Cigarette Smoke-Induced Emphysema in A/J mice is Associated with Pulmonary Oxidative Stress, Apoptosis of Lung Cells, and Global Alterations in Gene Expression

Supplementary Files

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Supplementary Methods

Antibodies and Reagents

InnoGenex™ Iso-IHC DAB kit (InnoGenex, San Ramon, CA); biotinylated anti-mouse IgG and peroxidase-conjugated streptavidin, Vectashield HardSet mounting medium and Vector RTU HRP-avidin complex (Vector Laboratories, Burlingame, CA); rabbit anti-surfactant protein C (SpC) antibody (Chemicon International, Inc., Temecula, CA); anti–caspase-3 polyclonal antibody (Idun Pharmaceuticals, San Diego, CA); anti-rabbit Texas red antibody, streptavidin-Texas red conjugated complex and DAPI (Molecular Probes Inc., Eugene, OR); biotinylated rabbit anti-mouse secondary antibody (DakoCytomation, Carpinteria, CA); Fluorescein-FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA); Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA); Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw Park, IL); halothane (Halocarbon Laboratories, River Edge, NJ); QuickHyb solution (Stratagene, Carlsbad, CA); normal mouse IgG1 (Sigma-Aldrich, St. Louis, MO); anti-CD34 antibody (Zymed Laboratories, Inc., South San Francisco, CA).

Exposure to Cigarette Smoke

A/J mice were divided into five groups: Group I mice (n = 80) were kept in filtered air environment, and Group II, Group III, Group IV, and Group V mice were exposed to 1 day, 8 days, 1.5 months (mos), and 6 mos CS (n = 35 mice/group), respectively, as previously described (20). Cigarette smoke was generated using an automated cigarette-smoking machine (Model TE-10, Teague Enterprises, Davis, CA) by burning 2R4F reference cigarettes (2.45 mg nicotine per cigarette; purchased from the Tobacco Health Research Institute, University of Kentucky, Lexington, KY). The cigarettes were kept in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water for 48 h before use in the study. The mice were

exposed to CS for 7 hours/day, 7 days/week for up to 6 months. Smoke generated by the TE-10 cigarette-smoking machine is a mixture of cigarette mainstream (11%) and side-stream smoke (89%), mimicking an exposure to environmental tobacco smoke. The cigarettes were smoked with standard puffs of 35 ml volume of 2-second duration. The smoke machine was adjusted to burn 5 cigarettes at one time. The total particulate matter in the exposure chambers was on an average 90 mg/m^3, and carbon monoxide concentration was 350 ppm. Age matched room filtered air exposed mice were used as control for all end points.

Bronchoalveolar Lavage and Phenotyping

For bronchoalveolar lavage and phenotyping, the mice (n = 7 per group) exposed to acute (1 day) and chronic (6 months) CS were anesthetized with 0.3 ml of 65 mg/ml pentobarbital 18 h post-CS exposure. The BAL fluid collected from the lungs of the mice with sterile phosphate buffered saline (3 × 1 ml containing 5 mM EDTA, 5 mM DTT, and 5 mM PMSF and the cocktail of protease inhibitors) was centrifuged at 1500 × *g* for 5 min at 4°C. The cell pellet was resuspended in 1 ml phosphate-buffered saline, and the total cell count was measured. Phosphate buffered saline containing 2×10^4 cells were cytocentrifuged (Shandon Southern Products, Pittsburgh, PA) onto glass slides and stained with Diff-Quick reagent. Differential cell counts were performed on 300 cells using standard morphological criteria (20).

