Pre–B-Cell Colony-enhancing Factor as a Potential Novel Biomarker in Acute Lung Injury

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Although the pathogenic and genetic basis of acute lung injury (ALI) remains incompletely understood, the identification of novel ALI biomarkers holds promise for unique insights. Expression profiling in animal models of ALI (canine and murine) and human ALI detected significant expression of pre–B-cell colony-enhancing factor (PBEF), a gene not previously associated with lung pathophysiology. These results were validated by real-time polymerase chain reaction and immunohistochemistry studies, with PBEF protein levels significantly increased in both bronchoalveolar lavage fluid and serum of ALI models and in cytokine- or cyclic stretch–activated lung microvascular endothelium. We genotyped two PBEF single-nucleotide polymorphisms (SNPs) in a well characterized sample of white patients with sepsis-associated ALI, patients with severe sepsis, and healthy subjects and observed that carriers of the haplotype GC from SNPs T-1001G and C-1543T had a 7.7-fold higher risk of ALI (95% confidence interval 3.01–19.75, $p < 0.001$). The T variant from the SNP C-1543T resulted in a significant decrease in the transcription rate (1.8-fold; $p < 0.01$) by the reporter gene assay. Together, these results strongly indicate that PBEF is a potential novel biomarker in ALI and demonstrate the successful application of robust genomic technologies in the identification of candidate genes in complex lung disease.

Keywords: gene expression; lung disease; single nucleotide polymorphism

Acute lung injury (ALI) is a refractory lung disease characterized by severe hypoxemia and unacceptably high mortality (30–50%) (1–3). The pathogenetic basis of ALI is incompletely understood; however, ALI survival appears to be influenced by the stress generated by mechanical ventilation (4) and by sepsis-associated factors, which initiate and amplify the inflammatory response in ALI (5). Emerging evidence also suggests that genetic factors are associated with susceptibility to ALI (6). Both a nonsynonymous single nucleotide polymorphism (SNP) in the surfactant protein B gene (7) and an intronic SNP in the angiotensin-converting enzyme gene (8) were reported to contribute to the susceptibility and outcome of patients with ALI. Associations with the susceptibility and mortality of septic shock, a frequent inciting cause of ALI, have been demonstrated with promoter polymorphisms in the tumor necrosis factor-α (TNF-α) (9) and CD14 genes (10). Additional studies are clearly needed to identify novel biochemical and genetic markers that may provide unique insights into pathogenic mechanisms and the genetic basis of ALI (11).

We used a high-throughput functional genomic approach, with extensive microarray-based lung gene expression profiling in canine, murine, and human ALI, to identify novel ALI candidate genes. In these studies, we observed significant increases in the expression of pre–B-cell colony-enhancing factor (PBEF), a relatively obscure cytokine with only 22 PubMed citations to date. PBEF was named after its effect on the maturation of B-cell precursors (12). Its expression in a human amniotic epithelial cell line *in vitro* was upregulated by the treatment of either mechanical force (13) or inflammatory cytokines (14). Recently, Jia and colleagues (15) reported that PBEF was significantly expressed in peripheral neutrophils of patients with sepsis, a frequent cause of ALI, and PBEF inhibited the neutrophil apoptosis. Despite these observations in nonlung tissues, the relevance of PBEF to the lung pathophysiology is unknown. The present study reports the first findings of PBEF expressed in lung tissues and overexpressed in ALI and the association of the PBEF with susceptibility to ALI. We postulated that PBEF might be a dual sensor to both the mechanical force and inflammatory stimuli to be involved in the pathogenesis of ALI. Our results from animal models and human patients with ALI and *in vitro* cell culture experiments strongly indicate that PBEF is a potential novel ALI biomarker and confirm the utility of genomic approaches to generate important insights into complex lung disease. A portion of the relevant results of these studies has been previously reported in abstract forms (16, 17).

METHODS

Animal Models of ALI

All animal models were institutionally approved. Canine Model 1 used unilateral saline lavage–induced lung injury (18), with the injured left and uninjured right lungs independently mechanically ventilated for 6 hours (8 ml/kg, 0 positive end-expiratory pressure, and 10 ml/kg and positive end-expiratory pressure 5 cm H_2O, respectively). Canine Model 2 used intrabronchially delivered endotoxin (LPS) with high V_T mechanical ventilation (6 hours, 17 ml/kg), as reported (19). Control animals received endobronchial saline with identical ventilation strategies. Lung tissues were processed for microarray analysis, and bronchoalveolar lavage (BAL)/serum was collected for protein analyses.

Two murine ALI models were described: (*1*) 24-hour spontaneous ventilation postintratracheal LPS and (*2*) 2-hour 17-ml/kg mechanical ventilation (19, 20). Control groups were spontaneously ventilated. Lung tissue and BAL were collected for microarray and protein analyses.

Human Acute Lung Injury BAL Samples

Human protocols were approved by the institutional review boards. Human BAL (n = 3 each) was obtained from patients with ALI (21, 22) and healthy control subjects.

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Gene Expression Profiling and Validation

The Affymetrix GeneChip (Affymetrix, Inc., Santa Clara, CA) microarray system was used as described previously (23). Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), Western blot, and real-time PCR were used to validate PBEF expression in animal lung tissues and human BAL, respectively.

Localization of PBEF Expression in Canine Lung

To evaluate the spatial localization of PBEF expression, we performed triple immunohistochemical staining in canine lung tissue (24, 25) using an anticanine PBEF polyclonal antibody (26) and antisera raised against factor VIII (to visualize vascular endothelium), neutrophils, and pro–surfactant protein C (alveolar epithelium). In addition, 4′6 diamidino-2-phenylinodole was used to visualize cell nuclei.

Western Blotting Analysis of PBEF Protein

The total protein content in each sample was quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). PBEF proteins were assessed by Western blotting with densitometric quantification.

Cytokine- or Mechanical Stress–induced Human Endothelial Cell Activation

Human lung microvascular endothelial cells (HMVEC-L) were exposed to cytokines for 4 hours or 18% cyclic stretch or static conditions for 48 hours plus or minus interleukin 1β (IL-1β) as previously described (27). PBEF content in cell lysates was analyzed as previously described.

Genotyping of PBEF Promoter SNPs

Leukocyte DNA and serum samples (n = 8 each) from subjects with sepsis-associated ALI, subjects with sepsis alone, and healthy control subjects were obtained (Johns Hopkins University, Medical College of Wisconsin) according to consensus diagnostic criteria (21, 22) with recording of Acute Physiology and Chronic Health Evaluation (APACHE) II scores (28). SNP discovery of the human PBEF gene was performed in 36 subjects (12/group) by direct DNA sequencing. Genotyping of the PBEF SNPs (T-1001G and C-1543T) in white subjects was performed using a restriction-site polymorphism assay and an Assays-by-Design Service SNP genotyping method (Applied Biosystems, Foster City, CA), respectively.

Transient Transfection Assays

A 272-bp fragment (either a T or G variant at the −1001 position) or a 147-bp fragment (either a C or T variant at the −1543 position) of the PBEF gene promoter was subcloned into pGL3 basic vector (Promega, Madison, WI) and transiently transfected into HMVEC-L. After 4-hour transfection, cell lysates were retrieved for luciferase activity determination.

Statistical Analysis

Statistical analyses were performed using SigmaStat (version 3.1, Systat Software, Inc., Point Richmond, CA) and/or Stata (version 8.0, StataCorp LP, College Station, TX).

