Supplemental Materials and Methods

Legends for Supplementary Figures

Supplemental Materials and Methods

Materials and Methods

Reagents

Bleomycin, and paraformaldehyde were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Media Tech Inc. (Washington, DC). X-vivo 10 medium was purchased from Biowhittaker (Wakersville, MA). Ketamine was purchased from Youhan Aynghaeng Co. (Seoul, Korea). The Advantage RT-for PCR kit was purchased from BD Biosciences (San Jose, CA), and the gene-specific relative RT-PCR kits were purchased from Intron (Seoul, Korea). M-MLV reverse transcriptase was purchased from Enzynomics (Seoul, Korea). HGF, TGF-β1, TNF-α, and MIP-2 ELISA kits were purchased from R&D Systems (Minneapolis, MN). The hydroxyproline assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China PR). The Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit was purchased from Promega (Madison, WI).

The antibodies used for Western blotting were as follows: cleaved caspase-3 antibody (Cell Signaling Technology, Inc., Danvers, MA), anti-PCNA, and anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Animal protocols

Specific pathogen–free male C57Bl/6 mice (Orient Bio, Sungnam, Korea) weighing 20-22 g were used in all experiments. The Animal Care Committee of the Ewha Medical Research Institute approved the experimental protocol. Mice were cared for and handled in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Mouse pharyngeal aspiration was used for administration of the test solution [17]. Briefly, animals were anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg, *i.p.*, respectively), and placed individually on a board in a near vertical position. The animal's tongue was extended with a lined forceps. The test solution (30 μl) containing bleomycin (5 U/kg body weight) was then placed posteriorly in the throat and aspirated into the lungs. Control mice were similarly administered sterile saline (0.9 % NaCl). Mice revived unassisted after approximately 10-20 min.

Two days after bleomycin stimulation, saline alone or 10 x 10^6 apoptotic or viable Jurkat cells in 50 μl saline were *i.t.* administered through pharyngeal aspiration [17, 18] and mice were euthanized 2 hours post-apoptotic cell instillation, and on days 3, 5, 7, 14, and 21 following bleomycin treatment. Animals were euthanized 2 hours after instillation of apoptotic or viable cells into the bleomycin-stimulated lungs (2 days).

For anti-HGF antibody inhibition experiments, 250 μg of either neutralizing rabbit polyclonal antibody to mouse HGF or normal goat IgG (Santa Cruz, CA) in 200 μl saline was *i.p.* coadministrated at the same time with *i.t.* instillation of 10 x 10^6 apoptotic Jurkat T cells in 50 μl saline, or 50 μl saline alone into the bleomycin-stimulated lungs (2 days), and mice were euthanized on days 3 following bleomycin treatment. To inhibit sequential induction of HGF, the anti-HGF antibody or isotype antibody was administrated once more sequentially at 5 days after bleomycin treatment and mice were euthanized on days 3 or 7 following bleomycin treatment.

BAL cells, lung tissue, and cell counts

Bronchoalveolar lavage (BAL) was performed through a tracheal cannula using 0.7 ml aliquots of ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM dextrose; pH 7.4) to a total of 3.5 ml for each mouse. BAL samples were centrifuged at 500 x *g* for 5 min at 4°C, and cell pellets were washed and resuspended in phosphate-buffered medium. Cell counts were determined using an electronic Coulter Counter fitted with a cell sizing analyzer (Coulter Model ZBI with a channelizer 256; Coulter Electronics, Bedfordshire, UK). Neutrophils and alveolar macrophages were identified by their characteristic cell

diameters. BAL cells were isolated and cytospins were made to assess phagocytic indices [19, 20]. After BAL, lungs were removed, immediately frozen in liquid nitrogen, and stored at -70°C.

Induction of apoptosis

Human T lymphocyte Jurkat cells were obtained from the American Type Culture Collection (Rockville, MD). Jurkat T cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C and 5% CO_2. Apoptosis was induced by UV irradiation at 254 nm for 10 min and incubated for 2.5 hours before use. Cells were approximately 70% apoptotic by evaluation of nuclear morphology by light microscopy [21].

