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Supplementary Materials for

Targeting Nrf2 Signaling Improves Bacterial Clearance by Alveolar Macrophages in Patients with COPD and in a Mouse Model

Christopher J. Harvey, Rajesh K. Thimmulappa, Sanjay Sethi, Xiaoni Kong, Lonny Yarmus, Robert H. Brown, David Feller-Kopman, Robert Wise, Shyam Biswal*

*To whom correspondence should be addressed. E-mail: sbiswal@jhsph.edu

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Supplementary Materials and Methods

Recruitment of Subjects: This study was approved by the institutional review board of Johns Hopkins University. Patients with COPD were recruited from clinical populations at Johns Hopkins Hospital and Johns Hopkins Bayview Medical Center. Patients were asked to volunteer for a bronchoscopy for collection of alveolar macrophages by BAL. We also collected BAL samples from patients who underwent bronchoscopy procedures for clinical indications. Informed consent was obtained from all patients. Inclusion criteria included 1) Adult patients with COPD: patients who were already scheduled to undergo bronchoscopy for a clinical indication. These patients had an $FEV_1/FVC < 0.70$, and a FEV_1 40-80% predicted; and a >10 pack-year smoking history (current and former smokers were included). Exclusion criteria included 1) pregnancy, 2) hemodynamic instability, 3) baseline hypoxemia (SpO2< 90% on up to 2 L/min via nasal cannula), and 4) bronchoscopy being performed 'urgently' or 'emergently' for therapeutic purposes. Demographic data are presented in Table 1.

The Broccoli Sprout Extract (BSE) study was approved by the institutional review board of Johns Hopkins University to test whether sulforaphane protects from airway hyper- reactivity in asthma. Volunteers, both healthy and individuals with asthma were recruited from the general population. Subjects were screened using a questionnaire, allergy skin testing, and routine methacholine (Mch) inhalation challenge. Venous blood was drawn from subjects prior to and

immediately after they consumed BSE containing 100 μMol of sulforaphane for 14 days. For our study, we used PBMCs isolated from healthy subjects.

Preparation of the broccoli sprouts beverage.

In the clinical study, 3 healthy human volunteers received rehydrated, lyophilized broccoli sprout extracts rich in sulforaphane produced by J.W. Fahey and P. Talalay, Department of Pharmacology and Molecular Sciences, Johns Hopkins University, School of Medicine. Broccoli (*Brassica oleracea italica*) sprouts were grown from specially selected seeds as previously described [1]. Briefly, seeds were surface disinfected and grown in a commercial sprouting facility (Sprouters Northwest, Kent, WA) under controlled light and moisture conditions. After 3 days of sprout growth, an aqueous extract was prepared at a food processing facility (Oregon Freeze Dry, Albany, OR). Sprouts were plunged into boiling deionized water and allowed to boil for 30 min. The aqueous extract contained about 5 μmol of glucoraphanin per ml. The extract was filtered, cooled to 37ºC, and treated with myrosinase, an enzyme released from a small amount of daikon (*Raphanus sativus*) sprouts, for 4 h in order to hydrolyze the glucosinolates to isothiocyanates. The levels of total isothiocyanate, sulforaphane, and residual precursor glucoraphanin were then quantified by cyclocondensation [2] and by direct HPLC [3, 4], respectively. The hydrolyzed aqueous extract was lyophilized. Sulforaphane content at time of use was 215 μmol/g of powder. The bulk powder was tested for microbial contaminants prior to release by Oregon Freeze Dry and again upon arrival in Baltimore, where it was stored in sealed bags at -20ºC. Daily allotments of powder were dissolved in 140 ml of mango juice (Jumex®,

Vilore Foods Co., Inc., San Antonio, TX) for daily distribution to study participants. The juice served to mask flavor and taste but had no effect on the stability of the sulforaphane, nor did the juice contribute any phase 2 enzyme inducer activity (data not shown). The daily doses, administered for 14 days, provided 100 μmol of sulforaphane. The mango juice alone was used as a "placebo".

Cell Culture: Human alveolar macrophages were purified from individual BAL samples by centrifugation and seeded onto 6-well tissue culture plates. After incubation for 2 h, non-adherent cells were removed and the adherent cells were incubated with R, S-sulforaphane (5 μM; LKT Laboratories, Inc., St. Paul, MN) and or DMSO in RPMI 1640 culture medium with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). After 16-20 h, adherent cells were scraped, suspended in RPMI 1640 culture media and plated onto 96-well tissue culture plates for bacterial phagocytosis and clearance assays. Cells obtained were >95% macrophages as determined by the morphologic appearance of Diff-Quick staining preparations. Percent viability of alveolar macrophages by trypan blue exclusion was >90%. For MARCO antibody-depletion, alveolar macrophages were incubated with human anti-MARCO antibody (1 μg/ml; (Hycult, Uden, The Netherlands) for 4 hours prior to bacterial challenge.

