Essential Role of Pre-B Cell Colony Enhancing Factor in Ventilator-Induced Lung Injury

Sang-Bum Hong, Yong Huang, Liliana Moreno-Vinasco, Saad Sammani, Jaideep Moitra, Joseph W. Barnard, Shwu-Fan Ma, Tamara Mirzapoiazova, Carrie Evenoski, Ryan R. Reeves, Eddie T. Chiang, Gabriel D. Lang, Aliya N. Husain, Steven M. Dudek, Jeffrey R. Jacobson, Shui Q. Ye, Yves A. Lussier, and Joe G.N. Garcia.

Online Data Supplement

SUPPLEMENTAL METHODS

Recombinant human PBEF. Recombinant human soluble PBEF/visfatin (rhPBEF), a 52 kDa protein containing 465 amino acid residues was purchased from PeproTech (Rocky Hill, NJ, USA). Purity was more than 98% by SDS-PAGE gel and HPLC analyses, and contained <0.01 ng LPS/ μg PBEF as determined by the *Limulus* amebocyte lysate method.

Transmigration and chemotaxis assay. Neutrophils were collected from retired breeder male Harlan-Sprague Dawley rats by intraperitoneal injection of 2.5% casein (5 ml). The resulting peritoneal fluid was recovered by PBS peritoneal lavage after 18hr, centrifuged and examined for total cell counts and cell differentiation. For chemotaxis assays, leukocytes were exposed to calcein-AM in the presence of plurionic acid for 1 hr at room temperature. The temperature was then elevated to 37°C for 30 min to cleave the calcein AM thereby reducing the rate of calcein diffusion out of the cells. The resuspended leukocyte suspension was placed above a Millipore Multiscreen-MIC Transwell plate (Billerica, MA) with 3 μm polycarbonate filters precoated with 4% bovine albumin. rhPBEF (10ng/ul) was placed below the filters in the receiving wells of the plate (in PBS) or casein (positive chemoattractant control). The receiving wells were assessed serially in a fluorescent plate reader with absorption at 494 nm and emission measured at 517 nm. Fluorescence was recorded at 5, 10, 15, 30 min and each subsequent 30 min period for up to 4hr. Aliquots of known numbers of neutrophils were counted in separate wells at each time point to account for any quenching of the fluorescence over time.

Experimental animal protocols. Animal procedures were conducted and approved following the recommendations of the Animal Care and Use Committee at the University of Chicago. C57BL/6J mice were obtained from Jackson Laboratories (Wilmington, MA) and housed under standard conditions (12h light-dark cycle, 25-27ºC, ~40% humidity) with free access to food and water throughout the duration of the experiments.

Generation of transgenic mice. The 129/Sv/Ola derived ES cell line (15) RR084 harboring the exon-trap vector pGT0lxf (16) in the 7^{th} intron of the murine PBEF gene was obtained from the NHLBI-funded Program In Genomic Applications BayGenomics Consortium (San Francisco, CA). Transgenic mice were produced by microinjection of the ES cells into blastocysts derived from B6/J mice, and screened by insertion junction-specific PCR on tail DNA. The gene-specific primers were In7 (forward; 5'-CGGATGCCTTAGCCTGAAGT-3') and VecIn7JR (reverse; 5'-GGGAGTGACACAGCAAATCA-3') giving a 458-bp product. The transgene-specific primer VecIn7JF (forward; 5'-CAGCAGCAGACCATTTTCAA - 3'), in conjunction with the primer VecIn7JR, produced a 284-bp knockout allele-specific product. All reactions were "hot-started" for 3 min at 93°C using the AmpliTaq™ Gold system (Applied Biosystems, Foster City, CA) including 1.5 mM $MgCl_2$, 500 μM dNTPs, and 200 nM of each primer, followed by 30 cycles of 15 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C. Founder mice were out-crossed to B6 mice for 4 generations to reach 85% congenic status. Five transgenic founders were obtained from 10 live births, of which 3 transmitted the transgene to the next generation. All outcrosses to B6 mice were viable, but none of the in-crosses were able to produce progeny homozygous for both knockout alleles. Mice derived from one founder (C10), hemizygous for the exon-trapped allele at N_4 backcross generation, are characterized in this report.

