

PBEF promotes the apoptosis of pulmonary microvascular endothelial cells and regulates the expression of inflammatory factors and AQP1 through the MAPK pathways

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Abstract. Pre-B cell colony-enhancing factor (PBEF) has been shown to have a variety of biological functions. Studies have proven that PBEF plays a functional role in acute lung injury (ALI). Therefore, in this study, we aimed to confirm the importance of PBEF in ALI. The effects of PBEF overexpression on the apoptosis of human pulmonary microvascular endothelial cells (HPMECs) were analyzed by flow cytometry, and the results indicated that PBEF promoted the apoptosis of HPMECs, which aggravated the development of ALI. Comparative experiments involving increasing and decreasing PBEF expression demonstrated that PBEF promoted the expression of inflammatory factors, such as interleukin (IL)-1 β , IL-6 and IL-8 in the HPMECs, thus intensifying the inflammatory response. PBEF also inhibited the expression of aquaporin 1 (AQP1), which caused a dysfunction and imbalance in water transport. Moreover, we also found that tumor necrosis factor (TNF)- α promoted the expression of PBEF in the HPMECs. After blocking the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, we found that PBEF regulated the expression of inflammatory factors and AQP1, mainly through the MAPK pathways. Taken together, these results demonstrate that the increase in intracellular PBEF expression promoted the apoptosis of HPMECs and the expression of inflammatory factors and thus enhanced the inflammatory response and inhibited the expression of AQP1, which resulted in abnormal water transport, diminishing the regulatory effects of AQP1 on water transport.

Introduction

Acute lung injury (ALI), a clinical syndrome caused by a variety of non-cardiogenic factors, is characterized by increased capillary permeability, pulmonary edema, atelectasis and refractory hypoxemia and is induced by injury to pulmonary capillary endothelial cells and alveolar epithelial cells; ALI has the potential to develop into acute respiratory distress syndrome (ARDS), which is associated with a mortality rate of 40-60% worldwide (1). ALI that manifests clinically as ARDS is a major cause of multiple organ dysfunction syndrome (MODS) (1). Inflammatory mediators, such as interleukin (IL)-1 β and IL-8, play key roles in the pathogenesis of ARDS, which is the primary cause of mortality in patients with these conditions.

Pre-B cell colony-enhancing factor (PBEF) is a highly conserved 52-kDa protein (2). It was originally cloned from a complementary DNA (cDNA) library of activated human peripheral blood mononuclear cells (PBMCs) and was identified as a secreted protein that enhances the effects of stem cell factor and IL-7 on pre-B cell colony formation. It is now evident that PBEF is a multifunctional protein, having nicotinamide phosphoribosyltransferase, adipokine and cytokine activities. Experimental and clinical analyses on the inflammatory aspects of PBEF conducted by Moschen *et al* (2) demonstrated that PBEF exhibited inflammatory and immune stimulatory activity, and that it regulated the expression of several inflammatory factors, such as tumor necrosis factor (TNF)- α , IL-1 β and IL-6. Ye *et al* (3) found that the level of PBEF in bronchoalveolar lavage fluid and blood plasma of animal and patients with ALI was significantly increased, suggesting that PBEF may be an indicator of lung injury. Bajwa *et al* (4) confirmed that the PBEF gene polymorphism was closely related to the prognosis of ARDS. Taken together, these data indicate that PBEF plays an important role in ALI. Ye *et al* (5) demonstrated that PBEF played an important role in the loss of barrier function in pulmonary vascular endothelial cells. Liu *et al* (6) found that IL-1 β promoted the expression of PBEF in pulmonary vascular endothelial cells, and that the secretion of inflammatory factors [IL-8, IL-16 and chemokine (C-C motif) receptor 3 (CCR3)] was involved.

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However, the exact mechanisms of PBEF-related pulmonary vascular endothelial cell injury remain unclear. Studies have proven that mitogen-activated protein kinases (MAPKs) are involved in the pathological process of lung injury (2,7,8); however, the association between the signal transduction pathways of the PBEF pro-inflammatory response in ALI and MAPK pathways remains unclear.

