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Cigarette Smoke Exposure Worsens Endotoxin-Induced Lung Injury and Pulmonary Edema in Mice

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Abstract

Introduction: Cigarette smoking (CS) remains a major public health concern and has recently been associated with an increased risk of developing acute respiratory distress syndrome (ARDS). Bronchoalveolar lavage (BAL) experiments in human volunteers have demonstrated that active smokers develop increased alveolar-epithelial barrier permeability to protein after inhaling lipopolysaccharide (LPS). Here we tested the hypothesis that short-term whole-body CS exposure would increase LPS-induced lung edema in mice.

Methods: Adult mice were exposed in a TeagueTE-10 machine to CS from 3R4F cigarettes at 100 mg/m^3 total suspended particulates for 12 days, then given LPS or saline intratracheally. Control mice were housed in the same room without CS exposure. Post-mortem measurements included gravimetric lung water and BAL protein, cell counts, and lung histology. Cytokines were measured in lung homogenate by ELISA and in plasma by Luminex and ELISA.

Results: In CS-exposed mice, intratracheal LPS caused greater increases in pulmonary edema by gravimetric measurement and histologic scoring. CS-exposed mice also had an increase in BAL neutrophilia, lung IL-6, and plasma CXCL9, a T-cell chemoattractant. Intratracheal LPS concentrated blood hemoglobin to a greater degree in CS-exposed mice, consistent with an increase in systemic vascular permeability.

Conclusions: These results demonstrate that CS exposure in endotoxin injured mice increases the severity of acute lung injury. The increased lung IL-6 in CS-exposed LPS-injured mice indicates that this potent cytokine, previously shown to predict mortality in patients with ARDS, may play a role in exacerbating lung injury in smokers and may have utility as a biomarker of tobacco-related lung injury.

Implications: Our results suggest that short-term CS exposure at levels that cause no overt lung injury may still prime the lung for acute inflammatory damage from a "second hit", a finding that mirrors the increased risk of developing ARDS in patients who smoke. This model may be useful for evaluating the acute pulmonary toxicity of existing and/or novel tobacco products and identifying biomarkers of tobacco-related lung injury.

Introduction

Acute Respiratory Distress Syndrome (ARDS) remains a major cause of mortality and morbidity, affecting nearly 200 000 patients each year in this country alone.[1] Chronic exposure to cigarette smoke (CS) has long been known to cause cardiovascular disease, malignancy and COPD. However, there is a growing awareness of the substantial risks of both active and passive CS exposure on acute pulmonary disease. During the last 5 years, CS has been associated with an increased risk of ARDS following blunt trauma,[2] blood product transfusion[3] and in non-pulmonary sepsis.[4] Lungs derived from cigarette smokers are more likely to result in primary graft dysfunction when transplanted,[5] and when studied ex vivo, lungs from heavy cigarette smokers have been found to have impaired alveolar fluid clearance and increased edema.[6]

Animal models of pulmonary toxicity from CS have usually focused on prolonged (>6 month) exposures, which recapitulate many of the inflammatory and destructive airway and alveolar changes seen in patients with COPD.[7] Notably, even short term exposure to CS in patients has been shown to alter platelet and endothelial function,[8] induce major changes in the kinetics of neutrophil trafficking through the pulmonary vasculature,[9] and increase epithelial permeability.[10] In mice, even a single 6 hour exposure to CS has been reported to modestly increase endothelial permeability and lung edema.[11] However, there is a lack of direct experimental evidence regarding the effects of CS exposure in animal models of acute lung injury. Furthermore, improved models are needed for evaluating the acute pulmonary toxicity of tobacco products, which have been under-appreciated in economic models of the public health impact of CS exposure.[12]

Our research group recently reported that healthy cigarette smokers compared to nonsmokers at baseline had equivalent levels of bronchoalveolar lavage protein (marker of alveolar-capillary permeability) and neutrophils, yet showed an exaggerated inflammatory response to nebulized endotoxin.[13] Here we developed a mouse model of moderate short-term CS exposure and measured gravimetric lung water and post-mortem indices of lung injury following administration of endotoxin. We hypothesized that CS-exposed mice would be more susceptible to endotoxin-induced lung injury, and also tested plasma and lung homogenate for biomarkers that might identify tobacco-related lung injury, a specific aim of this work funded by the FDA/NIH.

