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Severe disruption of the cytoskeleton and immunologically relevant surface molecules in a human macrophageal cell line in microgravity—Results of an in vitro experiment on board of the Shenzhou-8 space mission

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ABSTRACT

During spaceflight the immune system is one of the most affected systems of the human body. During the SIMBOX (Science in Microgravity Box) mission on Shenzhou-8, we investigated microgravity-associated long-term alterations in macrophageal cells, the most important effector cells of the immune system. We analyzed the effect of long-term microgravity on the cytoskeleton and immunologically relevant surface molecules. Human U937 cells were differentiated into a macrophageal phenotype and exposed to microgravity or 1*g* on a reference centrifuge on-orbit for 5 days. After on-orbit fixation, the samples were analyzed with immunocytochemical staining and confocal microscopy after landing. The unmanned Shenzhou-8 spacecraft was launched on board a Long March 2F (CZ-2F) rocket from the Jiuquan Satellite Launch Center (JSLC) and landed after a 17-day-mission. We found a severely disturbed actin cytoskeleton, disorganized tubulin

Abbreviations: CC, cell culture chamber; CMSEO, China manned space engineering office; DLR, German Aerospace Center; GESSA, General Establishment of Space Science Application; ESA, European Space Agency; EUE, experiment unique equipments; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; H/W, Hardware; ISS, International Space Station; JSLC, Jiuquan Satellite Launch Center; LFA-1, leukocyte function-associated antigen-1; LT, local time; MAC-1/CR3, macrophage antigen-1/complement receptor 3; μg, microgravity; PEEK, polyether ether ketone; PFA, paraformaldehyde; PMA, 12-O-tetradecanoylphorbol-13-acetate; PITC, payload integration and test center; TCC, test culture chambers; TUNEL, TdT-mediated dUTP- biotin nick end labeling; SIMBOX, Science in Microgravity Box; UTC, Coordinated Universal Time

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Gravisensitivity
Spaceflight

and distinctly reduced expression of CD18, CD36 and MHC-II after the 5 days in microgravity. The disturbed cytoskeleton, the loss of surface receptors for bacteria recognition, the activation of T lymphocytes, the loss of an important scavenger receptor and of antigen-presenting molecules could represent a dysfunctional macrophage phenotype. This phenotype in microgravity would be not capable of migrating or recognizing and attacking pathogens, and it would no longer activate the specific immune system, which could be investigated in functional assays. Obviously, the results have to be interpreted with caution as the model system has some limitations and due to numerous technical and biological restrictions (e.g. 23 °C and no CO_2 supply during in-flight incubation). All parameter were carefully pre-tested on ground. Therefore, the experiment could be adapted to the experimental conditions available on Shenzhou-8.

1. Introduction

The immune system is one of the most affected systems of the human body during spaceflight [1–3]. Immunological problems of spaceflight were already discovered during the first Apollo missions, when more than half of the astronauts suffered from bacterial or viral infections [4]. A reduced reactivity of blood lymphoid cells has also been observed in crew members of Skylab and Soyuz [5,6]. Recent studies describe a re-activation of the varicella zoster virus (VZV), a latent virus in the nervous system, in astronauts [7,8]. All these observations are alarming in view of long-term space missions. The high level of psychological and physical stress may explain the immune impairment observed before and after a mission, with physiological stress, confinement, isolation and disruption of the circadian rhythm as possible factors. Because of these obvious and severe effects on the human immune system, serious concerns arise as to whether spaceflight-associated weakening of the immune system ultimately precludes the expansion of human presence beyond the Earth's orbit [1]. Furthermore, the microbial environment of a spacecraft consists of numerous pathogenic microorganisms as well as species with bio-destructive properties, which can cause damage to materials (e.g. seals, the wiring and metallic surfaces) [9–14]. During spaceflight conditions, enhanced microbial proliferation, increased virulence and increased resistance to antibiotics have been observed [15–18]. The combination of a multilayered immune dysfunction with an altered endogenous microbial flora, particularly resistant and virulent bacteria, can be considered as a significant risk of serious infections during long-term space missions [9]. For this reason there is an urgent need to understand the cellular and molecular mechanisms by which altered gravity influences and changes the immune cell function. When considering the future of human spaceflight, we need to know which cellular and molecular mechanisms will provide therapeutic or preventive targets. Their discovery is the foundation for methods to sustain the immune system of astronauts during long-term space missions [3].

Cells of the immune system are exceptionally sensitive to microgravity [3]: During the first Spacelab-Missions in 1983, a pioneering discovery by Cogoli et al. which showed that isolated human lymphocytes fail to proliferate after several days in microgravity, provided the first strong evidence of cell sensitivity to long-term reduced gravity exposure [19]. Follow-up experiments clearly verified the depression of lymphocyte proliferation activation after mitogenic stimulation in long-term microgravity [20]. Microgravity also impaired monocyte function [2]: IL-2-receptor expression in monocytes was down-regulated [21] and the examination of gene expression in real microgravity demonstrated significant changes in gene induction associated with differentiation of monocytes into macrophages [22]. As demonstrated in different animal models and in samples from astronauts, the monocytes-macrophage system appeared to be affected during space flight [23–25]. In particular, phagocytosis and oxidative burst and degranulation capacity were reduced in monocytes isolated from astronauts before and after a mission when compared to control groups [25]. Recently, it has been shown that simulated weightlessness leads to massive alterations in the cytoskeleton of monocytes [26], which in turn influence motility. Accordingly, a severe reduction in the locomotion ability of monocytic cells in microgravity has been revealed during a recent ISS experiment [27].

During the SIMBOX (Science in Microgravity Box) mission on Shenzhou-8, we investigated microgravity-associated long-term alterations in macrophages, the most important effector cells of the immune system, which are responsible for attacking and killing bacteria and other foreign and pathogenic intruders in the human body. We analyzed s urface molecules, which are required for recognition of bacteria and cell–cell-communication, and investigated the cytoskeletal architecture after several days in microgravity. One of our questions was whether human macrophageal cells are able to adapt to a microgravity environment. For this reason, results from the SIMBOX experiment will be compared with subsequent parabolic flight and 2D clinostat experiments. During this mission, the China Manned Space Engineering Office (CMSEO) has for the first time cooperated with another nation in the use of Shenzhou – the core of China's human spaceflight program. The German Aerospace Center was responsible for the management of the German part of the mission, which consisted of the SIMBOX facility (built and operated by EADS Astrium), the mission procedures and the contributions of seven German universities in collaboration with two other universities, one in Denmark and one in Switzerland. The aim of the Shenzhou-8 mission was to test a docking maneuver with Tiangong-1, which meant the mission was a key step in China's human spaceflight program and important for building the Chinese space station, which is expected to be fully operational by 2020 [28].

2. Material and methods

2.1. SIMBOX incubator system

BIOBOX/SIMBOX is a programmable, space-qualified incubator for biology research in space, equipped with a 1*g* in-flight centrifuge for 1*g* control experiments (Fig. 1A and B). The incubator allows for fully automatic execution of biological experiments with limited use of commands during orbital flight in a controlled thermal environment. BIOBOX missions were performed on BION/FOTON and Space Shuttle missions. Astrium GmbH (Friedrichshafen, Germany) and Kayser Italia (Livorno, Italy) upgraded the BIOBOX to increased performance and capacity as a SIMBOX incubator. The SIMBOX incubator (internal volume 34 l, size $461 \times 551 \times 273$ mm^3, empty mass 16 kg, fully integrated mass 34.5 kg, max. power 130 W) accommodates 40 experiment unique equipments (EUEs) with 24 EUEs on the microgravity (μg)-platform and 16 EUEs on the 1*g*-centrifuge.

2.2. Mission profile

The unmanned Shenzhou-8 spacecraft was launched on October 31, 2011 at 21:58 UTC (November 1, 2011, 05:58 LT) on board of a Long March 2F (CZ-2F) rocket from the Jiuquan Satellite Launch Center (JSLC) in Inner Mongolia (Fig. 1C). The Shenzhou-8 spacecraft was automatically docked with the Tiangong-1 space module (launched on September 29, 2011) on November 2, at 17:28 UTC. After 12 days of being docked, Shenzhou-8 separated and a second rendezvous and docking were performed on November 14. On November 17, the capsule was autonomously de-orbited and landed at 12:38 UTC (20:38 LT) around 500 km north of Bejing. The SIMBOX was recovered immediately and transported by helicopter and jet aircraft transport to the PITC, Bejing. Total early retrieval time was 6 h. On arrival at PITC the SIMBOX was opened, and the EUEs were removed and inspected. The samples were recovered and stored in cold (4 °C) PBS until arrival in Zurich for analysis.

2.3. SIMBOX plunger units

The plunger experiment insert (Fig. 2) was developed by Astrium GmbH and is described in the *Astrium Space Biology Product Catalog* [29]: It allows medium exchange and chemical fixation of adherent cell cultures (Fig.2A–C).