Researchers have indicated that aquaporins (AQPs) play an important role in the pathogenesis of ALI (9,10). Studies have focused on vascular endothelial permeability; however, little information on the effects of the fluid-transport function of AQPs in the formation of pneumoniedema is available. Furthermore, the expression of AQPs in lung tissue in ALI remains a controversial topic. Certain studies have demonstrated a decrease in AQP expression in patients suffering from ALI (11,12), whereas in another study, Lai *et al* (13) demonstrated that inflammatory factors promoted the expression of AQP1 *in vitro*. In the present study, PBEF was upregulated using an overexpression plasmid, and TNF- α and siRNA were also used to investigate the association of PBEF with inflammatory factors and AQP activity. The mechanisms of ALI were further examined with the use of MAPK inhibitors. Our study of the pathological mechanisms of ALI provides information which may prove to be useful in the prevention and treatment of ALI.

Materials and methods

Cell line and cell culture. Human pulmonary microvascular endothelial cells (HPMECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; both from Gibco, Paisley, UK), 100 U/ml penicillin and 50 U/ml streptomycin (both from Sigma-Aldrich China, Inc., Shanghai, China) at 37°C in an incubator with 5% CO₂.

Construction of the PBEF-overexpressing plasmid. The PBEF-overexpressing plasmid was constructed by inserting the cDNA clone of PBEF reverse transcriptase from the HPMECs into the pEGFP-N1 vector [from Takara Biotechnology (Dalian) Co., Ltd., Dalian, China]. Total RNA was extracted using TRIzol reagent (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China) to prepare the cDNA clone of PBEF. Reverse transcription polymerase chain reaction (RT-PCR) was performed to retrieve the full-length mouse PBEF cDNA using cDNA and a reverse transcription kit (Promega Biotech Co., Ltd., Beijing, China). The PCR conditions were as follows: a pre-denaturation step at 94°C for 5 min, followed by 94°C for 30 sec, 58°C for 30 sec, 72°C for 90 sec for 30 cycles, and finally a complete cycle at 72°C for 10 min. The amplified products were analyzed by electrophoresis, and the specific 1,473-bp band of the amplified cDNA fragment was confirmed and collected. The cloned PBEF cDNA was inserted into the pEGFP-N1 vector with *Xba*I and *Bam*H I [Takara Biotechnology (Dalian) Co., Ltd.], sequenced and verified. The primers used for PBEF amplification were: PBEF-F, 5'-CCGCTCGAGATGAATCCTGCGGCAGAAGC-3' and PBEF-R, 5'-CGGGATCCCGATGATGTGCTGCTTCAGTTC-3'. Underlined primers indicate the restriction enzyme site.

Table I. siRNA sequences.

Name	Sense/antisense siRNA (5'→3')
PBEF-homo-514	GGCCAAAUUUUGUUAGAATT UUCUAACAAAUUUUGGCCTT
PBEF-homo-1056	GGGAUGGAGUAGAUUAUUAATT UUAAUAUCUACUCCAUCCTT
PBEF-homo-1279	GGGCCGAUUAUCUUUACAUTT AUGUAAAGAUAAUCGGCCCTT
Negative control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT

PBEF, pre-B cell colony-enhancing factor.

Design and synthesis of siRNA. The PBEF-targeting siRNA was designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences are presented in Table I.

Cell transfection and induction of PBEF expression by TNF- α . Cell transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions (Shanghai Invitrogen Biotechnology Co., Ltd.). Briefly, the cells were seeded in 6-well plates at a concentration of 1×10⁵ cells/ml (1 ml/well). When the cells reached 70% confluence 24 h after seeding, the plasmid (pEGFP-N1-PBEF) and PBEF siRNA were transfected into the cells at final concentrations of 4 µg/ml and 50 nM, respectively. The medium was changed 4–6 h after transfection. TNF- α (Peprotech, Inc., Rocky Hill, NJ, USA) was added at a concentration of 5.75 nM, as previously described (14), and was re-added every 24 h. The effects of the induction of PBEF expression were detected by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis after 72 h.

Determination of cell apoptosis. Cell apoptosis was evaluated by co-staining of the cells with Annexin V-FITC and propidium iodide (PI; BD Pharmingen, San Diego, CA, USA). Briefly, the cells were collected 72 h after transfection and resuspended in 0.5 ml binding buffer and stained with 5 µl Annexin V-FITC and 5 µl PI, and the solution was then incubated for 15 min at room temperature in the dark. Thereafter, the cells were immediately analyzed on a Coulter Epics XL Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA). The fluorochrome was excited at 488 nm by an argon ion laser, and Annexin V and PI emissions were monitored at 525 and 630 nm, respectively. In each analysis, 10,000 events were recorded. The dual parametric dot plots were used to calculate the percentage of non-apoptotic viable cells in the lower left quadrant (Annexin V-negative/PI-negative), early apoptotic cells in the lower right quadrant (Annexin V-positive/PI-negative), late apoptotic cells in the upper right quadrant (Annexin V-positive/PI-positive) and necrotic cells in the upper left quadrant (Annexin V-negative/PI-positive).

