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Cigarette smoke exposure worsens acute lung injury in antibiotic-treated bacterial pneumonia in mice

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Abstract

Evidence is accumulating that exposure to cigarette smoke (CS) increases the risk of developing Acute Respiratory Distress Syndrome (ARDS). *S. pneumoniae* is the most common cause of bacterial pneumonia, which in turn is the leading cause of ARDS. Chronic smokers have increased rates of pneumococcal colonization and develop more severe pneumococcal pneumonia than nonsmokers, yet mechanistic connections between CS exposure, bacterial pneumonia, and ARDS pathogenesis remain relatively unexplored. We exposed mice to 3 weeks of moderate whole-body CS or air, followed by intranasal inoculation with an invasive serotype of *S. pneumoniae*. CS exposure alone caused no detectable lung injury or BAL inflammation. During pneumococcal infection, CS-exposed mice had greater survival than air-exposed mice, in association with reduced systemic spread of bacteria from the lungs. However, when mice were treated with antibiotics after infection to improve clinical relevance, the survival benefit was lost, and CS-exposed mice had more pulmonary edema, increased numbers of BAL monocytes, and elevated monocyte and lymphocyte chemokines. CS-exposed antibiotic treated mice also had higher serum surfactant protein D and angiopoietin-2, consistent with more severe lung epithelial and endothelial injury. The results indicate that acute CS exposure enhances the recruitment of immune cells to the lung during bacterial pneumonia, an effect that may provide microbiologic benefit but simultaneously exposes the mice to more severe inflammatory lung injury. The inclusion of antibiotic treatment in pre-clinical studies of acute lung injury in bacterial pneumonia may enhance clinical relevance, particularly for future studies of current or emerging tobacco products.

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Keywords:

Pneumococcus

Acute lung injury

ARDS

Cigarette smoke

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Introduction

Acute Respiratory Distress Syndrome (ARDS) affects nearly 200,000 patients each year with high associated morbidity and mortality (64). While chronic exposure to cigarette smoke (CS) is a well-established causal factor in COPD and malignancy, there is increasing evidence of the substantial risks of both active and passive CS exposure on acute pulmonary disease, including ARDS. CS has now been associated with an increased risk of ARDS in the setting of trauma, transfusion, and non-pulmonary sepsis (10, 11, 75), as well as with primary graft dysfunction (pulmonary edema) after lung transplantation (18). In addition, lungs from cigarette smokers that were studied *ex vivo* have increased edema and reduced alveolar fluid clearance (84). Similarly, our research group recently reported that healthy human smokers exposed to nebulized lipopolysaccharide (LPS) have increased inflammatory cytokines and protein in bronchoalveolar lavage compared to non-smokers (48).

The most common etiology of ARDS is pneumonia, and the most frequent responsible pathogen in bacterial pneumonia is *Streptococcus pneumoniae* (53). CS exposure increases the incidence of pneumococcal pneumonia in patients (80) and the risk that it will be complicated by septic shock (25) and mortality (49). However, CS has also been shown to increase the risk of pneumococcal nasopharyngeal colonization (8, 13), and chronic CS exposure leading to COPD or emphysema reduces overall health and structural lung defenses against infection. Thus, it remains unclear how CS exposures limited in length and intensity may affect the natural history of pneumococcal pneumonia and ARDS.

Long-term (exceeding 6 months) heavy CS exposure in mice causes robust inflammation involving both innate and adaptive immune cells and produces alveolar destruction reminiscent of emphysema (38). Much less is known about how shorter-term exposures to more moderate levels of CS affect the severity of lung injury in response to acute infectious insults.

Given the rapidly evolving landscape of tobacco products, including e-cigarettes (14), there is a compelling need to develop improved models for testing the impact of both established and novel tobacco products on acute pulmonary complications, including ARDS. We recently reported that intra-tracheal LPS caused more severe neutrophilic lung injury in CS exposed mice compared to controls without CS exposure (29), analogous to our studies in human volunteers (48). Studies of CS exposure and pneumococcal infection in mice have yielded mixed results, with some researchers reporting that smoke exposure increases illness severity (70) and others reporting the opposite (5). Notably, these discrepant results may have reflected differences in the intensity of CS exposure or strain differences in response to CS itself (90). Importantly, mouse and rat models of cigarette smoke exposure followed by challenge with live bacterial pathogens have lacked antibiotic treatment (5, 7, 19, 26, 27, 36, 42, 52, 59, 70, 73, 79, 81), a cornerstone of the care of patients presenting to medical care with suspected infection (31).

For these studies, our initial objective was to test the effect of a limited and well-tolerated CS exposure on lung injury and mortality in mice during pneumococcal lung infection. We hypothesized that CS-exposed mice would have more severe lung injury and a higher mortality from pneumococcal pneumonia. Contrary to our hypothesis, CS exposed mice had improved survival, primarily related to a reduction in the extra-pulmonary dissemination of bacteria from the lungs. Therefore, our second objective was to test the effect of CS in antibiotic-treated mice with pneumococcal pneumonia, reasoning that there was more clinical relevance to include antibiotic therapy in these experiments, particularly since there is an increased emphasis on identifying patients at risk for developing ARDS when they present with pneumonia in the emergency department (45). A final objective of this work was to use our refined model to identify biomarkers that may be useful in evaluating the acute pulmonary toxicity of novel tobacco products.

Materials and Methods

Animals. Adult (8-10 week old) 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 models of acute lung injury (21, 29, 39).

Smoke exposure. Mice were exposed to smoke generated by a Teague TE-10 smoking machine using 3R4F cigarettes (47). The lipopolysaccharide (LPS) content of 3R4F Kentucky research cigarettes is in the middle of the range of 11 types of commercially available cigarettes tested at 9 pmol/mg (37). Following 5 days of 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. This CS exposure was designed to model the recent initiation of active smoking. In some experiments, mice were exposed for 2 hours daily to a lower CS concentration meant to mimic second hand smoke exposure, TSP 3 mg/m^3. This lower CS concentration was achieved by mixing concentrated sidestream smoke and fresh air into an aging chamber using an adjustable air amplifier and continuous monitoring of suspended particulate matter with a Sidepack AM 510 aerosol monitor (TSI incorporated, Shoreview MN). For context, a smoke-filled bar may reach TSP 1-2 mg/m^3 (71) while mouse models of CS exposure have used levels as high as 980 mg/m^3 (27).

Bacterial infection, antibiotic administration, and microbiology. Streptococcus pneumoniae serotype 19F (ATCC® 49619, Manassas, VA), was grown in brain-heart broth (Becton Dickinson 237500, Sparks, MD) and harvested at mid-log phase, spun down and re-

suspended in PBS at different dilutions. Mice were anesthetized deeply with isoflurane and inoculated intranasally with 50 µl of bacteria. In some experiments, ceftriaxone (150 mg/kg, i.p.) was administered every 12 hours beginning 12 hours after inoculation. This dose was selected based on known pharmacokinetics and proven efficacy in a mouse model of pneumococcal pneumonia (68). In other experiments, *S. pneumoniae* was delivered by intraperitoneal injection. Bacterial titers of BAL, blood, and spleen minced in 5 ml PBS were measured by serial dilution and plaque counting on sheep blood agar plates.

Oxygenation measurements during the experiments. Pulse oximetry was measured using the MouseOx+ cervical collar system (Starr Life Sciences), as we have done in prior studies (29). The mean SpO2 during five minutes of recording was calculated for each time point.

Lung injury endpoints. Mice underwent overdose of ketamine and xylazine, bilateral thoracotomy, and exsanguination by right ventricular puncture. The lungs were removed and homogenized in 1 ml PBS, and samples of blood, lung homogenate, and homogenate supernatant were weighed before and after desiccation. Systemic hemoglobin and hematocrit were measured with a Hemavet 950 cell counter (Drew Scientific Inc., Waterbury, CT). Another fraction of homogenate was assayed for hemoglobin concentration and the blood volume of the lung was calculated, permitting assessment of the excess extravascular lung water (i.e., pulmonary edema in the interstitial and air spaces above the level in normal mice of the same size) as in prior work (30, 72). In other animals, after exsanguination the trachea was cannulated and the lungs were lavaged twice with 250 µl of PBS. BAL cell count was measured with a Coulter counter, cytospin preparations of BAL fluid were made and stained with Hema 3 solution (Thermo Fisher Scientific, Waltham, MA), and 400 cells/mouse were analyzed at 100X magnification and classified as neutrophils, lymphocytes, or monocytic cells. 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.

Measurement of protein biomarkers of inflammation and lung injury. Cytokines were measured by Luminex using a 20-plex kit (Mouse Magnetic 20-Plex, ThermoFisher Scientific) and a custom multiplex kit from R&D (CCL7, CXCL12, ICAM-1, MMP-8, MMP-9, and TNF R1). In addition, biomarkers of lung endothelial and alveolar epithelial injury were measured with this same kit (Ang-2 and SP-D).

Statistical analyses. Comparisons between two groups were done with unpaired t-test or Mann-Whitney U-test (when data were not normally distributed). Comparisons of more than two groups were made with ANOVA or Kruskal Wallis. Repeated measures ANOVA was used for comparisons of multiple groups over more than one time point, and two way interaction terms were created for treatment group and time. Spearman or Pearson correlations were used depending on the normality of data distribution. Log-rank was used for survival analysis. $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed with Stata (StataCorp, College Station, TX) and graphs were produced in Prism (GraphPad, La Jolla, CA).

