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ORIGINAL PAPER

The Distinctive Sensitivity to Microgravity of Immune Cell Subpopulations

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Abstract Immune dysfunction in astronauts is well documented after spaceflights. Microgravity is one of the key factors directly suppressing the function of immune system. However, it is unclear which subpopulations of immune cells including innate and adaptive immune cells are more sensitive to microgravity. We herein investigated the direct effects of modeled microgravity (MMg) on different immune cells *in vitro*. Mouse splenocytes, thymocytes and bone marrow cells were exposed to MMg for 16 hrs. The survival and the phenotypes of different subsets of immune cells including $CD4^+$T cells, $CD8^+$T cells, $CD4^+Foxp3^+$ regulatory T cells (Treg), B cells, monocytes/macrophages, dendritic cells (DCs), natural killer cells (NK) were determined by flow cytometry. After splenocytes were cultured under MMg for 16h, the cell frequency and total numbers of monocytes, macrophages and $CD4^+Foxp3^+$T cells were significantly decreased more than 70 %. MMg significantly decreased the cell numbers of $CD8^+$ T cells, B cells and neutrophils in splenocytes. The cell numbers of $CD4^+$T cells and NK cells were unchanged significantly when splenocytes were cultured under MMg compared with controls. However, MMg significantly increased the ratio of mature neutrophils to immature neutrophils in bone marrow and the cell number of DCs in splenocytes. Based on the cell survival ability, monocytes, macrophages and $CD4^+Foxp3^+$Treg cells are most sensitive to microgravity; $CD4^+$T cells and NK cells are resistant to microgravity; $CD8^+$T cells and neutrophils are impacted by short term microgravity exposure. Microgravity promoted the maturation of neutrophils and development of DCs *in vitro*. The present studies offered new insights on the direct effects of MMg on the survival and homeostasis of immune cell subsets.

Keywords Immune cells · Microgravity · Modeled microgravity · Immune cell subpopulations · Innate immune cells · Adaptive immune cells · Spaceflight

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Introduction

The immune system has an essential role in protecting humans and animals from pathogen infections (Cogoli et al. 1984). Microgravity, an important characteristic in space that differs from that found on earth, is directly involved in the immune dysfunction found in astronauts after spaceflights. Microgravity has extensive impacts on immune cells, including affecting cellular development, cell survival, cytoskeleton, functions, and intracellular signal transduction pathways (Chapes et al. 1992;

Allebban et al. 1994; Crucian et al. 2013; Luo et al. 2014; Wang et al. 2014). Data from astronauts and rodents flown in space have shown that distribution of immune cell subsets changed, and peripheral lymphocyte numbers were decreased (Taylor 1993; Crucian et al. 2000, 2013).

However, our current understanding on the effects of microgravity on immune cells is still limited. In the current study, using a rotary bioreactor system (Wang et al. 2014), we studied the effects of microgravity on different immune cell subpopulations including thymocytes, T cells, $CD4^+CD25^+$ regulatory T cells (Treg), B cells, natural killer cells (NK), neutrophils, macrophages and dendritic cells (DC). The survival and phenotypes of mouse immune cells after exposing to modeled microgravity (MMg) were detected. We found that the responses to MMg of different immune subsets were not identical. Macrophages and monocytes were more sensitive to the microgravity than peripheral T cells. However, microgravity could promote the maturation of neutrophils and development of DCs in the *in vitro* assays. Thus, our studies showed that microgravity directly affects the survival and homeostasis of immune cell populations in distinctive manners.

Materials and Methods

Animals

Adult male C57BL/6 (B6) mice were purchased from the Vital River Experimental Animal Center (Beijing, China). All animals were housed in specific-pathogen free conditions, and before experiment, mice were allowed 1-wk to acclimate to the animal facility after delivery from the supplier. All experimental procedures were in accordance with the Institution Guidelines for the Care and Use of Laboratory Animals.

Immune Cell Isolation

Mouse splenocytes were isolated as reported previously (Zhu et al. 2014). Briefly, mouse spleens were aseptically removed and placed in RPMI-1640 medium (Gibco, USA). Splenocytes were isolated by mechanical dissociation through sterile nylon mesh, followed by red blood cell lysis (17mM Tris-HCl and 140mM NH_4Cl, pH 7.2). Cells were suspended in RPMI 1640 supplemented with L-glutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml), 2-ME (5×10^{-5} M), and 10 % FBS. Mouse bone marrow cells and thymocytes were harvested as reported previously (Sun et al. 2012; Chen et al. 2013).

mAbs and Chemical Reagents

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA): anti-mCD4-FITC, anti-mCD8-PE, anti-mTCRβ-PE, anti-mCD11b-FITC, anti-mCD11c-PE, anti-mB220-PE and anti-mFoxp3-PE. Anti-mCD8-PE-Cy5, anti-Gr1-PE, anti-mNK1.1-PE and anti-mF4/80-PE were obtained from ebioscience (San Diego, CA).

