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Regulation of Inflammatory Cytokine Expression in Pulmonary Epithelial Cells by Pre-B-cell Colony-enhancing Factor via a Nonenzymatic and AP-1-dependent Mechanism*

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Although our previous studies found Pre-B-cell colony-enhancing factor (PBEF) as a highly up-regulated gene in acute lung injury that could stimulate expressions of other inflammatory cytokines, the underlying molecular mechanisms remain to be fully elucidated. Growing evidence indicates that PBEF is a nicotinamide phosphoribosyltransferase involved in the mammalian salvage pathway of NAD synthesis. This study was designed to determine whether the effect of PBEF to stimulate expressions of inflammatory cytokines depends on its enzymatic activity. We prepared two human PBEF mutant (H247E and H247A) recombinant proteins and overexpressing constructs for their overexpressions in A549 cells and confirmed that enzymatic activities of both mutants were nearly or completely abolished. Two mutants stimulated interleukin-8 (IL-8) expression at both the mRNA level and protein level just as equally effective as the wild-type PBEF did. These effects were due to the increased transcription, not the mRNA stability, of the IL-8 gene. Reporter gene assays and gel shift experiments indicated that AP-1 transcription factor is required to mediate these effects. SB203580, a p38 MAPK pathway inhibitor, and JNK inhibitor 1 can attenuate these effects. Both PBEF mutants similarly stimulated the expression of two other inflammatory cytokines: IL-16 and CCR3. These results indicate that PBEF stimulated expression of IL-8, IL-16, and CCR3 via its non-enzymatic activity. This effect is AP-1-dependent, in part via the p38 MAPK pathway and the JNK pathway. This finding reveals a new insight, which may manifest a novel role of PBEF in the pathogenesis of acute lung injury and other inflammatory disorders.

Acute lung injury (ALI)[2] and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by inflammation of the lung parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators causing inflammation, hypoxemia, and frequently resulting in multiple organ failure (1, 2). Although ALI/ARDS was first described in 1967 by Ashbaugh *et al.* (3), its mortality and morbidity remain high (4). More studies are warranted to elucidate its molecular pathogenesis and to identify new diagnostic and therapeutic targets to ALI/ARDS.

In our previous study on animal models of ALI and human patients with ARDS, we identified pre-B-cell colony-enhancing factor (PBEF) as a biochemical and genetic marker in ALI (5). Our findings were confirmed and extended in a separate and larger population (>1000 patients) by Bajwa *et al.* (6). In further studies, we demonstrated that overexpression of PBEF can augment the expression of inflammatory cytokines and dysregulate pulmonary cell barrier function, whereas inhibition of PBEF expression by its cognate small interference RNA has the opposite effects (7–9). PBEF has been confirmed as a nicotinamide phosphoribosyltransferase (Nampt) involved in the mammalian salvage pathway of NAD synthesis (10, 11). Whether the enzymatic activity of PBEF is involved in its effect on cytokine stimulation is currently unknown. The signal transduction pathways underlying these effects are also not understood.

This study was designed to determine whether the effect of PBEF to stimulate expressions of inflammatory cytokines depends on its Nampt enzymatic activity and to dissect signal transduction pathways underlying these effects. We prepared two human PBEF mutant (H247E and H247A) recombinant proteins and overexpressing constructs for their overexpression in A549 cells and confirmed that enzymatic activities of both mutants were nearly or completely abolished. We determined whether two mutants in transfected A549 cells could similarly stimulate the expression of

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[2] The abbreviations used are: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; CCR3, chemokine (C-C motif) receptor 3; PBEF, pre-B-cell colony enhancing factor; Nampt, nicotinamide phosphoribosyltransferase; STAT3, signal transducers and activators of transcription 3; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; RT, reverse transcription; GFP, green fluorescent protein; eGFP, enhanced GFP; NMNAT, nicotinamide mononucleotide adenylyltransferase; EMSA, electrophoretic mobility shift assay; MBP, maltose-binding protein.

inflammatory cytokines such as IL-8, IL-16, and CCR3 as wild-type PBEF did. We also performed reporter gene assay and gel shift experiments to examine whether AP-1 transcription factor is required to mediate these effects. We also cultured transfected A549 cells in the absence or presence of SB203580, a p38 MAPK pathway inhibitor, and JNK inhibitor 1 to probe whether the p38 MAPK and JNK pathways are pertinent to the PBEF-mediated effect. Here we report that PBEF stimulated expression of IL-8, IL-16, and CCR via its nonenzymatic mechanism. This effect is AP-1-dependent, in part via the p38 MAPK pathway and the JNK pathway.

EXPERIMENTAL PROCEDURES

Materials—A rabbit anti-human PBEF polyclonal antibody was obtained from Bethyl Laboratories, Inc. (cat. no. A300–372A, Montgomery, TX). A rabbit anti-human IL-8 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (cat. no. sc-7922, Santa Cruz). A mouse anti-human β-actin monoclonal antibody (cat. no. A1978) was purchased from Sigma-Aldrich. Superscript III Reverse Transcriptase (cat. no. 18080044) was purchased from Invitrogen (Carlsbad, CA). *Pfu* DNA polymerase was purchased from Stratagene (cat. no. 600135). pGEX-6P-3 expression vector (cat. no. 27-4599-01) was purchased from Amersham Biosciences. pMAL Protein Fusion and Purification System and various restriction endonucleases were purchased from New England Biolabs, Inc. (Ipswich, MA). The inhibitor of p38 MAPK pathway, SB203580 (cat. no. 559389) was from EMD (San Diego, CA). The inhibitor of JNK pathway, JNK inhibitor 1 (cat. no. 159-600-R100) was from Enzo Life Sciences, Inc. (Plymouth Meeting, PA). Sources of other key reagents are specified in relevant texts.

Cell Culture—Human A549 cell, a lung carcinomatous type II alveolar epithelial cell line, was obtained from ATCC (cat. no. CCL-185™, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium with regular supplements at 37 °C in a humidified atmosphere of 5% CO_2, 95% air.

Plasmid Construction for Prokaryotic Expression—The human Nmnat 1 cDNA was amplified from A549 cell RNA by RT-PCR using the primers containing BamHI adaptors: forward, CGCGGATCCATGGAAAATTCCGAGAAGACT, and reverse, CGCGGATCCCCTATGTCTTAGCTTCTGCAGTG, and cloned into the pMAL-c4X vector. The human PBEF cDNA was amplified from pDNR-PBEF vector by PCR using the primer pair containing SalI adaptors TTAGTCGACATGAATCCTGCGGCAGAAGC and TTAGTCGACCTAATGATGTGCTGCTTCCAGTTC and cloned into the pGEX-6p-3 vector. Cloned human Nmnat 1 and Nampt cDNA were sequence-verified by matching NM_022787.3 and NM_005746.1, respectively.

