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## Effect of low doses of lipopolysaccharide prior to ozone exposure on bronchoalveolar lavage:

### Differences between wild type and surfactant protein A-deficient mice

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### SUMMARY

**Background**—Several aspects of the inflammatory response to a single insult, i.e., exposure to 2 ppm of ozone (O<sub>3</sub>) for 3 h or 6 h, are less pronounced in surfactant protein A deficient (SP-A <sup>-/-</sup>) mice (KO) than in wild type mice (WT). It was hypothesized that a mild insult, specifically low doses of lipopolysaccharide (LPS), would adversely affect host defense and differentially potentiate O<sub>3</sub>-induced injury in WT and KO mice.

**METHODS**—WT and KO mice were treated with different doses of LPS or LPS (2 ng) + O<sub>3</sub> (2 ppm) or filtered air (FA) for 3 h, then sacrificed 4 h following exposure (O<sub>3</sub>, FA) or 20 h after LPS treatment alone. Several endpoints of inflammation were measured in bronchoalveolar lavage (BAL).

**RESULTS**—1) At 20 h after LPS treatment alone, both WT and KO mice exhibited signs of inflammation, but with differences in the macrophage inflammatory protein 2 (MIP-2) response pattern, total cells (at 0.5 ng LPS) and basal levels of oxidized protein and phospholipids; 2) After LPS + O<sub>3</sub>, KO compared to WT showed decrease in polymorphonuclear leukocytes (PMNs) and MIP-2 and increase in phospholipids, and after LPS + FA an increase in total cells; 3) WT after LPS + FA showed an increase in SP-A with no further increase after LPS + O<sub>3</sub>, and an increase in oxidized SP-A dimer following O<sub>3</sub> or LPS + O<sub>3</sub>.

**CONCLUSIONS**—LPS treatment has negative effects on inflammation endpoints in mouse BAL long after exposure and renders KO mice less capable of responding to a second insult. LPS and O<sub>3</sub> affect SP-A, quantitatively and qualitatively, respectively.

### Keywords

polymorphonuclear leukocytes; cytokines; lipopolysaccharide; oxidation; macrophage; ozone

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“Duty is liberating. It forces you to transcend your own limitations and makes you do things that may not come naturally, but must be done, because they are right.”

*David Rockefeller*

## INTRODUCTION

Lipopolysaccharide (LPS) or endotoxin is a major component of the outer membrane of gram-negative bacteria, which consists of Lipid A, a core polysaccharide, and O antigen, and is a strong inducer of innate immunity. Inhalation of endotoxin results in the recruitment of inflammatory cells and enhances the production of a variety of proinflammatory cytokines. A significant number of studies have shown that an endotoxin response is mediated through the interaction of the CD14/TLR4/MD2 receptor complex, which is linked intracellularly to NF $\kappa$ B pathways involved in the production of cytokines in many cell types, especially macrophages<sup>1-4</sup>. Recent studies demonstrated that TLR-4 is also necessary for full development of ozone (O<sub>3</sub>) induced lung injury<sup>5,6</sup>. Inhalation of high concentrations (100  $\mu$ g) of LPS before O<sub>3</sub> exposure has been shown to potentiate the effect of O<sub>3</sub> and induce lung inflammation<sup>7,8</sup>.

O<sub>3</sub> is one of the major components of air pollution. It is a highly toxic constituent of photochemical smog and it reacts primarily with molecules at the air-fluid boundary because its low solubility in water limits its ability to diffuse into the tissues. The biological effects of O<sub>3</sub> are associated with oxidative stress in the lower respiratory tract and it may damage the epithelium, both directly and by triggering an inflammatory cascade that causes indirect damage<sup>9-11</sup>. O<sub>3</sub>-induced changes in the lung include an influx and activation of immune cells, increase in proinflammatory cytokines and chemokines, including the chemokines, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2<sup>7,8,12-14</sup>. O<sub>3</sub> exposure also causes surfactant dysfunction<sup>15,16</sup>, although the mechanism for this remains unclear.

Pulmonary surfactant is a complex mixture of lipids and proteins. In addition to preventing alveolar collapse, its components play an important role in the first line of defense against various insults. Surfactant protein A (SP-A) is a major protein component of lung surfactant involved broadly in surfactant-related functions and in innate immunity<sup>17-20</sup>. Concerning innate immunity, SP-A has been shown to enhance phagocytosis of a number of microorganisms<sup>21-27</sup>, regulate complement activation<sup>28</sup>, and augment the production of reactive oxygen and nitrogen species (RONS), a mechanism for killing pathogens<sup>22,29,30</sup>. Deficits in these functions in SP-A deficient (SPA<sup>-/-</sup>) (KO) mice make them susceptible to pneumonia<sup>27,30-32</sup>. Evidence also indicates SP-A involvement in the regulation of inflammation, particularly with regard to lung injury and repair, by regulation of proinflammatory cytokines<sup>2,14,33</sup>, clearance of apoptotic cells<sup>34,35</sup>, and regulation of collagen and matrix metalloproteinases<sup>36,37</sup>. Several studies have shown that various SP-A functions are adversely affected by oxidation<sup>38-41</sup> or nitration<sup>42-45</sup>. The authors recently reported that oxidation of SP-A increased immediately after O<sub>3</sub>, before the oxidation of other proteins<sup>14</sup>, and also that during aging in the rat, SP-A undergoes disproportionately increased carbonylation compared to other bronchoalveolar lavage (BAL) proteins, suggesting increased susceptibility to oxidation<sup>46</sup>. It was postulated that, on the one hand, decrements in SP-A function may occur due to oxidation and, on the other hand, SP-A, by way of this susceptibility to oxidation, may play a role in limiting tissue damage by scavenging excess RONS<sup>14</sup>.