Rotary Bioreactor System

Modeled microgravity effects were generated by culturing cells in an in-house developed MG-I rotary bioreactor system at 10 rpm as reported previously (Wang et al. 2014). Within the system, a 10-ml round-pan tank for cell culture is fixed on a steel shaft and can be driven to rotate by an electrical motor. The rotary velocity can be regulated in the range from 0 to 30 rpm. The tank is separated from outside by a pair of 0.2mm thick silicone membranes which are CO_2 and O_2 permeable on its right and left sides respectively. The reactor is a 3-dimensional suspension culture system that should be completely filled with 1640 medium so that the fluid and the vessel will rotate as a solid body to suspend cells in a state of constant free-fall and thereby reduce the effective gravitational force experienced by the cells (Luo et al. 2014). Before the bioreactor rotating, there should be without any bubbles in it, otherwise, the cell activity might be impaired by a great fluid shear force during rotating progress. In the present study, total 3×10^7 cells (3×10^6 cells/ml$\times$10ml) were added into the MG-I rotary bioreactor system and cultured in a humidified incubator at 37 °C and 5 % CO_2 for 16 h (Luo et al. 2014). During the experiments, the static cultured cells in the same vessel with the same concentration were used as control.

Cell Staining and Flow Cytometry

Cells were collected and washed twice with ice cold FACS buffer (PBS, pH 7.2, containing 0.1 % NaN_3 and 0.5 % BSA). 5×10^5 cells were resuspended in 100 uL FACS buffer and the non-specific staining was blocked by anti-mouse FcR mAb (2.4G2). The cells were then incubated with fluorochrome conjugated mAbs at 4 °C for 30 minutes (Chen et al. 2013). The intracellular Foxp3 expression in $CD4^+$T cells was stained as described previously (Chen et al. 2013). All isotype-specific fluorescence conjugated antibodies were used throughout as controls for non-specific cell surface binding in all FACS-based mea-

surements. Mouse cells were washed twice with FACS buffer and at least ten thousand cells were assayed using a FACS Calibur and Beckman Coulter Epics XL, and data were analyzed with CellQuest software.

Statistical Analysis

All data are presented as the mean±SD. Statistical analysis was performed with SPSS 11.0, using either the t-test or one-way ANOVA. A p value less than 0.05 was considered to be statistically significant.

Results

MMg Exposure on Thymocytes

T cells are all originated from hematopoietic stem cells, which migrate from bone marrow to the thymus via the cortical-medullary junction and interact extensively with the thymic stromal cells including thymic epithelial cells and fibroblasts (Fooksman et al. 2010). When first enter into the thymus from bone marrow, the progenitor cells express no CD4 and CD8 and are called double negative (DN) cells. Through positive selection, These DN cells progress to double positive (DP) cells, and then become CD4 or CD8 single positive cells (CD4SP and CD8SP respectively) through negative selection (Hashemi et al. 1999). In order to address whether microgravity exposure directly affects T cell development in the thymus, we cultured the freshly isolated thymocytes in a rotary bioreactor system for 16h to mimic microgravity, and then observed the subpopulations of T cells in thymus. As shown in Fig. 1, significantly decreased total cell number of thymocytes was observed after thymocytes exposed to MMg for 16h ($P<0.01$, Fig. 1A). However, the percentages of thymocyte subsets, including DN, DP, CD4SP and CD8SP were identical in MMg-exposure thymocytes as control group (1g) (Fig. 1B, C). These data suggest that MMg exposure resulted in impaired thymocyte survival. But thymocyte subsets have similar sensitive to MMg as evidenced the unchanged percentages of thymocyte subsets.

MMg Exposure on Peripheral T Cell Subpopulations

$CD4^+$ and $CD8^+$ T cells are two major subpopulations of T cells. We determined whether MMg exposure affected the composition of T cell compartments. After exposure to MMg for 16h, the percentage of $CD4^+$T cells in splenocytes was significantly increased ($P<0.05$, Fig. 2A, B), whereas the frequency of $CD8^+$T cells was comparable between MMg exposure and control group (Fig. 2A, B). However, the cell number of $CD8^+$T cells was decreased dramatically after MMg exposure ($P<0.001$, Fig. 2C), while no significant alteration were detected in $CD4^+$T cell number in this group. Thus, $CD4^+$ T cells were more resistant to microgravity than $CD8^+$ T cells. The increased percentage of $CD4^+$ T cells is likely caused by the decreased percentages of other immune cells possibly due to cell death. In addition, the frequencies of T cell receptor (TCR) in $CD4^+$ and $CD8^+$T cells were identical between control and MMg-treated group (Fig. 2D–F) supporting short-term exposure to MMg did not change the TCR expression on T cells. Furthermore, the MFI of TCR expression on $CD4^+$ and $CD8^+$ T cells were similar in MMg and I G groups, indicating that TCR expression are resistant to microgravity. These data collectively indicate that microgravity differentially attenuate the survival and homeostasis of T cell subsets.