Plasmid Construction for Eukaryotic Expression—The human PBEF cDNA was amplified from pDNR-PBEF vector by PCR using the following primers containing the following EcoRI adaptors: forward, TTAGAATTCATGAATCCTGCGGCAGAAGC; reverse, TTAGAATTCCTAATGATGTGCTGCTTCCAGTTC. An eGFP cDNA was amplified from pWPT-eGFP vector by RT-PCR using the following primer pair EcoRI adaptors: TTAGAATTCGCCACCATGGTGAGCAAGGGCGA and TTAGAATTCCTAGCTACTAGCTAGTCGAGA. They were subcloned into the pCAGGS vector and sequence-verified.

Site-directed Mutagenesis at Residue His-247 of PBEF—Two different point mutations H247A and H247E of PBEF were generated at the sequence coding for a histidine that is integral for enzymatic activity. The forward primers for each mutant contain an EcoNI site, TTACCTGTTCCAGGCTATTCTGTTCCAGCAGCAGAA**GCC**AGTACC(H247A) and TTACCTGTTCCAGGCTATTCTGTTCCAGCAGCAGAA**GAA**AGTACC(H247E), whereas the reverse primer contains a PpuM I site, TTAGGGTCCTTGAAGACGTTAATCCCAA. The bold letters indicate the mutated nucleotides. The PCR-amplified PBEF mutant products using wild-type pCAGGS-hPBEF as a template were cloned into the backbone of the pCAGGS-hPBEF and sequence-verified.

Prokaryotic Expression and Purification of Recombinant Human Nmnat 1, Nampt, and Mutant Nampt—MBP-Nmnat 1 expressed in *Escherichia coli* strain K12 TB1 or Nampt and mutant Nampts in *E. coli* BL21 were purified by affinity purification specific for MBP (cat. no. E8200, New England Biolabs) or glutathione *S*-transferase resin (cat. no. 27-0843-01, Amersham Biosciences), respectively.

Eukaryotic Expression of Recombinant Human PBEF and Mutant PBEFs in A549 Cells—A549 cells were transiently transfected with the pCAGGS-PBEF and mutant PBEF constructs using Lipofectamine™ 2000 (cat. no. 11668-019, Invitrogen). 48 h later, cell lysates were harvested for analysis.

Nmnat and Nampt Enzymatic Activity Assay—The activities of recombinant and cytoplasmic Nmnat and Nampt were assayed as described by Revollo *et al.* (12), except that their enzymatic activities were measured by monitoring the increase in absorbance at 340 nm caused by the reduction of NAD to NADH.

Isolation of RNA and RT-PCR Analysis—Total RNA from *in vitro* cultured cells was isolated using the TRIzol solution (cat. no. 15596-018, Invitrogen). RT-PCR was performed using the standard procedures with gene-specific primers (Table 1). β-Actin was used as a housekeeping gene control. PCR products were separated on a 1.5% agarose gel and stained by ethidium bromide (0.5 μg/ml). The band image was acquired using an Alpha Imager and analyzed by the AlphaEase™ Stand Alone Software (Alpha Innotech Corp., San Leandro, CA).

Western Blotting—Western blotting analysis was performed as described before (9).

IL-8 Messenger RNA Stability Analysis—A549 cells were transfected with pCAGGS-hPBEF, pCAGGS-mutant PBEFs (H247A and H247E), or pCAGGS vector for 24 h. RNA synthesis was blocked by addition of 5 μg/ml actinomycin D, a transcription inhibitor, and then total RNA was isolated at 0, 3, 6, 9, and 24 h. Semi-quantitative RT-PCR was performed for IL-8 and β-actin mRNA.

Construction of Human IL-8 Promoter 5′-Deletant Plasmids and Reporter Gene Assay—The 5′ deletion −1498 construct was amplified from genomic DNA of A549 cells by PCR using the following primers containing XmaI and XhoI sites: forward, TCCCCCCGGGTTTACTTTGCTATAGAATTCAG; reverse, CCGCTCGAGTGCTCCGGTGGCTTTTTATATC, and cloned into the pGL-3 enhancer reporter vec-

tor. The other 5′ promoter deletants were amplified using the PCR with pGL-3 −1498 hIL-8 plasmid as a template with the following primers: −162 hIL-8, 5′-TCCCCCCGGGACTCCG-TATTTGATAAGG-3′; −132 hIL-8, 5′-TCCCCCCGGGTG-TGATGACTCAGGTTTG-3′; −99 hIL-8, 5′-TCCCCCCGG-GGCCATCAGTTGCAAATCG-3′; −54 hIL-8, 5′-TCCCCC-CGGGATGAGGGTGCATAAGTTC-3′; downstream (underline indicates NcoI restriction site), CATGCCATGGTGGCT-TTACCAACAGTACCG (13–15), and cloned into pGL-3 enhancer reporter vector. The reporter gene assays were carried out using the Dual-Luciferase Reporter Assay System (cat. no. E1910, Promega, Madison, WI).

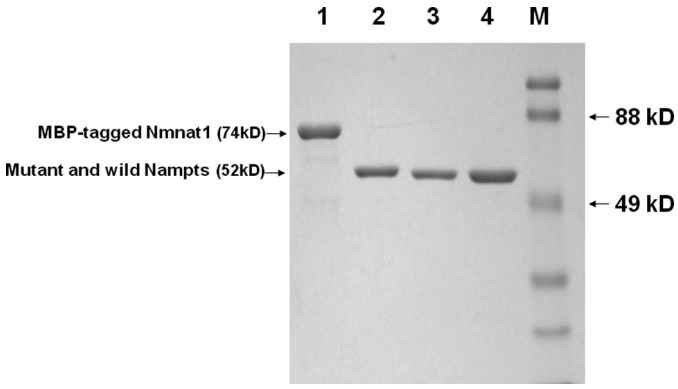

FIGURE 1. **Simple Blue™ staining of purified recombinant human MBP-tagged Nmnat 1, mutant, and wild-type Nampts separated by SDS-PAGE.** Recombinant human proteins were expressed in *E. coli* and purified as described under "Experimental Procedures." Two or three micrograms of each purified recombinant human protein were separated by 10% SDS-PAGE and stained with Simple Blue™ SafeStain. *Lane 1*, MBP-tagged recombinant human Nmnat 1; *lane 2*, recombinant human mutant Nampt (H247E); *lane 3*, recombinant human mutant Nampt (H247A); *lane 4*, recombinant human wild-type Nampt; *M*, prestained SDS-PAGE standards (Low Range, Bio-Rad).