There are two plungers which can be filled with any liquid and automatically activated to inject the liquid into the experimental volume. The plunger experiment insert is equipped with single slides which are optically clear for

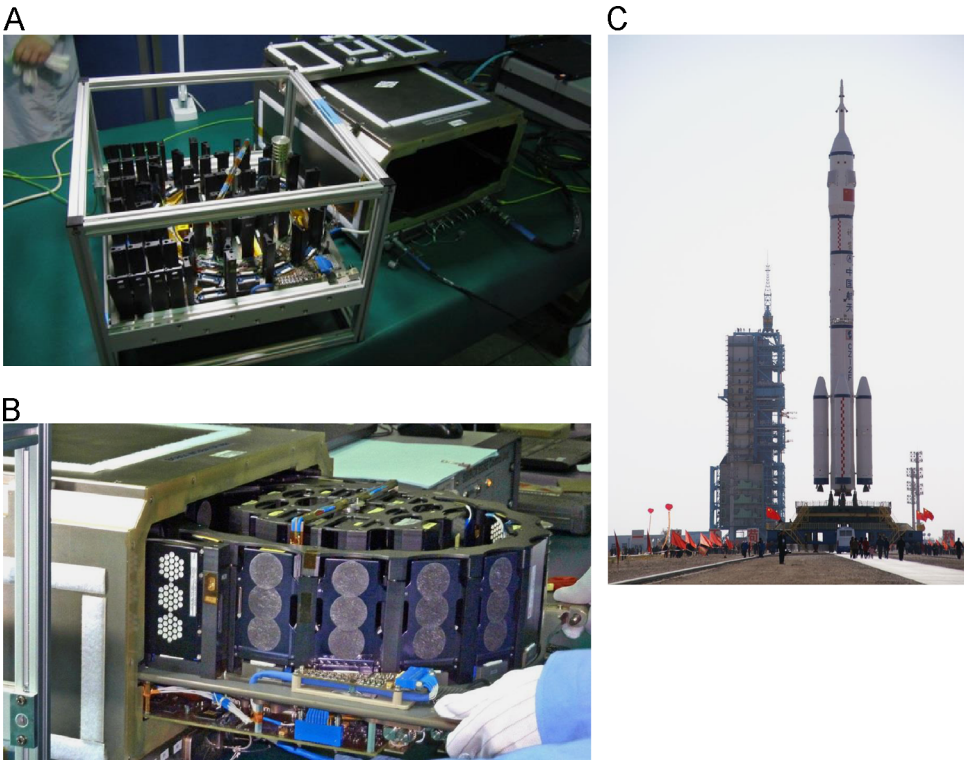

Fig. 1. (A) and (B) SIMBOX incubator system. Programmable, space-qualified incubator for biology research in space, which consists of a controlled thermal environment consisting of a 1*g* in-flight centrifuge for 1*g* control experiments. The SIMBOX incubator (internal volume 34 l, size $461 \times 551 \times 273$ mm^3, empty mass 16 kg, fully integrated mass 34.5 kg, max. power 130 W) accommodated 40 experiment units (EUEs) with 24 EUEs on the μg-platform and 16 EUEs on the 1*g*-rotor. (A) SIMBOX Incubator FM 601 (right) with the "intelligent tray" (left) before integration of the EUEs. (B) Integration of the intelligent tray, carrying the 1*g* reference centrifuge and μg positions (in the center), into the incubator (picture: DLR/Astrium). (C) Long March 2F carrying Shenzhou-8 space ship (take-off weight 497,000 kg, height 58 m, 4 booster with YF-20B engines and 4×814 kN thrust, first stage with four YF-20B main engines and 3256 kN thrust, second stage with one YF-25 and four YF-23 engines and 787 kN thrust) during Roll-Out on October 26, 2011 (picture: DLR/Astrium).

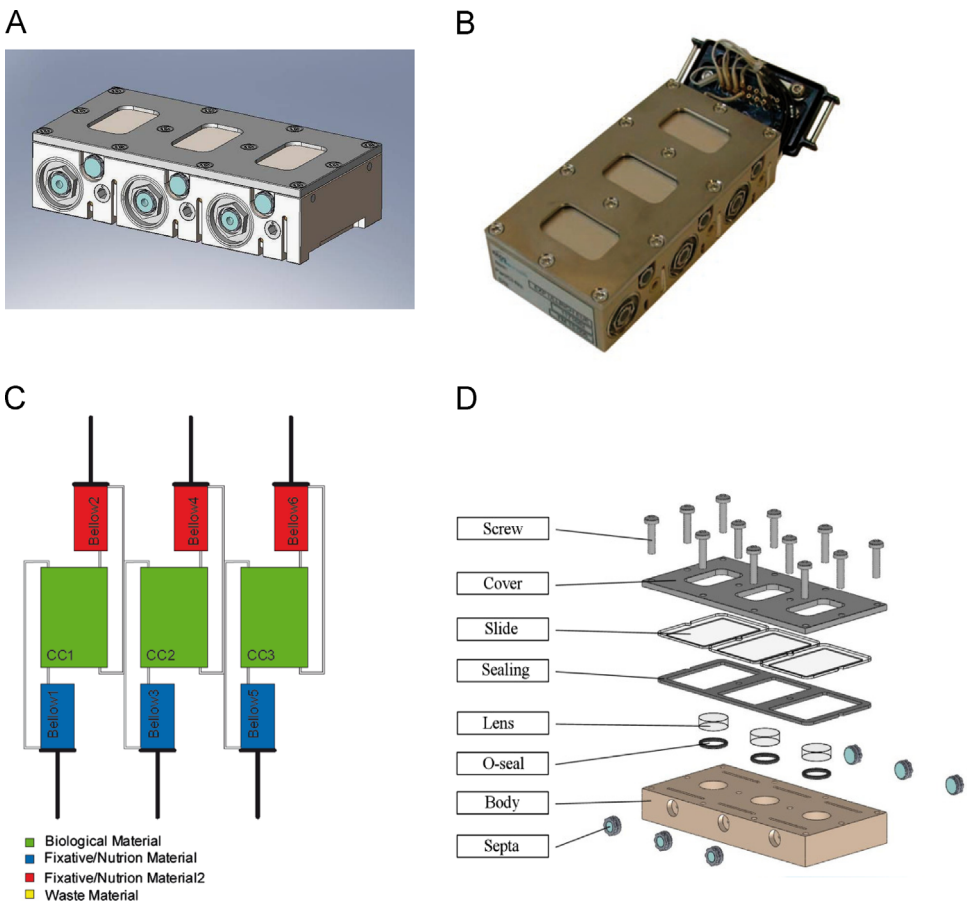

Fig. 2. Plunger unit and test culture chamber: (A) the Plunger unit EUE consisted of a support structure (housing made of PEEK), which includes three culture chambers and six supply units, two for each culture compartment and representing an independent loop. The culture chambers filled with medium are closed on the top of the housing by means of polycarbonate specimen window slides, where the adherent cells were attached. The housing is tightened by silicon sealing and covered by an aluminum plate (cover) fixed by screws. (B) Assembled Plunger Unit EUE before integration into the SIMBOX (picture: Astrium). (C) Filled plunger unit before activation. The Plunger Unit has three CCs in which the slides hold the cells. The chamber (covered by the window slide) contains the medium. During plunger activation, the medium was exchanged by emptying the plunger and the waste medium was pushed behind the empty plunger. For the first sequence of fluid exchange (fixation), plunger 1, 3 and 5 were activated, for the second sequence of activation (removal of fixative by PBS), plunger 2, 4 and 6 were activated. The bellow of each plunger contained a maximum volume of 620 μl, the specimen slides (SS) contained a minimum surface of $28.1 \times 19\ mm^2$. The plunger can be activated by $U=5$ V, $I=310$ mA, min. $t=2.5$ s. The inner volume of the Culture Compartment (CC) was 213 μl. (D) Test Culture Chamber (TCC) was constructed for mission scenario and biological tests during mission simulations. Part of the TCC are three windows inside the PEEK housing, which makes it possible to monitor and document the cells on the polycarbonate slides via microscopy during the various test procedures.

microscopic observation. The EUEs consisted of a support structure (housing made of PEEK) which includes three Culture Chambers (CCs) and six Supply Units (SU, plungers), two per culture compartment. Each CC has two Supply Units and represents an independent loop. The CCs are closed on the top of the housing by Specimen Slides (SS) made of polycarbonate, on which the adherent cells were attached. The chamber (covered by the window slide) contained the medium. The housing is tightened by silicon sealing and covered with an aluminum plate (cover), which is fixed with screws. The container lid of the Biorack standard type I container is mounted onto the housing. The Biorack standard is based on the accommodation of various Experiment Unique Equipments (EUEs) into Experiment Containers which provide the interface to facilities and support infrastructure [29]. The outer dimensions of Biorack standard type I containers are $90 \times 58 \times 24\ mm^3$, type II containers are $79 \times 79 \times 99\ mm^3$. The EUEs did not exceed the interior envelope of the closed Type I-Container with the dimensions ($L \times W \times H$) of $80 \times 40 \times 20\ mm^3$. The plunger unit is qualified for an unmanned capsule mission and for use on the International Space Station (ISS).

