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

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***Rationale*: Cigarette smoke (CS) is the primary cause of chronic obstructive pulmonary disease (COPD), an effect that is, in part, due to intense oxidant stress. Clearance of apoptotic cells (efferocytosis) is a critical regulator of lung homeostasis, which is defective in smokers and in patients with COPD, suggesting a role in disease pathogenesis.**

***Objectives*: We hypothesized that CS would impair efferocytosis through oxidant-dependent activation of RhoA, a known inhibitor of this process.**

***Methods*: We investigated the effect of CS on efferocytosis *in vivo* and *ex vivo*, using acute, subacute, and long-term mouse exposure models.**

***Measurements and Main Results*: Acute and subacute CS exposure suppressed efferocytosis by alveolar macrophages in a dose-dependent, reversible, and cell type–independent manner, whereas more intense CS exposure had an irreversible effect. In contrast, CS did not alter ingestion through the Fcγ receptor. The inhibitory effect of CS on apoptotic cell clearance depended on oxidants, because the effect was blunted in oxidant-resistant ICR mice, and was prevented by either genetic or pharmacologic antioxidant strategies *in vivo* and *ex vivo*. CS inhibited efferocytosis through oxidant-dependent activation of the RhoA–Rho kinase pathway because (*1*) CS activated RhoA, (*2*) antioxidants prevented RhoA activation by CS, and (*3*) inhibitors of the RhoA–Rho kinase pathway reversed the suppressive effect of CS on apoptotic cell clearance *in vivo* and *ex vivo*.**

***Conclusions*: These findings advance the hypothesis that impaired efferocytosis may contribute to the pathogenesis of COPD and suggest the therapeutic potential of drugs targeting the RhoA–Rho kinase pathway.**

Keywords: pulmonary disease, chronic obstructive; phagocytosis; macrophages, alveolar; superoxides; Rho-associated kinases

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Clearance of apoptotic cells is impaired in smokers and patients with chronic obstructive pulmonary disease (COPD). However, the mechanism by which cigarette smoke (CS) dysregulates apoptotic cell homeostasis and the implications for disease pathogenesis have not been explored.

What This Study Adds to the Field

CS inhibits clearance of apoptotic cells through oxidant-mediated activation of the RhoA–Rho kinase pathway, advancing the hypothesis that failed apoptotic cell clearance contributes to the pathogenesis of COPD and suggesting a new therapeutic target.

Intense oxidant stress is responsible for many of the deleterious effects of cigarette smoke (CS), the most important risk factor for the development of chronic obstructive pulmonary disease (COPD) (1). Each puff of a cigarette contains 10^{15} free radicals, including O_2^- and NO, that combine to form peroxynitrite, a potent mediator of lipid peroxidation (1). CS also acutely suppresses production of antioxidant defenses, such as glutathione, and may prevent adequate induction of antioxidants after chronic exposure (2, 3). The importance of oxidant stress in the pathogenesis of COPD is highlighted by (*1*) the known susceptibility of oxidant-sensitive mouse strains to develop CS-induced emphysema (4, 5), (*2*) the marked sensitivity of mice deficient in Nrf2 (a master transcription factor for 50 antioxidant and cytoprotective genes) to develop smoke-induced emphysema (6, 7), (*3*) the protective effect of strategies aimed at enhancing the antioxidant capacity in the murine lung (8), (*4*) epidemiologic studies linking dietary antioxidant intake with lung function (9, 10), and, although still controversial, the ability of *N*-acetylcysteine to decrease the incidence of COPD exacerbations (11).

CS may also contribute to the pathogenesis of COPD by interfering with the removal of apoptotic cells (termed *efferocytosis*) (12). Efferocytosis is a highly conserved process that plays an important role in the maintenance of lung homeostasis by regulating the inflammatory response (13–16), antiprotease activity (17), and several key growth factors (18, 19). Kirkham and colleagues first suggested that CS might negatively impact efferocytosis by showing that CS extract indirectly suppresses macrophage efferocytosis through modification of extracellular matrix proteins (20). In a series of human studies, Hodge and colleagues found that efferocytosis is not only defective in smokers, but is also defective in patients with COPD, thus suggesting a direct pathogenic link (21–23).

Efferocytosis depends on the interaction of a variety of apoptotic cell ligands, soluble bridging molecules, phagocytic

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receptors, and intact signaling pathways (24). These receptors ultimately regulate efferocytosis by altering the intracellular balance of several Rho GTPases, including Rac-1 and RhoA (25, 26). Rho GTPases are molecular switches that cycle between inactive (GDP-bound) and active (GTP-bound) configurations (27). Rac-1 is activated by the phosphatidylserine receptor(s) and the lipoprotein-related receptor and positively regulates efferocytosis by inducing formation of cell ruffles, which are characteristic of both efferocytosis and macropinocytosis (28–32). In contrast, active RhoA negatively regulates efferocytosis, through its downstream effector Rho kinase, by inducing the formation of stress fibers, focal adhesions, and cell spreading (25, 26).

Two important observations were made that led to the current study. First, Chiba and colleagues showed that CS increases RhoA activity, a negative regulator of efferocytosis, in the bronchial smooth muscle in the rat lung (33); and second, McPhillips and colleagues demonstrated that tumor necrosis factor (TNF)-α inhibits efferocytosis *in vitro* through oxidant-dependent activation of RhoA (34). Therefore, we hypothesized that CS would impair efferocytosis through oxidant-dependent activation of RhoA, and examined this possibility by both *in vivo* and *ex vivo* approaches. Our results indicate that CS impairs efferocytosis in a manner that can be reversible or permanent, depending on the conditions, and that during acute exposure CS inhibits efferocytosis through oxidant-dependent activation of the RhoA–Rho kinase pathway. Some of the results of these studies have been previously reported in the form of an abstract (35).

METHODS

Materials

See the online supplement for details.

Experimental Animals

Mice were housed and studied under institutional animal care and use committee–approved protocols at the animal facility of the National Jewish Medical and Research Center and the Veterans Administration Medical Center (Denver, CO). Experiments were performed on 8- to 12-week-old, age-matched, female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and ICR mice (Taconic, Hudson, NY), 1-year-old, age- and sex-matched FVB/N mice (36), and 4–month-old, age- and sex-matched mice overexpressing the human gene for extracellular superoxide dismutase (ecSOD OE) (37). TNF-α 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, Woodland, CA) in the core facility at the National Jewish Medical and Research Center (38). The CS was composed of 11% mainstream smoke and 89% side-stream smoke and delivered at a concentration ranging from 25 to 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, Lexington, KY).

Primary Cell Isolation and Culture

Human neutrophils, murine thymocytes, and alveolar macrophages were isolated as previously described (39–42). *See* the online supplement for additional details.

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, Manassas, VA) with 10% heat-inactivated fetal bovine serum (Gemini Bio Products, Sacramento, CA) and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 g/ml) (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C in 5% CO_2.

Induction of Apoptosis

Jurkat T cells, human neutrophils and murine thymocytes were induced to undergo apoptosis by ultraviolet irradiation as previously described (40, 43). *See* the online supplement for additional details.

IgG Opsonization

Jurkat T cells were opsonized by adding IgG anti-human CD45 antibody (BD Pharmingen, San Diego, CA), at 1 μg/L × 10^6 cells, to the medium and incubating at 4°C for 30 minutes before the experiment.

In Vitro Phagocytosis Assays

Phagocytosis was determined by visual inspection of samples and was expressed as the phagocytic index, as described (44). 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. *See* the online supplement for additional details.

In Vivo Phagocytosis Assays

Apoptotic thymocytes were instilled intratracheally as previously described (40, 42, 45). *See* the online supplement for additional details.

RhoA Activity Assay

See the online supplement for detailed methods.

Statistics

For *ex vivo* experiments, means were analyzed using analysis of variance (ANOVA) for multiple comparisons. When ANOVA indicated significance, 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 *t* test assuming equal variance was used. All data were analyzed with JMP (version 3) statistical software for the Macintosh (SAS Institute Inc., Cary, NC) and are presented as means ± SEM. *In vivo* experiments were analyzed by Wilcoxon rank sum test for matched pairs, or by the Mann-Whitney test for unmatched pairs. When three or more groups were analyzed, a Kruskal-Wallis test with Dunn's test for post hoc analysis was used. All animal data were analyzed with Prism 5 for Mac OS X (GraphPad Software Inc, La Jolla, CA) and are presented with box plots showing the median and ranges.

RESULTS

Cigarette Smoke Exposure Impairs Uptake of Apoptotic Cells (Efferocytosis) *in Vivo*

Efferocytosis is defective in both smokers and patients with COPD, suggesting that the inability to remove apoptotic cells may contribute to the pathogenesis of COPD. To determine whether CS also impairs efferocytosis in mice, we used our *in vivo* model, which has been used previously to evaluate apoptotic cell clearance by alveolar macrophages (40, 42, 45). C57BL/6J mice were exposed to air or CS for 5 hours at either 25 or 100 mg/m^3 total particulate matter (TPM). These doses were chosen, because 25 mg/m^3 TPM has been used to mimic secondhand CS exposure in mice (46) and 100 mg/m^3 TPM has been used in mouse models of emphysema (6). Ten million apoptotic thymocytes were then instilled intratracheally at time points ranging from 0 to 5 days after smoke exposure. One hour after instillation, whole lung lavage was performed and ingestions of apoptotic cells by alveolar macrophages were scored, using a phagocytic index. Two examples of apoptotic cells being ingested by alveolar macrophages are shown in Figure 1A.