RESULTS

Animal Models of Lung Injury

Evidence of the development of ALI varied among the different animal models. In the canine unilateral lavage model, lung injury was evident from the progressive increase in peak inspiratory pressure in the injured lung, eventually doubling by 6 hours, compared with minimal change in peak inspiratory pressure in the control lung. Because of the presence of the control lung

TABLE 1. PRE–B-CELL COLONY-ENHANCING FACTOR GENE EXPRESSION IN CANINE AND MURINE LUNG AND HUMAN BRONCHOALVEOLAR LAVAGE

Type	N	Control (*Mean* ± *SE*)†	ALI (*Mean* ± *SE*)†	RF‡	p Value
Canine	3*	312 ± 22	1807 ± 505	5.79	< 0.01
Murine	4	186 ± 14	396 ± 74	2.13	< 0.05
Human	3	597 ± 91	2190 ± 716	3.67	0.05

Definition of abbreviations: ALI = acute lung injury; RF = relative fold.

All expression profiling studies were performed on the Affymetrix GeneChip (Affymetrix, Inc.) system.

* Data were derived from 22 arrays (11 from controls, 11 from injured lungs) of 11 pairs of samples of different lung regions (apex, midbase, base in nondependent or dependent regions) from three canines as described in the main text.

† Mean ± SE of signal intensities reported by the Affymetrix MAS 5.0 (Affymetrix, Inc.) after the global normalization.

‡ RF increase in ALI vs. control.

Data were analyzed by the unpaired *t* test using the SigmaStat (version 3.1, SPSS). PBEF expressions in three injured, ventilated canine lungs were compared with three uninjured, ventilated control lungs; PBEF expression in four murine lungs that received intratracheal LPS treatment (24 hr) were compared with four controls; PBEF expression in BAL cells from three patients with ALI were compared with three healthy control subjects.

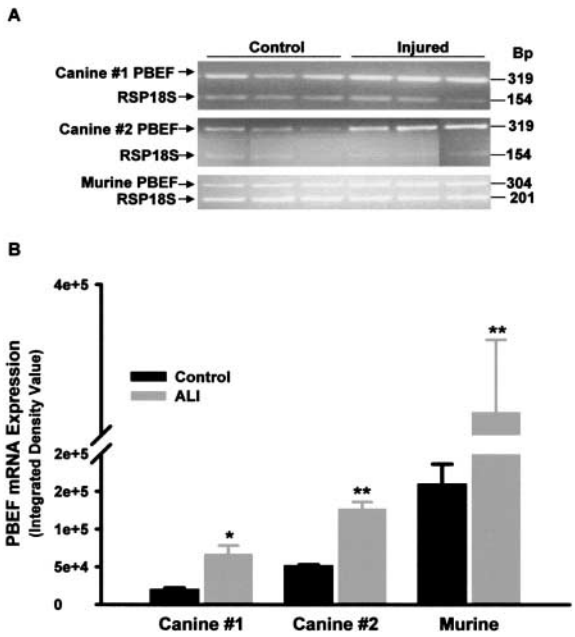

Figure 1. Validation of lung pre–B-cell colony-enhancing factor (PBEF) mRNA levels by semiquantitative duplex reverse transcriptase–polymerase chain reaction. (*A*) Agarose gel image of PBEF cDNA. Total RNA (0.5 μg) of each sample was reverse-transcribed, amplified by both PBEF and RPS18S specific primers, separated by 2% agarose gel electrophoresis, and visualized by ethidium bromide staining. (*B*) Densitometric analysis. Band density on agarose gel images was used as a measure of PBEF cDNA level. The band image was acquired using the Scanjet 7400c (Hewlett-Packard Co., Palo Alto, CA) and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp., San Leandro, CA). PBEF cDNA signal intensity was normalized to that of the internal control, RSP18S. Statistical comparative analysis of integrated density value (mean ± SEM) between control and acute lung injury (ALI) groups was performed using the paired *t* test for Canine Model 1 and an unpaired *t* test for Canine Model 2 and murine. Significantly higher lung PBEF expression was confirmed in the surfactant-depleted Canine 1 ALI model (3.32-fold increase, 67,617 ± 10,643 [ALI] vs. 20,384 ± 1,772 [control]; n = 3, $p < 0.05$), in the LPS-treated Canine 2 ALI model (2.46-fold increase, 127,630 ± 8,584 [ALI] vs. 51,933 ± 857 [control]; n = 3, $p < 0.01$), and in the LPS-murine ALI models (2.23-fold increase, 357,801 ± 23,878 [ALI] vs. 160,167 ± 26,104 [control]; n = 6, $p < 0.01$). *$p < 0.05$; **$p < 0.01$.

