Effects of Simulated Microgravity on Functions of Neutrophil-like HL-60 Cells

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Abstract Altered gravity, especially microgravity affects cellular functions of immune cells and can result in immune dysfunction for long-term, manned spaceflight and space exploration. The underlying mechanism, however, of sensing and responding to the gravity alteration is poorly understood. Here, a rotary cell culture system (RCCS) bioreactor was used to elucidate the effects of simulated microgravity on polymorphonuclear neutrophils (PMN)-like HL-60 cells. Alteration of cell morphology, up-regulation of (nitric oxide) NO production, enhancement of interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemotactic protein 1 (MCP-1) secretion, and diversity of cellular adhesion molecule expression were observed for the cells cultured in RCCS, leading to the up-regulated inflammatory immune responses and host defense. It was also indicated that such alterations in biological responses of PMNs mediated the reduced rolling velocity and decreased adhesion of PMN-like HL-60 cells on endothelial cells under shear flow. This work furthers the understandings in the effects and mechanism of microgravity on PMN functions, which are

potentially helpful for optimizing the countermeasures to immune suppression in the future long-term, manned spaceflight.

Keywords HL-60 · PMN · Effects of simulated microgravity · RCCS · Flow chamber

Introduction

Astronaut health is critical in spaceflight since such pathophysiological alterations of astronauts as immune dysfunctions or cardiovascular diseases usually take place during in-flight or post flight. Immune dysfunction under microgravity affects the astronaut health directly and leads to infection, as reported in many spaceflight programs and International Space Station (ISS). Taking Apollo program as an example, over 50 % of the crew members suffered illnesses during space missions, including respiratory infection, gastroenteritis, urinary tract infection, and skin infection (Hawkins and Zieglschmid 1975). Thus, understanding the effects of microgravity on the human immune system is essential to ensure the health, biosafety, and manned performance of astronauts during spaceflights. While scientists have worked intensively on preventing human infections in spaceflight, it is still an unsolved issue since the underlying mechanisms remain unclear in most contents.

Cellular responses under microgravity are crucial to understand the biological and physiological processes in space. PMNs, the predominant circulating phagocytes, are the first to reach the sites of infection and play a major role in producing resistance to bacterial infections (Sawyer et al. 1989), and also important in vascular injury during acute inflammatory responses (Varani and Ward 1994). Unlike other leukocytes which have been investigated extensively

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during spaceflight and under the effects of simulated microgravity (Sun et al. 2008; Long et al. 2009; Feurecker et al. 2013; Herranz et al. 2013; Long 2014), only quite a few studies have been reported about the effects of microgravity on PMN responses. During spaceflight or post-flight, the amount of PMNs is 1.5- to 1.85-fold increased to supplement the innate immunity dysfunction (Stowe et al. 1999; Kaur et al. 2004). PMN chemotactic dose is 10-fold reduced in the optimal dose response after landing and PMN adhesion to endothelial cells is enhanced before and after spaceflight (Stowe et al. 1999). Parabolic flight induces an increase in PMN number and the capability of PMNs to produce hydrogen peroxide (H_2O_2) in response to soluble stimuli (Kaufmann et al. 2009). These works seemingly indicate that the action of microgravity has enhanced the immune response of PMNs, which is inconsistent with the reduced capability of lymphocytes (Cogoli and Tschopp 1985; Konstantinova et al. 1993; Meshkov and Rykova 1995; Mills et al. 2002; Mills et al. 2008, 2013), monocytes (Kaur et al. 2005, 2008; Crucian et al. 2011), NK cells (Rykova et al. 1992; Konstantinova et al. 1993, 1995; Mehta et al. 2001), and macrophages (Armstrong et al. 1995; Hsieh et al. 2005; Ortega et al. 2012; Adrian et al. 2013; Wang et al. 2014, 2015; Brungs et al. 2015; Paulsen et al. 2015) under microgravity. The underlying mechanisms for such the difference remain unclear.