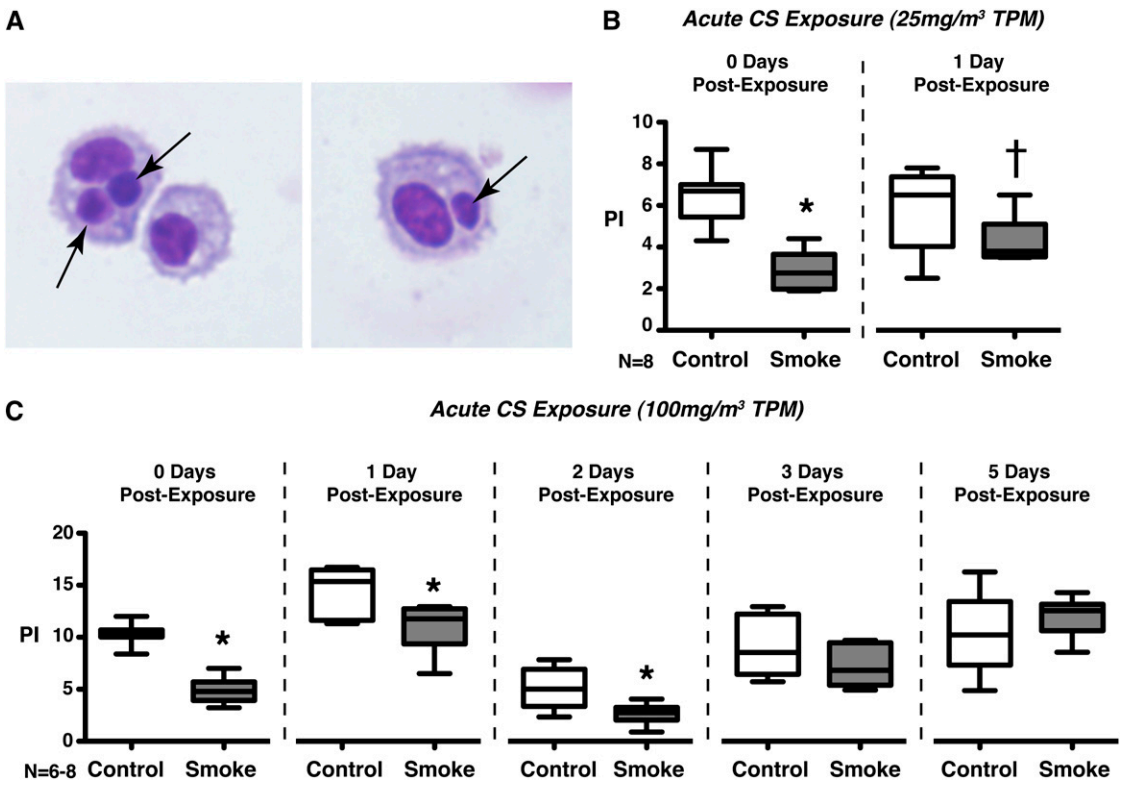

Figure 1. Cigarette smoke (CS) specifically impairs efferocytosis *in vivo*. To determine the effect of CS on efferocytosis *in vivo*, C57BL/6J mice were exposed to air or CS at either 25 mg/m^3 total particulate matter (TPM) or 100 mg/m^3 TPM for 5 hours and were then examined for their ability to remove apoptotic cells, using our *in vivo* clearance assay (42). At time points ranging from 0 to 5 days after CS exposure, 10 million apoptotic murine thymocytes were instilled intratracheally into anesthetized mice. Sixty minutes later, bronchoalveolar lavage was performed, cytospins were stained, and alveolar macrophage ingestions of apoptotic cells were quantified by calculating a phagocytic index (PI). (*A*) Photomicrographs (original magnification, ×40) show alveolar macrophages that have ingested instilled apoptotic thymocytes (*arrows*). (*B*) Low-dose CS (25 mg/m^3 TPM) decreased efferocytosis immediately postexposure (Day 0) and trended toward a decrease at 1 day postexposure, compared with air control. *Significantly different from Day 0 control (P = 0.008); †different from Day 1 control (P = 0.07). (*C*) Moderate-dose CS (100 mg/m^3 TPM) decreased efferocytosis at 0, 1, and 2 days postexposure. *Significantly different from Day 0 control (P = 0.02), Day 1 control (P = 0.03), and Day 2 control (P = 0.03).

Low-dose CS (25 mg/m^3 TPM) decreased efferocytosis immediately (Day 0) after CS exposure (Figure 1B; eight mice per group), but lost statistical significance at 1 day (P = 0.09; four mice per group). In contrast, moderate-dose CS (100 mg/m^3 TPM) decreased efferocytosis at all time points out to 2 days postexposure, but lost significance at 3 days or greater (Figure 1C; six to eight mice per group). Importantly, CS also decreased the clearance of apoptotic neutrophils at 1 day postexposure, indicating that the suppressive effect of CS was cell-type independent (*see* Figure E1 in the online supplement; eight mice per group). Therefore, acute CS exposure impairs efferocytosis *in vivo* in a reversible and cell type–independent manner.

We also tested the effect of CS on efferocytosis *in vivo*, using both subacute (Figure 2A) and chronic models (Figure 2B). In the subacute model, C57BL/6J mice were exposed to CS at 100 mg/m^3 TPM at 5 hours/day for 5 days and were then assessed for alveolar macrophage efferocytosis at 1 and 4 weeks postexposure. Efferocytosis was defective at 1 week but not at 4 weeks after CS exposure (Figure 2A; four to eight mice per group), indicating that the suppressive effect of CS was still reversible. We then examined efferocytosis in a chronic CS exposure model that is normally used to study the development of lung cancer (36). In these experiments, FVB/N mice were exposed to air or CS at 100 mg/m^3 TPM for the first week, and then increased to 250 mg/m^3 TPM for a total exposure period of 22 weeks. Mice were then returned to conventional caging for 20 weeks, before experimentation. Keith and colleagues previously reported on the development of tumors in these mice (36). Interestingly, alveolar macrophage efferocytosis continued to be defective in these long-term CS-exposed mice despite the fact that 5 months had lapsed since their last exposure (Figure 2B; four to six mice per group). Compared with air-exposed mice, the lungs of CS-exposed mice contained abundant clusters of pigmented macrophages and airway-associated inflammatory infiltrates consistent with terminal bronchiolitis (Figure 2C). Patches of pigmented macrophages containing at least 10 macrophages were significantly more common in the lungs of CS-exposed mice, compared with air-exposed mice. (Figure 2D). These results suggest that efferocytosis might become permanently defective at some critical level of CS exposure that causes lung disease reminiscent of the respiratory bronchiolitis that develops in human COPD. Because FVB/N mice were used in long-term experiments (Figures 2B and 2C) and C57BL/6J mice were used in shorter term experiments (Figures 1 and 2A), we cannot rule out the possibility that strain differences may have contributed to these results.

CS Exposure Impairs Efferocytosis *ex Vivo*

Ex vivo experiments were also performed on alveolar macrophages to confirm the suppressive effect of CS on efferocytosis. These were important experiments, because CS is known to increase the numbers of alveolar macrophages, which could falsely lower the alveolar macrophage phagocytic index by affecting the ratio of alveolar macrophages to apoptotic cells.

