Differential effects of *Rhodiola rosea* on regulatory T cell differentiation and interferon-γ production *in vitro* and *in vivo*

XI XU[1], PINGPING LI[1], PENG ZHANG[2], MING CHU[1], HONGJU LIU[2], XIAOPING CHEN[2] and QING GE[1]

[1]Key Laboratory of Medical Immunology, Ministry of Health, Department of Immunology, School of Basic Medical Sciences, Peking University Health Sciences Center, Beijing 100191; [2]State Key Laboratory of Space Medicine Fundamentals and Application, Chinese Astronaut Research and Training Center, Beijing 100094, P.R. China

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Abstract. *Rhodiola rosea* (*R. rosea*), a type of adaptogen, has been previously reported to exhibit immunostimulating activity in rodents and in human peripheral blood mononuclear cells (PBMCs) *in vitro*. To examine the effect of *R. rosea* on T cells under simulated microgravity, spaceflight analogs of human head-down bed rest (HDBR) at -6˚ and murine hind limb unloading (HU) were used. A decrease in the levels of interferon-γ (IFN-γ) and interleukin-17 (IL-17) and an increase in regulatory T (Treg) cells were observed in the placebo group following HDBR. The *R. rosea* treated HBDR group demonstrated further decreased IFN-γ production, however, *R. rosea* exhibited no effect on the ratio of circulating Tregs or Treg cell differentiation. By contrast, the treatment of *R. rosea* on human T cells *in vitro* did not alter IFN-γ secretion, however, Treg differentiation was significantly reduced. An *R. rosea*-induced upregulation of hypoxia-inducible factor 1α (HIF-1α) contributed to the suppression of Treg differentiation *in vitro*. Differences in the effect of *R. rosea in vitro* and *in vivo* were also observed using a mouse model of microgravity. The results of the current study suggest that *R. rosea* has differential modulatory effects on T cells *in vivo* and *in vitro* and care should be taken when evaluating the effects of *R. rosea* on the immune system.

Correspondence to: Professor Qing Ge, Key Laboratory of Medical Immunology, Ministry of Health, Department of Immunology, School of Basic Medical Sciences, Peking University Health Sciences Center, 38 Xue Yuan Road, Beijing, 100191, P.R. China
E-mail: qingge@bjmu.edu.cn

Professor Xiaoping Chen, State Key Laboratory of Space Medicine Fundamentals and Application, Chinese Astronaut Research and Training Center, 28 Beiqing Road, Beijing 100094, P.R. China
E-mail: xpchen2009@yahoo.com

Abbreviations: HDBR, head-down bed rest; PBMC, peripheral blood mononuclear cell; IFN-γ, interferon-γ; IL, interleukin; Treg, regulatory T cell; iTreg, induced regulatory T cell; *R. rosea*, *Rhodiola rosea;* HIF-1α, hypoxia-inducible factor 1α; HU, hind limb unloading; TGF-β1, transforming growth factor β1

Key words: Rhodiola rosea, regulatory T cells, interferon-γ, microgravity, hypoxia-inducible factor 1α

Introduction

Rhodiola rosea (*R. rosea*), a type of adaptogen, belongs to the *Crassulaceae* plant family, of the *Sedoideae* subfamily and *Rhodiola* genus. It has been used as traditional medicine in Europe, Asia and Russia for centuries (1). Greater than 20 compounds are present in the *R. rosea* root, including salidroside (rhodioloside), rosavins and p-tyrosol, which are understood to have important therapeutic activities (2). The commonly described functional activities include, performance enhancement, fatigue reduction, alleviation of depression symptoms, stimulation of the nervous system and prevention of high altitude sickness (3,4). *R. rosea* was previously demonstrated to have immunostimulatory potential in rodents *in vivo*, and in human peripheral blood mononuclear cells (PBMCs) *in vitro* (5-10). Additionally, *in vivo* administration of salidroside, the major component of *R. rosea*, enhanced the proliferation of murine T cells and the production of antibodies and cytokines, including interleukin (IL)-2, IL-4 and interferon-γ (IFN-γ) (5). *In vitro* administration of the aqueous extract of *Rhodiola imbricate* rhizome induced increased expression levels of IL-1β in human PBMCs, and of toll-like receptor-4 and granzyme-B in mouse splenocytes (10). Thus, *R. rosea* may potentially be used to enhance cellular immunity under microgravity conditions. However, it has not been previously reported whether *R. rosea* has an *in vivo* immune-modulating effect in humans (7,8). The effects of *R. rosea* on cytokine production by human T cells and the differentiation of regulatory T cells (Tregs) *in vivo* and *in vitro* is currently unknown.