A rotating wall vessel (RWV) bioreactor (RCCS) developed by NASA, has been proven to be an efficient way to simulate, at least partially, the effects of microgravity on cellular responses with lower cost compared with spaceflight (Goodwin et al. 1993; Baker and Goodwin 1997; Martin et al. 2000; Savary et al. 2001; Singh et al. 2010), even though how to stimulate the space microgravity environment on ground is a complicated issue and remains to be solved (Long 2014; Warnke et al. 2015). RCCS has been widely used for elucidating the effects of microgravity on lymphocytes (Bai et al. 2011), macrophages (Wang et al. 2014, 2015) and endothelial cells (Versari et al. 2007). To date, no data has been reported about PMNs cultured in RCCS, probably because PMNs isolated from whole blood exhibit a relatively short half-life (several hours) (Lefort et al. 2009; Carrigan et al. 2005) thereby precluding long-term culture. Alternatively, human promyelocytic leukemia cell line HL-60 has been widely used as a model for leukocytes to study their functions, since they can be differentiated into monocyte-like, eosinophil-like, or PMN-like cells using distinct inducing agents (Collins et al. 1977; Newburger et al. 1979; Miura et al. 2000; Tsiftoglou et al. 2003; Carrigan et al. 2005). Here we applied the RCCS bioreactor to unravel the effects of simulated microgravity on both intact HL-60 cells (promyelocytes) and dimethyl sulfoxide (DMSO)-differentiated HL-60 cells (PMN-like cells). Comparing these two types of HL-60 cells, we further

attempted to explain whether the effects of simulated microgravity regulates distinctly the different leukocytes and whether the increased number and activity of PMNs after spaceflight is linked to PMN maturation. We also used lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, to assess the ability of PMNs to defense pathogen infection under the effect of simulated microgravity. Cell morphology and functions such as NO production, inflammatory cytokine secretion, cellular adhesion molecule expression, and flow-induced adhesion were found to alter after exposure to the effects of simulated microgravity in this study.

Materials and Methods

Cell Culture, Differentiation, and Effects of Simulated Microgravity

Human promyelocytic leukemia HL-60 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin in 5 % carbon dioxide (CO_2) humidified air at 37 °C. The cells were induced to undergo differentiation to granulocytes by incubating them at a concentration of 5×10^5 cells/mL in the presence of 1.25 % DMSO for 5 days (Miura et al. 2000; Carrigan et al. 2005). For effects of simulated microgravity, RCCS developed by NASA was used (Savary et al. 2001; Singh et al. 2010). Since RCCS can only simulate microgravity to limited aspects, we keep the conservative term as effects of simulated microgravity. With rotating in a stable speed, medium inside RCCS would suffer very low shear forces and there would be mechanical equilibrium of gravity, shear forces and centrifuge forces. Under the effects of simulated microgravity, most cells cultured in the RCCS have 3-D growth without sedimentation and wouldn't collide to the rotating wall of RCCS. Both HL-60 and DMSO-differentiated HL-60 cells were cultured in suspension. They were seeded into a T25 tissue culture flask (standard static control) or disposable RCCS (RCCS-D vessels; Synthecon Inc., Houston, TX) at a concentration of 1×10^5 cells/mL in a 10-mL volume of culture medium. 1 µg/mL LPS was added in the medium in order to stimulate the cells in some cases. The RCCS was rotated at 15 rpm for 3 days in the same incubator with the control T25 flask. This rotating speed was chosen according our preliminary experiments which indicates that 15 rpm would be optimal for HL-60 cell growth in RCCS (supplemental Fig. 1). In those preliminary tests, we investigated cell proliferation, cell apoptosis, plate binding, transwell migration and adhesion molecules under different rotating speed, which varied from 5 rpm to 25 rpm.

However, no significant differences appeared in all tests except of significantly increased cell proliferation at 15 rpm compared to other rotating speed. It was notice that the speed of 15 rpm was also chosen in other studies as the rotating speed of RCCS (Yi et al. 2009; Zhang et al. 2010). Each experiment was repeated at least in triplicate.

Cell Morphology and Cytoskeleton Staining

Cells were harvested from RCCS or T25 flask, washed with phosphate-buffered saline (PBS), and fixed with 4 % paraformaldehyde at room temperature for 15 min. After being washed in PBS, cells were stained with 5 µg/mL FITC-conjugated phalloidin for cytochemical labeling for filamentous actin (F-actin). Cells were incubated at 37 °C for 60 min, rinsed with PBS twice, and re-suspended with a drop of ProLong Gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY). The cell suspension was then added onto glass slide and covered with coverslip. After curing for 24 h at room temperature in the dark, the images of stained cells were examined using a confocal laser-scanning microscope (Zeiss LSM710, Germany) with a 63× oil-immersion objective. Cell morphology was determined using Image J software, including its projected area and circularity (denoted as the $(4 \times \pi \times \text{area})/\text{perimeter}^2$).

Cell Apoptosis Analysis

Cell apoptosis analysis was performed using the Annexin V-PE apoptosis detection kit I (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. About 1×10^4 cells were measured for each experiment *via* a FACSCanto II flow cytometer (BD Biosciences) and analyzed with manufacturer-provided FACSDiva software. The Annexin V and 7-Amino-Actinomycin (7-AAD) negative cells were defined as intact healthy cells, whereas Annexin V positive and 7-AAD negative or both Annexin V and 7-AAD positive cells were taken as a measure of early or late apoptosis, respectively.