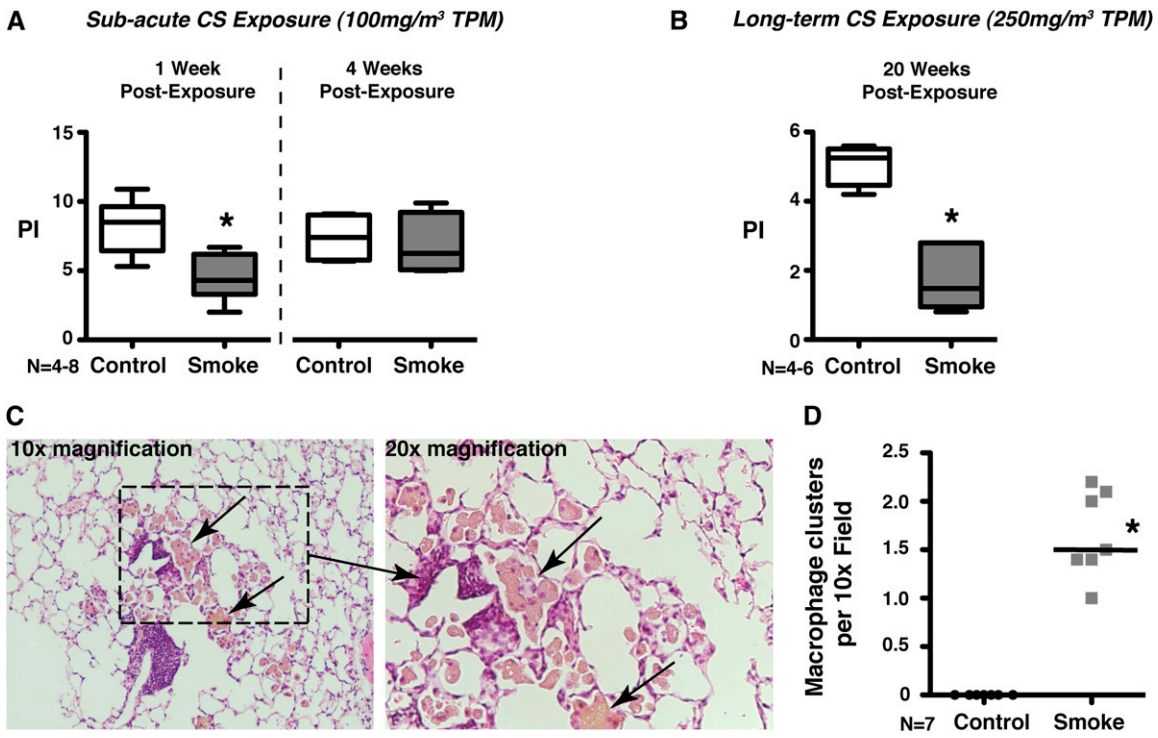

Figure 2. Subacute and long-term cigarette smoke (CS) impairs efferocytosis *in vivo*. (*A*) C57BL/6J mice were exposed to air or CS at 100 mg/m^3 total particulate matter (TPM) for 5 days at 5 hours/day in a subacute exposure model, and were then examined for their ability to ingest apoptotic cells *in vivo*. Subacute CS exposure decreased efferocytosis at 1 week, but not at 4 weeks postexposure. *Significantly different from Week 1 air control ($P = 0.02$). (*B*) In a long-term CS exposure model, FVB/N mice were exposed to air or CS at 100 mg/m^3 TPM for the first week, and then increased to 250 mg/m^3 TPM for a total exposure period of 22 weeks. Mice were then returned to conventional caging for 20 weeks before being examined for their ability to clear apoptotic human neutrophils. *Significantly different from control ($P = 0.02$; Mann-Whitney test). (*C*) Collections of pigmented macrophages (*arrows*) were diffusely present within the lungs of FVB/N mice exposed to CS (*left*: original magnification, ×10; *right*: original magnification, ×20). (*D*) Patches of macrophages containing 10 or more macrophages were significantly more common in the lungs of CS-exposed mice, compared with air-exposed mice. *Significantly different from control ($P = 02$).

Importantly, at 24 hours postexposure, CS did not significantly increase total leukocytes or leukocyte subsets in the bronchoalveolar lavage fluid (Table 1). These studies were performed with alveolar macrophages that had been exposed *in vivo* to air or CS at 100 mg/m^3 TPM for 5 hours. Whole lung lavage was then performed and alveolar macrophages were cultured *ex vivo* overnight before experimentation. The advantage of this approach is that it controls for the number and ratio of alveolar macrophages to apoptotic cells. We found that acute CS exposure decreased alveolar macrophage efferocytosis *ex vivo* (Figure 3A), even after 24 hours of culture without additional exposure to CS, echoing our *in vivo* results (Figure 1).

Using this *ex vivo* model, experiments were then performed to determine whether the suppressive effect of CS was specific for efferocytosis or could be generalized to other forms of phagocytosis. As in Figure 3A, alveolar macrophages were exposed to air or CS for 5 hours *in vivo*, cultured *ex vivo* overnight, and were then tested for their ability to ingest viable cells, apoptotic cells, or IgG-coated viable cells that are ingested through the Fcγ receptor (41). CS inhibited efferocytosis *ex vivo*, but had no effect on ingestion of viable or IgG-opsonized cells (Figure 3B). Therefore, CS suppresses efferocytosis *in vivo* and *ex vivo*, but has no effect on uptake through the Fcγ receptor.

TABLE 1. CELLULAR COMPOSITION OF BAL 24 HOURS AFTER EXPOSURE TO CIGARETTE SMOKE AT 100 mg/m^2 TPM FOR 5 HOURS

Cellular Subsets	Exposure Status	Mean ± SD
Leukocutes/mouse (n=4)	Non-smoked	$4.5 \times 10^5 \pm 2.4 \times 10^5$
	Smoked	$6.5 \times 10^5 \pm 2.7 \times 10^5$
Neutrophils/mouse (n=4)	Non-smoked	$3.0 \times 10^3 \pm 2.4 \times 10^3$
	Smoked	$5.8 \times 10^3 \pm 2.3 \times 10^3$
Macrophages/mouse (n=4)	Non-smoked	$4.4 \times 10^5 \pm 2.3 \times 10^5$
	Smoked	$6.4 \times 10^5 \pm 2.7 \times 10^5$
Lymphocytes/mouse (n=4)	Non-smoked	$3.4 \times 10^3 \pm 2.2 \times 10^3$
	Smoked	$5.6 \times 10^3 \pm 2.7 \times 10^3$

All comparisons of non-smoked *vs.* smoked were nonsignificant; Wilcoxon Test

CS Suppresses Efferocytosis through an Oxidant Stress–dependent Mechanism

Mouse strains vary in their sensitivity to oxidant stress and correspondingly vary in their propensity to develop emphysema when exposed to CS (4–7, 47, 48). We hypothesized that CS would have a diminished capacity to suppress efferocytosis in ICR mice, which are relatively resistant to oxidant stress and the development of CS-induced emphysema (4, 48), compared with the oxidant-sensitive C57BL/6J mice already tested in Figure 1 (5, 47, 48). ICR mice were exposed to air or CS at 100 mg/m^3 TPM for 5 hours and then tested for their ability to clear apoptotic cells *in vivo* at 0, 1, and 2 days postexposure (Figure 4A; seven or eight mice per group). Consistent with our hypothesis, CS nonsignificantly inhibited efferocytosis in ICR mice immediately postexposure ($P = 0.07$), whereas it impaired efferocytosis in the oxidant-sensitive C57BL/6 mice at all of these time points (Figure 1C).

We tested this hypothesis further using both pharmacologic and genetic overexpression strategies. C57BL/6J mice were

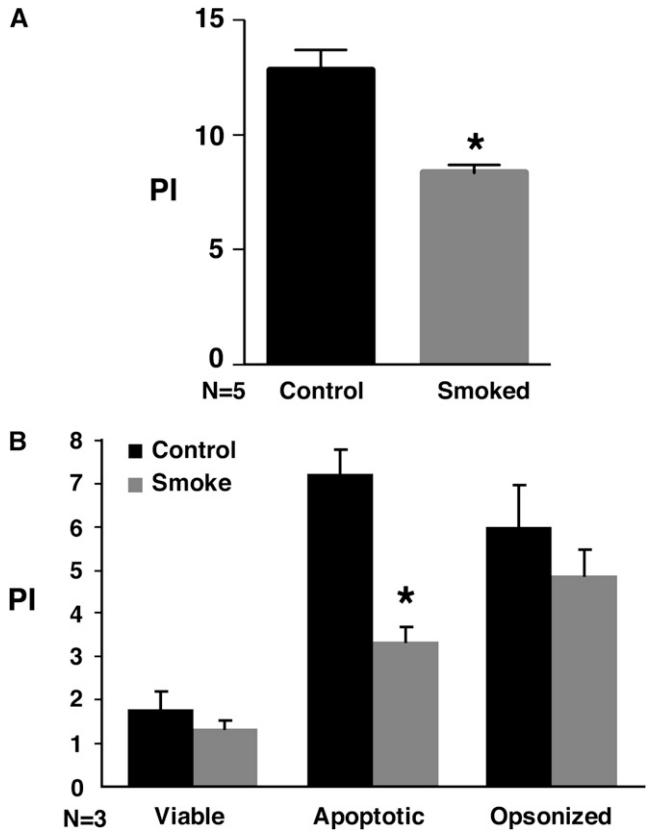

Figure 3. Cigarette smoke (CS) selectively suppresses efferocytosis *ex vivo*. (*A*) To determine whether CS suppressed efferocytosis *ex vivo*, C57BL/6J mice were exposed to air or moderate-dose CS (100 mg/m^3 total particulate matter [TPM]) for 5 hours. Whole lung lavage was then performed and alveolar macrophages were cultured *ex vivo* overnight. Alveolar macrophages were then examined for their ability to ingest apoptotic Jurkat T cells during a 60-minute coculture experiment. *Significantly different from Day 0 air control ($P = 0.002$). PI = phagocytic index. (*B*) To examine whether the suppressive effect of CS was specific for efferocytosis, C57BL/6J mice were exposed to CS and processed as in (*A*), and were then tested for their ability to ingest various targets. CS specifically decreased ingestion of apoptotic Jurkat T cells *ex vivo*, but had no significant effect on ingestion of viable or IgG-opsonized Jurkat T cells. *Significantly different from air control ($P = 0.003$).

treated with the superoxide dismutase mimetic and peroxynitrite scavenger, manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP), administered intraperitoneally at 5 mg/kg three times; once before and after CS exposure and then again the next morning before experimentation (49). Consistent with our hypothesis, CS suppressed efferocytosis *in vivo* at 1 day postexposure, but this effect was prevented by treatment with MnTBAP (Figure 4B; three or four mice per group). For genetic studies we used extracellular superoxide dismutase (ecSOD) overexpressor mice. In these mice ecSOD activity is approximately threefold higher and oxidant-dependent lung injury is attenuated after challenge with hyperoxia, hemorrhage, LPS, and bleomycin (37, 50–53). CS again impaired efferocytosis at 1 day postexposure, but this effect was not seen in ecSOD overexpressor mice (Figure 4C; seven or eight mice per group).