Results

S. pneumoniae produced dose-dependent lung injury. Mice underwent intranasal inoculation with between 1 x 10^7 and 1 x 10^8 colony forming units (cfu) of an invasive serotype of pneumococcus (19F), producing dose-dependent weight loss (**Fig. 1A**), hypothermia (**Fig. 1B**), arterial hypoxemia (**Fig. 1C**) and pulmonary edema as measured by excess extravascular lung water (**Fig. 1D**). A dose of 1x10^8 cfu resulted in approximately 60% mortality and severe lobar pneumonia in surviving mice, in contrast to 3x10^7 cfu which resulted in patchy pneumonia and no mortality (**Fig. 1E**). Doses of 2 x 10^8 cfu or greater were associated with severe hypothermia and death within 12-24 hours (data not shown).

Brief, mild cigarette smoke exposure did not affect pneumococcal lung injury. Mice were exposed for 2 days to 2 hours per day of sidestream cigarette smoke (CS) at 3 mg/m^3 total suspended particulate (TSP) to model second-hand smoke exposure (**Fig. 2A**). Following CS exposure, mice were inoculated with 5 x10^7 cfu *S. pneumoniae*. No difference was observed in weight loss (**Fig. 2B**), hypothermia (Fig. 2C), arterial oxygen saturation (**Fig. 2D**), or excess extravascular lung water (**Fig. 2E**).

More intense CS exposure improved survival during severe pneumococcal pneumonia. In order to model the recent initiation of active smoking, mice were exposed to 2.5 weeks of CS at 100 mg/m^3 TSP (**Fig. 3A**), an exposure we have previously demonstrated produces no significant inflammation as assessed by histology, BAL cellularity, or elevation in inflammatory cytokines (29). The day following the last CS exposure mice underwent inoculation with 1x10^8 cfu *S. pneumoniae*. We selected a higher bacterial inoculum here (than in **Fig. 2**) in order to model more severe pneumonia. Unexpectedly, CS-exposed mice had a significant survival benefit (**Fig. 3B**). The improved survival in the CS-exposed mice was associated with more

196 weight loss (**Fig. 3C**), less hypothermia (**Fig. 3D**), and a similar degree of arterial hypoxemia
197 (**Fig. 3E**), peripheral leukopenia (**Fig. 3F**), and pulmonary edema (**Fig. 3G**) in surviving mice.

198 *The survival benefit of CS exposure did not extend to severe non-pulmonary*
199 *pneumococcal infection.* In order to determine whether CS conferred a general protective effect
200 against severe pneumococcal infection, we developed an intraperitoneal (i.p.) inoculation
201 model. Although primary pneumococcal peritonitis is not nearly as common as pneumonia, it
202 represents approximately 1% of invasive pneumococcal disease (82). Mice were injected i.p.
203 with increasing doses of *S. pneumoniae*, with 50% survival obtained with 1×10^8 cfu (**Fig. 4A**).
204 Notably, mice either succumbed to this infection or rapidly recovered by 48 hours. In the next
205 set of experiments, we exposed mice to 2.5 weeks of CS or air (as in **Fig. 3A**), and the following
206 day, mice were inoculated with 1×10^8 cfu of *S. pneumoniae*, i.p. As shown in **Fig. 4B**, both air
207 and CS-exposed mice had high mortality with minimal lung injury in surviving mice (**Fig. 4C**).
208 Although measurement of pulmonary edema could not be accomplished in mice that had died,
209 the gross weight of the lungs did not differ between air and CS-exposure, suggesting a similar
210 degree of mild lung injury in both groups in this model of rapidly lethal pneumococcal peritonitis
211 (**Fig. 4D**).

212 *CS exposure reduces the systemic spread of infection during severe pneumococcal*
213 *pneumonia.* To determine whether the survival effect of CS in the pneumonia model was related
214 to the severity of lung injury, we repeated the experiment with the moderate smoke exposure
215 and intranasal pneumococcal inoculation (**Fig. 5A**) focusing on the 24-hour time point before the
216 survival curves separated. As shown in **Fig. 5B**, there was a significant improvement in
217 hypothermia in CS-exposed mice. Interestingly, arterial hypoxemia was significantly worse in
218 CS-exposed mice, opposite the survival benefit (**Fig. 5C**). However, BAL protein (**Fig. 5D**) and
219 lung water (**Fig. 5E**) did not differ significantly with CS exposure, indicating that the difference in
220 hypoxemia might be related to other factors such as differences in ventilation-perfusion

matching. Notably, the effect of the modest group temperature difference on oxygen-hemoglobin interactions is likely to be insignificant (61).

Because mice respond to overwhelming infection with hypothermia rather than fever (62), we suspected the survival difference might be due to a difference in systemic infection and therefore we measured bacterial loads in the blood (**Fig. 5F**) and spleen (**Fig. 5G**) at 24 hours. CS exposed mice had reduced systemic bacterial burden in pneumococcal pneumonia by several orders of magnitude. Notably, body temperature at 24 hours was inversely correlated with systemic bacterial load (log of blood cfu), Pearson r = -0.78, p=0.0004. To determine whether differences in systemic bacterial burden were due to a CS-induced reduction in airspace bacteria, we performed an additional experiment, identical to the protocol depicted in **Fig. 5A** except with a sacrifice time of 16 rather than 24 hours post-infection. As shown in **Fig. 6A**, CS-exposed mice again had significantly higher body temperature than air-exposed mice at this earlier time point. However, BAL bacterial loads were not different with regard to prior smoke exposure (**Fig. 6B**), indicating that both groups of mice had very high levels of pneumococcal airspace burden early after infection. Interestingly, BAL myeloperoxidase activity was significantly higher in CS-exposed mice (**Fig. 6C**), consistent with a more vigorous innate immune response within the airspaces.

A model of severe pneumococcal pneumonia treated with antibiotics. Because patients presenting with pneumonia and sepsis are uniformly treated with potent anti-pneumococcal antibiotics, we decided to enhance the clinical relevance of this model by treating infected mice with ceftriaxone, a third-generation cephalosporin with favorable pharmacokinetics and potent anti-pneumococcal activity. In preliminary experiments, we observed that a delay of 4 hours between infection and the first dose of antibiotics resulted in minimal lung injury and 100% survival, while a delay of more than 24 hours frequently resulted in severe and progressive lung injury and high mortality (data not shown). Therefore, we selected a ceftriaxone dose of 150

mg/kg and dosing frequency of 12 hours based on prior work in mice showing a favorable pharmacokinetic profile and efficacy against several strains of the pneumococcus (68). Mice were inoculated with $1x10^8$ *S. pneumonia* and treated with ceftriaxone beginning 12 hours post-infection for 3 doses as shown in **Fig. 7A**. Treated mice had more weight loss (**Fig. 7B**) and a significant improvement in hypothermia (**Fig. 7C**). Thus (as in **Fig. 3C**) weight loss and hypothermia, commonly assessed clinical parameters seemed discordant as regards the health of the animals. We therefore tested whether these parameters might be related in a counterintuitive manner. Interestingly, across both antibiotic treated and untreated mice, temperature was directly correlated with weight loss (% change from baseline, Spearman r = 0.68, p = 0.007), consistent with hypothermia reducing activity and/or metabolic rate. By 48 hours post-infection, antibiotic-treated mice had greatly reduced bacterial burden in BAL (**Fig 7D**) and reduced myeloperoxidase activity (**Fig. 7E**), indicating decreased degranulation of neutrophils and monocytes/macrophages. Histological analysis confirmed a major reduction in tissue neutrophils 48 hours post-infection in antibiotic-treated mice (**Fig. 7F**).

Prior moderate CS exposure increases lung injury in antibiotic-treated pneumococcal pneumonia. We next repeated the CS exposure shown earlier to have a survival benefit in untreated pneumococcal infection, this time treating all mice with ceftriaxone beginning 12 hours after bacterial inoculation (**Fig. 8A**). As shown in **Fig. 8B**, nearly all mice in both groups survived to 48 hours (25/25 CS-exposed vs. 22/25 air-exposed). CS-exposed mice had greater weight loss than air-exposed mice (**Fig. 8C**) and were less hypothermic (**Fig. 8D**). However, CS-exposed mice had more pulmonary edema as indicated by increased extravascular lung water (**Fig. 8E**). Histological analysis revealed moderate alveolar septal thickening in both groups with a shift from neutrophilic to monocytic inflammation in CS-exposed mice (**Fig. 8F-G**). Importantly, both air and CS-exposed antibiotic treated mice had *less severe* lung injury than air and CS-exposed non-antibiotic-treated mice (compare excess lung water in **Fig. 3G** and **8E**). Thus

antibiotics, rather than worsening lung injury, differentially reduced injury severity with regard to CS exposure.

Prior CS exposure changes the composition of inflammatory cells and cytokines in airspaces. At 48 hours post-infection, CS-exposed mice had a lower percentage of neutrophils and a higher percentage of monocytic cells in BAL with no change in the percentage of lymphocytes (**Fig. 9A**). Because overall BAL cell number trended higher in CS-exposed mice (mean 413 vs. 309, p = 0.29 by Mann-Whitney), the absolute numbers of neutrophils in BAL were similar in CS and air-exposed mice, while monocytic cells were significantly increased and lymphocytes trended higher relative to air-exposed mice (**Fig. 9B**). Notably, the CS exposure alone (without infection) did not result in any change in BAL cell number or composition (data not shown). We next measured the concentration of key chemokines and cytokines in BAL (**Fig. 9C**). KC (murine homologue of IL-8, a potent neutrophil chemoattractant) was detected at relatively low levels in both groups. In contrast, the monocyte chemokine MIP-1α (CCL3) and the lymphocyte chemokine CXCL9 were both significantly increased in BAL of CS-exposed mice relative to non-smoked mice (**Fig. 9C**). BAL levels of IL-6, MCP-1, MCP-2, MCP-3, and CXCL12 were consistent with a shift toward increased monocyte and lymphocyte chemokines in CS-exposed mice (**Table 1**), mirroring the cellular infiltrate in BAL and histology observed 48 hours post-infection.

