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Oxidized phospholipids impair pulmonary antibacterial defenses: Evidence in mice exposed to cigarette smoke

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ABSTRACT

Patients with COPD are associated with poor pulmonary anti-bacterial innate defenses, which increase the risk for frequent acute exacerbations caused by bacterial infection. Despite elevated numbers of phagocytes (macrophages and neutrophils), airways of patients with COPD show stable bacterial colonization. A defect in the phagocytic ability of alveolar macrophages (AMs) is one of the primary reasons for failure to clear the invading bacteria in airways of smokers and COPD patients and also in mice exposed to cigarette smoke (CS). Oxidative stress, as a result of CS exposure is implicated; however, the factors or mediators that inhibit phagocytic activity of AMs in lungs of smokers remain unclear. In the current study, we provide evidence that accumulation of oxidized phospholipids (Ox-PLs) mediate inhibition of phagocytic function of AMs in CS-exposed mice. Mice exposed to 6 months of CS showed impaired bacterial phagocytosis and clearance by AMs and elevated levels of Ox-PLs in bronchoalveolar lavage fluid (BALF), compared to mice exposed to room air. Intratracheal instillation of oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OX-PAPC) inhibited phagocytic activity of AMs and impaired pulmonary bacterial clearance in mice. *In vitro* studies demonstrated that exposure of J774 macrophages to OX-PAPC inhibited bacterial phagocytosis and clearance. However, pre-treatment of OX-PAPC with the monoclonal antibody EO6, which specifically binds to oxidized phospholipid but not native phospholipid, abolished OX-PAPC induced inhibition of bacterial phagocytosis and clearance. Incubation of BALF retrieved from CS-exposed mice impaired bacterial phagocytosis by J774 macrophages, which was abolished by pre-treatment of BALF with the EO6 antibody. In conclusion, our study shows that Ox-PLs generated following chronic CS exposure could play a crucial role in inhibiting phagocytic function of AMs and thus impair pulmonary anti-bacterial innate defenses in CS-exposed mice. Therapeutic approaches that augment pulmonary antioxidant defenses could be beneficial in reducing oxidative stress-driven impairment of phagocytosis by AMs in smokers and COPD patients.

1. Introduction

Chronic Obstructive Pulmonary Disease (COPD), which includes emphysema and chronic bronchitis, is the fourth leading cause of death worldwide [1]. COPD, caused primarily by cigarette smoking, is characterized by abnormal airway inflammation and progressive irreversible airflow obstruction [2]. Patients with COPD experience acute exacerbations, which result in further rise in airway inflammation and cause significant morbidity, mortality and faster rate of lung function decline [3]. Frequency of acute exacerbations of COPD increases with the severity of COPD and is mainly caused by microbial infections (bacteria and virus) [3]. It is estimated that 50% of AE-COPD are caused by bacterial infection, especially by *Hemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* [4]. Despite elevated numbers of phagocytes (macrophages and neutrophils) in the airways, COPD patients are associated with stable colonization of bacteria in the airways, which suggests that the ability of phagocytes to clear invading bacteria is attenuated [5,6]. The molecular mechanism by which cigarette smoke (CS) impairs phagocytic ability of alveolar macrophages remains unclear.

Mouse models exposed to chronic CS recapitulate features of impaired pulmonary innate immune defenses observed in smokers with COPD [7]. Evidence in experimental animals from our laboratory and others have shown that chronic exposure to CS impairs clearance of bacteria from lungs after infection with *H. influenzae*, or *P. aeruginosa*, and was not associated with a diminished

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inflammatory response [7–9]. Rather the pulmonary inflammation was heightened in lungs of mice exposed to CS plus bacteria, compared to mice exposed to room air plus bacterial infection. Numerous studies attribute impaired phagocytic ability of AMs as a major contributor to poor pulmonary innate anti-bacterial defense in patients with COPD and in CS-exposed mice [5,7–11].