Ventilator-induced murine lung injury models. We utilized two experimental models of ventilator-induce lung injury in these experiments. Our first approach (VILIa) utilized a VILI protocol with a tidal volume of 30 ml/kg designed to produce limited **lung injury** in order to allow assessment of potential synergism between delivered PBEF and mechanical ventilation-induced lung injury. Male C57BL/6J mice (8–12 wk old, 24.7 ± 2.0g) were weighed, anesthetized with ketamine/acepromazine, intubated using a 20 gauge catheter (Medex, Inc, Carlsbad, CA) and administered recombinant PBEF (20 μg/mouse in 1.5 μl water per gram body weight or vehicle equal volumes of water) via an intratracheal (IT) route ~30 min before being placed on mechanical ventilation as we have previously described (18). Mice were then connected to Harvard Apparatus ventilator (Boston, MA) at room air, tidal volume of 30 ml/kg, 65 breaths/minute, and positive expiratory pressure (PEEP) 0 cm H_2O for 4hr. Groups of mice were randomly allocated to either the spontaneous breathing group (SB) (*n*=4), the spontaneous breathing challenged with IT rhPBEF group (SB-rhPBEF) (*n*=6), the high tidal ventilation group (VILIa) (*n*=6), or high tidal ventilation with rhPBEF group (VILIa-rhPBEF) (*n*=6). Normal saline (0.2 cc) was given via intraperitoneal injection at the beginning and 2 hours later to all ventilated mice and peak inspiratory pressure was monitored continuously. Temperature was maintained with heat blanket to all mice during experiment.

In a separate set of experiments, our second approach utilized a VILI protocol employing a greater tidal volume (40 ml/kg) designed to produce more **severe lung injury** (VILIb) in order to assess potential protective effects of the single PBEF allele deletion in $PBEF^{+/-}$ mice on a B6/J murine background. PBEF transgenic mice ($PBEF^{+/-}$) and wild type controls ($PBEF^{+/+}$) at 18–22

week old (20–29 g) were anesthetized and ventilated at room air, tidal volume of 40 ml/kg at 65 breaths/minute with PEEP 0 cm H_2O for 4 hours. Wild type or $PBEF^{+/-}$ mice were randomly allocated into four groups and exposed to either spontaneous breathing, (SB-$PBEF^{+/+}$ and SB-$PBEF^{+/-}$), or to high tidal ventilation (VILIb-WT and VILIb-$PBEF^{+/-}$).

Bronchoalveolar lavage. Mice underwent bronchoalveolar lavage (BAL) of both lungs with Hank's balanced salt solution (1mL/mouse), and the recovered BAL fluid was used for assays as we have previously described (18). Total protein was measured using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) (18). Protein results were expressed as mg/ml to allow for comparison to other studies. Lavage samples were centrifuged at 500 x *g* on a refrigerated micro centrifuge for 20 min. The supernatant was decanted and aliquot for protein assays. The BAL cells were resuspended in Hank's balanced salt solution and counted using a hemacytometer using standard techniques.

Cytospin and differential counting. Re-suspended BAL cells from lung lavage were centrifuged using a Shandon Cytospin-2 at 600RPM for 15 min, fixed by air-drying, and stained with Diff-Quik dye for differential counts (18). Stained cell slides were evaluated and differentially counted for each individual animal (lymphocyte, neutrophil or a macrophage).

Cytokine assays*:* BAL fluid for cytokine measurements was stored at -80°C until analysis. A multiplex cytokine kit for IL-1-β, IL-6, KC, macrophage inflammatory protein-2 (MIP2) and tumor necrosis factor alpha (TNF-α) was obtained and the assay performed in accordance with manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Multiplex working solution (50 µl)