Cellular mediators of lung injury. In order to determine possible mediators of lung injury, we measured lung neutrophil elastase (NE), myeloperoxidase (MPO), and granzyme B. Notably, MPO is present in both neutrophils and monocytes (3 2, 50). Because inhibitory substances in lung homogenate precluded its use in the elastase and MPO enzymatic assays, we used cell-free BAL for these experiments. Although BAL NE did not differ between CS-exposed and air-exposed mice (data not shown), BAL MPO was significantly higher in CS-exposed mice (**Fig. 10A**), similar to the non-antibiotic pneumococcal model (**Fig. 6C**).

Granzyme B is a serine protease contained in the cytotoxic granules of lymphocytes (2). As shown in **Figure 10B**, lung homogenate levels of granzyme B trended higher in CS-exposed mice. Interestingly, the concentration of granzyme B was unrelated to the extent of pulmonary edema (excess extravascular lung water) in air-exposed mice (**Fig. 10C**), but was significantly associated with the extent of pulmonary edema in CS-exposed mice (**Fig. 10D)**.

Antibiotic treatment causes major changes in BAL cytokines in CS-exposed mice. Given that the effect of CS exposure on outcomes was so different in the untreated and antibiotic-treated models of pneumococcal pneumonia, we analyzed these model differences further by comparing the BAL cytokine profile of CS-exposed mice with and without antibiotic treatment. As show in **Table 2**, antibiotic treatment in CS-exposed mice was associated with significant reductions in the potent inflammatory molecules IL-1α, IL-17, TNF-α, and IL-1β, a marker of inflammasome activation. Interestingly, the greatest differences between antibiotic treated and untreated mice were neutrophil-associated KC (70-fold higher in untreated mice), and IL-6 (9-fold higher in untreated mice). In contrast, most monocyte (excepting MIP-1α) and lymphocyte chemokines were unchanged or trended *higher* with antibiotic treatment.

CS exposure increases lung epithelial and endothelial injury. Surfactant protein D (SP-D) is a product of alveolar epithelial type II cells that is released into the circulation during lung epithelial injury (57), is increased in the blood of patients with ARDS (33), and predicts worse outcomes in patients with ARDS (20, 83). Serum SP-D has also been shown to be increased during acute lung injury in rodents induced by nebulized LPS (28), bleomycin (24, 57), and hydrochloric acid (57). As shown in **Fig. 11A**, blood levels of SP-D in antibiotic-treated pneumococcal pneumonia (including both air and CS-exposed mice) were highly correlated with the degree of pulmonary edema (Spearman $r = 0.71$, $p < 0.0001$), consistent with its established role as a biomarker of alveolar epithelial injury. SP-D was significantly elevated in mice previously exposed to CS (**Fig. 11B**). Angiopoietin-2 (Ang-2) is released by vascular

endothelium by a variety of inflammatory insults and interferes with angiopoitein-1 signaling through Tie-2, increasing vascular permeability (63). Levels of Ang-2 in the blood of patients are associated with poor outcomes in sepsis-associated lung injury (9), and have been shown to predict the development of ARDS (3). As shown in **Fig. 11C**, CS-exposed mice had significantly higher blood levels of Ang-2, consistent with increased endothelial injury and permeability. Using different methods, other investigators have reported that CS exposure increases lung endothelial injury (40). Notably, CS exposure alone did not increase either SP-D or Ang-2 in uninjured mice (data not shown).

Biomarkers of CS-associated infection-related lung injury. A major goal of these studies was to identify potential biomarkers of smoking-related lung injury to be tested in future work with blood samples collected prospectively from a cohort of critically ill patients. Therefore, we measured several cytokines and molecules with well-established roles in inflammatory tissue injury in mouse serum samples 48 hours post-infection in the antibiotic-treated pneumococcal pneumonia model. As shown in **Table 3**, matrix metalloproteinases 8 and 9 were significantly increased in CS-exposed mice, along with the lymphocyte chemokine CXCL9, and the monocyte chemokine MIP-1α.

Discussion

The main findings of these experiments can be summarized as follows. First, several weeks of cigarette smoke (CS) exposure improved survival during subsequent challenge with pneumococcal pneumonia in mice. Second, this survival benefit was likely due to reduced dissemination of bacteria from the lungs into the systemic circulation, and did not generalize to extra-pulmonary pneumococcal sepsis. Third, when antibiotic treatment was introduced into the model of acute bacterial pneumonia, the survival benefit of CS exposure was lost, and CS-exposed mice instead suffered more severe lung injury relative to air-exposed control mice, including evidence of lung endothelial and alveolar epithelial damage. Fourth, CS-exacerbated lung injury was associated with increased accumulation of alveolar monocytes and monocyte-related airspace chemokines.

CS exposure is known to increase the risk of ARDS in trauma and in non-pulmonary sepsis (10, 11). Our group previously reported that healthy human smokers (compared to non-smokers) have increased BAL protein after inhaling nebulized lipopolysaccharide (LPS), a model of gram negative pneumonia (48). Similarly, we recently reported that short-term moderate CS exposure increases lung injury in response to intratracheal LPS in mice (29). Other investigators have reported similar results with LPS after short-term CS exposure in mice (40, 67). Although well-suited to experimental models, LPS lacks many characteristics of live bacteria, and even at high doses causes only mild lung injury in mice which are naturally resistant to endotoxin (22).

To the best of our knowledge, we here report for the first time that CS exposure improves survival in a mouse model of pneumonia employing live bacteria in the absence of antibiotics. Our CS exposure of 100 mg/m^3 for approximately 3 weeks causes no obvious BAL or histological inflammation or increase in inflammatory cytokines (29), making it moderate by

comparison to studies employing exposures of 250 mg/m^3 or greater which have consistently demonstrated significant inflammation from CS itself (26, 27, 42, 52). CS-exposed mice had no difference in lung injury or airspace bacterial burden but were less hypothermic and had decreased bacteremia by several orders of magnitude. Notably CS exposure provided no protection against death from pneumococcal peritonitis. These results are consistent with moderate CS exposure inducing an enhanced, localized innate immune response in the lung to invading lung pathogens that decreases translocation into the blood.

There are at least 13 published reports in which mice and rats have been exposed to cigarette smoke followed by bacterial challenge for which detailed methods are available (5, 7, 19, 26, 27, 36, 42, 52, 59, 70, 73, 79, 81). CS exposures (TSP) in these studies have ranged between 15 mg/m^3 and 980 mg/m^3 with total exposure durations from 4 days to 9 months. Several groups have reported that prior CS exposure increases bacterial loads following challenge with intratracheal *S. pneumoniae* (42) and *P. aeruginosa* (19, 73). However, other researchers have reported that CS exposed mice had either no change (36) or decreased lung bacteria following challenge with *H. influenza* (26, 27, 52), *P. aeruginosa* (5), and *S. pneumoniae* (5). Several methodological differences have been cited to explain these different results, including intensity and duration of CS exposure, size of the bacterial inoculum, and time points and tissues examined. Mouse strain, in particular, may be especially important, with well-characterized strain differences in physiologic responses to hypoxia and hypercapnea (1), CS-induced inflammation (90), and recently reported strain-dependent susceptibility to CS priming with endotoxin-induced lung injury (67). However, no study of bacterial pneumonia and CS in rodents has employed antibiotics, to our knowledge.

We are interested in the mechanisms by which CS predisposes patients to develop ARDS during critical illness (10, 11, 75). Recognizing that the survival results we obtained in mice were highly discordant from human studies demonstrating that smokers are at increased

risk of invasive pneumococcal disease (74) and death from pneumococcal pneumonia (6), we sought to improve the clinical relevance of our model. Patients with pneumonia are uniformly treated with broad spectrum antibiotics within 1-2 hours of presenting for medical care (31). Notably, treatment of serious pneumococcal infections with effective antibiotics releases large quantities of bacterial cell wall products over a short time and has been shown to produce a wave of inflammation that can worsen organ injury (43, 76, 77). This phenomenon is well-described in patients with pneumococcal meningitis and is the basis for co-administration of antibiotics and systemic glucocorticoids. In addition, all indications from our experiments without antibiotics were that the mice were dying of systemic infection, not due to the severity of the pneumonia, making it difficult to assess the effects of CS exposure on the degree of acute lung injury, which was our primary objective.

In our work developing the antibiotic-treated model of pneumococcal pneumonia, we found that 3 doses of ceftriaxone beginning 12 hours after infection nearly sterilized the airspaces by 48 hours, improved hypothermia, and significantly reduced lung neutrophils in naïve mice, as well as myeloperoxidase (MPO) levels in cell-free BAL, suggesting reduced degranulation of neutrophils and/or monocytes. The timing of the first dose of antibiotics was critical, with early initiation (under 6 hours) resulting in minimal lung injury and later initiation (after 24 hours) resulting in progressive hypothermia, hypoxemia, and death. The progressive organ injury phenotype observed with the later initiation of antibiotics is reminiscent of multiorgan failure that frequently develops in patients with septic shock despite the administration of effective antimicrobial therapy (31).

Applying antibiotic treatment to our moderate smoking model, we found that CS no longer significantly improved survival but instead caused greater lung injury in association with elevated numbers of monocytes and a trend toward increased lymphocytes. MPO levels were higher in the BAL of CS-exposed mice, suggesting either greater degranulation of neutrophils

(which would be consistent with reduced percentage of PMNs in BAL at 48 hours), or a predominantly monocytic source.