Blocking MAPK and AKT signaling pathways. SB 203580 (a p38 inhibitor), PD 98059 [an extracellular signal-

Table II. Primers used for quantitative PCR.

Primer name	Primer sequences (5'→3')	Product size (bp)	GenBank accession no.
PBEF-F	TGCTACAGAAGTTGACAAGAGATC	102	U02020
PBEF-R	CCCTGCTGGCGTCCTATG		
IL-1β-F	TGGCTTATTACAGTGGCAATG	134	NM_000576
IL-1β-R	GTGGTGGTGGAGATTG		
IL-6-F	TAGGACTGGAGATGTCTGAG	104	NM_000600
IL-6-R	GTGGAGAAGGAGTTCATAGC		
IL-8-F	CACAAACTTCAGAGACAGCAGAG	85	NM_000584
IL-8-R	ACACAGTGAGATGGTCCCTTC		
AQP1-F	ACTCATCTACGACTTCATCC	100	NM_001185060
AQP1-R	GGCATCCAGGTCATACTC		
GAPDH-F	TCTCTGCTCCCTGTTC	97	NM_002046
GAPDH-F	ACTCCGACCTTCACCTTC		

PBEF, pre-B cell colony-enhancing factor; IL, interleukin; AQP1, aquaporin 1.

regulated kinase (ERK)1/2 inhibitor], c-Jun N-terminal kinase (JNK) inhibitor II and LY 294002 [a phosphoinositide 3-kinase (PI3K) inhibitor] were all purchased from Calbiochem (Merck Millipore, Shanghai, China). The cells were exposed to the inhibitors at a final concentration of 20 µM for 1 h prior to transfection and induction of expression. DMSO (Sigma-Aldrich China, Inc.) was used as a negative control, as previously described (15).

RT-qPCR. Total RNA was extracted using TRIzol reagent (Shanghai Invitrogen Biotechnology Co., Ltd.) after the cells had been collected and quantified. This was followed by reverse transcription with 1 µg RNA. Quantitative (real-time) PCR was performed using the SYBR-Green dye method (SYBR-Green PCR Master Mix; Toyobo Co., Ltd., Osaka, Japan) under the follows conditions: 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, for 40 cycles. The reaction mixture (35 µl) contained 100 ng cDNA (20 µl). The primers used are presented in Table II. The experiments were repeated 3 times and analyzed by comparing the $2^{-\Delta\Delta Ct}$ values.

Western blot analysis. Total cellular proteins were extracted by incubating the cells in lysis buffer obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The protein concentrations of the cell lysates were determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. For SDS-PAGE, 10% gels were used, and equal amount of proteins were loaded per lane. Following electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Pierce Biotechnology, Inc.) and blocked with 5% non-fat milk in TBST buffer for 1 h. Subsequently, the membranes were incubated with primary antibodies [anti-PBEF (ab45890), anti-IL-1β (ab9722), anti-IL-6 (ab6672), anti-IL-8 (ab7747), anti-AQP1 (ab15080) and anti-GAPDH (ab181602) antibodies; Abcam, Shanghai, China] at a 1:1,000 dilution in 5% non-fat milk overnight at 4°C followed by secondary antibodies [goat

anti-rabbit IgG H&L (HRP); ab97051; Abcam] at a 1:2,000 dilution for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Pierce Biotechnology, Inc.).

Statistical analysis. Experiments were carried out at least in triplicate, and the results are expressed as the means ± SD. Statistical analysis was performed using the SPSS statistical package (SPSS 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). The differences between 2 groups were analyzed using a two-tailed Student's t-test, and those between 3 or more groups were analyzed by one-way analysis of variance (ANOVA) multiple comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Regulation of the intracellular expression level of PBEF. RT-qPCR was employed to evaluate the inhibitory effects of siRNA, and the results revealed that siRNA PBEF-homo-1279 inhibited PBEF gene expression by up to 85% at a concentration of 50 nM (data not shown). The results of RT-qPCR and western blot analysis revealed that the PBEF-targeting siRNA significantly inhibited the mRNA and protein expression of PBEF (Fig. 1). Moreover, transfection of the cells with the PBEF-overexpressing plasmid (pEGFP-N1-PBEF) increased the expression level of PBEF to a level 10-fold greater than that of the control group (Fig. 1). These results indicated the powerful effects of the PBEF-overexpressing plasmid and PBEF-targeting siRNA on the expression of PBEF. The effects of TNF-α on the overexpression and knockdown of PBEF were also investigated, and the results revealed that the expression of PBEF increased significantly with the presence of TNF-α, which indicated that TNF-α promoted the expression of PBEF in the HPMECs (Fig. 2). The effects of TNF-α on the expression of PBEF were further enhanced by combining TNF-α and transfection with the PBEF-overexpressing plasmid. However,

