# Supporting Information

## Lucas et al. 10.1073/pnas.1121075109

### SI Results

**JI-34 Treatment of Human Lung Microvascular Endothelial Cells (HL-MVEC) Affects Splice Variant SV1 Expression Levels.** As demonstrated in Table S1, the relative expression of SV1 in HL-MVEC treated with the growth hormone-releasing hormone (GHRH) agonist JI-34 is significantly elevated, being 2.9-fold higher than the control group. Moreover, we detected markedly elevated levels of mRNA for SV3 after treatment with pneumolysin (PLY), JI-34, or the combination of both (Table S1). cAMP response element-binding protein (CREB)-binding elements were found in the rat GHRH receptor (GHRH-R) promoter region, suggesting the possibility of cAMP-dependent induction of GHRH-R expression (1). On the other hand, CREB activation was also shown to be PKC-dependent (2, 3). These data allow us to speculate that both stimuli, JI-34 (cAMP) and PLY ($Ca^{2+}$-dependent conventional PKC activation), when applied to HL-MVEC, may lead to stimulation of GHRH-R gene expression and, as a result, an elevation of SV3 mRNA, as we observed.

**JI-34 Does Not Affect Levels of PLY-Induced Proinflammatory Cytokines That Directly Affect Permeability.** To investigate whether the protective effect of JI-34 was mainly mediated by its inhibitory effect on direct PLY-mediated barrier dysfunction in the alveolar epithelial and capillary endothelial compartments rather than by an inhibitory effect on inflammatory mediators affecting barrier integrity, such as TNF, IL-1β, IL-6, and VEGF, we also assessed its effects on pro- and anti-inflammatory mediator generation in the bronchoalveolar lavage fluid (BALF) of the treated animals ($n = 6$ per group). To that purpose, we performed a multiplex MCYTOMAG-70K assay (EMD Millipore), which makes use of magnetic bead technology to quantify 32 analytes consisting of cytokines, chemokines, and growth factors. As shown in Table S2, we could demonstrate that, at 24 h after PLY instillation, JI-34 does not affect the generation of important proinflammatory cytokines and growth factors that directly increase permeability, including TNF, IL-1β, IFN-γ, IL-6, and VEGF. Surprisingly, we found that, at 24 h, PLY induced generation of IL-5, a cytokine regulating the maturation, migration, and survival of eosinophils, and this activity was significantly decreased by JI-34. The GHRH agonist also significantly blunted PLY-induced G-CSF generation, although not to control levels. The combined treatment of JI-34 with PLY demonstrates an inhibition of PLY-induced generation of monocyte chemoattractant protein 1 (MCP-1), leukemia inhibitory factor (LIF), the eosinophil chemoattractant eotaxin, and monokine induced by IFN-γ (MIG), the latter of which is a T-cell chemoattractant. By contrast, cotreatment with JI-34 in PLY-instilled mice leads to a strong induction of macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and especially MIP-2, which are all neutrophil chemoattractants, suggested to be important for generating a sufficient host response to pneumococcal infection. JI-34 does not significantly influence LPS-induced CXC chemokine (LIX) and keratinocyte-derived chemokine (KC), which, in addition to attracting and activating neutrophils, also amplify the inflammatory cascade.

### SI Materials and Methods

**Cells.** HL-MVEC (Lonza) were grown in complete EBM-2 medium (Lonza) and used up to passage six. Experiments were performed in serum-free medium. The human bronchiolar epithelial cell line H441, derived from the pericardial fluid of a patient with papillary adenocarcinoma of the lung, was purchased from the ATCC. Cells were cultured in RPMI medium 1640 (Mediatech) with 10% FBS (HyClone) and incubated at 37 °C in an atmosphere of 95% $O_2$/5% $CO_2$. Medium was replaced every other day, and cultures were split three times a week in a subcultivation ratio of 1:10 by using trypsin/EDTA (Sigma-Aldrich).

**Mice.** Male 6- to 8-wk-old C57BL6 mice weighing 19–21 g were obtained from Harlan and kept at the animal facilities at Georgia Health Sciences University.

**PLY Purification.** PLY was purified from a recombinant *Listeria innocua* 6a strain expressing PLY in the laboratory of T.C. The batch of PLY used in this study had a specific activity of $1.25 \times 10^7$ hemolytic units/mg.