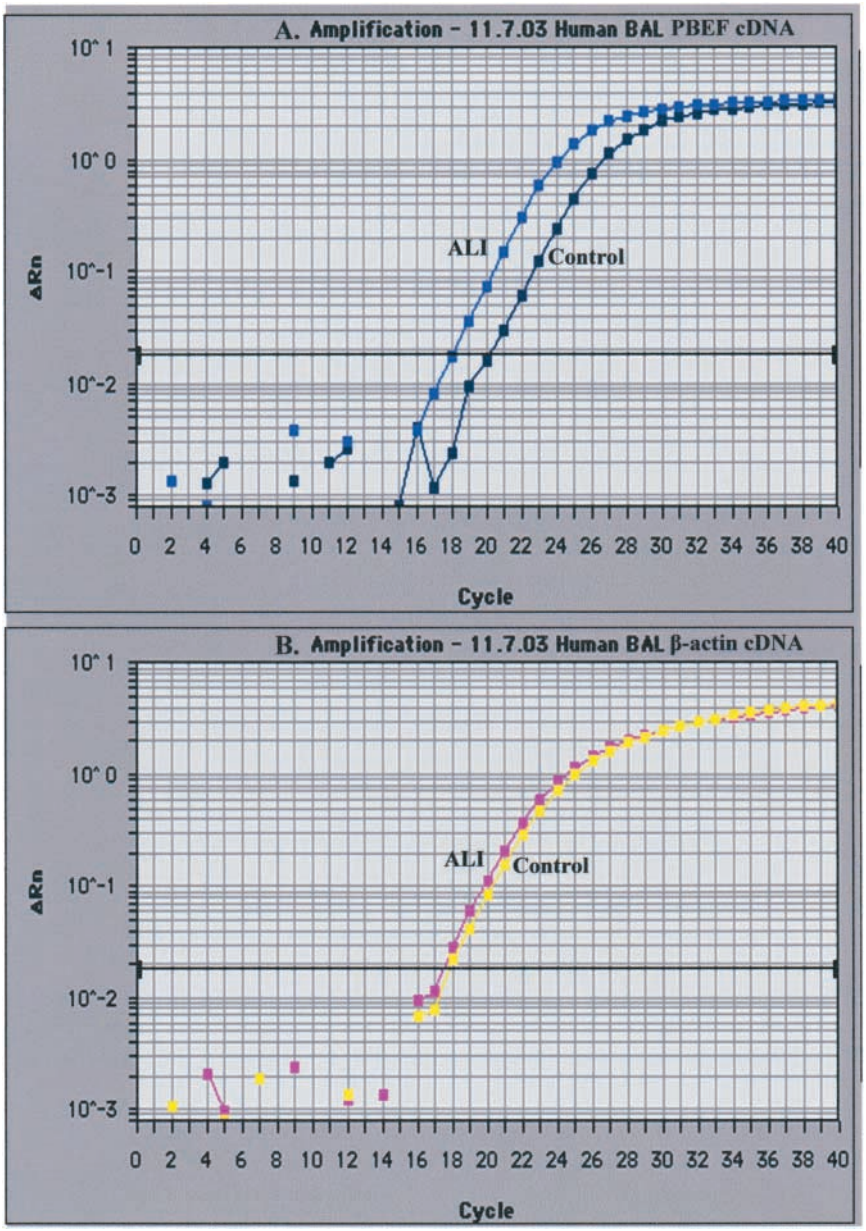

Figure 2. Representative real-time polymerase chain reaction (PCR) amplification plots comparing human bronchoalveolar lavage (BAL) PBEF (*A*) and β-actin (*B*) mRNA expression in patients with ALI and control subjects. Total RNA (0.5 μg) of each sample was reverse-transcribed and 10% of the cDNA product analyzed by real-time PCR using Assays-on-Demand gene expression products (Hs00237184_m1 for PBEF, Hs99999903_m1 for β-actin) and TaqMan Universal PCR Master Mix, No AmpErase UNG, on the ABI Prism 7700 sequence detection system (TaqMan). β-Actin was used as a housekeeping gene control. Each sample was run in five technical replicates. Relative quantitation using the comparative $\Delta\Delta C_T$ method was performed after demonstrating approximately equal efficiency between PBEF and β-actin mRNA assays. Significantly higher PBEF mRNA levels were confirmed in BAL cells from patients with ALI (4.83-fold increase; $n = 3$, $p < 0.001$) relative to their controls.

and the use of 100% oxygen, blood gases did not change substantially and Pa_{O_2} remained above 400 mm Hg even after unilateral injury. In one of these animals, we performed computed tomography imaging at 5 hours, which confirmed the involvement of the entire left lung in an injury pattern of dependent flooding and collapse and increased interstitial density throughout compared with the normal appearance and density of the control lung. In the canine LPS injury model, there were dramatic increases in shunt fraction, peak inspiratory pressure, and pulmonary artery pressure, and a sharp decrease in Pa_{O_2}/FI_{O_2} ratio beginning 60 minutes after LPS instillation and sustained out to 8 hours (19). BAL protein was markedly elevated. Computed tomgraphy studies revealed dependent volume loss, increased vertical gradients of lung density, and overall increased lung tissue volumes. BAL cell counts were not performed. In the murine LPS and high-V_T models, injury was documented by quantifying alveolar protein leak with Evan's blue extravasation. The canine LPS and murine LPS and high-V_T injury model findings have recently been published (19, 20).

Increased PBEF Expression in ALI: Microarray Studies

The first experiments in which PBEF was identified assessed lung gene expression in the canine unilateral lavage model of ALI (three animals, from which 22 lung tissue samples, including 11 injured and 11 uninjured, were obtained) with canine RNA cross-hybridized to individual human HG-U133A chips. Uniformly, the gene exhibiting the highest level of expression (5.79-fold increase; $n = 3$, $p < 0.01$) was PBEF (Table 1). Because this was the first demonstration of PBEF expression in lung tissues, we then evaluated PBEF gene expression in other ongoing models of lung injury for which microarray data were available. In the murine model of ALI induced by intratracheal LPS treatment (20), PBEF gene expression was unchanged in untreated control mice but significantly increased in mice 24 hours

after receiving intratracheal LPS treatment (2.13-fold increase; $n = 4$, $p < 0.05$). We further compared gene expression profiles in cells retrieved by bronchoalveolar lavage from patients with ALI and healthy control subjects. PBEF expression was significantly increased in human ALI (3.67-fold increase; $n = 3$, $p = 0.05$). These results are summarized in Table 1.

PBEF Expression in Lung Tissues: Validation Studies

To validate canine lung PBEF gene expression, we cloned the full-length canine PBEF cDNA (1,476-bp open reading frame encoding 491 amino acids) from canine lung tissues using RT-PCR based on the human PBEF sequence (26). On the basis of the cloned canine and published murine and human PBEF cDNA sequences, specific primers were next designed for either semiquantitative RT-PCR or real-time PCR. Consistent with the microarray results, significantly higher lung PBEF expression was observed in the surfactant-depleted canine ALI model (3.32-fold increase; $n = 3$, $p < 0.05$), in the LPS-murine ALI model (2.23-fold increase; $n = 6$, $p < 0.01$; Figure 1), and in BAL cells from human patients with ALI (4.83-fold increase; $n = 3$, $p < 0.001$) relative to controls (Figure 2). We further examined changes in PBEF gene expression using RT-PCR in lung tissue samples from the intratracheal LPS-treated canine ALI model versus intratracheal saline controls. There was a significant increase in PBEF expression in the LPS-injured canine lungs (2.46-fold increase; $n = 3$, $p < 0.01$; Figure 1). Western blot results also confirmed the increased PBEF protein expression in Canine Model 1 ALI lung tissues (2.02-fold increase; $n = 3$, $p < 0.05$; Figure 3). The expression of constitutively expressed genes, such as ribosomal protein S18 (RPS18) or β-actin, was unchanged. These results firmly corroborate the increased PBEF expression in both human and animal models of ALI detected by microarray profiling.

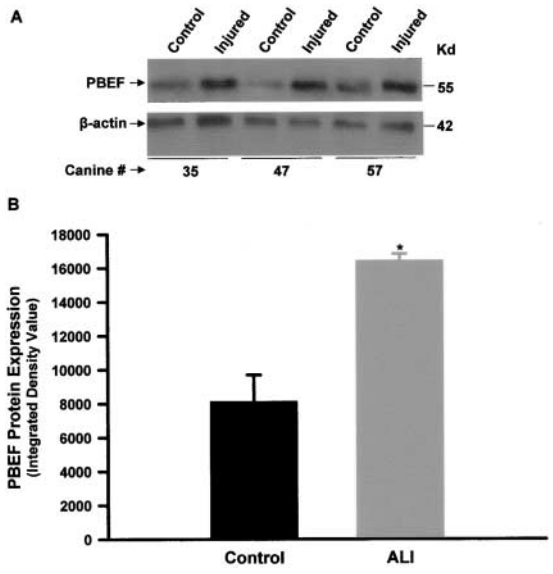

Figure 3. Validation of canine lung PBEF protein levels. (*A*) Western blot image of PBEF protein. Total protein (10 μg) of each sample was separated by 12% SDS-PAGE and immunodetected by Western blot analysis using the anticanine PBEF and β-actin antibodies. (*B*) Densitometric analysis: band density on Western blot images was used as a measure of PBEF protein level. The band image was acquired using the Scanjet 7400c and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp., San Leandro, CA). β-actin was included as a loading control. Statistical comparative analysis of integrated density value (mean ± SEM) between control and ALI groups was performed using the paired *t* test. The increased PBEF protein expression was observed in Canine 1 ALI left lung tissues (2.02-fold increase, 16,456 ± 370 [ALI] vs. 8,147 ± 1,545 [control]; $n = 3$, $p < 0.05$) relative to right control lung tissues. *$p < 0.05$.

