Atorvastatin improves bone marrow endothelial progenitor cell function from patients with immune-related hemocytopenia

Juan Qian¹#, Qian Shen²#, Chun-Xia Yan³, Hong Yin¹, Xin Cao¹, Zeng-Hua Lin¹, Yi-Feng Cai¹, Hong Liu¹

¹Department of Hematology, Affiliated Hospital of Nantong University, Nantong, China; ²Department of Oncology, Nantong Oncology Hospital, Nantong, China; ³Nantong University, Nantong, China.

Contributions: (I) Conception and design: J Qian; (II) Administrative support: H Liu; (III) Provision of study materials or patients: CX Yan, H Yin, XCao; (IV) Collection and assembly of data: Q Shen, ZH Lin; (V) Data analysis and interpretation: YF Cai; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

#These authors contributed equally to this work.

Correspondence to: Yi-Feng Cai; Hong Liu. Department of Hematology, Affiliated Hospital of Nantong University, Nantong 226001, China. Email: caiyf996@163.com; lhong6363@163.com.

Background: Immune-related hemocytopenia (IRH) is a type of autoimmune disease that targets bone marrow hematopoietic cells. This study investigated the influence of atorvastatin on the functional aspects of bone marrow endothelial progenitor cells (BM EPCs) in IRH patients.

Methods: BM EPCs were isolated from 15 patients with IRH and 20 normal controls. The isolated BM EPCs were characterized by flow cytometry. Cell counting kit-8, flow cytometry, and Transwell migration assays were used to determine the proliferation, apoptosis, and migration of BM EPCs, respectively. Protein levels were determined by western blot assay.

Results: The BM EPCs isolated from IRH patients showed reduced proliferation, increased apoptosis, and attenuated migratory ability compared to those from normal controls. Western blot analysis showed that the protein level of p-p38 was significantly increased, while that of Phosphorylated protein kinase B (p-AKT) was significantly decreased in the BM EPCs from IRH patients, compared to BM EPCs from healthy subjects. Cell proliferation and migration were significantly enhanced by atorvastatin, recombinant human thrombopoietin, and SB20358 compared to the untreated BM EPCs from IRH patients. Atorvastatin, Recombinat human thrombopoietin (TPO), and SB20358 treatment significantly suppressed the protein levels of p-p38 protein, but increased those of p-AKT in BM EPCS from IRH patients.

Conclusions: In summary, atorvastatin increases the number and function of BM EPCs in IRH patients by regulating the p38 and AKT signaling pathways.

Keywords: Immune-related hemocytopenia (IRH); atorvastatin; bone marrow endothelial progenitor cells (BM EPCs)

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Introduction

Immune-related hemocytopenia (IRH) is a type of autoimmune disease against bone marrow hematopoietic cells that were discovered in the 1990s, which damages the body’s normal hematopoietic function (1,2). Current research posits that immune-related cytopenia is caused by the imbalance of T lymphocyte regulation and the abnormal function of B lymphocytes, thereby producing autoantibodies that destroy or inhibit bone marrow hematopoietic cells, which ultimately leads to a decrease in peripheral blood cells (3). However, the pathogenesis remains poorly understood and the clinical management is challenging.

IRH is a special type of bone marrow failure syndrome. The bone marrow microenvironment is dynamic, and is composed of growth factors and stromal cells (3-5). Endothelial progenitor cells (EPCs) are one of the important
components and play a significant role in the bone marrow microenvironment. Previous studies have shown that EPCs regulate the transport and homing of hematopoietic stem cells (4,5), and are related to the delayed recovery of hematopoietic stem cells after transplantation (6-8). Moreover, the number and function of EPCs in patients with poor chimeric function after transplantation are significantly different from those with good chimeric function, and atorvastatin can improve this difference (9). Atorvastatin can also improve platelet function by regulating the function of EPCs in patients with immune thrombocytopenia (3). Recombinant human thrombopoietin (TPO) can improve the short-term efficacy of IRH patients and reduce the amount of platelet and erythrocyte infusion (10-12). Currently, there is no relevant report on whether the bone marrow EPCs (BM EPCs) in IRH patients are damaged, and whether atorvastatin can improve the function of BM EPCs in IRH patients.