**Peptide Analogs Preparation.** GHRH agonist JI-34, GHRH antagonist MIA-602, and bombesin/gastrin-releasing peptide antagonist RC-3940-II were synthesized in the laboratory of A.V.S. (4–6).

**Biochemicals.** Rabbit polyclonal anti-human myosin light chain 2 (MLC2), diphospho-MLC2 (T18/S19), mouse anti-human GAPDH, mouse anti-human β-actin mAb, and rabbit polyclonal anti–vascular endothelial (VE)-cadherin were from Cell Signaling Technology. Rabbit polyclonal anti–VE-cadherin (phospho-$Tyr^{658}$) was purchased from Invitrogen. Adenylate cyclase inhibitor SQ22536 as well as anti-mouse and anti-rabbit secondary antibodies conjugated to HRP were from Sigma-Aldrich.

**Total RNA Isolation and Real-Time RT-PCR Analyses.** Total RNA was extracted from HL-MVEC (control and treated) and normal human pituitary cells by using the NucleoSpin kit (Macherey-Nagel). The yield and quality of total RNA were determined spectrophotometrically by using 260-nm and 260/280-nm ratios, respectively. Two micrograms of RNA with a final volume of 40 μL was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen) with a Veriti 96-well thermal cycler (Applied Biosystems). We evaluated the mRNA expression for human GHRH, GHRH-R, SV1, SV2, SV3, SV4, and β-actin. Primers and probes used to detect human transcripts in these studies were described previously (7). All real-time PCR reactions were performed in the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Thermal cycling conditions were as described previously (8). All samples were run as described previously (9). The efficiencies of all primers (Invitrogen Life Technologies) and probes (Integrated DNA Technologies) were tested before the experiments, and they were all efficient in the range of 95–105%. Normal human pituitary was used as positive control, and β-actin was used as a housekeeping gene. Negative samples were run in each reaction consisting of no RNA in the reverse transcriptase reaction and no cDNA in the PCR. Two microliters of each amplification reaction was electrophoretically separated and visualized with the FlashGel DNA System (Lonza). The mathematical method described by Pfaffl (10) was used to evaluate the relative expression ratio for all genes normalized to β-actin, with the efficiencies for each set of primers and probes.

**Measurement of Transendothelial Electrical Resistance (TER).** TER in HL-MVEC monolayers [electrical cell-substrate impedance sensing (ECIS) system 1600R; Applied Biophysics] was measured as described previously (11, 12).

**In Vitro Vascular Permeability Assay.** A commercially available kit (EMD Millipore) was used to measure endothelial monolayer permeability to high molecular mass proteins by using 2,000-kDa

FITC-dextran, based on the Transwell model previously described (13). Briefly, HL-MVEC were seeded on collagen-coated Transwells at a density of $1 \times 10^5$ cells per well in a final volume of 200 μL of EBM-2 with supplements (Lonza). The inserts were placed overnight into 24-well plates containing 500 μL of medium. To measure agonist-induced endothelial cell permeability, complete medium was replaced for serum-free EBM-2, and 150 μL of FITC-dextran was added into the insert and incubated for 1 h. The insert was then removed, and 100 μL of medium was collected from the bottom chamber and transferred to a black 96-well plate. The fluorescent density of samples was analyzed on a Paradigm Microplate Fluorometer (Beckman-Coulter) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

**Western Blotting Procedure.** Immediately after treatment, HL-MVEC were washed twice with ice-cold PBS and lysed with lysis buffer [20 mM Tris·HCl (pH 7.6), 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM DTT, and protease inhibitor mixture]. The clear supernatants after centrifugation were mixed with SDS sample buffer and boiled for 5 min. Protein extracts were separated on SDS/PAGE, transferred to a nitrocellulose membrane, incubated with primary antibodies, and then incubated with HRP-conjugated secondary Ab. The immunoreactive proteins were visualized with LumiGLO solution (Cell Signaling) and were then exposed to X-ray film. The relative intensity of each protein band was quantified with ImageJ software (National Institutes of Health).

**Statistical Analysis.** All experimental data are presented as mean ± SD. Control samples and those obtained upon various stimuli were compared by unpaired Student's *t* test. For multiple group comparisons, one-way ANOVA was used. $P < 0.05$ was considered statistically significant.


