Supplemental Data

Acute Inflammation Induces IGF-1 to Mediate Bcl-2 and Muc5ac Expression in Airway Epithelial Cells

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METHODS

Generation of LPS Aerosols. LPS aerosols were generated by nebulization of LPS (*Pseudomonas aeruginosa* serotype 10, approximately 900,000 EU/mg; Sigma, St. Louis, MO) essentially as described previously (E1). Briefly, LPS aerosols were generated in a glove box connected to an exhaust system. LPS solutions were nebulized with a mini-HEART™ (Critical Care Concepts, Atlanta, GA) nebulizer followed by a diffusion drier (custom flow-through drier with Dri-rite™ dessicant) that dried the aerosol (high humidity saturated sample filters and impactor substrates). Aerosolized LPS was delivered to a 24-port nose-only inhalation chamber (In-Tox Products, Inc., Albuquerque, NM) operated at approximately 30 L/min.

Exposures were monitored by collecting exposure atmosphere on Teflon membrane filters (TEFLO, Pall-Gelman) that were weighed prior to and after sample collection. Filters were extracted with water and assayed by the Limulus assay described below. During exposure, aerosol concentration was analyzed in real-time with a RAM-S nephelometer to adjust dilutions and maintain the target exposure concentrations.

Particle Size Determination. Particle size distribution was determined with a 7-stage Mercer style impactor (In-Tox Products, Inc., Albuquerque, NM). The aerosol was withdrawn directly from the exposure chamber atmosphere. Particle size was approximately 1 μm with a geometric standard deviation of ~2.0.

Bronchoalveolar Lavage Collection and Cell Counts. At 72 h post-exposure mice were euthanized by injecting them with pentobarbital followed by exsanguination through the renal artery. The lung vasculature was perfused via an injection of 5 ml cold pathogen-free saline through the heart. The trachea was cannulated with an 18 gauge blunt needle and the lungs were removed. The lungs were lavaged three times using the same 0.5 ml of saline and inflated with 10% zinc formalin under a constant pressure of 25 cm H_2O for 3 h. BAL was centrifuged at 1200 RPM in a Beckman centrifuge at 4^{o}C for 5 minutes. Supernatant was collected and

aliquoted at -80^{o}C for cytokine analyses. The cell pellet was diluted in 0.5 ml of media and BAL cell number was enumerated under a microscope using a hematocytometer. Cytospin slides were prepared from 25,000 cells, stained with Hematoxylin/Giesma, and cell differential quantified as described previously (E2).

Histochemical Staining and Analysis. Histochemical staining for Alcian Blue and periodic acid Schiff (AB-PAS) was carried out as previously described (E3). Airway epithelial cell and mucous cell numbers per mm basal lamina (BL) were measured using the VisioMorph system (Visiopham A/S, Horsholm, Denmark) by counting the number of nuclei and mucous cells, respectively and dividing by the length of the BL. In all cases, quantification and morphometry was carried out by a person unaware of slide identity.

Luminex Analysis. The levels of cytokines and chemokines (IL-1β, IL-6, IL-9, IL-12, TNFα, IFNγ, Gro/KC, IP-10, MCP-1 and MIP-1α) in BAL fluid were determined using a multiplex assay kit (Lincoplex panel, Linco Research, Inc., St. Charles, MO) according to the manufacturer's instructions. Briefly, the beads were incubated first with diluted standards or BALF overnight and then with a detector antibody cocktail for 60 min each at room temperature. After two washes in PBS supplemented with 0.02% Tween 20, 0.1% BSA, and 0.02% NaN3, the beads were incubated for 30 min with fluorescent dye-conjugated streptavidin. Cytokine levels were measured using a flow cytometer and were analyzed with Flowmetrix software (Luminex, Ausitn, TX). Standard curves for each cytokine and chemokine were generated on a log-log plot for each assay, and the concentrations in each sample were calculated from the corresponding curve-fitting equations (E4). The data were analyzed using a Luminex 100-plate reader.

Muc5ac ELISA. Muc5ac levels in BAL fluid was detected using a specific sandwich ELISA. Briefly, 96-well plates were coated with 5 µg/ml Muc5ac antibody (MAB2011, Millipore Inc.,

Temeculla, CA) for 24 h at 4°C and were blocked with 2% BSA in PBS for 1 h at 37°C with intermittent thorough washing. After washing, samples and standard mucin preparation (Porcine Gastric Mucin, #M1778, Sigma Co.) diluted in PBS buffer with 1% BSA and DTT (15 mg/ml) were added and incubated for 1 h at 37°C. The bound Muc5ac was detected using biotin-conjugated wheat-germ lectin (Sigma Co., St Loius, MO) at 1µg/ml followed by incubation with streptavidin-peroxidase (Vectastain). The colorimetric detection was performed using TMB substrate (Sigma Co.) with absorbance measured at 450 nm using a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) plate reader with a correction factor measured at 640 nm. The relative quantities of Mac5ac were obtained by normalizing data to the control group.