The role of oxidant stress was examined with the *ex vivo* system described previously (Figure 3A and B), in which alveolar macrophages were exposed to air or CS *in vivo* for 5 hours and then removed and cultured *ex vivo* overnight before experimentation. Before each experiment, alveolar macrophages were pretreated *ex vivo* with *N*-acetylcysteine (NAC) overnight, or with MnTBAP for 4 hours before experimentation. At all concentrations tested, both *N*-acetylcysteine (Figure 4D) and MnTBAP (Figure 4E) prevented CS from inhibiting efferocytosis. These data indicate that, *in vivo* and *ex vivo,* CS impairs efferocytosis through an oxidant-dependent mechanism.

CS Impairs Efferocytosis through Oxidant Stress–dependent Activation of the RhoA–Rho Kinase Pathway

On the basis of previous studies (33, 34), we hypothesized that CS suppresses efferocytosis through oxidant-dependent activation of RhoA, a Rho GTPase that potently inhibits efferocytosis by activating its downstream effector, Rho kinase. To answer this question, we first measured RhoA activity in alveolar macrophages taken from C57BL/6J mice immediately and 1 day after exposure to CS for 5 hours, compared with untreated control mice. CS exposure increased RhoA activity 1 day postexposure, but had no significant effect immediately after exposure (Figure 5A). To determine whether CS-activated RhoA occurred through an oxidant-dependent mechanism, RhoA activity was measured in alveolar macrophages taken from C57BL/6J mice treated with and without MnTBAP (as described in Figure 4F). CS again increased RhoA activity, but not in the presence of MnTBAP (Figure 5B), confirming a role for oxidative stress in the activation of RhoA.

Finally, experiments were performed to determine whether increased RhoA activity was directly responsible for CS-impaired efferocytosis in alveolar macrophages. As in previous experiments, alveolar macrophages were isolated from the lungs of CS-exposed C57BL/6J mice, cultured overnight, and then pretreated with C3 transferase for 2 hours to inhibit RhoA, or with Y-27632 for 4 hours to inhibit the downstream effector Rho kinase. Both C3 transferase (Figure 5C) and Y-27632 (Figure 5D) prevented CS from inhibiting efferocytosis. Likewise, pretreatment of C57BL/6J mice with Y-27632 also prevented CS-impaired efferocytosis *in vivo* 24 hours after CS exposure (Figure 5E; four mice per group), but not immediately postexposure when RhoA activity is low (*see* Figure E2 in the online supplement). Taken together, these results demonstrate that acute exposure to CS impairs efferocytosis through oxidant-dependent activation of the RhoA–Rho kinase pathway. In contrast, at earlier time points CS may exert its suppressive effects through a RhoA-independent pathway.

CS Impairs Efferocytosis through a TNF-α–dependent Mechanism

In vitro, McPhillips and colleagues determined that TNF-α acted upstream to produce oxidants, activation of RhoA–Rho kinase, and impaired efferocytosis (34). Because the oxidant burden of CS is massive, we originally hypothesized that it alone would be enough to activate the RhoA–Rho kinase pathway and to suppress efferocytosis, and that CS-induced TNF-α would not contribute to the pathway. But to address this issue, we exposed wild-type and TNF-α receptor double knockouts (mice deficient in TNF receptors 1 and 2) to air or CS, and then tested their ability to clear apoptotic cells *in vivo*. Unexpectedly, these experiments showed that the suppressive effect of CS depended on intact TNF-α signaling 24 hours postexposure, but not immediately postexposure (Figure 6; five or six animals per group).

DISCUSSION

Factors that dictate whether a person who is exposed to CS will eventually develop COPD are far from understood. A great deal