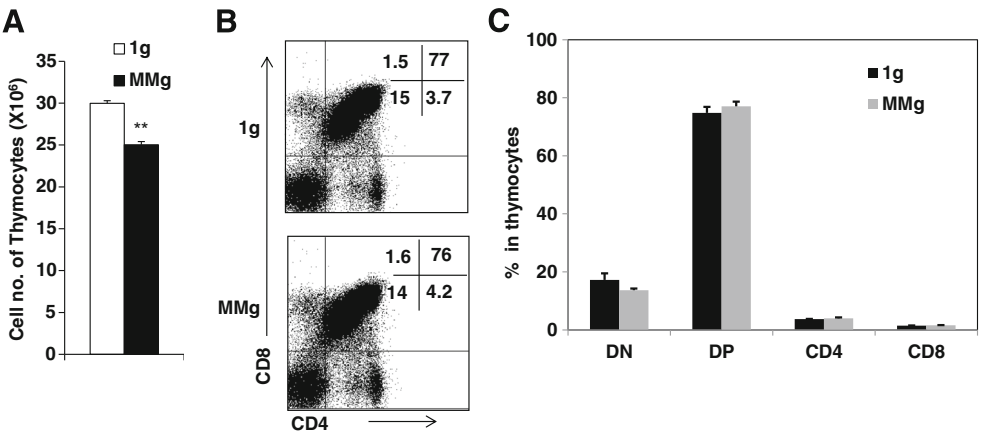

Fig. 1 The response of thymocyte subsets to MMg exposure

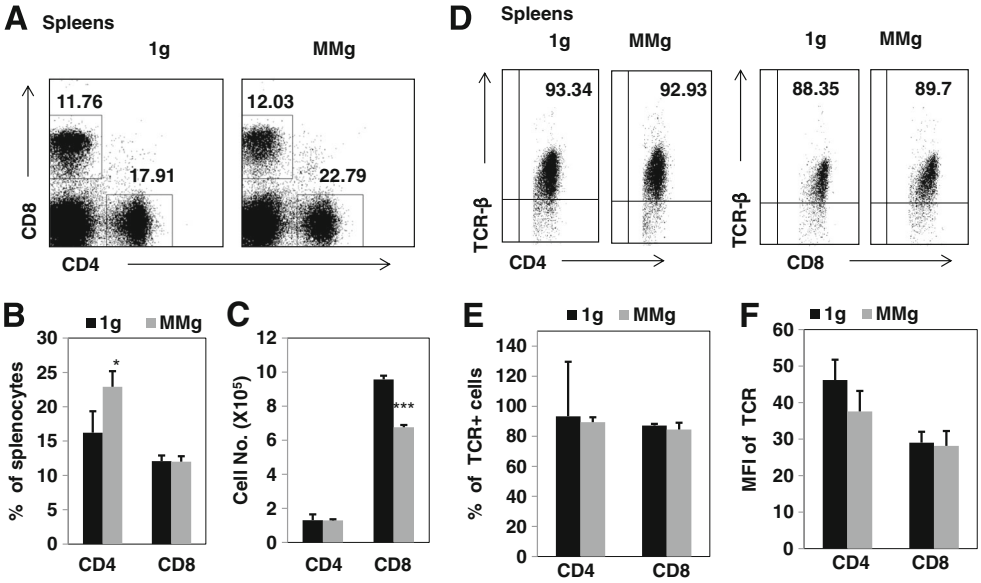

Fig. 2 Effects of MMg exposure on the subsets of peripheral T cells

Mouse $CD8^+$T cells are more sensitive to microgravity than $CD4^+$T cells in the respect of cell survival.