Localization of Macrophages in the Lungs

The macrophages in the lung tissues were stained using *Griffonia (Bandeiraea) simplicifolia* lectin I isolectin B4 (Vector Laboratories, Burlingame, CA) (21, 22). Briefly, the lung sections (n = 5 per group) were first incubated with 20% normal goat serum in TBS containing 0.1% BSA for 1 h, and then stained with biotinylated *Griffonia simplicifolia* lectin I isolectin B4 (10 μg/ml) in 10 mM HEPES buffer (pH 7.5) containing 150 mM NaCl for

overnight incubation at 4°C. After washing 3 times with HEPES buffer, the lung tissues were incubated for 30 min with avidin-alkaline phosphatase conjugate (Vector Laboratories, Burlingame, CA). Lectin stained macrophages were visualized by incubation with Dako liquid red permanent alkaline phosphatase reagent (DakoCytomation, Carpinteria, CA). Sections were counterstained with hematoxylin, and the images of the lung sections (10 per lung section) were captured using Nikon Eclipse E800 microscope (Nikon, Melville, NY) with a 20X lens. The lectin positive cells in the lung sections were counted manually.

Identification of Alveolar Apoptotic Cell Populations in the Lungs

To identify the different alveolar cell types undergoing apoptosis in the lungs, a fluorescent TUNEL labeling was performed on the lung sections from the CS-exposed (6 months) and age-matched air-exposed A/J mice, using the Fluorescein-FragEL DNA Fragmentation Detection Kit as described earlier (20). Apoptotic type II epithelial cells in the lungs were identified by incubating the TUNEL-labeled lung sections first with an anti-mouse SpC antibody and then with an anti-rabbit Texas red antibody (20). Apoptotic endothelial cells were identified by incubating the fluorescent TUNEL-labeled sections first with the anti- CD34 antibody and then with the biotinylated rabbit anti-mouse secondary antibody. The lung sections were rinsed with PBS and then incubated with the streptavidin-Texas red conjugated complex. Finally, DAPI was applied to all lung sections, incubated for 5 minutes, washed, and mounted with Vectashield HardSet mounting medium. DAPI and fluorescein were visualized at 330-380 nm and 465-495 nm, respectively. Images of the lung sections were acquired with the Nikon E800 microscope (Nikon) using 40X lens.

Oligonucleotide Microarray

Lungs were isolated and total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). The extracted RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA). The quality of the RNA was assessed using the RNA 6000 nano assay kits (Agilent Technologies, Palo Alto, CA). The isolated RNA was applied to Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) (n = 3 per group) as described previously (20). Scanned output files were analyzed using Affymetrix GeneChip Operating Software (GCOS) Version 1.3 and were independently normalized to an average intensity of 500. To identify the differentially expressed transcripts, pairwise comparison analyses were performed using the DMT 3.0 program (Affymetrix, Santa Clara, CA). Only genes that changed in at least 6 out of 9 comparisons, in which the absolute fold-change (FC) was greater than 1.5 ($P \leq 0.05$; Mann-Whitney test) were considered statistically significant with respect to differential gene expression. The following group comparisons were made: 1) 5 h CS vs 5 h air; 2) 8 days CS vs 8 days air; 3) 1.5 months CS vs 1.5 months air; and 4) 6 months CS vs 6 months air. In addition, for the air-exposed control dataset, the following group comparisons were made: 1) 8 days air vs 5 h air; 2) 1.5 months air vs 5 h air; and 3) 6 months air vs 5 h air (see supplementary Table 1). For all microarray experiments, RNA isolated from the age-matched filtered room air-exposed mice were used as control. NetAffx (11) was used to extract gene ontology information for the gene expression profiles. For genes with multiple probes, a single probe with the highest magnitude FC (positive or negative) was retained representing that gene. The data from the comparison of four different groups and timepoints were collated using Microsoft® Excel software. The group of over-expressed transcripts [1190 genes (5 h CS), 715 genes (8 d CS), 260 genes (1.5 months CS), and 246 genes (6 months CS)] in the lungs (CS vs. air) were analyzed for functional clusters using the gene ontology annotations or the scientific literature describing

those networks (see supplementary Table 1). The down-regulated transcripts [1840 genes (5 h CS), 730 genes (8 Days CS), 442 genes (1.5 months CS), and 236 genes (6 months CS)] were analyzed in an identical fashion (see supplementary Table 2). We preselected gene ontology categories of particular relevance to pulmonary emphysema. Microarray datasets were deposited to the Gene Expression Omnibus (GEO): http://www.ncbi.nlm.nih.gov/projects/geo/with accession #: GSE 8790. The genes that were commonly up-regulated in the lungs during all time points of CS exposure are represented in the supplementary Table 3. The total number of up-regulated or down-regulated genes in each functional category are shown in the supplementary Table 4. We compared (see supplementary Table 5) the gene expression profiles of 6 months CS-exposed A/J mice with the gene expression profile of chronic CS-exposed rats (24) and with the gene expression profile of lung tissues from human COPD (3, 17). Down-regulated genes whose deletion or overexpression has been shown to cause emphysema are listed in the supplementryl Table 6.