We found that blood levels of surfactant protein D (SP-D) were strongly correlated with the severity of lung injury (extravascular lung water) in mice during antibiotic-treated pneumococcal pneumonia. This finding is consistent with prior reports in patients that SP-D is an important prognostic biomarker in ARDS and an indicator of the degree of alveolar epithelial injury (20, 83). CS exposure was associated with elevated serum SP-D, consistent with greater lung epithelial injury. Additionally, elevated serum angiopoietin-2 suggests that CS-exposed mice suffered greater endothelial injury, similar to what has been reported by others in CS-exposed mice following challenge with endotoxin (7, 40) and *P. aeruginosa* (7). The combination of lung endothelial and alveolar epithelial injury is a well-established mechanism that leads to protein-rich pulmonary edema in experimental models (89) and in clinical studies (85, 86).

The pattern of BAL chemokines we observed in the antibiotic treated model is consistent with increased mobilization of monocytes and lymphocytes into the airspaces of CS-exposed mice during severe bacterial infection. The role of macrophages in CS-related lung inflammation is well-established. Macrophages have been shown to be activated by CS to release chemokines for monocytes, neutrophils, and lymphocytes, generate reactive oxygen species, and release elastolytic enzymes such as the matrix metalloproteinases. High intensity CS exposure in mice recruits monocytes into the lung within several days (12). Basilico and colleagues (5) recently reported that a CS exposure (100 mg/m^3 TSP for 6 weeks) similar to ours resulted in a reduced lung burden of *S. pneumoniae* and *P. aeruginosa* in association with increased bone marrow release of inflammatory Ly-6C^{hi} monocytes. These authors also reported that neutropenic mice (which as expected suffered very high bacterial burdens compared with wildtype), had bacterial loads reduced to wildtype levels by CS exposure. The

increased numbers of monocytes and macrophages that we observed in the lungs of CS-exposed mice are consistent with these data, and suggest that these cells may play an important role in the confinement of the infection to the lung.

Interestingly, we found that the severity of lung injury in CS-exposed (but not air-exposed) mice correlated with tissue levels of the lymphocyte serine protease granzyme B (2), suggesting that recruited lymphocytes may differentially impact acute bacterial inflammation in the setting of prior CS exposure. CD8+ T cells have long been associated with chronic smoking-related lung disease in patients (54, 65), and mice deficient in CD8+ T cells are protected against emphysema resulting from chronic CS exposure (44). BAL levels of granzyme B are increased in smokers and correlate with bronchial epithelial cell apoptosis (34). Similarly, NK cells isolated from the sputum of COPD patients have increased granzyme B expression, cytotoxicity, and expression of CXCR3 (78), a major T cell chemokine receptor (17). Although most studies have focused on chronic CS exposure, during intense CS exposure in mice, CD8+ T cells are recruited to the lung within only 3 days (51). Interestingly, in one study CXCR3 knockout mice were protected against both acute CS-induced T cell recruitment and lung injury (51). In our experiments, CS-exposed infected mice had significant elevations in BAL CXCL9, one of the major CXCR3 ligands and lymphocyte chemoattractants, previously shown to be increased in the sputum of patients with COPD (15).

In contrast, we observed a reduced percentage of neutrophils in BAL and low levels (<100 pg/ml) of the neutrophil chemokine KC (murine homologue of IL-8/CXLC8) at 48 hours in the antibiotic-treated model. BAL KC in CS-exposed mice was reduced over 70-fold with antibiotic treatment (relative to no antibiotics), IL-6 was reduced by nearly 10-fold, TNF-α was reduced over 4-fold, and IL-1β, a marker of inflammasome activation, was also significantly reduced. Meanwhile, levels of monocyte and lymphocyte chemokines generally remained similar or even trended higher compared to non-antibiotic treated mice. The results indicate that

462 ongoing bacterial presence in the lungs perpetuates intense neutrophil-dominated inflammation.
463 The omission of antibiotic treatment in animal models of severe pneumonia may thus limit their
464 applicability to the clinical setting, in which progressive organ dysfunction including ARDS
465 frequently occurs despite effective treatment of the causative pathogen (31) and reductions in
466 inflammatory cytokines such as IL-6 and IL-8 over time (58).

467 A major objective of this work was to identify biomarkers of CS-related acute lung injury.
468 As discussed above, SP-D and Ang-2 are established ARDS prognostic biomarkers reflecting
469 lung epithelial and endothelial injury, and we found that both biomarkers were elevated in the
470 blood of CS-exposed mice with bacterial pneumonia. Other investigators have emphasized the
471 role of CS smoke in causing lung endothelial injury (7, 40, 41, 66). Matrix metalloproteinase 9
472 (MMP-9, gelatinase B) was increased in the blood of CS-exposed mice by nearly 4-fold. MMP-9
473 is a collagenase expressed by many types of cells including neutrophils (55), monocytes (88),
474 and lymphocytes (87) with complex roles in lung inflammation and remodeling (4). MMP-8,
475 another collagenase expressed by neutrophils (56) and monocytes (16), was also significantly
476 elevated in the blood of CS-exposed infected mice. MMPs are known to be activated by CS
477 (69), and sputum MMP-8 distinguishes early stage COPD patients from active asymptomatic
478 smokers and non-smokers (35). Although both MMP-8 and 9 have been shown to be elevated
479 in the airspaces of patients with ARDS (23, 60), whether CS exposure differentially affects MMP
480 levels in smokers with ARDS is not yet known. Finally, the lymphocyte chemokine CXCL9 and
481 the monocyte chemokine MIP-1α, were elevated in the serum of CS-exposed infected mice. We
482 recently reported CS-associated increases in blood CXCL9 following injury with intratracheal
483 endotoxin (29), suggesting that diverse inflammatory stimuli may elicit common biomarker
484 signatures following CS exposure.

485 There are some limitations to these studies. Although the CS exposure is moderate in
486 duration, it does have clinical relevance to our published clinical studies showing an association

between ARDS and CS exposure (10, 11, 75). We have not determined the mechanism by which CS exposure reduces bacterial dissemination, but in light of the CS-associated increase in BAL MPO activity we hypothesize that it may involve a more robust innate immune response from macrophages, recruited monocytes and possibly lymphocytes. Also, we have not identified all of the potential mechanisms that account for the greater degree of pneumococcal lung injury in the antibiotic treated mice with CS exposure, although the cell and chemokine data indicate a major role for monocytes and monocyte derived chemokines in mediating the increase in lung endothelial and alveolar epithelial injury. Future experiments with broadly immunosuppressive agents such as corticosteroids and specific inhibition of lymphocyte and monocyte subsets using genetic manipulation may be helpful in elucidating these mechanistic pathways. We propose that this model of bacterial pneumonia and lung injury that develops in antibiotic treated mice has considerable clinical relevance to patients who often progress to develop ARDS in spite of appropriate antibiotic treatment (46) and should be valuable to other investigators who test novel therapeutics in pre-clinical models of ARDS.

In conclusion, compared to controls, moderate cigarette smoke exposure in mice over a three week period resulted in improved survival following bacterial pneumonia with *S. pneumoniae* in the absence of antibiotics, primarily explained by reduced bacteremia. However, when CS exposed mice with pneumococcal pneumonia were treated with antibiotics, as would usually be the case in the clinical setting, the degree of acute lung injury was greater in the CS-exposed mice, with evidence of more pulmonary edema and higher elevations of markers of alveolar epithelial injury (SP-D) and lung endothelial injury (Ang-2). The mechanisms for this greater lung injury in the antibiotic treated CS-exposed mice may be explained in part by a higher concentration of monocyte derived chemokines and monocytes. The antibiotic-treated *S. pneumoniae* model may be useful for future studies of the acute pulmonary impact of current

and emerging tobacco products, including the identification of biomarkers reflecting tobacco product-related lung injury.

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Author Contributions

J.G., C.C., S.N., and M.M. conceived of and designed the research. J.G., L.C., J.A., X.F., and N.T. performed the experiments. M.S. and S.S. conceived of, designed, and built the low concentration cigarette smoke generation and exposure system. J.G., S.N., C.C., and M.M analyzed the data, interpreted the results, prepared the figures, and drafted and edited the manuscript. All authors approved the final version of the manuscript.

Disclosures

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791

792

Figure Legends

Figure 1. *S. pneumoniae* dose response.

A-C: Mice inoculated intranasally with between 1 x10^7 and 1 x 10^8 cfu of *S. pneumoniae* developed dose-dependent weight loss, hypothermia, and arterial hypoxemia measured in freely moving mice. Data are mean +/-SD. n = 5 per dosing group, *P < 0.0001 for group, time, and interaction term (group*time) by repeated measures ANOVA; ^P <0.0001 for group, P = 0.23 for time, P = 0.0003 for interaction term; #P = 0.0001 for group, P < 0.0001 for time, P = 0.0004 for interaction term.

D: The severity of lung injury as assessed by pulmonary edema was greatest in the 1 x10^8 dose group. % by Mann-Whitney.

E: Representative low-power photomicrographs showing normal lung, patchy pneumonia 7 days following intranasal inoculation with 3 x 10^7 cfu *S. pneumoniae*, and profound lung consolidation with a dense inflammatory infiltrate following inoculation with 1 x 10^8 cfu *S. pneumoniae*.

Figure 2. Low-dose CS exposure did not affect pneumococcal lung injury.

A: Schematic depicting experimental procedures. Mice were exposed to low-dose sidestream smoke for 2 hours a day on subsequent days, then inoculated with 5x10^7 cfu *S. pneumoniae*.

B: Weight loss declined over time but was similar in air and CS-exposed mice. Data are mean +/-SD. n = 10 per group, *P = 0.60 for group, P < 0.0001 for time, P = 0.66 for interaction term (group*time) by repeated measures ANOVA.