Deleterious effects of CS in the lungs are primarily mediated by oxidative stress. It is estimated that one puff of CS consists of 10^{15} free radicals [12]. Epithelial lining fluid contains pulmonary surfactants, composed of complex mixtures of lipids and proteins, which are essential for normal lung function. Pulmonary surfactants consist of ~80–90% phospholipids, of which 85% is phosphocholine [13]. The phosphocholine, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), is a major phospholipid in mammalian cell membrane. Peroxidation of phospholipids, such as PAPC, leads to generation of OX-PAPC, which consists of a mixture of truncated oxidized phospholipids (Ox-PLs) such as POVPC, PGPC and PazPC and different types of low molecular weight aldehydes (e.g. 4-HNE, MDA) [14]. Levels of Ox-PLs, 4-HNE, and MDA are elevated in lungs of mice exposed to CS [15–17] and in COPD patients [18]. Additionally, reactive oxygen species generated at the site of inflammation due to NADPH oxidase and myeloperoxidase activity of phagocytes also mediate peroxidation of lipids and elevate Ox-PLs [19,20]. A growing body of evidence suggests that Ox-PLs play crucial roles in modulating the pulmonary innate immune response by interacting with Toll-like receptors [20–22]. However, whether accumulation of Ox-PLs affects pulmonary anti-bacterial defenses remains unclear. In the current study, we show that Ox-PLs inhibit bacterial phagocytosis by macrophages and impair pulmonary bacterial clearance. Using the IgM natural antibody EO6, which specifically binds to an epitope of Ox-PLs, we provide evidence that Ox-PLs accumulated in the lungs of mice exposed to chronic CS impair phagocytic function of macrophages.

2. Materials and methods

2.1. Material

1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) and OX-PAPC were obtained from Hycult biotech (Plymouth Meeting, PA); 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PazPC) were obtained from Avanti Polar Lipids (Alabaster, Alabama). Sterile and LPS free EO6 monoclonal antibody (an IgM) and mouse IgM were obtained from Avanti Polar Lipids. *P. aeruginosa* Xen 41, derived from the parental strain *P. aeruginosa* PAO1, was purchased from Caliper Life Sciences (Mountain View CA).

2.2. Cigarette smoke exposure

C57BL/6J mice (7–8 weeks) were exposed to CS for 6 months (5 h/day for 5 days per week) using a TE-10 smoke machine (Teague Enterprises) and 3R4F reference cigarettes with a total suspended particle concentration of 150 mg/m^3, as previously described [23]. At the end of the CS exposure, bronchoalveolar lavage was performed as described previously using 1 ml of PBS or RPMI-1640 for three lavages per mouse. The cell-free first fraction of bronchoalveolar lavage fluid (BALF) was used for measuring Ox-PLs. The cells from all three aliquots of BALF were pooled and used for total cell count and purification of alveolar macrophages.

2.3. Bacteria

P. aeruginosa (PA) was grown overnight in Luria Bertani (LB) medium and then subcultured for 2–3 h to mid-log phase at 37 °C. The culture was centrifuged at 3,000×*g* and the pellet was washed and resuspended in PBS, as described previously [7].

2.4. Cell culture

Alveolar macrophages were purified from BALF by adhesion to plastic plates as described previously [7]. J774A.1 (referred to as J774 in the text), a murine macrophage cell line, was procured from (ATCC) and was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. J774 macrophages were cultured for 24 h before bacterial phagocytosis and clearance assays.

2.5. Treatment of J774 macrophages

For bacterial phagocytosis and clearance experiments, J774 macrophages were treated with various doses (1, 10 or 50 μg/ml) of PAPC, OX-PAPC, POVPC, PGPC, or PazPC for 1 or 3 h. For neutralization of Ox-PLs with the EO6 antibody, the phospholipids were incubated with 50 μg/ml EO6 antibody or IgM for 10 min prior to exposing to J774 macrophages.

2.6. Cytotoxicity assay

J774 macrophages were exposed to increasing concentrations of OX-PAPC in serum-free media and cell viability was assessed by MTT assay, as described previously [17].

2.7. In vitro bacterial phagocytosis

AMs or J774 macrophages were seeded into a 96-well plate at a density of 1×10^5 per well and were cultured in RPMI 1640 without serum and antibiotics. The PA was added at a multiplicity of infection (MOI) of 20 onto the cells. To determine the bacterial uptake after 1 h, the cells were washed and incubated with gentamycin for 10 min to remove and kill the adherent and free bacteria. The cells were lysed in 0.1% triton-100 and the cell lysates aseptically plated and cultured in LB agar plates to measure the number of viable bacterial colony forming units (CFUs).

2.8. In vitro bacterial clearance

AMs or J774 macrophages were infected with PA (MOI: 20) for 4 h. The cell free culture medium was serially diluted and aseptically plated onto LB agar plates to quantify bacterial CFUs.