Supplementary Results

Increased Infiltration of Inflammatory Cells in the BAL Fluid and in the Lungs of Chronic CS-exposed Mice

Both acute (71.8 × 10^3 cells/ml BAL fluid of 1 day CS-exposed mice vs 42 X × 10^3 ± cells/ml BAL fluid of 1 day air exposed mice) and chronic (140 × 10^3 cells/ml BAL fluid of 6 months CS-exposed mice vs 44.3 × 10^3 cells/ml BAL fluid of 6 months air-exposed mice) exposure to CS increased the total number of inflammatory cells in the BAL fluid compared with the respective air-exposed animals (supplementary Fig. 1A and B). The total number of inflammatory cells in BAL fluid from 6 months CS-exposed mice (140 × 10^3 cells/ml BAL fluid) was significantly ($P \leq 0.05$) higher than the acute (1 day) CS-exposed mice (71.8 × 10^3 cells/ml BAL fluid). We did not see any significant difference in the total BAL inflammatory cell population between the air-exposed mice (1 day or 6 months). Among the inflammatory cell population, the predominant cell type were macrophages, constituting 87–90% of the total inflammatory cell population in the BAL fluid of the mice exposed to both acute and chronic CS. Other inflammatory cells such as polymorphonuclear leukocytes, eosinophils, and lymphocytes constituted 10–13% of the total inflammatory cells in the BAL fluid. Immunohistochemical staining with lectin revealed the presence of an increased number of macrophages (supplementary Fig. 1C and D) in the emphysematous lungs of 6 months CS-exposed mice (61.92 macrophages/10 fields) compared with lungs of their age-matched air-exposed counterparts (0.82 macrophages/10 fields).

Validation of Antioxidant Gene Expression by Real Time RT-PCR

Although inflammation and protease/antiprotease imbalance have been postulated to be critical in CS-induced emphysema, oxidative stress has been suspected to play an important role in COPD. Susceptibility of the lung to oxidative injury due to inhalation of CS depends largely on up-regulation of various Nrf2-regulated antioxidant and Phase II detoxification genes (20). Microarray analysis revealed reduced expression of multiple Nrf2-regulated antioxidant and Phase II detoxification genes in the emphysematous lungs of A/J mice compared to the acute CS-exposed lungs. We used real time RT-PCR analysis to confirm the relative expression of three classical antioxidant and Phase II detoxification genes such as HO-1, GCLc, and GSR. Real time RT-PCR results revealed a significantly ($P \leq 0.05$) reduced expression of these three antioxidant and Phase II detoxification genes in the lungs of the mice exposed to CS for 8 days, 1.5 months, and 6 months compared to the lungs of the mice exposed to CS for 1 day (see supplementary Fig. 2) and were in agreement with the microarray results (see supplementary Table 1).