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**Table S1. Relative expression ratios of mRNA for GHRH, SV1, and SV3 in HL-MVEC after a 30-min treatment with PLY (30 ng/mL), GHRH agonist JI-34 (1 μM), or both combined**

| | Relative expression ratio compared with controls | | |
|---|---|---|---|
| Treatment | GHRH | SV1 | SV3 |
| PLY | 1.53 ± 0.67 | 0.87 ± 0.39 | 5.43 ± 0.52* |
| JI-34 | 1.47 ± 0.45 | 2.91 ± 0.34* | 21.72 ± 0.93† |
| JI-34 + PLY | 0.51 ± 0.13 | 0.64 ± 0.21 | 9.64 ± 0.26† |

The expression of mRNA for GHRH-R, SV2, and SV4 was not detectable by real-time RT-PCR. Values are expressed as mean ± SE. *$P < 0.05$ and †$P < 0.01$ compared with control.

**Table S2. Concentrations of various analytes in BALF from C57BL6 mice at 24 h after PLY or vehicle instillation**

| Analyte | Control | PLY | JI-34/PLY |
|---|---|---|---|
| TNF | <3.2 | <3.2 | <3.2 |
| IFN-γ | <3.2 | 4.1 ± 0.4 | <3.2 |
| IL-1α | <3.2 | <3.2 | 6 ± 3 |
| IL-1β | <3.2 | 8 ± 3 | 17 ± 6 |
| IL-2 | 5 | 5 | 5 |
| IL-3 | <3.2 | 3.4 | <3.2 |
| IL-4 | <3.2 | <3.2 | <3.2 |
| IL-5 | <3.2 | 252 ± 42 | 15 ± 7* |
| IL-6 | 0 | 53 ± 11 | 38 ± 6 |
| IL-7 | 3.5 ± 0.2 | 4.8 ± 0.2 | 4.9 ± 0.3 |
| IL-9 | <3.2 | <3.2 | <3.2 |
| IL-10 | <3.2 | 12 ± 7 | 3 ± 2 |
| IL-12 p40 | <3.2 | 15 ± 3 | <3.2* |
| IL-12 p70 | <3.2 | <3.2 | <3.2 |
| IL-13 | <3.2 | 6.2 ± 1 | <3.2 |
| IL-15 | <3.2 | <3.2 | <3.2 |
| IL-17 | <3.2 | 4.5 ± 3 | <3.2 |
| VEGF | 17 ± 4 | 30 ± 3 | 33 ± 4 |
| G-CSF | 21 ± 3 | 490 ± 67 | 110 ± 23* |
| GM-CSF | <3.2 | <3.2 | <3.2 |
| M-CSF | <3.2 | <3.2 | <3.2 |
| RANTES | 4.1 ± 1 | 4.6 ± 1 | 5.0 ± 2 |
| Eotaxin | <3.2 | 14 ± 2 | 2 ± 0.1* |
| MIG | <3.2 | 10 ± 5 | 4 ± 1* |
| LIX | <3.2 | <3.2 | <3.2 |
| MIP-1α | <3.2 | <3.2 | 26 ± 3* |
| MIP-1β | <3.2 | <3.2 | 14 ± 2* |
| MIP-2 | <3.2 | <3.2 | 137 ± 21* |
| IP-10 | 5.5 ± 1 | 40 ± 13 | 25.5 ± 5 |
| KC | 10 ± 1 | 10 ± 2 | 10 ± 1 |
| MCP-1 | <3.2 | 38.5 ± 4 | <3.2* |
| LIF | <3.2 | 247 ± 32 | 1.6* |

Analytes (pg/mL) were assessed with the MCYTOMAG-70K assay (EMD Millipore). All groups were analyzed in sixfold, and values are expressed as mean ± SD. *$P < 0.02$ for JI-34/PLY treatment vs. PLY. IP-10, IFN-γ–induced protein 10; KC, keratinocyte-derived chemokine; LIF , leukemia inhibitory factor; LIX, LPS-induced CXC chemokine; MCP-1, monocyte chemoattractant protein 1; MIG, monokine induced by IFN-γ; RANTES, regulated upon activation, normal T-cell expressed and secreted.