Spaceflight changes the immune system in various ways. These include altered leukocyte distribution, altered serum cytokine levels, reduced functions of natural killer cells, granulocytes and monocytes, reduced leukocyte proliferation following activation, decreased delayed-type hypersensitivity to recall antigens, and latent viral reactivation (11-23). A number of studies have investigated strategies to monitor the

immune system during spaceflight and to develop countermeasures. To study whether *R. rosea* may enhance the functions of the immune system during spaceflights, the effect of *R. rosea* and its main component, salidroside, on human and mouse T cells was examined *in vitro* and *in vivo*. Head-down bed rest (HDBR) at -6˚ was used as a ground-based spaceflight model for the study of human T cells *in vivo,* and hind limb unloading (HU) was used as the *in vivo* mouse model.

Materials and methods

Ethical issues. The current study was approved by the Ethics Committee of China Astronaut Research and Training Center. Written consent was obtained from the subjects, who had been informed of the risks and the experimental details.

Subjects. Fifteen male volunteers of age 26.63±4.03, height 171.8±3.0 cm and weight 63.6±6.2 kg (all presented as means ± SEM) were recruited into the present study. All subjects were educated to a junior high level or above. The subjects were healthy and physically fit. Subjects with the following chronic or recent acute illnesses were precluded from the study: Skeletal muscle diseases; organic and functional diseases of psychiatry and neurology; and sleep disorders. No subjects had a history of cancer, hepatitis or any other relevant diseases, including autoimmune disorders. The final selection was based on typical clinical results comprising medical history, physical and psychological examination, complete blood count, blood chemistry analysis and several other tests. Eight subjects were randomly selected for the placebo group and seven for the RR group. The subjects in the RR group received *R. rosea* 0.5 g per day (prophylactic dose), twice a day from R1 to R7, then 1.0 g per day (therapeutic dose), twice a day from R8 to R45 during HDBR period. The dose of *R. rosea* was doubled for therapeutic purposes as changes in muscles and bones began to be significant following one week of bed rest. No medication, smoking, alcohol or caffeinated drinks were permitted during the study. Emergency medical surveillance and service were available throughout bed rest protocol.

Head-down bed rest (HDBR) protocol. The entire study included 45 days of HDBR, 10 days of adaptation prior to HDBR and 10 days of recovery following HDBR. During HDBR, the subjects were in a flat, resting, head-down position to -6˚ from horizontal. Lights were switched off between 22:00 and 06:00, with normal daylight illumination for the rest of the day. All the subjects were housed in an air-conditioned bedroom, maintained at 25±0.5˚C with a relative humidity of 60-70%. All dining, washing, urination and defecation activities were carried out in a bedridden state. Changing position around the body axis was permitted.

Human PBMC preparation. Sterile heparinized peripheral blood samples were obtained from 12 healthy volunteers and the 15 test subjects prior to (R-1), during (R15, R30 and R45) and following (R+9) the HDBR protocol at 6:00 a.m. PBMCs were collected by Ficoll-Hypaque density-gradient centrifugation.

Mice. Male C57BL/6 mice (age, 6-8 weeks old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and housed in a specific pathogen-free facility at the Chinese Astronaut Research and Training Center (Beijing, China). The mice were maintained under a 12-h light/dark cycle, 25±2˚C and access to food and water *ad libitum*. All mice were allowed to adapt to the environment for a minimum of 3 days following shipment and prior to the onset of the experiment. The experimental procedures used and the care of the animals were approved by the ethics committee of the Chinese Astronaut Research and Training Center.

Mouse hind limb unloading (HU) model. C57BL/6 mice at 8 weeks of age were randomly assigned to four groups, with 3 mice in each group as follows: Saline group; salidroside group; saline with HU group; and salidroside with HU group. Mice in the HU groups were suspended by their tails at a 30˚ head-down tilt with no load bearing on their hind limbs, with unlimited access to food and water. Mice without HU were housed individually in standard caging. The mice received salidroside 50 mg/kg/day by intragastric administration for 28 days prior to HU and for 14 days during HU. Immediately following the HU, the mice were sacrificed by cervical dislocation and cells from the spleens were collected for further experimentation.

Reagents. Salidroside was purchased from Sigma-Aldrich (St. Louis, MO, USA). The following monoclonal antibodies were used for staining: Anti-human cluster of differentiation 4-peridinin chlorophyll (CD4-PerCP) Cy5.5 (OKT4; BioLegend, Inc., San Diego, CA, USA); anti-human CD25-phycoerythrin (PE; MEM-181; QuantoBio, Beijing, China); anti-human forkhead box P3-Allophycocyanin (Foxp3-APC; PCH101) and anti-mouse Foxp3-APC (FJK-16s; eBioscience, Inc., San Diego, CA, USA); and anti-human IFN-γ-fluorescein isothiocyanate (FITC; 4S.B3), anti-mouse CD4-FITC (H129.19), anti-mouse CD25-PE (PC61) and anti-mouse IFN-γ-APC (XMG1.2; BD Biosciences, San Jose, CA, USA). The following antibodies and regents were purchased from BD Biosciences were used for cell cultures: Anti-human CD3 (HIT3a); anti-human CD28 (CD28.2); anti-mouse CD3 (145-2C11); anti-mouse CD28 (37.51); and protein transport inhibitor (containing Brefelding A). Recombinant human transforming growth factor (rhTGF)-β1 and rhIL-2 were purchased from R&D Systems China Co., Ltd. (Shanghai, China). The mouse IFN-γ enzyme linked immunosorbent assay (ELISA) kit was purchased from eBioscience.