was placed into each well and the appropriate cytokine standards and samples (50 µl) were added to wells of a filtered plate, and incubated at room temperature for 30 min on a plate shaker (set to 300 rpm) in the dark. Freshly diluted secondary/detection antibody (25 µl/well) was next added to the wells at room temperature for 30 min followed by streptavidin-PE (16 µg/ml in assay buffer). Bound beads were washed three times with 100µl of wash buffer After the last wash, 125 µl of assay buffer was added to each well, the plate placed for 1 min on a plate shaker set at 500 rpm and then for 3 min at the reduced speed of 300 rpm. Fifty µl of sample was analyzed on the Bio-Plex system (Bio-Rad) in accordance with the manufacturer's instructions. Data analyses for all assays were performed using the Bio-Plex Manager software. Evaluation of BAL PBEF levels was performed by using a C-terminal (human) enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Pharmaceuticals Inc. Belmont, CA) with a sensitivity of 1.89 ng/ml. ELISA plates were read using a Spectra MAX (Molecular Devices Corp, Sunnyvale, CA) instrument, and data analyzed using the SoftmaxPRO software (Molecular Devices Corp).

RNA isolation and microarray analysis. Lung total RNA was isolated from whole rodent lung tissue for microarray experiment as we have described previously (9) using the Affymetrix GeneChip platform and Expression Analysis Manual protocols (Affymetrix Inc., Santa Clara, CA). The signal intensity fluorescent images produced during Affymetrix GeneChip Mouse430_2 Array hybridizations were scanned using the Hewlett-Packard GeneArray Scanner G2500A. The 'Present' or 'Absent' calls of the probe sets in the expression chips were determined by GCOS (GeneChip® Operating Software) software. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus repository (GSE9368 for rhPBEF study and GSE9314 for $PBEF^{+/-}$ study). Chip quality

(RNA degradation, cDNA synthesis, hybridization, chip washing, scanning) was evaluated with GCOS, dChip (19) and Bioconductor 'affy' packages. All RNA samples and chips adopted in current study passed the quality criteria (data not shown). The intensities of probe sets were calculated by the GC Robust Multichip Average (GCRMA) package of Bioconductor software for normalization (20). To identify differentially-expressed genes, pairwise comparisons were conducted using Significance Analysis of Microarrays (SAM) (see the SAM online manual for detailed explanations of the blocking approach, http://www-stat.stanford.edu/~tibs/SAM) as we have previously described (12). Only probe sets present (determined by Affy 'P'-call) in three replicates of at least one group with mean intensity above 100 were used for further data mining. The gene filtering parameters and results were summarized in Supplementary Table E2. All differentially-expressed genes displayed at least 2 fold changes and are referred to as "dysregulated genes" when normal control animals are used as reference. For probe sets representing the same Entrez Gene or UniGene ID, only the probe set with lowest FDR or highest fold changes was included in the gene list.

Identification of Gene Ontology (GO) categories enriched with dysregulated genes. The functional profiles of dysregulated genes were represented by the biological processes in the GO database with the number of genes in each GO category compared to that of all genes in the Mouse432_2 chip to determine the significance of the GO category. The analysis was performed using Onto-Express (http://www.geneontology.org/GO.downloads.ontology.shtml), with the default selection of statistical method (hypergeometric distribution followed by false discovery rate correction). The lists of dysregulated genes were uploaded into Onto-Express to identify significant GO categories ($q \leq 0.05$ with 6 or more genes).

Ingenuity Pathway Analysis (IPA). Dysregulated genes were uploaded into the IPA software (http://www.ingenuity.com), a web-delivered application which utilizes the Ingenuity Pathways Knowledge Base (IPKB) containing a robust number of individually modeled relationships between gene objects (*e.g.* genes, mRNAs, proteins), in order to dynamically generate significant regulatory and signaling networks or pathways. The genes submitted for mapping to corresponding gene objects in the IPKB are called "focus genes." The significance of a canonical pathway is controlled by *p*-value, which is calculated using the right-tailed (referring to the overrepresented pathway) Fisher Exact Test for 2x2 contingency tables. This is done by comparing the number of 'Focus' genes that participate in a given pathway, relative to the total number of occurrences of those genes in all pathways stored in the IPKB. The significance threshold of a canonical pathway is set to 1.3, which is derived by $-\log_{10}$ [*p*-value], with *p* value ≤ 0.05.