Supplementary Discussion

Growth Factors

A dynamic coordinated interaction between growth factors, transcription factors, extra cellular matrix components, and integrin signaling pathways directs cell migration and lineage determination during lung development. Several growth factors have been implicated in the pathogenesis of pulmonary emphysema. Gene expression profiling revealed the expression of 14 growth factors and 85 transcription factors in the acute CS-exposed (1 day CS) lungs compared to the expression of 2 growth factors and 9 transcription factors in the emphysematous lungs (see GEO accession #: GSE 8790 and supplementary Table 1). Members of the family of transforming growth factor (TGF)-ß, platelet derived growth factors and the large family of fibroblast growth factors (FGF), are essential for several stages of mammalian lung development. TGF-ß plays an important role in pulmonary morphogenesis, function, and the pathogenesis of lung disease. The effect of TGF-ß is regulated via a selective pathway of TGF-ß synthesis and signaling that involves the activation of latent TGF-ß, specific TGF-ß receptors, and intracellular signaling via Smad molecules. Loss of integrin β6-mediated activation of latent TGF-β causes age-dependent pulmonary emphysema in mice (15). Disruption of platelet derived growth factor A (PDGF-A) in mice has been reported to lead to airspace enlargement (1). Disruption of transcription factor forkhead box F1 (Foxf1) in mice resulted in impaired alveologenesis (13). In the present study, we observed reduced expression of various growth factors and transcription factors including platelet derived growth factor receptor alpha, multiple genes constituting the TGF-β signaling pathway (see supplementary Table 1), and various forkhead box transcription factors during subchronic (1.5 months) and chronic CS exposure, which together may have contributed to the pathogenesis of pulmonary emphysema in A/J mice strain.

Ubiquitin-Proteasome Systems

The ubiquitin-proteasome complex is of particular importance in protecting cells against oxidative stress and is involved in a wide variety of key cellular processes including cell proliferation, cell differentiation, transcriptional regulation, antigen processing, stress response, degradation of oxidatively damaged and toxic proteins, and in the regulation of apoptosis (2). Acute CS exposure induced the expression of 49 genes constituting the ubiquitin-proteasome complex in the lungs of A/J mice (see supplementary Table 1). F-box only protein 32, an adapter protein, which binds the substrate to ubiquitin-proteasome complex was up-regulated in the emphysematous lungs of A/J mice. Proteasomes are involved in the degradation of mutant secretory protein, alpha1-antitrypsin Z (18, 19). However, very little is known about the role of proteosomes in the pathogenesis of pulmonary emphysema.

Heat Shock Proteins

The function of heat shock proteins (Hsps) upon exposure to environmental insults constitutes the most ubiquitous and evolutionarily conserved stress response. Heat shock proteins have been shown to enhance survival of cells during a wide variety of stress conditions, by inhibiting apoptotic machinery and by reducing the oxidative damage to proteins, DNA, and lipids (14). Similar to ubiquitin and proteasome complex, we observed reduction in the differential expression of 10 Hsps in the emphysematous lungs compared to the acute CS-exposed lungs. Hsp7 was the only Hsp up-regulated in the emphysematous lungs of A/J mice (see supplementary Table 1).

Down-regulated Genes

Similar to a gradual decline in the up-regualated genes (see supplementary Table 1), progression of CS-induced pulmonary emphysema is associated with a steady decline in the

downregulation of genes constituting multiple biologic pathways (see supplementary Table 2). Microarray analysis revealed the down-regulation of 1840, 730, 442, and 236 genes in the lungs of mice exposed to CS for 5 h, 8 days, 1.5 and 6 months, respectively. We have classified the down-regulated genes into different functional categories. The majority of down-regulated genes (see supplementary 2 and supplementary Table 4) during acute CS exposure belongs to the functional categories of transcription factors (172 genes), signal transduction pathways (138 genes), cell cycle/cell adhesion/cell proliferation (72 genes), ubiquitin-proteasome complex (52 genes), solute carriers and Phase III detoxification pathways (52 genes), genes involved in lipid metabolism (46 genes), inflammation (33genes) and cytoskeletal reorganization (32 genes). After subacute CS exposure (8 days CS), about 730 genes were down-regulated in the lungs of A/J mice. During 8 days CS exposure, there was a marked reduction in the number down-regulated transcription factors, solute carriers and phase III detoxification genes, cell survival genes, oncogenes, growth factors, inflammatory genes, humoral and immune response gens, apoptotic genes and genes constituting ubiquitin-proteasome complex, lipid metabolism, cytoskeletal reorganization and signal transduction pathways in the lungs of A/J mice (see supplementary Table 2 and supplementary Table 4). The number of down-regulated genes constituting ubiquitin-proteasome complex, solute carriers and phase III genes, lipid metabolism, cytoskeletal reorganization and various transcription factors was drastically reduced in the lungs after 1.5 months CS exposure. There was a drastic down regulation of genes constituting cell cycle/cell adhesion/cell proliferation and signal transduction pathways after 6 months CS exposure. However, the number of down-regulated genes constituting the extracellular matrix reorganization and Phase I detoxification genes in the lungs did not significantly altered during the entire period of CS exposure (see supplementary Table 2 and supplementary Table 4).