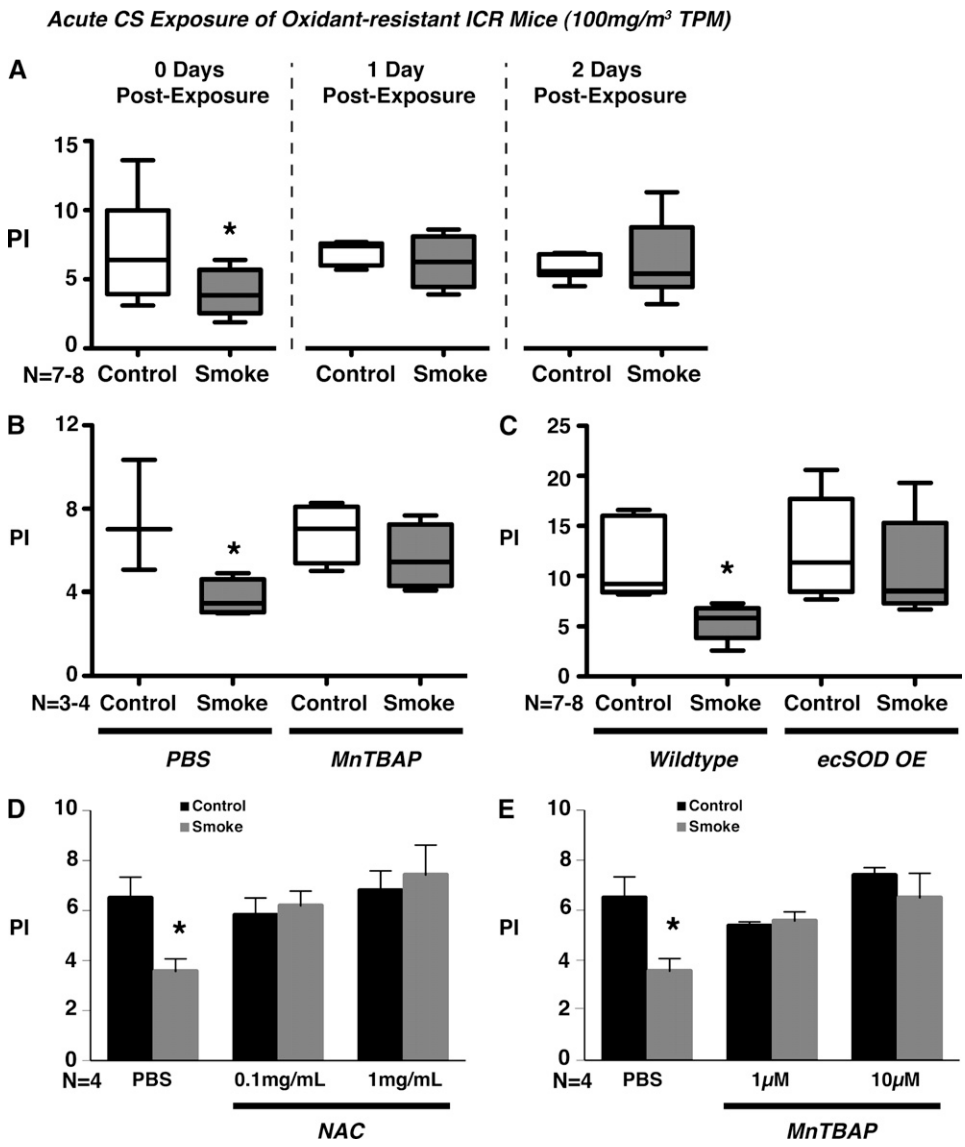

Figure 4. Cigarette smoke (CS) suppresses efferocytosis *in vivo* and *ex vivo* through an oxidant-dependent mechanism. To determine the effect of CS on efferocytosis *in vivo*, oxidant-resistant ICR mice were exposed to air or CS at 100 mg/m^3 total particulate matter (TPM) for 5 hours, and were then examined for their ability to remove apoptotic cells at time points ranging from 0 to 2 days postexposure (as in Figure 1C). (*A*) Moderate-dose CS (100 mg/m^3 TPM) trended toward a decrease in efferocytosis only immediately postexposure (Day 0), but had no effect at 1 and 2 days postexposure. *Nonsignificantly different from Day 0 control ($P = 0.07$). The role of oxidant stress was explored further in (*B*) mice treated with a superoxide dismutase (SOD) mimetic, manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP), and in (*C*) extracellular SOD–overexpressing (ecSOD OE) mice. (*B*) MnTBAP (5 mg/kg) or vehicle control was administered intraperitoneally to C57BL/6J mice three times: (*1*) immediately before and (*2*) after exposure to air or CS at 100 mg/m^3 TPM for 5 hours, and (*3*) again the next morning before experimentation. CS did not inhibit efferocytosis in mice pretreated with MnTBAP, as it was in mice that received vehicle control. *Significantly different from vehicle control ($P \leq 0.05$). (*C*) ecSOD OE mice were exposed to air or CS at 100 mg/m^3 TPM for 5 hours, and were then examined for their ability to remove apoptotic cells at 1 day postexposure. CS inhibited efferocytosis in wild-type mice, but not in ecSOD OE mice. *Significantly different from vehicle control ($P \leq 0.05$). To determine whether CS suppresses efferocytosis *ex vivo* through an oxidant-dependent mechanism. C57BL/6J mice were exposed to air or moderate-dose CS (100 mg/m^3 TPM) for 5 hours. Whole lung lavage was then performed and alveolar macrophages were cultured *ex vivo* overnight in the presence of (*D*) increasing concentrations of *N*-acetylcysteine (NAC) or phosphate-buffered saline (PBS) control, or (*E*) with increasing concentrations of MnTBAP (or PBS control) for 4 hours before experimentation. Alveolar macrophages were then examined for their ability to ingest apoptotic Jurkat T cells during a 60-minute coculture experiment. In both experiments, CS suppressed efferocytosis, but not in the presence of (*D*) NAC or (*E*) MnTBAP at all concentrations tested. (*D*) *Nonsignificantly different from phosphate buffered saline (PBS) control ($P = 0.08$). (*E*) *Significantly different from PBS control ($P \leq 0.05$). PI = phagocytic index.

of evidence suggests that CS unleashes imbalances in oxidative stress and protease activity (1, 54). But clearly, these imbalances are not sufficient to produce COPD in the majority of people; and, in most instances aside from α_1-antitrypsin deficiency, our knowledge has not translated into an ability to predict who will develop disease with time or who will have the most precipitous decline in lung function. Alternative hypotheses for COPD, such as the autoimmune hypothesis (55–58) or the cell death hypothesis (59, 60), have expanded our view of how COPD might develop, and importantly, in ways that fully complement or intersect with traditional views. The possibility that cell death plays an important role in the development of COPD is of increasing interest, because apoptotic cells are plentiful in the lungs of patients with COPD (41, 59, 61, 62) and in animal models of emphysema (60, 63–65). However, because apoptotic cells are normally removed as rapidly as they are produced (66), increased numbers of apoptotic cells in the lungs and sputum of patients with COPD suggest both that cell death may be increased (60, 67) and that removal of these cells by phagocytosis (efferocytosis) or egression may be decreased (23, 41, 68). Our data advance these concepts, because they show that CS directly results in impaired efferocytosis after acute, subacute, and long-term exposure; and they suggest that at some critical level of oxidant exposure coupled with oxidant sensitivity that this effect may become prolonged, or even permanent.

Using pharmacologic and genetic approaches *in vivo* and *ex vivo*, our data indicate that CS acutely impairs efferocytosis through an oxidant-dependent mechanism. Superoxide is one of the first free radicals produced from the reduction of oxygen to water. SODs convert superoxide to hydrogen peroxide in one of the most rapid reactions in nature, which is further neutralized to water by catalase or glutathione. Superoxide can also be

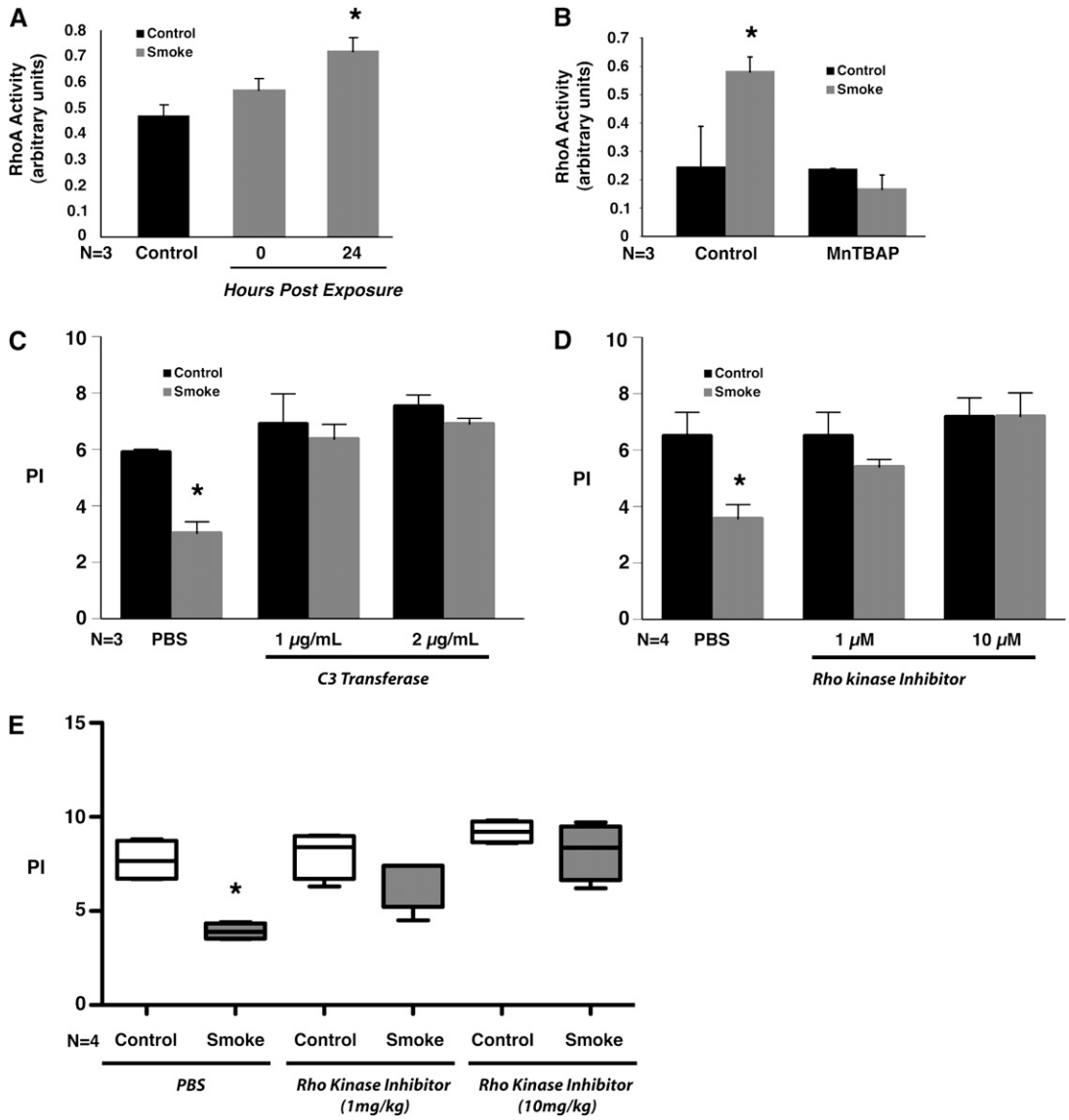

Figure 5. Cigarette smoke (CS) activates RhoA via an oxidant-dependent mechanism. We examined the hypothesis that CS suppresses efferocytosis through oxidant-dependent activation of RhoA, by (*A*) investigating the effect of CS on RhoA activation, and then by (*B*) determining whether RhoA activation could be prevented by the superoxide dismutase (SOD) mimetic manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP). Alveolar macrophages were collected 0 and 24 hours after exposure to air or CS (100 mg/m^3 total particulate matter [TPM] for 5 h) and then assayed for RhoA activity. (*A*) CS increased RhoA activity at 24 hours, but had no effect immediately postexposure. *Significantly different from air control ($P = 0.01$). (*B*) C57BL/J mice were pretreated with MnTBAP and exposed to CS as described in Fig. 4B, after which alveolar macrophages were collected by bronchoalveolar lavage 24 hours postexposure and assayed for RhoA activity. CS activated RhoA in the absence, but not the presence, of MnTBAP. *Significantly different from air control ($P \leq 0.02$). To determine whether acute exposure to CS impairs efferocytosis through activation of the RhoA–Rho kinase pathway, we first used the *ex vivo* system described in Fig. 3. Alveolar macrophages taken from air- or CS-exposed C57BL/6 mice were tested for their ability to ingest apoptotic cells in the presence or absence of (*C*) C3 transferase (a direct RhoA blocker) or (*D*) Y-27632 (a Rho kinase inhibitor). CS decreased alveolar macrophage efferocytosis, but not in the presence of (*C*) C3 transferase or (*D*) Y-27632. *Significantly different from air control ($P \leq 0.05$). (*E*) To determine the role of the RhoA–Rho kinase pathway in the ability of CS to impair efferocytosis *in vivo*, C57BL/6J mice were exposed to CS at 100 mg/m^3 TPM for 5 hours, and then assessed for their ability to clear instilled apoptotic cells the next day, after pretreatment with phosphate-buffered saline or the Rho kinase inhibitor Y-27632. At both doses used, Y-27632 prevented the suppressive effect of CS on efferocytosis, confirming that acute CS impairs efferocytosis through a RhoA–Rho kinase-dependent mechanism. *Significantly different from air control ($P \leq 0.05$). PBS = phosphate-buffered saline; PI = phagocytic index.

