it has been previously shown, studying cells under su g using the RPM is associated with fluid shear stress [40]. To avoid high fluid shear stress from traditional cell culture vessel (e.g. cell culture flask T75), which might affect T cell functions [88, 89], biocompatible custom-made microvessels were used to culture these cells [23]. To demonstrate that the obtained results throughout this study are due to the su g and not differences in fluid shear stress in both 2D and 3D conditions, we applied computational fluid dynamic simulation on an optimized model (Supplementary Figure S2) to visualize and calculate the fluid flow velocity under both conditions. As shown in Fig. 7A, velocity vectors of the fluid in 2D and 3D cultures under su g were visualized on the RPM at the velocity used throughout these experiments (60 deg/s). The maximum shear stress in these conditions was calculated and plotted as a function of time, showing constant maximum shear stress of approximately 1.2 × 0.01 dyn/cm² for both conditions (Fig. 7B). Our results indicate that differences in T cell behaviors observed in 2D and 3D cultured T cells on the RPM is unlikely due to the fluid shear stress in the microvessels.

Fig. 8 Schematic illustration of the proposed effects of microgravity on circulating and tissue-resident T cells
General discussion and conclusion

As of yet, there are limitations to studying the effects of microgravity on cells residing within tissues. Using 3D cell culture models, we are able to recapitulate a more physiologically relevant model of tissue-resident cells as opposed to circulating cells that are more accurately represented in traditional 2D cell culture models. 3D cell culture models have previously been used to study alteration in skin physiology and wound healing in cells exposed to microgravity [90, 91]. Given the important role that all T cells play, not just those in circulation, these 3D cell culture models will provide more precise information on how microgravity affects cells in their native environment. Our work indicates that T cells cultured using 3D models under the effects of sμg, and suggests that T cells residing in tissue are less affected by exposure to sμg than those circulating in the periphery. It seems that these cells are “protected” by their cellular environment, however, the underlying mechanisms involved in this remain unclear. A recent report has demonstrated a similar attenuation of the effects of sμg on the transcriptome of fibroblast cells cultured in 3D collagen matrices [91]. In addition, we observed nucleus irregularities in cells exposed to sμg in 2D cell culture but not in those that were cultured in 3D collagen matrices. We hypothesize that the protective mechanism could be attributed to mechanotransduction or mechanosensing of the T cells to their environment.

Along with the protection from the 3D microenvironment, we found that activated T cells were less subjective to alterations in their transcriptome due to exposure to sμg. We hypothesize that activated cells might be less subject to changes (have less cell plasticity) and therefore more stabilized gene expression compared to resting T cells under sμg conditions. Upon mapping our gene expression data to their chromosomal loci, we found attenuated gene activity on chromosome 19 in resting T cells exposed to sμg. These results were similar to a study in real microgravity using parabolic flights and sounding rockets [17]. On another note, it should be mentioned that starting and stopping the RPM may have effects on the cells [92]. However, there are currently no techniques for processing samples while the RPM is operating which would eliminate these technical artifacts. In addition, when using sμg, shear stresses always appear along the wall of the cell culture receptacle as it was experimentally shown through bulk population assessment of Pyrocystis noctiluca applied under sμg using the RPM [92]. In this study, the mechanoresponse of the cells is through bioluminescence emission and detectable levels were measured at certain RPM operating conditions [92]. Fortunately, computational studies show that only a small number of cells are exposed to higher shear stresses and presumably these particular cells will respond differently from the bulk population [40]. Furthermore, as a cautionary note, having static ground (1 g) controls does not decouple gravitational forces and fluid dynamic effects although, as discussed by Wuesta et al. (2017), the differences seen on mammalian cells exposed to sμg applied by the RPM are not a consequence of fluid dynamics alone [40]. Further studies should focus on determining whether the effect of 3D cell culture maintaining the transcriptome profile of T cells is a temporal effect. Shorter or longer culture periods would allow for the determination of a specific time point when this “protection” comes into play and when, if ever, it wears off and cells are again affected by microgravity.

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Data availability The datasets generated and analyzed for this study will be available upon request.

Declarations

Conflict of interest The authors declare no competing interest.

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