Inflammation Research

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Microgravity activates p38 MAPK-C/EBPβ pathway to regulate the expression of arginase and inflammatory cytokines in macrophages

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Abstract

Objective and design Molecular mechanisms of microgravity-caused immunosuppression are not fully elucidated. In the present study, we investigated the effects of simulated microgravity on macrophage functions and tried to identify the related intracellular signal pathways.

Material or subjects Primary mouse macrophages were used in the present study. The gene expression and function of IL-4-treated mouse macrophages were detected after simulated microgravity or 1 g control.

Methods Freshly isolated primary mouse macrophages were cultured in a standard simulated microgravity situation using a rotary cell culture system (RCCS-1) and 1 g control conditions. Real-time PCR, western blots and flow cytometry were used to investigate the related intracellular signals and molecule expression.

Results The arginase mRNA and protein levels in freshly isolated primary mouse macrophages under simulated microgravity using RCCS-1 were significantly higher than those under normal gravity. Meanwhile, simulated microgravity induced over-expression of C/EBPβ, a transcription factor of arginase promoter, and activation of p38 MAPK, which could increase C/EBPβ expression. Furthermore, upregulation of Interleukin-6 (IL-6) and down-regulation of IL-12 p40 (IL-12B) in LPS-stimulated macrophages were also detected after simulated microgravity, which is regulated by C/EBPβ.

Conclusions Simulated microgravity activates a p38 MAPK-C/EBPβ pathway in macrophages to up-regulate arginase and IL-6 expression and down-regulate IL-12B expression. Both increased arginase expression and decreased IL-12B expression in macrophages during inflammation could result in immunosuppression under microgravity.

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Introduction

Microgravity exposure could cause severe abnormalities in human physiology, including fluid shift, anemia, osteoporosis, immunosuppression, etc. [1–3]. In the 1960s and 1970s, 15 of the 29 Apollo astronauts reported bacterial or viral infections that occurred during flight or soon after return to earth [4]. Subsequent in-flight studies indicated that spaceflight was specifically associated with the reactivation of latent herpes viruses [5–11], which is indicative

of immunosuppression under microgravity. In addition, tests of the delayed-type hypersensitivity on cosmonauts demonstrated impaired cell-mediated immunity in space [12]. Dysfunction of immune cells represents an important mechanism for the immunosuppression under microgravity. Impaired proliferative responses of T cells to T cell receptor (TCR) agonists and decreased cytokines secretion by activated monocyte/macrophages have been observed under microgravity [13–19].

Arginase I expressed by myeloid cells has immunosuppressive roles. First, arginase-mediated depletion of intracellular L-arginine could limit the inducible nitric oxide synthase (iNOS)-catalyzed production of antimicrobial nitric oxide (NO), as this NO-producing reaction uses L-arginine as substrate. Second, arginase-mediated depletion of extracellular L-arginine could suppress T cell immune responses through down-regulation of TCRζ chain in activated T cells [20]. Whether microgravity alters the expression of arginase I was previously unknown. In the present study, we observed that microgravity could up-regulate arginase I expression in macrophage cells through p38 MAPK-C/EBPβ pathways.

Materials and methods

Cell culture and reagent treatment

Preparation and culture of primary mouse macrophages were performed as previously described [21]. In brief, 10–12 weeks male C57BL/6 mice, which were purchased from Beijing University Experimental Animal Center (Beijing, China), were each intraperitoneally injected with 1 ml of 3 % Brewer thioglycollate medium. Five days later, the total peritoneal cells were harvested, suspended in culture medium (Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 100 μg/ml penicillin and 0.1 mg/ml streptomycin), and cultured in plastic plates at 37 °C for 30 min. Nonadherent cells were washed away with warm PBS. The adherent cells (mostly macrophages) were detached from the plastic plates with an ice-cold solution of 1 mM EDTA in PBS. The collected macrophages were divided and cultured under either normal gravity or simulated microgravity. For the simulated microgravity condition, the macrophages were cultured in a rotary cell culture system (RCCS-1) manufactured by Synthecon, Inc. Disposable vessels (10 ml) were used in the rotary cell culture system [19]. Cytodex 3 microcarriers (GE healthcare) were supplemented in the rotary cell culture for the macrophages to adhere to. The rotation speed was initially set to 12 rpm, which is recommended by the manufacturer. As aggregates of cells/microcarriers formed in 1 h, the rotation speed was increased to 25 rpm to keep the aggregates in suspension. The aggregates disassembled 2 h later. The rotation speed was then decreased to 17 rpm. For the normal gravity control condition, the macrophages were cultured in plastic plates coated with gelatin/attachment factor (life technologies). After 24 h culture, the macrophages were either directly used for assays, or were stimulated with mIL-4 (10 ng/ml, R&D Systems) or LPS (100 ng/ml, Sigma) for subsequent assays. Animal protocols were approved by the Animal Ethics Committee of the Institute of Zoology, Beijing, China.