Generation and administration of PBEF neutralizing antibody (PBEF-Ab) Polyclonal anti-PBEF antibodies, used in the *in vivo* neutralization studies, were custom produced by Lampire Biological Laboratories, Inc. (Pipersville, PA) by immunization of a goat with full-length human recombinant PBEF protein. Antibodies were purified over a protein G column. 70µl of PBEFAb or saline were injected intratracheally. After delivery (30 min), mice were ventilated with room air (SB) or VILIb (40 ml/kg, for 4 hours). Boluses of sterile saline (200 µl) were given at the onset and after 2 h of ventilation, to maintain a mean arterial pressure greater than 60 mm Hg. This ventilation strategy maintains the blood gas parameters within a physiologic range (arterial pH of 7.3–7.5, HCO3 11–16 mmol/L) at the end of the experiment.

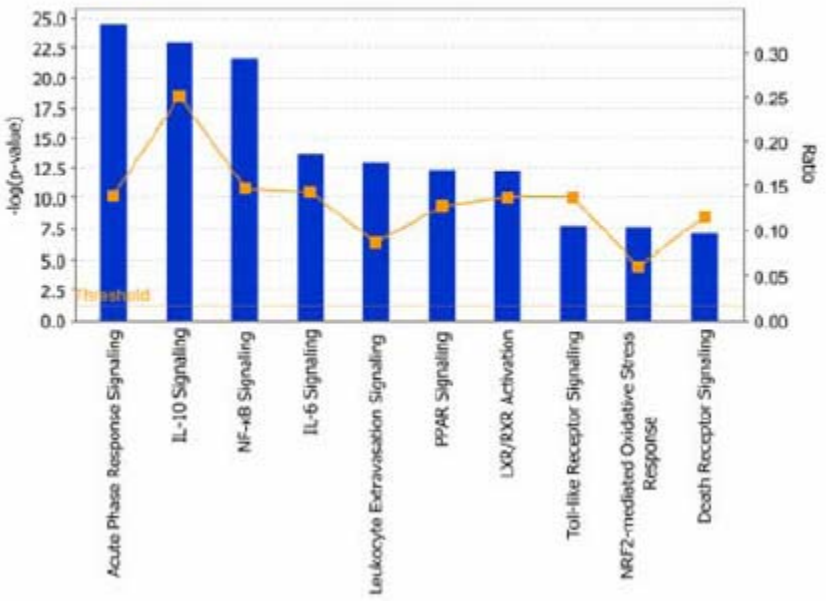
SUPPLEMENTARY FIGURES LEGENDS

Supplementary Figure E1: Ingenuity Pathway Analysis (IPA) of dysregulated genes in rhPBEF-challenged mice. The 493 dysregulated genes (see Supplementary Table E2, gene list 2) were generated by pairwise comparison between rhPBEF challenged and control animals. The gene list was submitted into Ingenuity software to identify canonical pathways enriched with the dysregulated genes. The threshold line represents the Fisher-exact *p*-value of 0.05. The ratio line represents the percentage of the dysregulated genes in the total number of genes in the corresponding pathway.

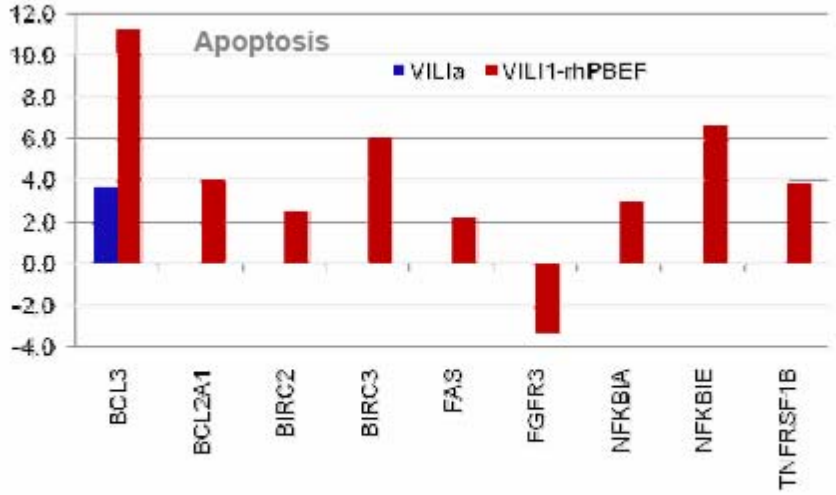
Supplementary Figure E2: Fold changes of the dysregulated genes induced by VILIa or VILIa-rhPBEF treatment in apoptosis pathway. The lists of dysregulated genes were generated using SAM software (Supplementary Table E2) and then submitted to Ingenuity Pathway Analysis software to identify canonical pathways enriched with dysregulated genes (Fig 6A).