Cell Cycle Analysis

Cell cycle analysis was assessed using FACSCanto II flow cytometer following the nuclei stained by a CycleTEST PLUS DNA Reagent Kit (BD Biosciences). Cell cycle distribution was obtained using FACSDiva software.

NO Production Analysis

Harvested 5×10^5 cells were incubated in 0.5 µM 4-Amino-5-methylamino- 2',7'-difluorofluorescein diacetate (DAF-FM DA, Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C.

Washed cells were analyzed for intracellular NO production using FACSCanto II flow cytometer.

Reactive Oxygen Species (ROS) Production Analysis

Another aliquot of harvested 5×10^5 cells were treated with CellROX Green Reagent (Life Technologies) at a final concentration of 0.5 µM for 30 min at 37 °C, and then washed with PBS. The intracellular ROS level was measured by FACSCanto II flow cytometer.

Phagocytosis of Latex Beads

The phagocytosis activity of the harvested cells was assessed based on the uptake of bacterial-sized 2-µm fluorescent latex beads (Sigma). 5×10^5 cells were mixed with 1×10^7 fluorescent latex beads in 0.5 mL PBS. The mixture was incubated at 37 °C for 30 min without shaking for 1 h. Then cells were washed with 1 mL PBS and spun down at $100 \times g$ for 2 min for three times to remove un-phagocytosed beads. The cells were then analyzed by FACSCanto II flow cytometer to determine the phagocytosis rate (denoted as the ratio of phagocytosed beads to total cells).

Cytokine Secretion Analysis

Culture supernatant was harvested from RCCS or control flask and used for enzyme-linked immunosorbent assay (ELISA). All supernatant samples were stored at -70°C until test. Interleukin-1 (IL-1β), IL-6, IL-8, macrophage inflammatory protein-1 alpha (MIP-1α) and MCP-1 levels in culture supernatant were measured with ELISA kits (R&D Systems, Minneapolis, MN) by following the manufacturer's protocol. Cytokine concentration was determined from a standard curve prepared from the provided controls. The sensitivities of the ELISA kits were as follows: IL-1β, 0.057 pg/mL; IL-6, 0.039 pg/mL; IL-8, 0.28 pg/mL; MIP-1α, 10 pg/mL; and MCP-1, 1.7 pg/mL.

Expression of Cellular Adhesion Molecules

Phycoerythrin (PE)-conjugated mouse IgG1 monoclonal antibodies (mAbs) against α_L integrin (CD11a, clone HI111), α_M integrin (CD11b, clone ICRF44), β_1 integrin (CD29, clone TS2/16), P-selectin glycoprotein ligand 1 (PSGL-1, CD162, clone KPL-1), and isotype-matched irrelevant mouse IgG1 (clone MOPC-21) were purchased from Biolegend (San Diego, CA). Five aliquots of cells harvested from RCCS or T25 flask were respectively incubated with the respective mAbs at a concentration of 10 µg/mL for 30 min on ice. Following washing, fluorescent cells were analyzed by FACSCanto II flow cytometer. Analysis was

done by gating on the cell population, and the geometric mean fluorescence intensity (GMFI) of the cells was used to present the expression of the four adhesion molecules. The GMFI of isotype-mediated binding was subtracted from the total binding.

Flow Chamber Assay

HL-60 cell rolling and adhesion on umbilical vein endothelial cell (HUVEC, ATCC) monolayer under shear flow were measured using a circular GlycoTech flow cell system (for a 35 mm tissue culture dish, #31-001, Gaithersburg, Maryland), respectively. HUVECs were cultured in a 35-mm tissue culture dish, stimulated with 100ng/ml TNF- α (R&D Systems) for 12 h and assembled onto the flow chamber with 2 cm length $\times$ 0.5 cm width $\times$ 0.01 inch height. For rolling velocity test, intact or DMSO-differentiated HL-60 cells were cultured in T-25 flask or RCCS bioreactor with 1 μ g/ml LPS for 3 days, re-suspended in Hank's balanced salt solution (HBSS, with Ca²⁺ and Mg²⁺) containing 1 % BSA at a concentration of 5×10^5 cells/mL, and perfused into the flow chamber at 1 dyne/cm² for 30 min. For shear-resistance test, the harvested cells were re-

suspended in the same buffer at a concentration of 2×10^6 cells/mL, perfused into the flow chamber at 0.1 dyne/cm² for 10 min, and incubated for 10 min without flow in order to allow cell adhesion to occur. After washing out non-adherent HL-60 cells at 0.1 dyne/cm², the shear stress increased stepwise from 1, 2, 4, 8, 16 to 32 dyne/cm² for 30 s each stress. The dynamic processes of both the rolling and shear-resistance tests were recorded at $10\times$ /NA0.25 objective using a CCD camera. The rolling velocity and adherent cell numbers at each stress were analyzed from the video using NIS-ELEMENT software (Nikon, Tokyo, Japan).