Alveolar macrophage and culture

Suspended alveolar macrophages from mice were over 95% viable as determined by trypan blue dye exclusion. Alveolar macrophages were isolated by adhesion (60 min) and cultured in serum-free X-vivo medium.

***In vitro* exposure of macrophages to stimulants for cytokine analysis**

Murine RAW 264.7 macrophages (American Type Culture Collection (ATCC) were plated at 10^6 cells/ml and incubated overnight in DMED (Media Tech Inc., Washington, DC, USA) supplemented with 10% heat-inactivated FBS plus penicillin-streptomycin-glutamine at 37°C in 5% CO_2. Before stimulation, the medium was replaced with serum-free X-vivo 10. The macrophages were stimulated with apoptotic or viable Jurkat T cells ($3x10^6$ cells/ml) and the supernatants were collected 18 h later. In some experiments, LPS at 1 ng/ml or bleomycin at 5 μg/ml was added to stimulate cytokine production. Apoptotic or viable cells, or apoptotic cells and anti-HGFR (c-Met) antibody (10 μg/ml) were added at the same time of the stimulus, then supernatants were collected 18 h later.

ELISA

BAL fluid samples were assayed using HGF, TGF-β1, TNF-α, and MIP-2 enzyme-linked immunosorbent assay (ELISA) kits as per the manufacturer's instructions. Alveolar macrophages (10^5/well) from the saline, bleomycin, bleomycin + apoptotic or viable cells groups were plated in a 24-well tissue culture plate and allowed to adhere for 60 min at 37°C. Wells were washed three times to remove nonadherent cells. Adherent cells were cultured for an additional 24 hours. After centrifugation, acellular

supernatants were harvested and HGF was measured by ELISA.

RT-PCR

Total RNA was isolated from lung tissue using TRIzol reagent as per the manufacturer's instructions. The concentrations and purities of the RNA samples were evaluated by spectrophotometry. Reverse transcription was conducted for 60 min at 42°C with 1 μg of total RNA using Advantage RT-for PCR kits. HGF-mRNA levels were determined using relative quantitative RT-PCR kits. The primer sequences used were mouse-specific HGF (sense 5′-ATC CAC GAT GTT CAT GAG AG-3′ and anti-sense 5′-GCT GAC TGC ATT TCT CAT TC-3′) and mouse-specific β-actin (sense 5′-GAT GAC GAT ATC GCT GCG CTG-3′ and anti-sense 5′-GTA CGA CCA GAG GCA TAC AGG-3′). cDNA was denatured for 5 min at 94°C and amplification was achieved in a thermocycler (Perkin Elmer GeneAmp PCR System 2400, Foster City, CA) with 24 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a 7-min final extension at 72°C. A total of 5 μl of each PCR sample was loaded on a 1.5% agarose gel stained with ethidium bromide. The relative fluorescence of HGF versus β-actin was analyzed by densitometry.

Measurement of total protein

Protein concentrations of the BAL samples were used as indicators of blood-pulmonary epithelial cell barrier integrity. Total protein content was measured according to the method of Hartree [22] using bovine serum albumin as a standard.

Western blot analysis

Lung tissue homogenate samples and total cell lysates (50 μg protein/lane) were separated on 10% SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred onto nitrocellulose paper and blocked for 1 hour at room temperature with Tris-buffered saline containing 3% BSA. Blocked membranes were incubated at room temperature for 1 hour with PCNA, or β-actin antibodies and visualized by chemiluminescence (ECL).

Measurement of hydroxyproline

Lung hydroxyproline content was measured using a hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China PR) as per the manufacturer's instructions.

Lung histology

Lung tissue was fixed with 10% buffered formalin with gentle perfusion through the trachea for 24 hours and was then embedded in paraffin. Sections 3 μm-thick were stained with hematoxylin-eosin (H&E) and with Masson's trichrome to evaluate inflammation and collagen deposition, respectively.