Supplementary Figure E3. Mapping of VILIa-rhPBEF-induced dysregulated genes in the NFκB pathway. The NFκB pathway was identified by IPA analysis (see Figure 6B). Shown are the expanded set of up- and down-regulated genes highlighted in red and green, respectively as induced by exposure to combined VILIa and rhPBEF challenge.

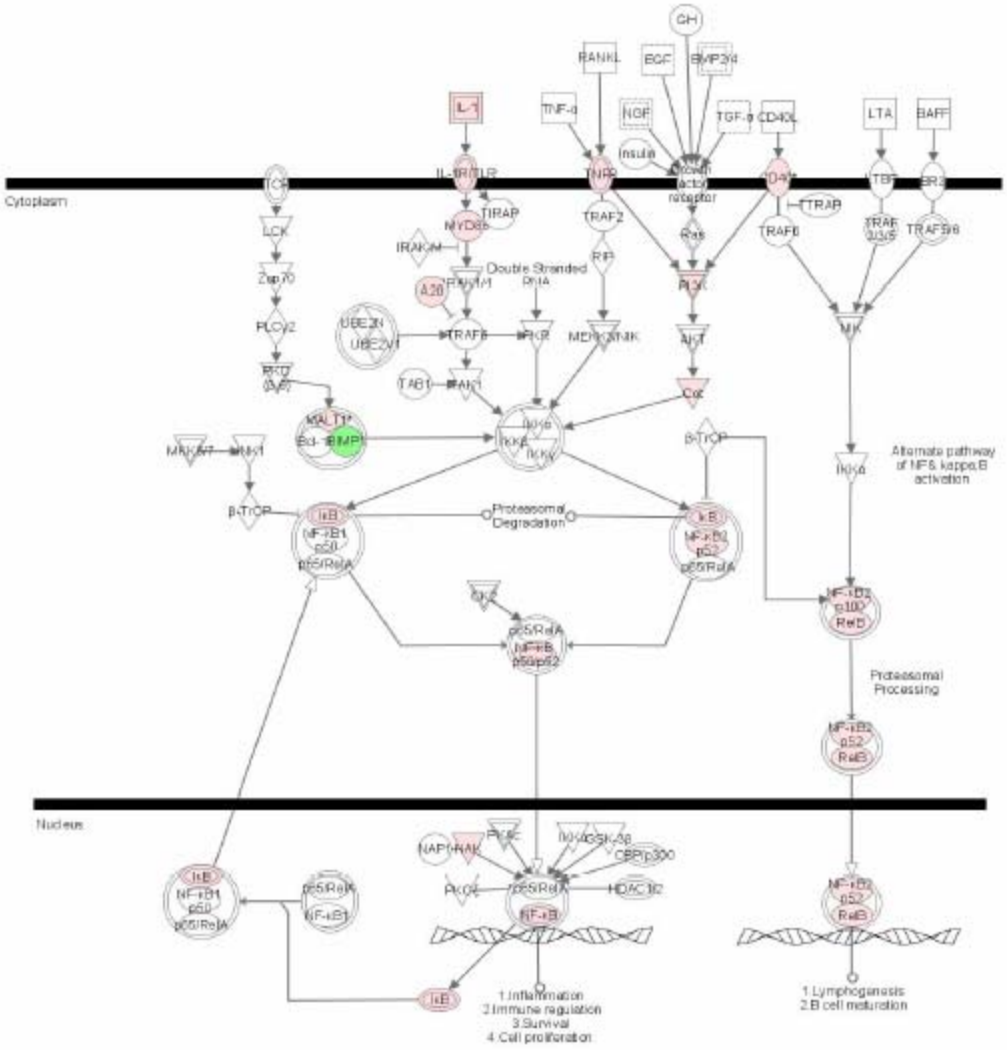
Supplementary Figure E4. Mapping of VILIa-rhPBEF-induced dysregulated genes in the leukocyte extravasation pathway. The leukocyte extravasation pathway was identified by IPA analysis (see Figure 6C). Shown are the expanded set of up- and down-regulated genes highlighted in red and green, respectively as induced by exposure to combined VILIa and rhPBEF challenge.