The purpose of this study was to evaluate proliferation, apoptosis, and migration of BM EPCs between healthy people and IRH patients, and to compare and determine whether atorvastatin and TPO treatment could affect the functions of BM EPCs in IRH patients. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-2459).

Methods
Patients and controls
In this prospective study, 15 IRH patients hospitalized in the Affiliated Hospital of Nantong University from January 2014 to December 2016 were included. A total of 20 BM of the sample from transplant donors were considered as healthy controls. This study was approved by the ethics committee of the Affiliated Hospital of Nantong University (No.: 20133492), and written informed consent was obtained from all subjects, in compliance with the Declaration of Helsinki (as revised in 2013).

Isolation, cultivation, and identification of BM EPCs
The segregation, growth and phenotyping of BM EPCs were proceeded as those that were reported previously (9). Briefly, BM mononuclear cells (BMMNCs) were grown with EGM-2-MV-SingleQuots (Lonza, Walkersville, MD) and 10% fetal bovine serum (Gibco, Rockville, MD) in a 37 °C humidified incubator with 5% carbon dioxide (CO₂) for 7 days. The cells were subsequently used in experiments in 24-well culture plates (Corning Incorporated, Corning, NY) that were pre-coated within fibronectin (Sigma, St. Louis, MO).

Pre-cultured and 7-day cultured BM EPCs were authenticated by performing with mouse antibody against human CD34, vascular endothelial growth factor receptor 2 (VEGFR2; CD309) and CD133 monoclonal antibody (BD Biosciences, San Jose, CA). The samples were then characterized with LSRFortessa (Becton Dickinson) to evaluate. Equivalent of isotype antibodies were applied as negative controls.

Chemicals and treatment
Atorvastatin, recombinant human thrombopoietin (TPO), and p38 inhibitor (SB203580) were purchased from Sigma-Aldrich (St. Louis, USA). BM EPCs were treated with 5 µM atorvastatin, 100 ng/mL TPO, or 10 µM SB203580 for 24 h, respectively, prior to undergoing further in vitro assays.

Measurement of proliferation of BM EPCs using the Cell Counting Kit-8 (CCK-8)
Cell proliferation of BM EPCs was performed by CCK-8 assay kit (Beyotime, Beijing, China). BM EPCs were seeded onto 96-well plates at a concentration of 2,000 cells/well. After a period of incubation, they were grown with 10 µL of the CCK-8 kit for 3 h at 37 °C. The proliferation of BM EPCs was confirmed by absorbance measurement at 450 nm.

Flow cytometry detection of apoptosis in BM EPCs
The Annexin V-FITC/PI Cell Apoptosis Detection Kit (Thermo Fisher Scientific) was performed to test the apoptosis of EPCs. Adherent cells were gathered, cleaned, and re-suspended in the cold binding buffer, and then diluted to a final concentration of 5×10⁵ cells/mL. Aliquots of 1x10⁴ cells were incubated with 0.5 µL of Annexin-V-FITC and 10 µL of PI per tube. After 15 min at room temperature, 400 µL of binding buffer was added before the flow cytometric analysis. For each sample, 1x10⁵ cells were analyzed on a FACS II flow cytometer (BD Biosciences, USA). CellQuest™ Pro software (BD Biosciences) was used to perform the flow cytometric analysis.

Transwell migration assay to detect cell migratory ability
The Boyden chamber test (Transwell, Costar) was
carried out to investigate the migration function of EPCs. Initially, adherent BM EPCs were to be exposed to different treatments for a 24-hour period. After detaching with trypsin/ Ethylene Diamine Tetraacetic Acid (EDTA), $2 \times 10^4$ cells in serum-free EGM-2 medium were coated in each of the upper chambers, and EGM-2 medium containing 10% FBS was covered in the lower chambers. After 24 h, the membranes were washed twice with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde. Remove the cells attached to the upper side of the membrane using a cotton swab. The membranes were then smeared with 0.25% crystal violet, and migrating cells were enumerated in six random 200× fields of view using an inverted microscope.