Materials and Methods

Mice

Adult female C57BL/6 mice were ordered from NCI (Frederick, MD), housed in pathogen-free housing and cared for in accord with NIH guidelines by the Laboratory Animal Resource Center of the University of California, San Francisco (UCSF). All experiments were conducted under protocols approved by the UCSF Institutional Animal Care and Use Committee. Group size was determined to ensure adequate statistical power based on our extensive experience with the endotoxin model and measures of lung injury.[14,15]

Smoke Exposure

Mice were exposed to smoke generated by a Teague TE-10 smoking machine using 3R4F cigarettes.[16] The 3R4F Kentucky research cigarettes have previously been analyzed for lipopolysaccharide (LPS) content[17] and found to be in the middle of the range of eleven types of commercially available cigarettes at 9 pmol/mg (range 7–16 pmol/mg). Following 5 days acclimation to increasing smoke concentrations of 20, 40, 60, 80, and 100 mg/m^3 total suspended particulates (TSP) for 2 hours a day, mice underwent 12 days of exposure to 100 mg/m^3 for 5 hours a day, with rest on weekends. Control mice were housed in the same room within the barrier facility but not exposed to smoke.

Endotoxin Administration

Lipopolysaccharide (LPS, Sigma) was administered at different concentrations in 30 µL of PBS by direct tracheal installation under ketamine and xylazine anesthesia, 24 hours following the last CS exposure. Mice were recovered in supplemental oxygen.

Lung Injury Endpoints

For gravimetric measurements, mice were euthanized 24 hours following intratracheal LPS or saline. Right ventricular puncture was performed and blood was collected into EDTA tubes. The lungs were removed and homogenized in 1 mL water. Samples of blood, lung homogenate, and homogenate supernatant were weighed before and after desiccation, and another fraction of homogenate was assayed for hemoglobin concentration, such that the blood volume of the lung could be accounted for.[18] As in prior work, excess extravascular lung water was calculated. In other animals, bronchoalveolar lavage (BAL) was accomplished by tracheal cannulation and lavage with 1 mL PBS. BAL cell count was performed with a Coulter counter, and systemic hemoglobin, hematocrit, and platelet count were measured with a Hemavet 950 cell counter (Drew Scientific Inc., Waterbury, CT). Cytospin preparations of BAL fluid were made and stained with Hema 3 solution (Thermo Fisher Scientific, Waltham, MA), and 200 cells/mouse were analyzed at 100X magnification and classified as neutrophils, lymphocytes, or monocyte/macrophages. BAL protein was measured with the BCA Protein Assay (Thermo Fisher Scientific). For histology, lungs were fixed by intratracheal installation of 1 mL 4% paraformaldehyde followed by overnight fixation, dehydration, paraffin embedding, and staining of 4 µm sections with hematoxylin and eosin. Forty randomly positioned fields were scored in 4 µm sections of left and right lungs at 60x for neutrophil count and septal thickening by an observer blinded to experimental condition. Pulse oximetry was measured using the MouseOx+ cervical collar system (Starr Life Sciences). Mice were monitored for 5 minutes per time point and the mean SpO2 was calculated.

Measurement of Cytokines

Plasma cytokines were measured by Luminex (Mouse Magnetic 20-Plex, ThermoFisher Scientific). In addition, IL-6, KC, CXCL9, and RAGE (receptor for advanced glycation end-products) were measured by Quantikine (R&D Systems) ELISA.

