SUPPLEMENTAL MATERIAL

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Supplemental Methods

Bitransgenic mice

Bitransgenic mice were generated by the crossing of Balb/c transgenic mice that harbor the reverse tetracycline transcriptional activator (rtTA, tetON system) under the control of the Clara Cell secreted protein (CCSP) promoter (CCTA line: a kind gift from Dr. J.A Whitsett)[1] with FVB transgenic mice that carry the human HO-1 transgene under the control of the tetracycline response element (TH77 line: CCSP-rTTA x TRE-hHO1). The latter was generated by microinjection of a $(TetO)_7$-CMV-human HO-1 transgene that consists of seven copies of the tet operator linked to a minimal CMV promoter, the human HO-1 cDNA, and SV40 polyadenylation signals. Animals were maintained in the pathogen-free Children's Hospital Animal Care facility and all animal experiments were approved by the Children's Hospital Boston Animal Care and Use Committee.

Hypoxic mouse model of PAH

Expression of human HO-1 in the lung was achieved by the addition of 1 mg/ml doxycycline (dox) (Sigma-Aldrich, Inc., St. Louis, MO) in the drinking water. After two days pretreatment with dox, animals were introduced to normobaric hypoxia (8.5% O_2) inside a chamber where oxygen was tightly regulated by an Oxycycler controller (Biospherix, Ltd., Lacona, NY). Nitrogen was automatically introduced as required to maintain the proper FiO_2 and ventilation was adjusted to keep CO_2 levels less than 8,000 ppm (0.8%). Ammonia was removed by charcoal filtration using an electric air purifier. Dox administration was either continued for the entire duration of the hypoxic exposure or terminated at two days or after seven days of hypoxia. Age and sex-matched littermates were exposed to identical conditions in hypoxia or normoxia and served as controls. The CCTA mouse line that lacks

the human HO-1 trangene, treated with dox, served as control to eliminate any potential effects imparted by dox itself, independent of human HO-1 expression.

CO treated mice inhaled the gas intermittently: 250 ppm for 1 hour prior to hypoxic exposure and then received 250 ppm for 1 hour twice daily, inside the hypoxic chamber for a total of 48 hours. A group of mice underwent i.p injections of 50 εmol/kg biliverdin IX hydrochloride (Frontier Scientific, Inc., Logan, UT) as previously reported,[2] prior to the onset of hypoxia and twice daily thereafter. Finally, a third group received both CO and biliverdin as above. Control mice were injected i.p with the same volume of PBS and inhaled room air or hypoxic air (8.5% O_2) without CO.

Hemodynamic and ventricular weight measurements

After hypoxic exposure at the indicated time periods, mice were anesthetized and hemodynamic and ventricular weight measurements were performed. Right ventricular systolic pressure (RVSP) was measured through a trans-thoracic route: a pressure transducer (ADI Instruments, Inc., Colorado Springs, CO) attached to a 23G needle was used and data were collected and analyzed using the PowerLab Software (ADI Instruments, Inc., Colorado Springs, CO).[3] Right ventricular (RV) hypertrophy was assessed by harvesting hearts, removing atria, dissecting the RV and deriving Fulton's Index, i.e the weight ratio of (right ventricle)/ (left ventricle and septum) [(RV)/(LV+S)].

Immunohistochemical analysis

Lungs were initially perfused with PBS through the right ventricle. The perfusion flow was kept at approximately 1ml/min by the use of a peristaltic pump with Platinum L/S 13 Masterflex silicone tubing. Lungs were then intratracheally inflated with 4% paraformaldehyde, fixed overnight at 4°C, then stored in 70% ethanol before embedding in paraffin. Lung tissue sections were deparaffinized and rehydrated. Immunohistochemical assessment of vascular remodeling was performed by staining for alpha-smooth muscle actin (anti-Ŭ-SMA antibody, Sigma-Aldrich, Inc., St. Louis, MO), a marker of smooth muscle

cells.[4] Endogenous peroxidase activity was inhibited with 3% H_2O_2 (Sigma- Aldrich, Inc., St. Louis, MO) in methanol. Next, the sections were incubated with a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA), treated with the avidinï biotin complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA), and stained with 3,3ô-diaminobenzidine substrate (KPL, Inc., Gaithersburg, MD). Slides were counterstained with 1% Methyl Green (Sigma-Aldrich, Inc., St. Louis, MO).