Intracellular cytokine staining and flow cytometry

Macrophages were fixed and analyzed for the intracellular production of cytokines (BD PharMingen) by staining with anti-mIL-6 and anti-mIL-12B Abs respectively and subsequent flow cytometry assay as described previously [22]. Anti-mF4/80-PE-Cy5 mAb was purchased from BD Biosciences PharMingen (San Diego, CA). Anti-mIL-12-PE and anti-mIL-6-PE mAbs were obtained from BioLegend (San Diego, CA).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from cell samples using EZNA® MicroElute Total RNA Kit (Omega Bio-Tek). One microgram total RNA was reverse transcribed into cDNA by oligo (dT) primers and AMV reverse transcriptase XL (Takara). Real-time PCR was performed with a CFX96 real-time PCR detection system (Bio-Rad). The PCR mixture was at a volume of 20 μl containing 10 μl SYBR Premix Ex Taq (Takara), 0.5 μM each of the primers, and 1 μl cDNA prepared as described [23]. PCRs were cycled 40 times after initial denaturation (95 °C, 2 min) with the following parameters: denaturation 95 °C for 15 s, annealing 60 °C for 20 s, and extension 72 °C for 15 s. The $2^{-\Delta\Delta CT}$ method was used to compare gene expression in different cell samples, with HPRT (hypoxanthine phosphoribosyltransferase) expression as the internal control [24, 25]. The following primers were used: HPRT, sense, 5′-AGTACAGCCCCAA AATGGTTAAG-3′, and antisense, 5′-CTTAGGCTTTGTA TTTGGCTTTTC-3′; IL-6, sense, 5′-GCAATGGCAATTC TGATTGTATG-3′, and antisense, 5′-AAGGACTCTGGCT TTGTCTTTCT-3′; IL-12B, sense, 5′-TCTTTGTTCGAAT CCAGCGCA-3′, and antisense, 5′-CGATCCTGAGCTTG CACGCA-3′; C/EBPβ, sense, 5′-GCCAAGAAGACGGTG GACAAGCT-3′, and antisense, 5′-CTTGAACAAGTTCC GCAGGGTG-3′; and YM1, sense, 5′-CAAGTTGAAGGC TCAGTGGCTC-3′, and antisense, 5′-CAAATCATTGTG TAAAGCTCCTCTC-3′; and arginase I, sense, 5′-CCAGA AGAATGGAAGAGTCAGTGT-3′, and antisense, 5′-GCA GATATGCAGGGAGTCACC-3′.

p38 MAPK activation

Macrophages were cultured under normal gravity, and were treated with anisomycin (25 μg/ml, Cell Signaling Technology) for 30 min, either with or without pretreatment with SB202190 (20 μM, Sigma; 60 min) and/or SP600125 (50 μM, Cell Signaling Technology; 40 min). After replacement of the culture medium by fresh medium, the macrophages were cultured for additional 4 h. The C/EBPβ and arginase I expression were then assayed by using the real-time PCR method described above [26].

Western blots

Whole cell lysates were prepared with RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma). The protein concentration was determined with a BCA protein assay kit (Beijing Cell Chip Biotechnology). Samples containing equal amounts of protein were resolved on 10 % SDS-PAGE. The proteins were transferred onto immobilon P polyvinylidene fluoride membranes (Millipore). Immunoblots were developed as described previously [27]. The following primary antibodies were used: anti-GAPDH (Proteintech No. 60004-1-Ig), anti-arginase I (Santa Cruz Biotechnology, No. sc-20150), anti-p-STAT6 (Cell Signaling Technology No. 9361), anti-C/EBPβ (LAP) (Cell Signaling Technology, No. 3087), anti-p-p38 (Cell Signaling Technology No. 9216), anti-p-ERK (Cell Signaling Technology No. 4370), and anti-p-JNK (Cell Signaling Technology No. 4668).

ELISA

The amounts of IL-6 and IL-12p40 in the culture supernatant of LPS-stimulated macrophages were assayed with mouse IL-6 ELISA MAX™ Deluxe kit (BioLegend, Inc.) and mouse IL-12/IL-23p40 Elisa kit (Dakewe Biotech Co., Ltd.), respectively. The manufacturer's instructions were strictly followed.

Statistical analysis

All data are presented as the mean ± SD. Student's unpaired t test for comparison of means was used to compare groups. A P value <0.05 was considered to be statistically significant.