For pre-flight testing of the mission scenario and optimization of the in-flight cell culture conditions, a Test Culture Chamber (TCC) was constructed (Fig. 2D), which provides the same culture conditions as the EUEs. A special part of the TCC are three windows inside the PEEK housing, which allow the monitoring and documenting of the cells on the polycarbonate slides with microscopy during the diverse test procedures. The fluids were exchanged manually and the device was without electronic components.

2.4. Pre-flight test and development program

Between 2008 and 2011, several pre-flight tests and developments for biocompatibility, chemical stability, medium optimization (buffer systems, supplements, sera, cell differentiation) and H/W sterilization were conducted. Viability tests were performed under the regular cell culture conditions, in the test culture chamber (TCC) under in-flight 1g cell culture conditions and in the 2D clinostat under the conditions of simulated microgravity. Further developments and standardizations were: test of different fixatives and fixative concentrations, establishment of antibody staining procedures for immunocytochemistry, establishment of a water-jet slide cutting procedure, establishment and testing of cell and sample transport scenarios. The standardized mission scenario was finally verified during a matching and mission scenario test at PITC in April 2011.

2.5. Biological logistics and principles

Materials for the mission were either ordered from Germany or Switzerland directly to China or imported by Astrium GmbH. Human U937 cells and temperature sensitive materials were transported by team members on board Lufthansa flights to Beijing as in-cabin transport with special permission. Human U937 cells were not irradiated during security checks based on special permission. The material was transported from Beijing to Jiuquan Satellite Launch Center by train or by a special military transport flight.

All logistical, technical and biological procedures were compiled in a "Mission Handbook" with the status of an administrative directive of the university. Procedures were completely standardized and tested beforehand; margins were known and experimentally validated. Biological procedures were planned with a minimum of 200% reserve for Beihang University and the Payload Integration and Test Center (PITC), Beijing, and with a 400% reserve for the Jiuquan Satellite Launch Center (JSLC) for possible launch scrub events. All logistic, technical and biological procedures were planned with a tested and validated secondary or tertiary protocol; logistic transport procedures were redundant. The entire team was trained and tested in the required primary and secondary procedures. During the mission, operations started at Beihang University and PITC on October 9, 2011 and at JSLC on October 15, 2011 and ended at LSLC on November 1, 2011, at Beihang on November 9, 2011 and at PITC on November 18, 2011.

2.6. Cell culture, macrophageal differentiation and sample preparation

U937 cells (ATCC CRL-1593.2) are a human monocytic cell line and preserves the main monoblastic characteristics of monocytes including the ability to differentiate into a macrophageal phenotype. U937 cells were cultured in RPMI-1640-medium with or without 20 nM HEPES (Biochrom), supplemented with 10% fetal calf serum (FCS, Biochrom) or 10% human serum (HS, Biowest), 1% glutamine (200 mM) (PromoCell) and 1% penicillin/streptomycin (10.000 IU/ml) (Gibco). Subcultivation was done at a cell density of 1×10^6 cells/ml. Stimulation and differentiation was performed by adding 25 nM phorbolmyristylacetate (PMA) (Sigma-Aldrich) in dimethylsulfoxid (DMSO, 0.1%) (Sigma-Aldrich) at a cell density of 0.5×10^6 cells/ml. Differentiation medium was supplemented with 10% human serum, 1% glutamine (200 mM) and 1% penicillin/streptomycin (10.000 IU/ml). Cells were differentiated on polycarbonate (Makrolon) slides into a macrophageal phenotype for 72 h at 37 °C with 5% CO_2. For mission scenario tests, cells were differentiated for 72 h with fetal calf or human serum in RPMI 1640 medium with or without 20 mM HEPES, integrated into the TCCs and cultured for 5 or 8 days. Afterwards cells were harvested and stained with propidium iodide (PI) and/or trypan blue. Viability and cell numbers were analyzed by flow cytometry (BD FACS Canto II) and manual counting using a Neubauer cell counting chamber.

After incubation the medium was changed and the slides were immediately integrated into the EUEs (plunger units). Inside the EUEs the slides were bedded in 0.5 ml fully CO_2 saturated RPMI 1640 medium with 10% human serum, 1% glutamine (200 mM), 1% penicillin/streptomycin (10,000 IU/ml) and 0.1% amphotericin (0.25 mg/ml) (PromoCell). Bellow 1,3 and 5 of the EUEs were filled with RPMI 1640, 10% human serum, 1% penicillin/streptomycin (10,000 IU/ml), 0.1% amphotericin (0.25 mg/ml), 1% glutamine (200 mM), 1% PFA and 0.6% sucrose. Bellow 2,4 and 6 of the EUEs were filled with PBS, 1% penicillin/streptomycin (10,000 IU/ml) and 0.1% amphotericin (0.25 mg/ml). 2 EUEs (FM005 and FM006, 6 chambers) were prepared for the 0g-position, 1 EUE (FM 002, 3 chambers) was prepared for the 1g-position inside the SIMBOX. The gravity vector of the 1g-position was perpendicular to the surface of sample slides (*z*-axis).

2.7. On-orbit experiment execution

The timeline of experiment execution on orbit is summarized in Table 1: After handover of the EUEs to Astrium/DLR, the EUEs were stored in standard lab incubators with active temperature control. The EUEs were integrated into the SIMBOX on October 31, 2011 at 10:45 UTC in a temperature-conditioned room (22–23 °C). The SIMBOX was closed at 13:46 UTC and the set flight command was given at 14:08 UTC (active temperature control). During the unpowered transport from the laboratory to the spacecraft and installation in the spacecraft (total time 3 h 06 min), the temperature was always above 21 °C.

Shenzhou-8 launch was on October 31, 2011, 21:58:00 UTC and the spacecraft attained orbit at 22:08 UTC. The SIMBOX timeline started at 22:34 UTC by means of a command given by Shenzhou-8 once it reached the orbital flight condition. That time was understood as "Experiment Zero Time" to which all timings given in the timeline were referred. Active temperature control was set at 23 °C. The centrifuge speed for the 1g reference centrifuge was 74.40 rpm. Plunger 1,3 and 5 of all three EUEs were activated between 120:50:00 and 120:55:20 (h:min:s) of the timeline sequences of 40 s. Plunger 2,4 and 6 of all

Table 1
Time-line for experiment execution on orbit.

Date and time	Event
October 31, 2011, 10:45 UTC	EUEs integrated into the SIMBOX (22–23 °C)
October 31, 2011, 13:46 UTC	SIMBOX closed
October 31, 2011, 14:08 UTC	Set flight command (active temperature control, 23 °C). Unpowered transport from the laboratory to the spacecraft and installation in the spacecraft (total time 3h06min, temperature always above 21 °C)
October 31, 2011, 21:58 UTC	Shenzhou-8 launch
October 31, 2011, 22:08 UTC	Spacecraft attained orbit
October 31, 2011, 22:34 UTC	Start of SIMBOX timeline (Experiment Zero Time, to which all timings given in the timeline were referred) Timeline: 000:00:00 (hours:min:sec)
November 5, 2011, 23:24 UTC - November 5, 2011, 23:29 UTC	Plunger 1,3 and 5 of all three EUEs were activated in sequences of 40 s Timeline: 120:50:00–120:55:20 (hours:min:sec)
November 6, 2011, 01:24 UTC - November 6, 2011, 01:29 UTC	Plunger 2,4 and 6 of all three EUSs were activated in sequences of 40 s Timeline: 122:50:00–122:55:22 (hours:min:sec)
November 17, 2011, 09:40 UTC	"Set cooling" command
November 17, 2011, 11:12 UTC	Re-entry
November 17, 2011, 11:32 UTC	Incubator shut off
November 17, 2011, 12:38 UTC	Capsule landed, sample recovery (within 6 hours)

three EUSs were activated between 122:50:00 and 122:55:22 (h:min:s) of the timeline sequences of 40 s. Thus, human macrophageal U937 cells were cultivated for 5 days inside the SIMBOX on board Shenzhou-8 in microgravity (μg) and 1*g* conditions, fixed with 1% paraformaldehyd (PFA)/sucrose (Sigma-Aldrich) solution for 2 h and stored in PBS (Biochrom) on board at 23 °C until landing. After landing, the polycarbonate slides were removed, washed and then stored in PBS at 4 °C for 2 weeks until analysis. On November 17 the "set cooling" command was given at 09:40:59 UTC, re-entry was at 11:12:46 UTC and the incubator was shut off at 11:32:28 UTC. The capsule landed on 12:38 UTC and sample recovery was conducted within 6 h.

2.8. Ground control experiment execution

2 EUEs (FM001 and FM004, 6 chambers) were prepared for the "0*g*-position", 1 EUE (FM 003, 3 chambers) was prepared for the "1*g*-position" inside the SIMBOX. The gravity vector of the "1*g*-position" during the ground control experiments was perpendicular to the surface of sample slides (in the *z*-axis) and therefore perpendicular to the gravity vector of Earth. The ground control experiment was executed according to the flight scenario. The EUEs were integrated into the SIMBOX on November 2, 2011 at 13:35 UTC in a temperature-conditioned room (24 °C). The SIMBOX was closed at 15:15 UTC and the set flight command was given at 15:29 UTC (active temperature control). The SIMBOX timeline started at 15:56:39 UTC ("Experiment Zero Time"). Active temperature control was set at 23 °C. Timeline execution was similar to the on-orbit experiment. Due to a technical centrifuge problem, the "centrifuge off" command was given on November 10 at 07:26:55 UTC. The "set cooling" command was given on November 19 at 03:05:35 UTC and the incubator was shut off at 03:15:31 UTC.