Localization of PBEF Expression in Canine Lung

To evaluate spatial localization of PBEF expression, we performed triple immunohistochemical staining in canine lung tissue samples from the unilateral lavage injury model and colocalized PBEF expression in vascular endothelial cells, neutrophils, and type II alveolar epithelial cells. Antibodies to factor VIII (an endothelial marker), neutrophils, and ProSPC (a type II alveolar epithelial cell marker) were obtained commercially. Figure 4 depicts strong canine PBEF expression in the vascular endothelium (Figure 4C) within infiltrating leukocytes in the surfactant-depleted, injured canine lung (Figure 4F) and type II alveolar epithelial cells (Figure 4I), whereas PBEF immunoreactivity was minimally detectable in the uninjured but ventilated control canine lung (Figures 4B, 4E, and 4H, respectively). Figures 4A, 4D, and 4G demonstrated background staining of injured/ventilated lungs with only secondary antibodies. Preimmune serum did not show any significant staining of PBEF in an injured/ventilated canine lung (data not shown). These results indicate that lung vascular endothelial cells, type II alveolar epithelial cells, and infiltrating neutrophils overexpress PBEF in the injured lung.

PBEF Protein Levels in ALI Animal Models and Human Patients

To examine the potential utility of PBEF as an ALI biomarker, we measured BAL and serum levels of PBEF protein in animal models of ALI and humans with ALI using Western blots. PBEF protein levels were significantly increased in canine LPS ALI BAL fluid (2.23-fold increase; $n = 3$, $p < 0.01$) and serum (2.01-fold increase; $n = 3$, $p < 0.01$; Figure 5). PBEF protein levels were increased in BAL fluid obtained from the two murine models: that induced by high V_T ventilation without LPS (2.62-fold increase; $n = 7$, $p < 0.01$) and LPS-mediated lung injury (1.67-fold increase; $n = 3$, $p < 0.05$; Figure 5). Finally, PBEF protein levels in human patients with ALI were significantly increased in BAL (4.96-fold increase; $n = 3$, $p < 0.01$) and serum (2.25-fold increase; $n = 8$, $p < 0.01$) relative to healthy control subjects (Figure 6). These results support PBEF as a potential biomarker in ALI and further validate the microarray-based enhanced PBEF expression in animal and human ALI.

Inflammatory Cytokine-induced/Mechanical Stress–induced PBEF Protein Expression in Human Lung Endothelial Cells

To examine effects of inflammatory stimuli (LPS, TNF-α, and IL-1β) and mechanical stress (18% cyclic stretch) on PBEF protein expression and to confirm our immunohistochemical localization of PBEF immunoreactivity, we next challenged human lung microvascular endothelium with LPS (50 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml), and 18% cyclic stretch in the absence or presence of IL-1β (10 ng/ml), and evaluated PBEF protein content in cellular lysates by Western blotting and densitometric quantification. Each cytokine produced significantly increased PBEF protein expression (2.2- to 4.2-fold increases; Figure 7), indicating that enhanced PBEF expression occurs in response to cytokines implicated in the pathogenesis of ALI. To further confirm the increased PBEF expression observed in mechanical ventilation models of murine and canine ALI (Table 1; Figures 1–5), we applied 18% cyclic stretch to human lung microvascular endothelium *in vitro* for 48 hours and observed significantly augmented PBEF protein expression (3.1-fold increase; $n = 6$, $p < 0.05$) relative to static controls (Figure

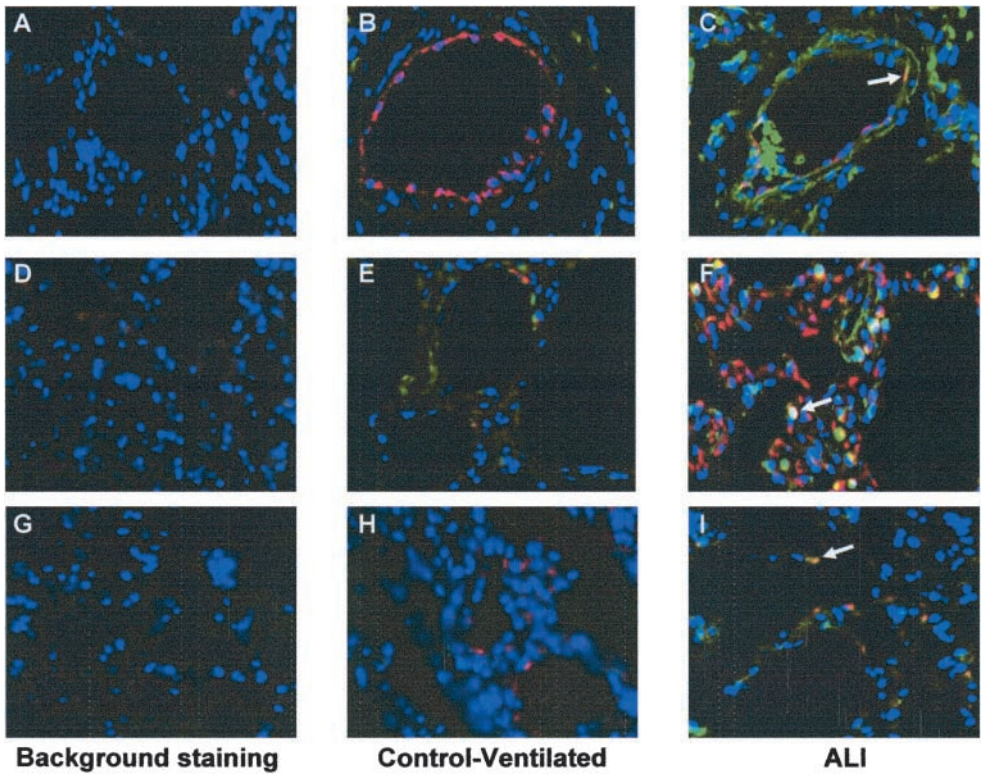

Figure 4. Immunohistochemistry localization of increased PBEF in canine ALI. *A*, *D*, and *G* show background staining with only secondary antibodies of injured/ventilated canine lungs. *B*, *E*, and *H* demonstrate mild sporadic staining of PBEF in uninjured/ventilated canine lung detected by a rabbit anticanine PBEF affinity purified polyclonal antibody followed by a goat antirabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) (*green*). *C*, *F*, and *I* demonstrate significant staining of PBEF (*green*) in injured/ventilated canine lung with positively stained endothelial cells identified by a rabbit–anti-human von Willebrand factor followed by a goat–anti-rabbit IgG conjugated with Texas red (*C*, *red*). Neutrophils were identified by a rat–anti-mouse neutrophil antibody followed by a goat–anti-rat IgG conjugated with Texas red (*F*, *red*) and type II epithelial cells identified by a rabbit–anti-human proSPC followed by a goat–anti-rabbit IgG conjugated with Texas red (*I*, *red*) indicated by arrows, respectively. *Blue* indicates nuclei staining by 4′6 diamidino-2-phenylinodole. Preimmune serum did not show any significant staining of PBEF in an injured/ventilated canine lung (data not shown). Images were magnified at 400×.

8). The combination of both 18% cyclic stretch and IL-1β seems to further increase PBEF expression in HMVEC-L (3.7-fold increase; $n = 6$, $p < 0.01$; Figure 8). Thus, increases in mechanical stress appear to contribute to increased PBEF expression in lung tissues and in HMVEC-L with inflammatory stimuli exerting a possible additive effect.