EMSA—Electrophoretic mobility shift assay (EMSA) was performed essentially as described by Wang *et al.* (16) except the probe AP-1/IL-8 (GTG TGA TGA CTC AGG TTT G) (17) was labeled with Biotin instead of a radioisotope, and the DNA-binding reactions were carried out using the LightShift® chemiluminescent EMSA kit (cat. no. 20148, Pierce).

Cignal™ Reporter Assay for AP-1-dependent GFP Reporter Activity—The Cignal™ Reporter Assay for AP-1-dependent GFP reporter activity was performed following the manufacture's manuals (Cignal™ Reporter Assay Kits, cat. no. CCS-011G, SABiosciences, Frederick, MD). Plasmids of interest (200 ng) plus Cignal reporter (100 ng) were transfected into A549 cells using Lipofectamine 2000 (cat. no. 31985-062, Invitrogen). 48 h later, the transfected cells were directly visualized under a fluorescence microscope.

Statistical Analysis—Statistical analyses were performed using the Sigma Stat (ver 3.5, Systat Software, Inc., San Jose, CA). Results are expressed as means ± S.D. of three samples from at least two independent experiments. Stimulated samples were compared with controls by unpaired Student's *t* test. $p < 0.05$ was considered statistically significant.

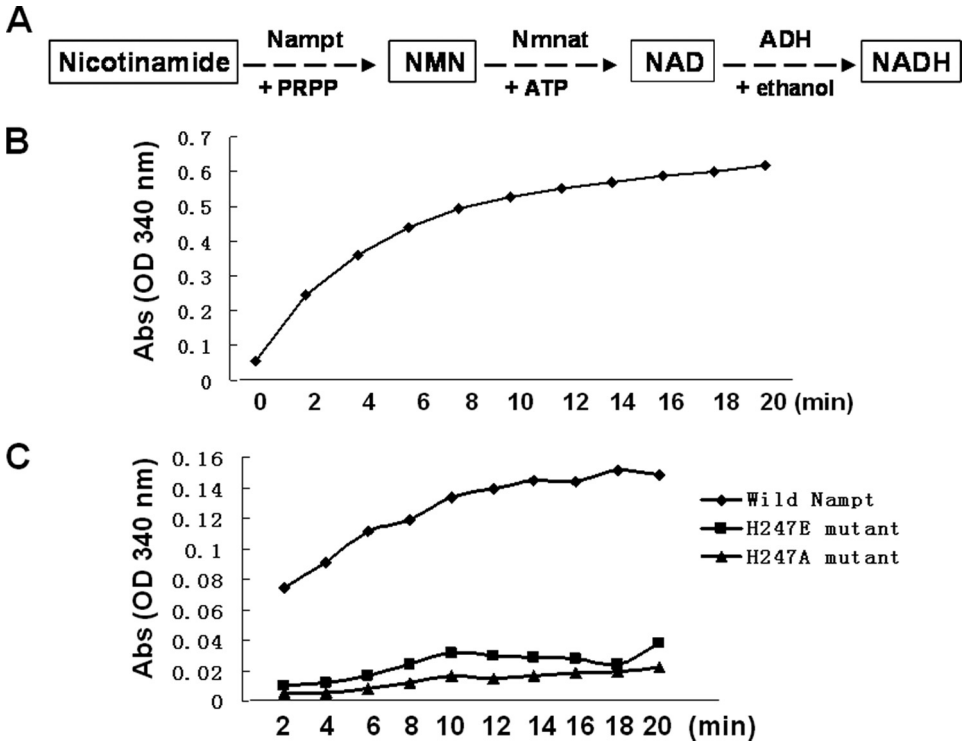

FIGURE 2. **An *in vitro* enzymatic activity assay of purified recombinant human wild-type and mutant Nampt proteins.** *A*, the scheme of the mammalian NAD biosynthesis reactions in the enzyme-coupled spectrometric assay. Nicotinamide is first converted to NMN by Nampt and then to NAD by Nmnat. The synthesized NAD *in vitro* was converted to NADH by alcohol dehydrogenase (*ADH*), and the enzymatic activity is indicated by the production of NADH at different time points as measured at 340 nm by a spectrometer. *B*, the enzymatic activity of purified MBP-tagged human Nmnat 1. The substrate for the MBP-tagged human Nmnat 1 is NMN. *C*, the enzymatic activity of purified recombinant human wild-type and mutant Nampt proteins. The starting substrate in this reaction is nicotinamide. Purified recombinant human wild-type and mutant Nampts and MBP-tagged recombinant human Nmnat 1 are used as the enzyme sources.

RESULTS

Purification and Enzymatic Activity Assay of Recombinant Human MBP-tagged Nmnat 1, Wild-type, and Mutant Nampts in Vitro—To establish the assay of Nampt activity, we first prepared enzymatic components (Nmnat 1 and Nampt) as well as two Nampt mutants (H247E and H247A). As shown in Fig. 1, human MBP-tagged Nmnat 1 and human wild-type and mutant Nampts display a single band with the right molecular weight, respectively, on a Simple Blue™-stained gel separated by SDS-PAGE, indicating a purity >95%. Two human Nampt mutants (H247E and H247A) were prepared based on the work by Wang and his colleagues (18). The H247E mutant of Nampt retains low enzymatic activity, whereas the H247A mutant has no detectable activity.

As depicted in Fig. 2*A*, analysis of Nampt enzymatic activity in the enzyme-coupled spectrometric assay requires three enzymes: Nampt,

Nmnat1, and alcohol dehydrogenase. MBP-tagged Nmnat 1 displayed a normal enzymatic activity to catalyze the synthesis of NAD from NMN (Fig. 2*B*). Therefore, we were confident that MBP-tagged Nmnat 1 could be used for the analysis of Nampt enzymatic activity. As presented in Fig. 2*C*, recombinant wild-type Nampt had a higher activity that can catalyze the synthesis of NAD from nicotinamide while two mutant human Nampts showed a very low or nearly no enzymatic activity.