Some of the down-regulated genes in the lungs of CS-exposed A/J mice whose deletion or overexpression have been shown to contribute to the pathogenesis of pulmonary emphysema are listed in the Table 4 of the online supplement. Cigarette smoke down-regulated elastin, lysyl-oxidase like 1, fibrillin 1, latent transforming growth factor beta binding protein 4, integrin beta 6, lysosomal acid lipase, macrophage-colony stimulating factor, platelet derived growth factor α, interleukin 1ß, and toll-like receptor 4 in the lungs of A/J mice. Elastin, lysyl oxidase like-1 and fibrillin 1 play an important role in the extracellular matrix reorganization of the lung. Elastic fibers are components of extracellular matrix of mammalian lungs and are predominantly found in the alveoli. The main component of elastic fibers is an amorphous polymer composed of protein elastin, known as tropoelastin in its monomeric form. Polymerization of elastin requires an initial step of oxidative deamination of lysine residues, catalyzed by a lysyl oxidase (5). Destruction of alveolar elastic fibers is implicated in the pathogenic mechanism of emphysema in adults (6). Deletion of elastin gene in mice caused a defect in the branching of the terminal airways. The branching defect is accompanied by fewer distal air sacs that are dilated with attenuated tissue septae, a condition reminiscent of emphysema (25). Mice lacking lysyl oxidase like-1 do not deposit normal elastic fibers and develop enlarged airspace of the lungs with concomitant tropoelastin accumulation (12). Microfibrils, made up of fibrillins and microfibril-associated glycoproteins, are thought to serve as a scaffold that guides elastin deposition (6). Mice deficient in fibrillin-1 develop destructive emphysema and show marked dysregulation of transforming growth factor-beta activation and signaling, resulting in apoptosis in the developing lung (16). Perinatal antagonism of TGF-ß attenuates apoptosis and rescues alveolar septation in vivo (16). Loss of integrin αvß6-mediated TGF-ß has been shown to cause MMP12-dependent emphysema (15). Mice lacking integrin ß6 develop age-related emphysema that is completely

abrogated either by transgenic overexpression of ß6 integrin subunit that support TGF-ß activation, or by the loss of MMP12 (15). In addition, disruption of the gene encoding the latent transforming growth factor- ß binding protein 4 causes abnormal lung development (23). Overexpression of lysosomal acid lipase disrupts the formation of lamellar bodies and alveolar structure in the lung (8). Deletion of lysosomal acid lipase gene causes respiratory inflammation and destruction in the lung (9). Administration of macrophage–colony stimulating factor aggravated elastase-induced emphysema in mice (4). Deletion of platelet derived growth factor alpha gene in mice causes pulmonary emphysema due to complete failure of alveogenesis (1, 10). TLR4 deficient mice developed spontaneous-age dependant emphysema, which was associated with increased up-regulation of NADP(H) oxidase 3, enhanced oxidative stress, and apoptosis of alveolar septal cells (26). Transgenic overexpression of interleukin-1 ß in respiratory epithelial cells of adult mice causes lung inflammation, enlargement of distal airspaces, mucus metaplasia, and airway fibrosis in the adult mouse (7).