DNA damage and apoptosis in lung tissue

DNA fragmentation of apoptotic cells was detected in the lung tissue using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit, according to the manufacture's protocols. Briefly, section of formalin-fixed, paraffin-embedded lung tissue was examined by TUNEL in each mouse. The samples were biotinylated at 37°C for 1 h and the endogenous peroxidase were blocked in 0.3% H_2O_2. The samples were treated with Streptavidin-HRP at room temperature for 30 min and 3,3'-diaminobenzinine (DAB) and cells were counted with the light microscope. Positive staining is indicated by black-brown. Almost whole fields of each section were studied at an original magnification of 200-fold. In each field, we counted the number of positive cells in each field and calculated the means of all the numbers per field in each group.

Caspase-3 and Caspase-9 Activities

The bioactivity of caspase-3 and 9 was measured with a Fluorometric Assay Kit (ABcam, Cambridge, UK). In brief, 25 μg lung homogenate samples were added to reaction buffer and then incubated with caspase-3 substrate DEVD-AFC, or caspase-9 substrate LEHD-AFC. The fluorescence of the cleaved substrates was determined at an excitation wave length of 400 nm and an emission wave length of 505 nm.

Immunohistochemistry for HGF and PCNA

Four μm-sections were obtained from formalin-fixed, paraffin-embedded tissues. Slides were deparaffinized twice in xylene for 5 minutes and rehydrated through graded ethanol solutions to distilled water. Antigen retrieval was performed by heating the sections in a microwave oven in 10 mM citrate buffer (pH 6.0). Sections were then immunostained using an automated machine (Bond™ Automated Immunohistochemistry, Leica, Wetzlar, Germany) and the Bond Polymer Detection System with counterstain (Leica). The process included endogenous peroxidase blocking with 3% H_2O_2 for 5 min, incubation with primary antibodies for HGF (H-145, rabbit polyclonal, Santa Cruz Biotechnology, CA) or control rabbit IgG, and PCNA (ab2426, rabbit polyclonal,

Abcam, Cambridge, UK) at room temperature for 30 minutes, incubation with polymeric HRP-linker antibody conjugates as secondary antibodies, and expression using 3,3'-diaminobenzidine. The number of PCNA-positive cells was counted in three randomly-chosen microscopic fields per tissue section at a magnification of 200× by an observer who was blinded to the animal group assignment.

Statistical analysis

Values are expressed as mean ± SEM. Analysis of variance (ANOVA) was applied for multiple comparisons and Tukey's post hoc test was applied where appropriate. Student's *t* tests were used for comparisons of two sample means. A p value of <0.05 was considered statistically significant. All data were analyzed using JMP software (SAS Institute, Cary, NC).

References

(Note: reference numbers correspond to reference list in main articles)

17. Rao GVS, Tinkle S, Weissman DN, Antonini JM, Kashon ML, Salmen R, Battelli LA, Willard PA, Hoover MD, Hubbs AF. Efficacy of a technique for exposing the mouse lung to particles aspirated from the pharynx. *J Toxicol Environ Health A* 2003;66:1441-1452.

18. Huynh, M.L., Fadok, V.A., and Henson, P.M. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J. Clin. Invest.* 109:41-50.

19. Moon C, Lee YJ, Park HJ, Chong YH, Kang JL. N-acetylcysteine inhibits RhoA and promotes apoptotic cell clearance during intense lung inflammation. *Am J Respir Crit Care Med* 2010;181:374-387.

20. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma: persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997;156:737-743.

21. Hoffman PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, Ridley AJ, Fadok VA, Henson PM. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol*

2001;155:649-659.

22. Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 1972;48:422-427.

Legends for Supplementary Figures

Supplementary Figure 1. Effects of *in vivo* exposure to apoptotic cells into unstimulated lungs on production and mRNA expression of HGF. Two hours after intratracheal instillation of saline alone (Sal), or apoptotic Jurkat T cells (ApoJ) into unstimulated lungs, bronchoalveolar lavage was performed. (A, C) HGF production in BAL fluid samples and alveolar macrophage cultures was quantified by ELISA. (B) HGF mRNA levels in lung homogenates were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (D) Phagocytic indices (PIs) were measured in BAL alveolar macrophages. Values represent means ± SEM from groups of five mice. * Significantly different from saline control, $p<0.05$.