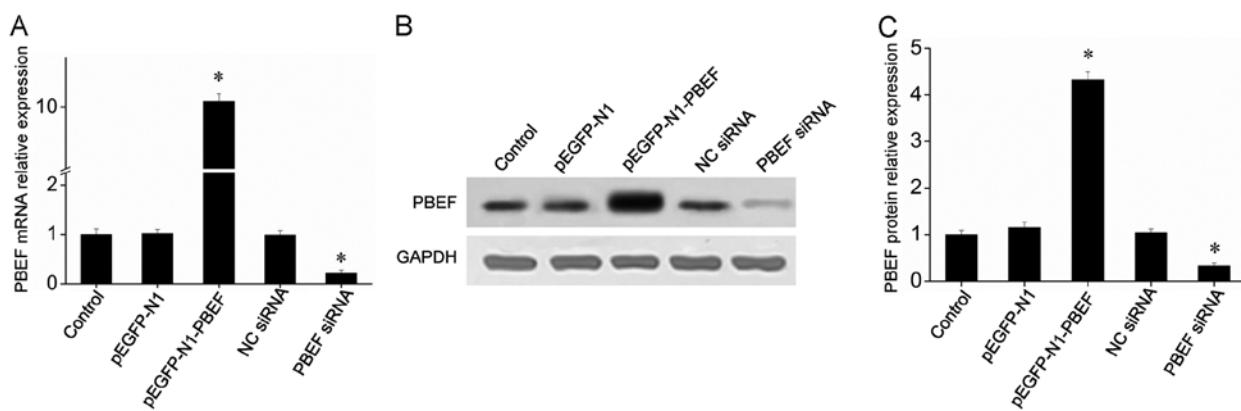


Figure 1. Overexpression and silencing of pre-B cell colony-enhancing factor (PBEF). (A) The changes in PBEF mRNA expression were detected by RT-qPCR. (B and C) The changes in PBEF protein expression were detected by western blot analysis. Cells were seeded in 6-well plates at a concentration of 1×10^5 cells/ml (1 ml/well). When the cells reached 70% confluence 24 h after seeding, the plasmid (pEGFP-N1-PBEF) and PBEF siRNA were transfected into the cells at final concentrations of 4 μ g/ml and 50 nM, respectively. The medium was changed 4–6 h after transfection. GAPDH served as a control for the equal loading of proteins. Each bar represents the means \pm SD from 3 samples (* $P < 0.05$ vs. control). NC, negative control.