Results

Microgravity increased arginase I expression through up-regulation of C/EBPβ

To study whether microgravity alters the expression of arginase I in macrophage cells, we cultured primary mouse macrophages under normal gravity and simulated microgravity, respectively, and assayed the arginase I expression. The arginase I expression in macrophages under simulated microgravity was significantly higher than in those under normal gravity (Fig. 1a). Consistently, the protein amount of arginase I in macrophages under simulated microgravity was larger (Fig. 1b). We were interested in how microgravity up-regulated the expression of arginase I. The induced arginase I expression could be caused by activation of the transcriptional factor STAT6 [28, 29]. We therefore assayed the amounts of phosphorylated STAT6, which is the activated form of STAT6 [28], in macrophages cultured under the two gravity conditions. Phosphorylated STAT6 was undetectable in macrophages under either gravity condition (Fig. 1c). This indicates that microgravity did not induce the activation of STAT6. The arginase I promoter could also be activated by the transcriptional factor C/EBPβ [30]. We therefore assayed the amounts of C/EBPβ. Larger amount of C/EBPβ was detected in macrophages under simulated microgravity compared with the normal gravity condition (Fig. 1d).

The 8-bromo-cAMP-induced binding of C/EBPβ to arginase I promoter could enhance IL-4-induced arginase I expression [30]. Since microgravity increased the expression of C/EBPβ, which may bind to the arginase I promoter, we hypothesized that the IL-4-induced arginase I expression might be higher under simulated microgravity than under normal gravity. Indeed, the IL-4-stimulated mouse macrophages under simulated microgravity expressed higher level of arginase I than those under normal gravity (Fig. 2a). The IL-4-induced YM1 expression, which is one of the up-regulated molecules in M2 macrophages and is controlled by STAT6 activation [28, 29], was similar under the two gravity conditions (Fig. 2b). In addition, the IL-4-induced STAT6 activation (phosphorylation) was identical under the two gravity conditions (Fig. 2c). Altogether, the results indicate that microgravity increased the expression of C/EBPβ, which might consequently up-regulate the expression of arginase I in mouse macrophages.
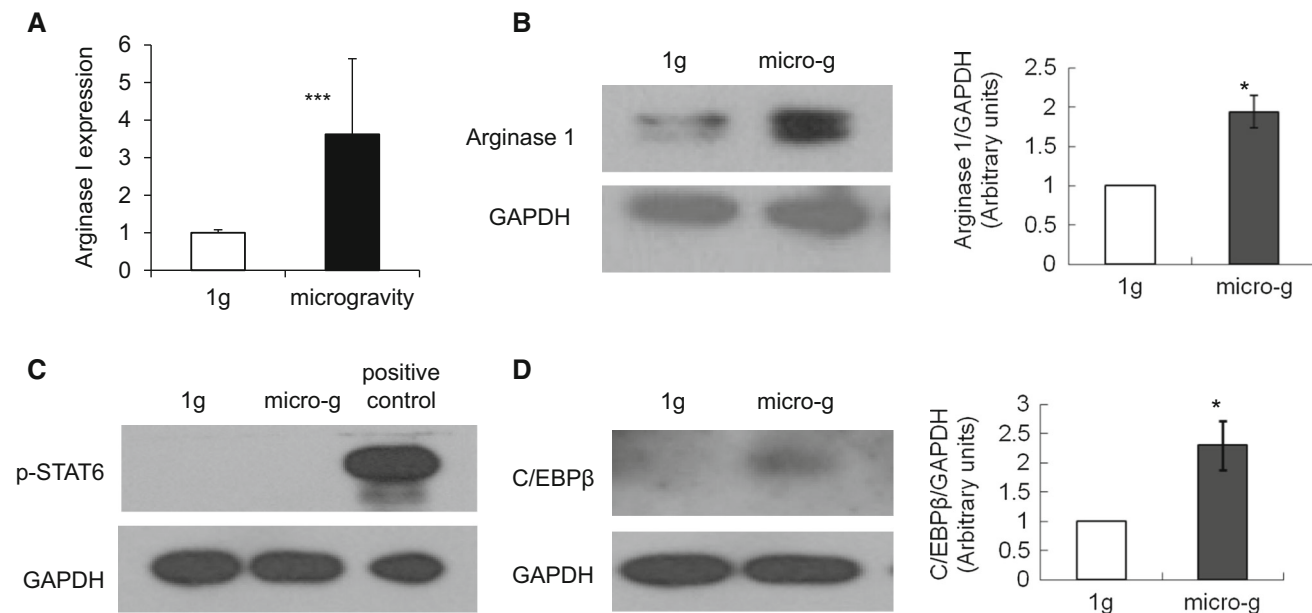
Fig. 1 Microgravity increased arginase I expression through up-regulation of C/EBPβ. **a** The arginase I expression in macrophages cultured under either simulated microgravity or normal gravity (1 g). Data are the mean ± SD of three independent experiments. ***$P < 0.001$. **b** Immunoblots for arginase I, **c** p-STAT6 and **d** C/EBPβ in macrophages cultured under either simulated microgravity (micro-g) or normal gravity (1 g). Results presented are one representative of three independent experiments showing identical results. Positive control: IL-4-stimulated macrophages. Results of densitometry measurement of repeat experiments were also shown. *$P < 0.05$ compared with the control