Statistical Analysis

Comparisons between two groups were done with unpaired *t* test or Wilcoxon rank sum (when data were not normally distributed). $p < .05$ was considered to be statistically significant. Comparisons of more than two groups were made with ANOVA or Kruskal Wallis. All statistical analyses were performed with Stata-13 (StataCorp, College Station, TX).

Results

In order to model ARDS in mice, we administered increasing doses of LPS by the intratracheal route. LPS was associated with

a dose-dependent increase in excess extravascular lung water (Supplementary Figure A). Similarly, increasing doses of LPS were associated with increased blood hemoglobin concentration, suggesting that LPS delivered to the lung induced an increase in systemic endothelial permeability. Notably, LPS administered intravenously[19] or intraperitoneally[20] has previously been reported to cause acute elevations in the concentration of blood hemoglobin. Both blood leukocyte count and platelet count declined with increasing doses of LPS, consistent with prior reports of intravenous and intratracheal LPS administration.[21–23] Systemic white blood cell count and platelet count were inversely correlated with excess lung water over the range of LPS doses tested, consistent with an important role for neutrophil and platelet sequestration in LPS-induced lung injury (Supplementary Figure A).

To model a modest CS exposure, mice underwent habituation for 5 days with 2 hour sessions of increasing CS concentration, followed by 12 days of CS (Supplementary Figure B). The day following the last CS exposure, mice underwent intratracheal installation of either saline control or LPS at 20 mg/kg. CS exposed mice gained weight similar to air exposed controls and had no evidence of toxicity such as impaired eating or drinking behavior, indicating that the CS was well-tolerated. CS exposed mice treated with saline control had higher levels of systemic hemoglobin, consistent with multiple prior reports of polycythemia in healthy smokers.[24,25] At 24 hours following intratracheal LPS, both air and CS-exposed mice exhibited an acute increase in systemic hemoglobin concentration consistent with hemoconcentration, an effect that was greater in CS-exposed mice (Figure 1). Similarly, LPS reduced systemic platelet and white blood cell counts (Supplementary Figure B), effects that were exaggerated in smoke-exposed mice. Together, the data suggest that CS exposed mice are more susceptible to LPS-induced increases in systemic endothelial permeability, possibly in part related to acute decreases in blood platelets and neutrophils.

CS exposed mice had a greater increase in pulmonary edema as measured by excess extravascular lung water (Figure 2). The concentration of protein in the bronchoalveolar lavage, an indicator of alveolar capillary barrier permeability, was not increased by CS alone, but was increased by LPS to a similar degree in CS-exposed and control mice (Supplementary Figure C). LPS induced BAL neutrophilia, with a higher increase in CS-exposed mice. Notably, this modest CS exposure alone did not appear to recruit neutrophils into the alveolar compartment. Lymphocytes were rarely detected in all groups (<2% of analyzed cells, data not shown). Oxygen saturation, measured in freely moving mice, trended lower in LPS-treated mice that had previously been exposed to CS in parallel with the greater degree of pulmonary edema (Supplementary Figure C), although this difference did not reach statistical significance. Histological analysis (Figure 3) revealed that smoke exposure alone resulted in no measurable increase in tissue neutrophils or septal thickening. LPS induced an increase in lung neutrophils and alveolar septal thickening, with a significantly greater increase in CS-exposed mice (Supplementary Figure D), consistent with the exaggerated BAL neutrophilia and lung edema.

Because one of the aims of this work was to identify potential biomarkers for susceptibility to lung injury in patients previously exposed to CS, we performed multiplex analysis of 20 inflammatory biomarkers in mouse plasma. As shown in the Supplementary Table, most analytes were undetectable or detected at very low levels in all groups. MCP-1, a potent mononuclear cell attractant, was mildly elevated by LPS but was not significantly affected by CS exposure (Supplementary Figure E1). In contrast, CXCL9, a T-cell chemoattractant, was increased by LPS to a higher degree in CS-exposed mice following LPS instillation (Supplementary Table and Figure 4). This result was confirmed with ELISA which showed high concordance with the multiplex results (Supplementary Figure E2–3).