2.9. In vivo bacterial clearance

Mice were treated intratracheally with OX-PAPC or PAPC at a dose 200 μg/mouse. After 2 h, mice were intranasally inoculated with PA (10^5 CFU in 50 μl of PBS). After 2 h, bacterial burden was measured in BALF as described [7]. For assessing *ex vivo* bacterial phagocytosis, AMs were isolated from BALF 2 h after OX-PAPC or PAPC treatment.

2.10. Measurement of Ox-PLs

The levels of Ox-PLs in BALF was measured by ELISA using EO6 antibody as described elsewhere [19].

2.11. Statistical analysis

Statistical comparisons were performed by paired Student *t* tests. A value of $P < 0.05$ was considered statistically significant.

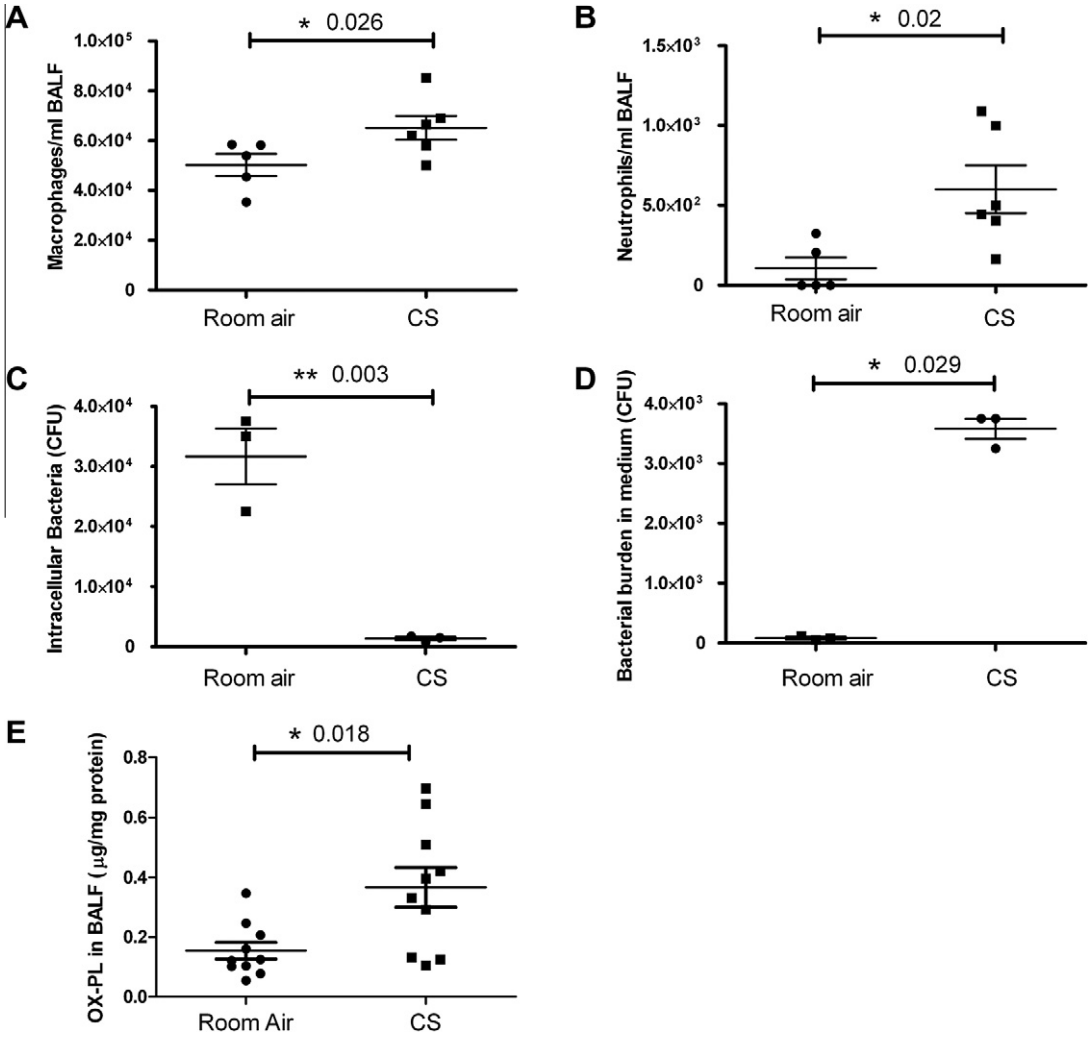

Fig. 1. Cigarette smoke exposure increases oxidized phospholipids and inhibits bacterial phagocytic activity of alveolar macrophages. (A and B) Number of macrophages and neutrophils in the lungs of mice exposed to 6 months of CS or room air. (C) Intracellular bacteria in AMs, as indicator of bacterial phagocytosis, 1 h after incubation with *PA*. AMs were isolated from mice exposed to 6 months of CS or room air. AMs were pooled from 5 mice and assay was performed in triplicate. (D) Bacterial concentration in the AMs culture medium 4 h after incubation of bacteria. Data presented as mean intracellular bacteria CFU ± SD. (E) Levels of oxidized phospholipids (Ox-PL) in the BALF retrieved from mice exposed to 6 months of CS or room air ($n = 10$). *Statically significant when compared to room air.

3. Results and discussion

3.1. Accumulation of Ox-PLs impair bacterial clearance in lungs

The mechanism by which oxidative stress inhibits pulmonary antibacterial defenses remains unclear. Due to previous observations that Ox-PLs may modulate the innate immune responses, we postulated that Ox-PLs generated in lungs following exposure to CS, may inhibit phagocytic activity of AMs and impair pulmonary bacterial clearance.