Statistical Analysis

Data were expressed as mean $\pm$ SEM of at least three independent experiments. All data were tested for their deviations from the Gaussian distribution using the Kolmogorov-Smirnov test. Unpaired two-tail Student's t-test and Mann-Whitney test (depending on the results of the normality test) were used to assess the statistical significance of the differences. Values of $p < 0.05$ were considered statistically significant.

Fig. 1 DMSO treated HL-60 differentiation. (a–b) Confocal images of intact (a) and DMSO-differentiated (b) HL-60 cells. F-actin was labeled by FITC-conjugated phalloidin (green) while the nucleus was stained with DAPI (blue). (c–d) Mac-1 expression on intact (c) and DMSO-differentiated (d) HL-60 cells

Fig. 2 Cell projected area (**a**) and circularity (**b**) of intact and DMSO-differentiated HL-60 cells cultured in T-25 flasks (closed bars) or in the RCCS bioreactor (open bars) with or without LPS stimulation.

Data are presented as mean $\pm$ SEM of total 50–100 cells in each case. *, $p < 0.05$; **, $p < 0.01$, indicating statistically significant difference between the cells in flask and in RCCS

Results

Differentiation of PMN-like HL-60 Cells

The DMSO-differentiated HL-60 cells showed the alterations of cell morphology and actin cytoskeleton (Fig. 1a–b). It was indicated that, after DMSO treatment, the cells tend to present multiple nuclei, to form peripheral

pseudopodia, and to yield high F-actin expression co-localized with those pseudopodia, which demonstrate the phenotypes of PMN-like cells. As a marker of PMN-like differentiation, undifferentiated HL-60 cells presented essentially no macrophage-1 antigens (Mac-1, integrin $\alpha_M\beta_2$, CD11b/CD18) while DMSO treated HL-60 cells expressed a widely distributed Mac-1 (Fig. 1c–d).

Fig. 3 Cell proliferation (**a**), apoptosis (**b-c**), and cell cycle (**d-f**) of intact and DMSO-differentiated HL-60 cells cultured in flasks (closed bars) or in the RCCS (open bars) with or without LPS stimulation. (**a**) Cell proliferation folds after 72 h culture. (**b-c**) Annexin V flow

cytometry analysis for early (**b**) or late (**c**) apoptosis, respectively. (**d-f**) Cell cycle analysis of G_0/G_1 (**d**), S (**e**), and G_2 (**f**) phases. Data are presented as mean $\pm$ SEM in triplet

Effects of Simulated Microgravity on Cell Morphology and Cytoskeleton

We examined the morphological changes of intact and DMSO-differentiated HL-60 cells under the effects of simulated microgravity, which are in the presence or absence of LPS stimulation (Fig. 2). In the absence of LPS stimulation, cell project area was 1.4-fold increased in RCCS for the intact HL-60 cells, and 0.9-fold decreased for DMSO-differentiated HL-60 (Fig. 2a). Cell circularity was 1.1-fold enhanced for both the intact and the DMSO-differentiated cells in RCCS (Fig. 2b). After being treated with LPS, no significant difference in cell project area or cell circularity was found between the cells cultured in flask and in RCCS (Fig. 2). We also investigated the changes of cytoskeleton under the effects of simulated microgravity. F-actin proteins were always polymerized around the cell membrane, and no significant difference was found between the suspending HL-60 cells cultured in flask and in RCCS in our experimental settings (data not shown).

Effects of Simulated Microgravity on Cell Proliferation, Apoptosis and Cell Cycle

Furthermore, we analyzed the proliferation, apoptosis and cell cycle of the HL-60 cells in different conditions (Fig. 3). No significant differences were found in cell proliferation between the cells in flask and in RCCS even though the proliferation multiple is slightly lower for cells in RCCS (Fig. 3a). Similar ratios of early and late apoptosis were also observed for both types of cells, in which the apoptosis rate for DMSO-differentiated cells seemed to present the wide variation with large standard errors in the presence or absence of LPS-stimulation (Fig. 3b–c). For cell cycle, the percentages of three phases for both types of cells were comparable in the distinct stimulations even though the G₂-phase percentage is slightly higher for the cells in RCCS than those in flask (Fig. 3d–f). Thus, cell proliferation, apoptosis, and cycle were indifferent between the cells in flask and in RCCS.