SNP Discovery and Allelic Associations of Human PBEF Promoter SNPs T-1001G and C-1543T in Sepsis-associated ALI

Because our findings strongly implicated PBEF as a novel candidate gene in ALI, we next examined whether common variants in the human PBEF gene might be associated with susceptibility to sepsis-associated ALI. Direct DNA sequencing in 36 subjects with ALI, subjects with sepsis, and healthy control subjects identified 11 PBEF SNPs (*see* Table E2 in the online supplement) with a T-1001G transversion in the human PBEF gene immediate promoter (−1 to –3000 bp) having the highest degree of representation in 12 subjects with ALI (40% minor allelic frequency). The PBEF (T-1001G) SNP was genotyped in a case-control population of white subjects with sepsis-associated ALI ($n = 87$), subjects with sepsis alone ($n = 100$), and healthy control subjects ($n = 84$); relevant characteristics of the study population are presented in Table 2. The T-1001G SNP was in Hardy-Weinberg equilibrium (HWE; $p = 0.50$). The G-allele frequency observed among both subjects with ALI (30%) and among subjects with sepsis without ALI (23%) was significantly higher compared with the frequency observed in the healthy control group (12%; $p < 0.001$ and $p = 0.01$, respectively). Although the G-allele frequency was higher among subjects with ALI compared with subjects with sepsis without ALI, the difference was not statistically significant. A second SNP, C-1543T, was also in HWE ($p = 0.46$; Table 3). The T-allele frequency observed among subjects with ALI (20%) was significantly lower than the frequency observed in the healthy control group (31%; $p < 0.05$); no significant difference was observed when comparing the T-variant frequency among subjects with sepsis (24%) with healthy control subjects ($p = 0.136$). The difference in the T-allele frequency between subjects with sepsis-associated ALI and sepsis only was not statistically significant.

A significant association was observed between the PBEF (T-1001G) genotype and subjects with ALI and subjects with sepsis compared with healthy control subjects ($p < 0.001$ and $p = 0.004$, respectively). However, the difference in the genotype frequency between ALI and sepsis was not statistically significant. Because a dominant-G model fit the data best, the GT and GG genotypes were included as a single risk group. In a univariate analysis, carriers of the G allele had a 2.75-fold increased risk of ALI compared with control subjects ($p = 0.002$). Multiple logistic regression analysis using relevant clinical risk factors revealed that, after controlling for age and sex and other comorbidity factors (cancer, immunosuppression, liver disease, end-stage renal failure, chronic obstructive pulmonary disease, alcohol abuse, diabetes, congestive heart failure, anemia, acute renal failure), the G-mutant allele remained an independent risk factor for ALI susceptibility (odds ratio 2.16, 95% confidence interval [CI] 1.01–4.62) but not for sepsis without ALI. The G allele was not associated with mortality among patients with sepsis-associated ALI or sepsis only after controlling for age (odds ratio 1.26, 95% CI 0.47–3.38) and sex (odds ratio 1.67, 95% CI 0.59–4.73). For both sepsis and sepsis-associated ALI, the APACHE II score was the single best predictor of mortality

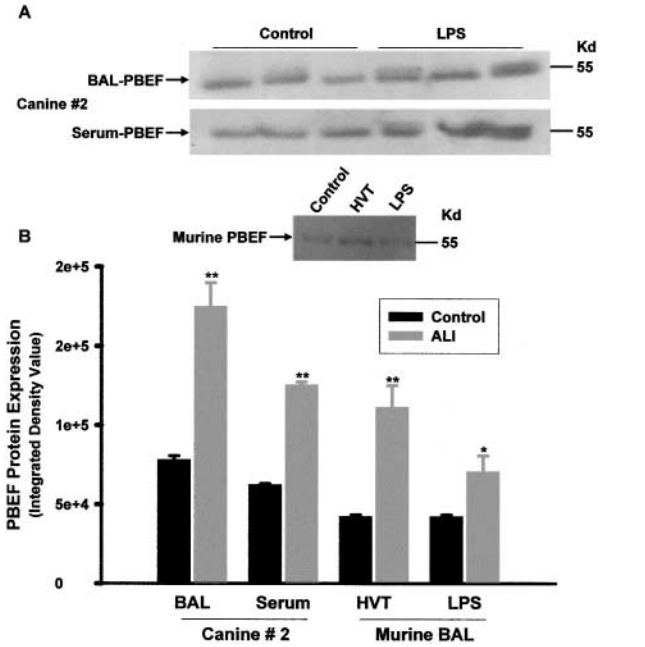

Figure 5. Analysis of PBEF protein levels in canine BAL/serum and murine BAL. (*A*) Western blot image of PBEF protein. Total protein (10 μg) of each sample was separated by 12% SDS-PAGE and immunodetected by Western blot using the anticanine PBEF antibody. Also shown is a representative murine experiment (one of four replicate experiments). (*B*) Densitometric analysis: band density on Western blot images, used as a measure of PBEF protein level, was acquired using the Scanjet 7400c and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp.). Statistical comparative analysis of integrated density value (mean ± SEM) between control and ALI groups was performed using the unpaired *t* test. PBEF protein amount was significantly increased in BAL fluid (2.23-fold increase, 174,999 ± 14,721 [ALI] vs. 78,488 ± 2,161 [control]; n = 3, $p < 0.01$) and serum (2.01-fold increase, 125,608 ± 1,695 [ALI] vs. 62,646 ± 401 [control]; n = 3, $p < 0.01$) in the canine LPS ALI model as well as in BAL fluid obtained from both a murine model of ALI induced by high V_T ventilation without LPS (2.62-fold increase, 111,586 ± 13,475 [ALI] vs. 42,580 ± 789 [control]; n = 7, $p < 0.01$) and a murine model of LPS-mediated lung injury (1.67-fold increase, 70,907 ± 9,835 [ALI] vs. 42,580 ± 789 [control]; n = 3, $p < 0.05$). HVT = high tidal volume. *$p < 0.05$; **$p < 0.01$.

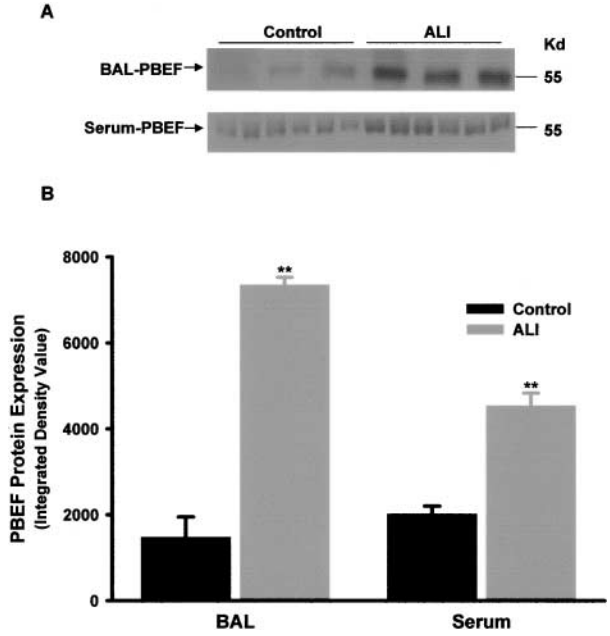

Figure 6. Analysis of human BAL- and serum-PBEF protein levels by Western blot. (*A*) Western blot image of PBEF protein. Total protein (10 μg) of each sample was separated by 12% SDS-PAGE and immunodetected by Western blot using the anticanine PBEF antibody. (*B*) Densitometric analysis: band density on Western blot images was used as a measure of PBEF protein level. The band image was acquired using the Scanjet 7400c and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp.). Statistical comparative analysis of integrated density value (mean ± SEM) between control and ALI groups was performed using the unpaired *t* test. PBEF protein levels in human ALI BAL (4.96-fold increase, 7,353 ± 171 [ALI] vs. 1,482 ± 466 [control]; n = 3, $p < 0.01$) and serum (2.25-fold increase, 4,543 ± 285 [ALI] vs. 2,020 ± 179 [control]; n = 8, $p < 0.01$) were significantly increased relative to healthy controls. **$p < 0.01$.

in this population (data not shown). A borderline association was observed between the PBEF (C-1543T) genotype and ALI ($p = 0.059$), but no association was observed between the PBEF (C-1543T) genotype and sepsis ($p = 0.226$).