PBEF Augments IL-8 mRNA Level and IL-8 Protein Secretion in A549 Cells via Its Nonenzymatic Activity—To examine whether PBEF regulates IL-8 expression via its Nampt activity, we transfected A549 cells with pCAGGS-hPBEF (H247E, HE) and pCAGGS-hPBEF (H247A, HA), which overexpress two human PBEF mutants, as well as positive and negative control constructs to assay their effects on IL-8 mRNA level and IL-8 protein secretion. As shown in Fig. 3*A*, the cell lysate from A549 cells transiently transfected with two pCAGGS-PBEF mutants, pCAGGS vector only and pCAGGS-eGFP, had very low Nampt activities, whereas those cells with wild-type pCAGGS-hPBEF showed a higher Nampt enzymatic activity. However, overexpression of two mutant PBEFs similarly and significantly augmented the IL-8 expression at both mRNA and protein levels as wild-type PBEF (Fig. 3, *B* and *C*). The controls, pCAGGS vector only and pCAGGS-eGFP, had no effect. Expression of a housekeeping gene, β-actin, was not affected by any treatment. These results indicate that PBEF can augment IL-8 expression in A549 cells via its nonenzymatic activity.

PBEF Induces IL-8 Expression in A549 Cells Not via an Increase of Its mRNA Stability—To examine whether the increase of IL-8 gene expression by PBEF in A549 cells is due to an increase of its gene transcription or mRNA stability, we carried out a transcriptional blockade study. Actinomycin D (5 μg/ml), an RNA polymerase II inhibitor, was added to A549 cell culture 24 h after transfections with different constructs. The IL-8 mRNA level was analyzed by RT-PCR. As presented in Fig. 4 (*A* and *B*), the rate of IL-8 mRNA decay in all five groups was about the same. β-Actin expression was again used as a housekeeping gene control. It should be pointed out that only one-third of the normal control total RNA amount from wild-type and mutant PBEF groups was used for the RT-PCR analysis. These data suggest that PBEF induces IL-8 expression in A549 cells not due to an increase of its mRNA stability.

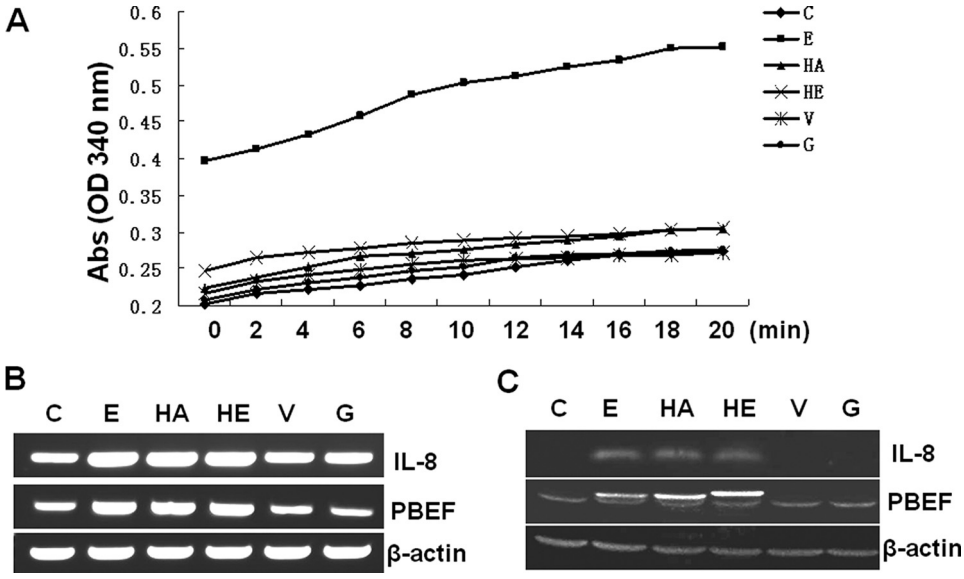

FIGURE 3. **Analysis of the IL-8 expression and Nampt enzymatic activity in A549 cells transfected with pCAGGS-hPBEF, -mutant PBEFs, pCAGGS vector and pCAGGS-eGFP.** *A*, a representative gel image of IL-8, PBEF, and β-actin mRNA detections. A549 cells were transfected with the reagent only (control, *C*), pCAGGS-hPBEF (*E*), pCAGGS-mPBEF (H247A, HA), pCAGGS-mPBEF (H247E, HE), pCAGGS vector (*V*), and pCAGGS-eGFP (*G*) for 48 h. Total cell RNA was reverse-transcribed, amplified by PCR using the gene-specific primers (see Table 1), separated by 1.5% agarose electrophoresis, and visualized with ethidium bromide. β-Actin was used as a housekeeping gene control. The displayed image is one of three replicate samples and is typical of those obtained. *B*, a representative Western blotting image of IL-8 and PBEF protein detections. A549 cells were transfected as described in *A*. Secreted IL-8 and cell lysate PBEF and β-actin proteins were immunodetected. The displayed image is one of three replicate samples and is typical of those obtained. *C*, time course of the Nampt enzymatic activity in transfected A549 cells. A549 cells were transfected as described in *A*. 40 μg of cell lysates of each transfected A549 cells was employed to assay the Nampt enzymatic activity.

PBEF Increases AP-1 Binding to the IL-8 Promoter to Activate Transcription in A549 Cells—The result in Fig. 4 suggests that overexpression of PBEF augments IL-8 expression at the transcriptional level. To confirm this notion and to define the regions of the IL-8 promoter involved in PBEF-mediated effect, we prepared several luciferase reporter plasmids containing serial deletions of the proximal region of the IL-8 gene promoter as shown in Fig. 5*A*. A549 cells were transiently co-transfected with pCAGGS-hPBEF, -mutant PBEF vectors (H247E and H247A), -eGFP vector, pCAGGS vector, and luciferase reporter plasmids containing different IL-8 gene promoter deletants. Luciferase activities were measured after 48 h of the transient transfections. As shown in Fig. 5*B*, PBEF and mutant PBEFs induced luciferase activity of the −162/IL-8 by 4- to 6-fold compared with control (PBEF *versus* control: 71.07 ± 7.98 *versus* 17.35 ± 4.29,