Plasma KC, the murine homologue to IL-8 and a potent neutrophil chemoattractant, was also elevated following exposure to LPS (Supplementary Table, Supplementary Figure E4). Given (1) the insensitivity of the multiplex for KC (limit of detection 80 pg/ml), (2) the well-established importance of KC in neutrophil chemotaxis, and (3) the observed BAL and tissue neutrophilia in CS-exposed mice following LPS injury, we performed ELISA analysis of KC in plasma, lung homogenate, and BAL. As shown in Supplementary Figure E, ELISA confirmed that plasma KC was elevated following LPS injury but not differentially with regard to prior CS exposure. Interestingly, lung homogenate KC trended lower in CS exposed mice and BAL KC was significantly lower following CS exposure, suggesting simple chemotactic KC gradients do not explain the increased BAL and tissue neutrophilia observed in CS exposed LPS injured mice.

Elevated levels of IL-6 in plasma[26,27] and in lung[28] have been shown to predict poor outcomes in patients with ARDS. Plasma levels of IL-6 1 day following intratracheal LPS were minimally elevated in the multiplex luminex analysis (Supplementary Table). Therefore we measured IL-6 in lung homogenate. As shown in Figure 4, lung IL-6 was significantly higher in LPS-injured mice previously exposed to CS.

Finally, in order to test whether the increase in lung water in CS-exposed LPS injured mice was associated with increased alveolar

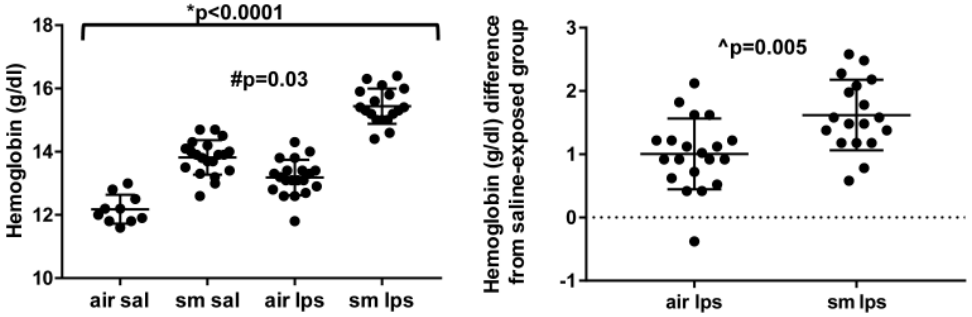

Figure 1. Systemic hemoglobin was increased by cigarette smoking (CS) exposure (air sal vs. sm sal), an effect previously described in healthy smokers. Lipopolysaccharide (LPS)-injury increased hemoglobin concentration acutely and to a greater degree in CS exposed mice as demonstrated by a significant statistical interaction (left panel) and by the difference between the hemoglobin of air or CS-exposed LPS-injured mice and the means of the corresponding saline-exposed control groups (right panel). *by ANOVA, # by interaction, ^by Wilcoxon rank-sum.

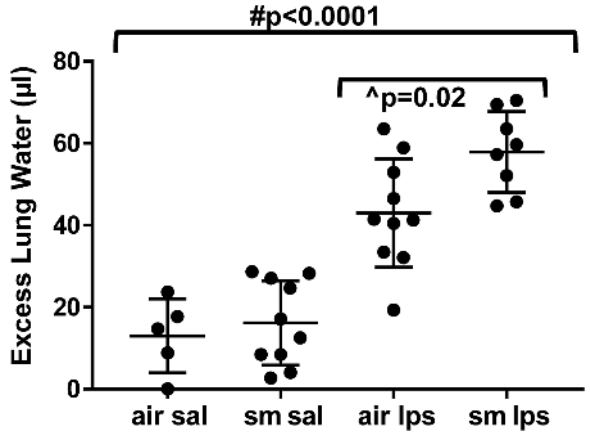

Figure 2. Smoke exposure alone caused no lung injury as assessed by extravascular lung water, yet lipopolysaccharide (LPS) induced significantly more lung injury in smoke-exposed mice. #by Kruskal Wallis, ^by Wilcoxon rank-sum.