2.9. Sample analysis

Polycarbonate slides were cut by a water-jet method into 16 T-shaped pieces. Each piece was stained individually. In order to make a division between dead (necrotic/apoptotic) and living cells before fixation occurred, slides were stained with CellMask-deep red plasma membrane stain (Invitrogen) and TUNEL reagent (Fluorescein-12-dUTP, Roche). In addition, cells were labeled with different mono- and polyclonal primary antibodies against the cytoskeleton components actin (Invitrogen), vimentin (Abcam) and alpha-tubulin (Sigma), migration- and adhesion-associated surface molecules CD54 (Cellsignaling,) CD18 (Acris), CD11a (Abcam) and CD11b (Abcam), cell interaction and antigen presentation-associated molecules MHC-I, MHCII and CD86 (Abcam) and phagocytosis-associated molecules CD36 (Abcam) and CD64 (Novus Biological) in concentrations according to the manufacturers protocols. First antibodies were subsequently labeled, after blocking with 1% BSA in PBS for 1 h, by species specific secondary antibodies, 1:1000 in 0.5% BSA in PBS, carrying the following fluorochromes Alexa-Fluor405 or Alexa-Fluor568 (Invitrogen). Slide pieces were examined by confocal laser scanning microscopy (Leica, SP5). Exclusively cells positive for CellMask and negative for TUNEL were further analyzed, since these represent the living cells in the experiment. Digital image analyses was performed using Imaris software (Bitplane).

2.10. Statistical analysis

Each group contained the analysis of 200–1000 cells, shown in box-plots. Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad, San Diego, CA, USA), Wilcoxon-test, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. Pre-flight tests: Cell culture optimization and viability during mission conditions

The material of the sample slides (polycarbonate) required biocompatibility tests and optimization of the cell culture and differentiation conditions in order to achieve an optimal density of the adherent macrophageal human U937 cells on the samples slide surfaces. Different culture volumes and stimulation times with PMA were tested, and density of adherent macrophageal human U937 on polycarbonate slides was analyzed (Fig. 3A). Macrophageal differentiation was induced in a culture of

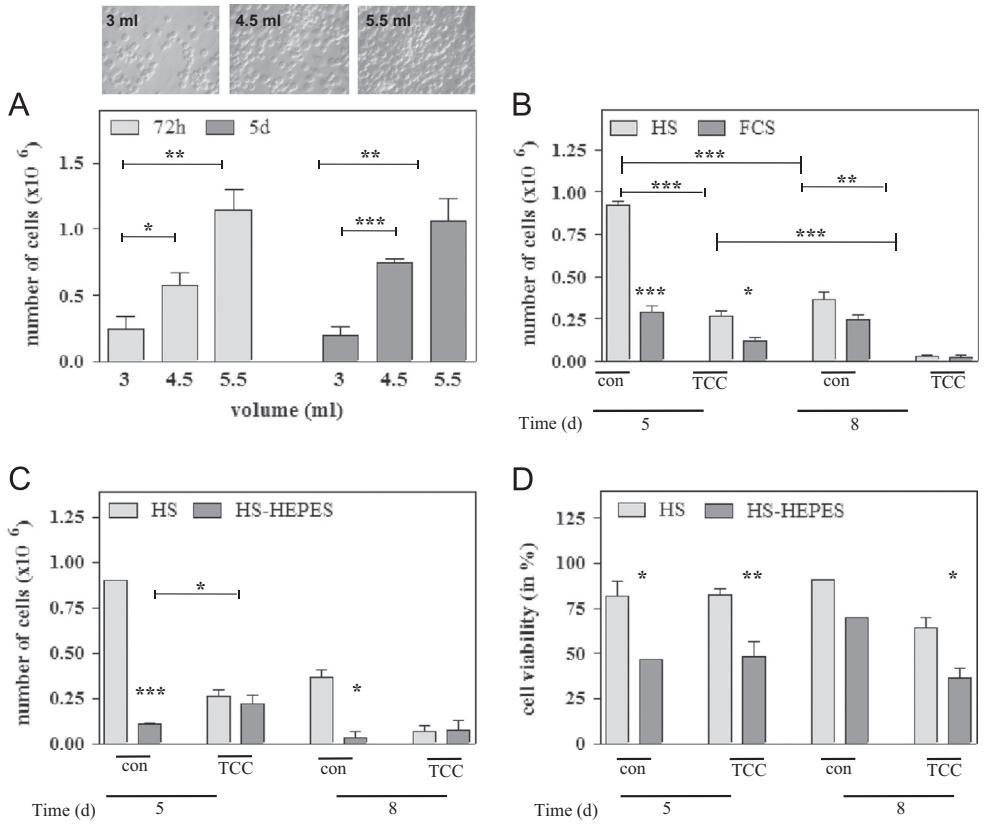

Fig. 3. (A) Cell density optimization on polycarbonate sample slide. Different culture volumes and stimulation times with PMA were tested, and the density of adherent macrophageal human U937 on polycarbonate slides was analyzed. Macrophageal differentiation was induced by 25 nM PMA in a culture volume between 3.0 and 5.5 ml and for a stimulation time of 72 h or 5 days. Further conditions were: 0.5×10^6 cells/ml in human serum, RPMI-1640 medium without HEPES, 5% CO_2, 37 °C. Top: Transmitted light microscopy (DIC) pictures of sample slides with macrophageal human U937 cells attached after 72 h stimulation. (B)–(D) Long-term viability during simulated flight conditions. Macrophageal differentiated human U937 cells were cultured for 5 or 8 days in the test culture chamber (TCC) under flight conditions (23 °C, no gas exchange) or under standard cell culture conditions (con, 37 °C, 5% CO_2). The medium was supplemented with 10% human or fetal calf serum (B), with or without 20 mM HEPES (C,D). The number of adherent cells on one polycarbonate slides (B) and (C) or cell viability (D) was shown. One to six independent experiments were performed. Data is expressed as mean with standard error (± SE). Significance was calculated using the Wilcoxon test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

0.5×10^6 cells/ml in human serum and RPMI-1640 medium without HEPES, 5% CO_2 and 37 °C by 25 nM PMA in a volume between 3.0 and 5.5 ml and for a stimulation time of either 72 h or 5 days. As shown in fig. 3A, macrophageal differentiation on polycarbonate slides was possible and the number of adherent macrophageal cells increased significantly with the culture volume ($0.2467 \pm 0.08819 \times 10^6$ cells with 3 ml volume vs. $0.5733 \pm 0.09387 \times 10^6$ cells with 4.5 ml volume vs. $1.150 \pm 0.1443 \times 10^6$ cells with 5.5 ml volume). An almost confluent monolayer of adherent macrophageal cells was achieved by using 5.5 ml of cell suspension (Fig. 3A top). Additionally, no significant loss of cells after 5 days of cultivation could be observed ($1.150 \pm 0.1443 \times 10^6$ cells after 5 days vs. $1.063 \pm 0.1674 \times 10^6$ cells after 72 h).

Furthermore, long-term viability during flight conditions was tested. Macrophageal differentiated human U937 cells were cultured for 5 or 8 days in the test culture chamber (TCC) under flight conditions (23 °C, no gas exchange) and under standard cell culture conditions (con, 37 °C, 5% CO_2). The medium was supplemented with 10% human or fetal calf serum (Fig. 3B), with or without 20 mM HEPES (Fig. 3C and D). The number of adherent cells on polycarbonate slides (Fig. 3B and C) and cell viability (Fig. 3D) were analyzed. As shown in Fig. 3B and C, the number of adherent macrophageal cells differed significantly between normal conditions (con) and in-flight culture conditions in the test culture chambers (TCC). After 5 days of culture under flight conditions (23 °C, no gas exchange) with no additional medium supplementation, the number of adherent macrophageal cells was reduced from 0.2900 ± 0.03512 to $0.1167 \pm 0.02404 \times 10^6$ cells compared to the control cell culture conditions (with CO_2 supplementation, 37 °C). Even under optimum culture conditions, cell density on polycarbonate slides was only 35% after 8 days of culture compared to 5 days of culture ($0.9250 \pm 0.01500 \times 10^6$ cells after 5 days vs. $0.2650 \pm 0.03374 \times 10^6$ cells after 8 days). To improve the culture conditions, human serum was tested against fetal calf serum (Fig. 3B). Supplementation of the culture medium with human serum significantly increased the number of adherent macrophageal cells on the sample slides after 5 days of cultivation under standard culture conditions as well as under flight conditions in the TCCs