Morphometric analysis

Alveolar/distal pulmonary arterioles of 50-100 ε m in diameter, not associated with bronchi, from lung sections immunostained with Ŭ-SMA (as described above), were captured with light microscopy. At least 10 representative pulmonary arterioles were chosen from three different sections from each animal. Morphometric analysis of medial vessel wall thickness was performed using the software package Metamorph v.6.2r (Universal Imaging, Downingtown, PA). The entire vessel area including the lumen was identified as ñTotal Areaò and the area of brown-color (Ŭ- SMA stained) that represents the medial smooth muscular layer was labeled ñThreshold Areaò. Medial Wall Thickness Index was determined by using the quotient of Threshold Area x 100 over Total Area [(%Threshold Area x 100)/ total Area].

Isolation of alveolar macrophages

Animals were anesthetized with 2,2,2-tribromoethanol (avertin, Sigma-Aldrich, Inc., St. Louis, MO) after exposure for the indicated time periods in hypoxia. Bronchoalveolar lavage fluid (BALF) was obtained through intratracheal instillation of 4 x 0.85 ml PBS and filtered via a 35 ε m cell strainer to exclude contamination from epithelial cells that appeared in clusters. Red blood cells were lysed using ammonium chloride lysis buffer (Sigma-Aldrich, Inc., St. Louis, MO). More than ninety percent of the cells isolated this way appeared to be of the monocyte/macrophage lineage and this was confirmed by cell-specific markers in flow cytometry (below). Isolated cells were used for RNA extraction, flow cytometry, or immunocytochemistry.

Flow cytometry

Total white blood cell (WBC) counts of BALF isolated cells were accessed by hemocytometer counting using Kimura stain,[5] and reconfirmed by flow cytometry analysis [6] by the use of FITC-anti mouse CD45 antibody (BD Biosciences, Franklin Lakes, NJ) and flow cytometry absolute count standard beads (Bangs Laboratories, Fishers, IN). Differential WBC analysis was performed using APCï anti-mouse F4/80 (eBioscience, Inc., San Diego, CA), PE-anti-mouse Ly-6G/Ly-6C (BD Biosciences, Franklin Lakes, NJ), and Pacific Blue-anti-mouse CD3 (eBioscience, Inc., San Diego, CA) antibodies specific for macrophages, neutrophils, and T cells, respectively. Expression profile of BALF isolated alveolar macrophages was assessed by APC-anti mouse F4/80 (eBioscience, Inc., San Diego, CA),[7] FITC-anti-mouse CD11c, and PE-anti-mouse CD45 (BD Biosciences, Franklin Lakes, NJ) in separate analyses. Fizz1 expression was assessed by performing fixation with parafolmadelhyde, intracellular permeabilization and staining with primary rabbit anti-mouse-Fizz1 antibody (Abcam, Cambridge, MA) followed by secondary rabbit FITC-conjugated anti-rabbit antibody (BD Biosciences, Franklin Lakes, NJ). In order to evaluate the IL-10-expressing alveolar macrophages, BALF-isolated cells were incubated with Golgi inhibitor (monensin, BD Biosciences, Franklin Lakes, NJ) fixed and permeabilized, and stained with APC-conjugated monoclonal antibody against murine IL-10 (BD Biosciences, Franklin Lakes, NJ). The proper isotype controls were used in each case. The flow cytometry events were acquired in a MoFlo Legacy Cell Sorter (Beckman Coulter, Inc., Brea, CA) and analyzed with the use of Summit Software (Summit Software, Inc., Fort Wayne, IN).

Cytospin preparation and Immunocytochemistry

BAL was performed and the cell suspension was cytocentrifuged at 300g for 5 min using the Shandon Cytospin 4 (Thermo Fisher Scientific, Inc., Waltham, MA). Slides were air-dried overnight, stained with Hema stain set (Fisher Diagnostics, Middletown, VA), and evaluated

under light microscope. Immunocytochemistry for Fizz1 or iNOS was performed by immersion of the slides in 2% parafolmadehyde, incubation with blocking serum, followed by incubation at 4°C overnight with rabbit polyclonal anti-mouse-Fizz1 antibody (Abcam, Cambridge, MA), or rabbit polyclonal anti-mouse iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Goat biotinylated anti-rabbit secondary antibody (Cell Signaling Technology, Inc., Boston, MA) and FITC-avidin conjugate (Vector Laboratories, Inc., Burlingame, CA) were further used. Primary alveolar macrophages stimulated with 100 ε g/ml LPS *E.coli* and 100 U/ml INF-ɔ for 48 hours served as positive controls. Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Inc., Waltham, MA) and samples were observed with fluorescent microscopy.