Fig. 2 The IL-4-induced arginase I expression in macrophages was increased under simulated microgravity. The arginase I (**a**) and YM1 (**b**) expressions in IL-4-stimulated macrophages under either simulated microgravity or normal gravity (1 g) were detected by real-time PCR. Data are the mean ± SD of three independent experiments. ***$P < 0.001$. The macrophages were treated with IL-4 (10 ng/ml) for 24 h. **c** Immunoblots for p-STAT6 in IL-4-stimulated macrophages under either simulated microgravity (micro-g) or normal gravity (1 g). Control: non-stimulated macrophages. Results of densitometry measurements were also shown
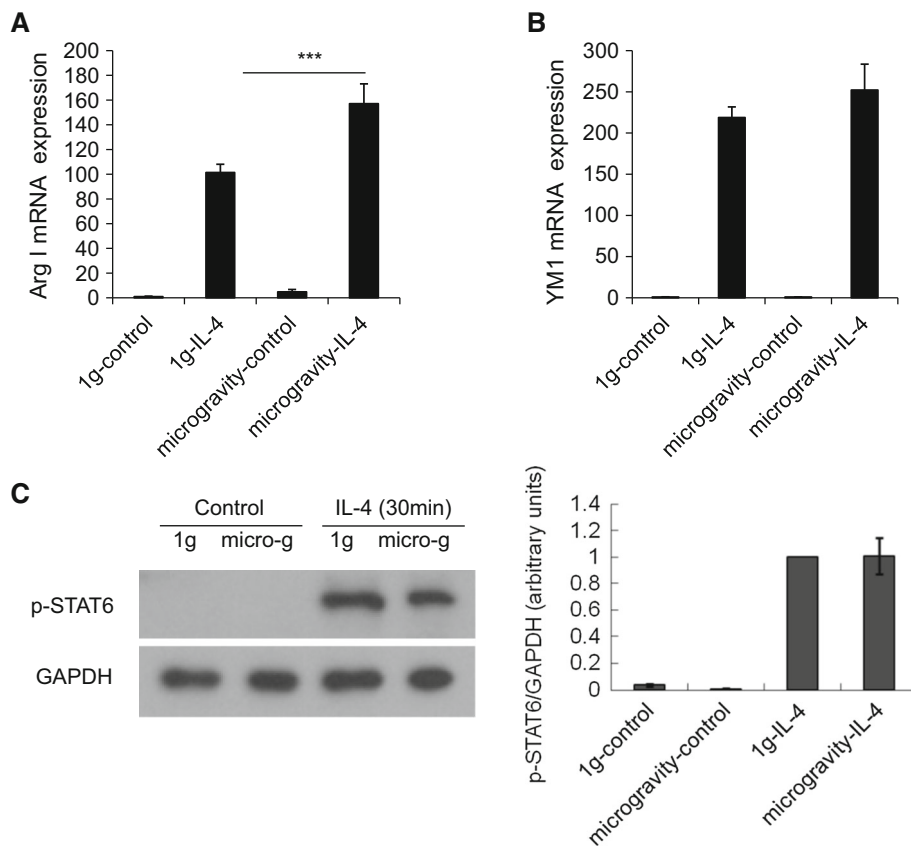
Microgravity increased C/EBPβ expression through p38 activation

We next investigated the mechanisms of the C/EBPβ induction by microgravity. Previous studies showed that C/EBPβ expression could be induced by p38 MAPK, JNK or ERK activation in different experimental settings [31]. Furthermore, the p38, JNK and ERK MAPK pathways were proposed to be signaling pathways of microgravity [32]. We therefore assayed whether these pathways were activated by simulated microgravity. Phosphorylated JNK and phosphorylated ERK, the activated forms of JNK and ERK, were undetectable in macrophages under either gravity condition (Fig. 3a). This indicates that neither JNK nor ERK was significantly activated by simulated microgravity. Phosphorylated p38 MAPK, the activated form of p38 MAPK, was undetectable in macrophages under normal gravity, but was clearly detected in macrophages under simulated microgravity (Fig. 3a). This indicates that p38 was activated by simulated microgravity.