TABLE 1
Primers and products sizes

Product	5′ Primers	3′ Primers	Size (bp)	Accession no.
PBEF	AAGCTTTTTAGGGCCCTTTG	AGGCCATGTTTTATTTGCTGACAAA	319	NM_005746
IL-8	ATGACTTCCAAGCTGGCCGT	CCTCTTCAAAAACTTCTCCACACC	297	NM_000584
β-Actin	CAAACATGATCTGGGTCATCTTCTC	GCTCGTCGTCGACAACGGCTC	487	NM_001101
IL-16	TAGTGCCAAGGTACAAACAGGTG	GGGTCTCAAACTCAGATGCCTAT	280	NM_172217
CCR3	AGCCCCTAAAGCAGCACTAA	TGATAGCTTAGGCGTCACCA	228	NM_001837

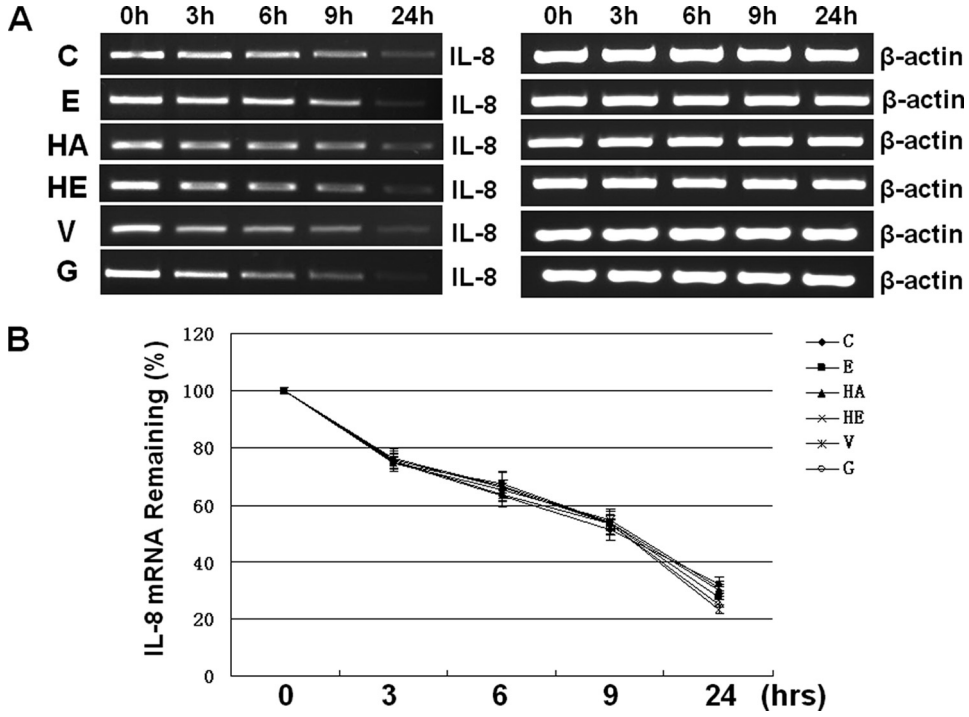

FIGURE 4. **Determination of IL-8 mRNA stability in transfected A549 cells by RT-PCR.** A549 cells were transfected with the reagent only (control, *C*), pCAGGS-hPBEF (*E*), pCAGGS-mPBEF (H247A, *HA*), pCAGGS-mPBEF (H247E, *HE*), pCAGGS vector (*V*), and pCAGGS-eGFP (*G*) for 24 h. The transfected cells were further incubated in the presence of 5 μg/ml actinomycin D, and then total RNA was isolated at 0, 3, 6, 9, and 24 h. RT-PCR was used to semi-quantitate IL-8 mRNA as described in Fig. 3*A*. *A*, representative RT-PCR gel image of IL-8 and β-actin mRNA detections. *B*, the percentage of IL-8 mRNA remaining from transfected A549 cells treated with 5 μg/ml actinomycin D at different time points as indicated.

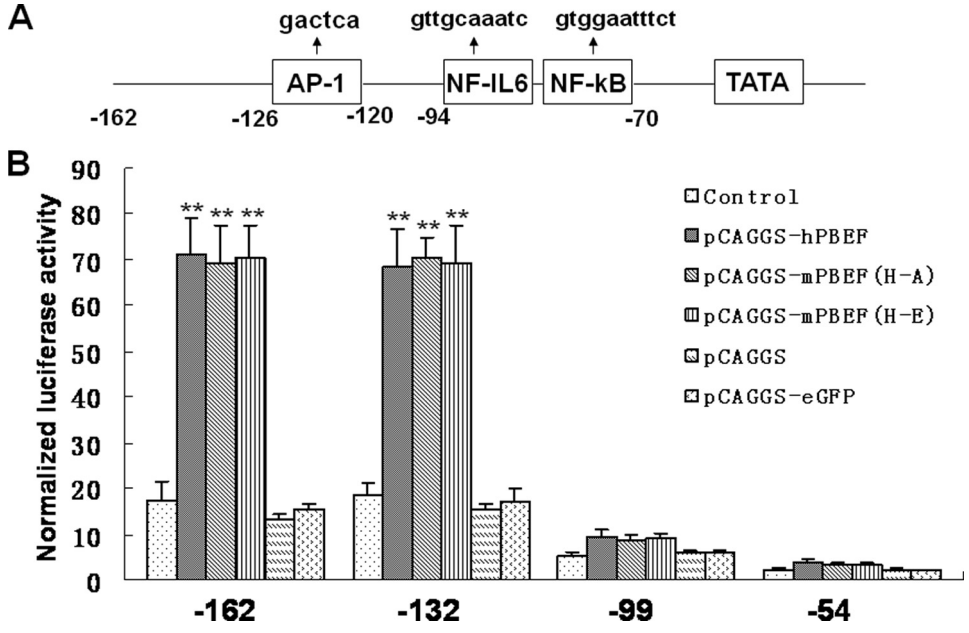

FIGURE 5. **Report gene assay for the effects of PBEF and mutant PBEFs on 5′ deletants in the IL-8 gene promoter.** A549 cells were transiently transfected with the reagent (*C*), pCAGGS-hPBEF (*E*), pCAGGS-mPBEF (H247A, HA), pCAGGS-mPBEF (H247E, HE), pCAGGS vector (*V*), pCAGGS-eGFP (*G*), and different 5′ deletant plasmids of IL-8 gene promoter. For each sample, firefly luciferase activity is presented as normalized to *Renilla* luciferase activity. Results from each group are presented as mean ± S.D. of three samples from two separate experiments. *A*, the schematic representation of the 5′ flanking region of IL-8 gene. *B*, the normalized luciferase activity of PBEF and mutant PBEFs on 5′ deletions in the IL-8 promoter sequence.