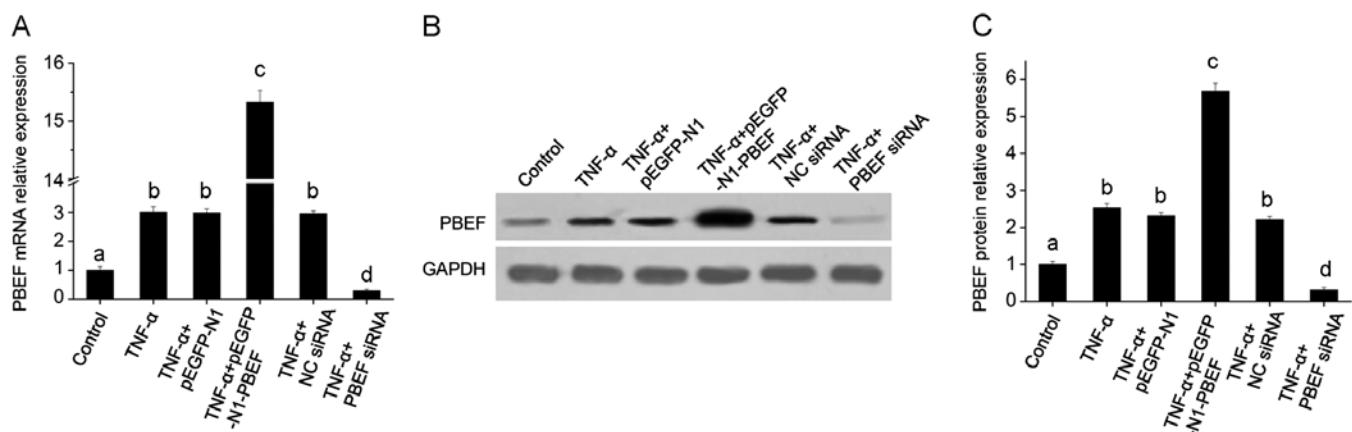


Figure 2. Effects of tumor necrosis factor (TNF)- α on pre-B cell colony-enhancing factor (PBEF) expression. TNF- α was added at a concentration of 5.75 nM, and then re-added every 24 h. The effect of the induction of PBEF expression was detected by (A) RT-qPCR and (B and C) western blot analysis after 72 h. GAPDH served as a control for the equal loading of proteins. Each bar represents the means \pm SD from 3 samples, and bars labeled with different letters (a–d) indicate a statistically significant difference between groups (* $P < 0.05$ vs. the control). NC, negative control.

the TNF- α -induced overexpression of PBEF was blocked by PBEF siRNA (Fig. 2). Taken together, our results suggest that TNF- α has an enhancing effect when combined with the PBEF-overexpressing plasmid and an antagonistic effect when combined with PBEF siRNA.

Overexpression of PBEF promotes HPMEC apoptosis. To examine the effects of PBEF overexpression on HPMEC apoptosis, flow cytometric analysis was employed on the cells co-stained with Annexin V-FITC and PI. The results revealed that the overexpression of PBEF significantly promoted both the early- and late-phase apoptosis of the HPMECs (Fig. 3). These results suggest that an increase in the PBEF expression level causes apoptotic cell death, and this in turn, may lead to the development of ALI.

PBEF regulates the expression of inflammatory factors and AQPs. To examine the effects of PBEF expression on inflammatory factors and AQPs, RT-qPCR and western blot analysis were employed to measure the mRNA and protein levels of PBEF

in the HPMECs. The results indicated that PBEF overexpression significantly increased the mRNA and protein expression of IL-1 β , IL-6 and IL-8, and this was further enhanced by the addition of TNF- α (Fig. 4). The mRNA and protein expression of AQP1 was inhibited by the overexpression of PBEF and this effect was enhanced by TNF- α (Fig. 4). Furthermore, the inhibition of PBEF using siRNA downregulated the mRNA and protein levels of IL-1 β , IL-6 and IL-8 and increased the mRNA and protein level of AQP1. Taken together, these results indicate that PBEF enhances the mRNA and protein expression of IL-1 β , IL-6 and IL-8, and inhibits the mRNA and protein expression of AQP1.

PBEF regulates the expression of inflammatory factors and AQPs through the MAPK pathways. The regulatory effects of MAPKs on inflammatory factors and AQPs have previously been demonstrated (16). Therefore, in this study, experiments were also carried out to investigate the association between the PBEF-regulated expression of inflammatory factors and AQPs, and the activation of the MAPK and PI3K pathways.

