Cigarette Smoke Impairs Clearance of Apoptotic Cells Through Oxidant-dependent Activation of RhoA.

Tiffany R. Richens, Derek J. Linderman, Sarah A. Horstmann, Cherie Lambert Yi-Qun Xiao, Robert L. Keith, Darren M. Boé, Konosuke Morimoto, Russell P. Bowler, Brian J. Day, William J. Janssen, Peter M. Henson and R. William Vandivier

Online Data Supplement

METHODS

Mn (III) tetrakis (5, 10, 15, 20-benzoic acid) (MnTBAP) was provided by Dr. Brian J. Day (National Jewish Medical and Research Center, Denver, CO, USA). Y-27632, a specific inhibitor of RhoA Kinase, was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA) N-Acetyl-L-cysteine was obtained from Sigma-aldrich Chemical Co. (St. Louis, MO). Cell permeable C3 transferase and the G-LISA RhoA Activation Assay were obtained from Cytoskeleton Inc. (Denver, CO). Purified Mouse Anti-Human CD45 was obtained from BD Biosciences (Franklin Lakes, NJ).

Experimental animals

Mice were housed and studied under institutional animal care and use committee-approved protocols at the animal facility of National Jewish Medical and Research Center and the Veterans Administration Medical Center (Denver, CO). Experiments were performed on 8- to 12-wk-old, age-matched, female C57BL/6J (Jackson Laboratories, Bar Harbour, ME) and ICR (Taconic, Hudson, NY) mice, 1 year old, age- and sex-matched FVB/N mice (Jackson Laboratories)(1), and 4 month old, age- and sex-matched mice overexpressing the human gene for extracellular superoxide dismutase (ECSOD OE) (2). TNFα double receptor knockout mice (B6;129S-*TNFrsf1a*^{tm1Imx}*TNFrsf1b*^{tm1Imx}/J; stock # 003243) and controls (B6129SF2/J; stock # 101045) were purchased from Jackson Laboratories.

Cigarette smoke exposure

Mice were exposed to CS in a TE-10c smoking chamber (Teague Enterprises, Davis, CA) in the core facility at National Jewish Medical and Research Center(3). The CS was composed 11% mainstream and 89% side-stream smoke and delivered at a concentration ranging from 25-120 mg/m^3 total particulate matter (TPM), depending on the experiment, for 5 hours/day, 5 days/week. 3R4F research cigarettes were purchased from the Kentucky Tobacco Research and Development Center (University of Kentucky).

Primary cell isolation and culture

Neutrophils were isolated and prepared from normal blood using Percoll gradient centrifugation, as previously described (4).

Murine thymocytes were isolated from the thymi of 3- to 4-wk-old, female C57BL/6J mice, by passing thymi through a 40-μm strainer (Fisher Scientific, Pittsburgh, PA) to separate individual cells.

Murine alveolar macrophages were obtained by performing bronchoalveolar lavage (BAL) using 10mL of ice-cold PBS with 5mM EDTA. Samples were pooled and cells were added to a 96-well plate at a density of 100,000 cells/well. The cells were cultured in DMEM (MediaTech Inc, Manassas, VA) with 10% FBS (Gemini Bio Products; Sacramento, CA) and supplemented with 2mM L-glutamine, 100U/ml penicillin, and 100g/ml streptomycin (Sigma-Aldrich), and incubated at 37°C in 10% CO_2 for 30 minutes. Media was change after 30 minutes and cells were incubated overnight prior to experimentation.

Cell lines and culture

The human Jurkat leukemia T cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (MediaTech Inc.) with 10% heat-inactivated FBS (Gemini Bio Products) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin (Sigma-Aldrich and incubated at 37°C in 5% CO_2.