Acute lung injury has been shown to alter the levels of pulmonary surfactant in BAL, although questions remain about the mechanisms responsible for this, because both increases and decreases in the levels of phospholipids have been reported after LPS insults<sup>16,47-49</sup>. The lipids in surfactant, while primarily responsible for the reduction of surface tension can also influence the behaviour of immune cells. Surfactant lipids suppress

the activation of immune cells and inhibit the production and release of proinflammatory cytokines<sup>50,51</sup>. However, both SP-A and the surfactant lipids can be oxidized by O<sub>3</sub> or endogenous RONS<sup>39,40,44,52–55</sup>, altering their function and potentially contributing to the inflammation resulting from O<sub>3</sub> exposure.

Exposure to O<sub>3</sub> is associated with changes in innate immunity and inflammatory processes in both KO and wild type (WT) mice, but differences between the two types were observed, indicating a role of SP-A in the first line of defense. Because exposure to multiple toxicants may result in injuries or responses that are not predicted by evaluation of exposure to individual toxicants, the present study was undertaken in order to investigate the effect of a low dose of LPS prior to O<sub>3</sub> exposure in WT mice with intact defenses and SP-A KO mice in which the innate host defense molecule, SP-A, has been ablated. The focus was on the early events occurring 4 hours after O<sub>3</sub> treatment, a time point that in previous studies<sup>14</sup> showed significant changes in several relevant endpoints.

## OBJECTIVES

The aim of the study was to determine whether mice treated with a low concentration of LPS are sensitized, altering their response to an oxidative stress such as that produced by the environmental pollutant O<sub>3</sub>.

## MATERIALS AND METHODS

### Animals

The study was conducted on pathogen-free male WT and SP-A KO male mice on the C57BL/6 genetic background. WT mice were obtained from Jackson Laboratories (Bar Harbor, ME) and kept under standard environmental conditions in the animal facility at the Penn State College of Medicine. KO mice were maintained as specific pathogen-free mice under barrier/sterile conditions in the animal facility at the Penn State College of Medicine. Both WT and KO mice were fed rodent chow and tap water ad libitum. The body weight of the mice was in the range of 20–25 g. The Institutional Animal Care and Use Committee at the Penn State College of Medicine approved this study.

### LPS dose response

Five to six week old WT male mice (n=4 mice/time point) received a single dose (0.2, 0.5, 1, 2, 50, or 200 ng) of smooth LPS (*Escherichia coli* 055:B5; Sigma, St. Louis, MO) in 50 µl saline intrapharyngeally. Control mice received equal volumes of saline intrapharyngeally, in parallel. Treatments were administered by placing the 50 µl dose in the pharynx of anaesthetized mice. The mice aspirated the bolus and recovered from the instillation with no obvious ill effects. Based on the WT data, two low doses of LPS shown to have stimulatory effects on inflammation were selected for study in KO mice. Five to six week old KO mice (n=4 mice/time point) received a single dose of LPS (0.5 or 2 ng/mouse/50 µl in saline). Control mice received equal volumes of saline intrapharyngeally. Both WT and KO mice were sacrificed at 20 h after LPS treatment by anaesthetization with halothane and exsanguination. The 20 h time point was selected on the basis of a number of reports that showed LPS-induced injury to occur within 24 h of LPS treatment<sup>56–58</sup>. The lungs were subjected to BAL with normal saline.

### LPS treatment followed by O<sub>3</sub> exposure in WT and KO mice

The 2 ng dose of LPS was selected for subsequent experimentation because it provided an effect on several outcomes from a low concentration of LPS. Five to six week old WT and KO mice (20–25 g) (n=4 mice/time point) received a single dose of LPS (2 ng) in 50 µl

saline intrapharyngeally. Control mice received equal volumes of saline intrapharyngeally, in parallel. Thirteen hours after LPS treatment, the animals were exposed to either filtered air (FA) or O<sub>3</sub> at a concentration of 2 ppm for 3 hours. These exposures were conducted in parallel at room temperature and 50% humidity as described elsewhere<sup>14</sup>. In brief, four mice were put in glass containers with wire mesh lids and then placed in a closed glass exposure chamber. The O<sub>3</sub> was generated by an electric discharge ozonizer (Model OZ2SS-SS, Ozotech, Yreka, CA) and its concentration was monitored with an ultraviolet ozone analyzer (Model 400A, Advanced Pollution Instrumentation, San Diego, CA). This system is highly efficient at delivering O<sub>3</sub> in specific concentrations between 0.1 ppm and 10 ppm<sup>59</sup>. Four hours after the termination of exposure, the mice were sacrificed (total time of 20 hours after LPS treatment) by anesthetization and exsanguination. The lungs were subjected to BAL with normal saline.

### Cell count and biochemical studies of BAL fluid

BAL fluid was obtained by instilling the lung 3 times with a volume of saline equal to 80% of lung vital capacity (for a total of 1.5 ml) through a tracheal cannula. The total fluid recovery was approximately 90% of the instilled volume and did not differ significantly between the experimental and control groups. The BAL fluid was centrifuged (150 × g, 5 min, 4°C) and the cell pellet was resuspended in 0.9% sodium chloride (NaCl). On each sample a total cell count was performed using a haemocytometer and a cytocentrifuge preparation was used to obtain differential cell counts. The cell-free supernatant was frozen and stored at a temperature of -80°C for subsequent biochemical analysis. Total protein concentration was determined using the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). For determination of total phospholipids, a 100 µl sample of BAL supernatant was lyophilized and assayed using the Phospholipids B Assay (WAKO Chemicals Inc, Richmond, VA).