epithelial injury, we measured the plasma concentration of RAGE, a protein highly expressed in alveolar type I cells, released into the blood in the presence of lung injury,[29,30] and associated with impaired active epithelial fluid transport in patients with ARDS and in mouse models.[31,32] As shown in Supplementary Figure E8, plasma RAGE was higher in air-exposed LPS challenged mice than in CS exposed LPS challenged mice, suggesting that the magnitude of alveolar epithelial injury was *lower* in CS-exposed mice despite the increased level of pulmonary edema.

Discussion

Cigarette smoke exposure increases the risk of ARDS in patients following a number of insults.[33] Previous studies of CS exposure in mice have mostly focused on prolonged heavy exposures that produce major structural lung changes.[7] However, even passive CS exposure in patients increases the risk of ARDS following trauma.[2] Thus the first objective was to determine whether a more modest CS exposure in mice predisposed to increased inflammatory lung injury in response

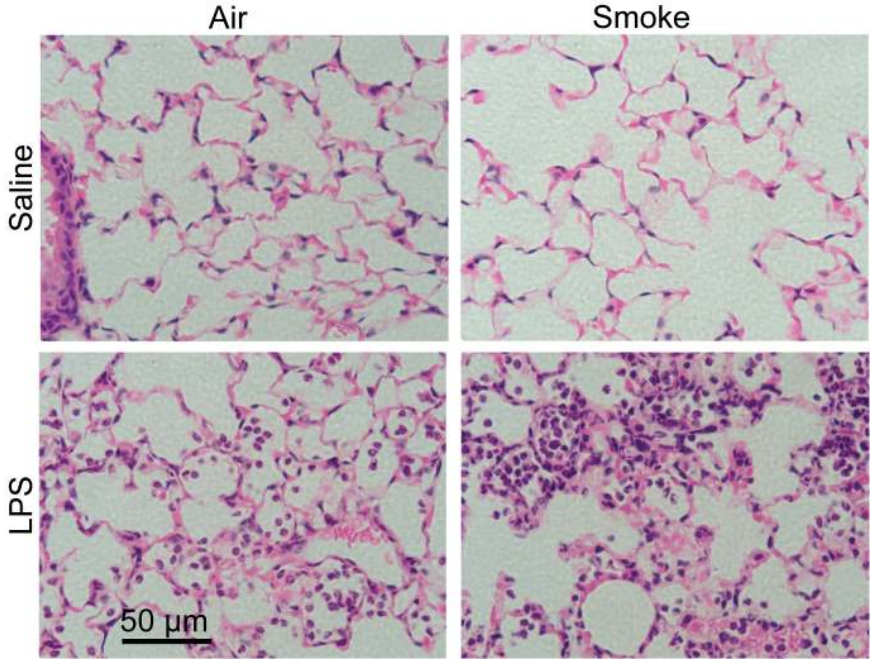

Figure 3. Representative photomicrographs of 4 µm hematoxlyin-eosin stained lung sections 24 hours after LPS or saline instillation. Smoke-exposed and air-exposed mice receiving saline showed no significant inflammation. LPS installation increased septal thickening and cellular inflammation to a greater extent in smoke-exposed mice.