Murine alveolar macrophages and bone marrow (BM)-derived macrophages were isolated from $Nrf2^{+/+}$, $Nrf2^{-/-}$, $Keap1^{f/f}$[5], Lysm-$Keap1^{-/-}$ mice (specific deletion of Keap1 in myeloid cells) was created by crossing with mice bearing Cre recombinase under the control of lysozyme M promoter as described in the

supplemental material (Fig. S6). BMDM were isolated by culturing bone marrow cells in the presence of 10 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech) for 7 days. Murine macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Mouse embryonic fibroblasts (MEFs) were isolated from *Nrf2*$^{+/+}$, *Nrf2*$^{-/-}$, and *Keap1*$^{-/-}$ mice as previously described [6, 7] and were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 1% penicillin–streptomycin.

The human THP-1 cell line (American Type Culture Collection, Manassas, VA) was grown in RPMI 1640 medium supplemented with 10% non-heat-inactivated fetal bovine serum and β-mercaptoethanol per manufacturer's instructions. Macrophage differentiation occurred through supplementation with 20 ng/ml Phorbol 12-myristate 13-acetate (Sigma Aldrich, St. Louis, MO) for 48 h.

Bacterial Strains: Sputum isolates of nontypeable *H. influenzae* (Ref# 11P6H1) and *P. aeruginosa* (Ref# 19P207) obtained from patients with COPD at the Department of Veterans Affairs, Western New York Healthcare System were used [8-11]. For bacterial growth, *H. influenzae* was cultured on chocolate agar or grown in brain heart infusion broth supplemented with 20 μg/ml NAD and 10 μg/ml hemin at 35°C in 5% CO2. *P. aeruginosa* was cultured on LB agar plates or in LB broth at 37°C. For FITC-labeling of bacteria, heat inactivated *PA* and *NTHI* (10^9 CFU/ml) were resuspended in 1 ml of labeling buffer (0.1 M NaHCO3, pH 9.2) and incubated with fluorescein isothiocyanate (FITC) (Sigma) under constant stirring in the dark at room temperature for 1 h. Finally, bacteria were

washed three times with PBS and dialyzed overnight against PBS. FITC- labeled bacteria were resuspended with PBS at a concentration of 10^9/ml.

Clearance of Bacteria *In vitro*: After treatment, macrophages (10^5 cells) were incubated with bacteria (*PA* or *NTHI*) for 4 h. Subsequently, 100 µL of cell free culture medium was aseptically plated and cultured on tryptose blood and or chocolate agar plates at 37°C. The number of bacterial colonies was counted after 24 h.

Phagocytosis Assay: Bacterial phagocytosis was quantified using a flow cytometry analysis. Macrophages were incubated with FITC-labeled bacteria (*PA* or *NTHI*) at a bacteria/alveolar macrophage ratio of 20:1 for 4 h at 37°C with continuous gentle rotation. At the end of the incubation, cells were washed three times with cold PBS and resuspended in PBS containing 0.2% (wt/vol) trypan blue to quench fluorescence caused by the binding of bacteria to the surface of the cells and 1% (vol/vol) paraformaldehyde to fix the cells. Flow cytometry was conducted using a FACSCalibur machine (BD Biosciences, San Diego, CA) and CellQuest software (BD Biosciences). Phagocytic activity was expressed as the mean fluorescence intensity (MFI) obtained using FlowJo software (Tree Star, Inc., Ashland, OR). For bacterial binding assay, macrophages were treated with cytochalasin D prior to incubation with FITC-*PA* and analyzed by FACS for the acquisition of fluorescence as an indication of macrophage association with bacteria.

Intracellular Killing: To determine intracellular killing, macrophages were treated with vehicle (DMSO) or sulforaphane (10 µM) for 24 hours. Cells were

then washed and the medium was replaced with serum-free medium. After the medium replacement, approximately 10^5 CFUs of *PA* were added to each well. After 1 h, the medium was removed and cells were washed, and the gentamicin-containing medium was added to kill bound, unphagocytosed bacteria. Cells were lysed at the indicated time points, and lysates were plated to determine viable intracellular bacteria.