IFN-γ production. Human PBMCs were stimulated with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 2 days. Protein transport inhibitor was added 4 hours prior to intracellular IFN-γ staining. Mouse splenocytes were stimulated with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 2 days. The supernatants were collected and the concentrations of IFN-γ were measured using the mouse IFN-γ ELISA kit.

Helper T cell differentiation. Human PBMCs were stimulated by anti-CD3 and anti-CD28 under induced regulatory T cell (iTreg)-inducing conditions (rhIL-2, 5 ng/ml; and rhTGF-β1, 10 ng/ml) with various doses of salidroside (0, 10, 30, 50 and 100 μg/ml). After 5 days, the cells were

collected and stained with CD4 and CD25 antibodies, then intracellular staining of Foxp3 was conducted according to the manufacturer's protocols and the cells were examined by FACSCalibur flow cytometry (BD Biosciences).

Mouse splenocytes were stimulated by anti-CD3 and anti-CD28 under iTreg-inducing condition (rhIL-2, 2 ng/ml; and rhTGF-β1, 1 ng/ml). iTreg-skewing cells were directly stimulated for 3 days prior to intracellular Foxp3 staining.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from iTreg-skewing PBMCs using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol, and cDNA was obtained using the FastQuant RT kit (Tiangen Biotech Co., Ltd., Beijing, China). RT-qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on an iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.), with each sample in triplicate. The primers used for measurement were as follows: Forward, 5'-TTCACCTGAGCCTAATAGTCC-3' and reverse, 5'-CAAGTCTAAATCTGTGTCCTG-3' for hypoxia inducible factor-1α (HIF-1α); and forward, 5'-GCACCGTCAAGGCTG AGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3' for GAPDH. PCRs were performed for 40 cycles of 95˚C for 20 sec, 56˚C for 20 sec and 72˚C for 20 sec. The quantification was based on ΔΔCq calculations and were normalized to GAPDH as the reference gene (24).

Statistical analysis. Statistical analysis of the results was performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between the placebo group and RR group were analyzed by repeated measures analysis of variance (ANOVA), with time and treatment as two factors for repeated measures and were further evaluated using Bonferroni correction. Unpaired or two-tail paired t test was further used to evaluate the significance of the differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of R. rosea on human T cells in the HDBR model. To investigate the *in vivo* effects of *R. rosea* on human T cells under simulated microgravity, placebo- and RR-treated groups underwent a 45-day HDBR protocol (25). The subjects in the RR group received *Rhodiola rosea* 0.50 g (prophylactic dose) twice a day from R1 to R7, then 1.0 g (therapeutic dose) twice a day from R8 to R45 during the HDBR period. As presented in Fig. 1A, the percentages of total T cells, $CD4^+$ and $CD8^+$ T cells in the peripheral blood of the placebo group did not change until 9 days following the completion of bed rest (R+9), where the percentages were significantly different to those at day R45 (Fig. 1A) (25). The changes included a significant increase in the total T and $CD4^+$ T cells and a decrease in $CD8^+$ T cells on R+9 compared with R45 (P=0.008, P=0.013 and P=0.013, respectively). Consistently, an ~14% increase in CD4:CD8 ratio was observed at R+9 compared with R45 (P=0.015, Fig. 1A) (25). Compared with the placebo group, the adaptogen RR-treated group exhibited a similar pattern of changes in the T cell subsets (Fig. 1B).

The percentage of circulating Treg cells ($CD4^+$ $CD25^+$ $Foxp3^+$ $CD127^-$) in the HDBR PBMCs was observed to be increased at R45 compared with R-1 (P=0.026) and had returned to the baseline levels at R+9 (Fig. 1C) (25). Similar Treg changes were observed in the group treated with RR (Fig. 1C). A late increase in circulatory Tregs was observed, whereas, the percentage of iTregs was increased at R15 compared with R-1, however, there was no change in iTreg levels at R45(Fig. 1C) (25). The pattern of T cell levels in the RR group was not significantly different to the placebo group (Fig. 1C). The differentiation of iTregs at various time points was induced by stimulation of PBMCs with anti-CD3, anti-CD28 and TGF-β1 under 1g conditions for 3 days (26).