Consistent with our previous studies, CS exposure for 6 months significantly increased infiltration of AMs and neutrophils to the lungs of mice compared to room air (RA) (Fig. 1A and B). We examined *ex vivo* bacterial phagocytosis and clearance by AMs isolated from mice exposed to 6 months of CS or RA. Consistent with previous studies [7–10], we found that AMs from CS-exposed mice showed marked deficiency in phagocytosis of PA compared to RA-exposed mice (Fig. 1C). To verify that impaired phagocytosis correlates with diminished bacterial clearance, we incubated AMs with PA and quantified the viable bacteria remaining in the culture medium after 4 h. The bacterial concentration in the culture media of AMs isolated from CS-exposed mice was markedly higher compared to RA-exposed mice (Fig. 1D), suggesting that AMs from CS-exposed mice exhibit impairment in elimination of bacteria. Chronic oxidative stress increases levels of Ox-PLs in airways and has been shown to interact with components of the innate immune system to dysregulate immune responses [19,20]. Therefore, we assessed the levels of Ox-PLs in BALF from mice exposed to CS for 6 months. The levels of Ox-PLs were significantly elevated in the BALF from mice exposed to CS compared to RA as measured by ELISA (Fig. 1E). Because the airway lining fluid is highly diluted by the BALF, it is likely that the levels of Ox-PLs reported are underestimated. A recent study also reported elevated levels of Ox-PLs in BALF of mice after short-term CS exposure [15]. Additionally, systemic oxidative stress caused by cigarette smoking is shown to increase the levels of Ox-PLs in peripheral blood monocytes of smokers [24]. Taken together, this data demonstrate that impaired phagocytic activity of AMs in CS-exposed mice is associated with elevated accumulation of Ox-PLs in lungs.

To determine whether accumulation of Ox-PLs affects pulmonary bacterial clearance, mice were treated intratracheally with either OX-PAPC or un-oxidized PAPC and 2 h later infected with PA. OX-PAPC that was generated by air oxidation of synthetic PAPC consists of a mixture of oxidized and truncated Ox-PLs. Pulmonary