Quantitative PCR

RNA from total lung or from alveolar macrophages was isolated using the Qiagen RNAeasy mini and micro extraction kit, respectively (Qiagen, Hilden, Germany). One ε g of total DNA-digested RNA was used for cDNA synthesis (Superscript III oligo dT primer kit, Invitrogen Corporation, Carlsbad, CA). The following primers were used in the PCR reaction: human HO-1; fwd: 5ô GCAGTCAGGCAGAGGGTGATA-3ô rev: 5ô AGCCTGGGAGCGGGTGTTGAG-3ô Ym1; fwd: 5ô GCAGAAGCTCTCCAGAAGCAATCCTG-3ôrev: 5ôATTGGCCTGTCCTTAGCCCAACTG-3ô Fizz1; fwd: 5ôGCTGATGGTCCCAGTGAATAC-3ô rev: 5ôCCAGTAGCAGTCATCCCAGC-3ô Arginase-1; fwd: 5ôCAGAAGAATGGAAGAGTCAG-3ô rev: 5ô CAGATATGCAGGGAGTCACC-3ô Arginase-2; fwd: 5ôCACGGGCAAATTCCTTGCGTCC-3ô rev: 5ôGGTTGGCAAGGCCCACTGAACG-3ô Mannose Receptor, C type 1 (MR): fwd: 5ô TTTCCATCGAGACTGCTGC-3ô rev: 5ôACCAAAGCCACTTCCCTTC-3ô iNOS; fwd: 5ô TCCTGGAGGAAGTGGGCCGAAG-3ô rev: 5ôCCTCCACGGGCCCGGTACTC-3ô IL12b; fwd: 5ô GGAGGGGTGTAACCAGAAAGGTGC-3ô rev: 5ô CCTGCAGGGAACACATGCCCAC-3ô TNFa; fwd: 5ôGCCCACGTCGTAGCAAACCACC-3ô rev: 5ôCGGGGCAGCCTTGTCCCTTG-3ô CCL2 (MCP-1); fwd: 5ô

GGCTGGAGCATCCACGTGTTGG-3ô; rev: 5ôTTGGGGTCAGCACAGACCTCTCTC-3ô; IL-6; fwd: 5ôCAAAGCCAGAGTCCTTCAGAG-3ô; rev: 5ôCACTCCTTCTGTGACTCCAGC-3ô; IL-10; fwd: 5ô GCGCTGTCATCGATTTCTCCCCTG-3ô; rev: 5ô GGCCTTGTAGACACCTTGGTCTTGG-3ô; PDGF-BB: fwd: 5ô GGGAGCAGCGAGCCAAGACG-3ô; rev: 5ôTGCCCACACTCTTGCCGACG-3ô; CD80 (B7-1); fwd: 5ôGGGAAAAACCCCCAGAAG-3ô; rev: 5ô CCCGAAGGTAAGGCTGTTG-3ô; CD86; fwd: 5ô CAGCCTAGCAGGCCCAG-3ô; rev: 5ô GGCTCTCACTGCCTTCACTC-3ô Ribosomal Protein S9 (Rps9) with forward primer 5ôGCTAGACGAGAAGGATCCCC-3ôand reverse primer 5ô. CAGGCCCAGCTTAAAGACCT -3ôserved as housekeeping gene. Annealing was carried out at 60ęC for 30 sec, extension at 72ęC for 30 sec, and denaturation at 95ęC for 30 sec for 40 cycles. Analysis of the fold change was performed based on the Pfaffl method.[8]

BAL fluid cytokine profile

The BALF supernatant was analyzed using a multiplex mouse cytokine kit (FGFƀ, MIP-1Ŭ, IL-1ƀ, IL-17, IL-2, IL-13, IL-4, TNF-Ŭ, IL-12, INF-ɔ) (Invitrogen Corporation, Carlsbad, CA) in the Luminex 200Ê System (Luminex Corporation, Austin, TX). BALF supernatant samples from animals treated with either dox or regular water in normoxia versus two and four days in hypoxia were analyzed in duplicate. Standard Luminex protocol was followed as suggested by the manufacturer.