MMg Exposure on $CD4^+CD25^+$Treg Cells

Immunosuppressive $CD4^+CD25^+Foxp3^+$Treg cells play crucial roles in the maintenance of self-immune tolerance and keep host proper intensity of immune response (Qu and Zhao 2007; Zheng et al. 2009; 2009). Decreased numbers and/or functional deficiency of $CD4^+CD25^+Foxp3^+$Treg cells result in development of a variety of autoimmune diseases like multiple sclerosis and type I diabetes (Wang et al. 2006; Zeng et al. 2012; Miyara et al. 2014). Thus, the altered $CD4^+CD25^+Foxp3^+$Treg cells may be involved in the impaired function of astronauts' immune system. We detected the potential direct impacts of MMg on immunosuppressive $CD4^+Foxp3^+$Treg cells. Interestingly, when mouse splenocytes were cultured under MMg condition for 16h, a significantly lower proportion and cell number of $CD4^+Foxp3^+$Treg cells were observed in MMg exposure group compared with control group ($P<0.01$, Fig. 3), suggesting that $CD4^+CD25^+Foxp3^+$Treg population is more sensitive to microgravity than $CD4^+CD25^+$ T effector cells.

MMg Exposure on Mature B Cells and NK Cells

As we know, B lymphocytes and NK cells differentiate from hematopoietic stem cells through a series of distinct stages. Early B and NK cell development proceeds in bone marrow until immature cells migrate into the secondary lymphoid tissues, such as spleens and lymph nodes. B cells are critical for antibody production and immune regulation (Stolp et al. 2014). NK cells are important for host defense against tumor (Cichocki et al. 2014). In order to understand whether the microgravity impacts on B and NK cell subpopulations, we cultured the splenocytes and bone

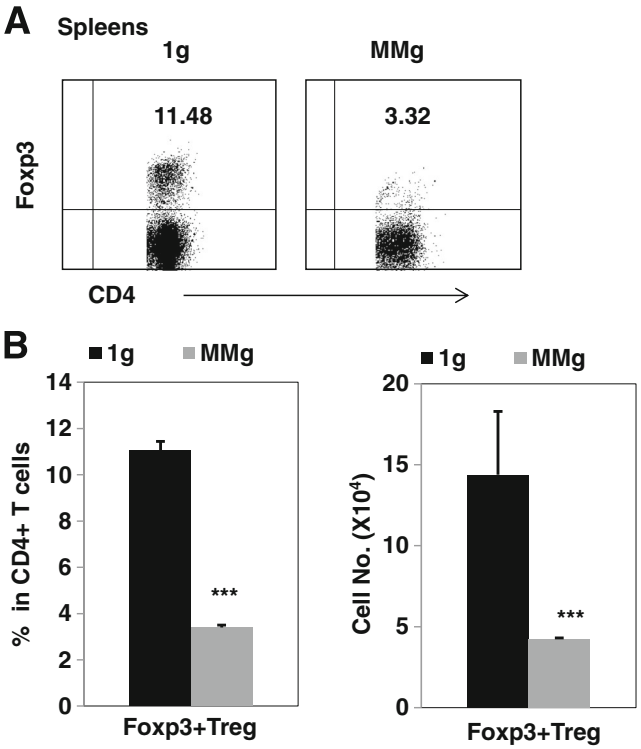

Fig. 3 Decreased percentages and cell numbers of splenic $CD4^+Foxp3^+$Treg cells after MMg exposure