### Cytokine ELISAs

The concentration of MCP-1 and MIP-2 was measured on cell-free BAL samples with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, McKinley Place, NE). In brief, 500 µl of cell-free BAL were lyophilized and then reconstituted to 100 µl with the assay diluent provided in the kit. Assays were performed according to the manufacturer's recommendations, and the plates were read at 450 nm in a SPECTRA Fluor Plus ELISA plate reader (Tecan US, Research Triangle Park, NC) after the appropriate colour development period. The detection limits of these ELISA kits are 1.5 pg/ml for MIP-2 and 2 pg/ml for MCP-1.

### SP-A Dot Blot

SP-A levels in the BAL were determined by protein dot blot. Protein dot blotting was done by diluting 25 µl of the cell-free BAL fluid in 975 µl of 0.02 M tris-buffered saline (pH 7.5; TBS) solution and applying 200 µl aliquots to Bio-Rad Trans Blot transfer medium (nitrocellulose, 0.45 µm) under vacuum in a 96 well dot blot apparatus (Bio-Rad, Hercules, CA). Blots were blocked overnight in 0.01 M Na/K phosphate buffered saline (pH 7.2; PBS) containing 1% bovine serum albumin (BSA). Following blocking, blots were incubated in a solution containing polyclonal rabbit anti-SP-A IgG<sup>60</sup> (1:10,000) in PBS with 1% BSA, 0.05% Tween-20 for 1 h at RT and washed 3 times for 10 min with PBS, 0.5% Tween-20. The blots were then incubated for 1 h at room temperature with secondary antibody (goat anti-rabbit IgG HRP conjugate; 1:25,000) (Bio-Rad) and then washed as in the previous step. Antibody binding was detected by enhanced chemiluminescence (ECL). Blots were incubated for 1 min with 10 ml of each ECL solution (PerkinElmer Life Sciences, Boston, MA), and then wrapped in cellophane wrap, the excess solution removed, and exposed to

Kodak X-Omat XAR film (Eastman Kodak Co., Rochester, NY). The film was developed and SP-A levels were quantified by laser densitometry.

### Detection of oxidized protein in BAL

Oxidized proteins were detected using the OxyBlot Oxidized Protein Detection Kit (Intergen, Purchase, NY) as described previously<sup>14</sup> with certain modifications. This kit detects carbonyl groups that have been introduced into proteins through oxidation. In brief, 25  $\mu$ l of BAL samples were denatured by adding an equal volume of 12% SDS. Samples were then derivatized with 2.5  $\mu$ l of 10X 2, 4-dinitrophenylhydrazine (DNPH) solution and incubated for 10 min at room temperature. Derivatization was stopped with the addition of 25  $\mu$ l of neutralization solution. Samples were then analyzed by dot blot. Aliquots containing the DNPH-derivatized proteins were brought up to a volume of 500  $\mu$ l with 0.01 M phosphate buffered saline (pH 7.2) and 200  $\mu$ l of each sample was blotted onto nitrocellulose by vacuum using a 96-well dot-blot apparatus (Bio-Rad). Immunodetection of oxidized proteins was performed according to the manufacturer's instructions, although the rabbit anti-DNP and goat anti-rabbit IgG (HRP-conjugated) antibodies supplied were used at one half of the recommended concentrations. ECL was used to detect antibody binding and the blots were exposed to XAR film (Eastman Kodak Co., Rochester, NY). The film was developed and carbonylated protein levels were quantified by laser densitometry.

### Oxidized and non-oxidized SP-A protein in BAL by Western Blot

BAL samples in 200  $\mu$ l aliquots were concentrated by lyophilization. Protein samples were then subjected to electrophoresis on 12.5% SDS polyacrylamide gels. Two separate gels were run and then the separated proteins were transferred to nitrocellulose membrane (nitrocellulose, 0.45  $\mu$ m) for SP-A detection and on polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for oxidized SP-A in a buffer of 25 mM Tris base, 192 mM glycine, 20% methanol. Immunodetection of SP-A protein was made as described in the previous section of SP-A BAL protein dot blots. Oxidized SP-A protein was detected as described previously<sup>61</sup>, with some modifications. The membrane was dehydrated for 1 min with 100% methanol and then washed for 5 min in 0.02 M Tris (pH 7.5) with 20% methanol, 5 min with 2 N hydrochloric acid (HCl), and then treated with 100  $\mu$ g DNPH/ml in 2 N HCl for 5 min. The membrane was again washed 3 times with 2 N HCl, 7 times with 100% methanol, and once with 0.02 M TBS (5 min each wash). The membrane was blocked overnight in 5% powdered milk (Carnation) in 0.02 M TBS, 0.05% Tween-20. All the post-transfer steps were made in 100 ml of solution at room temperature. Immunodetection of oxidized proteins was made, as described in the previous section on BAL protein dot blots.

### Statistical analysis

All analyses for the linear trend model between dose and main outcome and non-parametric comparisons were performed using SAS V9.1 (SAS Institute, Cary, NC) and a p-value of less than 0.05 was considered significant. LPS dose was converted to log scale prior to analyses in order to satisfy the distributional assumption associated with the linear model. All the other data were analyzed by t-test (Sigma Stat; SPSS; Chicago, IL).

## RESULTS

### Behavioural Observations

Both WT and SP-A mice exposed to different doses of LPS appeared to behave similarly to those treated with saline. The mice that received a low dose of LPS followed by O<sub>3</sub> exposure behaved differently from those that received LPS followed by FA exposure. Shortly after the start of the O<sub>3</sub> exposure period the fur of these mice became ruffled, and after 30 min to 1

hour, the O<sub>3</sub>-exposed mice became less active, curled up, and appeared to sleep for the duration of the exposure period. Following the exposure, their activity returned to normal within the first hour. Mice exposed to LPS and FA remained active throughout the exposure period.