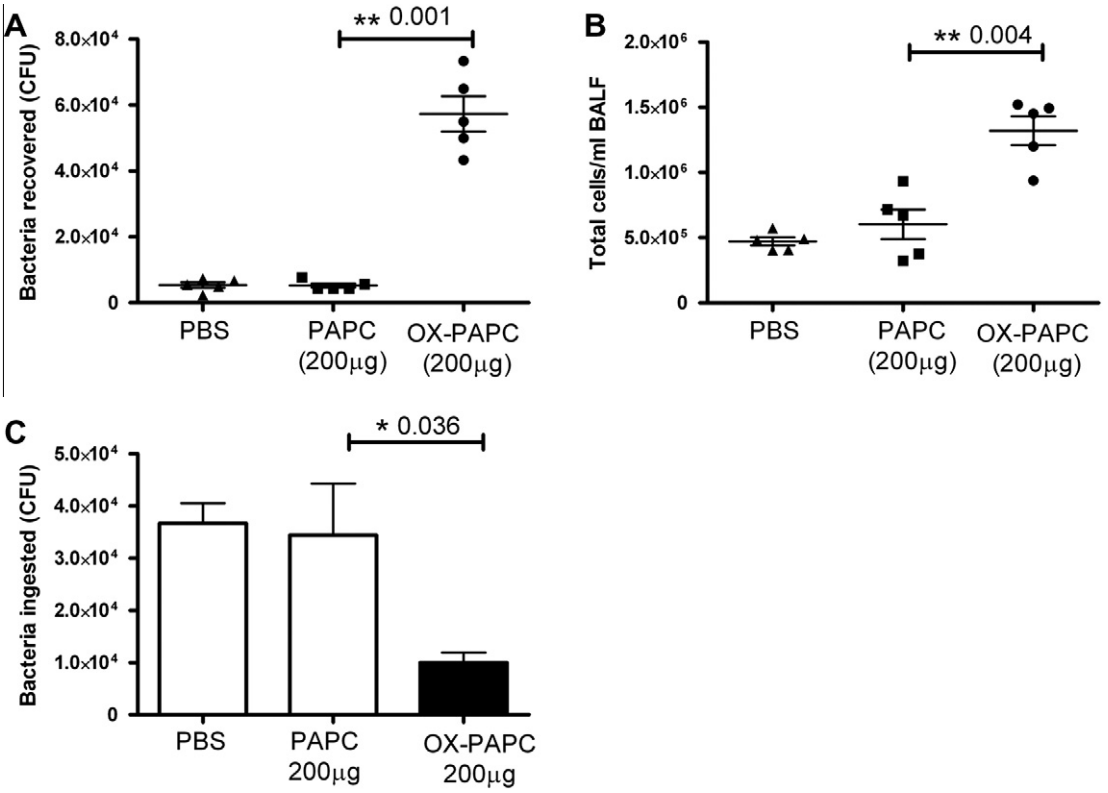

Fig. 2. Instillation of oxidized phospholipids inhibits phagocytic activity of AMs and impairs pulmonary bacterial clearance in mice. Mice were challenged with PAPC (200 μg in 50 μl PBS/mouse), OX-PAPC (200 μg in 50 μl PBS/mouse), or PBS by intratracheal instillation. After 2 h, mice were challenged with *PA*, and bacterial burden in BALF was assessed 2 h post infection. (A) Bacterial burden in BALF from mice treated with PAPC or OX-PAPC. Data presented as a dot plot (bacterial CFU/ml BALF) corresponding to each individual mouse. (B) Total inflammatory cells in BALF of mice. (C) Bacterial phagocytosis by AMs isolated 2 h after PAPC or OX-PAPC treatment. AMs were pooled from 5 mice and assay was performed in triplicate. Data presented as mean intracellular bacteria CFU ± SD. *Statistically significant compared to PAPC.

bacterial burden was assessed in BALF 2 h post PA infection. The bacterial burden in the BALF of mice treated with OX-PAPC was markedly higher compared to un-oxidized PAPC (Fig. 2A), suggesting a diminished clearance in OX-PAPC exposed mice. However, bacterial burden in mice exposed to un-oxidized PAPC was comparable to PBS-treated controls. Pulmonary inflammation was significantly elevated in OX-PAPC treated mice compared to PAPC or PBS challenged mice following PA infection (Fig. 2B). To address whether OX-PAPC influences AM function, we assessed bacterial phagocytic activity and clearance by AMs retrieved from mice 2 h after OX-PAPC or PAPC intratracheal administration. AMs from OX-PAPC treated mice exhibited diminished bacterial phagocytosis and clearance compared to AMs from PAPC treated mice (Fig. 2C). Taken together, the results suggest that accumulation of Ox-PLs in lungs could impair anti-bacterial innate immune defenses, mainly by diminishing the phagocytic activity of AMs.