The levels of cytokines produced by the activated T cells were examined after stimulation of HDBR PBMCs with anti-CD3 and anti-CD28 under 1g conditions for 2 days. The levels of IFN-γ and IL-17A in the placebo group exhibited a gradual decrease during HDBR, reaching the lowest level at R45 (P=0.05, 0.003 vs. R1, repeated measures ANOVA; 25.0%±26.2% and 53.8%±20.3%, respectively; Fig. 1D) (25). Unlike the findings of previous post-flight and HDBR studies (11,22,27), the current study did not observe a decrease in IL-2 expression in the placebo group (Fig. 1D) (25). No consistent or significant changes were observed in the production of IL-4 by T cells (data not shown). In the RR group, the levels of IFN-γ production by T cell were reduced upon anti-CD3 and anti-CD28 stimulation (R15). The decrease to IFN-γ levels was significantly enhanced in the RR group compared with the placebo group at R15 (P=0.046, repeated measures ANOVA; Fig. 1E) (25). There was no difference in the IL-17A and IL-2 levels between the placebo and the RR groups (Fig. 1E).

Effect of R. rosea on human T cells in vitro. To investigate whether the suppressive effect of *R. rosea* on T cell function may also be observed *in vitro* under 1g conditions, normal human PBMCs were treated with salidroside, the main component of *R. rosea*, and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies. In contrast to the *in vivo* results obtained from the HDBR experiment, no significant alterations of IFN-γ production was observed between salidroside-treated or untreated PBMCs (Fig. 2A). Notably, a significant decrease in iTreg cell differentiation was observed in the cells treated with salidroside (Fig. 2B). The inhibition of iTreg differentiation by salidroside was dose-dependent, as an increase in salidroside concentration resulted in a further decrease to the iTreg cell percentage, with the lowest percentage of T cells observed following 100 μg/ml salidroside treatment (P=0.019 vs. 0 μg/ml; Fig. 2B). These results suggest that, in contrast to the *in vivo* effect, salidroside may have a direct and suppressive impact on regulatory T cell differentiation *in vitro*.

As the signaling pathways downstream of CD28 and the TGF-β1 receptor are crucial to iTreg differentiation, the concentration of anti-CD28 antibody and TGF-β1 were titrated to examine whether salidroside increased the effects of these two pathways. As presented in Fig. 3A and B, the optimal concentration for induction of iTreg differentiation in the absence of salidroside was 1 μg/ml for anti-CD28 antibody and 10 ng/ml for TGF-β1. Compared with cells that received anti-CD28 or TGF-β1 only, salidroside treatment significantly