Immunofluorescent Staining and Image Analysis. Lung sections were deparaffinized, hydrated in graded ethanol and deionized water, then washed in 0.05% v Brij-35 in Dulbecco's PBS (pH 7.4). The antigens were unmasked by treating with Digest-All kit (Zymed Laboratories, San Francisco, CA) at a 1:3 dilution of trypsin to diluent at 37°C for 10 min. Sections were then blocked using 0.2% Triton X-100 with 0.2% Saponin in a blocking solution containing 3% IgG-free BSA, 1% Gelatin and 2% normal donkey serum followed by incubation with anti-Bcl-2 (N19, Santa Cruz Biotech, CA), anti-Muc5ac (MAB2011, Millipore, CA), anti-IGF-1 (H70, Santa Cruz Biotech, CA), or isotype controls at 1:200 dilution. The immunolabeled cells were detected using F(ab)2-fragments of respective secondary antibodies conjugated to either DylightTM-549 or DylightTM-649 (Jackson Immunoresearch, West Grove, PA) at 1:1000 dilution and mounted with 4',6-diamidino-2-phenylindole (DAPI) containing Fluormount-GTM (SouthernBiotech, Birmingham, AL) for nuclear staining. Digitized images of fluorescently-labeled sections were captured using Axioplan 2 imaging system (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped with a ORCA-ER CCD camera (Hamamatsu Photonics, Japan) coupled with Lambda DG-4 wavelength switch (Sutter Instrument, Novato, CA) and acquisition software Slidebook™ 5 (Intelligent Imaging Innovations, Inc. Denver, CO). For three-dimensional multi-channel image

processing images were visualized using Fiji image processing package of ImageJ (E5). To visualize the labeled epithelial cells, a rendering mode was used, which shows the maximum intensity projection (i.e., the maximum intensity of all layers along the viewing direction) of the recorded three-dimensional stack.

For cytometry, cells were grown on Lab-Tek-II 8-chamber slides (Nalge Nunc International, Rochester, NY) and treated with 10ng/ml of IL-1β or were left untreated and were fixed using 3% paraformaldehyde with 3% sucrose in PBS and antigens were retrieved by steaming slides in 10mM citrate buffer for 0.5 h. Slides were blocked and immunostained as described above. Quantification of Bcl-2-positive, IGF-1-positve and Muc5ac-positive cells was performed using the NIH Image J or VisioMorph software (Visiopham A/S, Horsholm, Denmark) with minimum of 300 cells counted per treatment group.

Quantitative RT-PCR. RNA was isolated from the snap-frozen right lungs of animals using TRIzol as described previously (E5) whereas RNA from cultured cells was extracted using the RNeasy kit (Qiagen, Valencia, CA). The primer/probe sets for Bcl-2, Muc5ac , IGF-1, and CDKN1B were obtained from Applied Biosystems (Foster City, CA) and were amplified by quantitative real-time PCR using RT-PCR Master Mix (Applied Biosystems, Foster City, CA) in the ABI-PRISM 7900HT Real-Time PCR System. Relative quantities were calculated by normalizing averaged CT values to that of CDKN1B or 18s RNA to obtain ΔCt, and the relative standard curve method was used for determining the fold change as described previously (E6).

Western blot analysis. Protein was extracted by homogenization in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 5 mM EDTA) supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) at 1:100 final concentration. Protein concentration was determined using the BCA kit (Pierce, Thermo Fisher Scientific, Rockford, IL) and 100 μg of protein lysate was analyzed. The Bcl-2 and β-

actin were detected using appropriate peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Perkin Elmer, Waltham, MA) using the FujiFilm Image Reader LAS-4000 (Valhalla, NY).