3.2. EO6 antibody blocks Ox-PLs induced cell death in macrophages

In macrophages, the scavenger receptor CD36 recognizes and phagocytize oxidized but not un-Ox-PLs [25]. Recently, other scavenger receptors, such as MARCO, have been identified to bind Ox-PLs [26]. The chemical reactivity of specific truncated Ox-PLs greatly determines the uptake and target protein interaction in macrophages [27]. To unequivocally address the effects of Ox-PLs on phagocytic function of macrophages, we tested whether blocking the reactive oxidized motif of Ox-PLs using the IgM monoclonal antibody EO6 inhibits the biological effects of Ox-PLs [19,20]. The EO6 antibody binds to the PC head group of oxidized PC containing phospholipids but does not bind to the PC of the native unoxidized phospholipids such as PAPC, as well as other as to other lipid peroxidation products such as 4HNE, or MDA [28]. It also inhibits the binding and uptake of Ox-PLs by macrophages[29]. To test the efficacy and specificity of the EO6 antibody, we assessed cytotoxicity induced by OX-PAPC neutralized with IgM or EO6 antibody. J774 macrophages were incubated with increasing doses (1, 10, 50, 100 and 300 μg/ml) of OX-PAPC, and cell death was assessed 24 h post-treatment by MTT assay. OX-PAPC at concentrations of 100 and 300 μg/ml caused significant cytotoxicity, however PAPC at similar doses showed no cytotoxicity (Fig. 3A). Pretreatment of J774 macrophages with EO6 antibody prior to OX-PAPC treatment completely abolished the OX-PAPC induced cytotoxicity (Fig. 3B), suggesting the efficiency of the EO6 antibody to block the biological effects of OX-PAPC under conditions used.

3.3. EO6 antibody abolishes OX-PAPC mediated inhibition of bacterial phagocytosis by macrophages

Next, we evaluated if neutralization of Ox-PLs by the EO6 antibody inhibited OX-PAPC induced impairment of phagocytosis and bacterial clearance. J774 macrophages were exposed to increasing doses of OX-PAPC or specific Ox-PLs, POVPC, PGPC or PazPC, for 3 h and then challenged with PA for 1 h. Cells were washed with culture media to remove any oxidized-phospholipids prior to incubation with the bacteria. OX-PAPC and specific truncated phospholipids inhibited bacterial phagocytosis in a dose-dependent manner (Fig. 3C). Next, we determined whether the effect of OX-PAPC on macrophage phagocytosis was reversed after removal of OX-PAPC. J774 macrophages were treated with OX-PAPC or PAPC for either 1 or 3 h and then the phospholipids were washed