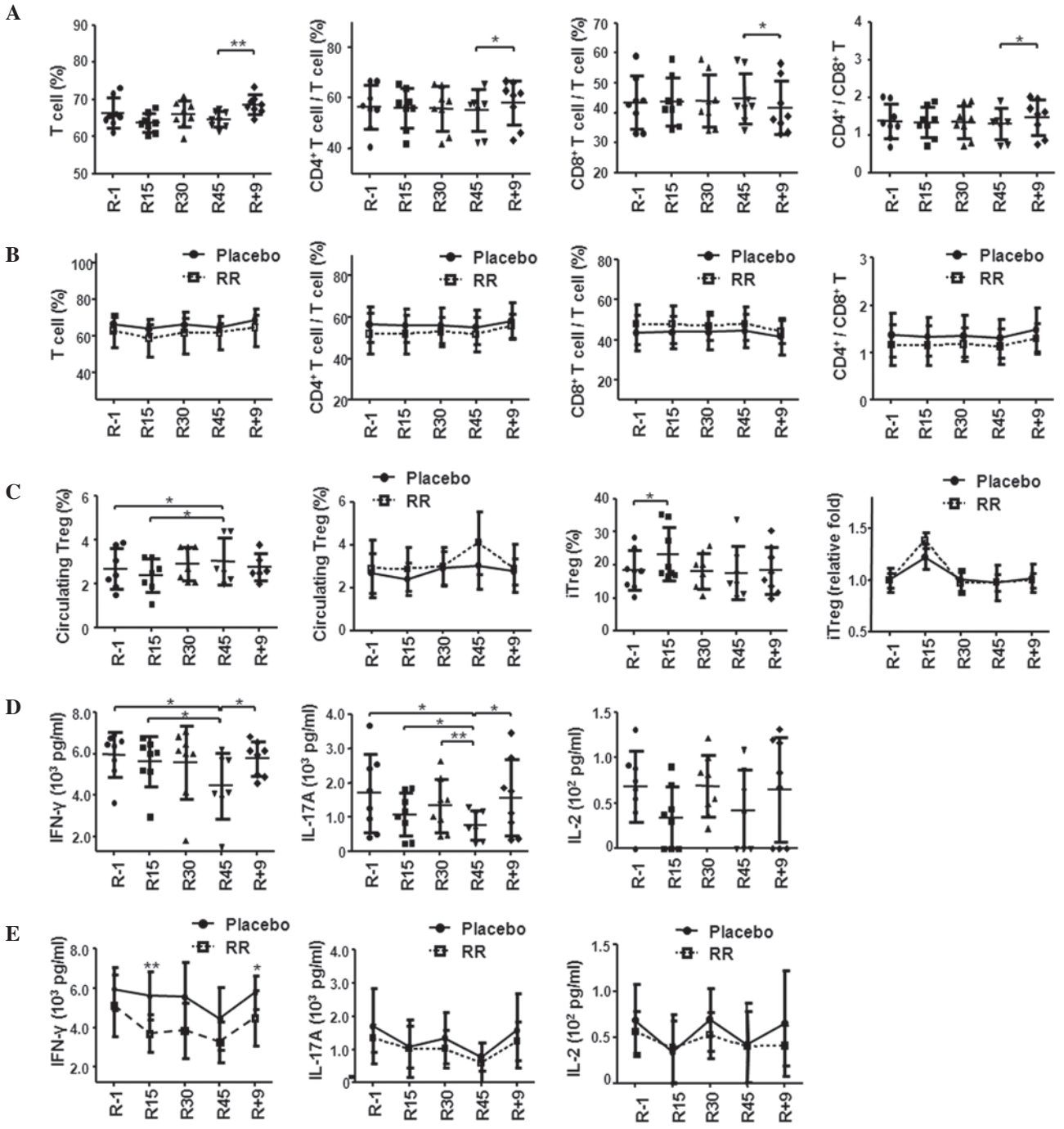

Figure 1. RR reduced the secretion of IFN-γ by T cells but did not alter the differentiation of iTregs in 45-day HDBR. (A) Analysis of total T cell, $CD4^+$ T cell and $CD8^+$ T cell percentages within the placebo group. (B) Comparison of total T cell, $CD4^+$ T cell and $CD8^+$ T cell percentages between the placebo and RR treated groups. (C) The changes in circulating Treg cells and iTreg cells within the placebo group and between the placebo and RR treated groups. (D) Changes to T cell-derived cytokines within the placebo group. PBMCs were stimulated by anti-CD3 and anti-CD28 antibodies for 2 days. The supernatants were analyzed for cytokines IFN-γ, IL-17A and IL-2. (E) The comparison of IFN-γ, IL-17A and IL-2 secretion between the placebo and RR treated groups. Data are presented as the mean ± standard deviation. The statistical significance between any two time points within the placebo group or between the placebo and RR-treated groups within any single time point was calculated by two-tailed Student's t-test. $^*P<0.05$, $^{**}P<0.01$. The placebo group is indicated as a solid line and the RR-treated group as a dashed line. RR, *Rhodiola rosea*; INF-γ, interferon-γ; iTregs, induced T regulatory cells; HBDR, head-down bed rest; IL, interleukin; CD, cluster of differentiation; PBMCs. peripheral blood mononuclear cells; R, number of HDBR days.

reduced iTreg differentiation (P=0.046 and P=0.016, respectively). Increasing the concentrations of anti-CD28 or TGF-β1 did not abolish the suppressive effect of salidroside. The suppression of Treg differentiation *in vitro* was not due to the inhibition of T cell survival or proliferation by salidroside treatment (data not shown).

R. rosea, specifically, salidroside, was previously reported to increase HIF-1α expression and its nuclear translocation in cardiomyocytes, fibroblasts, kidney and liver cells (28-30). HIF-1α was also previously observed to suppress Treg differentiation by promoting the glycolytic activity of T cells and by binding Foxp3 to promote its proteasomal degradation (31,32).