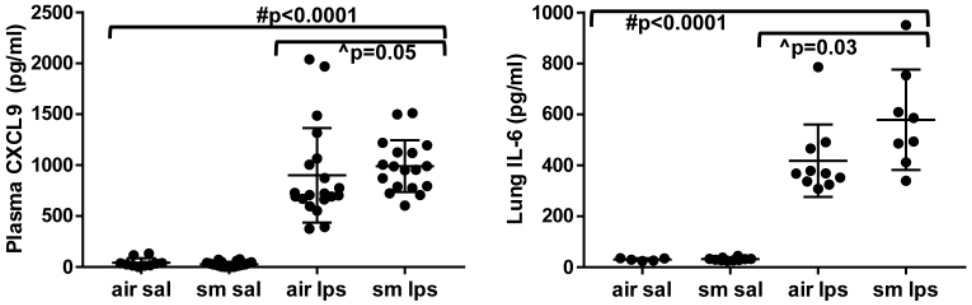

Figure 4. Levels of Interleukin-6 and the T-cell chemoattractant CXCL9 were not affected by smoke alone and were increased by lipopolysaccharide (LPS) to a greater degree in smoke-exposed mice. #by Kruskal Wallis, ^by Wilcoxon rank-sum.

to tracheal instillation of LPS, a model of gram negative bacterial lung injury. The second objective was to identify potential biomarkers of tobacco-related lung injury. The main findings can be summarized as follows: (1) three weeks of modest CS exposure resulted in more pulmonary edema and an increase in neutrophilic associated-lung injury following LPS, and (2) CS exposure also resulted in elevated levels of IL-6 and CXCL-9, potential biomarkers of tobacco-related lung injury.

In a recent study from our research group, healthy smokers and non-smokers underwent BAL 6 hours after inhaling nebulized LPS. Smokers had evidence of increased alveolar-capillary barrier permeability to protein in association with higher BAL neutrophil counts and higher plasma concentrations of the inflammatory biomarkers IL-8 and MMP-8.[13] Here, we report that CS-exposed LPS-treated mice have increased neutrophilic inflammation in BAL and lung parenchyma and an increase in the quantity of pulmonary edema as measured by extravascular lung water.

BAL protein, though increased following LPS administration, was not higher in CS-exposed mice. How can this result be explained when extravascular lung water was significantly elevated in the cigarette-smoke exposed mice injured with LPS? Extravascular lung water may be located in the interstitial or alveolar spaces, as edema fluid crosses first the endothelial barrier and accumulates in the interstitial space and then may cross the epithelial barrier and accumulate in the alveolar space. Prior work by our laboratory using radiolabeled albumin has shown that intratracheal LPS given 18 hours after a smaller "priming" dose of LPS in mice resulted in a substantial increase in endothelial barrier permeability but no change in the epithelial barrier permeability to protein,[14] a finding that was consistent with prior studies we carried out with alveolar and intravenous endotoxin in sheep at 4 and 24 hours.[34] In the current studies, it is likely that the increase in pulmonary edema in CS-exposed mice was mostly confined to the lung interstitial space because (1) arterial hypoxemia was mild in both groups, consistent with a limited quantity of alveolar edema, (2) there was histological evidence of more interstitial lung edema based on the significantly thicker alveolar septae, and (3) both groups had only modestly elevated levels of BAL protein, indicating that that the epithelial barrier remained largely intact, thus limiting the extravasation of high protein pulmonary edema fluid into the airspaces. Two additional lines of evidence suggest that the endothelial barrier (rather than epithelial barrier) was a major site of CS-enhanced inflammatory injury: (1) LPS-induced hemoconcentration was significantly greater in the CS-exposed mice, and (2) plasma RAGE, a well-established marker of alveolar epithelial injury[29–31] was significantly *lower* in the CS-exposed (as compared to air-exposed) LPS-injured mice.