C: Core body temperature was not different 48 hours post-infection, P = 0.11 by Mann-Whitney.

D: Arterial hypoxemia did not differ between air and CS-exposed mice, P = 0.1 by unpaired t-test.

E: Pulmonary edema 48 hours post-infection was moderate and did not differ based air and CS-exposed mice, P = 0.43 by unpaired t-test.

Figure 3. Moderate-dose CS exposure increases survival in severe pneumococcal pneumonia.

A: Schematic depicting smoke exposure followed by intranasal inoculation with $1x10^8$ cfu *S. pneumoniae*.

B: CS-exposed mice had a significant survival advantage through sacrifice at 48 hours. *by Log-Rank test. n= 20 mice per group.

C: Weight loss was slightly greater over time in surviving CS-exposed mice. Data are mean +/- SD, n = 20 per group. ^ P = 0.09 for group, P <0.0001 for time, P = 0.001 for interaction term (group*time) by repeated measures ANOVA.

D: Hypothermia in surviving mice was less severe in CS-exposed mice over time. Data are mean +/- SD, n = 20 per group. #P = 0.4 for group, P < 0.0001 for time, P = 0.0003 for interaction term by repeated measures ANOVA.

E: Arterial hypoxemia in surviving mice worsened over time but did not differ according to prior CS exposure. Data are mean +/-SD, n = 20 per group. %P = 0.67 for group, P < 0.0001 for time, P = 0.48 for interaction term by repeated measures ANOVA.

F: Peripheral leukopenia at 48 hours among surviving mice was similar, P = 0.44 by Mann-Whitney.

G: Pulmonary edema in surviving mice was similar 48 hours post-infection in CS and air-exposed mice, P = 0.8 by Mann-Whitney.

Figure 4. Prior CS exposure does not protect against intraperitoneal *S. pneumoniae*.

A: Survival of naïve mice with increasing doses of i.p. *S. pneumoniae.* n=5 per group.

B: Prior CS exposure had no effect on 24 hour survival following i.p. challenge with 1 x 10^8 cfu *S. pneumoniae*. n=20 per group, P = 0.68 by Log-Rank test.

C: Pulmonary edema was minimal in both CS and air-exposed surviving mice 24 hours after i.p. *S. pneumoniae*, P = 0.32 by unpaired t-test.

D: Lungs extracted from dead mice did not differ in weight based on prior CS exposure, suggesting a similar degree of pulmonary edema, P = 0.68 by unpaired t-test.

Figure 5. Prior CS exposure reduces bacteremia during pneumococcal pneumonia.

A: Schematic depicting smoke exposure and infection.

B: CS-exposed mice were less hypothermic than air-exposed mice. *by unpaired t-test.

C: Arterial hypoxemia was more severe in CS-exposed mice, in contrast to the survival benefit. *by unpaired t-test.

D: BAL protein, a gross measure of the permeability of the alveolar-capillary barrier, did not differ with regard to prior CS exposure 24 hours following pneumococcal inoculation, P = 0.62 by unpaired t-test.

E: Pulmonary edema was not different in CS and air-exposed mice at 24 hours, P = 0.7 by unpaired t-test.

F: Prior CS exposure reduced recoverable pneumococci in blood by several orders of magnitude. *by Mann-Whitney.

G: Splenic pneumococci were also reduced by prior CS exposure. *by Mann-Whitney.

Figure 6. The CS-associated reduction in bacteremia is not due to a reduced pneumococcal burden within the airspaces.

A: CS-exposed mice were less hypothermic than air-exposed mice at 16 hours post-infection. *by unpaired t-test.

B: Airspace pneumococcal burden at 16 hours post-infection was similar in air and CS-exposed mice.

C: Myeloperoxidase activity within BAL was significantly higher in CS-exposed mice. ^by Mann-Whitney.

Figure 7. A model of pneumococcal pneumonia treated with antibiotics.

A: Schematic depicting experimental protocol. Mice were inoculated with *S. pneumoniae* and then injected with saline or ceftriaxone 150 mg/kg, i.p. at 12, 24, and 36 hours post-infection, followed by sacrifice at 48 hours.

B-C: Mice treated with antibiotics had greater weight loss and less hypothermia. Data are mean +/- SD, n = 7-8 per group. *P = 0.05 for group, P < 0.0001 for time, P = 0.17 for interaction term by repeated measures ANOVA; ^P = 0.0001 for group, P < 0.0001 for time, P = 0.24 for interaction term.

D: Recoverable pneumococci in BAL were greatly reduced by 48 hours with antibiotic treatment. #by Mann-Whitney.

E: BAL myeloperoxidase activity was significantly reduced 48 hours post-infection in antibiotic-treated mice. %P =0.007 compared with No Abx, P = 0.006 compared with Uninfected by Mann-Whitney.

F: Representative high-power photomicrographs showing neutrophil-predominant inflammation 48 hours post untreated infection, reduced in mice treated with antibiotics but still clearly present relative to uninfected mice.

Figure 8. CS exposure increases lung injury in pneumococcal pneumonia treated with antibiotics.

A: Schematic depicting experimental procedures. Mice were exposed to moderate CS or air, then infected with $1x10^8$ cfu *S. pneumoniae* and treated with ceftriaxone 150 mg/kg i.p. at 12, 24, and 36 hours post-infection, followed by sacrifice at 48 hours.

B: Survival did not differ between CS and air-exposed mice. n = 25 mice per group, P = 0.08 by Log-Rank test.

C: Weight loss was greater in CS-exposed mice over time. Data are mean +/- SD, n = 25 mice per group. *P <0.0001 for group and time, P = 0.004 for interaction term (group*time) by repeated measures ANOVA.

D: CS-exposed mice were less hypothermic but this difference decreased with time as air-exposed mice gained body temperature during antibiotic treatment. Data are mean +/- SD, n = 25 mice per group. ^P <0.0001 for group, time, and interaction term (group*time) by repeated measures ANOVA.

E: Pulmonary edema was significantly greater in mice previously exposed to CS. #by Mann-Whitney.

F: Representative high power photomicrograph of an H&E stained section from an air-exposed mouse 48 hours post-infection showing an inflammatory infiltrate composed mostly of neutrophils (dotted arrows) and monocytes/macrophages (solid arrow).

G: CS exposed mice had a subtle increase in septal thickening and greater numbers of monocytes/macrophages (solid arrows) relative to neutrophils (dotted arrow).

Figure 9. CS exposure prior to pneumococcal pneumonia changes the cellular composition of airspace inflammation.

A: CS exposure increases the percentage of monocytes/macrophages in the BAL at the expense of neutrophils, while the percentage of lymphocytes is unchanged. *by Mann-Whitney.

B: Given the trend toward higher BAL cell counts in CS-exposed mice, the total number of BAL neutrophils was unchanged, while total BAL monocytes/macrophages were significantly increased, and total BAL lymphocytes trended higher. ^by unpaired t-test.

C: The concentration of key neutrophil, monocyte/macrophage, and lymphocyte chemokines was measured in BAL and corrected for total protein. KC was detected at very low levels and not different with regard to CS exposure. However MIP-1α and CXCL9 were significantly increased in CS-exposed mice. *by Mann-Whitney.

Figure 10. Prior CS exposure increases inflammatory cell cytotoxic proteins during pneumococcal pneumonia.

A: CS-exposed mice had greater myeloperoxidase activity than air-exposed mice. *by Mann-Whitney.

B: Lung levels of Granzyme B, a serine protease contained in cytotoxic lymphocyte granules, were not significantly increased by prior CS exposure, P = 0.14 by Mann-Whitney.

C: Granzyme B levels were unrelated to the level of pulmonary edema in air-exposed mice, Spearman r = -0.07, p = 0.81.

D: In contrast, Granzyme B levels predicted the extent of pulmonary edema in CS-exposed mice, Spearman r = 0.58, p=0.04.