Western blot analysis

Protein concentration from BALF or total lung was determined by the Bradford assay. BALF samples were concentrated with 20% trichloroacetic acid (TCA, Sigma-Aldrich, Inc., St. Louis, MO) overnight, washed with ice-cold acetone and resuspended in SDS-containing loading dye. Twenty ɛg of protein was electrophoresed on 13.3% denaturing polyacrylamide gel prior to wet transfer to 0.2 ɛm PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Briefly, after blocking with 5% bovine serum albumin (BSA) in phosphate buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST) for an hour at room temperature, the membranes

were incubated with rabbit polyclonal anti-mouse Fizz1 antibody (Abcam, Cambridge, MA), rabbit polyclonal anti-human and anti-mouse HO-1 antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA), rabbit polyclonal anti-mouse HO-2 antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) or goat polyclonal anti-mouse IgA Ab (Millipore, Billerica, MA) at 4°C overnight. The membranes were then incubated with 40 ng/ml of peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (Santa Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, for 30 min at room temperature followed by reaction with Lumi-Light ECL substrate (Thermo Fisher Scientific, Inc., Waltham, MA).

For IL-10 detection in alveolar macrophages, cells were collected from BALF and centrifuged at 400g for 5 min. Cells were washed with PBS prior to lysis in 20 ul of RIPA buffer supplemented with protease inhibitor cocktail. Ten ug protein per lane was loaded on 13.3% polyacrylamide gel, wet transfer was performed at 200 mAmp for 2-3 hrs, followed by blocking with 2.5% BSA in PBST (0.1% Tween 20) for 30 min, and incubation with rat anti-mouse IL-10 antibody (Abcam, Cambridge, MA) overnight at 4°C with continuous shaking. Anti-mouse ɓ-actin monoclonal antibody (R&D Systems, Minneapolis, MN) was used as internal control and densitometric analysis was performed with the NIH ImageJ program.

Griess reaction and arginase activity assay

In the Griess reaction, nitrite and nitrate concentration in BALF supernatant were measured by the Total NO/Nitrite/Nitrate Assay (R&D Systems, Minneapolis, MN). Proteins were removed before analysis with ultrafiltration using 10,000 molecular weight (MW) cut-off filters (Amicon Ultra; Millipore, Billerica, MA). Supernatants from RAW 264.7 macrophages stimulated with 100 ɛg/ml LPS or 100 U/ml INF-ɔ for 48 hours served as positive controls.

Arginase activity in BALF-isolated alveolar macrophages was assessed utilizing the Quantichrom arginase assay kit (Bioassay Systems, Hayward, CA). Briefly, 10^5 cells per sample were harvested, washed, and lysed with 10 mM Tris.HCl (pH 7.4) containing 0.4%

(w/v) Triton X-100 and protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany). Cell lysate samples were analyzed for arginase activity in duplicate.

Primary alveolar macrophage culture

For cell culture experiments, BALF was obtained through intratracheal instillation of 5 x 1 ml Hankôs Balanced Salt Solution (without calcium and magnesium) supplemented with 10 mM EDTA and 1 mM HEPES and filtered twice via a 35 εm cell strainer to exclude contamination of epithelial cells. $3.5x10^5$ macrophages per well were seeded in 48-well tissue-culture plates in a volume of 0.25 ml macrophage complete medium (DMEM/10: Dulbeccoôs Modified Eagle Medium) (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% (v/v) FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin). Cells were incubated at 37°C for 4 hours and then their medium was replaced with serum-free DMEM prior to hypoxic exposure or IL-4 stimulation.

Macrophage activation and CO treatment

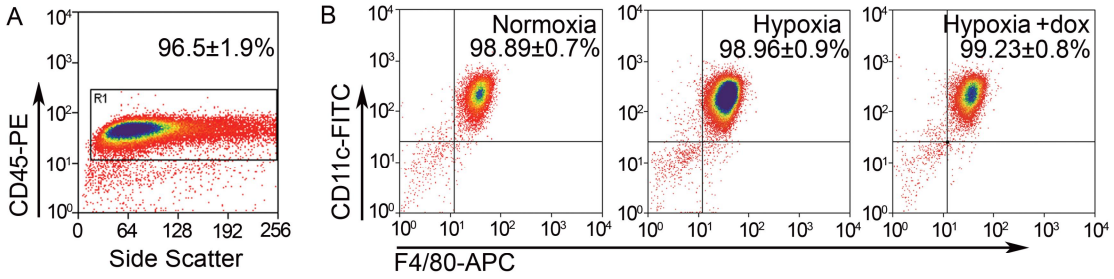
Macrophages were cultured for 48 hours at 0.5-1% O_2 (pO_2 in the media was 14-18 torr) in a hypoxic work station (in Vivo2, Ruskinn Technology, Ltd., Bridgend, UK) and/or Billups chambers that were flushed with a mix of 0.5% O_2 and 5% CO_2 (N_2 balance). Alternatively, macrophages were stimulated with 20 ng/ml murine recombinant interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) in order to be polarized towards M2.[9, 10] CO treatment was performed in Billups chambers that were flushed with a mix of 0.5% O_2, 5% CO_2, 500 ppm CO (N_2 balance). Cells were pretreated with CO for 1 hour in normoxia prior to hypoxic exposure. Cell viability was greater than 80% in all groups as assessed by trypan blue exclusion.