Animals and Treatments: $Nrf2^{+/+}$ and $Nrf2^{-/-}$ C57BL/6J mice were housed under controlled conditions for temperature and humidity, using a 12-hour light/dark cycle. At 8 weeks of age, mice were exposed to CS for (2.5 h/day for 5 days/week) for 1 week or or 6 months using a TE-10 smoke machine (Teague Enterprises) and 2R4F reference cigarettes with a total suspended particle concentration of 250 mg/m^3 as previously described [12]. Following exposure to CS, mice were inoculated with *PA* or *NTHI* at a dose of 10^6 CFU in 50 µl of PBS intranasally. For sulforaphane administration (0.5 mg/mouse of R, S-sulforaphane in PBS), Aeroneb Lab nebulizer (Aerogen, Inc., Galway, Ireland) designed specifically for small animals was used. All animal protocols were conducted as approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Bronchoalveolar lavage in mice: BAL fluid analysis and cell counts with differential staining were carried out as described previously [13]. For analyzing bacterial colonization in lungs, 100 µl of BAL fluid were diluted serially in sterile 0.9% NaCl and aseptically plated and cultured on appropriate (blood [*PA*] or chocolate [*NTHI*]) agar plates. After 24 h, the number of CFU was counted.

Antibodies and Flow Cytometry: Antibodies against MARCO were purchased from Santa Cruz Biotechnology (mouse) and Hycult (human), and Nrf2 antibody was purchased from Santa Cruz (mouse and human). Flow cytometry analysis was performed using a Beckton Dickinson FACS-Calibur Flow cytometer (BD, Franklin Lakes, NJ). Analysis of flow cytometry data was performed using FLOWJO software (Tree Star, Inc., Ashland, OR).

siRNA Transfection in Human Alveolar Macrophages: COPD alveolar macrophages plated at 80% confluence in a 96-well plate were transfected with Nrf2 siRNA (Dharmacon) and or control ssRNA using JetPI (Polyplus Transfection-SA, Illkirch, France) macrophage transfection reagent according to manufacturer's protocol. Knockdown of the Nrf2 gene and Nrf2 target genes were quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) 24 h after transfection.

Microarray and Quantitative real-time RT-PCR: Total RNA was extracted from macrophages using the RNeasy Mini Kit according to the manufacturer's recommended protocol (Qiagen Inc., Valencia, CA). Quality assessment was determined by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100. Processing of templates for GeneChip analysis and data analysis was in accordance with methods described previously [6]. Quantitative real-time RT-PCR analyses were conducted using assay on demand probe sets (Applied Biosystems), and reactions were analyzed using the ABI 7000 Taqman system. GAPDH was used for normalization.

Identification of AREs in the Promoter of MARCO: To identify the presence and location of AREs in the MARCO (Accession No. 17167) promoter, the 2-kb upstream region from the translation start site was downloaded from the NCBI database (www.ncbi.nlm.nih.gov). AREs in this region were identified as previously described [14, 15].

Plasmids and Mutagenesis: The 5′ flanking region of the mouse MARCO promoter region was PCR amplified from genomic DNA isolated from murine blood, cloned into pCR2.1 (Invitrogen), and subsequently cloned into the pGL3 Basic vector (Promega, Madison, WI, USA) [14, 15]. Two deletion constructs (− 1708 to +76 and − 1005 to + 76) were generated. The primers used for amplification were as follows: AAAACCACTGAGGCA (ARE1-2 forward), ATGGAACCCAGAG (ARE1 forward), and GATTTCCATGTGGGTGGAAC (reverse primer for all constructs).

Individual AREs identified in the mouse MARCO promoter region were PCR amplified from the ARE1–2 constructs and ligated into pCR2.1 (Invitrogen). A Kpn1-Xho1 fragment from this construct was cloned into the pTAL luciferase reporter vector (BD Biosciences, San Jose, CA, USA). The forward primers used for amplification were as follows: ARE1 TCCCCCACTTCTGATGATGT, ARE 2 AAAACCACTGAGGCATCGAC. The reverse primers used for amplification were as follows: ARE1 GTTCCACCCACATGGAAATC, ARE2 CACAAACCTCTGGGTTCCAT. Mutated (mu) ARE sequences were generated by using a site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA)

according to manufacturer's instructions. Primers containing the mu-ARE sequences (mu-ARE2, CTTAATGCACAAA*CC*AAAAGGCATTCAG and mu-ARE1, ATATGTATCCTG*CC*ACCTGGCACCAT) were used. The mutation in each promoter was verified by sequencing. The NQO1 ARE was used as a positive control [16].