Haplotype-weighted analysis of T-1001G and C-1543T SNPs revealed four haplotypes: GC, GT, TC, and TT (Table 4). Among the haplotypes, the frequency of the GC haplotype was twofold or more higher in both the ALI and sepsis samples, suggesting its role as an ALI and/or sepsis susceptibility haplotype. The TT haplotype appeared protective and was less than twofold lower in the ALI group and 1.7-fold lower in the sepsis group. Univariant logistic regression analysis revealed that the GC haplotype conferred a 7.71-fold higher risk of ALI (95% CI 3.01–19.75, $p < 0.01$) and a 4.84-fold higher risk of sepsis (95% CI 1.97–11.90, $p < 0.01$). Carriers of the TT haplotype had a 0.84-fold lower risk of ALI, but the difference did not reach significance. No significant risks were observed for haplotypes GT and TC for either ALI or sepsis. The difference in weighted haplotype frequencies between ALI and sepsis was not statistically significant.

To examine whether the T-1001G or T-1543C polymorphisms directly alter gene transcription, we performed a transient luciferase reporter gene assay by transfecting T-1001 variant or G-1001 variant-pGL3 basic vector as well as C-1543 variant or T-1543 variant-pGL3 basic vector into HMVEC-L for 4 hours. No significant difference in luciferase activities between T variant–1001–containing and G variant– 1001–containing pGL3 basic vector constructs was observed. However, the T variant in the PBEF gene promoter SNP C-1543T resulted in nearly a twofold decrease in the reporter gene expression (Figure 9).

DISCUSSION

Candidate gene identification in a complex lung disorder such as ALI poses a serious challenge because of the heterogeneity in inciting stimuli and the lack of available linkage studies. We applied emerging functional genomic technologies, specifically DNA microarray profiling and genotyping, to the study of the ALI pathogenesis in hope of providing mechanistic insights and identifying novel biomarkers and therapeutic targets. Gene expression profiling in lung tissue from animal models of ALI identified PBEF as a highly upregulated gene in ALI, and results were reinforced and validated by several complementary approaches (molecular cloning of canine PBEF, RT-PCR, immunohistochemical analysis). Furthermore, PBEF protein levels were significantly increased in BAL, serum, and lung tissues from canine, murine, and human ALI models, suggesting its potential as a biomarker.

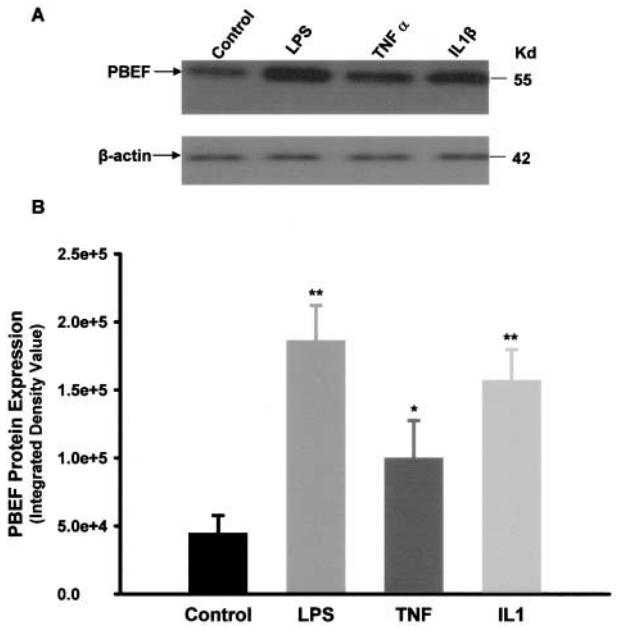

Figure 7. PBEF protein levels in cytokine-challenged human lung microvascular endothelial cells (HMVEC-L). (*A*) Western blot image of PBEF protein. Total protein (10 μg) of each sample was separated by 12% SDS-PAGE and immunodetected by Western blot using the anticanine PBEF antibody. The experiment shown is one of six replicate experiments and is typical of those obtained. (*B*) Densitometric analysis: band density on Western blot images was used as a measure of PBEF protein level. The band image was acquired using the Scanjet 7400c and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp.). Statistical comparative analysis of integrated density value (mean ± SEM) between control and each treated group was performed using the unpaired *t* test. These results demonstrate significantly increased PBEF protein expressions in HMVEC-L treated with LPS (4.15-fold increase, 186,513 ± 25,577; n = 6, $p < 0.01$); TNF-α (2.23-fold increase, 100,174 ± 27255; n = 6, $p < 0.05$), IL-1β (3.50-fold increase, 157,143 ± 22454; n = 6, $p < 0.01$) relative to controls (44,925 ± 12721, n = 6). TNF-α = tumor necrosis factor α. *$p < 0.05$; **$p < 0.01$.

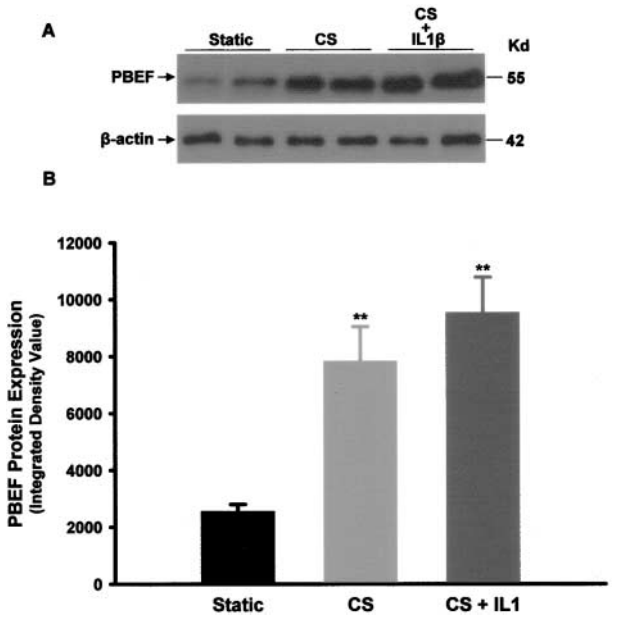

Figure 8. PBEF protein levels in mechanical stress–challenged HMVEC-L. (*A*) Western blot image of PBEF protein. Total protein (10 μg) of each sample was separated by 12% SDS-PAGE and immunodetected by Western blot using the anticanine PBEF and β actin antibodies. The experiment shown is two of four replicate experiments and is typical of those obtained. (*B*) Densitometric analysis: band density on Western blot images was used as a measure of PBEF protein level. The band image was acquired using the Scanjet 7400c and analyzed by the AlphaEase Stand Alone software (Alpha Innotech Corp.). β-actin was included as a loading control. Statistical comparative analysis of integrated density value (mean ± SEM) between control and the CS- or CS plus IL-1β–treated group was performed using the paired *t* test. The application of 18% cyclic stretch to HMVEC-L *in vitro* for 48 hours significantly augmented PBEF protein expression (3.06-fold increase, 7,840 ± 1,200; n = 6, $p < 0.05$) relative to static controls (2,560 ± 244, n = 6). Cyclic stretch plus IL-1β seems to further increase PBEF expression in HMVEC-L (3.73 fold increase, 9,546 ± 1230; n = 6, $p < 0.01$) relative to static controls (2,560 ± 244, n = 6). CS = cyclic stretch. **$p < 0.01$.

