Online Data Supplement

The effect of Class II Transactivator (CIITA) mutations on bleomycin induced lung inflammation and fibrosis.

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Detailed methods:

Cell isolations:

Fibroblasts were isolated from the lungs of 19 day old CIITA C-/- and CIITA G-/- mice and wild-type controls (C57BL/6J) (Jackson Laboratory, ME) essentially as previously described(6). Briefly, lungs were removed from mice, minced into small pieces (1-2 mm^3) and digested with Puck's Saline A (1 lung equivalent/ ml) containing 0.05% trypsin and 0.001% DNase at 37^o C for 20 min. The digest was filtered through a 100-μm Nucleopore filter to remove large tissue fragments, centrifuged (300 x g, 10 min), and the cell pellet washed once with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mg/ml fungizone, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete media) and cells were seeded in three flasks (25 cm^2). To enrich for mesenchymal cell types, cells were allowed to adhere for 2 h before the medium was replaced. After 1 week, the cells were released with trypsin, collected by centrifugation, re-suspended in complete medium and counted. Cells were then seeded at 1 x 10^6 cells/flask (25 cm^2) and incubated for 8-14 days with the medium changed every 3 days. The cells were passed a second time following the procedure described above and again seeded at 1 x 10^6 cells/flask (25 cm^2) for experiments that follow.

Cell maintenance and treatment protocols:

Mouse lung fibroblasts were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone). Cells were plated in 6-well culture plates at density 2x10^5 cells per well and incubated at 37^o C with 5% CO_2 for 16-24 hours. In several studies, mouse cells were treated with IFN-γ. Cells were plated in p35 tissue culture dishes at 4x10^5 cells/dish for mRNA studies

Transfection and luciferase assay:

Transfections were performed in serum-free DMEM using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Three hours after transfection, cells were transferred to DMEM with 10% FBS and harvested 24 hours later. After cell lysis, luciferase activities were assayed using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells. The ratio of experimental to control (reporter plasmid with empty vector) was calculated for each experiment with the control values set at 1. Values from three or more experiments were averaged and presented in this paper.

Bleomycin Experiments:

Adult (8-week old) CIITA or C57BL/6 mice were anesthetized with isoflurane and treated with bleomycin (0.1 U/100 μl saline/20 gm mouse) or saline (0.14 M NaCl) by intratracheal instillation as described (24). Animals were euthanized at 3–14 days post bleomycin instillation, as indicated in the text. A minimum of four animals was used for each group. Mice were weighed at 0, 5, 7, 10 and 14 days post instillation. Animals were anesthetized with I.P. injection of 0.1 ml phenobarbitol. The thoracic cavity was opened, the dorsal aorta was cut and the lungs were perfused with sterile saline followed by freshly prepared 4% paraformaldehyde in phosphate buffered saline (pH 7.4). The lungs were inflation fixed by intratracheal

instillation of 4% paraformaldehyde. The trachea was ligated just caudal to the larynx and the lungs were excised and placed in 4% paraformaldehyde at 4°C for 16h. The lungs were processed by dehydration in ethanol followed by xylene and embedded in paraffin for histology. Approximately 10-20 sections were prepared from each block by serially sectioning. For some experiments, the entire right lung was placed in RNALater and processed for RNA analysis as described above.

RNA isolation and real-time PCR:

Cells or lung tissue were harvested and RNA was extracted using an RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on ABI Prism 7700 sequence detection PCR machine (Applied Biosystems) according to manufacturer's protocol. The oligonucleotide primers (forward and reverse) and Taqman probes are described previously (6, 15).

Histology:

Mouse lung sections were stained with Masson's trichrome and picrosirius red (Sigma) according to standard techniques and parallel sections were stained for collagen type I and cell surface markers. The sections were then blocked with 10% normal goat serum (Vector Laboratories, Inc) for 1 hour at room temperature and then incubated with purified antibodies, collagen α1(I) (Rockland) (1:200), CD3 (clone 145-2C11, BD Bioscience)(1:100 dilution), CD45 (clone 30-F11, BD Bioscence)(1:250), F4/80 (clone C1:A3-1, AbD Serotech)(1:50 dilution). An IgG isotype (BD Pharmingen) at an identical dilution to antibody was used as controls. Staining was achieved using an appropriate biotinylated secondary antibody (Vector Laboratories, Inc, Burlingame, CA) for 30 min at room temperature. Sections were incubated with ABC-AP reagent (Vector Laboratories, Inc.) and developed using the Vector Red alkaline phosphatase kit (Vector Laboratories). Sections were counterstained with Gill 3 Hematoxylin (Thermo Shandon). Digital images, 6 per slide, were acquired using an Olympus IX-70 microscope. The amount of red or blue staining was quantified using Image-Pro Plus (Media Cybernetics) or ImageJ (NIH). The disease index, an estimate of the proportion of the lung that is diseased was used to describe the extent of the disease process as described previously (25).

Bronchoalveolar Lavage (BAL) cell analysis:

After anesthetizing mice, bronchoalveolar lavages (BALs) were collected using two 1 ml aliquots of PBS. Cells were centrifuged and re-suspended in 1 ml PBS. A small portion of the cells (~10 μl) were counted by hemocytometer to determine total cells recovered. The remainder of the cells were collected by centrifugation and used for RNA isolation and the supernatant fluid (BALF) was used to analyze active TGF-β.

Active BAL Fluid TGF-Beta Analysis

In brief, BALF or standard concentrations of TGF-β1 were applied to 2 x 10^4 mink lung epithelial cells (MLEC) stably transfected with a plasminogen activator inhibitor-1 promoter–luciferase reporter construct plated at a density of 2 x 10^4 per 96 well in complete DMEM until adherent (~3 hours)(26). Media was aspirated, washed twice with PBS, and supplemented with 100 μL without serum. BALF (100 μl) or human TGF-β (50-1000 pg/ml), serving as a standard, was added to each well in triplicate and incubated at 37°C for 20 hours. Cells were lysed in 1x reporter lysis buffer (Promega, Madison, WI) and frozen at -80° C overnight. Cell lysates were read directly on an auto-injecting BioTek plate reader using 100 μL of luciferase assay substrate (Promega, Madison, WI). The average luciferase unit of BAL fluid replicates were translated into active TGF-β concentrations using the average of standard triplicates and reported as picograms per milliliter.

Collagen Extraction and Sircol Assay for Collagenous Protein

Lungs were minced, homogenized and subjected to neutral salt extraction in RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, and protease inhibitors) for 24 hr with agitation by rotation at 4^{o}C. Homogenates were pelleted by centrifugation and resuspended in 0.5M acetic acid and agitated by stirring for 24 hr at 4^{o}C. Supernatants were collected and stored at -80^{o}C. An equivalent amount of supernatant, relevant to the mass of tissue used in the extraction, was used in the Sircol assay (Biocolour, U.K). Briefly, RIPA extract, acetic acid extract, or rat-tail collagen (standard) was incubated with 1 ml of Sircol reagent for 30 min with shaking. The samples were centrifuged at >15,000 x g for 10 min. Supernatant was decanted and residual dye was removed using a cotton swab. Pellets were dissolved in 1 ml of 0.1 M KOH for 10 min and subjected to spectrophotometric analysis at A=540 nm. BSA standards served as negative control for non-specific binding of Sircol dye.