DNA Transfection and Luciferase Activity: Cells were transfected at 80% confluence using Lipofectamine 2000 (Invitrogen), and luciferase activity was assessed as previously described [14, 15]. A ratio of luciferase activity to *Renilla* luciferase activity was determined to normalize for transfection efficiency.

Chromatin Immunoprecipitation Assay: BMDM macrophages were isolated and derived from Keap1 LysM and Keap1$^{fl/fl}$ mice, and the chromatin immunoprecipitation (CHIP) assay was performed using a commercially available kit (Upstate Biotechnology, Lake Placid, NY, USA) as previously described [14]. The MARCO primer sequences were as follows: (1) ARE1 forward, TGCTATTAACAAAGATCTCT; reverse, CCAGGCACCCATATCTCAGT; (2) ARE2 forward, TCCTCACAGATATGGAGCTC; reverse, TCTGCTTGCTGCCTAGAGGT.

ChIP assay was performed using either anti-Nrf2 or anti-RNA Pol2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

MARCO shRNA: THP-1 cells were infected with lentivirus particles containing MARCO shRNA (Open Biosystems, Huntsville, AL). Puromycin supplemented

medium ensured positive selection of lentivirus plasmid-expressing cells, and knockdown of MARCO mRNA and protein was confirmed by both real-time PCR and western blot.