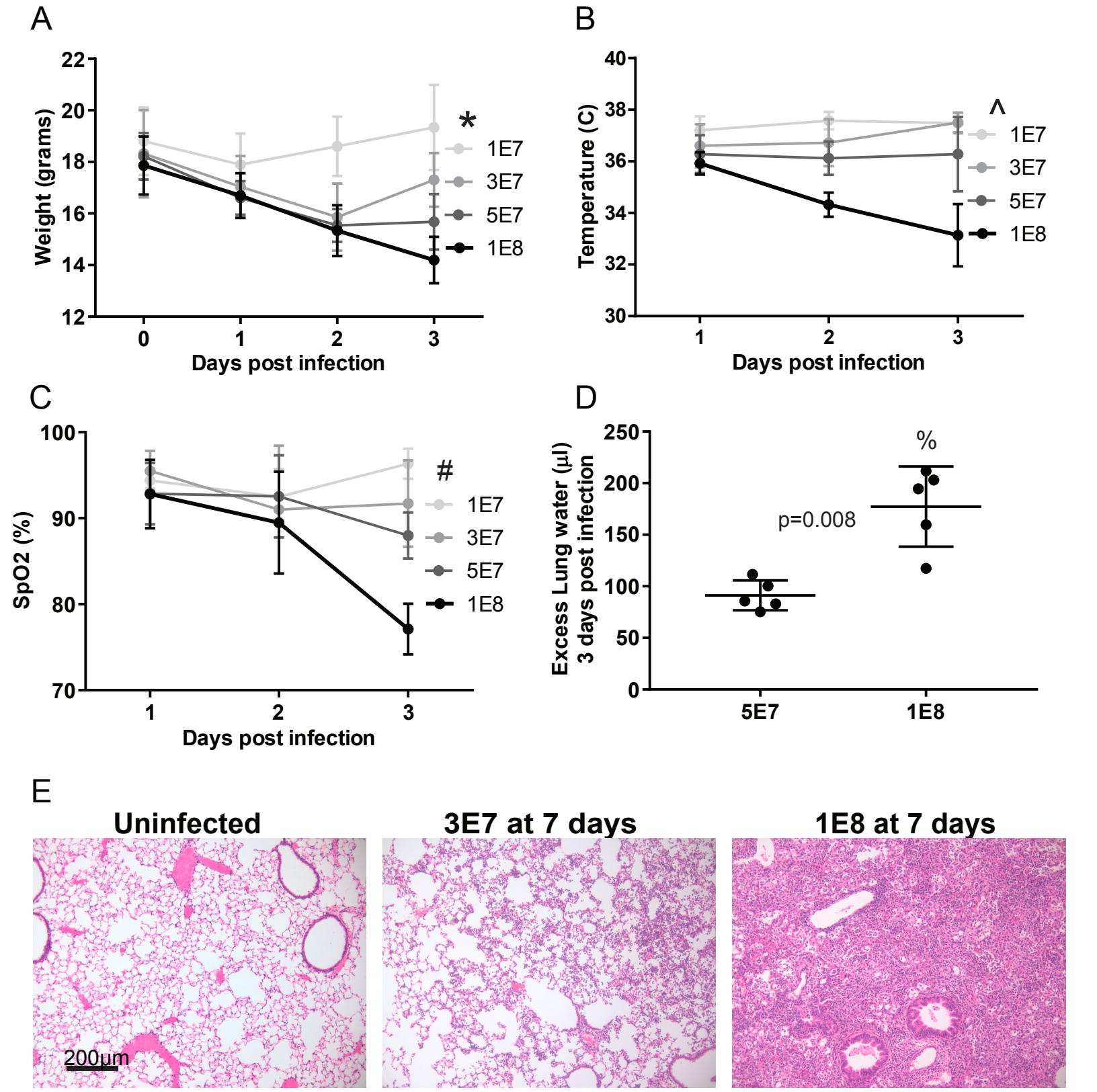
Figure 11. Prior CS exposure increases markers of alveolar epithelial and endothelial injury in the blood during pneumococcal pneumonia.

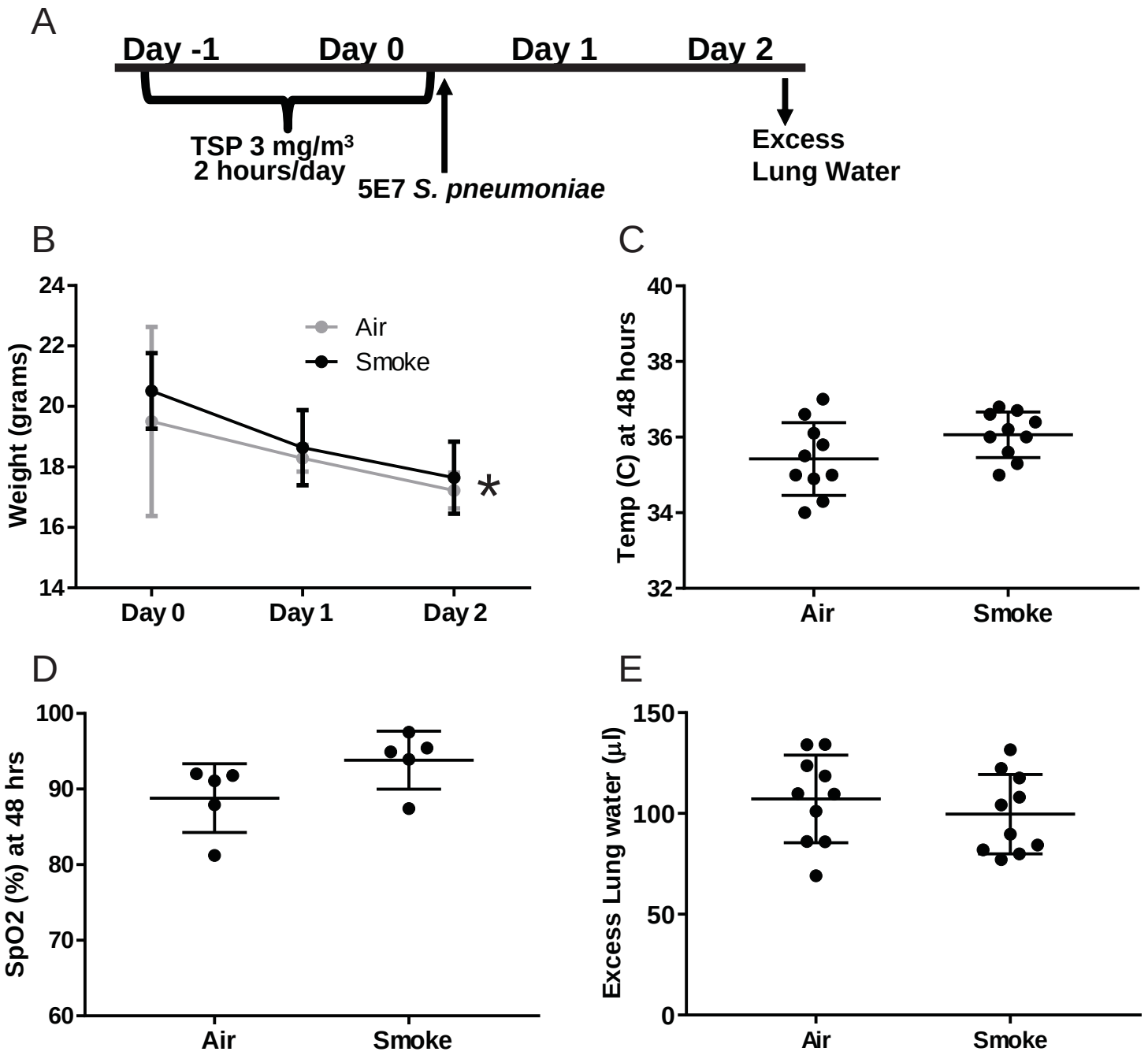
A: Plasma surfactant protein D (SP-D) was highly correlated with the severity of lung injury (extravascular lung water) across CS and air-exposed mice, Spearman r = 0.71, p<0.0001.

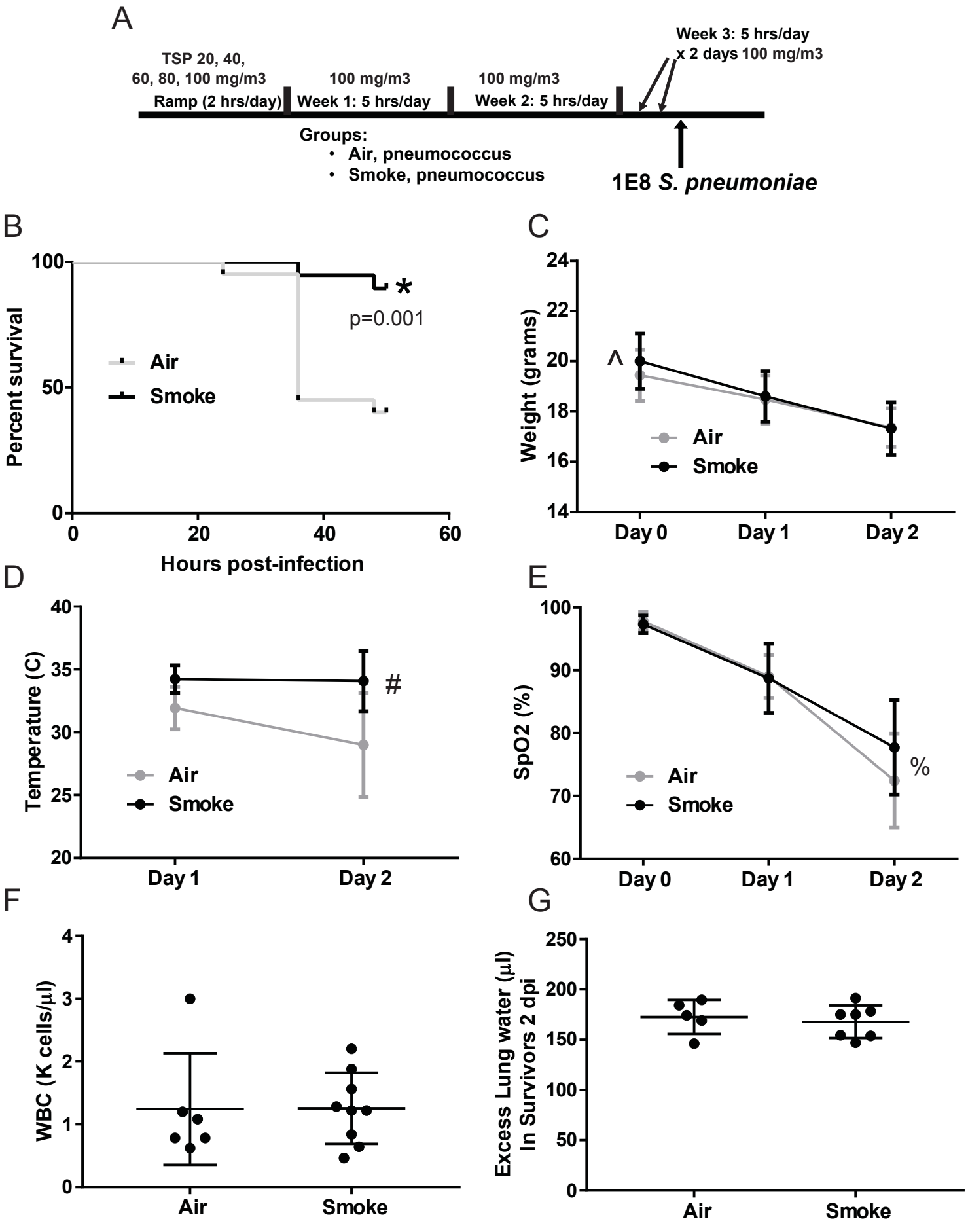
B: Serum SP-D was significantly higher 48 hours post-injury in CS-exposed mice. *by Mann-Whitney.