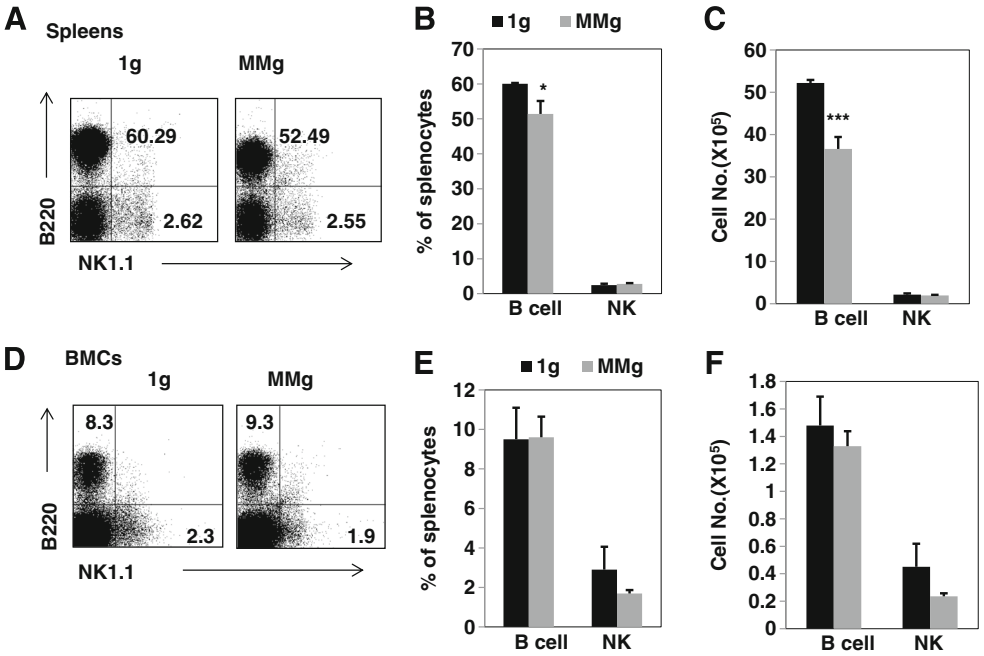

Fig. 4 Effects of MMg exposure on the B cells and NK cells in spleens and BMCs

marrow cells in a rotary culture condition for 16hrs. The frequency and number of $B220^+$ B lymphocytes in splenocytes were significantly decreased in MMg exposure group ($P<0.05$, Fig. 4A–C). But the levels of NK cells were similar in both MMg and control groups (Fig. 4A–C). The same trend of B cells and NK cells were observed in bone morrow cells (Fig. 4D–F). These data indicate that mature B cells in the periphery were more sensitive to MMg exposure compared with NK cells.

MMg Exposure on Innate Immune Cells

Monocytes/macrophages and DCs have crucial and distinct roles in tissue homeostasis and immunity, but they also contribute to a broad spectrum of pathologies and are thus attractive therapeutic targets (Hu et al. 2012). They have a common origin in hematopoietic stem cells and develop along distinct differentiation pathways in response to internal and external cues. Macrophages play an essential role in