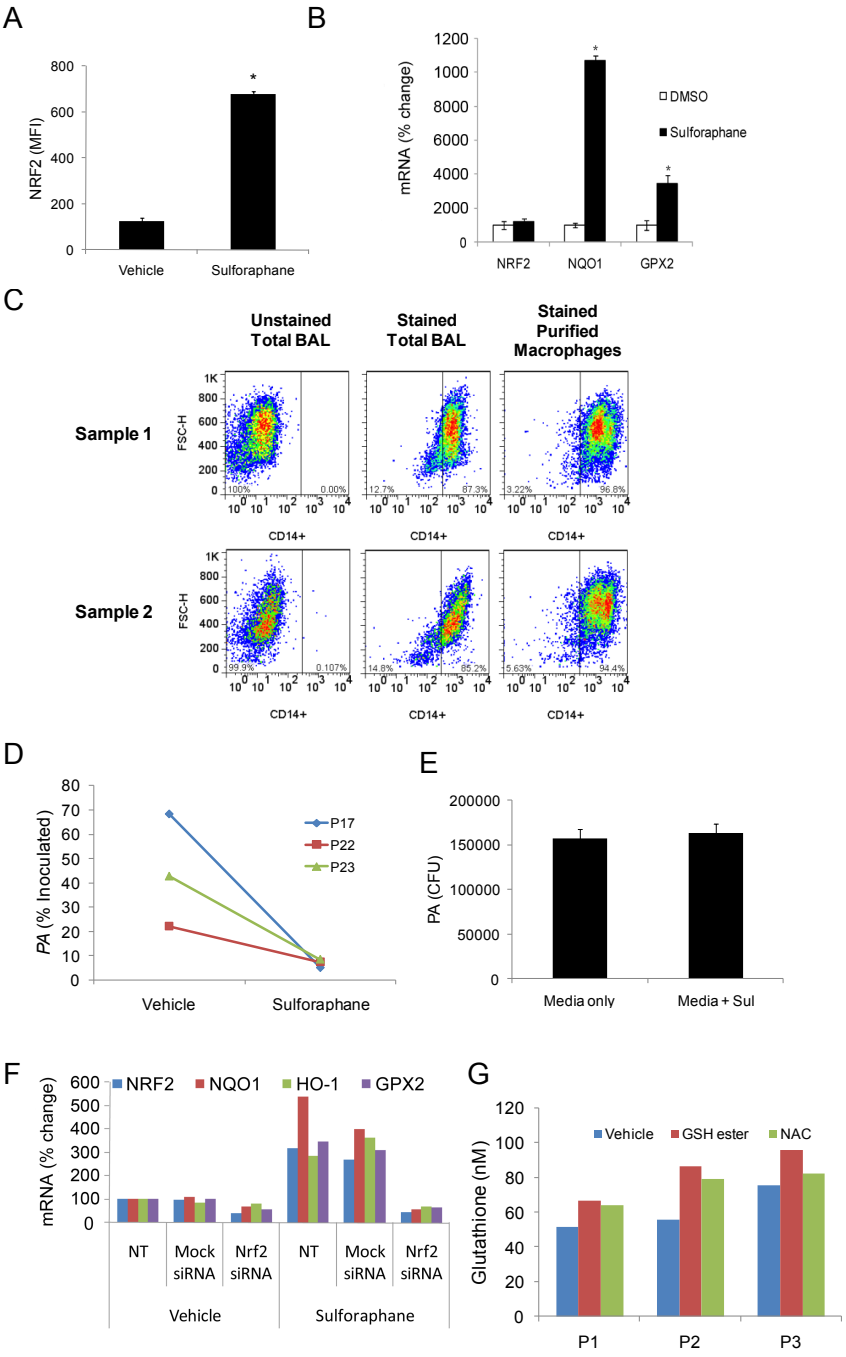

Figure S1: Sulforaphane stabilizes Nrf2 expression in COPD alveolar macrophages and enhances Nrf2-dependent gene expression. (**A**) Nrf2 protein levels in vehicle or sulforaphane treated COPD alveolar macrophages as measured by flow cytometry. (**B**) mRNA expression of Nrf2 and Nrf2-regulated antioxidant genes NQO1 and GPX2 in COPD macrophages after sulforaphane treatment. (**C**) Representative cytograms of adherent purified alveolar macrophages obtained from two human subject broncho-alveolar lavage fluid stained for macrophage marker CD14. (**D**) *PA* colonization in the culture medium of vehicle- or sulforaphane-treated alveolar macrophages from non-COPD patients. (**E**) *PA* colonization in cell-free medium with or without sulforaphane. (**F**) mRNA expression of Nrf2 and Nrf2-regulated antioxidant genes NQO1,HO-1 and GPX2 in COPD macrophages transfected with mock siRNA, Nrf2 siRNA or no treatment (NT, untransfected) prior to sulforaphane treatment. (**G**) Intracellular glutathione levels in COPD macrophages after treatment with vehicle, NAC, GSH ester, or sulforaphane.