PASMC proliferation assay

Mouse primary pulmonary artery smooth muscle cells (PASMCs) were cultured ($2x10^3$ cells per well) in a volume of 100 µl of DMEM (GIBCO, Invitrogen Corporation, Carlsbad,

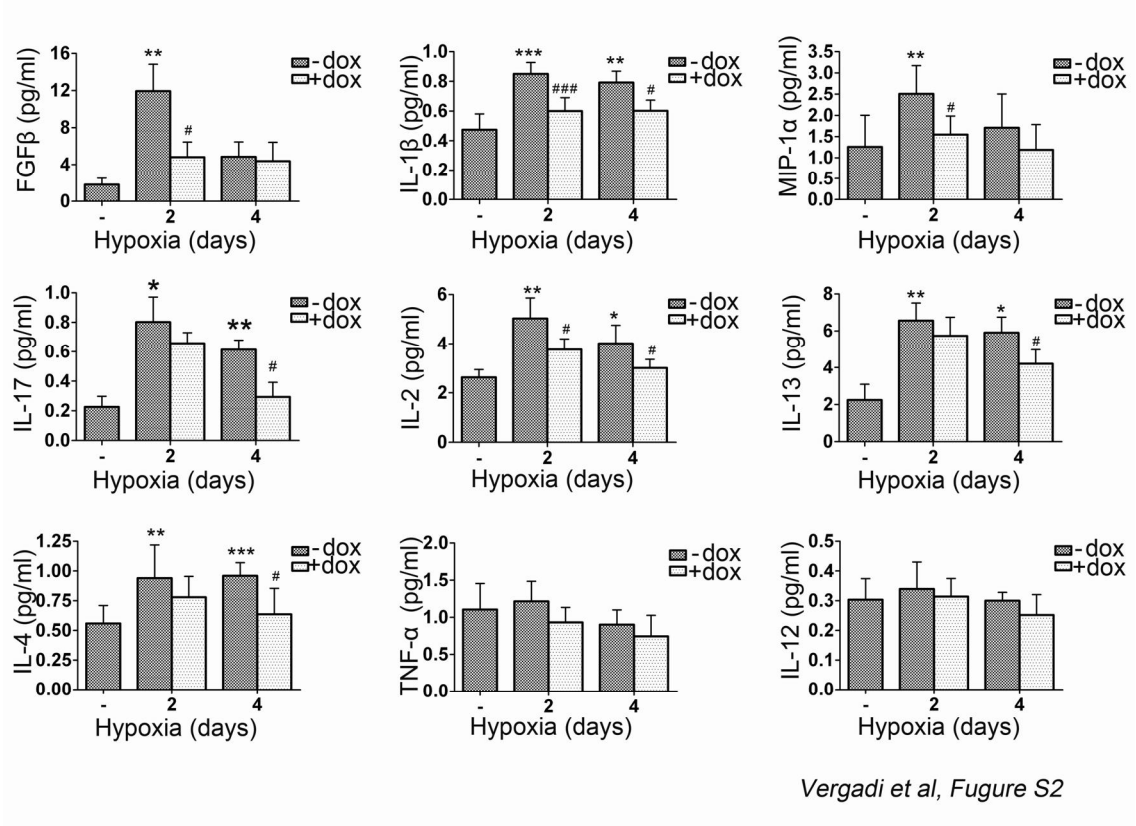
CA) supplemented with 10% FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, using 96-well tissue culture plates. Two days prior to proliferation assay, the medium was replaced with DMEM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Macrophage-conditioned media, diluted two-fold with fresh low-serum media, were then applied to PASMCs and the cultures were incubated for an additional three days. Cell proliferation was assessed by cell proliferation reagent WST-1 (Roche Diagnostics, Mannheim, Germany) by applying 10 µl of WST-1 reagent to each well and measuring OD_{440}-OD_{690} after two hours of incubation at 37^0C. Treatment of PASMCs with 25 ng/ml PDGF-BB served as a positive control. Fresh cell culture medium (DMEM) or medium equilibrated in 0.5% Oxygen for 48 hours were used as negative controls. Mouse recombinant IL-10 (R&D Systems, Minneapolis, MN) was used in the range of 1-100 ng/ml.