### LPS dose response study for WT mice

The analysis of BAL revealed several changes in the mice treated with LPS compared to the saline treated controls (Figure 1). These changes included a significant increase ( $p < 0.05$ ) in the mean total BAL cell count (Fig. 1A), in the percentage of polymorphonuclear (PMN) leukocytes (Fig. 1B), and in the mean level of MIP-2 (Fig. 1C) starting for each end point at LPS dosage of  $\geq 0.2$  ng,  $\geq 0.5$  ng, and at  $\geq 1$  ng, respectively. Total protein (Fig. 1D) and its levels of oxidation (Fig. 1E), measured by carbonyl content for total oxidized protein, were also higher in LPS treated mice and reached significance ( $p < 0.05$ ) at doses of LPS of 1 ng and higher. SP-A protein levels in BAL measured by protein dot blot for the most part remained at normal levels with a decrease and increase observed at 0.2 and 50 ng LPS, respectively, (Fig. 1F). No consistent change was observed in total phospholipids at any dose of LPS (not shown). The increases correlated with LPS dose (Figs. 1A, 1B, 1C) for total cells ( $r^2 = 0.688$ ,  $p < 0.0001$ ), percentage of PMNs ( $r^2 = 0.689$ ,  $p < 0.0001$ ), and the level of MIP-2 protein ( $r^2 = 0.633$ ,  $p < 0.0001$ ). For the other parameters, no correlation was observed.

### Comparison of LPS effect in WT and KO mice

KO mice LPS treated (0.5 ng or 2.0 ng/mouse) also showed signs of inflammation in the BAL (Figure 2). Significant differences were observed for both LPS doses in several endpoints (Figs. 2A–2D) compared to measurements in the BAL of control saline-treated mice. Control values for total oxidized protein (Fig. 2E) and total phospholipids (Fig. 2F) differed from one another so control values at were set at 100% and the LPS samples were calculated as a percentage of the control. Oxidized protein values increased after LPS and there was a trend toward greater increases in KO mice, although statistical significance was observed only at 0.5 ng LPS. With respect to phospholipids, LPS administration was associated with reduced levels and significant differences occurred at 0.5 ng LPS for WT, and at 2 ng LPS for KO mice.

On comparison of the KO data and WT data certain differences emerged: 1) Significant differences in the total number of cells (Fig. 2A) were observed between WT and KO at exposure to 0.5 ng of LPS but not 2 ng of LPS, indicating an inability of KO to respond to low levels of LPS, but this inability was overcome in the face of a more severe insult (i.e. 2 ng LPS); 2) PMN recruitment as assessed by the percent of PMNs (Fig. 2B) in BAL is gradual in WT; significant differences were observed between the two doses of LPS and between the LPS treated animals and the controls. For the KO no significant differences between the two doses of LPS were observed although PMN recruitment was increased in LPS treated animals compared to the controls. A decrease in macrophage/monocyte cell count in WT and KO (not shown) followed a pattern similar to that described for PMNs; 3) MIP-2 levels (Fig. 2C) for each group, for the most part, tended to parallel the observations made for PMNs, except for a lack of significant difference in WT between mice treated with 0.5 ng of LPS and controls. Taken together, these observations indicate that the response of KO mice to low levels of LPS is limited; 4) With regard to BAL protein (Fig. 2D), although no differences were observed between WT and KO mice in total protein levels, there was a trend toward greater changes in total oxidized protein in KO mice than in WT mice; 5) A difference (decrease) in total phospholipids (Fig. 2F) was observed between control and LPS treated mice, but not between WT and KO mice.

### Effect of O<sub>3</sub> or FA on BAL cells and cytokines in LPS treated WT and KO mice

Exposure of WT mice to LPS (2 ng) + O<sub>3</sub> resulted in significant increases ( $p < 0.05$ ) in several parameters in the BAL (Figure 3) compared to LPS + FA exposure. These differences included significant changes in the total cell count (Fig. 3A), levels of MIP-2 (Fig. 3C), and MCP-1 (not shown), total protein (Fig. 3D), and total oxidized protein (Fig. 3E) in BAL. No significant differences were observed between the groups of WT mice in the percentage of PMNs (Fig. 3B) or phospholipids (Fig. 3F).

Exposure of KO mice to LPS (2 ng) + O<sub>3</sub> resulted in significant ( $p < 0.05$ ) increases compared to the LPS + FA treated group, in the levels of MIP-2 (Fig. 3C) and MCP-1 (not shown), phospholipids (Fig. 3F), total protein (Fig. 3D) and oxidized protein (Fig. 3E). This LPS response pattern was similar to that observed in the WT mice, except that in the KO mice a significant increase in phospholipid levels in response to LPS + O<sub>3</sub> was observed compared to the LPS + FA mice, but no change in total BAL cell count.

Significant differences were observed between WT and KO mice after LPS + O<sub>3</sub> treatment in the percent of PMNs (Fig. 3B), MIP-2 (Fig. 3C), and phospholipid (Fig. 3F). No changes were observed between WT and KO mice with regards to the levels of MCP-1 (not shown), total protein (Fig. 3D), and total oxidized protein (Fig. 3E). Differences between WT and KO mice after LPS + FA were observed only for total cells (Fig. 3A).

### The impact of LPS and O<sub>3</sub> on SP-A content and levels of oxidized SP-A

Small but significant increases in total SP-A content were observed in response to LPS + FA compared to FA alone (Fig. 4A). Similar increases were observed in response to LPS + O<sub>3</sub> compared to O<sub>3</sub> alone, indicating that LPS is the dominant factor for this increase. When the levels of oxidized SP-A were examined, significant differences were observed in the dimeric form of SP-A in response to O<sub>3</sub> and in response to LPS + O<sub>3</sub> (Fig. 4B). No significant differences were observed between LPS + FA and FA alone, indicating that changes in the oxidation state of SP-A are primarily due to O<sub>3</sub> exposure.