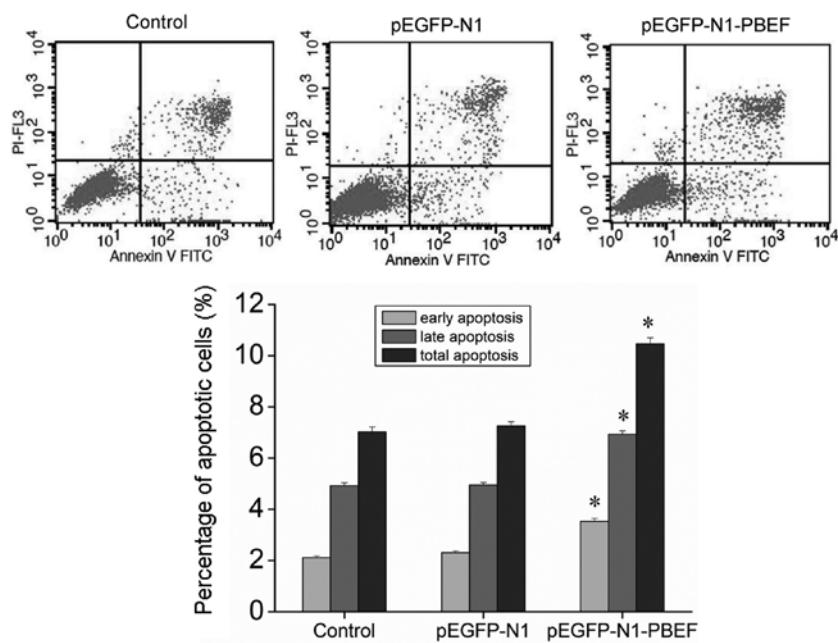


Figure 3. Effect of pre-B cell colony-enhancing factor (PBEF) expression on human pulmonary microvascular endothelial cell (HPMEC) apoptosis, as measured by Annexin V-FITC-PI staining and flow cytometric analysis. The results are presented as the means \pm SD, ($n=3$); * $P<0.05$ vs. control.

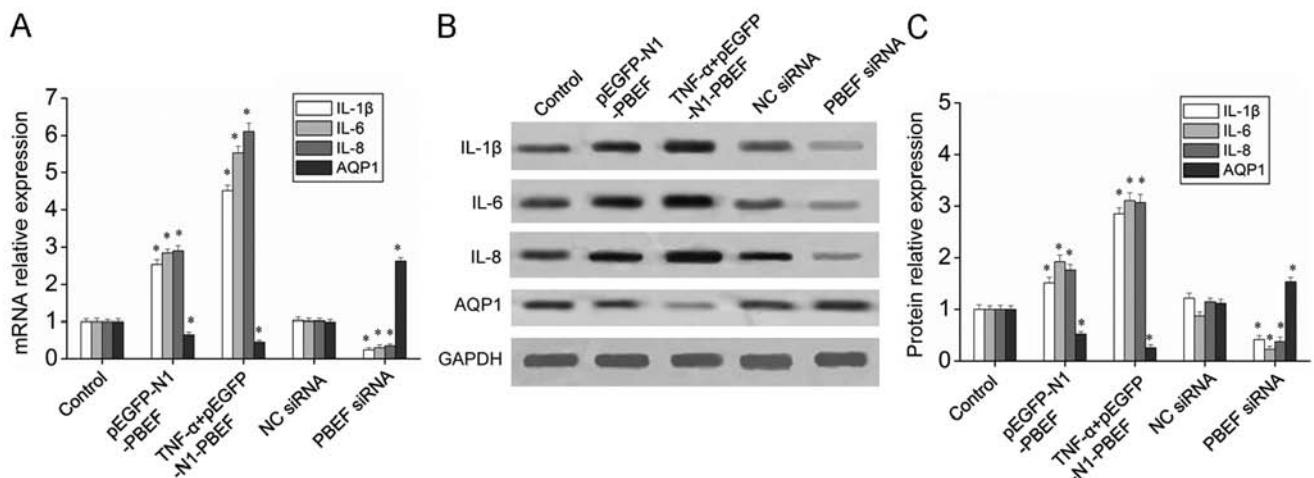


Figure 4. Effect of pre-B cell colony-enhancing factor (PBEF) expression on inflammatory factor and aquaporin (AQP1) expression. The expression of interleukin (IL)-1 β , IL-6, IL-8 and AQP1 at the (A) mRNA and (B and C) protein level was detected by RT-qPCR and western blot analysis, respectively. GAPDH served as a control for the equal loading of proteins. Each bar represents the means \pm SD from 3 samples (* $P<0.05$ vs. control).

Inhibitors of several signaling pathways (p38, ERK, JNK and PI3K) at non-cytotoxic concentrations (20 μ M) were introduced into PBEF-overexpressing HPMECs that were treated or not with TNF- α . The mRNA and protein levels of the inflammatory factors and AQP1 were measured by RT-qPCR and western blot analysis. The results (Fig. 5) revealed that the expression of the inflammatory factors, IL-1 β , IL-6 and IL-8, was significantly downregulated in the presence of the p38 inhibitor (SB 203580), as well as in the presence of the ERK inhibitor (PD 98059). However, the JNK inhibitor (JNK inhibitor II) decreased only the expression level of IL-8, and no significant changes were observed in the expression of inflammatory factors in the presence of the PI3K inhibitor (LY 294002). These results indicate that PBEF regulates the expression of inflammatory factors and that the MAPK

pathways, particularly p38 MAPK, are involved. The expression of AQP1 was upregulated significantly by the p38, ERK and JNK inhibitors, indicating that the regulatory effects of PBEF on AQP1 are dependent on the MAPK pathways. Taken together, our results suggest that PBEF regulates the expression of inflammatory factors and AQPs through the MAPK pathways.