To confirm that p38 MAPK activation could lead to C/EBPβ over-expression in our experimental setting, we treated macrophages with a p38 MAPK activator and assayed the C/EBPβ expression. The treatment with anisomycin, an activator of p38 and JNK [33], significantly increased the C/EBPβ expression (Fig. 3b). To specifically activate p38 MAPK, we treated macrophages with anisomycin combined with a specific JNK inhibitor (SP600125) [33, 34]. Macrophages treated with anisomycin plus SP600125 expressed even higher level of C/EBPβ than those treated with anisomycin alone (Fig. 3b). The induction of C/EBPβ expression by anisomycin plus SP600125 treatment could be reversed by a specific p38 inhibitor, SB202190 (Fig. 3b). These results indicate that p38 activation increased C/EBPβ expression while JNK activation inhibited it in primary mouse macrophages. Thus, the microgravity-induced p38 activation in macrophages may lead to C/EBPβ over-expression.

We further assayed whether p38 MAPK activation in macrophages could increase arginase I expression. As shown in Fig. 3c, p38 MAPK activation in macrophages by
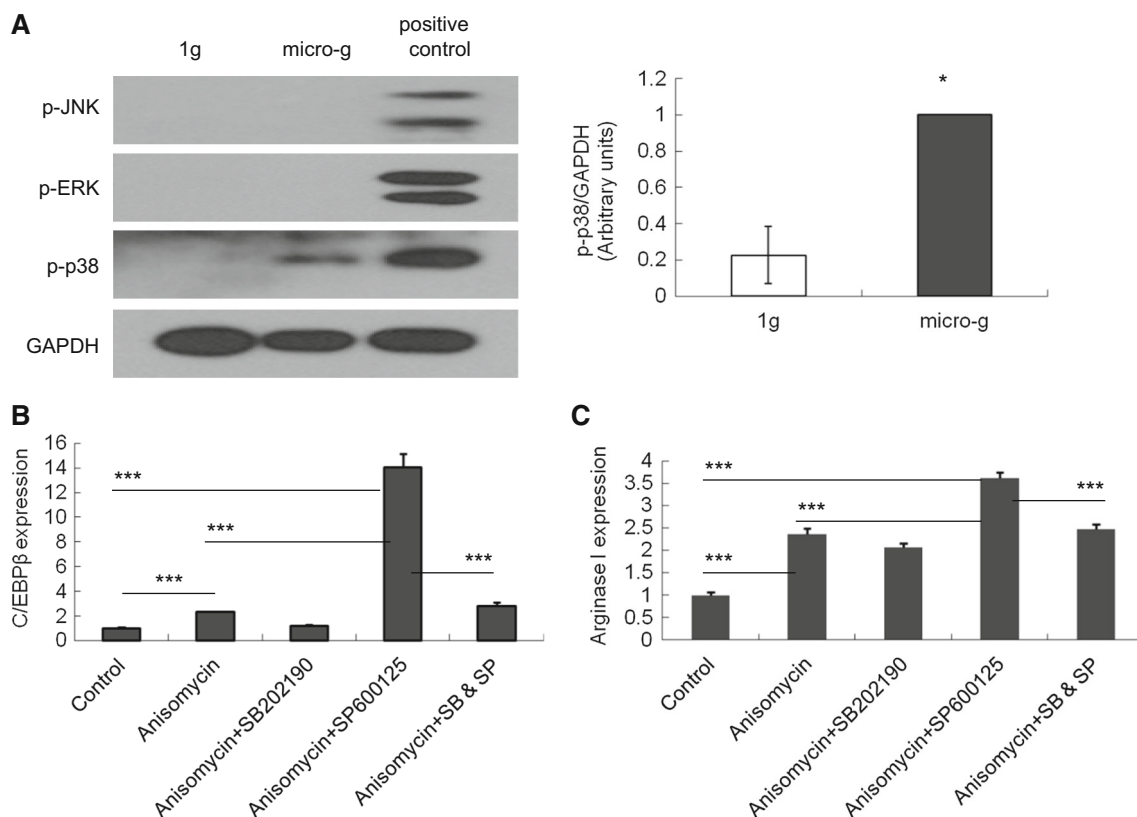

Fig. 3 Microgravity increased C/EBPβ expression through p38 MAPK activation. **a** Immunoblots for p-p38 MAPK, p-ERK and p-JNK in macrophages cultured under either simulated microgravity (micro-g) or normal gravity (1 g). Results presented are one representative of three independent experiments showing identical results. Positive control: LPS-stimulated macrophages. Results of

densitometry measurement of repeat experiments were also shown. *$P < 0.05$. The C/EBPβ (**b**) and arginase I (**c**) expression in macrophages treated either with anisomycin alone or with anisomycin plus SB202190 and/or SP600125. Data represent one of three independent experiments showing identical results. ***$P < 0.001$

the treatment with anisomycin plus SP600125 significantly increased arginase I expression. This increase could be reversed by SB202190, a specific p38 inhibitor (Fig. 3c). The results suggest that p38 MAPK activation in macrophages induced C/EBPβ over-expression, which then increased arginase I expression.