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Supplementary Legends

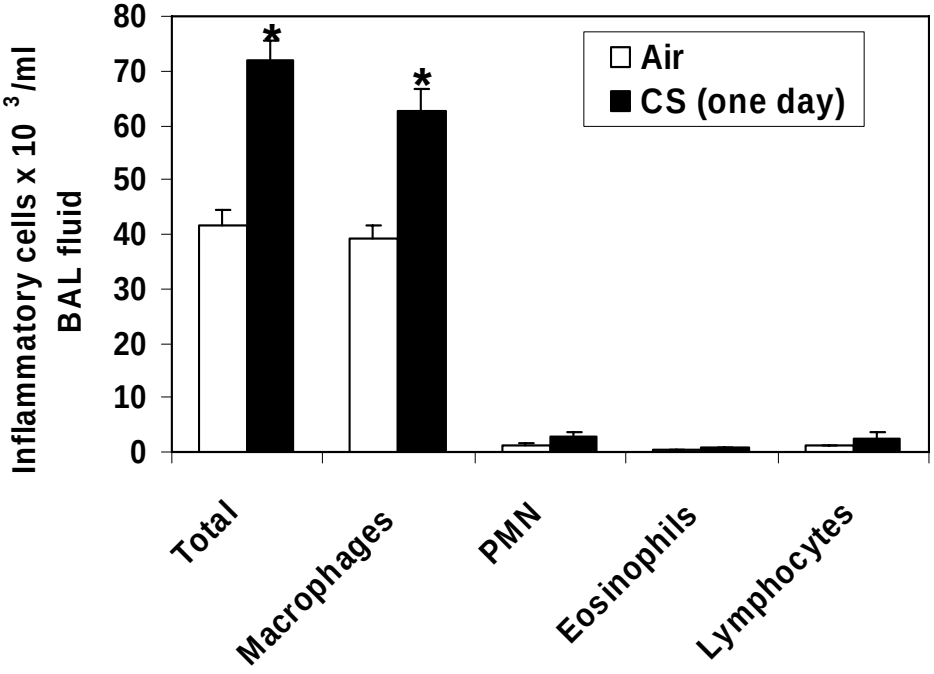
Supplementary Figure 1. Inflammatory response in the lungs of CS-exposed A/J mice. There were a significantly increased number of inflammatory cells in the BAL fluids from the lungs of acute (1 day CS-exposed mice) (A) and chronic (6 months) CS-exposed (B) animals compared to their respective age-matched air-exposed mice. However, the total number of inflammatory cells in BAL fluid of 6 months CS-exposed animals was 2-fold higher than inflammatory cells in BAL fluid of acute CS-exposed animals (n = 8 mice/group). Data are mean ± SEM. $P \leq 0.05$. C) Histochemical staining of macrophages in the lung tissues. Lectin staining was used to detect the macrophages in the lung tissues. Arrows indicate the macrophages stained positively with lectin in the lungs of 6 months CS-exposed mice. Images were taken using 20X lens. (D) The number of macrophages was significantly increased in the lung tissues of 6 months CS-exposed A/J mice. Few macrophages were detected in the lung tissues of age-matched air-exposed control mice (n = 6 mice/group). Values are represented as mean ± SEM, $P \leq 0.05$.

Supplementary Figure 2. Validation of antioxidant and Phase II detoxification genes by real time RT-PCR. Real time RT-PCR was used to validate the expression of three typical antioxidant and Phase II detoxification genes such as HO-1 (A), GCLc (B), and GSR (C) in the lungs of mice exposed to CS for various time points. Results are mean ± SEM derived from three different air- or CS-exposed lungs (n = 3 mice).