$n = 3$, $p < 0.01$; H247A *versus* control: 69.06 ± 8.46 *versus* 17.35 ± 4.29, $n = 3$, $p < 0.01$; H247E *versus* control: 70.49 ± 7.04 *versus* 17.35 ± 4.29, $n = 3$, $p < 0.01$), while there was no significant difference between pCAGGS vector or pCAGGS-eGFP and the control groups (pCAGGS vector *versus* control: 14.28 ± 1.11 *versus* 17.35 ± 4.29, $n = 3$, NS; pCAGGS-eGFP *versus* control: 15.33 ± 1.18 *versus* 17.35 ± 4.29, $n = 3$, NS). A 5′ deletion to −132 bp did not affect the response of IL-8 promoter to wild-type and mutant PBEF overexpression (PBEF *versus* control: 68.45 ± 8.41 *versus* 18.68 ± 2.56, $n = 3$, $p < 0.01$; H247A *versus* control: 70.19 ± 4.76 *versus* 18.68 ± 2.56, $n = 3$, $p < 0.01$; H247E *versus* control: 69.27 ± 8.38 *versus* 18.68 ± 2.56, $n = 3$, $p < 0.01$), there was also no significant difference between pCAGGS vector, pCAGGS-eGFP, and the control groups (pCAGGS vector *versus* control: 16.09 ± 1.12 *versus* 18.68 ± 2.56, $n = 3$, NS; pCAGGS-eGFP *versus* control: 17.08 ± 2.88 *versus* 18.68 ± 2.56, $n = 3$, NS). However, further deletion to −99 nucleotides and −54 nucleotides reduced the basal activity of the promoter by 2- to 3-fold and almost completely abolished the wild-type PBEF and mutant PBEF-induced luciferase activity (−132/IL-8 *versus* −99/IL-8 in PBEF group : 68.45 ± 8.41 *versus* 9.57 ± 1.47, $n = 3$, $p < 0.01$; −132/IL-8 *versus* −99/IL-8 in H247A group: 70.19 ± 4.76 *versus* 8.66 ± 1.30, $n = 3$, $p < 0.01$; −132/IL-8 *versus* −99/IL-8 in H247E group: 69.27 ± 8.38 *versus* 9.18 ± 1.14, $n = 3$, $p < 0.01$), indicating that the sequence between −132 and −99 nucleotides is critically involved in the wild-type PBEF- or mutant PBEFs-mediated IL-8 gene activation. This region contains a functional AP-1 binding site. These data suggest that AP-1 is required in the wild-type PBEF- or mutant PBEFs-augmented IL-8 gene expression.

To corroborate the above finding, we performed EMSA to determine whether PBEF and mutant PBEFs overexpression engendered an increase in the abundance of transcription factor AP-1 that recognizes this region of the IL-8 promoter. As shown in Fig. 6, PBEF and mutant PBEFs overexpression significantly increased the binding of AP-1 compared with those in Control, pCAGGS

vector only, and pCAGGS-eGFP groups. The binding to AP-1 site was sequence-specific, because it was competed out by the addition of 200-fold unlabeled cognate oligonucleotide. In addition, Fig. 7 showed that 48 h after co-transfection with GFP Cignal reporter under the control of AP-1 transcription factor, similar numbers of GFP-positive cells were observed in pCAGGS-hPBEF, pCAGGS-H247A, and pCAGGS-H247E groups, further validating the AP-1-dependent regulation of gene expression by PBEF in a non-enzymatic way.

p38 MAPK Pathway and the JNK Pathway Are Involved in the PBEF-augmented IL-8 Transcription in A549 Cells—To investigate signal transduction pathways in the PBEF-augmented IL-8 transcription, we cultured transfected A549 cells in the absence or presence of the p38 MAPK pathway inhibitor, SB203580, and the JNK pathway inhibitor, JNK inhibitor 1, or their combinations and analyzed their reporter gene activities. As presented in Fig. 8, PBEF and mutant PBEF overexpression induction of luciferase activity of IL-8 promoter were significantly attenuated by the treatment of SB203580 (PBEF group: 48.78 ± 1.81 *versus* 60.52 ± 5.36, $n = 3$, $p < 0.05$; H247A group: 47.41 ± 5.33 *versus* 62.80 ± 4.58, $n = 3$, $p < 0.05$; H247E group: 46.38 ± 4.06 *versus* 59.63 ± 5.44, $n = 3$, $p < 0.05$). Similarly, JNK inhibitor 1 was also inhibited luciferase activity of IL-8 promote stimulated by PBEF and mutant PBEF overexpression, although the inhibition was not significant in PBEF and H247E overexpression groups (PBEF group: 51.33 ± 4.49 *versus* 60.52 ± 5.36, $n = 3$, NS; H247A group: 49.74 ± 5.63 *versus* 62.80 ± 4.58, $n = 3$, $p < 0.05$; H247E group: 51.40 ± 4.78 *versus* 59.63 ± 5.44, $n = 3$, NS). When a combination of these two inhibitors was used, the inhibition became more significant (PBEF group: 40.50 ± 4.82 *versus* 60.52 ± 5.36, $n = 3$, $p < 0.01$; H247A group: 42.93 ± 2.56 *versus* 62.80 ± 4.58, $n = 3$, $p < 0.01$; H247E group: 41.23 ± 3.59 *versus* 59.63 ± 5.44, $n = 3$, $p < 0.01$). These data suggest that wild-type PBEF- or mutant PBEF-mediated augmentation of IL-8 expression is in part via the p38 MAPK pathway and the JNK pathway.

Two Mutant PBEFs Also Stimulate the Expression of Two Other Inflammatory Cytokines in A549 Cells—To determine whether two mutant PBEFs can also augment the expression of other inflammatory cytokines, we quantitated mRNA levels of IL-16 and CCR3 in A549 cells transfected with the same groups of constructs. As presented in Fig. 9, overexpression of mutant PBEFs similarly and significantly enhanced the expression of IL-16 and CCR3 gene at the mRNA level in A549 cells as the wild-type PBEF, whereas there was no significant difference between pCAGGS-eGFP or pCAGGS vector and the control groups. These data suggest that PBEF could augment the expression of inflammatory cytokines IL-16 and CCR3 in A549 cells via its nonenzymatic activity.