Microgravity-induced C/EBPβ over-expression may regulate the expression of inflammatory cytokines in macrophages

In addition to regulating arginase I expression, C/EBPβ may regulate the expression of several inflammatory cytokines [31]. For example, C/EBPβ positively regulates the expression of IL-6 while negatively regulates the expression of IL-12B (IL-12 p40 subunit) [31, 35]. Since the amount of C/EBPβ increased significantly under simulated microgravity, we hypothesized that the expression of IL-6 and IL-12B might alter in this condition. Indeed, the LPS-induced IL-6 expression in macrophages was increased under simulated microgravity, while the LPS-induced IL-

12B expression was decreased (Fig. 4a, b). Consistently, IL-6 secretion by LPS-stimulated macrophages was increased under simulated microgravity, while IL-12B secretion was decreased (Fig. 4c, d). Differences in LPS-induced expression of inflammatory cytokines could be caused by differences in LPS-induced signal transduction. However, it was previously found that the LPS-induced activation of NF-κB and MAPK pathways in macrophages remained unchanged under simulated microgravity [19]. The microgravity-induced C/EBPβ over-expression may contribute, at least partly, to the up-regulation of IL-6 and down-regulation of IL-12B in macrophages under simulated microgravity.

As we found that microgravity increased C/EBPβ expression through p38 activation, we further assayed whether p38 MAPK activation with anisomycin plus SP600125 [33] in macrophages could alter the expression of IL-6 and IL-12B. As shown in Fig. 5, p38 MAPK activation by the treatment with anisomycin plus SP600125 significantly increased the IL-6 mRNA expression in macrophages with or without LPS stimulation, while this