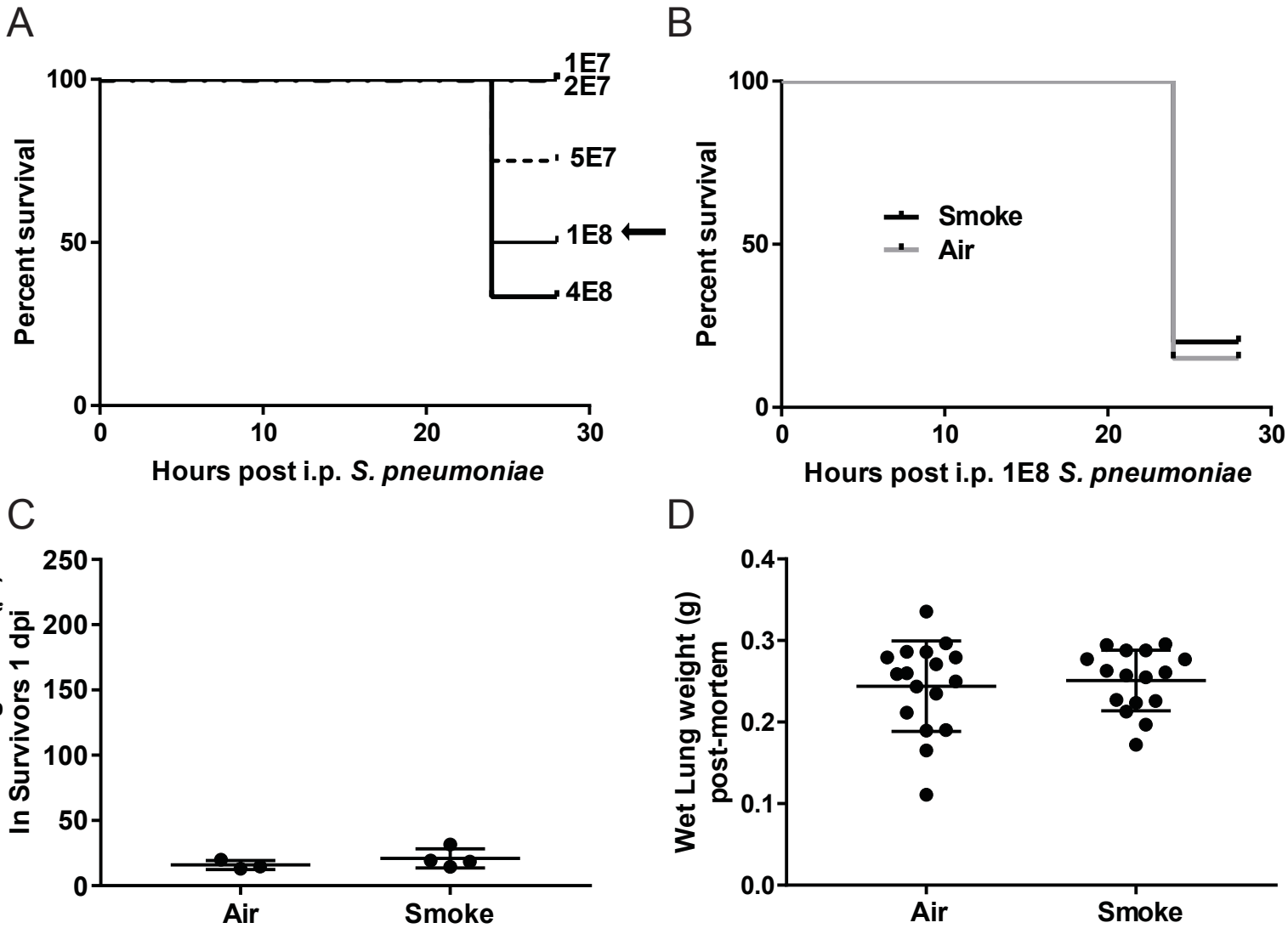
C: Serum Ang-2, a marker of endothelial injury, was significantly higher in CS-exposed mice. *by Mann-Whitney

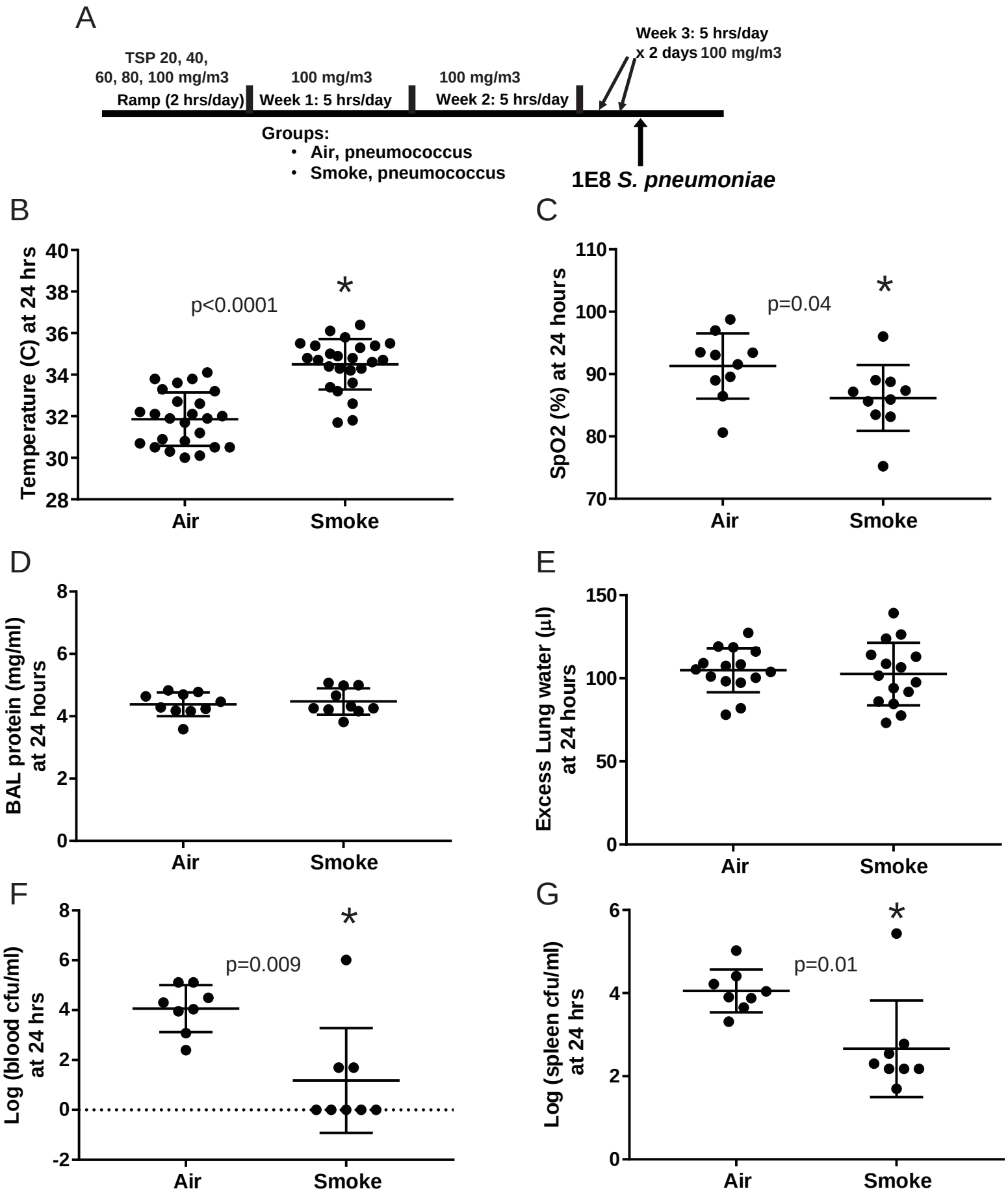
931


A
Weight (grams)
22
20
18
16
14
12
0
1
2
3
Days post infection
*
1E7
3E7
5E7
1E8
B
Temperature (C)
40
38
36
34
32
30
1
2
3
Days post infection
^
1E7
3E7
5E7
1E8
C
SpO2 (%)
100
90
80
70
1
2
3
Days post infection
#
1E7
3E7
5E7
1E8
D
Excess Lung water (µl)
3 days post infection
250
200
150
100
50
0
5E7
1E8
%
p=0.008
E
Uninfected
3E7 at 7 days
1E8 at 7 days
200µm