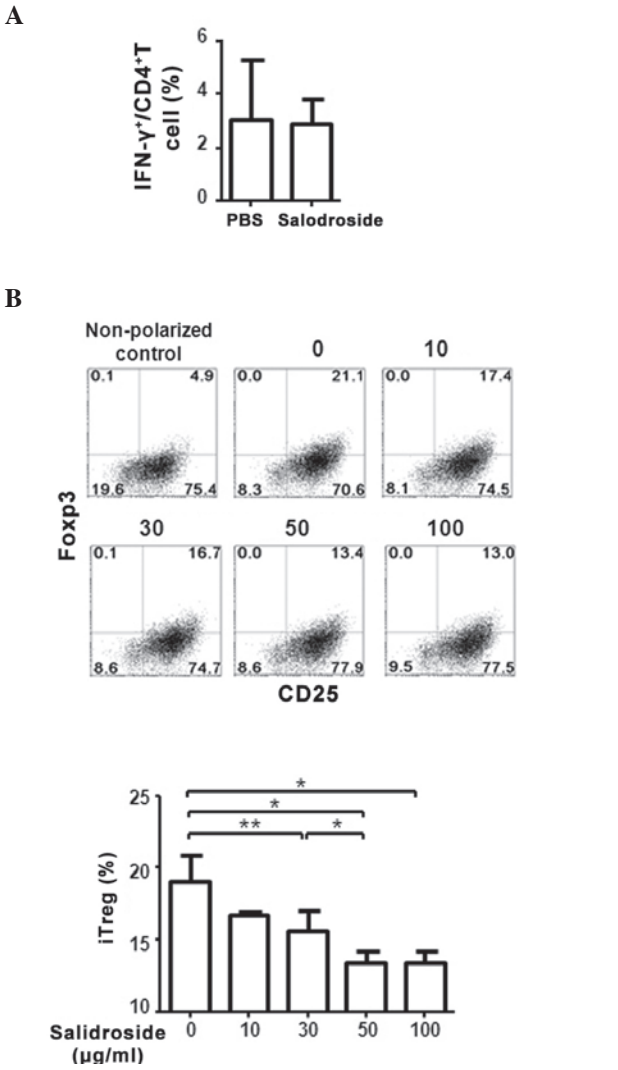

Figure 2. Salidroside treatment of human PBMCs *in vitro* suppressed the differentiation of iTreg cells. (A) Human PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies for 2 days in the presence or absence of 50 μg/ml salidroside. The intracellular level of IFN-γ in $CD4^+$ T cells was measured by flow cytometry. The experiment was performed 3 times using different blood donors. (B) The suppression of iTreg differentiation by salidroside is dose-dependent. Human PBMCs were cultured in regulatory T cell-induction media with increasing concentrations of salidroside. Following 5 days of treatment, cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 antibodies. The flow cytometry results were shown in the left panel with the numbers indicating the amount of salidroside (μg/ml). Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, comparisons between the two subsets by unpaired Student t-test. PBMC, peripheral blood mononuclear cell; iTreg, induced regulatory T cell; CD, cluster of differentiation; INF-γ, interferon-γ; Foxp3, forkhead box P3.

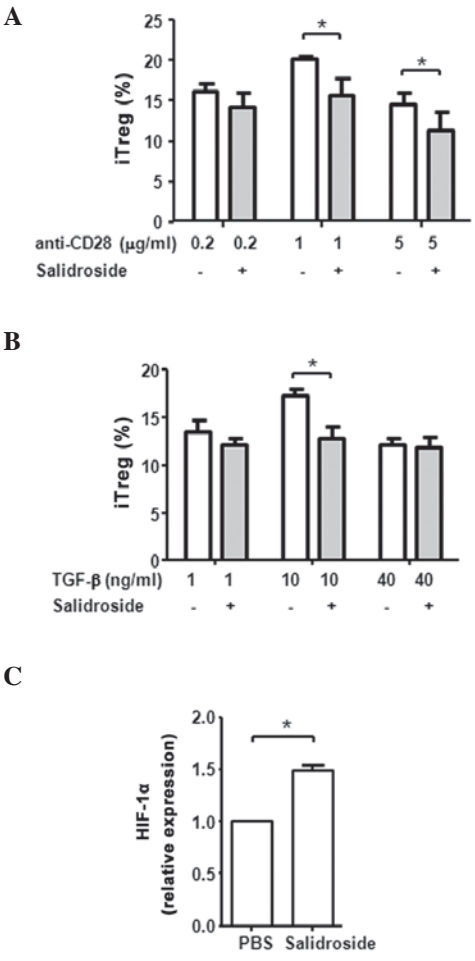

Figure 3. Salidroside induced the upregulation of HIF-1α in human PBMCs. Salidroside did not affect the signaling downstream of CD28 or TGF-β receptor. Human PBMCs were cultured in Treg induction media in the presence or absence of 50 μg/ml salidroside. The concentrations of anti-CD28 antibody and TGF-β1 were titrated from (A) 0.2 μg/ml to 5 μg/ml and (B) 1 ng/ml to 40 ng/ml. (C) Salidroside facilitated the upregulation of HIF-1α. Human PBMCs were cultured in Treg induction media in the presence or absence of 50 μg/ml salidroside. The total RNAs were collected following 5 days and the expression of HIF-1α was measured by reverse transcription-quantitative polymerase chain reaction. The experiments were repeated a minimum of 3 times. Data are presented as the mean ± standard deviation. *P<0.05. HIF-1α, hypoxia inducible factor 1α, PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation; TGF-β1, transforming growth factor β1; Treg, regulatory T cell; iTreg, induced Treg; PBS, phosphate-buffered saline.

Thus, the current study examined whether the treatment of salidroside alters the expression of HIF-1α in T cells when cultured in regulatory T cell inducing conditions. As presented in Fig. 3C, the HIF-1α mRNA levels in PBMCs were significantly increased by salidroside *in vitro*, compared with PBS treated cells (P=0.010).