Supplemental Figures and Figure Legends

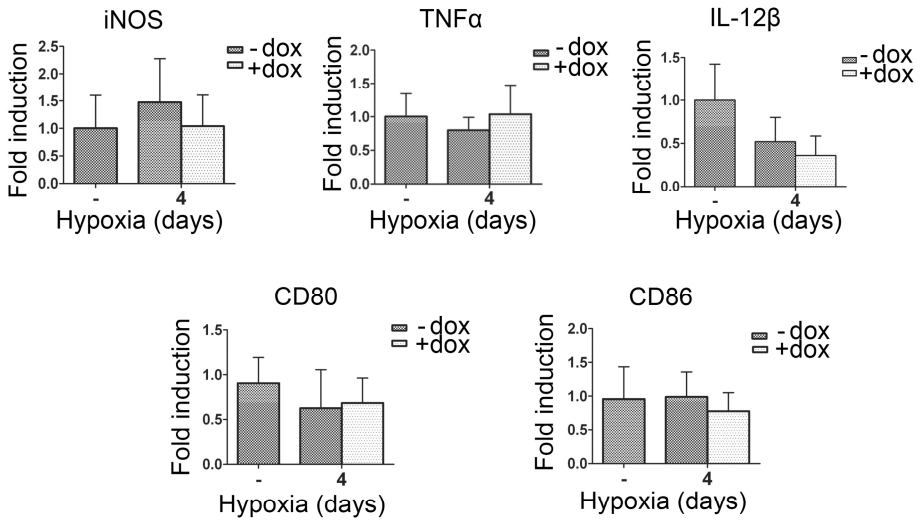


Vergadi et al, Figure S1

Supplemental Figure 1. Analysis of BALF cell content in hypoxic mice. [A] More than 95% of the cells isolated (gated for Side and Forward Scatter) were CD45(+), i.e white blood cells. [B] More than 98% of CD45 (+) cells expressed the macrophage cell surface antigens F4/80 and CD11c, irrespective of treatment.

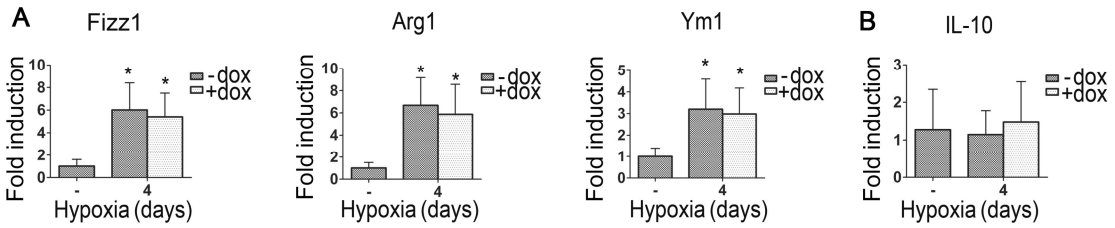


Supplemental Figure 2. Chemokine and cytokine profile in the BALF of hypoxic mice. Chemokine/cytokine profile in the BALF of mice exposed in hypoxia for two and four days in the absence (-) or presence (+) of dox. Upregulation of FGFb, IL-1b, MIP-1Ŭ, IL-13, IL-4, IL-17 and IL-2 in hypoxia and the suppressive effect of HO-1 expression (+dox). Note that the Th1 related cytokines, TNF-Ŭ and IL-12, were not upregulated in hypoxia as compared to normoxic mice. (Numbers represent mean +/-SD, with a minimum of 6 animals per time point or treatment group). *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001. #:relative to hypoxia ï dox; #p<0.05, ###p<0.001.