Effects of Simulated Microgravity on NO/ROS Production and Phagocytosis

We further tested the NO/ROS production and phagocytosis ability of HL-60 cells in order to elucidate the effects of simulated microgravity on host defense against bacterial pathogens (Fig. 4). As compared with those in flask, NO production for intact HL-60 cells in RCCS was 0.6 ~ 0.7-fold decreased in the absence ($p < 0.01$) or presence ($p = 0.08$) of LPS-stimulation but it was 1.4 ~ 1.8-fold increased for DMSO-differentiated HL-60 cells in RCCS in the absence ($p = 0.07$) or presence ($p = 0.05$) of LPS-stimulation (Fig. 4a), demonstrating a modest effects of simulated microgravity on NO production and a reverse impact between intact and DMSO-differentiated HL-60 cells. By contrast, no impact on ROS production (Fig. 4b) or phagocytosis (Fig. 4c) was found between the cells in flask and in RCCS.

Effects of Simulated Microgravity on Cytokine Secretion

To assess if exposure of HL-60 cells to the effects of simulated microgravity has a significant impact on cytokine secretion, we used ELISA assay to profile the secretion of IL-1 β , IL-6, IL-8, MIP-1 α and MCP-1 using the supernatants collected from culturing HL-60 cells in RCCS (Fig. 5). Cytokine concentration of culture supernatant remained a low level without LPS stimulation (Fig. 5a–e). Specifically, a 3.6-fold increased IL-8 secretion was observed for both intact and DMSO-differentiated HL-60 cells and a 2.6-fold MCP-1 secretion was enhanced for intact HL-60 cells when they are cultured in RCCS (Fig. 5c, e). By contrast, levels of cytokine secretion were increased dramatically in the presence of LPS stimulation through Toll-like receptor (TLR) pathway. Here IL-1 β , IL-8, and MCP-1 secretions were enhanced 2.6-, 3.9- and 2.3-fold, respectively, in the supernatants of intact HL-60 cells in RCCS compared with those in flask (Fig. 5a, c, e). Moreover, a 1.7-, 3.7- and 1.7-fold increased IL-6, IL-8, and

Fig. 4 Intracellular NO (a) and ROS (b) production, and phagocytosis activity (c) of intact and DMSO-differentiated HL-60 cells cultured in flasks (closed bars) or in the RCCS (open bars) with or without LPS stimulation. Data are presented as mean $\pm$ SEM in triplet

Fig. 5 IL-1 β (a), IL-6 (b), IL-8 (c), MIP-1 α (d), and MCP-1 (e) levels in culture supernatant of intact and DMSO-differentiated HL-60 cells cultured in flasks (closed bars) or in the RCCS (open bars) with or without LPS stimulation measured with ELISA kits in biological

triplicate and technical duplicate. Data are presented as mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$, indicating statistically significant difference between the cells in flask and in RCCS

MCP-1 secretion were observed for DMSO-differentiated HL-60 cells in RCCS (Fig. 5b–c, e). No significant change was found in the concentration of MIP-1 α in different conditions (Fig. 5d) and the negative values of IL-1 β for intact cells with or without LPS stimulation was attributed to the lower values compared with the ELISA nonspecific control binding.

Effects of Simulated Microgravity on the Expression of Adhesion Molecules

Adhesion molecules such as integrins and selectins play critical roles for the immune system in leukocyte trafficking and migration, immunological synapse formation, co-stimulation, and phagocytosis (Ley et al. 2007). To investigate if the effects of simulated microgravity has a significant impact on adhesion molecule expression of HL-60 cells, we used flow cytometry to determine the presence of lymphocyte function-associated antigen-1 (LFA-1, integrin $\alpha_L\beta_2$, CD11a/CD18, Fig. 6a), Mac-1 (Fig. 6b), very late antigen 4 (VLA-4, integrin $\alpha_4\beta_1$, CD49d/CD29, Fig. 6c), and PSGL-1 (Fig. 6d) at the cell surface respectively. We found a 1.6- or 1.7-fold LFA-1 up-regulation on intact HL-60 cells cultured

with or without LPS in RCCS compared with those in flask (Fig. 6a). For DMSO-treated HL-60 cells, Mac-1 expression was 3.5- or 2.2-fold enhanced for the cells cultured with ($p < 0.05$) or without ($p = 0.08$) LPS in RCCS. No impacts on VLA-4 expression were found for both types of the cells in the absence or presence of LPS stimulation (Fig. 6c). PSGL-1 expression seemed to be reduced in all the cases at a different content, as exemplified the remarkable decrease for intact cells with LPS stimulation (Fig. 6d).