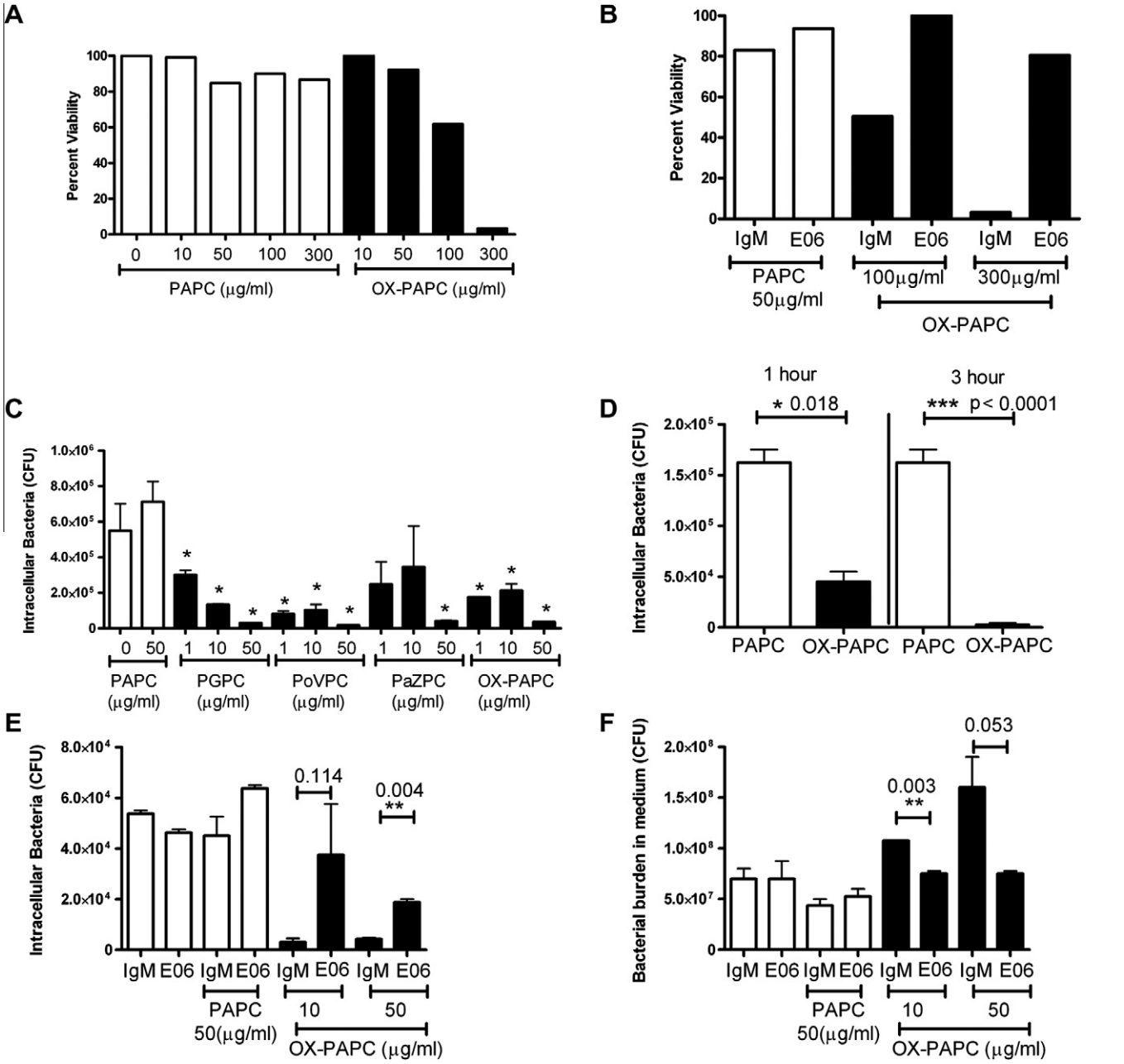

Fig. 3. EO6 blocks OX-PAPC induced inhibition of bacterial phagocytosis in J744 macrophages. (A) Dose response studies for OX-PAPC induced cytotoxicity. J774 macrophages were incubated with increasing doses of OX-PAPC or PAPC for 24 h and cell death was measured by MTT assay. Data presented as % cell death compared to untreated cells. (B) Cytotoxicity induced by EO6- or IgM-treated OX-PAPC (100 or 300 μg/ml) or PAPC in J744 macrophages. (C) Dose response studies for OX-PAPC induced impairment of bacterial phagocytosis. J774 macrophages were incubated with increasing doses of OX-PAPC, PoVPC, PGPC, PazPC or PAPC for 3 h, and bacterial phagocytosis was measured 1 h after PA inoculation. Data presented as intracellular bacterial CFU. (D) Bacterial phagocytosis by J774 macrophages 1 or 3 h after OX-PAPC. After incubation with OX-PAPC, J774 macrophages were washed to remove OX-PAPC and challenged with PA 6 h later. (E and F) EO6 blocks OX-PAPC induced impairment in bacterial phagocytosis. (E and F) Bacterial phagocytosis (E) and clearance (F) by J774 macrophages at 1 h and 4 h respectively following exposure exposed to EO6- or IgM-treated OX-PAPC (100 or 300 μg/ml) or PAPC for 3 h. *Statistically Significant compare to PAPC; $P < 0.01$.

out. After 6 h, the cells were incubated with PA, and bacterial phagocytosis was assessed. We observed impaired bacterial phagocytosis by J774 macrophages as early as 1 h post-OX-PAPC, which further worsened by continued exposure for 3 h (Fig. 3D), suggesting that the deficit in phagocytosis persists even after Ox-PLs are removed. Next, we incubated OX-PAPC with the EO6 antibody or IgM prior to exposure to J774 macrophages, and assessed bacterial phagocytosis and clearance. Compared to IgM, incubation with the EO6 antibody nearly completely attenuated OX-PAPC induced inhibition of bacterial phagocytosis and clearance by J774 macrophages (Fig. 3E and F). The EO6 antibody had no effect on the phagocytic activity of macrophages exposed to PAPC.