($0.9250 \pm 0.01500 \times 10^6$ cells vs. $0.2900 \pm 0.03512 \times 10^6$ cells under standard culture conditions and $0.2650 \pm 0.03374 \times 10^6$ cells vs. $0.1167 \pm 0.02404 \times 10^6$ cells in the TCCs). After 8 days in culture, human serum improved long-term survival of adherent macrophageal cells under standard culture conditions, but not under flight conditions in the TCCs ($0.02533 \pm 0.008253 \times 10^6$ cells vs. $0.02137 \pm 0.01144 \times 10^6$ cells). Due to the lack of gas exchange during flight conditions, a buffer system capable of keeping the pH constant under flight conditions is necessary. HEPES buffers are known to maintain the pH very well and to dissociate less in decreased temperatures, rendering HEPES a more effective buffer for maintaining enzyme and protein structures at a lower temperature [30,31]. We thus cultured adherent macrophageal cells (1) under standard conditions and (2) under flight conditions in the TCC with supplementation of 20 mM HEPES (Fig. 3C). Surprisingly, the number of adherent cells decreased significantly under standard culture conditions and did not change in the TCCs ($0.9000 \pm 0.00042 \times 10^6$ cells vs. $0.1068 \pm 0.01106 \times 10^6$ cells during standard culture conditions and $0.2597 \pm 0.03454 \times 10^6$ cells vs. $0.2200 \pm 0.04531 \times 10^6$ cells in the TCCs, Fig. 3C). While cell viability in all previous reported experiments was nearly 100% with no significant differences between the groups (data not shown), cell viability after HEPES supplementation decreased significantly during standard culture conditions as well as during flight conditions in TCCs ($81.65\% \pm 8.15\%$ vs. $46.70\% \pm 4.959 \times 10^{-5}\%$ during standard culture conditions and $82.52\% \pm 3.186\%$ vs. $47.87\% \pm 8.857\%$ in TCCs, Fig. 3D). In further experiments it was investigated whether a medium exchange after 5 days of cultivation could enhance cell number and viability after 8 days of total cultivation time, but revealed no significant differences with or without medium exchange after 5 days (data not show). Because addition of HEPES buffer kept a higher risk of cell loss and viability (Fig. 3CD), no HEPES buffer was used in flight conditions in the SIMBOX experiment. Additionally, medium exchange after 5 days was not advantageous, though the two possible in-flight fluid injections were used for addition of the fixative and for the subsequent removal of the fixative in order to avoid prolonged fixation that could damage epitopes for immunocytochemical detection. Human macrophageal U937 cells were cultured in human serum instead of calf serum, beginning from the time point of macrophageal differentiation due to the increased viability and cell number. According to viability and cell number, the time point of fixation was set to 5 days. To exclude dead cells from analysis, we applied a staining method, which was utilized to differentiate between living and apoptotic/necrotic cells at the time point of fixation (TUNEL–CellMask staining) and combined this with digital image analysis. The final protocol was: U937 cells were differentiated into the macrophageal phenotype on polycarbonate slides by 25 nM PMA in 0.1% DMSO at a cell density of 0.5×10^6 cells/m in RPMI-1640 medium supplemented with 10% human serum and 200 mM glutamine for 72 h at 37 °C with 5% CO_2. Inside the EUEs the slides were incubated in 0.5 ml fully CO_2 saturated RPMI-1640 medium with 10% human serum and 200 mM glutamine at 23 °C without CO_2. Fixation was performed after 5 days incubation with 1% PFA and 0.6% sucrose at 23 °C for 2 h. Fixed cells were stored on board in PBS for 12 days at 23 °C until landing.

3.2. Mission events and sample recovery

During the mission, experiment operations started at Beihang University and PITC on October 9, 2011 and at JSLC on October 15, 2011. The operations ended at JSLC on November 1, 2011, at Beihang on November 9, 2011 and at Beihang University and PITC on November 19, 2011. Biological and technical pre-flight procedures were nominal. Off-nominal events during logistic and transports could be compensated by previously planned redundant and secondary procedures. Proliferation, differentiation, adherence, morphology and viability of the cells were always nominal, and no contamination occurred. Sample preparation and integration into the EUEs was nominal. The temperature profile was stable during the entire implementation process and during the space flight. The centrifuge speed was stable and within tolerance levels. Due to a technical malfunction, most of the bellows in the EUEs of the flight and of the ground control experiment were not activated. In the ground control experiment, the centrifuge had been shut off ahead of schedule on November 10, 2011, at 07:26:55 UTC because of a technical problem. The centrifuge in the flight experiment operated nominally. After landing of Shenzhou-8 on November 17, 2011 at 12:38 UTC, recovery of the SIMBOX and transport to the PITC was very fast (early retrieval time 6 h). Removal and on-site processing of samples (washing and storage in PBS at 4 °C) were nominal. Due to the plunger unit malfunction, only slide 2/FM 006 (0g-position) from the flight experiment and slide 2/FM 001 and slide 1/FM 003 from the ground experiment could be recovered. The samples arrived at Astrium Friedrichshafen, Germany, on November 25, 2011, and they were handed over for analysis on the same day. The cells fixated on slide 2/FM001 were lost during the transport from China to Germany due to an unknown reason. Including one sample slide from the ground control experiment (control slide no. 59), all recovered slide were cut into 16 pieces and used for immunocytochemical analysis (including negative controls and reserve pieces). Post-flight analysis was limited by the total amount of recovered intact sample material, which allowed less analysis parameters than originally planned. The mean fluorescence intensity of 200–1000 single cells was analyzed by confocal laser scan microscopy and Imaris software and the median of the measured cellular fluorescence intensities is demonstrated in Figs. 4–6.

3.3. Severe disturbance of the cytoskeleton in microgravity

U937 cells are a human monocytic cell line and preserves the main monoblastic characteristics of monocytes including the ability to differentiate into a macrophageal phenotype, which expresses specific surface molecules (MHCI, MHCII, CD11, CD14) and has the ability of phagocytosis. The maximum of adherence is reached 72 h after induction with PMA, whereas after removal of

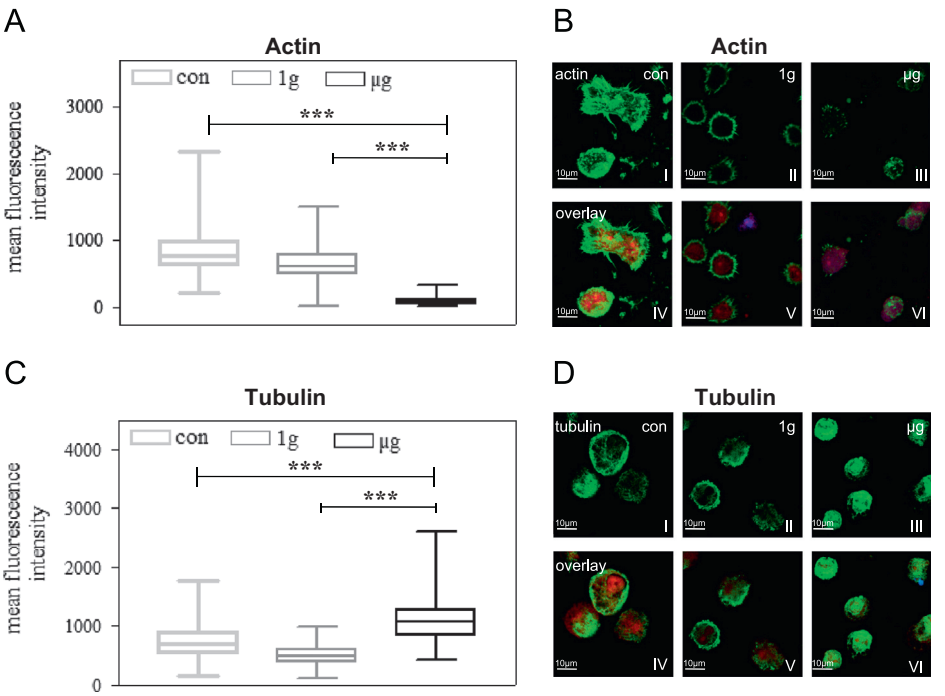

Fig. 4. Confocal microscopy analysis of the cytoskeletal proteins actin (A, B) and tubulin (C, D). Demonstrated are the standard cell culture control (con), 1g hardware control (1g) and the microgravity sample (µg). Exposure time to different gravity conditions was 5 days in all cases. Only CellMask-positive and TUNEL-negative cells were analyzed. (B) and (D) Single stain (top) of cytoskeletal protein (actin or tubulin) and overlay (bottom) of all stainings (red: CellMask, blue: TUNEL, green: cytoskeletal protein with phalloidin-Alexa Fluor568 for actin or mouse-anti-alpha-tubulin and anti-mouse-AlexaFluor568 for tubulin). Each group represents analysis of the mean fluorescence of 200–1000 individual cells from one recovered slide, shown in box-plots (A and C). Data are expressed as the median of mean single cell fluorescence intensities with the smallest observation (sample minimum), lower quartile, median, upper quartile and largest observation (sample maximum). Statistical analysis was performed with Graph Pad Prism 5, Wilcoxon-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PMA, retro-differentiation starts around day 9–10 [32,33]. During PMA-induced differentiation, monocytic U937 cells changed their morphology from a round shape with short microvilli and kidney-shaped nuclei to adherent, flat cells with long pseudopodia. Because cytoskeletal functions are not only indispensable for the differentiation process but also for crucial macrophageal functions such as migration and phagocytosis, we investigated the main cytoskeletal structures actin, tubulin and vimentin, which are all required for migration and adhesion, intracellular transport and structural stability. In Fig. 4A the confocal microscopy analysis of actin staining is shown. Exclusively living CellMask-positive and TUNEL-negative cells were analyzed regarding the mean fluorescence intensity of the whole cell: A slightly decreased actin fluorescence could be detected in Imaris analysis of the 1g ground hardware control cells integrated in the EUEs (1g) compared to standard cell culture control (con) (Fig. 4A). Importantly, an excessive loss of actin fluorescence staining could be observed in the microgravity sample (µg) compared to both controls samples (con and 1g). A morphological view of the actin cytoskeleton in all three groups is depicted in Fig. 4B, I–VI (I–III: single actin stain [green]; IV–VI: overlay of Aktin [green], cell mask-deep red [red] and TUNEL [blue]). In standard culture condition (I, IV), long pseudopodia, a flat shape and intense actin signals were present. High fluorescent signals and long pseudopodia could be seen also in the 1g ground hardware control sample (II,V), however some cells rounded up. After 5 days microgravity (µg) actin fluorescence was almost lost, whereas some pseudopodia could be still detected (III,VI). In CellMask staining, indicating regular cellular structures, no changes were evident between the groups.