Effects of Simulated Microgravity on HL-60-Endothelial Interaction

Finally, we investigated the rolling and adhesion of HL-60 cells on HUVEC monolayer under shear flow in order to assess if the effects of simulated microgravity regulates significantly HL-60 cell-endothelial cell interactions. Here intact or DMSO-differentiated HL-60 cells were pre-incubated by LPS in T25 flask or RCCS while HUVECs were pre-stimulated with TNF- α , prior to integrate them into a flow chamber assay. For HL-60 rolling velocity, no significant differences were found for intact HL-60

Fig. 6 Expression of cellular adhesion molecules LFA-1 (a), Mac-1 (b), VLA-4 (c), and PSGL-1 (d) of intact and DMSO-differentiated HL-60 cells cultured in flasks (closed bars) or in the RCCS (open bars) with or without LPS stimulation by flow cytometry analysis. Negative values in some cases derived from the non-positive statistics between total and isotype-matched control binding. Data are presented as mean $\pm$ SEM of 3–6 independent experiments. *, $p < 0.05$; **, $p < 0.01$, indicating statistically significant difference between the cells in flask and in RCCS

cells after flask or RCCS exposure. After pre-incubation with DMSO, differentiated HL-60 cells yielded a 0.8-fold reduced rolling velocity in RCCS (Fig. 7a), which is consistent with the reduced expression of PSGL-1 and enhanced expression of Mac-1 (*cf.* Fig. 6b, d). For HL-60 shear-resistant adhesion, it was indicated that under a low shear stress ($1\sim 2$ dyne/cm²), more intact HL-60 cells remained adhered in RCCS (Fig. 7b), presumably due to the enhanced expression of LFA-1 (*cf.* Fig. 6a). Such difference was eliminated when applying high shear stress. By contrast, the 0.7-fold decreased adhesion of DMSO-differentiated HL-60 cells was observed in RCCS at 8 dyne/cm² and no adhesion differences were found between the cells in flask and in RCCS at other given shear stress (Fig. 7c).

Discussion

In this study, we demonstrated the effects of simulated microgravity on the innate immune responses of HL-60 cells. Such stimulation plays distinct roles on the intact and DMSO-differentiated HL-60 cell. It has a reverse impact on cell project area (Fig. 2a) and NO production (Fig. 4a), and affects different production or expression of cytokines (Fig. 5) and adhesion molecules (Fig. 6). These results provide an insight how altered gravity regulates the functions of neutrophils and the underlying mechanisms which mediate the innate immune responses for long-term, manned spaceflight.

Fig. 7 Shear-induced rolling and adhesions of HL-60 cells on HUVECs. (a) Rolling velocities of intact and DMSO-differentiated HL-60 cells cultured in flasks (closed bars) or in the RCCS (open bars) with LPS stimulation rolling on HUVECs pre-stimulated by TNF- α . (b–c) Number of adherent intact (b) and DMSO-differentiated (c) HL-60 cells cultured in flasks (closed bars) or in the RCCS

(open bars) per field of view (FOV) under stepwise shear stress of $\tau = 1, 2, 4, 8, 16$, and 32 dyne/cm² for 30 s each. Data are presented as mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$, indicating statistically significant difference between the cells in flask and in RCCS

Table 1 Summaries of effects of simulated microgravity on immune cells and endothelial cells

Cell Type	Methods	Cytokine	Adhesion molecules	NO	Oxidative burst	Reference
Neutrophils	Parabolic flight Spaceflight HDTBR	IL-8 ↑ G-CSF ↑	LFA-1 ↑ Mac-1 ↓ or ↑ L-selectin ↓ or ↑ ICAM-1 ↓		H ₂ O ₂ - or ↑ Oxidative burst capacity - or ↑	Stowe et al. 1999; Kaur et al. 2004; Kaufmann et al. 2009; Ortega et al. 2009; Kaufmann et al. 2011; Feuerecker et al. 2013; Current study
HL-60	RWV	IL-1β ↑ IL-6 - IL-8 ↑ MIP-1α - MCP-1 ↑	LFA-1 ↑ VLA-4 - PSGL-1 ↓	NO ↓	ROS -	
DMSO- differentiated HL-60	RWV	IL-1β - IL-6 ↑ IL-8 ↑ MIP-1α - MCP-1 ↑	LFA-1 - Mac-1 ↑ VLA-4 - PSGL-1 ↓	NO ↑	ROS -	Current study
Monocytes	Spaceflight	IL-6 ↓ TNF-α ↓ IL-10 ↓ IL-1β ↑ or ↓ IL-8 ↑ or ↓	LFA-1 ↓ L-selectin ↓			Mills et al. 2002; Kaur et al. 2008; Crucian et al. 2011;
Macrophages	RWV Clinostat Parabolic flight Spaceflight	IL-6 ↑ or ↓ IL-12B ↓ TNF-α ↓ IL-1β -	ICAM-1 ↑ Mac-1 ↓ PECAM-1 ↑ CD44 -	NO ↓	ROS ↓	Armstrong et al. 1995; Hsieh et al. 2005; Adrian et al. 2013; Wang et al. 2014; Brungs et al. 2015; Paulsen et al. 2015; Wang et al. 2015;
T lymphocytes	RWV; Spaceflight	IFN-γ ↓ or ↑ IL-4 ↓ IL-6 ↑ TGF-β ↑ IL-17 ↑ IL-2 ↓ IL-1β ↑ IL-10 ↑ MIP-1α ↑	L-selectin - LFA-1 ↓ ICAM-1 ↓			Cooper and Pellis 1998; Mills et al. 2002; Bai et al. 2011; Gridley et al. 2013;
Endothelial cells	RPM RWV	IL-6 ↓ or ↑ TNF-α ↓ IL-1α ↓ IL-8 ↓ or ↑ bFGF ↓	ICAM-1 ↓ E-selectin ↓ VCAM-1 ↓	eNOS ↑ NO ↑		Versari et al. 2007; Ulbrich et al. 2008; Griffoni et al. 2011; Grenon et al. 2013;