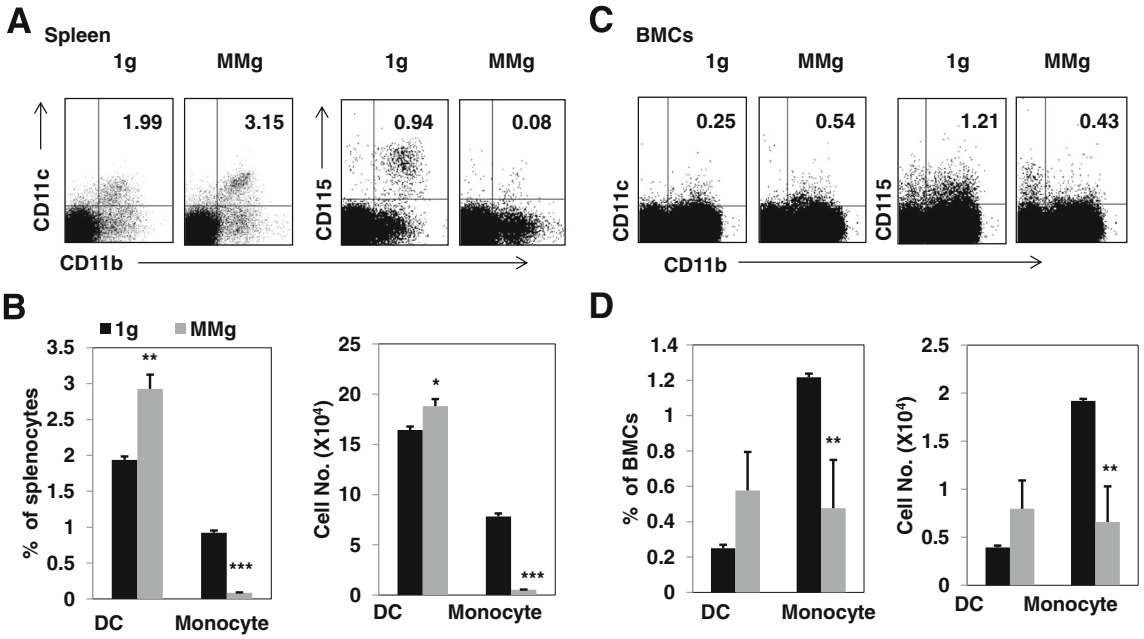

Fig. 5 Effects of MMg on DCs and Monocyte in spleens and BMCs

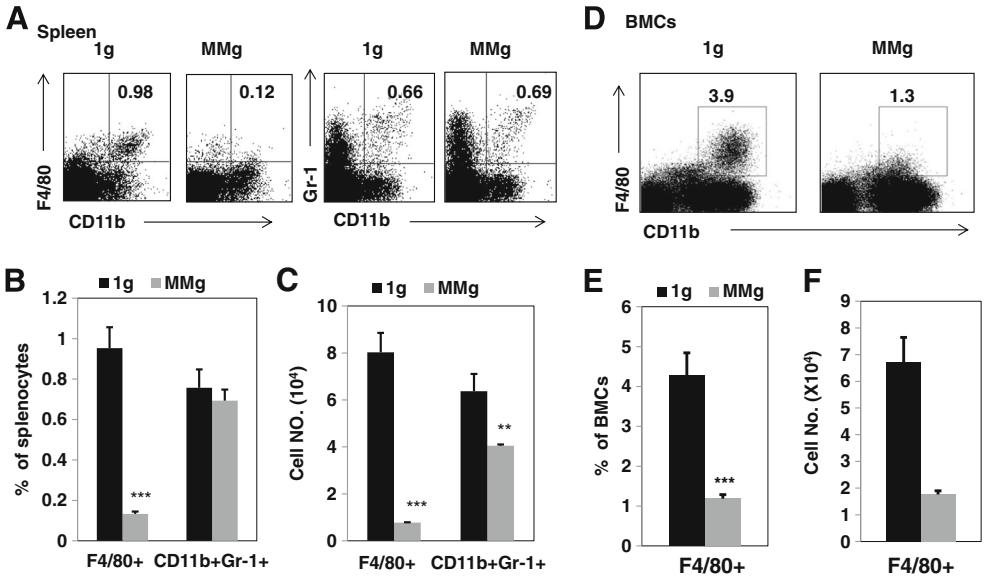

Fig. 6 Effects of MMg on macrophages and neutrophils in spleens and BMCs

tissue homeostasis, they orchestrate the initiation and resolution phases of both innate and adaptive immunity, with significant impact on protective immunity and immune-mediated pathological damage. Neutrophils represent the first immunologic barrier against invading pathogens, and neutropenia predisposes to infection (Crucian et al. 2013; Sun et al. 2014). Neutrophils are one of the earliest immune cells recruited to the sites of infection and inflammatory response. Therefore, we are interested in exploring the potential effects of microgravity on innate immune cell populations.

After culture of freshly isolated mouse splenocytes and bone marrow cells in rotary bioreactor system for 16h, we analyzed the cell numbers and the phenotypes of $CD11b^+CD11c^+$ DCs and $CD11b^+CD115^+$monocytes in spleens and bone marrow. As shown in Fig. 5, after 16h static culture, the percentage and cell number of DCs were significantly higher both in splenocytes (Fig. 5A, B) and bone marrow cells compared with the control (Fig. 5C, D), while the population of monocytes was decreased. In addition, we have detected monocytes and DCs with different time points with MMg treatment. The results showed that in splenocytes, monocytes and DCs expressed exposure-time dependent manner of MMg effects (Fig. s1A). These results indicated that MMg exposure may promote DC development and differentiation.

Our recent studies demonstrated that short-term treatment with microgravity caused significantly decreased TNF-α production (Wang et al. 2014), but whether microgravity affects macrophage development was remain unknown. As shown in Fig. 6, the frequency and number of $CD11b^+F4/80^+$ macrophages in splenocytes were reduced after cultured in MMg exposure for 16h ($P<0.001$, Fig. 6A–C). The total cell number of $CD11b^+Gr1^+$