converted to hydrogen peroxide or the hydroxyl radical through a number of pathways (69). Superoxide may combine with nitric oxide to produce the hydroxyl radical, nitrogen dioxide, nitrogen trioxide, and peroxynitrite, which are known to directly damage cells through their effects on proteins, lipids, and DNA. The importance of superoxide in the pathogenesis of emphysema is highlighted by the fact that overexpression of CuZn-SOD (SOD1), the most prevalent SOD in the lung, protects mice from the development of emphysema in both the CS and elastase models (8). In our studies, the acute suppression of efferocytosis by CS was completely abrogated by the potent SOD mimetic MnTBAP, or by overexpression of ecSOD (SOD3), the second most prevalent SOD in the lung, indicating the importance of superoxide. In studies by McPhillips and colleagues (34), TNF-α inhibited efferocytosis in murine and human macrophages through an NADPH oxidase–independent pathway that depended on cytosolic phospholipase A_2–mediated release of arachidonic acid and subsequent production of superoxide. The fact that exogenous hydrogen peroxide alone was capable of inhibiting efferocytosis suggests that degradation of superoxide to hydrogen peroxide would be sufficient to inhibit efferocytosis, but does not preclude the involvement of other free radicals in their system. Therefore, superoxide plays an important role both in the regulation of efferocytosis and in the pathogenesis of emphysema.

Our data suggest a link between oxidative stress and activation of the RhoA–Rho kinase pathway, but they do not definitively determine the relative importance of CS-associated oxidative versus oxidative stress downstream of TNF-α. Increasingly, local and systemic activation of the RhoA–Rho

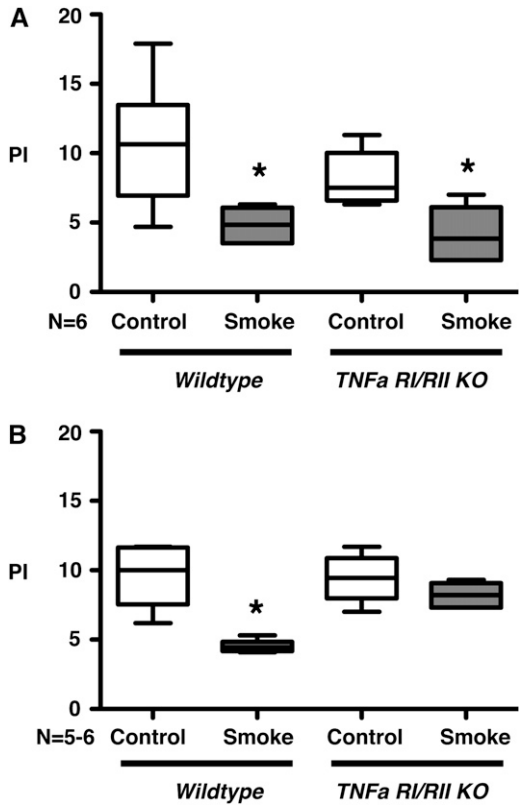

Figure 6. Acute cigarette smoke (CS) exposure impairs efferocytosis *in vivo* through a tumor necrosis factor (TNF)-α–dependent mechanism. To determine whether CS suppressed efferocytosis *in vivo* through a TNF-α–dependent mechanism, wild-type and TNF-α receptor double knockouts (mice deficient in TNF receptors 1 and 2; TNFa RI/RII KO) were exposed to air or CS, and then tested their ability to clear apoptotic cells (*A*) immediately or (*B*) 24 hours after exposure. *Significantly different from air control ($P \leq 0.05$).

kinase pathway is recognized as a downstream consequence of CS exposure. For instance, CS exposure *in vivo* increases RhoA activity in rat bronchial smooth muscle cells (33), and *in vitro*, CS increases RhoA activity in Calu-3 bronchial epithelial cells, resulting in increased permeability (70). The effect of CS on the RhoA–Rho kinase pathway also appears to be systemic, because CS exposure activates Rho kinase in the forearm vasculature of human subjects, leading to a higher degree of vasoreactivity (71). More broadly, reactive oxidant species activate the RhoA–Rho kinase pathway in several settings, both in an NADPH oxidase–dependent and –independent manner (34, 69, 72–74); but the converse has also been observed, that reactive oxygen species may down-regulate RhoA activity (75). So reactive oxygen species may retain the ability to activate or deactivate Rho GTPases, depending on the context. This dichotomy may in part be explained by the presence of a novel redox-sensitive regulatory motif [GXXXXGK(S/T)C] in 50% of all Rho GTPases that contain cysteine, including RhoA, Rac-1, and cdc42 (69). This motif facilitates dissociation of GDP in the presence of superoxide, hydrogen peroxide/Cu^{2+}, and nitrogen dioxide, which would be expected to inactivate these Rho GTPases. However, it could also result in Rho GTPase activation, because, in the presence of a free radical scavenger, like ascorbate, these Rho GTPases may then associate with GTP.

On the other hand, McPhillips and colleague demonstrated *in vitro* that TNF-α suppresses efferocytosis through cytosolic phospholipase A_2–mediated production of oxidants (34). Because our data show that CS-impaired efferocytosis also depends on intact TNF-α signaling, we cannot rule out the possibility that oxidants may act at two points in the pathway; early when associated with CS and later at a point downstream of TNF-α. The role of TNF-α in this pathway is certainly relevant because of its potential importance in the development of COPD (76–81).

Activation of RhoA certainly may not be the only mechanism by which CS impairs efferocytosis. For example, we found that CS inhibited efferocytosis immediately after exposure, but that this was not associated with activation of RhoA. The mechanism(s) for this acute effect of CS is not known, but it may involve the ability of CS to inhibit the activation of the Rac-1 guanine nucleotide exchange factor, Vav-1 (82). This could also have important consequences for RhoA activation, because of the ability of Rac-1 and RhoA to reciprocally regulate each other (27). CS also decreased efferocytosis at later time points. In addition to a role for oxidative stress, this may be due to the ability of CS to increase protease–antiprotease imbalance or to decrease lung collectins such as surfactant protein A, surfactant protein D, or mannose-binding lectin (22, 83, 84), which all play important roles in the regulation of apoptotic cell clearance (41, 42, 85). In humans, Hodge and colleagues have demonstrated that smoking decreases a number of receptors involved with efferocytosis, including CD31, CD91, and CD44 (21), all of which could have contributed to impaired efferocytosis, especially at later time points. Finally, Kirkham and colleagues showed that CS decreases efferocytosis through posttranslational modification of extracellular matrix proteins, indicating that indirect effects of CS could be just as crucial (20).

We found that the effect of CS on efferocytosis was variable depending on the mouse strain, and that the most intense exposure regimen caused an irreversible defect in efferocytosis. Oxidant-sensitive C57BL/6J mice took longer to recover their ability to clear apoptotic cells after CS exposure, compared with the oxidant-resistant ICR mice. Similarly, C57BL/6J mice are also more susceptible to develop emphysema in response to CS, compared with ICR mice. Perhaps this increase in sensitivity to oxidative stress in C57BL6J mice is due to a polymorphism present in the promoter of Nrf2 (86), a master transcription factor for antioxidant and detoxification genes with a known role in the development of emphysema (6, 7). Of greatest interest, efferocytosis continued to be defective in FVB/N mice 20 weeks after the most intense CS exposure, suggesting that efferocytosis becomes permanently impaired at some critical level of exposure (perhaps mixed with strain). Because these experiments were performed with FVB/N mice and not C57BL/6J mice, we do not know the relative contribution of mouse strain and smoke exposure to these findings. Nonetheless, these data demonstrate that efferocytosis becomes irreversibly impaired at a point when permanent structural damage, consistent with COPD, would have been expected to occur.

Important questions left unanswered concern whether impaired efferocytosis is simply a marker of CS exposure, an important factor in the pathogenesis of COPD, or whether the presence or absence of reversibility indicates a transition from injured to diseased lung. Moreover, if failed efferocytosis contributes to COPD pathogenesis, is there a role for drugs known to enhance efferocytosis, such as peroxisome proliferator–activated receptor γ agonists (87, 88), macrolide antibiotics (22, 89, 90), and corticosteroids (91)? Because CS impairs efferocytosis, at least in part, by activating the RhoA–Rho kinase pathway, is there a therapeutic role for Rho kinase inhibitors or for statins, which both block the RhoA–Rho kinase pathway and increase efferocytosis (40, 92)? Epidemiologic studies showing that statins decrease the incidence of

COPD exacerbations (93, 94) and prevent the development of emphysema in CS-exposed rats (95) suggest that the RhoA–Rho kinase may ultimately be a therapeutic target worth pursuing.