The results of the tubulin staining are demonstrated in Fig. 4C and D (I–III: single stain alpha tubulin [green]; IV–VI overlay of tubulin [green], CellMask [red] and TUNEL [blue]). In standard culture conditions (con), a normal distribution of fine fibrillary tubulin filaments with missing signals in the nucleus region and an organized structure beginning in the centrosome could be detected (I, IV). In 1g ground hardware control samples (II,V), tubulin staining was reduced and some cells lost the fine fibrillary structure of tubulin, whereas the nuclear region was still free of tubulin and the structural integrity and organization appeared intact. In contrast to the control groups (con, 1g), increased fluorescent signal and disorganized tubulin skeleton could be observed in the microgravity sample (µg) (III,VI). Tubulin protein appeared to be accumulated and clumped, and the whole cell including the nuclear region was filled with tubulin fibers, with the exception only of vacuole-like regions. Vimentin staining demonstrated no change in structure or concentration (data not shown).

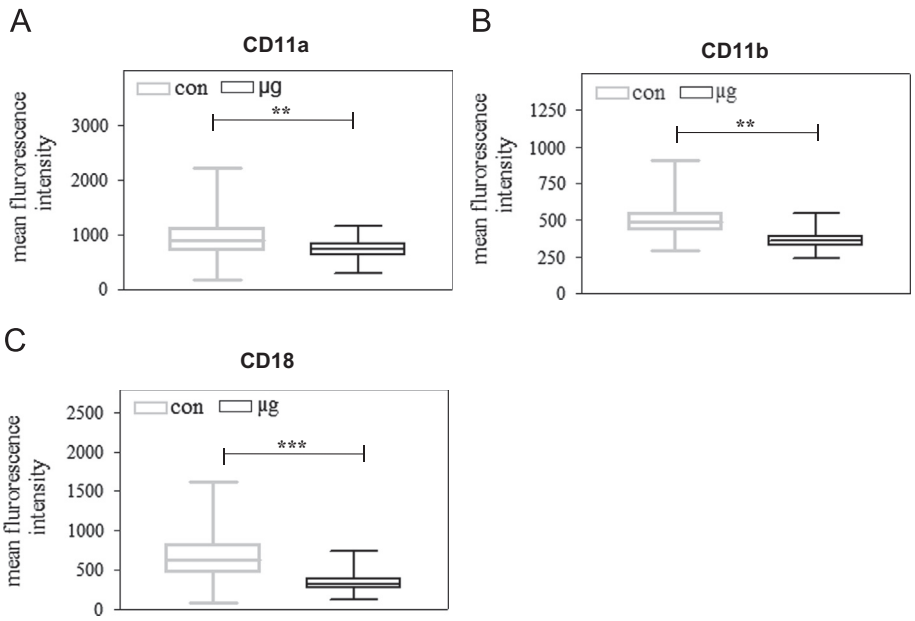

Fig. 5. Confocal microscopy analysis of CD11a (A), CD11b (B) and CD18 (C). The figure shows the standard cell culture control (con) and the microgravity sample (µg). Exposure time to different gravity conditions was 5 days in all cases. Only CellMask-positive and TUNEL-negative cells were analyzed. Each group represents analysis of the mean fluorescence of 200–1000 individual cells from one recovered slide, shown in box-plots (A–C). Data are expressed as the median of mean single cell fluorescence intensities with the smallest observation (sample minimum), lower quartile, median, upper quartile and largest observation (sample maximum). Statistical analysis was performed with Graph Pad Prism 5, Wilcoxon-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

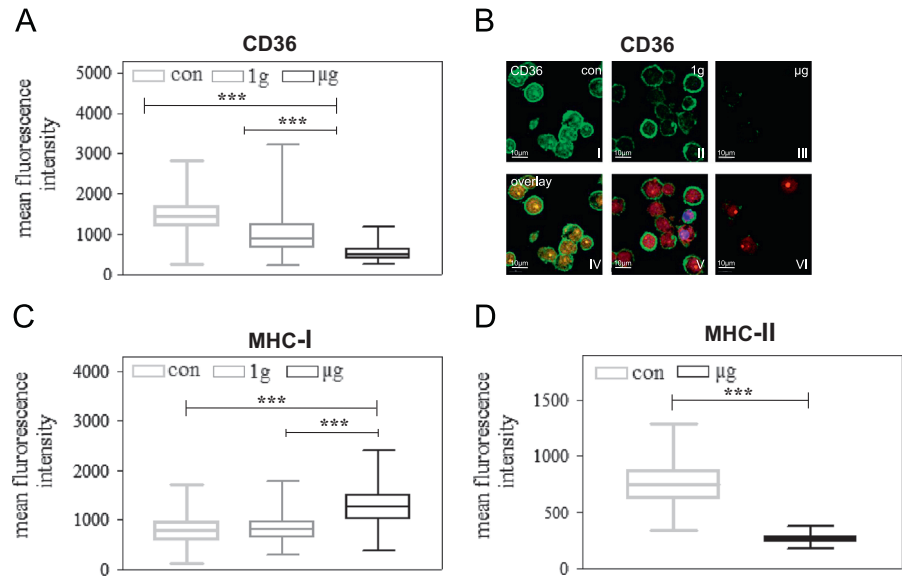

Fig. 6. Confocal microscopy analysis of the CD36 (A), MHC-I (C) and MHC-II (C). The figure shows the standard cell culture control (con), 1g hardware control (1g) and the microgravity sample (µg). Exposure time to different gravity conditions was 5 days in all cases. Only CellMask-positive and TUNEL-negative cells were analyzed. (B) Single stain (top) of CD36 and overlay (bottom) of all stainings (red: CellMask, blue: TUNEL, green: mouse-anti-CD36 and anti-mouse-AlexaFluor568). Each group represents analysis of the mean fluorescence of 200–1000 individual cells from one recovered slide, shown in box-plots (A, C, D). Data are expressed as the median of mean single cell fluorescence intensities with the smallest observation (sample minimum), lower quartile, median, upper quartile and largest observation (sample maximum). Statistical analysis was performed with Graph Pad Prism 5, Wilcoxon-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Down-regulation of CD18 in microgravity

Cell migration, adhesion and communication are indispensable functions of macrophages and required cytoskeletal elements and integrins (such as LFA-1, Mac-1 or ICAM-1), which mediate the cell–cell-contact or the cell–matrix-contact. The integrins LFA1 (CD11a/CD18) and CR3/Mac1 (CD11b/CD18) share a common beta-chain (CD18) and bind to ICAM-1 on endothelial cells and T cells. CR3/Mac-1 is mainly expressed on macrophages, dendritic cells

and neutrophils, where it fulfills a function as a pattern recognition receptor and therefore plays a key role in the innate immune response. In Fig. 5, the analysis of CD11a, CD11b and CD18 during standard cell culture conditions at 1*g* (con) and during microgravity conditions (μg) is shown. All chains of the LFA-1 and CR3/Mac-1 receptor decreased in microgravity (μg), but while the alpha chain of the receptors (CD11a and CD11b) were only slightly decreased (916.4 ± 26.5 vs. 723.2 ± 12.20 for CD11a, 500.7 ± 4.441 vs. 360.7 ± 5.129 for CD11b), down-regulation of the beta chain CD18 (652.6 ± 13.51 vs. 332.5 ± 7.639) was very distinct. We thus suppose that key macrophageal functions such as adhesion and pattern recognition could be disturbed in microgravity through the down-regulation of CD18.

3.5. Down-regulation of CD36 and MHC-II in microgravity

Finally, we analyzed macrophageal surface molecules, which are responsible for antigen-presentation (MHC-I/II) and cell–cell-communication with T lymphocytes, as well as CD36, an important macrophageal scavenger receptor, binding many ligands such as oxidized low density lipoprotein (oxLDL), oxidized phospholipids, long chain fatty acids and collagen.