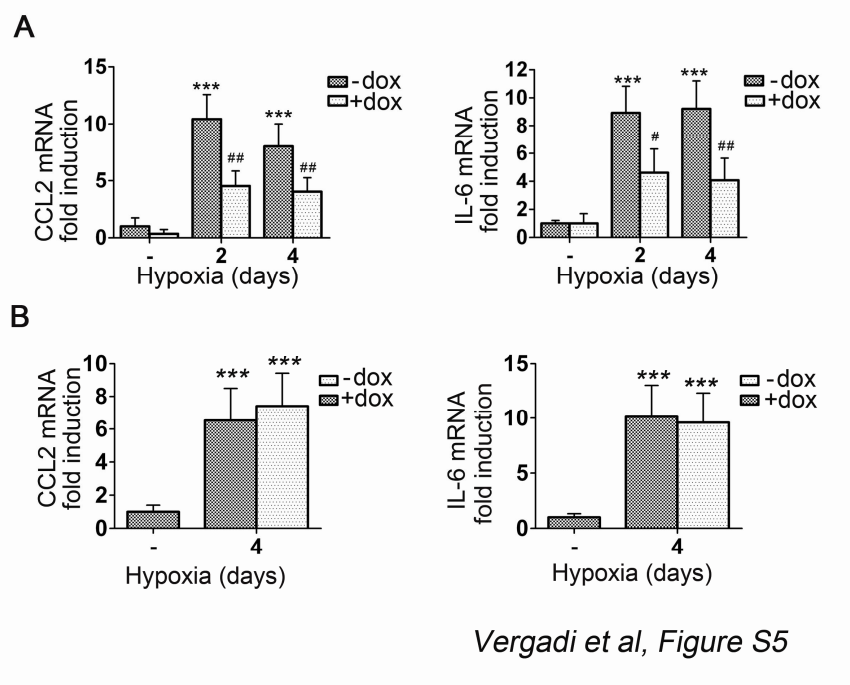


Vergadi et al, Figure S3

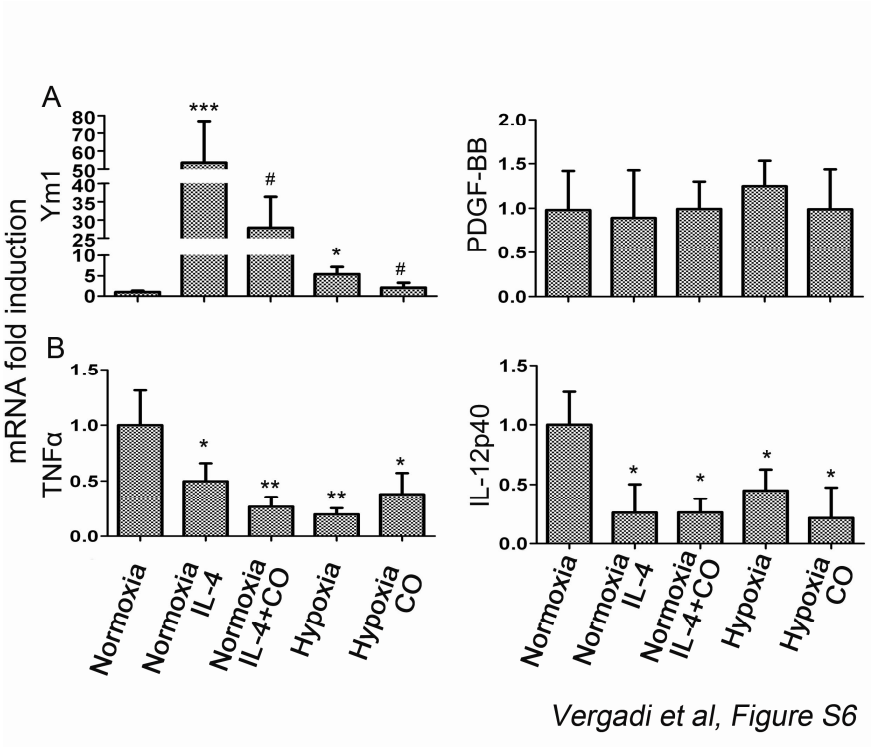
Supplemental Figure 3. Expression levels of M1 markers in hypoxic alveolar macrophages. mRNA levels of M1 markers, iNOS, TNFŭ, IL-12ƀ, CD80, and CD86, were assessed through qPCR in alveolar macrophages isolated from normoxic animals or animals exposed to hypoxia for four days with or without dox treatment. Values are shown relative to normoxia.