Conflict of Interest Statement: T.R.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.J.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.-Q.X. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L.K. is a coinvestigator of a patent for the use of prostacyclin analogues for the chemoprevention of cancer. D.M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.P.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.J.D. is a consultant for Aeolus Pharmaceuticals, a cofounder of the company, and holds equity in Aeolus Pharmaceuticals. B.J.D. is an inventor on nine patents covering the use and composition of matter of metalloporphyrins. B.J.D. received $200,000 in research grants from Aeolus Pharmaceuticals from 2005 to 2008. B.J.D.'s institution, National Jewish Health, has a license agreement for rights to patients on metalloporphyrins of which B.J.D. is an inventor. W.J.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.W.V. has received grant funding from GlaxoSmithKline of $150,000 per year for 2 years.

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References

1. MacNee W. Oxidants/antioxidants and COPD. *Chest* 2000;117(5, Suppl 1):303S–317S.
2. Rahman I, Li XY, Donaldson K, Harrison DJ, MacNee W. Glutathione homeostasis in alveolar epithelial cells *in vitro* and lung *in vivo* under oxidative stress. *Am J Physiol* 1995;269:L285–L292.
3. Li XY, Donaldson K, Rahman I, MacNee W. An investigation of the role of glutathione in increased epithelial permeability induced by cigarette smoke *in vivo* and *in vitro*. *Am J Respir Crit Care Med* 1994; 149:1518–1525.
4. Cavarra E, Bartalesi B, Lucattelli M, Fineschi S, Lunghi B, Gambelli F, Ortiz LA, Martorana PA, Lungarella G. Effects of cigarette smoke in mice with different levels of α_1-proteinase inhibitor and sensitivity to oxidants. *Am J Respir Crit Care Med* 2001;164:886–890.
5. Bartalesi B, Cavarra E, Fineschi S, Lucattelli M, Lunghi B, Martorana PA, Lungarella G. Different lung responses to cigarette smoke in two strains of mice sensitive to oxidants. *Eur Respir J* 2005;25:15–22.
6. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke–induced emphysema in mice. *J Clin Invest* 2004;114:1248–1259.
7. Iizuka T, Ishii Y, Itoh K, Kiwamoto T, Kimura T, Matsuno Y, Morishima Y, Hegab AE, Homma S, Nomura A, *et al.* Nrf2-deficient mice are highly susceptible to cigarette smoke–induced emphysema. *Genes Cells* 2005;10:1113–1125.
8. Foronjy RF, Mirochnitchenko O, Propokenko O, Lemaitre V, Jia Y, Inouye M, Okada Y, D'Armiento JM. Superoxide dismutase expression attenuates cigarette smoke– or elastase-generated emphysema in mice. *Am J Respir Crit Care Med* 2006;173:623–631.
9. Tabak C, Smit HA, Rasanen L, Fidanza F, Menotti A, Nissinen A, Feskens EJ, Heederik D, Kromhout D. Dietary factors and pulmonary function: a cross sectional study in middle aged men from three European countries. *Thorax* 1999;54:1021–1026.
10. Butland BK, Fehily AM, Elwood PC. Diet, lung function, and lung function decline in a cohort of 2512 middle aged men. *Thorax* 2000;55: 102–108.
11. Sutherland ER, Crapo JD, Bowler RP. *N*-Acetylcysteine and exacerbations of chronic obstructive pulmonary disease. *COPD* 2006;3:195–202.
12. deCathelineau AM, Henson PM. The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. *Essays Biochem* 2003;39:105–117.
13. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE_2, and PAF. *J Clin Invest* 1998; 101:890–898.
14. Hoffmann PR, Kench JA, Vondracek A, Kruk E, Daleke DL, Jordan M, Marrack P, Henson PM, Fadok VA. Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses *in vivo*. *J Immunol* 2005;174:1393–1404.
15. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J Clin Invest* 2002;109:41–50.
16. McDonald PP, Fadok VA, Bratton D, Henson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-β in macrophages that have ingested apoptotic cells. *J Immunol* 1999;163:6164–6172.
17. Odaka C, Mizuochi T, Yang J, Ding A. Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response. *J Immunol* 2003;171:1507–1514.
18. Golpon HA, Fadok VA, Taraseviciene-Stewart L, Scerbavicius R, Sauer C, Welte T, Henson PM, Voelkel NF. Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. *FASEB J* 2004;18:1716–1718.
19. Morimoto K, Amano H, Sonoda F, Baba M, Senba M, Yoshimine H, Yamamoto H, Ii T, Oishi K, Nagatake T. Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice. *Am J Respir Cell Mol Biol* 2001; 24:608–615.
20. Kirkham PA, Spooner G, Rahman I, Rossi AG. Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products. *Biochem Biophys Res Commun* 2004;318:32–37.
21. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN. Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2007;37:748–755.
22. Hodge S, Hodge G, Jersmann H, Matthews G, Ahern J, Holmes M, Reynolds PN. Azithromycin improves macrophage phagocytic function and expression of mannose receptor in COPD. *Am J Respir Crit Care Med* 2008;178:139–148.
23. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol* 2003;81:289–296.
24. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* 2006;129:1673–1682.
25. Leverrier Y, Ridley AJ. Requirement for Rho GTPases and PI 3-kinases during apoptotic cell phagocytosis by macrophages. *Curr Biol* 2001; 11:195–199.
26. Tosello-Trampont AC, Nakada-Tsukui K, Ravichandran KS. Engulfment of apoptotic cells is negatively regulated by Rho-mediated signaling. *J Biol Chem* 2003;278:49911–49919.
27. Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell* 2004; 116:167–179.
28. Bratton DL, Henson PM. Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Curr Biol* 2008;18:R76–R79.
29. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. *Nature* 2007; 450:435–439.
30. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* 2007;450:430–434.
31. Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, Kwon TH, Park RW, Kim IS. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ* 2008;15:192–201.
32. Kinchen JM, Cabello J, Klingele D, Wong K, Feichtinger R, Schnabel H, Schnabel R, Hengartner MO. Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in *C. elegans*. *Nature* 2005;434:93–99.
33. Chiba Y, Murata M, Ushikubo H, Yoshikawa Y, Saitoh A, Sakai H, Kamei J, Misawa M. Effect of cigarette smoke exposure *in vivo* on bronchial smooth muscle contractility *in vitro* in rats. *Am J Respir Cell Mol Biol* 2005;33:574–581.
34. McPhillips K, Janssen WJ, Ghosh M, Byrne A, Gardai S, Remigio L, Bratton DL, Kang JL, Henson P. TNF-α inhibits macrophage