## DISCUSSION

The authors have previously reported<sup>14</sup> that WT mice and KO mice of the same genetic background showed signs of inflammation in BAL fluids after exposure to O<sub>3</sub> (2 ppm) for three or six hours. Several aspects of the inflammatory response were less pronounced in KO mice, and it was speculated that this occurs because in the absence of SP-A the alveolar macrophage is not primed by SP-A, rendering it less capable of mounting an effective inflammatory response. Other researchers reported that high dose LPS (100 µg) inhalation, prior to O<sub>3</sub> exposure of WT mice, appeared to sensitize the mice to O<sub>3</sub> differentially at different postnatal ages, with regard to inflammatory markers<sup>8</sup>. The present study was designed to investigate the hypothesis that low doses of instilled LPS adversely affect the inflammatory processes in the lung and potentiate differentially the impact of O<sub>3</sub>-induced injury in mice with intact innate immunity (i.e., WT mice) and those with compromised innate immunity (i.e., KO mice).

The results showed that: 1) a low dose of LPS (2 ng) alters the cellular, cytokine, total protein and oxidized protein content of the BAL of WT mice at 20 h after LPS treatment; 2) differences are observed between WT and KO mice after LPS exposure (0.5 or 2 ng) in the total cell count, the MIP-2 response pattern, and the base levels of oxidized protein and phospholipid content; 3) when 3 h ozone exposure follows 13 h after LPS (2 ng) treatment, differences between WT and KO mice are observed in PMNs, MIP-2 protein and phospholipid content of BAL; 4) in WT an increase in total SP-A is observed in response to LPS + FA or LPS + O<sub>3</sub> but not in response to O<sub>3</sub> alone. An increase in oxidized SP-A dimer

is observed in response to O<sub>3</sub> or LPS + O<sub>3</sub> but not in response to LPS + FA; 5) LPS treatment has no apparent impact on behaviour, whereas during the course of O<sub>3</sub> exposure, both WT and KO mice become less active. These findings together allow the conclusion that: 1) low level LPS insult can have long lasting effects on BAL composition; 2) a second stimulus may result in more pronounced effects than those produced by each stimulus alone; and 3) SP-A plays a role in the regulation of inflammation.

The differences between the WT and KO mice in response to LPS are long-lasting, as they were detectable 20 h after LPS treatment, and are limited to the total BAL cell count and the pattern of the MIP-2 response. Although the reasons for this are not fully understood, these findings support the theory that in WT mice the alveolar cells, and most likely the macrophages, in the face of a weak threat (i.e. 0.5 ng LPS) can, via small non-significant changes in MIP-2 expression, mediate a significant recruitment of cells into the alveolar space. When the insult is stronger (i.e., 2 ng LPS), increases in molecules such as MIP-2, a macrophage-produced neutrophil chemoattractant molecule, are necessary for adequate cell recruitment. When the activity of the alveolar cells is compromised with regard to the first line of defense, as could be the case with the KO mice, the low level threat (0.5 ng LPS) appears to be perceived as a higher level threat (i.e. 2 ng LPS) and this results in an increase in molecules such as MIP-2. The increased MIP-2 production in the KO mice in response to a low level insult, failed to translate to the increase of total cells in the alveolar space seen in the WT mice that could presumably help to eliminate the perceived threat. Together these observations are consistent with the idea that KO mice exhibit a compromised and/or limited response compared to WT mice. It is possible that the alveolar macrophages of the KO mice, in contrast to those of the WT mice, are not adequately prepared to effectively mount a response to LPS. It has been postulated that the alveolar macrophages in the absence of SP-A are hypoactive due to lack of priming by SP-A<sup>18</sup>. Inability of KO mice alveolar macrophages to function at a level similar to that of the WT mice has been observed recently with regards to phagocytosis of *K. pneumoniae*<sup>27</sup> and in several other studies where SP-A KO mice have been used. These include the inability of SP-A in KO mice to clear *P. aeruginosa* infection<sup>32,62</sup> and group B streptococcus<sup>63</sup>, and the ability of SP-A to modulate inflammatory processes, and minimize lung injury and mortality after experimental bone marrow transplantation<sup>64-66</sup>.

When a second insult (i.e. O<sub>3</sub> exposure) was imposed upon the LPS-treated mice, a significant decrease in PMNs and MIP-2 protein content was observed in the BAL of KO mice compared to WT mice, although both types of mice showed a significant increase in MIP-2 compared to LPS + FA treated mice. In contrast, when a single insult was present (either LPS (2 ng) or O<sub>3</sub> exposure<sup>14</sup>) no differences in MIP-2 protein or PMNs (in LPS only) were observed between WT and KO mice, even though both types showed increase in MIP-2 protein and PMNs compared to non-treated controls. In the earlier O<sub>3</sub> study<sup>14</sup>, although no difference between WT and KO mice was observed in response to O<sub>3</sub> in BAL MIP-2 protein, the MIP-2 mRNA content was significantly lower in the KO than in the WT mice. These observations indicate that prior LPS treatment may up-regulate MIP-2 expression via a translational control. On the other hand O<sub>3</sub> exposure in the KO mouse may negatively affect posttranscriptional processes involved in MIP-2 expression. These possibilities could explain the lower levels of MIP-2 protein observed in response to LPS + O<sub>3</sub> in the KO mice compared to the WT mice, and implicate SP-A in the regulation of MIP-2.