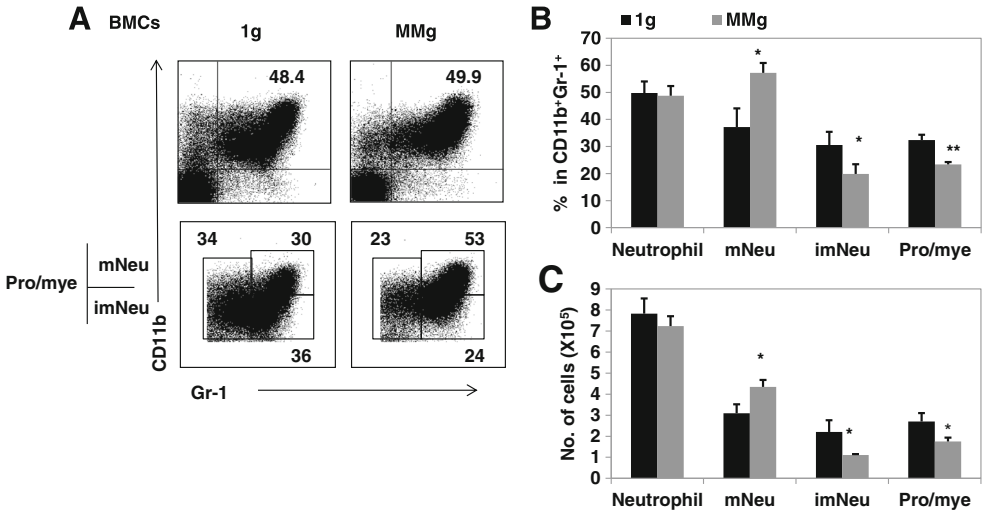

Fig. 7 Microgravity promotes the maturation of neutrophils in BMCs

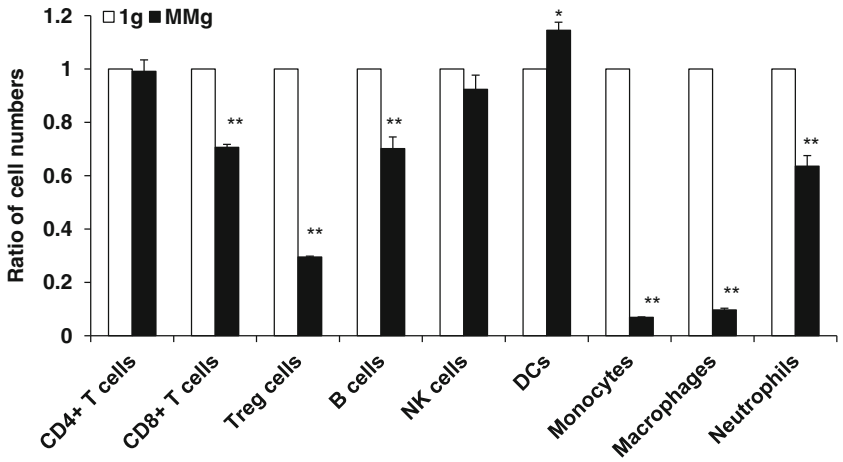

Fig. 8 The different responses of immune cell subsets to MMg exposure

neutrophils were also significantly decreased although the proportion of neutrophils were comparable with control group ($P<0.01$, Fig. 6A–C). Furthermore, we analyzed the population of macrophages in bone marrow cells and the results were identical to the splenocytes (Fig. 6D–F), showing the remarkable decreased $CD11b^{+}F4/80^{+}$macrophages after exposure to MMg.

MMg Promotes the Maturation of Myeloid Neutrophils *in vitro*

Neutrophils develop and mature in the bone marrow. They are fully equipped with a variety of granules, which contain proteases that enable the neutrophils to deliver lethal hits against invading microorganisms. As granulocytic differentiation proceeds in bone marrow through GMPs ($CD34^{hi}CD16/32^{hi}$), $CD11b^{int}Gr\text{-}1^{int}$ promyelocytes/myelocytes, $CD11b^{low}Gr\text{-}1^{hi}$ immature neutrophils (imNeu), and $CD11b^{hi}Gr\text{-}1^{hi}$ mature neutrophils (mNeu) (Yamada et al. 1982; Liu et al. 2013). The results showed that bone marrow $Gr1^{+}CD11b^{+}$ neutrophils cultured with MMg exposure had identical cell number and proportion as control group (Fig. 7A–C). But interestingly, $CD11b^{hi}Gr\text{-}1^{hi}$ mNeu cultured with MMg exposure had higher cell percentage and absolute cell number, while the $CD11b^{low}Gr\text{-}1^{hi}$ imNeu cells and $CD11b^{int}Gr\text{-}1^{int}$ Pro/mye cells were significantly reduced compared to the control group ($P<0.05$, Fig. 7A–C). These results indicate that microgravity can promote the maturation of myeloid neutrophils *in vitro*.

Different Sensitivity to MMg of Immune Cell Subpopulations

In order to identify the sensitivity to microgravity of different immune cell populations, we calculated the survival percentages of each immune cell subpopulation after splenocytes exposed to MMg for 16h. As shown in Fig. 8, different immune cell subpopulations decreased in different degrees after exposure to MMg, in terms of cell survival. Monocytes and macrophages are most sensitive to microgravity, while $CD4^{+}$T cells and NK cells are most resistant to microgravity ($P<0.01$, Fig. 8). Among T cells, $CD4^{+}Foxp3^{+}$Treg cells are most sensitive to microgravity ($P<0.01$, Fig. 8). B cells and NK cells showed mild response to microgravity. In contrast to the decreased cell numbers of other immune cells under MMg, DCs showed increased cell number after splenocytes were cultured under MMg ($P<0.01$, Fig. 8). Thus, microgravity significantly altered the cellular components of splenocytes.

Discussion

In recent years, microgravity has been indicated a direct factor for the immune dysfunction in spaceflights (Cogoli et al. 1984; Mermel 2013). However, knowledge about the adaptation of the immune cell subpopulations to microgravity is still limited, and the data from experiments involving space flown animals as well as astronauts varied time to time (Taylor et al 1986; Gould et al. 1987; Allebban et al. 1994; Crucian et al. 2000; Pecaut et al. 2000; Mills et al. 2001; Borchers et al. 2002; Crucian et al. 2013). The changes in immune system caused by microgravity may be due to the changes of microenvironments, endocrine factors and others. In present study, in order to offer insight about the sensitivity of immune cells to microgravity, we used the MG-I rotary bioreactor system to generate the effect of microgravity and cultured the freshly isolated immune cells under this conditions to see the direct effects of microgravity on immune cells *in vitro* (Luo et al. 2014). The rotary cell culture system (the rotary bioreactors) used here is just a model system for mimicking some biological