3.4. Treatment of BALF with EO6 antibody inhibits impairment of bacterial phagocytosis by macrophages

Next, we determined whether elevated levels of Ox-PLs in BALF retrieved from mice exposed to CS (as shown in Fig. 1C) influences phagocytic function of macrophages. To address this, J774 macrophages were incubated with BALF obtained from mice exposed to 6 months of either CS or RA for 3 h, washed to remove the BALF, and incubated with PA for 1 h. Bronchoalveolar lavage in mice was performed with serum-free RPMI culture medium. Bacterial phagocytosis was significantly reduced in J774 macrophages exposed to BALF retrieved from CS exposed mice when compared

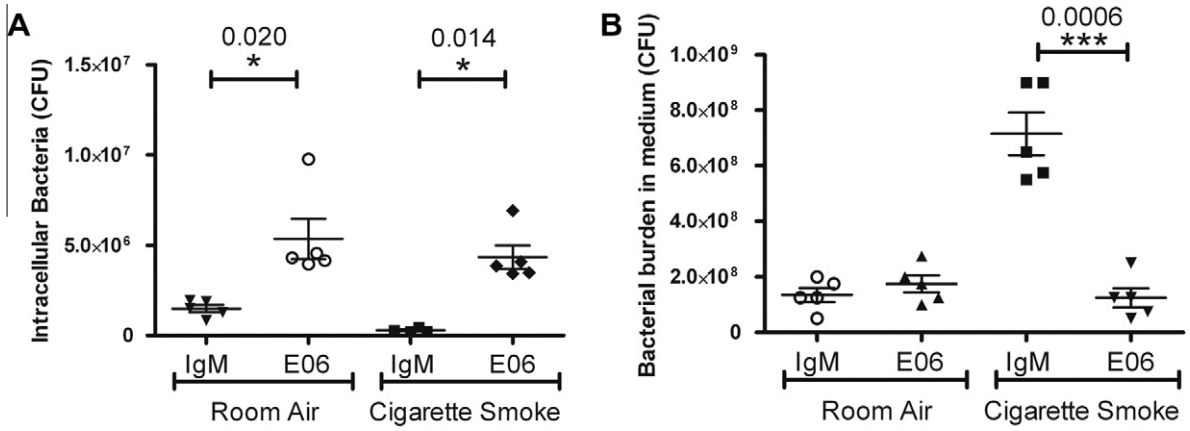

Fig. 4. Treatment of BALF retrieved from CS-exposed mice with the EO6 antibody inhibits impairment of bacterial phagocytosis by macrophages. BALF retrieved from mice exposed to 6 months of CS or room air was treated with either the EO6 antibody or IgM and subsequently incubated with J774 macrophages for 3 h. Bacterial phagocytosis (A) and clearance (B) was measured in BALF-exposed J774 macrophages 1 or 4 h after incubation with PA. *Statistically significant compared to IgM; †Statistically significant compared to room air.

to room air. However, when BALF from CS-exposed mice was treated with EO6 for 10 min prior to incubation with J774 macrophages, the bacterial phagocytic activity and clearance was significantly increased, compared to IgM-treated BALF (Fig. 4A and B). We observed a moderate increase in bacterial phagocytosis in J774 macrophages exposed to RA BALF treated with EO6 compared to IgM. However, the bacterial clearance by J744 macrophages exposed to either EO6- or IgM-treated BALF was similar. Taken together, the data suggest that Ox-PLs in BALF of CS exposed mice could inhibit bacterial phagocytic activity of macrophages.

In conclusion, our study provides evidence that accumulation of Ox-PLs in lungs as a result of CS-induced oxidative stress could be a major contributor to defective phagocytic activity in macrophages from smokers and patients with COPD. We found that Ox-PLs, which are elevated in BALF of CS exposed mice, inhibit phagocytic activity of macrophages, and the biological effects of Ox-PLs can be blocked by the EO6 antibody. The underlying mechanisms of how Ox-PLs impair phagocytic function are unclear. It is plausible that interaction of Ox-PLs with membrane proteins such as scavenger receptors could disrupt binding and phagocytosis [25]. A recent study reported that Ox-PLs stimulate redox signaling and induce a distinct phenotypic change in macrophages [30]. Whether this change in macrophage phenotype affects its phagocytic function warrants further investigation. Therapeutic approaches that could decrease oxidative stress, such as augmenting either the Nrf2 signaling pathway [23,31] or exogenous antioxidants, could be effective in improving pulmonary anti-bacterial innate defenses in smokers and patients with COPD by reducing the generation of Ox-PLs.

Acknowledgments

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