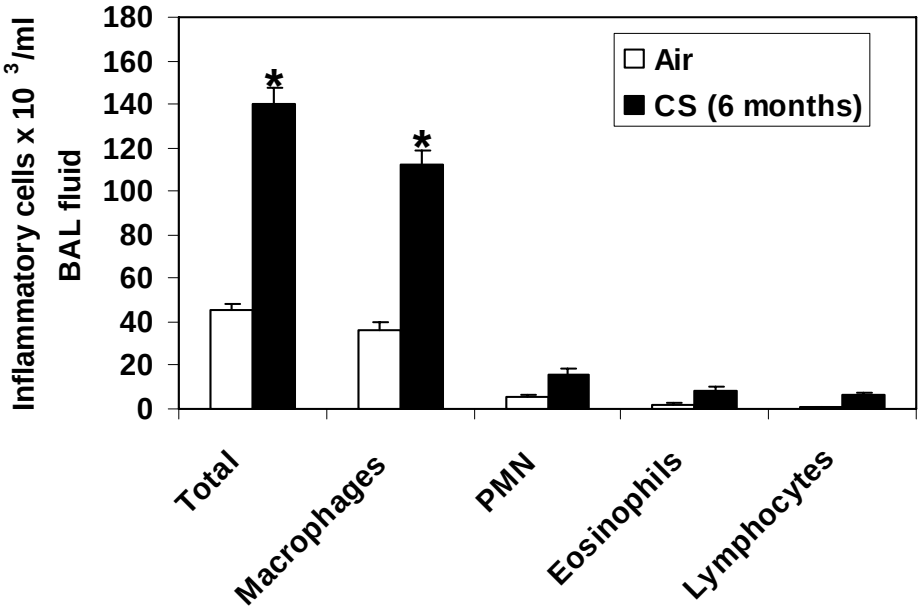
Supplementary Figures

Figure 1

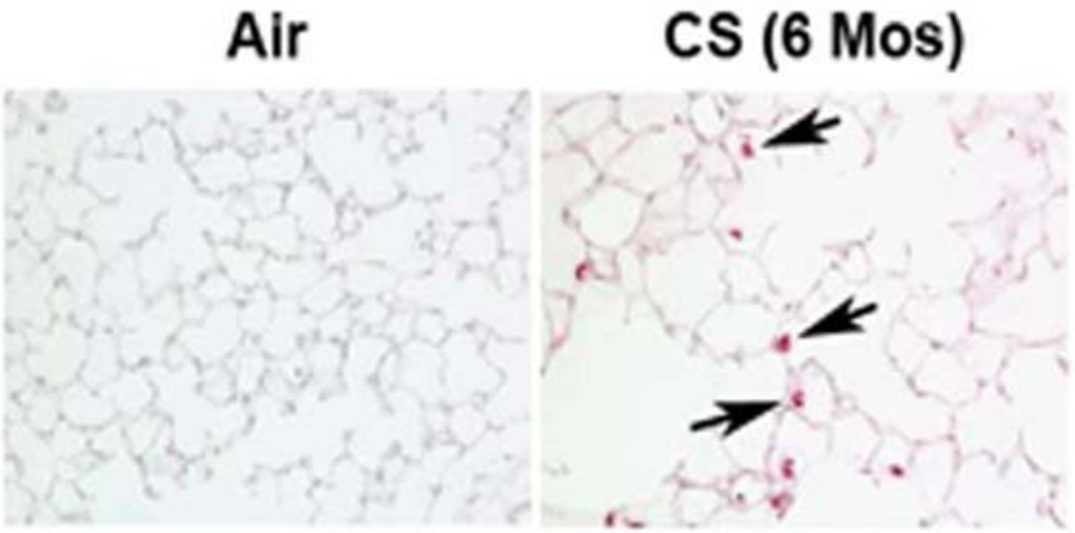
A)



B)



C)


Air
CS (6 Mos)

D)