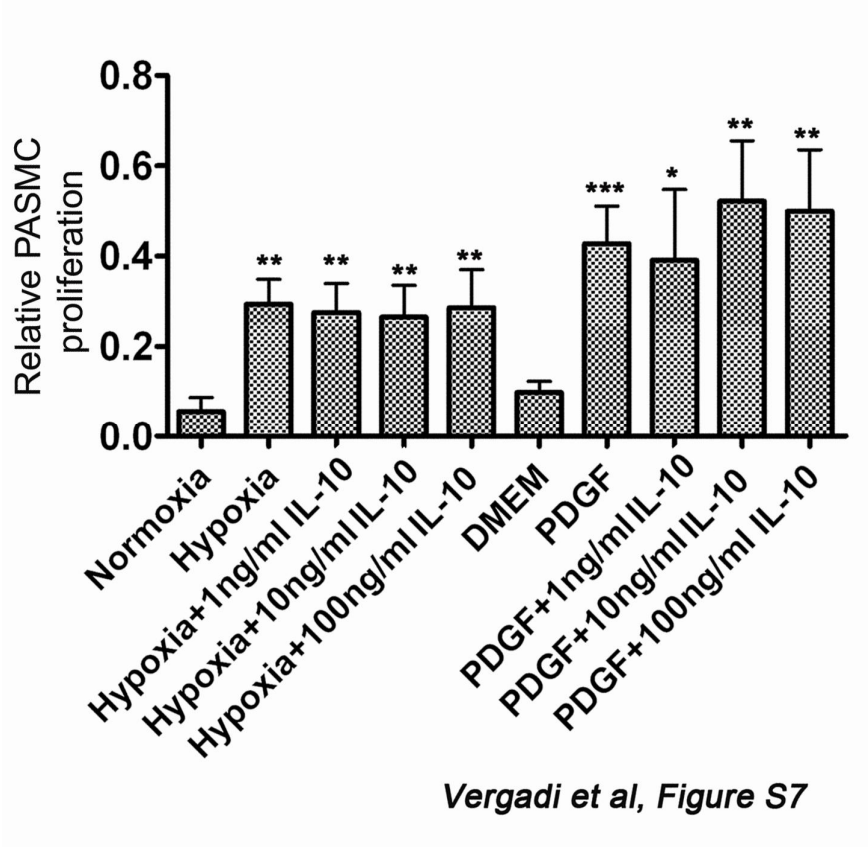
Supplemental Figure 4. The anti-inflammatory effects of dox-treatment are hHO-1 dependent. The CCTA transgenic line that harbors the tetracycline transactivator but lacks the human HO-1 transgene was used to assess potential biologic effects of dox treatment. [A] Markers of alternative activation, Fizz1, Arg1, and Ym1, were not suppressed in hypoxic CCTA animals treated with dox. [B] Concordantly, the anti-inflammatory mediator, IL-10, was not upregulated in dox-treated CCTA animals. *: relative to normoxia; *p<0.05.



Supplemental Figure 5. Suppression of CCL2 and IL-6 levels in the hypoxic lung is HO-1 dependent [A] CC77 animals. The hypoxic induction of CCL2 and IL-6 mRNA levels in the lung was suppressed by dox treatment. [B] CCTA animals. CCL2 and IL-6 mRNA levels were not suppressed in hypoxic CCTA animals lacking the hHO-1 transgene. Numbers represent mean +/-SD, with a minimum of 6 mice per group. *: relative to normoxia, ***p<0.001. $^{\#}$:relative to hypoxiaï dox; $^{\#}$p<0.05, $^{\#\#}$p<0.01.



Supplemental Figure 6. Cytokine and growth factor profile of *in vitro* stimulated primary alveolar macrophages. [A] mRNA levels of the M2 marker, Ym1, but not the growth factor, PDGF-BB, were upregulated in primary alveolar macrophages stimulated with 20 ng/ml IL-4 or hypoxia (0.5% O_2) and suppressed upon CO treatment. [B] The mRNA levels of M1 specific markers, TNF-Ŭ and IL-12p40 subunit, were downregulated in alveolar macrophages stimulated with 20 ng/ml IL-4 or hypoxia (0.5% O_2) with or without 250 ppm CO. Numbers represent mean +/-SD, at least 5 mice donors per group. *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001. $^{\#}$:relative to hypoxia or normoxia + IL-4; $^{\#}$p<0.05.



Supplemental Figure 7. IL-10 is not sufficient to block hypoxic macrophage-derived signals for PASMC proliferation. PASMC cultures were inclubated with media conditioned by either normoxic [Normoxia] or hypoxic [Hypoxia] alveolar macrophages in the presence or absence of IL-10 at the indicated final concentrations, and their proliferation rate assessed. Stimulation of PASMC proliferation by 25 ng/ml PDGF-BB served as a control. *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001.

Supplemental References

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