clearance of apoptotic cells via cytosolic phospholipase A_2 and oxidant-dependent mechanisms. *J Immunol* 2007;178:8117–8126.
35. Richens TR, Linderman DJ, Xiao YQ, Morimoto K, Day BJ, Henson PM, Vandivier RW. Cigarette smoke impairs removal of apoptotic cells (efferocytosis) through oxidant-dependent activation of RhoA [abstract]. *Am J Respir Crit Care Med* 2008;177:A622.
36. Keith RL, Miller YE, Hudish TM, Girod CE, Sotto-Santiago S, Franklin WA, Nemenoff RA, March TH, Nana-Sinkam SP, Geraci MW. Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. *Cancer Res* 2004;64:5897–5904.
37. Folz RJ, Abushamaa AM, Suliman HB. Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J Clin Invest* 1999;103:1055–1066.
38. Martin RJ, Wexler RB, Day BJ, Harbeck RJ, Pinkerton KE, Chu HW. Interaction between cigarette smoke and mycoplasma infection: a murine model. *COPD* 2006;3:3–8.
39. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 1989;83:865–875.
40. Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, Xiao YQ, Kench JA, Henson PM, Vandivier RW. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol* 2006;176:7657–7665.
41. Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, Henson PM. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest* 2002;109:661–670.
42. Vandivier RW, Ogden CA, Fadok VA, Hoffmann PR, Brown KK, Botto M, Walport MJ, Fisher JH, Henson PM, Greene KE. 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* 2002;169:3978–3986.
43. Savill JS, Henson PM, Haslett C. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. *J Clin Invest* 1989;84:1518–1527.
44. Fadok VA, Savill JS, Haslett C, Bratton DL, Doherty DE, Campbell PA, Henson PM. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* 1992;149:4029–4035.
45. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, Henson PM, Noble PW. Resolution of lung inflammation by CD44. *Science* 2002;296:155–158.
46. Yuan H, Wong LS, Bhattacharya M, Ma C, Zafarani M, Yao M, Schneider M, Pitas RE, Martins-Green M. The effects of secondhand smoke on biological processes important in atherogenesis. *BMC Cardiovasc Disord* 2007;7:1.
47. Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzo H, Triantafillopoulos A, Whittaker K, Hoidal JR, Cosio MG. The development of emphysema in cigarette smoke–exposed mice is strain dependent. *Am J Respir Crit Care Med* 2004;170:974–980.
48. Yao H, Edirisinghe I, Rajendrasozhan S, Yang SR, Caito S, Adenuga D, Rahman I. Cigarette smoke–mediated inflammatory and oxidative responses are strain-dependent in mice. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1174–L1186.
49. Oury TD, Thakker K, Menache M, Chang LY, Crapo JD, Day BJ. Attenuation of bleomycin-induced pulmonary fibrosis by a catalytic antioxidant metalloporphyrin. *Am J Respir Cell Mol Biol* 2001;25:164–169.
50. Bowler RP, Arcaroli J, Abraham E, Patel M, Chang LY, Crapo JD. Evidence for extracellular superoxide dismutase as a mediator of hemorrhage-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L680–L687.
51. Bowler RP, Arcaroli J, Crapo JD, Ross A, Slot JW, Abraham E. Extracellular superoxide dismutase attenuates lung injury after hemorrhage. *Am J Respir Crit Care Med* 2001;164:290–294.
52. Bowler RP, Nicks M, Tran K, Tanner G, Chang LY, Young SK, Worthen GS. Extracellular superoxide dismutase attenuates lipopolysaccharide-induced neutrophilic inflammation. *Am J Respir Cell Mol Biol* 2004;31:432–439.
53. Bowler RP, Nicks M, Warnick K, Crapo JD. Role of extracellular superoxide dismutase in bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L719–L726.
54. Vandivier RW, Voelkel NF. The challenges of chronic obstructive pulmonary diseases (COPD): a perspective. *COPD* 2005;2:177–184.
55. Cosio MG. Autoimmunity, T-cells and STAT-4 in the pathogenesis of chronic obstructive pulmonary disease. *Eur Respir J* 2004;24:3–5.
56. Feghali-Bostwick CA, Gadgil AS, Otterbein LE, Pilewski JM, Stoner MW, Csizmadia E, Zhang Y, Sciurba FC, Duncan SR. Autoantibodies in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;177:156–163.
57. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, Bakaeen F, *et al.* Antielastin autoimmunity in tobacco smoking–induced emphysema. *Nat Med* 2007;13:567–569.
58. Taraseviciene-Stewart L, Burns N, Kraskauskas D, Nicolls MR, Tuder RM, Voelkel NF. Mechanisms of autoimmune emphysema. *Proc Am Thorac Soc* 2006;3:486–487.
59. Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med* 2001;163:737–744.
60. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;106:1311–1319.
61. Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, D'Armiento J. Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. *Am J Respir Crit Care Med* 2001;163:786–791.
62. Yokohori N, Aoshiba K, Nagai A. Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. *Chest* 2004;125:626–632.
63. Aoshiba K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* 2003;28:555–562.
64. Massaro GD, Radaeva S, Clerch LB, Massaro D. Lung alveoli: endogenous programmed destruction and regeneration. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L305–L309.
65. Clark H, Palaniyar N, Strong P, Edmondson J, Hawgood S, Reid KB. Surfactant protein D reduces alveolar macrophage apoptosis *in vivo*. *J Immunol* 2002;169:2892–2899.
66. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251–306.
67. Tuder RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, Flores SC. Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am J Respir Cell Mol Biol* 2003;29:88–97.
68. Uller L, Persson CG, Erjefalt JS. Resolution of airway disease: removal of inflammatory cells through apoptosis, egression or both? *Trends Pharmacol Sci* 2006;27:461–466.
69. Heo J, Campbell SL. Mechanism of redox-mediated guanine nucleotide exchange on redox-active Rho GTPases. *J Biol Chem* 2005;280:31003–31010.
70. Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. Cellular mechanisms of mainstream cigarette smoke–induced lung epithelial tight junction permeability changes *in vitro*. *Inhal Toxicol* 2007;19:13–22.
71. Noma K, Goto C, Nishioka K, Hara K, Kimura M, Umemura T, Jitsuiki D, Nakagawa K, Oshima T, Chayama K, *et al.* Smoking, endothelial function, and Rho-kinase in humans. *Arterioscler Thromb Vasc Biol* 2005;25:2630–2635.
72. Dada LA, Novoa E, Lecuona E, Sun H, Sznajder JI. Role of the small GTPase RhoA in the hypoxia-induced decrease of plasma membrane Na,K-ATPase in A549 cells. *J Cell Sci* 2007;120:2214–2222.
73. Jackson EK, Gillespie DG, Zhu C, Ren J, Zacharia LC, Mi Z. α_2-Adrenoceptors enhance angiotensin II–induced renal vasoconstriction: role for NADPH oxidase and RhoA. *Hypertension* 2008;51:719–726.
74. Jin L, Ying Z, Webb RC. Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. *Am J Physiol Heart Circ Physiol* 2004;287:H1495–H1500.
75. Nimnual AS, Taylor LJ, Bar-Sagi D. Redox-dependent downregulation of Rho by Rac. *Nat Cell Biol* 2003;5:236–241.
76. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530–534.

77. Kuschner WG, D'Alessandro A, Wong H, Blanc PD. Dose-dependent cigarette smoking–related inflammatory responses in healthy adults. *Eur Respir J* 1996;9:1989–1994.
78. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, Yuki H, Kato S, Tomoike H. The relationship between chronic hypoxemia and activation of the tumor necrosis factor-α system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1179–1184.
79. Churg A, Dai J, Tai H, Xie C, Wright JL. Tumor necrosis factor-α is central to acute cigarette smoke–induced inflammation and connective tissue breakdown. *Am J Respir Crit Care Med* 2002;166:849–854.
80. Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. Tumor necrosis factor-α drives 70% of cigarette smoke–induced emphysema in the mouse. *Am J Respir Crit Care Med* 2004;170:492–498.
81. D'hulst AI, Bracke KR, Maes T, De Bleecker JL, Pauwels RA, Joos GF, Brusselle GG. Role of tumour necrosis factor-α receptor p75 in cigarette smoke–induced pulmonary inflammation and emphysema. *Eur Respir J* 2006;28:102–112.
82. Minematsu N, Shapiro SD. Inhibitory effect of cigarette smoke on macrophage phagocytosis of apoptotic polymorphonuclear leukocytes *in vitro* and *in vivo* [abstract]. *Am J Respir Crit Care Med* 2008;117:A762.
83. Betsuyaku T, Kuroki Y, Nagai K, Nasuhara Y, Nishimura M. Effects of ageing and smoking on SP-A and SP-D levels in bronchoalveolar lavage fluid. *Eur Respir J* 2004;24:964–970.
84. Honda Y, Takahashi H, Kuroki Y, Akino T, Abe S. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. *Chest* 1996;109:1006–1009.
85. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 2001;194:781–795.
86. Cho HY, Jedlicka AE, Reddy SP, Zhang LY, Kensler TW, Kleeberger SR. Linkage analysis of susceptibility to hyperoxia: Nrf2 is a candidate gene. *Am J Respir Cell Mol Biol* 2002;26:42–51.
87. Asada K, Sasaki S, Suda T, Chida K, Nakamura H. Antiinflammatory roles of peroxisome proliferator–activated receptor γ in human alveolar macrophages. *Am J Respir Crit Care Med* 2004;169:195–200.
88. Majai G, Sarang Z, Csomos K, Zahuczky G, Fesus L. PPARγ-dependent regulation of human macrophages in phagocytosis of apoptotic cells. *Eur J Immunol* 2007;37:1343–1354.
89. Hodge S, Hodge G, Brozyna S, Jersmann H, Holmes M, Reynolds PN. Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages. *Eur Respir J* 2006;28:486–495.
90. Yamaryo T, Oishi K, Yoshimine H, Tsuchihashi Y, Matsushima K, Nagatake T. Fourteen-member macrolides promote the phosphatidylserine receptor–dependent phagocytosis of apoptotic neutrophils by alveolar macrophages. *Antimicrob Agents Chemother* 2003;47:48–53.
91. Liu Y, Cousin JM, Hughes J, Van Damme J, Seckl JR, Haslett C, Dransfield I, Savill J, Rossi AG. Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. *J Immunol* 1999;162:3639–3646.
92. Janssen WJ, McPhillips KA, Dickinson MG, Linderman DJ, Morimoto K, Xiao YQ, Oldham KM, Vandivier RW, Henson PM, Gardai SJ. Surfactant proteins A and D suppress alveolar macrophage phagocytosis via interaction with SIRPα. *Am J Respir Crit Care Med* 2008;178:158–167.
93. Mancini GB, Etminan M, Zhang B, Levesque LE, FitzGerald JM, Brophy JM. Reduction of morbidity and mortality by statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers in patients with chronic obstructive pulmonary disease. *J Am Coll Cardiol* 2006;47:2554–2560.
94. Soyseth V, Brekke PH, Smith P, Omland T. Statin use is associated with reduced mortality in COPD. *Eur Respir J* 2007;29:279–283.
95. Lee JH, Lee DS, Kim EK, Choe KH, Oh YM, Shim TS, Kim SE, Lee YS, Lee SD. Simvastatin inhibits cigarette smoking–induced emphysema and pulmonary hypertension in rat lungs. *Am J Respir Crit Care Med* 2005;172:987–993.