The published literature on PBEF is sparse, and our studies provide the first observation that PBEF is significantly upregulated in the lung and in other models of lung injury (16, 17, 26). PBEF was first isolated from an activated peripheral blood lymphocyte cDNA library and found to be involved in B-cell precursor maturation (12). Subsequently, dysregulated PBEF gene expression was described in human fetal membranes of severe chorioamnionitis (14), with increased expression in an amniotic epithelial cell line following challenge with inflammatory cytokines (LPS, IL-1β, TNF-α, and IL-6) (14) and during IFN-γ–induced maturation of pre–B cells (29) in a B lymphoma cell line (30) and in IFN-induced preterm labor gestational membrane (31). Recombinant PBEF protein significantly increased expression of IL-6 and IL-8 in amniotic epithelium (32, 33). Despite the findings of PBEF expression in nonlung tissues, the molecular physiologic and pathophysiologic relevance of PBEF to lung pathophysiology is unknown. The robust expression of PBEF in murine and canine models of ALI in our study suggests that PBEF may be an inflammatory signal transducer in the pathogenesis of ALI. Immunohistochemical colocalization studies revealed increased PBEF expression in lung endothelium, type II alveolar epithelial cells, and infiltrating neutrophils, and upregulation of PBEF expression in inflammatory cytokine-stimulated HMVEC-L *in vitro*. These results strongly support a potentially important role for PBEF in the inflammatory lung processes observed in ALI. Several clinical studies implicate a complex network of inflammatory cytokines and chemokines in mediating, amplifying, and perpetuating the lung injury process (34). The immunohistochemical colocalization of increased PBEF expression in infiltrating neutrophils and lung endothelium suggests a novel role for PBEF as a signal transducer during lung inflammation. This notion is supported by a recent report (15) that PBEF expression is significantly increased in circulating peripheral blood neutrophils derived from patients with sepsis, including data that convincingly demonstrated that PBEF inhibits neutrophil apoptosis. Because the rate of clearance of apoptotic neutrophils is associated with resolution of neutrophilic lung inflammation (35), prolonging neutrophil survival via PBEF inhibition of apoptosis may sustain neutrophilic inflammation and contribute to the pathogenesis of ALI and other neutrophil-mediated disorders.

In addition to inflammatory cytokines, another clinically relevant stimulus for PBEF expression is increased mechanical stress, a major contributing factor to both ALI mortality and ventilator-associated ALI (3, 36). The application of 18% cyclic

TABLE 2. DEMOGRAPHIC AND COMORBID INFORMATION ON SUBJECTS WITH SEPSIS-ASSOCIATED ALI, SUBJECTS WITH SEPSIS, AND HEALTHY CONTROL SUBJECTS

Variable	Control (*n = 84*)	Sepsis (*n = 100*)	ALI (*n = 87*)
Age, mean ± SD	32.9 ± 10.5	59.7‡ ± 19.2	52.0‡ ± 16.9
Sex, M/F	40/44	48/52	51/36
APACHE II, mean ± SD	NA	21.3 ± 8.7	23.4 ± 6.9§
60-d survival,*L/D (%)†	84/0 (100%)	71/23 (75.5%)‡	55/26 (67.9%)‡
Cancer, %	NA	22	17
Immunosuppression, %	NA	16.5	29.9
Liver disease, %	NA	10.9	11.5
ESRF, %	NA	9.9	8
COPD, %	NA	12.1	4.5
Alcohol abuse, %	NA	8.8	12.6
Diabetes, %	NA	19.8	19.5
CHF, %	NA	9.8	6.9
HIV-positive, %	NA	1.1	4.6
Anemia, %	NA	26.4	17.2
ARF, %	NA	8.8	25.3
Use of vasopressors, %	NA	47.1	35.2
Insult			
Lung, %	NA	37.5	70.1
UTI, %	NA	15.4	2.3
Other, %	NA	37.4	28.7

Definition of abbreviations: ALI = acute lung injury; ARF = acute renal failure; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; ESRF = end-stage renal failure; UTI, urinary tract infection.

All subjects are white. "Sepsis" denotes severe sepsis or septic shock.

* Data were not available for nine and six patients in sepsis and ALI groups, respectively.

† Percentage of survival in each group.

‡ Sepsis or ALI vs. control; $p < 0.01$.

§ Data were not available for one patient.

stretch to HMVEC-L *in vitro* for 48 hours resulted in significant augmentation in PBEF protein expression (3.1-fold), which is consistent with the increased PBEF gene expression observed in distended human fetal membranes *in vitro* (13). The PBEF promoter contains two nuclear factor κB binding elements that may potentially participate in conferring mechanical stretch responsiveness (14). Besides demonstrating the survival benefit of a lung-protective ventilatory strategy, the landmark Acute Respiratory Distress Syndrome Network study also highlighted a marked reduction in the number of neutrophils and the concentration of proinflammatory cytokines released into the airspaces of the injured lung (34). Studies are ongoing to establish the potential contribution of PBEF to the pathogenesis of ALI. At a minimum, however, increased PBEF protein expression, either in BAL fluid or serum, has promise as a novel and useful biomarker to assist in the clinical diagnosis of inflammatory lung disease (37–39).

Given that our genomic studies identified PBEF as a viable candidate gene and potential biomarker in ALI, we selected two PBEF promoter variants (T-1001G and C-1543T) and conducted genetic studies to test for an association between these PBEF SNPs and sepsis-associated ALI. Both T-1001G and C-1543T SNPs conform to the HWE.

The HWE implies maintenance of allele and genotype frequencies in a steady state from generation to generation. Departures from HWE can be used as an indication of population genetic features in a sample and also can be used to judge potential genotyping errors. Both allele and genotype frequencies of T-1001G and C-1543T SNPs were in HWE ($p = 0.50$ and $p = 0.46$, respectively), suggesting that genes of subjects used in this study were picked independently from the gene pool, which is equivalent to picking patient samples at random from the population. It also supports that the genotyping work was correctly performed in this study using two well established techniques (restriction-site polymorphism and 5′ nuclease assays). Only after HWE was verified for each SNP did we initiate the individual SNP or haplotype association tests. For the T-1001G SNP, a significantly higher frequency of the minor G allele and TG/GG genotypes and lower frequencies of the minor T-allele and CT/TT genotypes in the C-1543T SNP were associated with ALI. The former also was associated with sepsis without ALI. Multiple logistic regression analysis revealed that, after controlling for 12 other risk factors, the G-variant allele from T-1001G remains an independent risk factor for ALI susceptibility. Haplo-