Induction of apoptosis

Apoptosis was induced in Jurkat T cells by exposure to UV irradiation at 312 nm (Fotodyne Inc. Hartland, WI) for 12.5 minutes and cultured in RPMI 1640 (MediaTech Inc.) with 10% FBS (Gemini Bio Products) at 37°C in 5% CO_2 for 3 hours before use. Human neutrophils were cultured in RPMI 1640 (MediaTech Inc.) with 10% FBS (Gemini Bio Products) at 37°C in 5% CO_2 for 18 h before use. Thymocytes were cultured in RPMI 1640 (MediaTech Inc.) with 10% FBS (Gemini Bio Products) at 2 x 10^6 cells/ml at 37°C in 5% CO_2 for 3 h. Murine thymocytes were exposed to ultraviolet irradiation at 254 nm for 10 minutes and were cultured in RPMI 1640 (MediaTech Inc.) with 10% FBS (Gemini Bio Products) at 2 x 10^6 cells/ml at 37°C in 5% CO_2 for 3 hours. All cells were approximately 70% apoptotic by nuclear condensation at the time of experimentation, as described previously (5, 6). Jurkat T cells were opsonized by adding 1 ug/1x10^6 cells of IgG anti-human CD45 antibody (BD Biosciences) to the media and incubating at 4°C for 30 minutes prior to the experiment.

IgG Opsonization

Jurkat T cells were opsonized by adding 1 ug/1x10^6 cells of IgG anti-human CD45 antibody (BD Biosciences) to the media and incubating at 4°C for 30 minutes prior to the experiment.

In vitro phagocytosis assays

Phagocytosis was determined by visual inspection of samples and was expressed as the phagocytic index (PI), as described(7). A minimum of 300 alveolar macrophages was counted per condition in duplicate. In all cases, during analysis, the reader was blinded to the sample identification.

One hundred thousand alveolar macrophages (from cigarette smoke exposed mice or air control mice) were pretreated with one of the following: 0–10 μM MnTBAP, 0–10 μM Y-27632, 0.1-1 mg/mL NAC, or 1-2 mg/mL C3 transferase for 4 h before experimentation. Experiments included media controls as well as viable and opsonized, viable Jurkat T cell. Phagocytosis assays were then performed with 1 x 10^6 apoptotic Jurkat T cells/well at 37°C in 10% CO_2 for 2 hours. The wells were washed 5 times with PBS and stained with modified Wright's Giemsa (Fisher Scientific).

In vivo phagocytosis assays

To test the effect of MnTBAP on uptake of apoptotic cells in mice exposed to cigarette smoke, mice were divided into four groups and treated as follows: 1) Air control group, treated with PBS by i.p. injection; 2) Air MnTBAP group, treated with MnTBAP (5 mg/kg) by i.p. injection; 3) CS control group, treated with PBS by i.p. injection; 4) CS MnTBAP group, treated with MnTBAP (5 mg/kg) by i.p. injection. Mice were treated MnTBAP three times: before smoke exposure, immediately following smoke exposure and the next morning before the time of experimentation.

To test the effect of Y-27632 on uptake of apoptotic cells in mice exposed to CS, mice were divided into six groups: CS exposed treated with PBS, Y-27632 (1mg/kg) or Y-27632 (10

mg/kg) by gavage and air exposed treated with PBS, Y-27632 (1mg/kg) or Y-27632 (10 mg/kg) by gavage. Mice were treated once 4h prior to experimentation. The instillation of apoptotic thymocytes was done 20 hours after completion of CS exposure.

Apoptotic thymocytes were instilled intratracheally as previously described (5, 8, 9). Briefly, mice were anesthetized with Avertin, following which 10 x 10^6 apoptotic thymocytes or human neutrophils, suspended in 50 µl of PBS, were instilled intratracheally using a modified animal feeding needle (Fisher Scientific). Sixty minutes later, whole lung bronchoalveolar lavage was performed with a total of 3 ml of ice-cold PBS. Lavage cells were fixed and stained with modified Wright's Giemsa (Fisher Scientific). Phagocytosis was determined by visual inspection of samples, as previously described and was expressed as a PI. A minimum of 300 alveolar macrophages was counted blindly.

RhoA Activity Assay

At indicated timepoints, mice lungs were lavaged with 10 ml cold PBS containing 5mM EDTA. Two million cells were plated per well of a 6-well plate in DMEM + 10% FBS + PSG. Cells were incubated at 37ºC with 10% CO_2 for 30 minutes until the cells were adherent. Iced cells were then washed with 3 mL cold PBS and then lysed in 70 µl of ice-cold Cell Lysis Buffer. In some experiments mice were pre-treated with MnTBAP at 5 mg/kg prior to CS exposure and again immediately after CS exposure.