In Fig. 6 the expression of CD36a and MHC-I in standard cell culture conditions at 1*g* (con), in 1*g* ground hardware control (1*g*) and in microgravity conditions (μg) is displayed. MHC-II expression as present in standard cell culture conditions at 1*g* (con) and in microgravity conditions (μg) is compared. CD36 expression was slightly reduced in 1*g* ground hardware control samples (1*g*) compared to standard cell culture conditions (con), but decreased distinctly under microgravity (μg) (Fig. 6A). Images of CD36 expression and distribution in all three groups are shown in Fig. 6B (I–III: single actin stain [green]; IV–VI: overlay of CD36 [green], CellMask [red] and TUNEL [blue]). During standard culture conditions CD36 is homogenously distributed between different cells (I, IV), whereas in 1*g* ground hardware control samples (1*g*), CD36 expression is more heterogeneously (II, V) expressed. CD36 expression during microgravity was reduced highly significant (III, VI). Image analysis quantified the loss of CD36 expression in microgravity (539.7 ± 18.27 in μg sample vs. 1445 ± 9.331 in standard culture control vs. 1012 ± 21.28 in 1*g* hardware controls). MHC-II expression was decreased in microgravity compared to standard culture controls (751.5 ± 6.474 in standard culture controls vs. 262.8 ± 2.245 in microgravity sample), whereas expression of MHC-I was increased in microgravity compared to standard culture controls and 1*g* ground hardware controls.

4. Discussion

It is well known that the immune system of astronauts is severely dysregulated in microgravity, a situation that represents a major challenge to the health and performance of astronauts [1–3]. However, the underlying molecular mechanisms are far from being fully understood. There is thus an urgent need to elucidate the underlying molecular alterations during immune dysfunction in microgravity, identify key mechanisms and test potential counteractive interventions. In previous experiments we were able to identify rapid gravi-sensitive alterations in cells of the immune system. [34,35]. During the SIMBOX mission on SHENZHOU-8, we investigated microgravity-associated long-term alterations of the cytoskeleton and of immunologically relevant surface molecules in macrophageal cells, which are the most important effector cells of the immune system.

During the SIMBOX mission, different species (e.g. human cells, algae, plants, worms) were accommodated in the incubator and the incubator temperature had to maintain viability of all biological systems on board. Macrophages are tissue-resident cells, which are accustomed to experience temperatures far below the body core temperature of 37 °C (e.g. in the extremities where the physiological surface temperature can drop below 30 °C). In the pre-flight test program, we therefore investigated the viability, morphology and functionality of macrophageal differentiated human U937 under temperature conditions provided by the SIMBOX mission (23 °C). We found that human U937 macrophageal cells were viable, morphologically intact and functional at 23 °C. However, their life-span was reduced due to the sub-optimal conditions, which limited the experiment time to 5 days on orbit. Thus, due to technical limitations resulting from the mission scenario and the SIMBOX incubator, cell culture conditions differed significantly from those usually used in laboratories (no gas exchange, no CO_2 supplementation, temperature 25 °C or lower, limited volume of cell culture medium). In addition, interaction of diverse materials with the cell culture, cell culture medium, supplements and fixatives had to be tested. To optimize the biological conditions under these logistical and technical limitations and requirements, several parameters such were tested extensively. These included culture medium, medium supplements, buffer systems, concentration and incubation time of chemical differentiation inductors, cell density, medium volume, and cultivation time with/without new medium supply. For post-flight analysis, a staining method to exclude apoptotic/necrotic cells from quantification was established. Finally, a special cutting method (water jet-cutting) had to be established for the polycarbonate slides, as well as several staining protocols together with different fixation methods and times. Due to the very short time period for hardware development and biological testing, special devices (such as the TCC) and compromises in the test plan and parameters were necessary, although the entire team was working according to an extremely difficult test and development plan. Nevertheless, the total available test time was too short to allow further improvements of the hardware systems, which caused significant risk of malfunction as occurred during the mission. Other risks resulted from logistical and administrative difficulties during transport and experiment preparation, such as procedures and restriction given by the different customs, import and export regulations, air carriers, courier services, supply companies, etc. Therefore, detailed planning with redundant and alternative logistical and administrative procedures was an indispensable requirement for the success of the mission. Additionally, extensive training of all

Table 2
Challenges and limitations during development, testing, execution and analysis of the experiment.

Parameter	Challenges/limitations	Results/consequences
Experiment conditions before fight		
Very short time frame for hardware development and biological testing	Special devices (such as the TCC) and compromises in the biological test plan, all parameter were tested standardized before the mission	No biological problems in experiment execution and analysis discovered
Total available test time was too short to allow further improvements of the hardware systems	Risk of technical malfunction	Technical malfunction occurred during flight
Logistic and administrative difficulties during transport and experiment preparation (e.g. customs, import and export regulations, air carriers, courier services, supply companies)	Detailed planning, with redundant and alternative procedures, required, logistic and administrative problems occurred frequently	Import/export regulations are often not considering international scientific collaborations, flexible and extraordinary solutions were often required, always excellent support from DLR and CMSEO
Familiarization with different styles of planning, organization, communication and decision making	Extensive training of all team members, including training in alternative procedures	Excellent team spirit, dedication and enthusiasm between the German and the Chinese scientists
Storage of mission cell culture between matching test and mission (6 month, −180 °C)	100% dead cells after thawing of the primary and reserve cell culture stock	Reason could not be identified, reserve transports were required
Reserve cell transport from Germany to Beijing (commercial flight, in-cabin)	Customs and health control clearance in China required, permission and support from the airline required	Successful transport, import and cell culture establishment
Set-up of new cell culture laboratories at PITC and JSCL	Establishment of new cell culture laboratories, implementation of local requirements and procedures	Successful, no problems
Experiment conditions in-flight		
• No optimal cell culture conditions • (23 °C, no gas exchange)	Risk of loss of cell number and viability	Extensive test and optimization program
Test of CO_2-independent buffer systems	Risk of loss of cell number and viability	No advantage in cell number and viability, no CO_2-independent buffer systems used
In-flight cell culture optimization (buffer systems, medium supplements and serum type)	Optimal conditions in morphology, adherence and viability achieved, culture limited to 5 days	Test of possible extension of cell culture with one medium exchange after 5 days
Test of medium exchange after 5 days	No advantage in cell number and viability	No medium exchange, the two possible in-flight fluid injections were used for addition of the fixative and for the subsequent removal of the fixative
Long fixation duration (day 5 until day 17)	Fixative was removed by PBS in-flight after 2 h fixation	Fixation was stable until landing
Risk of contamination	Sterilization of SIMBOX plunger units and assembly in strictly sterile conditions	Test of sterilization procedures and decision for ethylen oxide sterilization
Dead cells have to be excluded from analysis	Establishment of a staining method to differentiate between living and apoptotic/necrotic cells at the time point of fixation (TUNEL/CellMask staining), combined with digital image analysis	Only living and morphologically intact cells (at the time point of fixation) were analyzed
Sample preparation and integration	Maintain time-line of sample preparation and assembly of plunger units	Successful late access
Temperature profile	Stable during the entire implementation process and during the space flight	Temperature in the biological system constant
Centrifuge speed	Stable and within tolerance levels	Gravity force in the in-flight 1g-reference constant
Activation of plunger units (activation of in-flight-fixation)	Technical malfunction during the flight: most of the bellows were not activated, fluids were spilled, regular function of chamber 2/FM 006 (0g-position), chamber 2/FM 001 (ground), bellow 1 of chamber 1/FM 003 (ground), malfunction of all other chambers	Only one sample slide of the μg-group and two sample slides of the ground control group could be recovered, in-flight-1g-group was lost, post-flight analysis was limited and allowed less analysis parameters
Experiment conditions post-flight		
Sample recovery and stabilization	Rinsing and storage in PBS	Successful early retrieval
Transport to home laboratory for analysis by courier serivice	Cells on slide 2/FM 001 were lost during transport due to unknown reasons	One sample slide of μg-group, one sample slides of 1g-ground control group available for analysis
Water-jet-cutting and staining	According to standardized protocol	Samples were successful stained and analyzed

team members, including training in alternative procedures, was one of the most important success conditions. Due to flexible and redundant planning, consideration of alternatives and sufficient reserves, it was possible to compensate for all off-nominal events on ground. Table 2 gives a summary of the challenges and limitations during

development, testing, execution and analysis of the experiment.