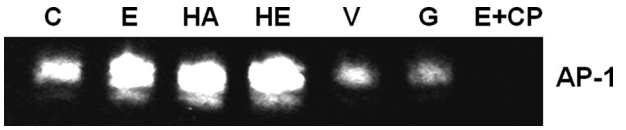

FIGURE 6. **EMSA of IL-8 AP-1-binding complexes from transfected A549 cells.** *A*, A549 cells were transfected with the reagent (*C*), pCAGGS-hPBEF (*E*), pCAGGS-mPBEF(H247A, HA), pCAGGS-mPBEF (H247E, HE), pCAGGS vector (*V*), and pCAGGS-eGFP (*G*) for 48 h. 10 μg of nuclear extract protein in each sample was incubated with biotin-labeled AP-1 oligonucleotide. The bindings were visualized by a chemiluminescence. *E+CP*, nuclear extract from A549 cells transfected with pCAGGS-hPBEF (*E*) was used to bind to biotin-labeled AP-1 probe in the presence of their unlabeled 200-fold excess probe (cold probe, *CP*).

DISCUSSION

Although accumulating evidence indicate that PBEF is a pleiotropic protein involved in a number of physiological processes from bacteria to human and its dysregulation has been implicated in the susceptibility and pathogenesis of acute lung injury, sepsis, cancer, diabetes, atherosclerosis, and other diseases (19), only the Nampt enzymatic function of PBEF has been well substantiated (10, 11). Whether all functions of PBEF depend on its Nampt enzymatic activity remains to be fully elucidated. The data from this study provided the first evidence that PBEF can regulate the expression of inflammatory cytokines such as IL-8, IL-16, and CCR3 via its nonenzymatic function. In addition, our data revealed a new insight into the signal transduction of PBEF-mediated augmentation of inflammatory cytokine expression, which is AP-1-dependent, in part via p38 MAP kinase pathway and JNK pathway.

PBEF cDNA was first cloned by Samal and his colleagues in 1994 and initially named pre-B-cell colony-enhancing factor (20). That PBEF could be a nicotinamide phosphoribosyltransferase was first shown by Martin *et al.* (11) in 2001 in bacteria. Rongvaux *et al.* (10) verified that, similarly to its microbial counterpart, the mammalian PBEF is a Nampt, catalyzing the condensation of nicotinamide with 5-phos-

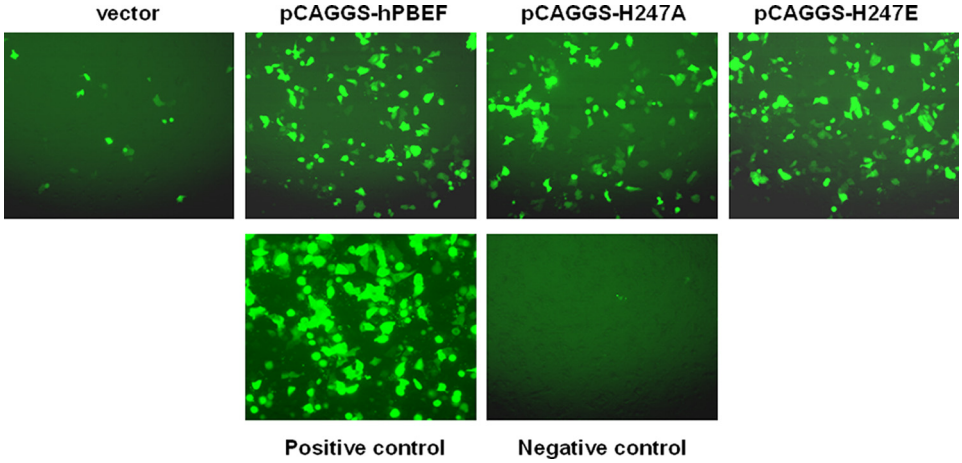

FIGURE 7. **The effects of pCAGGS, -hPBEF, -H247A, and -H247E on AP-1-dependent GFP reporter activity.** The A549 cells were separately co-transfected with pCAGGS+Cignal reporter, pCAGGS-hPBEF+Cignal reporter, pCAGGS-H247A+Cignal reporter, pCAGGS-H247E+Cignal reporter, and Cignal-positive or -negative control. After 48-h transfection, the samples were directly analyzed under a fluorescence microscope using an excitation wavelength at 488 nm and detection at 530 nm, ×100.

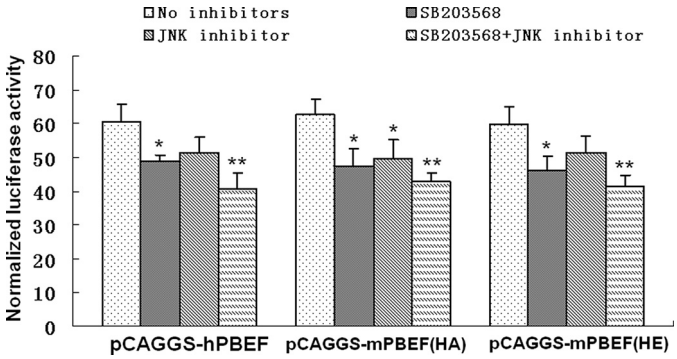

FIGURE 8. **Reporter gene assays for the effects of PBEF and mutant PBEFs on 5′ deletants in the IL-8 gene promoter in the presence of inhibitors.** A549 cells were transiently transfected pCAGGS-hPBEF (*E*), pCAGGS-PBEF H247A (*HA*), pCAGGS-PBEF H247E (*HE*), and pGL-3-IL-8–132 for 48 h before the treatment without or with 3 μM SB203580 and 5 μM JNK inhibitor or combination for 6 h. For each sample, firefly luciferase activity is presented as normalized to *Renilla* luciferase activity. Results from each group are presented as mean ± S.D. of three samples from two separate experiments. *, $p < 0.05$ and **, $p < 0.01$.