Supplementary Figure 2. *In vivo* production and mRNA expression of HGF in bleomycin-stimulated lungs are increased by apoptotic cell clearance. Two hours after intratracheal instillation of saline alone (BLM+Sal), apoptotic HeLa cells (BLM+ApoH), or viable HeLa cells (BLM+ViaH) into bleomycin-treated lungs (day 2), bronchoalveolar lavage was performed. (A, C) HGF production in BAL fluid samples and alveolar macrophage cultures was quantified by ELISA. (B) HGF mRNA levels in lung homogenates were analyzed by relative quantitative RT-PCR and normalized to β-

actin mRNA levels. (D) Phagocytic indexes (PIs) were measured in BAL alveolar macrophages. Values represent means ± SEM from groups of five-ten mice. * Significantly different from saline control, $p<0.05$. $^{+}$Significant differences between the BLM+ApoH group versus the BLM+Sal or BLM+ViaH group, $p<0.05$.

Supplementary Figure 3. Anti-HGF antibody abrogated the anti-inflammatory and anti-apoptotic effects of apoptotic cell instillation. Rabbit anti-HGF neutralizing antibody (anti-HGF Ab) or normal goat IgG (Isotype Ab) was *i.p.* coadministrated with saline (Sal) alone or apoptotic Jurkat T cells (ApoJ) into bleomycin-stimulated lungs (2 days), and mice were euthanized on days 3 following bleomycin treatment. To inhibit sequential induction of HGF, the anti-HGF antibody or isotype antibody was administrated once more sequentially at 5 days after bleomycin treatment and mice were euthanized on days 7 following bleomycin treatment. (A) TNF-α, (B) MIP-2, and (C) TGF-β1 levels in BAL fluid were quantified by ELISA. (D) Neutrophil and (E) total protein levels in BAL fluid. (F, G) Caspase-3 and 9 activities in lung tissue. Values represent means ± SEM from groups of 5 mice. $^{+}$Significant differences between the groups, $p<0.05$.

Supplementary Figure 4. Contribution of HGF to the inhibition of proinflammatory cytokines which occurs after apoptotic cell treatment to LPS or bleomycin-stimulated macrophages *in vitro*. (A-E) RAW 264.7 macrophages were treated with 1 ng/ml LPS or 5 μg/ml bleomycin at the same time, apoptotic cells, viable Jurkat cells, or apoptotic cells and anti-HGFR were added. Supernatants were collected 18 h later**.** (A, D) TNF-α, (B, E) MIP-2, and (C) HGF levels in the conditioned medium were quantified by ELISA. Values represent means ± SEM of five separate experiments. $^{+}$ $p<0.05$.

Supplementary Figure 5. Proliferating cell nuclear antigen (PCNA) protein expression and immunostaining in lung tissue. Mice were *i.t.* instilled with saline (BLM+Sal) or apoptotic Jurkat T cells (BLM+ApoJ) 2 days after bleomycin treatment and lungs were harvested on days 3-21. (A) Lung tissue homogenates were immunoblotted for PCNA. Relative values of PCNA versus β-actin are indicated below the gel. (B) Representative photomicrographs showing PCNA-positive cells on days 7 and 21 after bleomycin treatment. Dark brown nuclei indicate positive staining. Original magnifications: ×200. (C) Relative-quantification of histological assessment of PCNA-positive cells. Values represent means ± SEM from groups of five mice. * Significantly different from control, $p<0.05$. $^{+}$Significant differences between the BLM+ApoJ group versus the BLM+Sal

group, $p<0.05$.

Supplementary Figure 6. HGF receptor antibody further enhances TGF-β1 production induced by apoptotic cells. RAW macrophages were pretreated 10 μg/ml anti-HGFR antibody or 10 μg/ml IgG for 1 hour and then stimulated with apoptotic Jurkat cells for 18 hours to detect TGF-β1 production. TGF-β1 levels in the conditioned medium were measured by ELISA. Values represent means ± SEM of five separate experiments, *$p<0.05$.