**Western blot assay**

The cultured EPCs were cleaned and hatched in radioimmunoprecipitation assay buffer containing inhibitors of proteases (Roche, China). For protein level determination, we used a bicinchoninic acid (BCA) kit (Thermo Scientific, Waltham, USA). Proteins were electrophoretically separated by 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred to polyvinylidene difluoride (Millipore, Bedford) membranes. The membrane was then blotted with 5% bovine serum albumin and overnight with antibodies against p-p38 total p38, p-Akt, total Akt and β-actin (Cell Signaling Technology) at dilutions indicated by the manufacturer. Anti-rabbit or anti-mouse secondary antibodies and an ECL chemiluminescence detection system (Pierce Biotechnology Inc, Rockford, IL) were applied to scan and semi-quantitatively analyze the proteins according to the manufacturer's instructions.

**Statistical analysis**

SPSS20.0 statistical software was used for analysis. The data were expressed as mean ± standard deviation (SD). Comparisons between/among groups were analyzed by the Student’s $t$ test or one-way ANOVA followed by Bonferroni’s multiple comparison test. $P<0.05$ was considered statistically significant.

**Results**

**Characterization of the isolated BM EPCs**

The morphology of the isolated BM EPCs from normal subjects and IRH patients was observed under a light microscope (Tianzhuo Instrument Equipment Co., Ltd., China). After incubation for 7 days, the cells were spindle-shaped, connected end-to-end, and densely arranged like paving stones (Figure 1A,B). Flow cytometry analysis of the surface markers showed that the positive rates of CD34,
CD133, and CD209 were 70.3%, 57.8%, and 40.4%, respectively (Figure 1C).

**Comparison of proliferation and apoptosis of BM EPCs between normal subjects and IRH patients**

After culturing, the number of BM EPCs was counted by light microscopy. The results showed that the number of BM EPCs isolated from IRH patients was significantly lower than that of BM EPCs isolated from normal subjects (Figure 2A). The CCK-8 assay was performed to determine the cell proliferation of BM EPCs. As shown in Figure 2B, the cell proliferation of BM EPCs isolated from IRH patients was significantly impaired compared to that of BM EPCs isolated from normal subjects (Figure 2B). Flow cytometry analysis showed that the apoptosis of BM EPCs isolated from IRH patients was significantly increased compared to that of BM EPCs isolated from normal subjects (Figure 2C).

**Comparison of migratory potential of BM EPCs between normal subjects and IRH patients**

The Transwell migration assay analyzed the migratory potential of BM EPCs from normal subjects and IRH patients. As shown in Figure 3, the number of migrated BM EPCs isolated from IRH patients was significantly lower than that of BM EPCs isolated from normal subjects (Figure 3).

**Western blot analysis of p-p38, p38, p-AKT, and AKT protein levels in BM EPCs from normal subjects and IRH patients**

As shown in Figure 4, compared to that of BM EPCs from healthy subjects, the protein level of p-p38 was significantly increased, while the protein level of p-AKT was significantly decreased in the BM EPCs from IRH patients. These results suggest that the p38 pathway is activated and the
Figure 3  Transwell migration assay analyzed the migratory potential of BM EPCs from normal subjects and IRH patients (crystal violet staining, 100×). N=3. *P<0.05. BM, bone marrow; EPCs, endothelial progenitor cells; IRH, immune-related hemocytopenia.

Figure 4  Western blot analysis of p-p38, p38, p-AKT, and AKT protein levels in BM EPCs from normal subjects and IRH patients. N=3. **P<0.01. BM, bone marrow; EPCs, endothelial progenitor cells; IRH, immune-related hemocytopenia.
AKT pathway is inhibited in BM EPCs from IRH patients.

**The effects of atorvastatin, TPO, and SB20358 on the cell proliferation and migration of BM EPCs from IRH patients**

The BM EPCs from IRH patients were treated with atorvastatin, TPO, and SB20358, respectively, and the cell proliferation and migration of these cells were determined using CCK-8 and Transwell migration assays. As shown in Figure 5A, cell proliferation and migration were significantly enhanced by atorvastatin, TPO, and SB20358 compared to the untreated BM EPCs from IRH patients (Figure 5A). Similarly, atorvastatin, TPO, and SB20358 also markedly increased the migratory potential of BM EPCs isolated from IRH patients (Figure 5B).