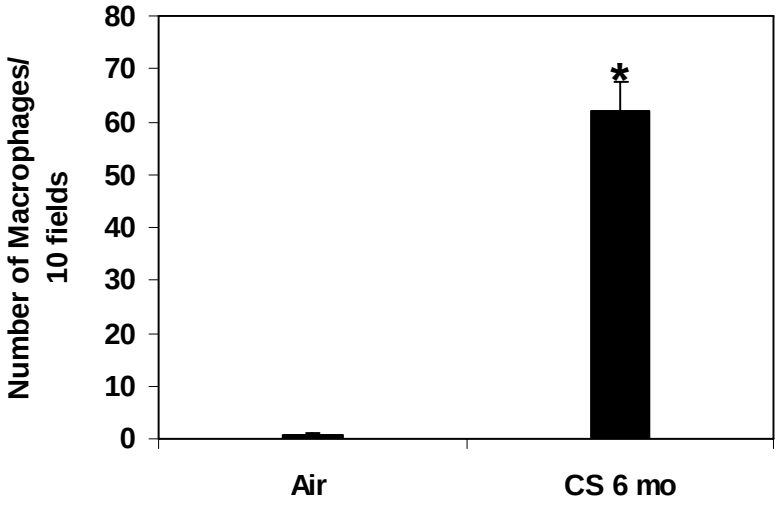

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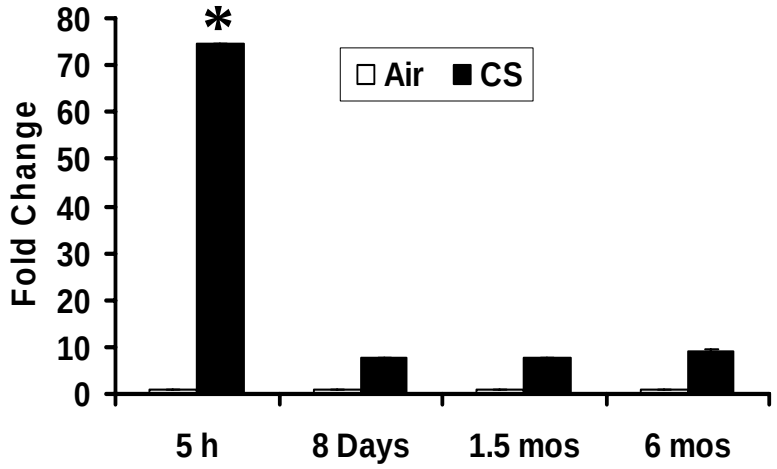
Figure 2

A)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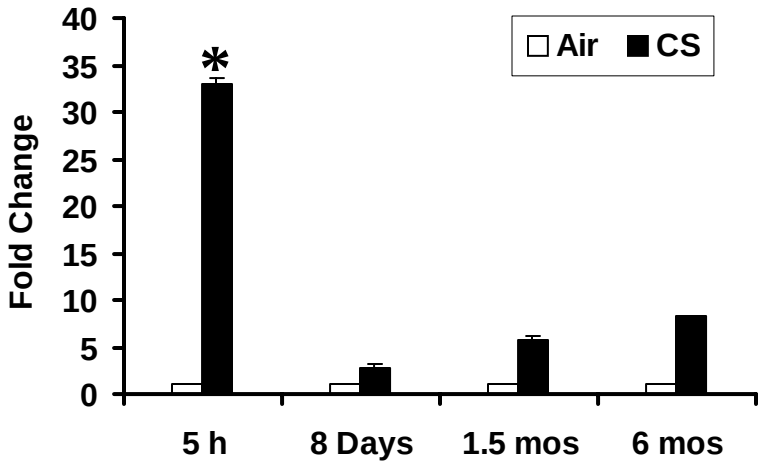
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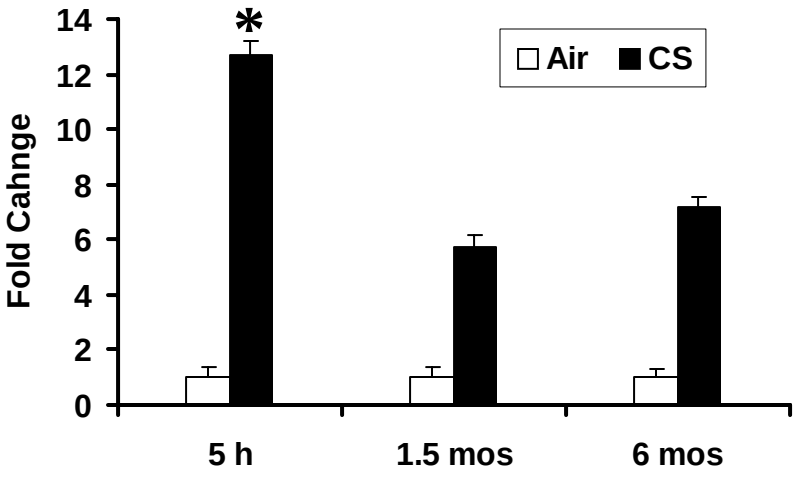
Air
CS
5 h
8 Days
1.5 mos
6 mos

C)


14
12
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Fold Cahnge
*
Air
CS
5 h
1.5 mos
6 mos