Discussion

ARDS is still one of the main causes of death of patients in the intensive care unit (ICU), with a mortality rate of 40-60% (1); it is proving difficult to reduce the incidence and mortality rate of ARDS. The prevention of ALI is the main measure which must be taken in order to reduce the incidence of ARDS. There have

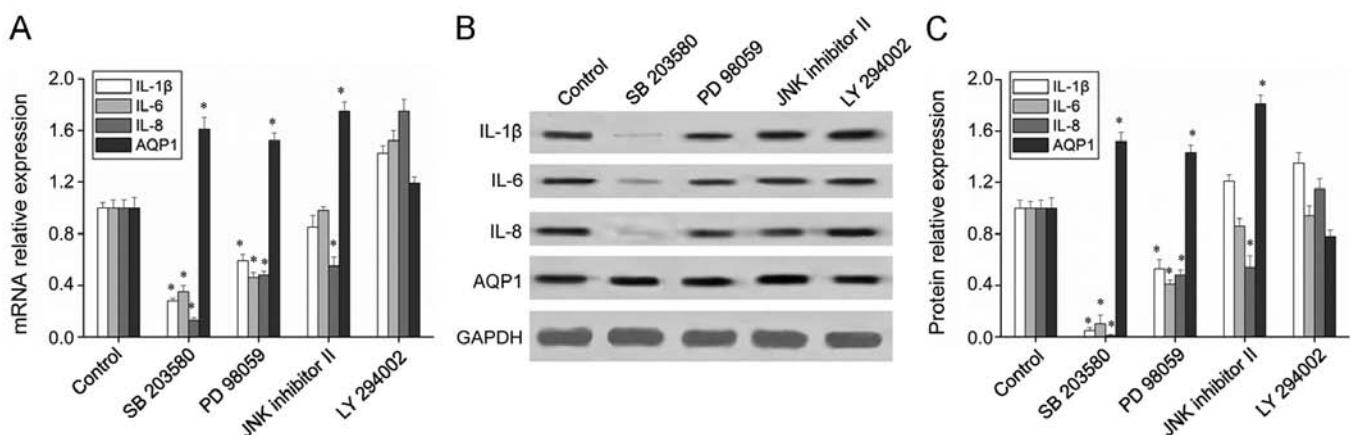


Figure 5. Effects of pre-B cell colony-enhancing factor (PBEF) overexpression on inflammatory factor and aquaporin (AQP1) expression. Human pulmonary microvascular endothelial cells (HPMECs) were transfected with the PBEF-overexpressing plasmid (pEGFP-N1-PBEF) and then exposed to different signaling inhibitors, which include SB 203580 for p38, c-Jun N-terminal kinase (JNK) inhibitor II, PD 98059 for extracellular signal-regulated kinase (ERK) and LY 294002 for phosphoinositide 3-kinase (PI3K). Cells were exposed to the inhibitors at a final concentration of 20 μ M for 1 h prior to transfection and the induction of PBEF expression. DMSO was used as a negative control. (A) mRNA expression; (B and C) protein expression. Each bar represents the means \pm SD from 3 samples (* P <0.05 vs. control).

been many important developments in the study of the pathogenesis, treatment strategies and other aspects of ALI (17,18). Studies have emphasized the important role which PBEF plays in the development of ALI. For instance, Kamp *et al* (19) proved that PBEF is a candidate gene which is related to ALI through genome-wide association studies (GWAS). The present study focused on investigating the important role of PBEF in ALI and its association with inflammatory signaling pathways and pulmonary water transport. Our results revealed that TNF- α promoted the expression of PBEF in HPMECs, which was consistent with the results of Hector *et al* (14). Moreover, Wang *et al* (20) previously reported that TNF- α enhanced the hyperbaric oxygen-induced visfatin expression through the JNK pathway in human coronary arterial endothelial cells.