HDTBR, head-down-tilt bed rest; RWV, rotating wall vessel (i.e., RCCS used in the current work); RPM, random positioning machine

IL, interleukin; G-CSF, granulocyte-macrophage colony-stimulating factor; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemotactic protein 1; TNF-α, tumour necrosis factor-α; IFN-γ, Interferon-γ; TGF-β, transforming growth factor-β; bFGF, basic fibroblast growth factor

LFA-1, lymphocyte function-associated antigen-1; Mac-1, macrophage-1 antigen; ICAM-1, VLA-4, very late antigen 4; PSGL-1, P-selectin glycoprotein ligand 1; ICAM-1, intercellular cell adhesion molecule-1; PECAM-1, platelet/endothelial cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1

NO, nitric oxide; eNOS, endothelial nitric oxide synthase

H₂O₂, hydrogen peroxide; ROS, reactive oxygen species

Legend: ↑, increased; ↓, decreased; -, unchanged

The cytoskeletal proteins have been proposed as potential gravity sensor, and significant changes of cytoskeletal network have been observed under microgravity environment both during spaceflight and in ground-based simulation techniques (Vorselen et al. 2014). Although we have not seen obvious disorganization of the F-actin under the effects of simulated microgravity, the cell shape changes of HL-60 cells (Fig. 2) could be considered as a sign for cytoskeletal change. It is rather remarkable that most changes happen in non-LPS group, but not in LPS group. Since LPS could also induce actin polymerization and localization in PMNs (Zemans and Arndt 2009), it might induce a potential compensation to the effects of simulated microgravity.

The amount of PMNs is increased during spaceflight or post-flight (Stowe et al. 1999; Kaur et al. 2004), probably due to the enhanced PMN maturation and release from the bone marrow or the decreased PMN apoptosis. Comparing the responses of intact and DMSO-differentiated HL-60 cells under the effects of simulated microgravity, no clear evidence is found for PMN maturation between them (Figs. 2–7). The apoptosis rate remains unchanged under RCCS exposure compared to 1g (Fig. 3b, c), which is also inconsistent with the hypothesis. Increased IL-8 secretion is observed in all cases (Fig. 5c), which could be the reason of PMN increase after spaceflight, since IL-8 might induce more PMNs released from the bone marrow (Loos et al. 2009; Van Eeden and Terashima 2000).

Impaired immune responses under microgravity have been reported for other types of leukocytes such as T lymphocytes, monocytes, macrophages as well as endothelial cells. Interestingly, an enhanced immune response is reported for PMNs under microgravity, as seen in our work and those clues from literatures. Table 1 summarize our data and further compare this work with published findings, as below.

NO is one of the important messengers and effector molecules, which have close relationship with immune function and can nonspecifically inhibit or kill bacteria, fungi, viruses, and parasites (Chen et al. 2014). In addition to the direct toxicity, NO could also influence many physiological processes from DNA transcription and replication to protein synthesis and secretion (Pavanelli et al. 2010). NO production is demonstrated to be involved in mechanotransduction of mechanically stimulated bone cells (McGarry et al. 2008) and endothelial cells (Rizzo et al. 1998), and regulated by the effects of simulated microgravity (Random Positioning Machine) in endothelial cells (Grenon et al. 2013) and macrophages (Hsieh et al. 2005). NO is synthesized by a class of NADPH-dependent NO synthases (NOSs) and regulated by arginase (Shatanawi et al. 2015). NO production is significantly increased by up-regulating endothelial NOS (eNOS) in endothelial cells

under the effects of simulated microgravity (Grenon et al. 2013), and decreased due to the enhanced arginase I expression in macrophages (Wang et al. 2015). Here we observe that NO production is decreased for intact HL-60 cells but slightly increased for DMSO-differentiated HL-60 cells under the effects of simulated microgravity (Fig. 4a). This reverse impact might be a result of differentially expressed NOS and arginase isoforms during neutrophil maturation (Kumar et al. 2010).