These data demonstrate that salidroside has a significant effect on iTred differentiation, however, the effects are different *in vitro* and *in vivo*. Salidroside directly suppressed the differentiation of iTregs *in vitro* under 1g conditions. However, when *R. rosea* was administered *in vivo* during the HDBR model, it did not significantly alter iTreg differentiation, though the actual T cell differentiation assay was also performed *in vitro* under 1g condition.

Effect of R. rosea on murine T cells in HU model. To investigate whether similar differences are also observed in the murine system, a 14-day HU mouse model was used. Mice received salidroside at 50 mg/kg/day by intragastric administration for 28 days prior to HU and 14 days following HU. The mice were subsequently sacrificed and splenic T cells were cultured under various conditions at 1g. The control groups included saline treatment with and without HU and salidroside treatment without HU. There was no difference in the levels of IFN-γ in T cells following anti-CD3 and anti-CD28 stimulation between the saline with and without HU groups, and

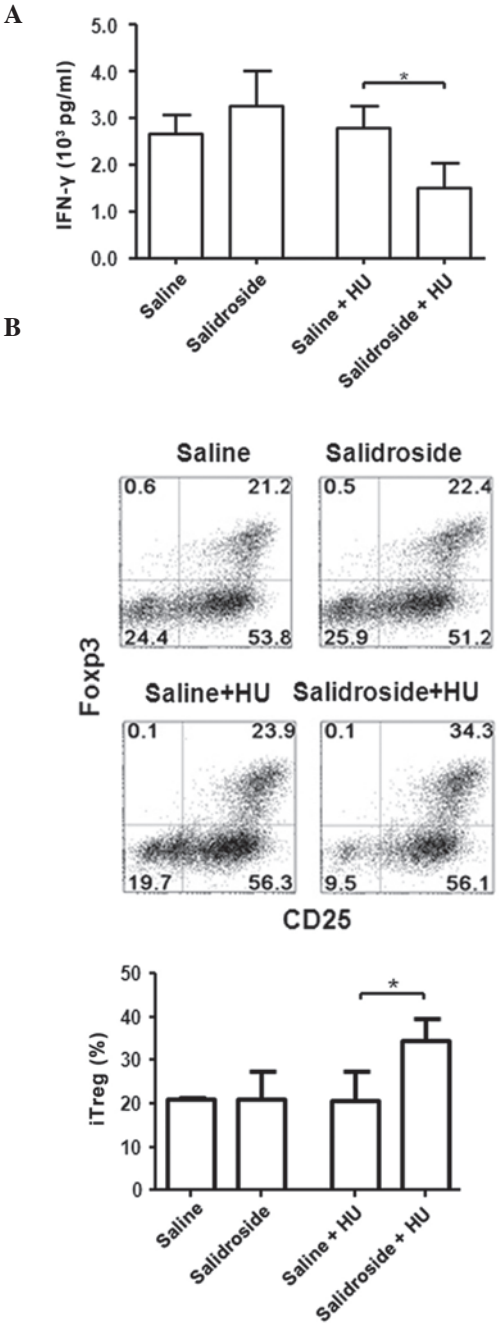

Figure 4. Salidroside reduced the production of IFN-γ by T cells and increased the differentiation of iTregs in HU mouse model. C57BL/6 mice were randomly assigned to 4 groups: saline and salidroside groups, with and without HU. Mice received salidroside (50mg/kg/day) daily by intragastric administration for 28 days before HU and 14 days during HU. At the end of HU, splenocytes were collected and stimulated with anti-CD3 and anti-CD28. (A) The concentrations of IFN-γ in the supernatant were measured by enzyme-linked immunosorbent assay. (B) The splenocytes were also cultured in regulatory T cell polarizing conditions and the differentiation of iTreg ($CD4^+Foxp3^+CD25^+$) cells was measured. Similar results were obtained from 3 independent experiments. Data are presented as the mean ± standard deviation. *P<0.05. IFN-γ, interferon-γ; iTreg, induced regulatory T cell; HU, hind limb unloading; CD, cluster of differentiation; Foxp3, forkhead box P3.