Fig. 4 Microgravity up-regulated IL-6 and down-regulated IL-12B in LPS-stimulated macrophages. The IL-6 (**a**) and IL-12B (**b**) expressions in LPS-stimulated macrophages under either simulated microgravity or normal gravity (1 g) were determined by real-time PCR. The IL-6 (**c**) and IL-12B (**d**) secretions by LPS-stimulated macrophages under either simulated microgravity or normal gravity (1 g) were assayed by ELISA. The macrophages were treated with LPS for 4 h (**a**–**c**) or 24 h (**d**). Data are the mean ± SD of three independent experiments. ***$P < 0.001$. **$P < 0.01$. Control: non-stimulated macrophages
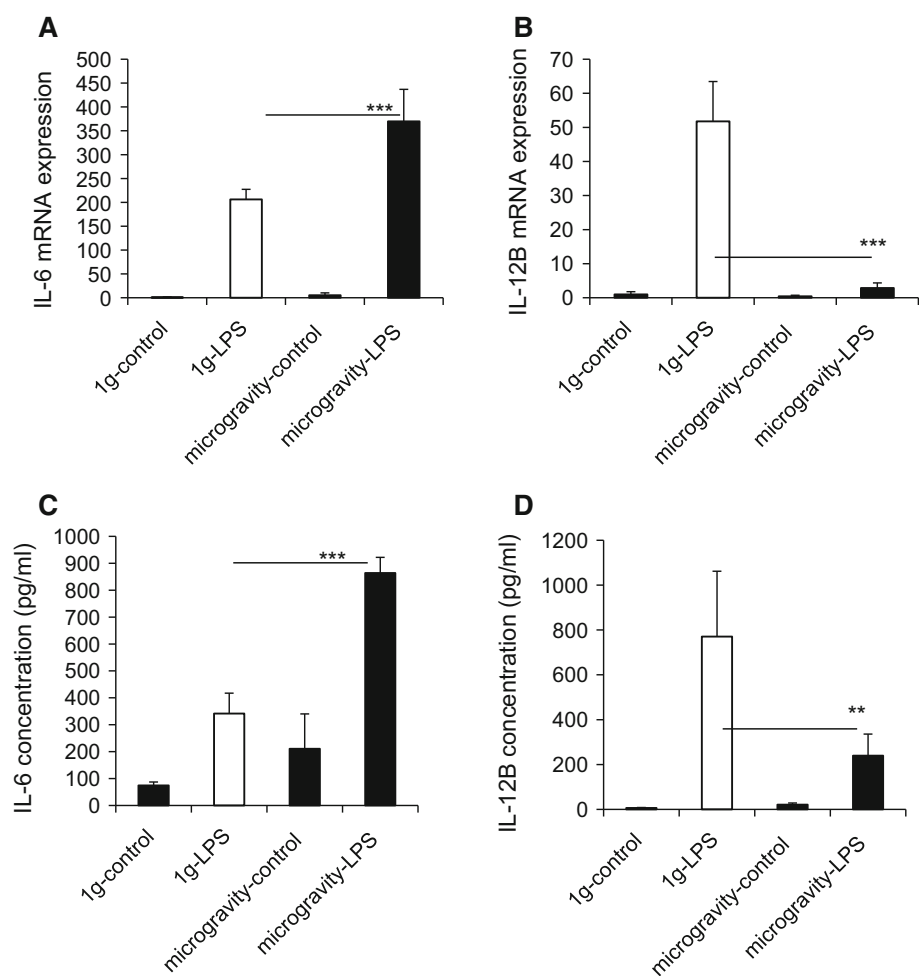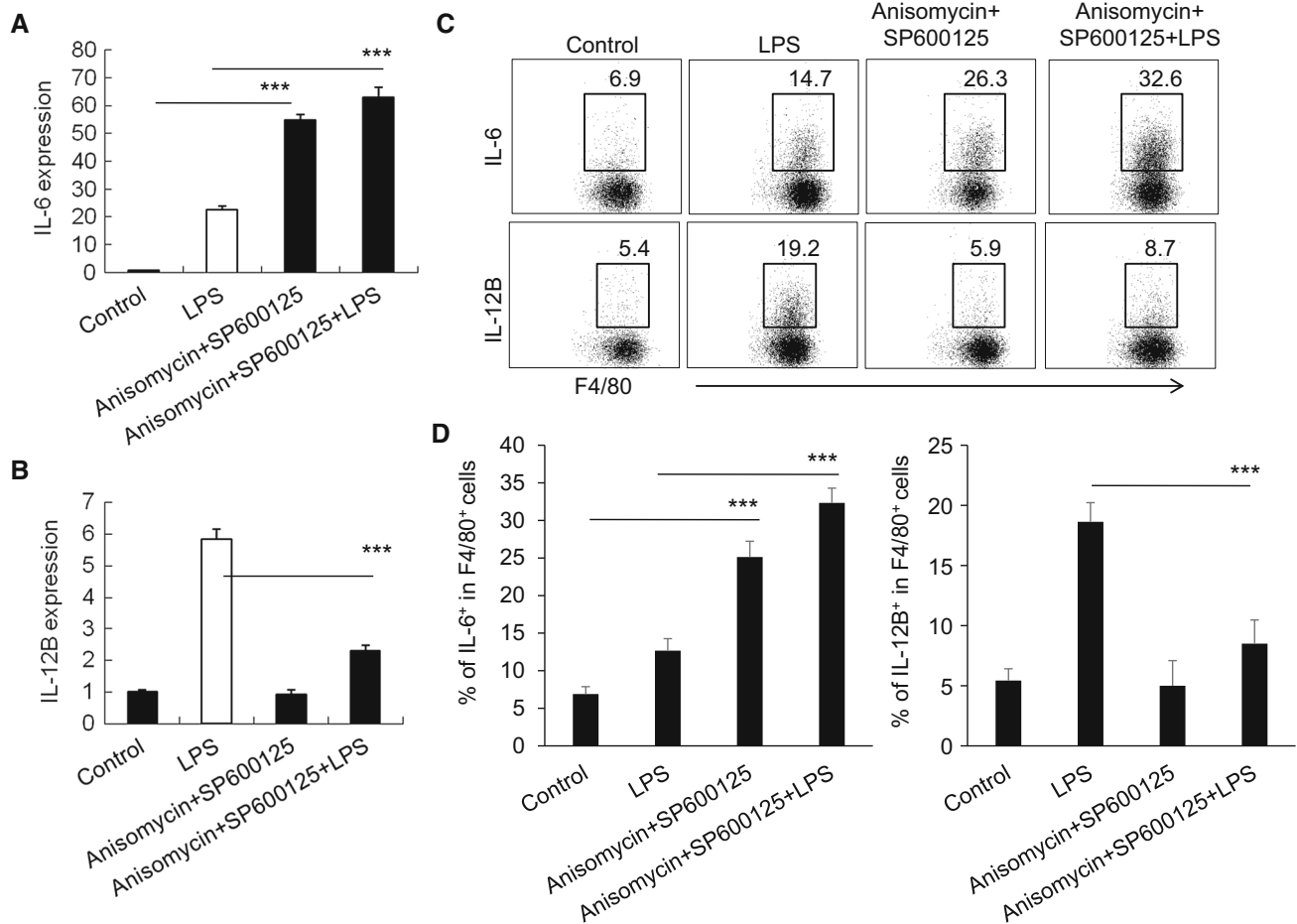
Fig. 5 p38 MAPK activation up-regulated IL-6 and down-regulated IL-12B in LPS-stimulated macrophages. Macrophages were stimulated with LPS for 4 h, with or without previous treatment with anisomycin plus SP600125. The mRNA expressions of IL-6 (**a**) and IL-12B (**b**) were determined by real-time PCR. (**c**, **d**) The protein expressions of IL-6 and IL-12B in these treated macrophages were determined by intracellular staining flow cytometry assays. ***$P < 0.001$. Control: non-stimulated macrophages
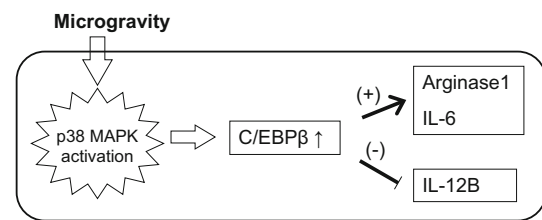
treatment decreased the LPS-stimulated IL-12B mRNA expression in macrophages as determined by real-time PCR ($P < 0.001$, Fig. 5a, b). Consistent with the mRNA expression, p38 MAPK activation by anisomycin plus SP600125 significantly increased the IL-6 protein expression in macrophages with or without LPS stimulation, whereas the treatment with anisomycin plus SP600125 decreased the LPS-stimulated IL-12B protein expression as assessed by intracellular staining flow cytometry ($P < 0.001$, Fig. 5c, d). These results suggest that the microgravity-induced p38 activation may contribute to the up-regulation of IL-6 and down-regulation of IL-12B in macrophages under simulated microgravity.