The data from the earlier O<sub>3</sub>-exposure study<sup>14</sup> indicate also that translation of MIP-2 may be halted entirely in the KO mouse, as assessed by the lack of difference in mRNA content between O<sub>3</sub>-exposed and control KO mice. Moreover, although LPS and O<sub>3</sub> may each have an individual impact on MIP-2 production, the mechanisms via which this occurs are likely



to differ or overlap. In the WT mice the change in MIP-2 levels with the combined exposure (LPS + O<sub>3</sub>) was greater than the sum of changes resulting from individual exposures (LPS or O<sub>3</sub> alone). The MIP-2 values for the controls were 36 pg/ml ± 16, for LPS treated 148 pg/ml ± 38, and for those following O<sub>3</sub> exposure (without prior LPS treatment) 627 pg/ml ± 205<sup>14</sup>. However, after exposure to both LPS and O<sub>3</sub>, MIP-2 levels reached 1,234 pg/ml ± 277, raising the possibility of synergism between the two agents. This synergism appears to be significantly compromised in the KO mouse, indicating a role for SP-A in the regulation of inflammation, and specifically the production of MIP-2.

Increased SP-A content in WT mice was observed in response to LPS and in agreement with the earlier O<sub>3</sub> study<sup>14</sup> no differences in SP-A content were observed in response to O<sub>3</sub> exposure, indicating that the SP-A increase observed in response to LPS + O<sub>3</sub> is primarily mediated by LPS. On the other hand, an increased level of oxidized SP-A was observed in response to O<sub>3</sub>, in both the present study and the earlier report<sup>14</sup>, but not in response to LPS, indicating that the oxidative stress mediated by O<sub>3</sub> exposure is responsible for the increased content of oxidized SP-A in response to LPS + O<sub>3</sub>. The differences in SP-A content and oxidized SP-A observed in response to LPS and O<sub>3</sub>, both separately and in combination, indicate further that each agent operates via a different mechanism, and that LPS affects SP-A quantitatively, perhaps by interfering with its regulation, whereas O<sub>3</sub> affects SP-A qualitatively by increasing oxidation of its dimeric form. At first glance it appears that LPS and O<sub>3</sub> work at cross-purposes in terms of SP-A, as LPS increases the production and O<sub>3</sub> increases the oxidation of SP-A. Recent studies have shown that oxidized SP-A exhibits reduced function<sup>27,39-41</sup> and therefore even if the SP-A content is increased, the functional SP-A content may not change under the experimental conditions of this study. SP-A, however, has been shown to be oxidized earlier than the total BAL protein content<sup>14,46</sup>, indicating that SP-A may play a protective role in response to an oxidative stress by scavenging RONS and serving as a sacrificial antioxidant<sup>67,68</sup>. According to this scenario, SP-A may serve as the sacrificial molecule, as its high susceptibility to oxidation may scavenge reactive oxidants and protect other molecules from becoming inactivated by oxidation. Alternatively, as postulated previously<sup>14</sup> the oxidation of dimeric SP-A may be important in signalling, and thus some of the defects observed in the KO mice may be due to lack of SP-A-mediated signalling in time of stress. Moreover, deficits of immune cell function, in both macrophages and neutrophils, and in the regulation of RONS production have been observed in KO mice, thereby implicating SP-A in these processes<sup>14,29-31</sup>.

In summary, the findings of this study lead to several conclusions: 1) A low dose of LPS can have a long-lasting effect on several aspects of BAL properties including cell count, cytokines, protein content, and other indices. 2) SP-A plays a role in the processes of inflammation in response to LPS and/or O<sub>3</sub> exposure. LPS appears to have an impact on SP-A regulation, affecting the quantitative content of SP-A and the O<sub>3</sub> effect on SP-A dimer oxidation, thus altering SP-A qualitatively, which may have an impact on its function. 3) SP-A appears to play a role in the regulation of MIP-2 perhaps via its ability to “prime” alveolar macrophages for an appropriate response in the face of an insult. LPS and O<sub>3</sub> exposure probably modulate MIP-2 expression via different mechanisms. 4) Exposure to a low dose of LPS and O<sub>3</sub> revealed some unexpected outcomes that were not observed with either insult alone, indicating that these agents operate via different and/or overlapping mechanisms. Finally, the present findings support the theory that in the absence of SP-A, alveolar macrophages may not be able to elicit an appropriate response to insulting agents, implicating SP-A in a role of adequately preparing or priming macrophages to respond.