Lu and colleagues have previously reported that whole body CS exposure increases susceptibility to LPS-induced lung injury in mice, primarily mediated by oxidative stress-mediated inhibition of RhoA and focal adhesion kinase.[11] Notably, the single 6 hour CS exposure employed in that study significantly increased lung edema (measured by wet to dry ratio) and BAL inflammation even in the absence of LPS. Interestingly, that study also showed that cultured lung endothelial monolayers exposed to CS extract had an exaggerated increase in permeability following exposure to LPS. Importantly, acute systemic endothelial dysfunction has been well documented in humans following limited exposure to CS.[8] In the context of these studies, our results suggest that relatively brief exposure to CS predisposes both lung and systemic endothelium to greater barrier permeability following a second inflammatory stimulus.

Lung levels of the inflammatory cytokine IL-6 discriminate ARDS from cardiogenic pulmonary edema,[35] and elevated lung and plasma IL-6 have been shown to predict poor outcomes in patients with ARDS.[26–28] Therefore, we measured IL-6 in lung homogenate, finding higher levels in CS-exposed LPS-treated mice. To our knowledge, this is the first report that lung IL-6 is associated with more severe inflammatory lung injury following CS exposure. Although the mechanistic contributions of IL-6 in tissue injury are complex,[36] lung levels of IL-6 have previously been correlated with neutrophil activation in ARDS[37] and IL-6 can directly stimulate neutrophil degranulation.[38] Thus IL-6 may play a key role in exacerbating lung injury in patients previously exposed to cigarette smoke.

Given the lung and BAL neutrophilia observed in CS exposed LPS injured mice, we also measured plasma and lung levels of KC, the murine homologue of the potent neutrophil chemoattractant IL-8. Plasma levels did not differ between CS and air exposure. Surprisingly, CS-exposed mice had lower levels of BAL KC despite more pronounced neutrophilia, suggesting the importance of alternative mechanisms of neutrophil recruitment. One possibility is that low levels of endotoxin or other components of CS "prime" the endothelium to be more responsive to a subsequent insult, a phenomenon previously reported with repeated exposures to LPS by our group[14] and others.[39,40]

In testing for novel biomarkers of tobacco-related lung injury that might be measured in the plasma of critically ill patients, we carried out Luminex analysis of murine plasma focusing on a panel of inflammatory cytokines. Although most of the assayed biomarkers were at undetectable or very low concentration in all groups, CXCL9 was increased substantially following LPS exposure, and was higher in CS-exposed mice. CXCL9 is a potent chemokine made by macrophages and fibroblasts that binds CXCR3 predominantly on T-cells (though also reported to be expressed on epithelial, endothelial, and smooth muscle cells).[41] CXCR3 ligands including CXCL9 have been associated with diffuse alveolar damage (the pathological finding in ARDS) following lung transplant,[42] though to our knowledge have not yet been assessed in more common types of ARDS. Interestingly, CXCL9 has been found to be elevated in mouse lung parenchyma following acute CS exposure,[43] and in the serum[44] and sputum of patients with COPD.[45] Furthermore, CXCR3 knockout mice have reduced numbers of BAL neutrophils and less lung injury following an intense 3 day CS exposure.[46] Taken together, these findings suggest that CXCL9 may also have potential utility as a biomarker of tobacco-related lung injury.

In conclusion we report here that 3 weeks of moderate whole body CS exposure in mice predisposes to more pulmonary edema and inflammatory lung injury following intratracheal LPS, in association with evidence of increased pulmonary and systemic endothelial permeability. These results provide experimental support for the clinical data that patients exposed to CS are at greater risk for developing ARDS, probably in part because of a greater increase in lung endothelial permeability in the presence of a clinically relevant insult. This model may be useful for evaluating the acute pulmonary toxicity of novel tobacco products, and for developing biomarkers of tobacco-related lung injury. Future work seeks to extend these findings using live bacterial and viral pathogens following CS exposure of varied intensity and nicotine concentration.

Supplementary Material

Supplementary data are available at *Nicotine & Tobacco Research* online.

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Declaration of Interests

None declared.

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