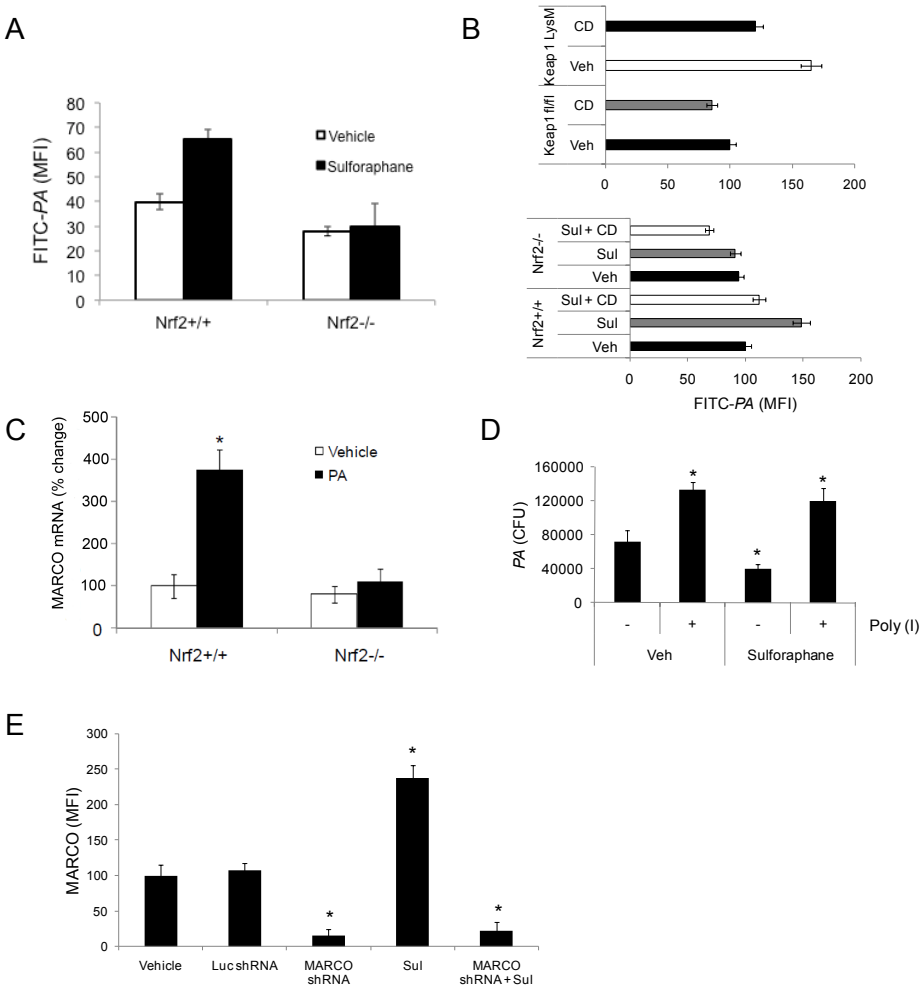

Figure S2: Nrf2-dependent increase in bacterial uptake is dependent on scavenger receptor MARCO-mediated bacterial binding. (**A**) Uptake of FITC-*PA* by vehicle or sulforaphane treated alveolar macrophages from Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice. (**B**) Flow analysis of macrophages treated with cytochalasin (2 µM) 45 min after incubation with FITC-PA. (**C**) MARCO mRNA expression in macrophages from Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice 4 h after incubation with *PA*. (**D**) *PA* colonization (CFU's) in culture medium of THP-1 macrophages treated sulforaphane or vehicle after incubation with poly (I) (10 µg/ml), a scavenger receptor inhibitor. Data represented as CFU's. (**E**) MARCO expression by FACS in vehicle- or sulforaphane-treated THP-1 cells following stable transfection of luciferase or MARCO shRNA.

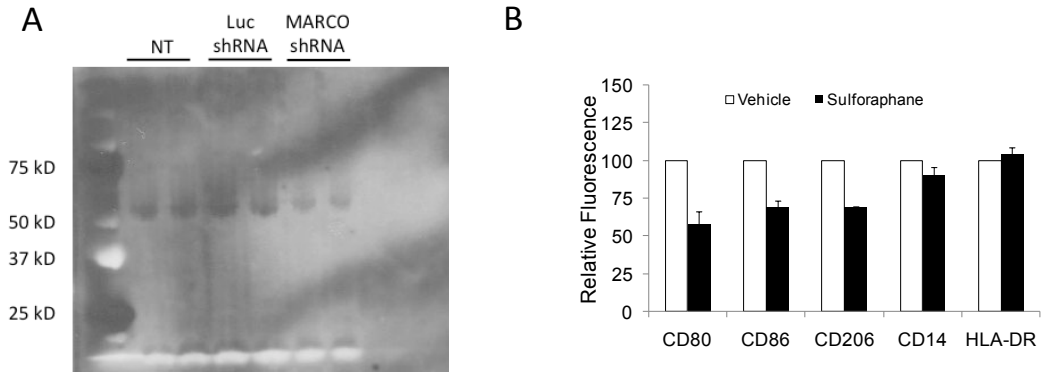

Figure S3: Determination of antibody specificity and macrophage activation. **(A)** Western blot of MARCO using PLK-1 antibody in untransfected (NT), luciferase shRNA transfected (Luc shRNA), or MARCO shRNA transfected THP-1 macrophages. **(B)** Surface expression of CD80, CD86, CD206, CD14, and HLA-DR as measured by flow cytometry in vehicle and sulforaphane treated COPD alveolar macrophages.