Under the restricted conditions of cell cultivation inside the SIMBOX, we could achieve a sufficient cell number and viability for at least 5 days. In order to improve the cell culture conditions, we tested different serum supplements, HEPES buffering and substitution of L-glutamine with the more stable glutamine dipeptide glutamax. We also assessed the lowest effective dose of PMA to minimize possible delayed toxic effects of PMA. Addition of human serum resulted in a higher cell number in several cases under standard or hardware cell culture conditions. Whereas different concentrations of PMA (25–100 nM) did not influence the density of macrophageal differentiated cells, the highest number of viable cells in long-term survival tests (5 and 8 days after differentiation) was achieved with 25 nM PMA (data not shown). Phorbolesters were used in many different concentrations such as 50 ng/ml, 2–200 nM or 0.1 μg/ml, which were all effective [32,36–39]. The use of HEPES in cell cultures as a CO2-independent buffer system is very common [31,37,40,41]. Since its dissociation decreases with temperature, HEPES is one of the best buffers for maintaining enzymes and cellular functions [30,31]. However, we observed a distinct cell loss under standard culture conditions (up to 90%) after supplementation with 20 mM HEPES. A possible explanation for this phenomenon could be the temperature-dependent phototoxicity of HEPES [42,43]. It was reported that the quality of differentiation, the number of differentiated cells and the expression of differentiation markers decreased in a HEPES-containing medium [44]. We also observed that U937 monocytes did not differentiate into the macrophageal phenotype in the presence of HEPES (data not shown). Interestingly, HEPES-dependent hydrogen peroxide production occurred during light irradiation of HEPES-supplemented cell cultures [45]. L-glutamine is an essential amino acid, which is unstable at a physiological pH, but is required especially for highly proliferating cell lines with ineffective glucose metabolism. Aside from its instability, it builds up toxic ammonia during metabolism, so that the culture medium has to be changed every 3 days [46]. To prevent this toxicity, use of the stable glutamine dipeptide glutamax is common in eliminating the need for frequent medium exchanges. However, we found that number and viability of macrophageal U937 cells in TCC long-term cultures were reduced after addition of glutamax. These results showed that standard cell culture conditions cannot be easily transferred to experimental hardware conditions for space experiments, which means they have to be modified and/or newly developed. After completion of all tests, we decided to use 25 nM PMA for differentiation of human U937 cells into the macrophageal phenotype in RPMI-1640 medium supplemented with human serum at a density of 0.5×10^6 cell/ml in 5.5 ml culture medium for 72 h, with subsequent cultivation for 5 days with L-glutamine without HEPES. Under these conditions it was possible to maintain between 40% and 50% of the initial cell number with high viability until the time point of fixation.

To distinguish the effects of microgravity from the effects due to cell cultivation inside the H/W (e.g. the SIMBOX plunger units) or due to the launch acceleration or vibration, cell culture ground controls, H/W ground controls and baseline controls should be performed, additionally to 1*g* in-flight centrifuge controls. A baseline control group, which is fixed directly before the onset of microgravity would represent the status after launch acceleration and vibration. Possible effects of the launch could influence all on-board samples and should therefore be quantified by a baseline control group and considered for interpretation of the 1*g* and the μg in-flight group. The comparison between cell culture ground controls and H/W ground controls clarifies the influence of the H/W alone on the biological system. One must be aware of the fact that all microgravity research platforms are associated with limitations in the sample number, sample volume and sample accessibility. It is unavoidable that cells are exposed to conditions that deviate from the cell culture conditions in a ground laboratory. Therefore, results from a space experiment should be interpreted very carefully in the light of appropriate control experiments. Due to the limitations in the sample number during the SIMBOX experiment, a baseline control could not be performed, whereas cell culture controls, H/W ground controls and H/W-1*g*-in-flight controls were part of the experiment. Due to the technical malfunction and the low number of retrieved samples, the presented results are first indications only, obtained from a cell line in vitro.

Although a technical malfunction hampered the final scientific success of our experiment, a significant progress was made: A biological mission scenario for the cultivation and analysis of adherent differentiated macrophageal cells was developed and standardized. The mission scenario can be applied to further experiments in space and to different research platforms, and it can be adapted to primary human macrophages. In future experiments, additional control groups (baseline group), a larger sample size and different time points during the microgravity experiment will enhance the scientific return significantly. Despite of the limited direct scientific return of this experiment, it can facilitate an even higher scientific return based on the experience made and based on the standards and biological scenario developed.

The model system of macrophageal cells differentiated from the U937 cell line has its biological limitations compared to primary macrophages. For example, although they maintained their capability of phagocytosis, they did not generate a respiratory burst response [38,39,47]. In order to engulf material, the cell builds pseudopodia, temporary cytoplasm-filled projections of the cell wall, which require a special network of actin filaments and microtubules at the front or leading edge of cell. Microtubules provide mechanical stability and are scaffolds for motor proteins, thus allowing cell organelles and other cellular components to move through the cell cytoplasm [48]. Whereas the intermediary filament vimentin [49] was not altered in microgravity, we detected a massive loss of actin filaments and disorganized tubulin proteins. This could possibly result in a reduced ability to migrate and build pseudopodia for phagocytosis. Accordingly, previous studies reported that actin and microtubule proteins were influenced by short- and long-term real or simulated microgravity in glial cells, neuronal cells, lymphocytes and myocytes [50–53]. In previous studies in real [51] and simulated microgravity

[52], it was demonstrated that microtubules were shortened and were extending from poorly defined microtubule organizing centers (MTOCs) [51] and lost their radial array [52]. However, adaption and re-organization of the cytoskeleton was observed after 1–2 days [51,52]. Thus, it was assumed that low gravity induced only transient alterations in the cultured cells, which were able to adapt to the gravity vector changes and to regain normal activity [50]. In our study, we detected indications of a severely disturbed cytoskeleton after 5 days in microgravity. Because of the experiment design as an end-point measurement due to the limited space for experiment units, we are not able to draw conclusions about adaptation.

In this study, we investigated the influence of long-term microgravity on the expression of immunologically relevant surface molecules. We detected that macrophageal U937 cells lost nearly completely the scavenger receptor CD36, which binds to collagen, oxidized LDL, lipoproteins and apoptotic cells [54–57]. Because organization and function of the cytoskeleton is important for CD36 receptor clustering, ligand addition and receptor mediated signaling and internalization [58], it can be assumed that the disturbance of actin filaments contributed to the loss of the CD36 receptor on the cell surface in microgravity [59]. The expression of MHC-II, but not of MHC-I, was also severely reduced in microgravity, which could result in a disturbed capability of antigen-presentation and binding to CD4-positive T lymphocytes and therefore in failure of adaptive immune system activation. Binding to T lymphocytes could be also hampered by the reduced expression of LFA-1 (CD11a/CD18). Down-regulation of Mac-1 (CD11b/CD18), an important pattern recognition and complement receptor [60–62], would disturb complement-mediated phagocytosis and would therefore be a key step in innate immune response.

Our results suggested that key elements of the molecular architecture in human macrophageal cells were severely disturbed after 5 days of microgravity. The disturbed cytoskeleton, the loss of surface receptors for bacteria recognition and activation of T lymphocytes, the loss of an important scavenger receptor and of antigen-presenting molecules represent a functional macrophage phenotype, which is no longer capable of migrating, recognizing and attacking pathogens, or activating the specific immune system. Since macrophages exist frequently as resident and phagocytosing cells that only slowly renew in the parenchyma of different organs such as the brain, skin and bone, there is a potential danger that microgravity down-regulates the innate immune system in key tissues and organs after a few days in microgravity. Needless to mention, results should be interpreted very carefully and with caution because of the limitations of the model system used and due to several technical and biological limitations. However, if our results are even partially true for the in vivo situation in astronauts, the consequence would be a clear limitation on human presence to the Earth's orbit [1] if appropriate countermeasures are not available. More importantly perhaps, such findings would demonstrate that the Earth's gravity field has been and remains one of the most important conditions known today for cellular and complex life.

The experiment during the SIMBOX mission demonstrated that it is possible to set up a complex space life science experiment within 3 years, from the idea until the spaceflight. The experiment demonstrated, that all important tasks, including hardware development and testing, development and standardization of the biological pre-flight and post-flight procedures, mission scenario tests and the very demanding logistics, can be mastered within this time frame. Even more important, all challenges were solved in an inter-cultural cooperation with different styles and procedures in planning, organization, communication and decision making. From the viewpoint of the authors, the “team spirit”, the dedication and the enthusiasm of all project partners from science, industry and agencies were the key driving force for the successful execution of this experiment.

Authors' contributions

O.U. developed the study idea, concept and the overall study design in addition to planning, coordinating and supervising the entire study and mission while also executing and editing the manuscript. K.P. wrote the manuscript, coordinated and supervised the mission, and developed and carried out the pre- and post-flight experiments and analysis. K.P. supervised S.K. and S.H. N.G. participated in the development and execution of the pre- and post-flight experiments and analysis. A.K., E.H., S.E., S.H., D.M.S., D.S., J.B. and A.W. participated in the development of the pre- and post-flight experiments. O.U., S.T. and K.P. planned, coordinated and supervised the biological operations during the SIMBOX mission. O.U., S. T. and D.M.S. performed the biological operations at Jiuquan Satellite Launch Center. N.G., S.E., C.S., S.T. and O. U. performed the biological operations at the Payload Integration and Test Center and Beihang University, Beijing. K.P. and D.M.S. performed the mission test campaign at the Payload Integration and Test Center and Beihang University, Beijing. D.M.S. contributed to development and testing of the experimental hardware. C.D. contributed to the establishment of staining procedures and image processing work flow during post-flight analysis. M.B. performed the technical operations with the experimental hardware and contributed to the planning and execution of the mission. F.Z. contributed to the study idea and supervision of the procedures at Beihang University. F.G. contributed to study idea and edited the manuscript.

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