Discussion

In the present study, we demonstrated that microgravity activated a p38-C/EBPβ pathway in macrophages to regulate the expression of arginase I, IL-6 and IL-12B (Fig. 6).

Fig. 6 Schematic diagram of the effect of microgravity on macrophage function through a p38 MAPK pathway. Microgravity caused activation of p38 MAPK and over-expression of C/EBPβ expression in macrophages which subsequently promoted arginase and IL-6 production and inhibited IL-12 production. *Upper arrow* over-expression, *plus* up-regulation, *minus* down-regulation

The finding that p38 MAPK was activated by simulated microgravity in primary mouse macrophages is consistent with a previous study using RAW264.7 macrophage cell line [36]. Importantly, this is the first report showing that the transcriptional factor, C/EBPβ, was induced by

microgravity. C/EBPβ in cells of the monocytic lineage is known to regulate different genes involved in proliferation, differentiation and immune responses [31]. Therefore, by inducing C/EBPβ expression, microgravity might modulate the proliferation, differentiation and immune responses of macrophage cells.

Through arginine depletion, arginase I in myeloid cells plays immunosuppressive roles, which include reduction of NO production and suppression of T cell immune responses [20]. We are the first to show that arginase I expression in macrophages was up-regulated under microgravity. The microgravity-increased arginase I expression may contribute to immunosuppression under microgravity. It was previously found that LPS plus IFN-γ-stimulated RAW264.7 cells produced less NO under simulated microgravity than under normal gravity [18]. This may be caused by higher arginase I expression under simulated microgravity. The finding that LPS-induced IL-12B expression and production in macrophages were significantly decreased under simulated microgravity is consistent with a previous study showing that LPS plus IFN-γ-stimulated RAW264.7 cells secreted less IL-12 under simulated microgravity [18]. IL-12 is an important proinflammatory cytokine which induces IFN-γ production by T cells and drives T_H1 differentiation [37]. These functions are essential for the control of intracellular pathogens such as *Mycobacterium tuberculosis* and various viruses. Impaired IL-12 production by activated macrophages under microgravity may lead to impaired T_H1 differentiation of T cells and failure to control intracellular pathogens.

Similar to our finding that the LPS-induced IL-6 expression and production in mouse macrophages were increased under simulated microgravity, LPS-stimulated peritoneal macrophages of spaceflight rats were found to secrete more IL-6 than ground controls [38]. IL-6 is a cytokine with both pro- and anti-inflammatory properties [39]. It inhibits the production of neutrophil-attracting chemokines by resident tissue cells, while augments the production of monocyte- and T cell-attracting chemokines. Increased IL-6 production by activated macrophages under microgravity may promote the recruitment of monocytes and T cells to sites of inflammation, but inhibit the neutrophil recruitment.

In summary, we found that microgravity activated a p38 MAPK-C/EBPβ pathway in macrophages to up-regulate arginase I and IL-6 expression and down-regulate IL-12B expression. Importantly, both increased arginase I expression and decreased IL-12B expression in macrophages under microgravity could result in immunosuppression.

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