## Acknowledgments

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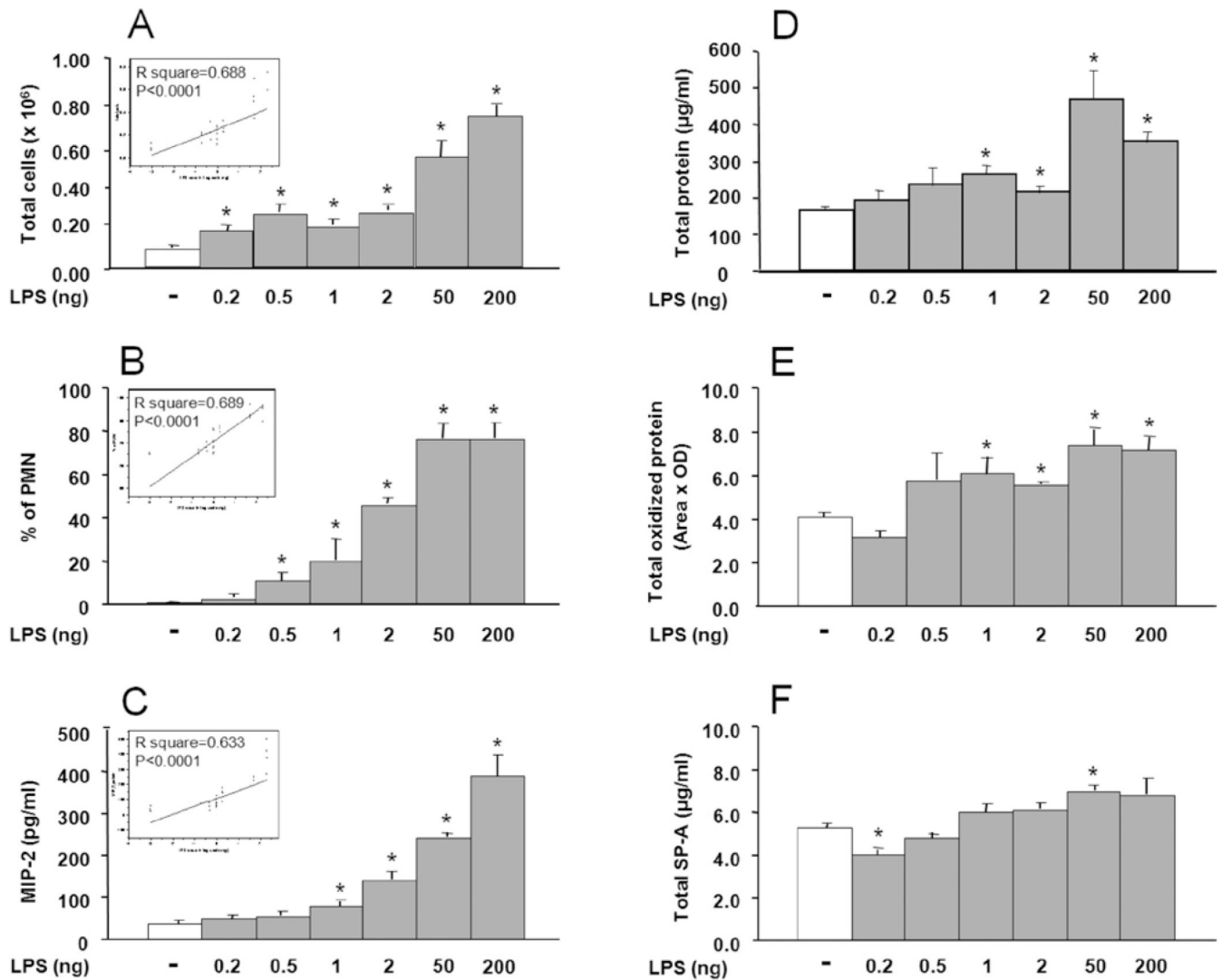
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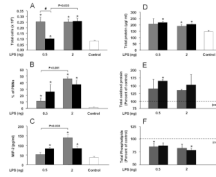
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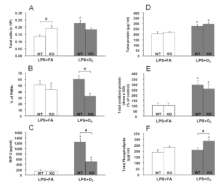
**FIGURE 1.**

Lipopolysaccharide (LPS) effect on total cell and polymorphonuclear leukocyte (PMN) count, macrophage inflammatory protein 2 (MIP-2) content, total protein and total oxidized protein, and total surfactant protein A (SP-A) in bronchoalveolar lavage (BAL) of wild type (WT) mice. The mice received LPS at different concentrations (0.2, 0.5, 1, 2, 50, and 200 ng) intrapharyngeally in 50  $\mu$ l saline. Control animals received 50  $\mu$ l saline in parallel. BAL was collected 20 h after LPS treatment. Values are expressed as mean  $\pm$  S.D., for total cells (A), PMNs (B), MIP-2 (C), total protein (D), total oxidized protein (E), and total SP-A protein (F). Statistically significant ( $p \leq 0.05$ ) differences between LPS treated (gray bar) and control (open bar) mice ( $n=4$  for each group) are indicated by an asterisk (\*). The figure in the box represents the correlation between LPS dose and the indicated read-outs.