effect of microgravity, which is the best available for studying the effects of microgravity on cells. Haiying Luo et al found that 16hrs was the ideal time point to detect the difference between MMg and I G groups. Nevertheless, it is worthy to be noted whether these findings are consistent with those in the real microgravity needs to be demonstrated. The results showed that the survival and homeostasis of immune cell populations could be directly affected by MMg exposure for 16hrs, and moreover, the different subsets have different responses to MMg. Among all the detected immune cell subpopulations, Treg cells, macrophages, and monocytes are the most sensitive subsets to MMg exposure, and both their cell frequency and total numbers decreased significantly. T cells and B cells were affected mildly. NK cells in the spleen showed no detectable changing in both cell frequency and total numbers after exposure to MMg. Mature neutrophils in the spleen significantly decreased after exposure to MMg. The neutrophil development in bone marrow was promoted by MMg. To our surprise, DCs was obviously increased after splenocytes exposed to MMg for short term.

As the main part of adaptive immunity, T cells play an important role in various humoral and cellular immune responses. In this study, it was found that the frequency and number of $CD4^+$T cells did not show detectable alteration after cultured under MMg condition in terms of the cell survival, while $CD8^+$T cells were significantly decreased, indicating that $CD8^+$T cells were more sensitive to microgravity than $CD4^+$T cell. In spite of these results, it has been already demonstrated that the functions of T cells can be inhibited by microgravity directly. Furthermore, our recent studies demonstrated that The concanavalin A-induced activation and cell proliferation of both $CD4^+$ and $CD8^+$ T cells were significantly suppressed by pre-exposing to MMg, and this suppression was in an exposure-time dependent manner (Luo et al. 2014). Therefore, it seems that the detection of T cell functions is more sensitive parameter to evaluate the effects of microgravity on immune cells.

As an important subset of T cells, immunosuppressive $CD4^+CD25^+$Treg cells play crucial roles in the maintenance of self-immune tolerance (Zhang and Zhao 2007; Miyara et al. 2014). $CD4^+CD25^+$Treg cells mainly differentiate in thymus and migrate to peripheral tissues (Gratz and Campbell 2014). In this study, it was found for the first time that frequency and total cell number of the peripheral $CD4^+CD25^+$Treg cells were down regulated significantly by MMg. These results indicated that the astronauts might be in the high risk to have a variety of autoimmune diseases after their spaceflight because of the $CD4^+CD25^+$Treg cell deficiency caused by microgravity.

Monocytes and macrophages are important components of the immune system. Impaired cytokine secretion by activated monocyte/macrophages under microgravity has been reported (Limouse et al. 1991; Schmitt et al. 1996). We recently reported that LPS-induced TNF-α expression in macrophages was suppressed under MMg condition, and this microgravity-reduced TNF-α production may be mediated by the induction of HSF1 activity (Wang et al. 2014). Here, the seriously decreasing survival and homeostasis of monocyte/macrophages were detected. Up to now, there has no report about the effect of microgravity on DCs. In present study, it was demonstrated for first time that MMg could promote the differentiation of DCs in the spleen. Whether this expansion of DCs had normal function needs to be studied. Nevertheless, both dysfunction and abnormal frequency of monocyte/macrophages might cause functional suppression of innate immunity directly in one hand, and in the other hand, made adaptive immunity abnormal indirectly.

The survival of mature neutrophils was decreased after splenocytes were cultured under MMg condition in terms of the cell survival. However, the granulocytic differentiation in bone marrow was affected by MMg, during which the mNeu had higher cell percentage and absolute cell number, while the imNeu and Pro/mye cells were reduced compared to the control group. These results suggested that MMg might promote the development of granulocyte Whether MMg can cause its function abnormality need to be studied in the future.

In summary, microgravity could directly affect the survival and homeostasis of immune cells and different immune subset has different sensitivity to MMg effect. With the cell survival as the detection parameter, the microgravity-sensitivity rank of immune cell subpopulations is summarized as following: Monocytes and macrophages > $CD4^+Foxp3^+$Treg cells > $CD8^+$T cells and neutrophils > $CD4^+$T cells and NK cells. The present study offered new insight for the alteration of immune cell components caused by microgravity. The conclusion that monocytes/macrophages and $CD4^+Foxp3^+$ Treg cells are most sensitive to MMg provide potential cellular targets for us to efficiently prevent microgravity-caused immune alteration.

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Competing interests The authors declared that no competing interest exists.

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