The remainder of the protocol was followed as described in the GLISA RhoA Activation Assay Biochem Kit manual (Cytoskeleton, Denver, CO). Briefly, cell lysates were collected and snap frozen in liquid nitrogen. Protein concentrations were determined for each sample using the

Precision Red Advanced Protein Assay Reagent (Cytoskeleton), then equalized. The cell lysates were added to the Rho plate, binding Rho-GTP. The plate was washed, an antigen-presenting buffer was added, followed by primary and secondary antibodies. The reaction was detected using a horseradish peroxidase detection reagent followed by the stop buffer. The plate was read immediately by measuring absorbance at 490 nm on a microplate spectrophotometer.

Statistics

For ex vivo experiments, means were analyzed using ANOVA for multiple comparisons. When ANOVA indicated significance, a Dunnett's method was used to compared groups with an internal control. For all other experiments in which two conditions were being compared, a Student's *t-test* assuming equal variance was used. All data were analyzed using JMP (version 3) Statistical Software for the Macintosh (SAS Institute Inc., Cary, North Carolina, USA) and are presented as the mean ± SEM. *In vivo* experiments were analyzed using a Wilcoxon Rank Sum Test for matched pairs, or a Mann-Whitney Test for unmatched pairs. When three or more groups were analyzed, a Kruskal-Wallis Test with a Dunn's Test for post hoc analysis was used. All animal data were analyzed using Prism 5 for Mac OS X (GraphPad Software Inc, La Jolla, CA) and are presented with box-plots showing the median and ranges.

For in vivo experiments, medians were analyzed by a Wilcoxon Rank Sum Test where mice were matched and a Mann Whitney Test when mice were not. Post hoc analysis was performed using a Dunn's Test.

REFERENCES

E1. Keith, R. L., Y. E. Miller, T. M. Hudish, C. E. Girod, S. Sotto-Santiago, W. A. Franklin, R. A. Nemenoff, T. H. March, S. P. Nana-Sinkam, and M. W. Geraci. 2004. Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. *Cancer Res* 64(16):5897-904.

E2. Folz, R. J., A. M. Abushamaa, and H. B. Suliman. 1999. Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J Clin Invest* 103(7):1055-66.

E3. Martin, R. J., R. B. Wexler, B. J. Day, R. J. Harbeck, K. E. Pinkerton, and H. W. Chu. 2006. Interaction between cigarette smoke and mycoplasma infection: a murine model. *Copd* 3(1):3-8.

E4. Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 83(3):865-75.

E5. Morimoto, K., W. J. Janssen, M. B. Fessler, K. A. McPhillips, V. M. Borges, R. P. Bowler, Y. Q. Xiao, J. A. Kench, P. M. Henson, and R. W. Vandivier. 2006. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol* 176(12):7657-65.

E6. Savill, J. S., P. M. Henson, and C. Haslett. 1989. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. *J Clin Invest* 84(5):1518-27.

E7. Fadok, V. A., J. S. Savill, C. Haslett, D. L. Bratton, D. E. Doherty, P. A. Campbell, and P. M. Henson. 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* 149(12):4029-35.

E8. Teder, P., R. W. Vandivier, D. Jiang, J. Liang, L. Cohn, E. Pure, P. M. Henson, and P. W. Noble. 2002. Resolution of lung inflammation by CD44. *Science* 296(5565):155-8.

E9. Vandivier, R. W., C. A. Ogden, V. A. Fadok, P. R. Hoffmann, K. K. Brown, M. Botto, M. J. Walport, J. H. Fisher, P. M. Henson, and K. E. Greene. 2002. Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J Immunol* 169(7):3978-86.