**The effects of atorvastatin, TPO, and SB20358 on p-p38, p38, p-AKT, and AKT protein levels in BM EPCs from IRH patients**

Further western blot analysis showed that atorvastatin, TPO, and SB20358 treatment significantly suppressed p-p38 protein levels, but increased p-AKT protein levels in BM EPCs from IRH patients (Figure 6).

**Discussion**

In recent years, with growing research and the development of technology, the pathogenesis and treatment of IRH has made some progress. For example, some studies have found that, compared with the normal population, natural killer (NK) cells in IRH patients are reduced, and NK cells are unable to maintain the steady state of the immune system (13,14). Current research suggests that the imbalance of T lymphocyte regulation results in the abnormal function of B-lymphocytes, as well as T helper cell 17 (Th17), Th9, and Regulatory cell (Treg) cells (15). Moreover, infections caused by the invasion of pathogenic microorganisms are also important causes (16-18). In terms of the specific signaling pathways, the B cell receptor (BCR) pathway of peripheral blood B-lymphocytes in IRH patients is enhanced, and the number and function of CD22 are also enhanced (19,20). The expression of the transcription factor, Transcription factor 2 (Bach2), is also different between IRH patients and normal controls, suggesting that Bach2 is also related to the occurrence of IRH (21). EPCs play an important role in the bone marrow microenvironment. Atorvastatin can increase the number of platelets by improving the function of endothelial cells in patients with immune thrombocytopenia (3). TPO is a thrombopoietic growth factor produced by the liver that expressly regulates the release of platelets. Studies have found that recombinant human thrombopoietin (rhTPO) can reduce platelet and red blood cell transfusions in IRH patients (12,22).

To study the relationship between BM EPCs and the pathogenesis of IRH, we compared the function of BM EPCs from IRH patients and normal controls after isolation and culturing. The results showed that the proliferative capacity of BM EPCs in newly treated IRH patients decreased and the proportion of apoptosis increased. BM EPCs and hematopoietic stem cells have a common origin and play an important role in the construction and maintenance of hematopoietic niches. If BM EPCs are
abnormal, hematopoietic stem cells will be depleted (23), indicating that the function of hematopoietic stem cells may be abnormal due to abnormal bone marrow endothelial cells, which in turn leads to IRH.

The p38 mitogen activated protein kinases (p38 MAPK) and AKT signaling pathways play important roles in cell differentiation, apoptosis, migration, and proliferation (24,25). The expression of p-p38 and p-AKT was further tested to observe whether the expression of the p38 and AKT pathways was altered in the BM EPCs from IRH patients. We found that the protein level of p-p38 was significantly increased, while the protein level of p-AKT was markedly decreased in the BM EPCs from IRH patients compared to those of BM EPCs from healthy subjects. These results suggest that the p38 pathway is activated and the AKT pathway is inhibited in BM EPCs from IRH patients.

Previous studies have shown that statins can improve the number and function of BM EPCs in patients with different diseases. For example, statins can increase the number and function of EPCs both in vitro and in vivo by down-regulating p38 MAPK (26,27) or up-regulating AKT signaling (28-30). Atorvastatin improves the BM EPCs of immune thrombocytopenia patients by down-regulating the p38 MAPK pathway and up-regulating the AKT pathway in number and function. In vitro experiments have shown that atorvastatin improves the number and function of BM EPCs in patients with transplanted liver dysfunction by down-regulating the p38 MAPK pathway. To study the effect of atorvastatin on the number and function of BM EPCs in IRH patients, atorvastatin and other drugs were used to treat BM EPCs and to detect the expression of p-AKT and p-p38. The results showed that atorvastatin, p38 inhibitor (SB203580), and TPO treatment can markedly increase cell proliferation and significantly promote cell migration ability. These results indicated that atorvastatin and TPO improved the cell function of EPCs by inhibiting the p38 signaling pathway and activating the AKT signaling pathway, which is consistent with the findings of previous studies.

Conclusions

In summary, atorvastatin increases the number and function of BM EPCs in IRH patients by regulating the p38 and AKT signaling pathways. Further studies are required to confirm the detailed role of atorvastatin in the treatment of IRH.
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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (No.: 20133492), and written informed consent was obtained from all subjects, in compliance with the Declaration of Helsinki (as revised in 2013).

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