**FIGURE 2.**

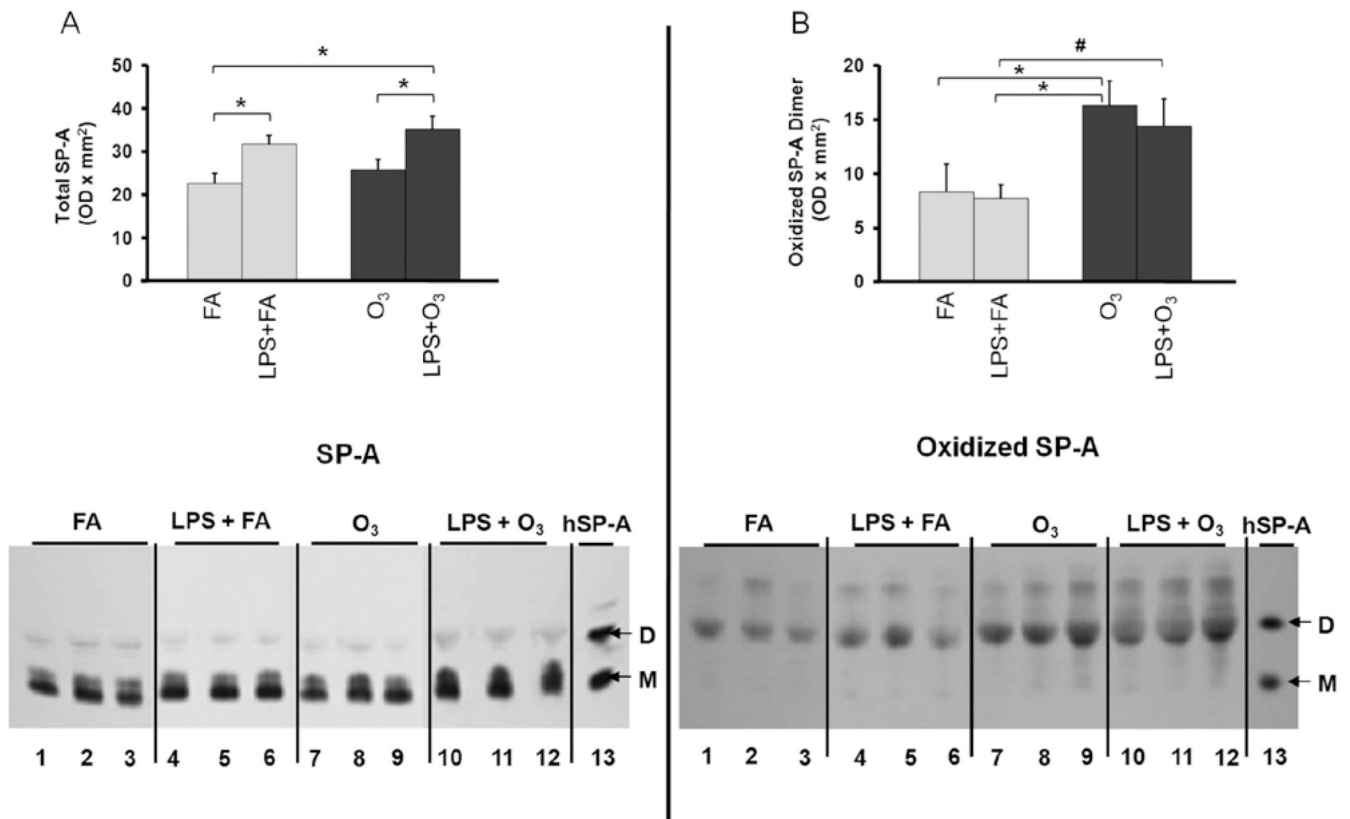
Lipopolysaccharide (LPS) effect on total cell and polymorphonuclear leukocyte (PMN) count, macrophage inflammatory protein 2 (MIP-2) content, total protein and oxidized protein, and total phospholipids in bronchoalveolar lavage (BAL) of surfactant protein A deficient (*SP-A*<sup>-/-</sup>) mice (KO) in comparison with wild type (WT) mice. The mice received intrapharyngeal instillation of selected LPS doses (0.5 ng and 2 ng) in 50  $\mu$ l saline. Control mice received 50  $\mu$ l saline in parallel. BAL was collected 20 h after LPS treatment. Values are expressed as mean  $\pm$  S.D. (n=4) for WT (gray bars), KO (black bars). Filtered air (FA) control values are represented by a single bar (open bar) when no differences were seen between KO and WT mice (panels A, B, C, D). For panels E and F, where differences in basal levels between WT and KO were observed, the control values were set to 100% (dotted line). Data are shown for total cells (A), percentage of PMNs (B), MIP-2 (C), total protein (D), total oxidized protein (E), and total phospholipids (F). Statistically significant ( $p \leq 0.05$ ) differences between LPS treated and control mice are indicated by an asterisk (\*), and between WT and KO mice by the sign (#) above a connecting bar. Differences between LPS doses in WT or KO are shown by a connecting bar with the p-value above the bar.



**FIGURE 3.**

Effect of lipopolysaccharide (LPS) + filtered air (FA) or LPS + ozone (O<sub>3</sub>) on total cell count, polymorphonuclear leukocyte (PMN) count, MIP-2, phospholipids, total protein, and total oxidized protein content in the bronchoalveolar lavage (BAL) of wild type (WT) and surfactant protein A deficient (SP-A <sup>-/-</sup>) mice (KO). The mice received 2 ng of LPS intrapharyngeally in 50  $\mu$ l saline, and 13 h later they were exposed to either FA or O<sub>3</sub> (2 ppm for 3 h). BAL was collected 4 h following the conclusion of FA or O<sub>3</sub> exposures, or a total time of 20 h after LPS treatment. Values are expressed as mean  $\pm$  S.D. (n=4) for LPS+FA (open bars), and LPS + O<sub>3</sub> (black bars). Data are shown for total cells (A), percentage of PMNs (B), MIP-2 (C), total protein (D), total oxidized protein (E), and phospholipids (F). Significant ( $p \leq 0.05$ ) differences between LPS + FA and, LPS + O<sub>3</sub> treated mice are indicated by an asterisk (\*), and between WT and KO with the pound sign (#) above a connecting bar ( $p \leq 0.05$ ).



**FIGURE 4.**

The effects of lipopolysaccharide (LPS) with or without ozone (O<sub>3</sub>), O<sub>3</sub> alone or filtered air (FA) alone on the level of surfactant protein A (SP-A) (Panel A) and oxidized SP-A (Panel B) in the bronchoalveolar lavage (BAL) of wild type (WT) mice. The mice received 2 ng of LPS intrapharyngeally in 50  $\mu$ l saline, then 13 h later they were exposed to either FA (gray bars) or O<sub>3</sub> (2 ppm for 3 h) (black bars) and subjected to BAL 4 h after ozone exposure or 20 h after LPS treatment. SDS gel electrophoresis was performed on concentrated samples from 200  $\mu$ l of BAL fluid and the proteins transferred to membranes as described in Methods. Levels of SP-A (Panel A; top) and oxidized SP-A (Panel B; top) were measured by densitometry of Western blots. For each condition n=6 except for FA, where n=5. At the bottom of panels A and B a representative immunoblot (Panel A) and an oxyblot (Panel B) are shown. A reference lane is shown that contains immunostained human alveolar proteinosis SP-A (hSP-A) with bands of monomeric (M) and dimeric (D) SP-A marked in each panel. The position of the oxidized SP-A dimer bands was determined by comparison with an identical immunoblot stained with an antiserum to SP-A. The values depicted are the mean  $\pm$  S.D. Significant ( $p \leq 0.05$ ) differences between LPS + FA and LPS + O<sub>3</sub> treated mice are indicated by the pound sign (#) and other comparisons by an asterisk (\*).