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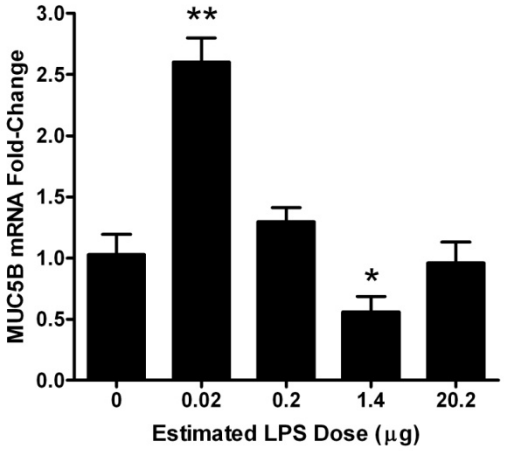

Figure E1. Muc5b mRNA levels in lung tissues of mice exposed to LPS as analyzed by qRT-PCR and the fold-change (2^{-ddCT}) calculated after normalizing to respective CDKN1B levels. The data is shown as fold-change over control mice (0 μg of LPS). Data shown as mean ± SEM; n = 6 per group; * $p < 0.05$, ** $p < 0.01$.

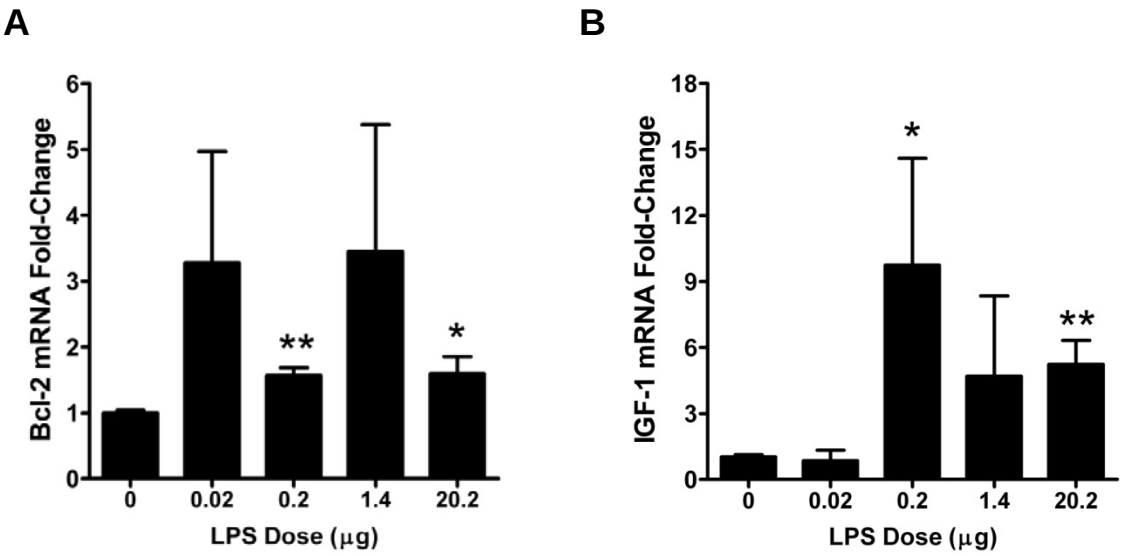

Figure E2. Bcl-2 and IGF-1 mRNA levels in lung tissues of mice exposed to LPS as analyzed by qRT-PCR. The fold-change (2^{-ddCT}) was calculated after normalizing to the respective 18s RNA levels. The data is shown as fold-change over control mice (0 μg of LPS). Data shown as mean ± SEM; n = 3/group; * $p < 0.05$, ** $p < 0.01$.

z-S-Image 1. A z-stack video of a representative lung section from mice exposed to 20.2 µg aerosolized LPS and immunostained for Bcl-2 (green) and Muc5ac (red) showing co-expression in airway epithelial cells, DAPI-stained nuclei (blue). Video was captured at 2 frames per second (fps) with 14 frames of 0.3 µM spanning 4.2 µM; (scale – 10 µM).

z-S-Image 2. A 3-D rendering view of the lung section from mice exposed to 20.2 µg aerosolized LPS and immunostained for Bcl-2 (green) and Muc5ac (red) showing co-expression in airway epithelial cells, DAPI-stained nuclei (blue). Video was captured at 7 fps of 4.2 µM thick image with 360º view moving at 1º per view; (scale – 10 µM).

z-S-Image 3. A z-stack video of a representative lung section from mice exposed to 20.2 µg aerosolized LPS and immunostained Bcl-2 (green) and IGF-1 (red) showing co-expression in airway epithelial cells, DAPI-stained nuclei (blue). Video was captured at 2 frames per second (fps) with 14 frames of 0.3 µM spanning 4.2 µM; (scale – 10 µM).

z-S-Image 4. A 3-D rendering view of the lung section from mice exposed to 20.2 µg aerosolized LPS and immunostained for Bcl-2 (green) and IGF-1 (red) showing co-expression in airway epithelial cells, DAPI-stained nuclei (blue). Video was captured at 7 fps of 4.2 µM thick image with 360º view moving at 1º per view; (scale – 10 µM).