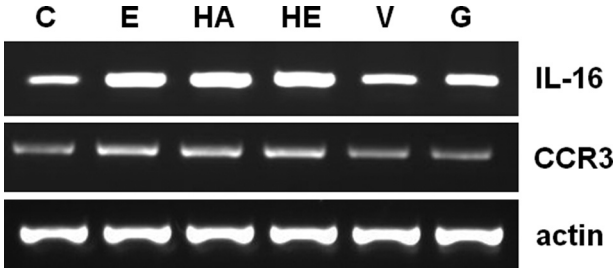

FIGURE 9. **RT-PCR analyses of IL-16, CCR3, and β-actin mRNA levels in A549 cells.** A549 cells were transfected with the reagent (*C*), pCAGGS-hPBEF (*E*), pCAGGS-mPBEF (H247A, *HA*), pCAGGS-PBEF (H247E, *HE*), pCAGGS vector (*V*), and pCAGGS-eGFP (*G*) for 48 h. RT-PCR semi-quantitation of IL-16, CCR3, and β-actin mRNA was carried out as described with IL-8 in Fig. 3, except that IL-16- and CCR3-specific primers were used as listed in Table 1.

phoribosyl 1-pyrophosphate to yield nicotinamide mononucleotide, an intermediate in the biosynthesis of NAD (Fig. 1). A study by Revollo *et al.* (12) demonstrated that Nampt is the rate-limiting component in a salvage pathway of the mammalian NAD biosynthesis. Hasmann and Schemainda (22) identified an inhibitor to PBEF, FK866, which inhibits its Nampt activity to reduce the cellular NAD synthesis to trigger cell apoptosis in HepG2 human liver carcinoma cells. Thus FK866 has been applied in clinical trials to treat cancer patients (23). van der Veer *et al.* (24) proved that it is due to the enhanced Nampt activity of PBEF that cellular lifespan of human primary SMCs, human clonal SMCs, and fibroblasts derived from a patient with Hutchinson-Gilford progeria syndrome can be lengthened. Recent work by Revollo *et al.* (25) revealed that Nampt regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. In short, it has been well documented that PBEF is a nicotinamide phosphoribosyltransferase, and its enzymatic activity is attributed for its various functions. However, Li *et al.* (26) recently reported that extracellular PBEF protected macrophages from endoplasmic reticulum stress-induced apoptosis by activating an IL-6/STAT3 signaling pathway via a nonenzymatic mechanism. Our study here provided seven lines of evidence to support PBEF augmenting expressions of inflammatory cytokines IL-8, IL-16, and CCR3 via its nonenzymatic mechanisms. Firstly, two human PBEF mutants similarly increased IL-8 mRNA level as wild-type PBEF (Fig. 3). Secondly, we found that this effect was not due to the increased IL-8 mRNA stability (Fig. 5), suggesting that a transcriptional mechanism may be operative. Both mutants behaved like the wild-type PBEF. Thirdly, augmentation of IL-8 expression by both mutants and the wild-type PBEF is AP-1-dependent, because the reporter gene assay indicated that, without a functional AP-1 binding site, theses effects were abrogated (Fig. 5). Fourth, gel shifting assays indicated that overexpression of two PBEF mutants similarly increased the binding of AP-1 to the IL-8 gene promoter as the wild-type PBEF (Fig. 6). Fifth, an AP-1-driven GFP Cignal reporter assay revealed a similar number of GFP-positive cells in pCAGGS-hPBEF-, pCAGGS-H247A-, and pCAGGS-H247E-transfected A549 cells (Fig. 7). Sixth, SB203580, a p38 MAPK pathway inhibitor, and JNK inhibitor 1 attenuated the augmentative effects of both two mutants and the wild-type PBEF on IL-8 transcription similarly (Fig. 8). Seventh, both PBEF mutants similarly stimulated the expression of two other inflammatory cytokines: IL-16 and CCR3 as the wild-type PBEF. It indicates that this is a general mechanism underlying PBEF's role in the regulation of inflammatory cytokines, not restricted to the IL-8 gene alone. PBEF is joined in a unique protein club in which protein members not only function as enzymes but also display other functions unrelated to their enzymatic activities. Recently, Xu *et al.* (27) found that myosin II regulatory light-chain kinase had a myosin II regulatory light-chain phosphorylation-independent function in complex with c-Src and Pyk2. This nonenzymatic function of myosin II regulatory light-chain kinase was essential for neutrophil transmigration during sepsis-induced lung injury. The results by Zhai *et al.* (28) implicate nicotinamide mononucleotide adenylyltransferase (NMNAT), which can catalyze the NAD formation using nicotinamide mononucleotide as a substrate, as a stress-response protein that acts as a chaperone for neuronal maintenance and protection. This chaperone activity of NMNAT is independent of its enzymatic activity, *i.e.* the NAD synthesis activity.

ALI/ARDS is characterized by an acute inflammatory process in the airspaces and lung parenchyma. Evidence from several clinical studies indicates that a complex network of inflammatory cytokines play a major role in mediating, amplifying, and perpetuating the lung injury process (29). The pro-inflammatory cytokines IL-1β, tumor necrosis factor-α, and IL-8 have been identified in bronchoalveolar lavage fluids from ARDS patients. IL-8 has been identified as one of biomarkers of ALI/ARDS mortality (30). Studies of models of acute inflammation have established IL-8 as a key mediator in neutrophil-mediated acute inflammation (31). Wang *et al.* reported that IL-16 was a critical factor in the development of inflammation-mediated renal injury and may be a therapeutic target for prevention of ischemia-reperfusion injury of the kidney (32). Animal studies suggest that CCR3 is a prominent mediator of allergic responses and that antagonizing the receptor will lead to a reduction in airway inflammation (33). Our previous study on animal models of ALI and human patients with ARDS identified PBEF as a biochemical and genetic marker in ALI (5). Our subsequent studies found that PBEF could modulate IL-1β and the role of tumor necrosis factor-α in their inductions of other

inflammatory cytokines as well as directly regulate the expression of IL-8, IL-16, and CCR3. This study provided a new insight into the signal transduction process in PBEF-mediated augmentation of inflammatory cytokine expression, *i.e.* an AP-1-dependent nonenzymatic mechanism in part via p38 MAPK and JNK pathways. These data lend further support that PBEF might be an important signal transducer or initiator in the inflammation process to regulate the synthesis of IL-8 or other inflammatory cytokines, and PBEF could play a critical role as an inflammatory factor during the pathogenesis of ALI. PBEF expression is up-regulated in a variety of other acute and chronic inflammatory diseases such as sepsis (34), rheumatoid arthritis (35), inflammatory bowel disease (36), and myocardial infarction (21). The mechanism revealed in this study may be generally applicable to explain some novel roles of the dysregulated PBEF gene in the pathophysiology of those inflammatory diseases or processes.

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Regulation of Inflammatory Cytokine Expression in Pulmonary Epithelial Cells by Pre-B-cell Colony-enhancing Factor via a Nonenzymatic and AP-1-dependent Mechanism

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