It has been demonstrated that endothelial cell injury is an important marker of ALI, and that the degree of injury is closely related to the prognosis of ALI patients (21). Therefore, the study of the link between HPMEC dysfunction and ALI has attracted increasing attention. In the present study, the influence of PBEF overexpression on HPMEC apoptosis was detected by flow cytometry, and the results revealed that PBEF overexpression promoted HPMEC apoptosis, which was consistent with the findings of the study by Martin *et al* (22) and de Souza *et al* (23). In addition, Martin *et al* (22) found that ALI was related to an increase in cell apoptosis in lung tissue, a delay in inflammatory cell apoptosis, and the exacerbation of lung injury. However, during the healing process, the apoptosis of proliferous endothelial cells and fibroblasts occurred, and the lung tissue was reconstructed. de Souza *et al* (23) found that the increase in endothelial/epithelial cell apoptosis and the decrease in inflammatory cell apoptosis were closely related to the occurrence of ALI. Gao *et al* (24) also demonstrated that PBEF silencing using siRNA inhibited the expression of inflammatory cytokines and decreased the apoptosis mediated by FasL in HPMECs.

It has also been demonstrated that PBEF plays an important role in inflammatory signaling pathways and endothelial cell permeability; however, the exact mechanisms remain unclear (25). In the present study, we demonstrated that PBEF

promoted the expression levels of the inflammatory factors, IL-1 β , IL-6 and IL-8, which resulted in the enhancement of the inflammatory response. Liu *et al* (6) and Li *et al* (26) proved that PBEF promoted the expression of IL-8 in human pulmonary alveolar epithelial cells. Ognjanovic *et al* (27) indicated that recombinant PBEF increased the expression of IL-6 and IL-8 in amniotic epithelial cells. PBEF has also been found to induce the mRNA and protein expression of IL-1, IL-6, IL-10 and TNF- α in PBMCs and that of IL-1 β , IL-6 and TNF- α in CD14 monocytes (2). Therefore, PBEF and some corresponding inflammatory factors may have mutually promoting effects and may exhibit a 'waterfall-like' chain reaction in HPMECs. Our results confirmed that PBEF inhibited the expression of AQP1 in HPMECs. AQP1 is a crucial molecule in maintaining the balance of water movement between blood vessels and interstitial fluid (9). In a previous study using an animal model of lipopolysaccharide-induced ALI, the expression of AQP1 was decreased, and pneumoniedema with ALI aggravation were observed (12). In a murine model of pneumoniedema induced by viral infection, it was found that the expression of AQP1 and AQP5 in the mouse lungs was decreased, which then recovered when inflammation became slighter (11).

The mechanisms of the PBEF-regulated inflammatory response remain elusive. In the present study, we found that PBEF regulated the expression of inflammatory factors mainly through the MAPK pathways, which was consistent with the conclusion drawn by Moschen *et al* (2). However, they also found that PBEF regulated the expression of IL-10 and TNF- α through the PI3K pathway. Liu *et al* (7) demonstrated that the visfatin-induced monocyte chemoattractant protein (MCP)-1 and IL-6 production involved the p38 MAPK, PI3K and ERK1/2 pathways in human umbilical vein endothelial cells. They also found that PBEF induced the expression of IL-8, IL-16 and CCR3 through the p38 and JNK pathways in pulmonary epithelial cells. Taken together, these results suggest that p38 MAPK is the main pathway through which PBEF regulates inflammatory factors.

The molecular mechanisms through which PBEF affects the expression of AQP1 have yet to be clarified. In this study, we found that the expression of AQP1 increased after blocking

p38, ERK and JNK, which indicated that PBEF regulates the expression of AQP1 mainly through the MAPK pathways. Therefore, it may be inferred that, in the pathological process, the increase in PBEF expression inhibits AQP1 expression and causes pneumoniedema, mainly through the MAPK pathways. It was also confirmed in this study that the MAPK pathways have a close association with AQP1. Similarly, Umenishi *et al* (28) demonstrated that the activation of the ERK, p38 and JNK pathways, as well as the hypertonicity response element in the AQP1 promoter, was involved in hypertonicity-induced AQP1 expression in mouse inner-medullary collecting duct (mIMCD)-3 cells. MEK/ERK mediated the UVB-induced AQP1 downregulation and water-permeability impairment in human retinal pigment epithelial cells, which was also confirmed by Jiang *et al* (29). Furthermore, Shankardas *et al* (30) found that AQP1 played an important role in human corneal endothelial cell (HCEC) proliferation and migration through the ERK signaling pathway.

In conclusion, our study demonstrated that PBEF regulated the pathological process of ALI by promoting HPMEC apoptosis and regulating the expression of inflammatory factors and AQP1 through the MAPK and PI3K pathways. These results provide valuable experimental evidence which may be used in future studies on the biological function, molecular mechanisms and clinical application potential of PBEF in ALI pathogenesis.

Acknowledgements

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