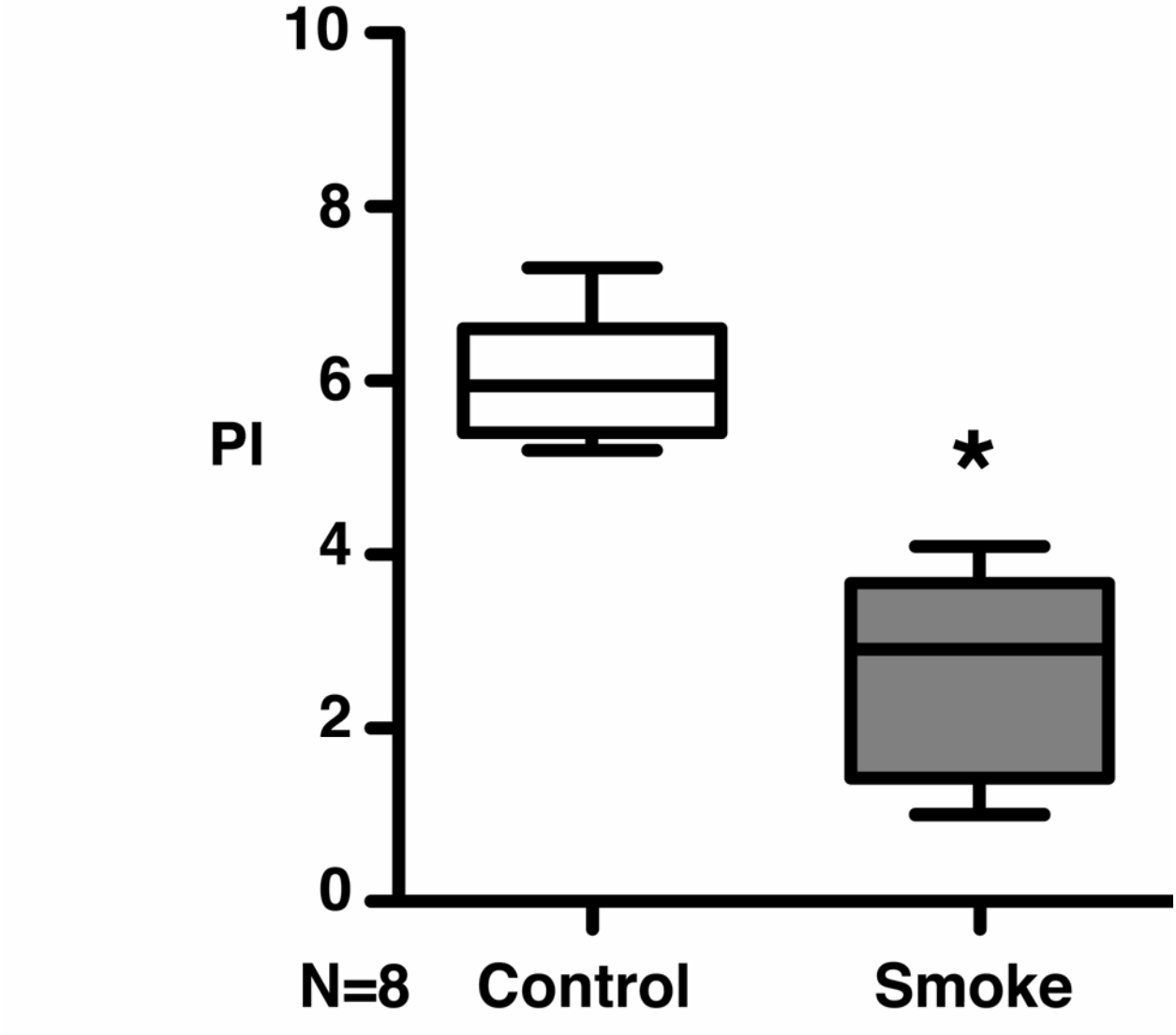
FIGURE LEGENDS

Supplemental Figure 1 (E1). CS impairs efferocytosis *in vivo*, independent of apoptotic cell type. Moderate-dose CS (100mg/m^3 TPM) decreased efferocytosis of human neutrophils *in vivo* at 1 day post-exposure. * significantly different from day 1 air control ($P \leq 0.05$).

Supplemental Figure 2 (E2). Acute CS exposure immediately impairs efferocytosis through a RhoA/Rho kinase-independent pathway. To determine whether acute CS exposure immediately impairs efferocytosis through a RhoA/Rho kinase-dependent pathway, C57BL/6J mice were pretreated with the Rho kinase inhibitor, Y27632, exposed to CS at 100mg/m^3 TPM for 5h, and

were then immediately assessed for their ability to clear instilled apoptotic cells. At this time point, Y-27632 did not prevent the suppressive effect of CS on efferocytosis, confirming that acute CS exposure impairs efferocytosis through a RhoA/Rho kinase independent mechanism. * significantly different from air control ($P \leq 0.05$).

Supplemental Figure 1



Supplemental Figure 2