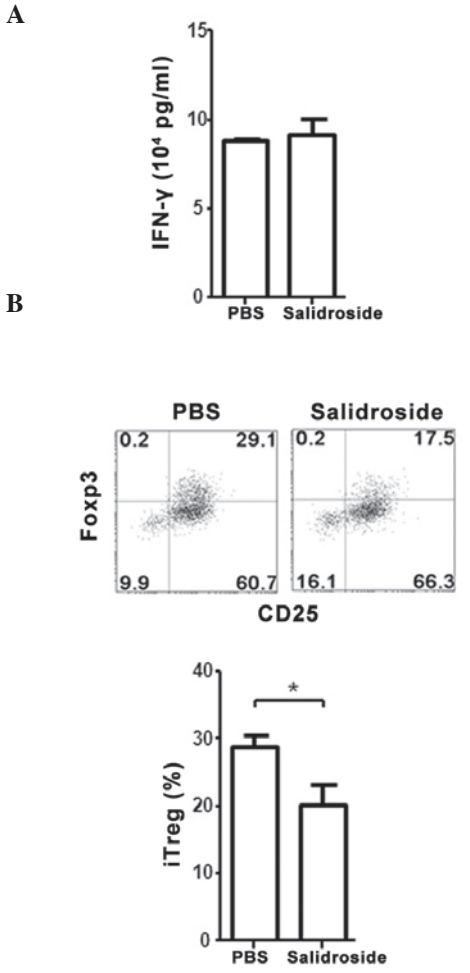

Figure 5. Salidroside suppressed the differentiation of mouse iTreg cells *in vitro*. (A) The splenocytes from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of salidroside. The production of IFN-γ was measured by enzyme-linked immunosorbent assay. (B) The splenocytes were also cultured in iTreg polarizing conditions with or without salidroside. The differentiation of iTreg cells was measured by flow cytometry. Data are presented as the mean ± standard deviation. *P<0.05. iTreg, induced regulatory T cells; CD, cluster of differentiation; IFN-γ, interferon-γ, Foxp3, forkhead box P3; PBS, phosphate-buffered saline.

in the salidroside without HU group (Fig. 4A). However, the salidroside with HU group exhibited a significant reduction in IFN-γ production by T cells, compared with the saline with HU group (P=0.032), a similar pattern to the results obtained from human HDBR samples (Fig. 4A). The differentiation of iTregs was also similar among saline groups with or without HU, and the salidroside group without HU. However, the salidroside with HU group exhibited a significant increase in the level of iTreg differentiation (Fig. 4B).

Effect of R. rosea on murine T cells in vitro. The direct effect of salidroside on murine T cells *in vitro* was also investigated. Similar to human PBMCs, mouse splenic T cells treated with salidroside showed no significant difference in the levels of IFN-γ, however, compared with PBS treatment, a significant decrease in iTreg differentiation was observed (P=0.034; Fig. 5A and B).

Discussion

Various studies have previously reported that *R. rosea* has anti-stress and immunostimulatory activities (3-10). Thus, the

current study investigated whether *R. rosea* may improve the function of the immune system during spaceflight. *R. rosea* exhibited differential effects *in vitro* and *in vivo*. The administration of *R. rosea in vivo* decreased the production of IFN-γ by human T cells following simulated microgravity (HDBR). The treatment with *R. rosea in vitro*, however, did not change the production of IFN-γ by T cells. Similarly, the differentiation of iTregs was not altered in *R. rosea*-treated human or mouse cells following microgravity simulation, whereas, iTreg differentiation was significantly decreased when *R. rosea* was directly added into the T cell culture. These differences suggest that *R. rosea* may have a direct suppressive effect on regulatory T cell differentiation *in vitro* and may have an indirect impact on regulatory T cell differentiation by the production of Th1 type cytokines under microgravity conditions *in vivo*. It is possible that the differences in doses and durations of *R. rosea* treatments *in vitro* and *in vivo* may account for the different effects demonstrated. This may be difficult to confirm as T cells cultured *in vitro* for >40 days may need multiple rounds of T cell receptor-mediated activation and the presence of cytokines to promote cell survival. It is also possible that the different modulatory effects that were observed following *R. rosea* treatment occurred as a result of the different experimental conditions used for the *in vivo* microgravity model and the *in vitro* 1g model. However, this is unlikely as PBMCs derived from humans/mice with or without microgravity were eventually cultured in the same culture conditions as the *in vitro* experiment (anti-CD3 and anti-CD28 with or without TGF-β1). In addition, mice receiving saline and *R. rosea* under 1g conditions exhibited similar levels of IFN-γ production and iTreg differentiation (Fig. 3). This further suggests that *R. rosea* may have differential modulatory functions on T cells directly (*in vitro*) and indirectly under microgravity (*in vivo*).

Regarding the direct suppressive effect of *R. rosea* on iTregs, these data suggest a casual link between *R. rosea*-promoted HIF-1α transcription in T cells and a reduction in iTreg cell differentiation. Whether *R. rosea* may alter HIF-1α transcription *in vivo* is awaiting further investigation.

Collectively, the results of the present study obtained from human and mouse T cells indicate that *R. rosea* has a direct negative impact on the differentiation of regulatory T cells *in vitro*. Thus, the increase in Treg differentiation and decrease in IFN-γ production by *R. rosea in vivo* under microgravity conditions is probably due to the effect of *R. rosea* on cells other than T cells. Whether they are antigen presenting cells or even cells from neuronal pathways remains unclear. The results of the current study do not support an immunostimulatory effect of *R. rosea* and suggest that *R. rosea* may not improve T cell immunity under microgravity *in vivo*.

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