TABLE 3. FREQUENCIES AND ASSOCIATIONS OF PRE–B-CELL COLONY-ENHANCING FACTOR T-1001G AND C-1543T SINGLE NUCLEOTIDE POLYMORPHISMS IN SUBJECTS WITH SEPSIS-ASSOCIATED ACUTE LUNG INJURY, SUBJECTS WITH SEPSIS, AND HEALTHY CONTROL SUBJECTS

Factor	Control (*% frequency*)	Sepsis (*% frequency*)	p Value†	ALI (*% frequency*)	p Value‡	p Value§
T-1001G						
N	82	75		69		
TT genotype	64 (78)	44 (55)		29 (42)		
GT genotype	16 (20)	33 (44)	0.004	39 (57)	< 0.001	NS
GG genotype	2 (2)	1 (1)		1 (1)		
T allele	144 (88)	115 (77)		97 (70)		
G allele	20 (12)	35 (23)	0.01	41 (30)	< 0.001	NS
C-1543T						
N*	83	98		75		
CC genotype	42 (51)	56 (57)		47 (63)		
CT genotype	31 (37)	37 (38)	0.226	26 (35)	0.059	NS
TT genotype	10 (12)	5 (5)		2 (2)		
C allele	115 (69)	149 (76)		120 (80)		
T allele	51 (31)	47 (24)	0.136	30 (20)	0.026	NS

Definition of abbreviations: ALI = acute lung injury; NS = not significantly different.

All subjects are white. "Sepsis" denotes severe sepsis or septic shock.

* T-1001G was genotyped by a Restriction Site Polymorphism method, whereas C-1543T was genotyped by an Assays-by-Design Service-SNP genotyping method (Applied Biosystems).

† Sepsis vs. control.

‡ ALI vs. control.

§ ALI vs. sepsis.

TABLE 4. HAPLOTYPE-WEIGHTED ANALYSIS OF PRE–B-CELL COLONY-ENHANCING FACTOR T-1001G AND C-1543T SINGLE NUCLEOTIDE POLYMORPHISMS IN SUBJECTS WITH SEPSIS-ASSOCIATED ACUTE LUNG INJURY, SUBJECTS WITH SEPSIS, AND HEALTHY CONTROL SUBJECTS

Haplotype Frequencies				
Haplotype*	ALI	Sepsis	Control	Total
GC	34.635	27.635	14.28	76.55
GT	67.365	79.365	93.72	240.45
TC	2.365	3.365	2.72	8.45
TT	21.635	27.635	47.28	96.55
Total	126	138	158	422
		χ^2 test: $p < 0.01$		
Logistic Regression Analysis, *OR* (*95% CI*)				
Haplotype*	ALI	Sepsis	p Value‡	
GC	7.71† (3.01–9.75)	4.84† (1.97–11.90)	NS	
GT	2.09 (0.74–5.87)	2.63 (0.74–9.37)	NS	
TC	2.46 (0.69–8.75)	2.73 (0.86–8.67)	NS	
TT	0.84 (0.33–2.10)	1.04 (0.45–2.39)	NS	

Definition of abbreviations: ALI = acute lung injury; CI = confidence interval; NS = not significantly different; OR = odds ratio.
*In each haplotype, the first allele is from single nucleotide polymorphism T-1001G and the second allele is from C-1543T.
† ALI or sepsis (dependent variable) vs. their haplotypes (independent variable); $p < 0.01$.
‡ Haplotypes of ALI (dependent variable) vs. those of sepsis (independent variable).

type analysis revealed four possible haplotypes (GC, GT, TC, TT; Table 4) from two SNPs. Among them, carriers of the GC haplotype had a 7.71-fold higher risk of ALI and a 4.84-fold higher risk of sepsis (both $p < 0.01$). Trends of differences in the minor allele, genotype, and haplotype frequencies comparing subjects with sepsis-associated ALI and subjects with sepsis were evident but not statistically significant. This finding may reflect, in part, a limited sample size, which prevented the detection of a difference between sepsis and ALI groups. An alternative postulate is that this locus is more strongly associated with the sepsis severity rather than ALI susceptibility. For example, we observed a slightly higher APACHE II score (a measure of severity) in patients with sepsis-associated ALI compared with patients with sepsis only, although the difference didn't achieve significance. Our ongoing recruitment of additional patients will allow us to test this hypothesis in the future. In addition, further analysis of DNA from patients with ALI from causes other than sepsis may be necessary to distinguish whether the haplotype GC is a risk factor or the haplotype TT a protective factor specific for ALI, rather than for severe sepsis, which frequently leads to ALI.

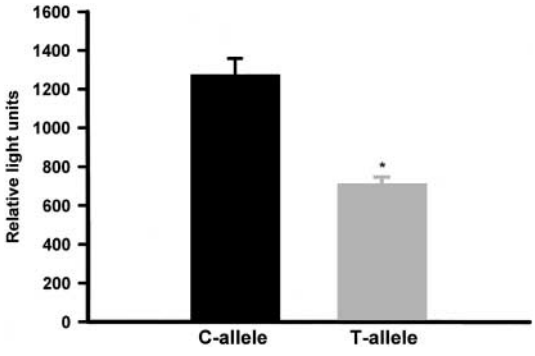

Figure 9. Measurement of C-1543C variant-pGL-3 or T-1543T variant-pGL-3 basic vector firefly luciferase activity in transiently transfected HMVEC-L. C-1543 variant-pGL-3 or T-1543 variant-pGL-3 basic vector firefly luciferase reporter constructs were cotransfected with the renila luciferase controls into HMVEC-L for 4 hours. Cell lysates were harvested for luciferase activity assay. Renila luciferase activity was assayed as a base for the transfection efficiency normalization. The activity was expressed as relative light unit. The T variant in the PBEF promoter C-1543T single nucleotide polymorphism resulted in a decreased luciferase reporter gene expression (1.80-fold decrease, 710 ± 37; n = 6, $p < 0.01$) relative to the C-variant controls (1,275 ± 84, n = 6). *$p < 0.01$.

Preliminary studies addressing the functionality of the T-1001G variant using the luciferase reporter gene assay did not demonstrate a significant role for this variant in gene transcription regulation (data not shown). However, the T variant in the C-1543T SNP, 542 bp upstream from T-1001G in the PBEF promoter region, resulted in nearly a twofold decrease in the reporter gene expression (Figure 9). The frequency of the T allele was significantly lower in subjects with ALI (20%, n = 75) than that in normal control subjects (31%; n = 83, p = 0.026). This result is consistent with our observations from animal models of ALI, human subjects with ALI, and *in vitro* cell culture experiments, and suggests that higher expression of PBEF is implicated in the pathogenesis of sepsis-associated ALI. These results further suggest that genetically determined increased PBEF expression contributes to susceptibility to ALI.

In summary, using a candidate gene approach and a series of diverse cellular, animal, and human studies, we identified PBEF as a potential novel biomarker and candidate gene in sepsis- and mechanical stress–induced inflammatory lung disease, such as ALI. Although further studies are required to both define the pathophysiologic role of altered PBEF expression in ALI and to more clearly link PBEF variants to ALI susceptibility, our results strongly support that PBEF may be a potential novel biomarker in ALI. Finally, this study underscores the powerful potential of using genomic approaches to deciphering the genetic basis of complex lung disorders.

Conflict of Interest Statement: S.Q.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.P.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.Z.-W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.B.E. does not have a

financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.G.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.C.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.G.N.G does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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