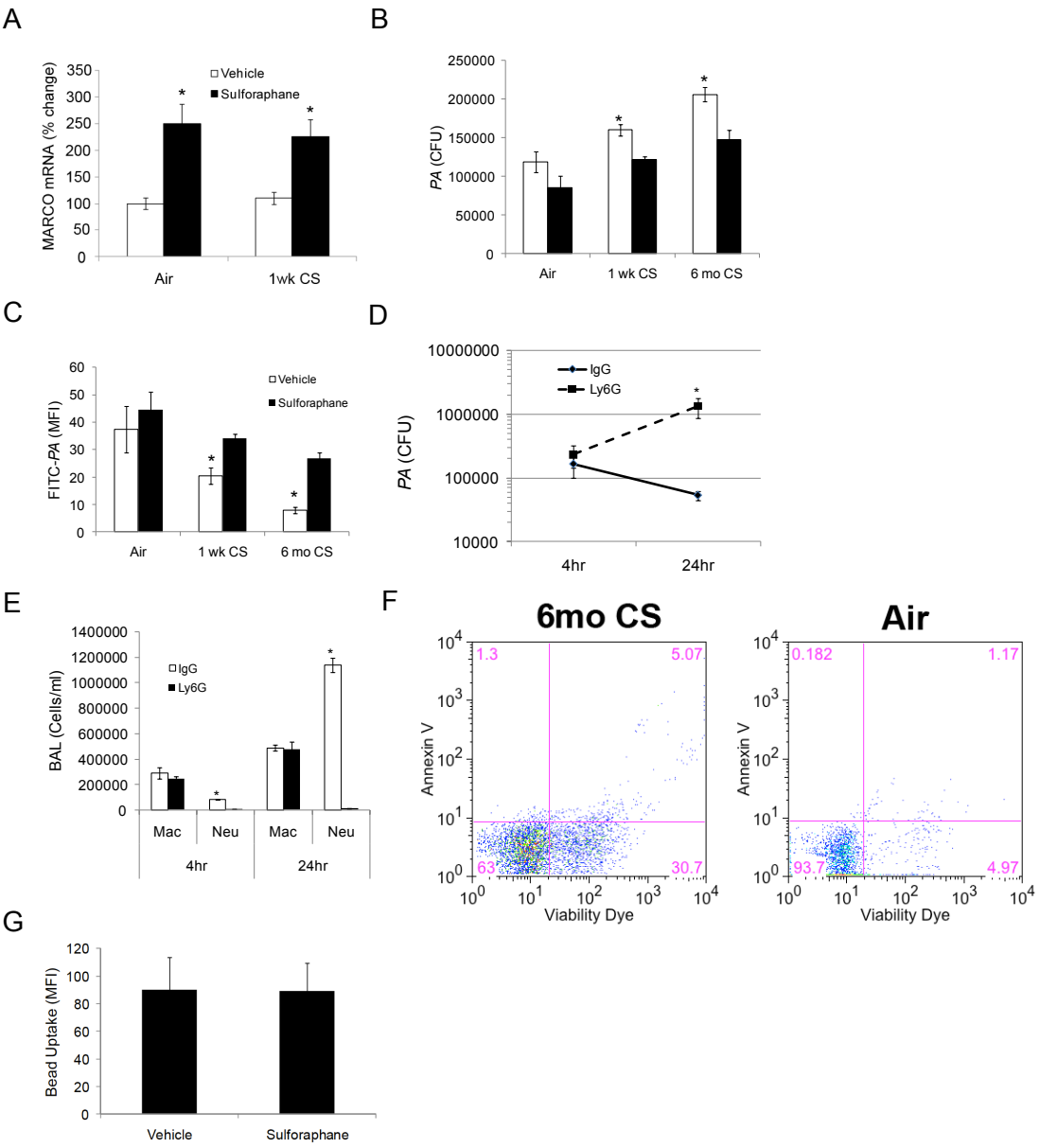

Figure S4: Macrophage response and ex vivo analyses. (**A**) MARCO expression (mRNA) in alveolar macrophages isolated from mice exposed to cigarette smoke or air after treatment with bacteria (*PA*) or sulforaphane. (**B**) *ex vivo PA* clearance by vehicle or sulforaphane treated alveolar macrophages isolated from mice exposed to room air, 1 week, or 6 months of CS. (**C**) ex vivo uptake of FITC-*PA* by vehicle or sulforaphane treated alveolar macrophages isolated from mice exposed to filtered-air, 1 week, or 6 months of CS. (**D**) Bacterial colonization and (**E**) Inflammatory cells in broncho-alveolar fluid at 4 h and 24 h post-infection in control (IgG) mice or mice depleted of neutrophils by intraperitoneal administration of anti-Ly6G antibody. (**F**) Incorporation of propidium iodide viability dye and annexin V staining on total BAL cells from 6mo cigarette smoke or air exposed mice. Data are representative cytograms. (**G**) Uptake if polystyrene beads by vehicle or sulforaphane treated Nrf2$^{+/+}$ macrophages.