The capacity of ROS release and pathogen phagocytosis is also important in innate immune response. ROS release is reduced in macrophages under the effects of simulated microgravity because of significantly reduced spleen tyrosine kinase Syk phosphorylation (Adrian et al. 2013; Brungs et al. 2015). The oxidative burst of the neutrophils is increased immediately upon landing after a 5–11 day spaceflight mission compared to 10 days before launch (Kaur et al. 2004), but the spontaneous H₂O₂ production by PMNs after 30 subsequent parabolas remains unchanged. The phagocytic capacity of PMNs does not reveal significant changes after 5 day spaceflight mission or 30 subsequent parabolas, but increased after 9-, 10-, and 11-day spaceflight missions. We have not observed ROS release or phagocytic capacity alteration under the effects of simulated microgravity in this work (Fig. 4b–c), probably because the loading time (72 h) is not sufficiently long to lead significantly effects in HL-60 cells.

Varied profiles of cytokine secretion have been seen previously in spaceflight mission and by the effects of simulated microgravity. Specifically, cytokine production of monocytes, macrophages and endothelial cells is mostly reduced under microgravity, and distinct cytokines secreted by T lymphocytes respond differently to microgravity environment. Our study reveal the up-regulation of IL-1 β , IL-8, and MCP-1 in intact HL-60 cells and the enhanced secretion of IL-6, IL-8, and MCP-1 in PMN-like HL-60 cells under the effects of simulated microgravity. These cytokines are potent chemotactic and activating factors of PMN, monocytes, macrophages and lymphocytes, and responsible for fever and acute inflammatory response. The increased levels of these cytokines could lead to the activation of immune cells and the up-regulation of inflammatory immune responses. It should also be noticed that IL-8 could regulate the expression and activation of LFA-1 and Mac-1 (Li et al. 2013), therefore presenting the influence on adhesion molecules expression (Fig. 6a–b) and HL-60-endothelial interaction (Fig. 7).

LFA-1 and Mac-1 are two β_2 integrins expressed on PMNs, which mediate PMN recruitment cascade by binding to intercellular adhesion molecule (ICAM-1) (Ley et al. 2007). Here we observe the increased LFA-1 and Mac-1 expressions on intact and PMN-like HL-60 cells under the effects of simulated microgravity respectively (Fig. 6a–b),

which could be responsible for the slightly enhanced adhesion (Fig. 7b) and reduced rolling velocity (Fig. 7a). We also notice the modest decreased PSGL-1 (Fig. 6d), which might result in disturbing selectin-PSGL-1 interaction (Jia et al. 2009; Xiao et al. 2012) and integrin activation (Yago et al. 2010), and then causing reduced adhesion (Fig. 7c). The static adhesion of PMNs to endothelial cells is enhanced before and after spaceflight in previous study (Stowe et al. 1999), but the PMN recruitment occurs in blood flow and is regulated by shear stress, suggesting that the flow chamber assay could reveal more physiological information than static adhesion assay.

Taken together, this work presents the effects of simulated microgravity on intact and PMN-like HL-60 cells for the first time, and the outcomes could well supplement those, very limited studies on PMN functions from spaceflights. Unlike other leukocytes, PMN-like cells respond to microgravity with the enhanced immune responses to some extent. They exhibit slightly enhanced NO production and up-regulation of IL-6, IL-8, and MCP-1. These data is in consistence with the increased PMN amount and cytokine secretion found in spaceflight (Stowe et al. 1999; Kaur et al. 2004). The reduced rolling velocity and decreased adhesion of PMN-like cells on endothelial cells under shear flow point out PMN recruitment might not be effective under microgravity, especially when the adhesion molecules on endothelial cells are decreased (Grenon et al. 2013). This work also implies that the effects of microgravity environment could be stimulated, at least partially, using a ground-based methodology with much low cost to predict how immune cells respond to the altered gravity. Physical principles, numerical simulations, human physiological tests, and spaceflight missions (Sun et al. 2008; Long et al. 2009; Feuerecker et al. 2013; Long 2014) are required to validate such ground-based predictions in the future works. Our findings furthers the understandings in the effects and mechanism of microgravity on PMN functions, which are potentially helpful for optimizing the countermeasures to immune suppression in the future long-term, manned spaceflight.

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