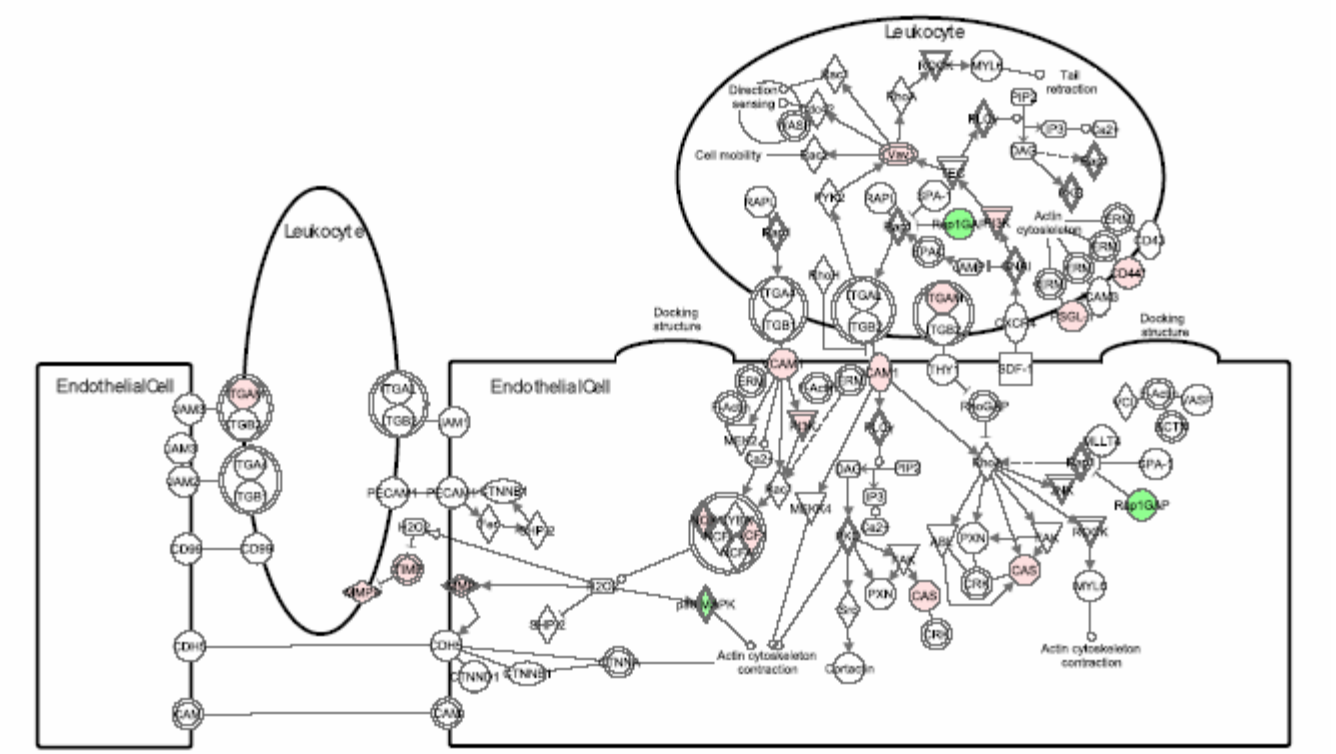
Supplementary Figure E1



Supplementary Figure E2



Supplementary Figure E3



Supplementary Figure E4