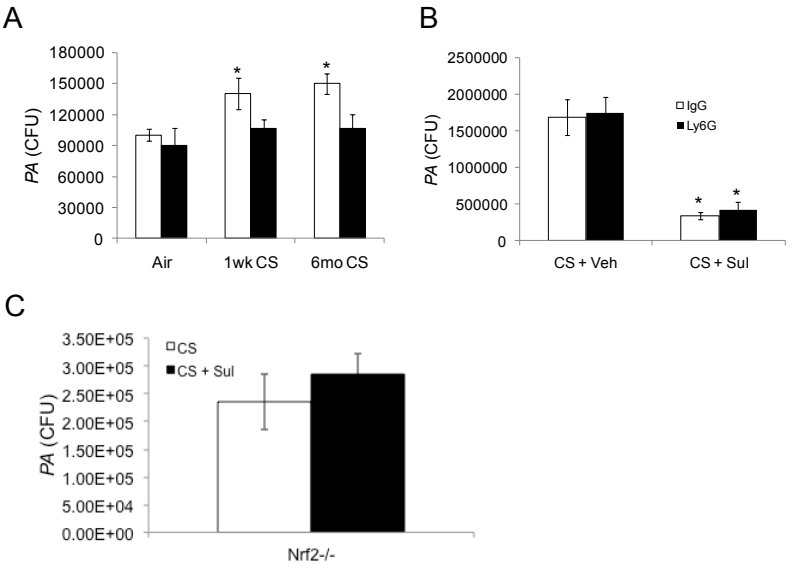

Figure S5: Sulforaphane decreases lung bacterial CFUs in $Nrf2^{+/+}$ mice. (**A**) Bacterial burden in lungs of CS (6 months) or air-exposed wild-type mice treated with sulforaphane or vehicle 4 h after *PA* infection. Data are represented as mean ± SEM of CFUs; n= 5 / group. * Significant compared to air or CS alone ($p < 0.05$). (**B**) Bacterial burden in the lungs of CS-exposed mice treated with control (IgG) or anti- Ly6G antibody following vehicle or sulforaphane administration. * Significant compared to CS alone ($p < 0.05$). * Significant compared to vehicle ($p < 0.05$). (**C**) Bacterial burden in the lungs of CS (1wk) or CS (1wk) plus sulforaphane treated $Nrf2^{-/-}$ mice.

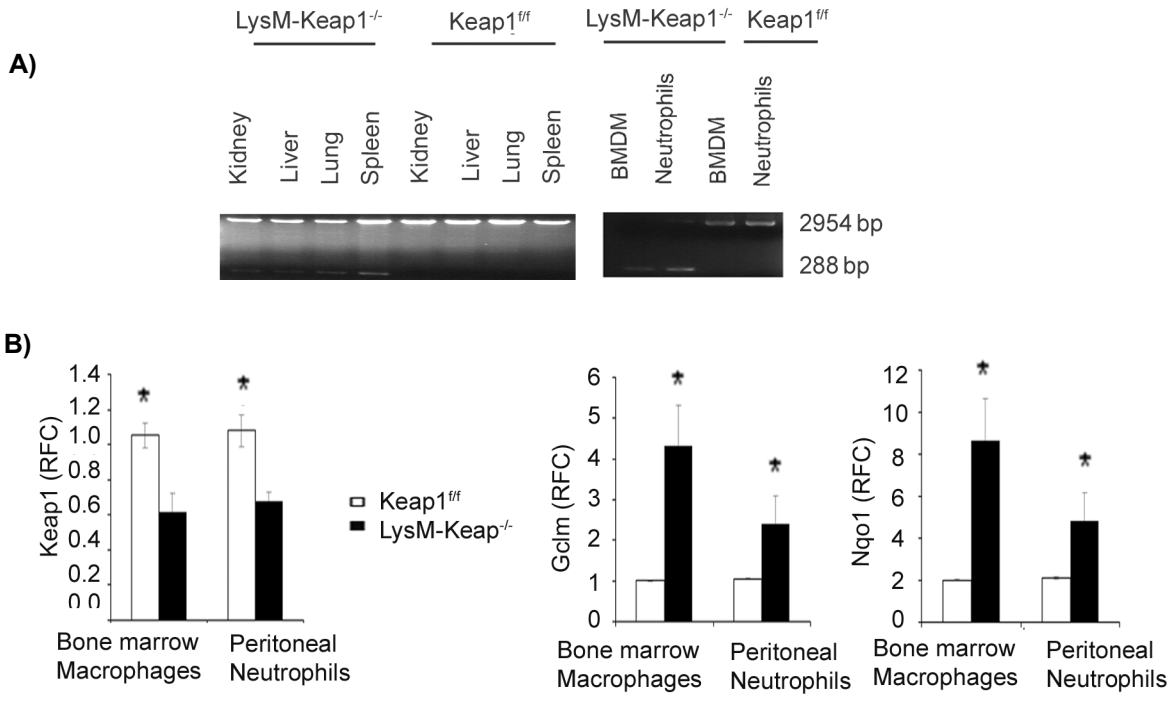

Figure S6: Generation and characterization of Lysm-Keap1$^{-/-}$ conditional knockout mice. (A) Specific recombination of the conditional Keap1 allele in the LysM-Keap1$^{-/-}$ mice lungs, liver, kidney, spleen, bone marrow macrophages, and neutrophils. The 288 bp band represents exons 2 and 3 deleted Keap1 allele and 2954 bp band represents the floxed or the wild-type allele. No deletion was detected in the macrophages and neutrophils from Keap1$^{f/f}$ mice. (B) mRNA expression by qRT-PCR of Keap1, Gclm, and Nqo1 genes in bone marrow macrophages and peritoneal neutrophils from Keap1$^{-/-}$ and Keap1$^{f/f}$ mice. * $p < 0.05$.

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