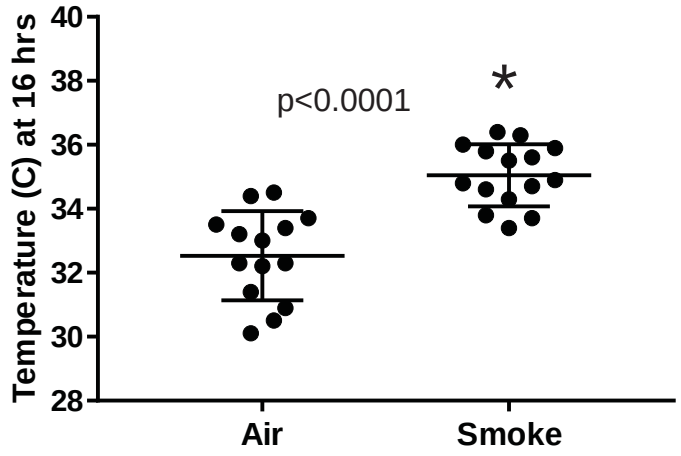

A
Day -1
Day 0
Day 1
Day 2
TSP 3 mg/m3
2 hours/day
5E7 S. pneumoniae
Excess
Lung Water
B
Weight (grams)
24
22
20
18
16
14
Air
Smoke
*
Day 0
Day 1
Day 2
C
Temp (C) at 48 hours
40
38
36
34
32
Air
Smoke
D
SpO2 (%) at 48 hrs
100
90
80
70
60
Air
Smoke
E
Excess Lung water (µl)
150
100
50
0
Air
Smoke


A
TSP 20, 40,
60, 80, 100 mg/m3
Ramp (2 hrs/day)
100 mg/m3
Week 1: 5 hrs/day
100 mg/m3
Week 2: 5 hrs/day
Week 3: 5 hrs/day
x 2 days 100 mg/m3
Groups:
• Air, pneumococcus
• Smoke, pneumococcus
1E8 S. pneumoniae
B
Percent survival
100
50
0
0
20
40
60
Hours post-infection
*
p=0.001
Air
Smoke
C
Weight (grams)
24
22
20
18
16
14
^
Air
Smoke
Day 0
Day 1
Day 2
D
Temperature (C)
40
35
30
25
20
#
Air
Smoke
Day 1
Day 2
E
SpO2 (%)
100
90
80
70
60
%
Air
Smoke
Day 0
Day 1
Day 2
F
WBC (K cells/µl)
4
3
2
1
0
Air
Smoke
G
Excess Lung water (µl)
In Survivors 2 dpi
250
200
150
100
50
0
Air
Smoke

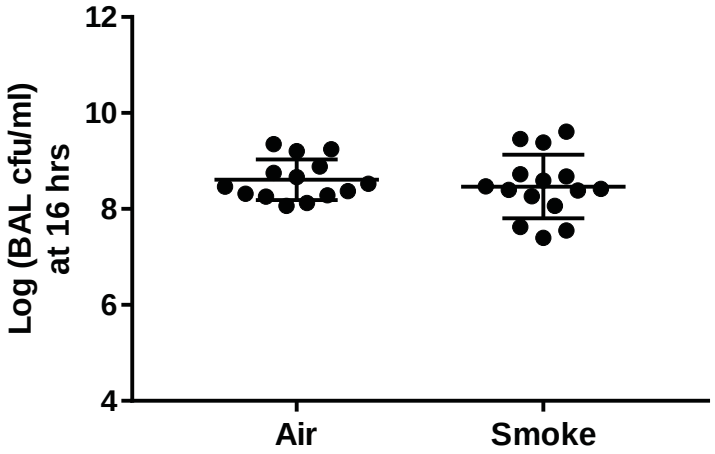

A
100
50
0
Percent survival
1E7
2E7
5E7
1E8
4E8
0
10
20
30
Hours post i.p. S. pneumoniae
B
100
50
0
Percent survival
Smoke
Air
0
10
20
30
Hours post i.p. 1E8 S. pneumoniae
C
250
200
150
100
50
0
Excess Lung water (µl)
In Survivors 1 dpi
Air
Smoke
D
0.4
0.3
0.2
0.1
0.0
Wet Lung weight (g)
post-mortem
Air
Smoke


A
TSP 20, 40, 60, 80, 100 mg/m3
Ramp (2 hrs/day)
100 mg/m3
Week 1: 5 hrs/day
100 mg/m3
Week 2: 5 hrs/day
Week 3: 5 hrs/day
x 2 days 100 mg/m3
Groups:
• Air, pneumococcus
• Smoke, pneumococcus
1E8 S. pneumoniae
B
Temperature (C) at 24 hrs
40
38
36
34
32
30
28
p<0.0001
*
Air
Smoke
C
SpO2 (%) at 24 hours
110
100
90
80
70
p=0.04
*
Air
Smoke
D
BAL protein (mg/ml) at 24 hours
8
6
4
2
0
Air
Smoke
E
Excess Lung water (µl) at 24 hours
150
100
50
0
Air
Smoke
F
Log (blood cfu/ml) at 24 hrs
8
6
4
2
0
-2
p=0.009
*
Air
Smoke
G
Log (spleen cfu/ml) at 24 hrs
6
4
2
0
p=0.01
*
Air
Smoke

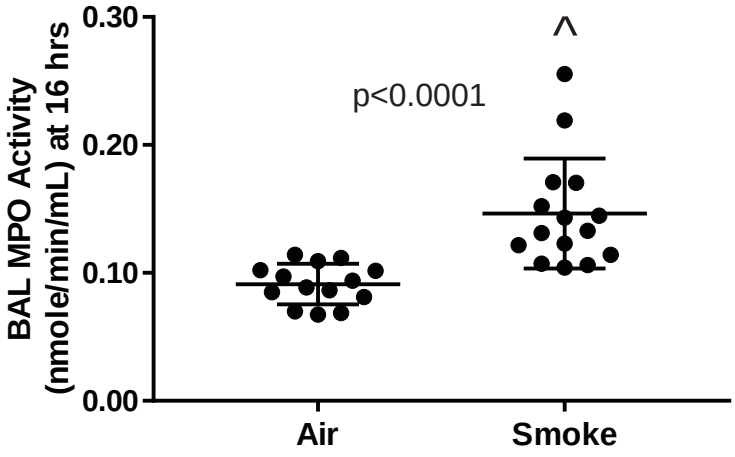
A

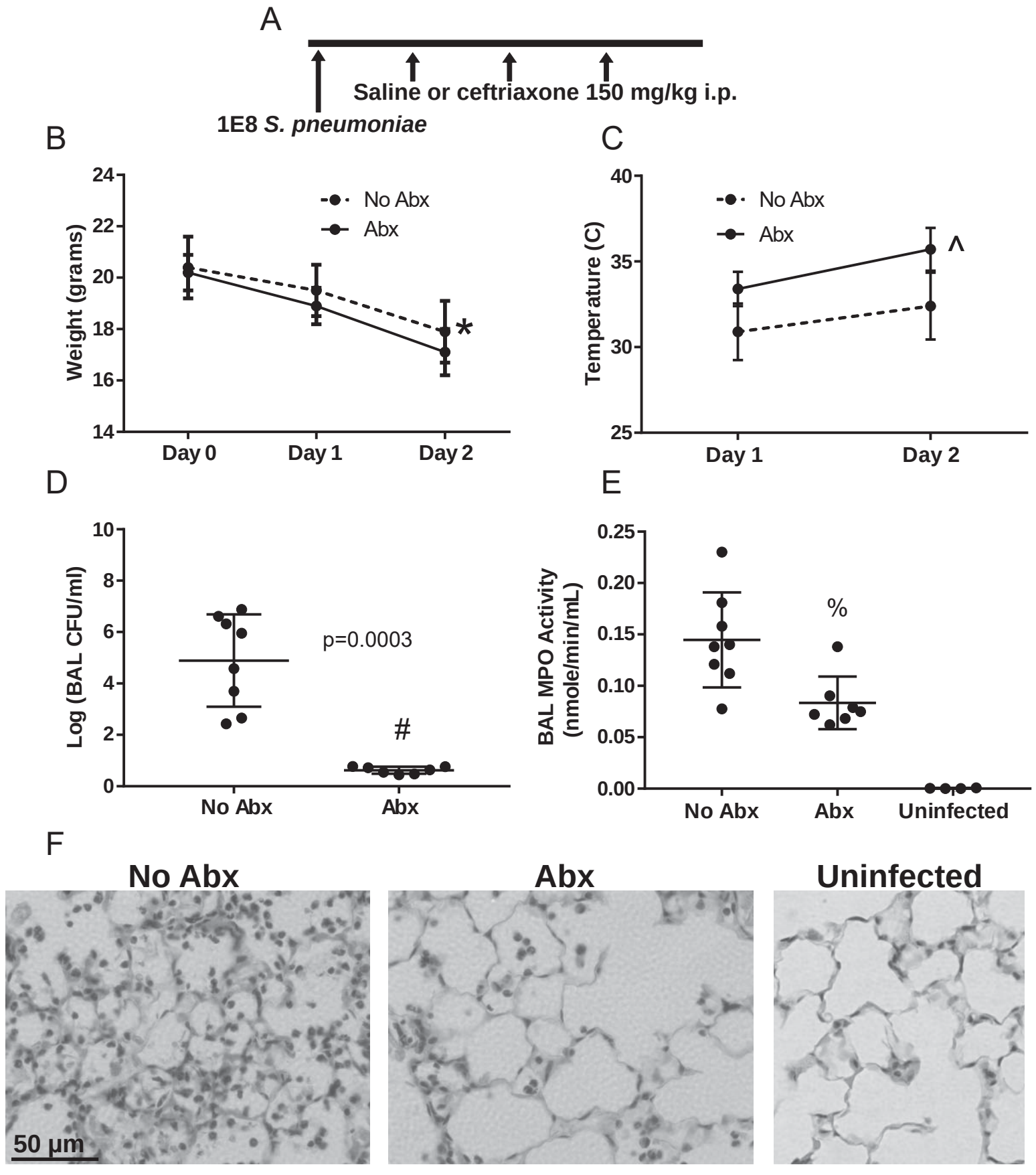

Temperature (C) at 16 hrs
40
38
36
34
32
30
28
p<0.0001
*
Air
Smoke

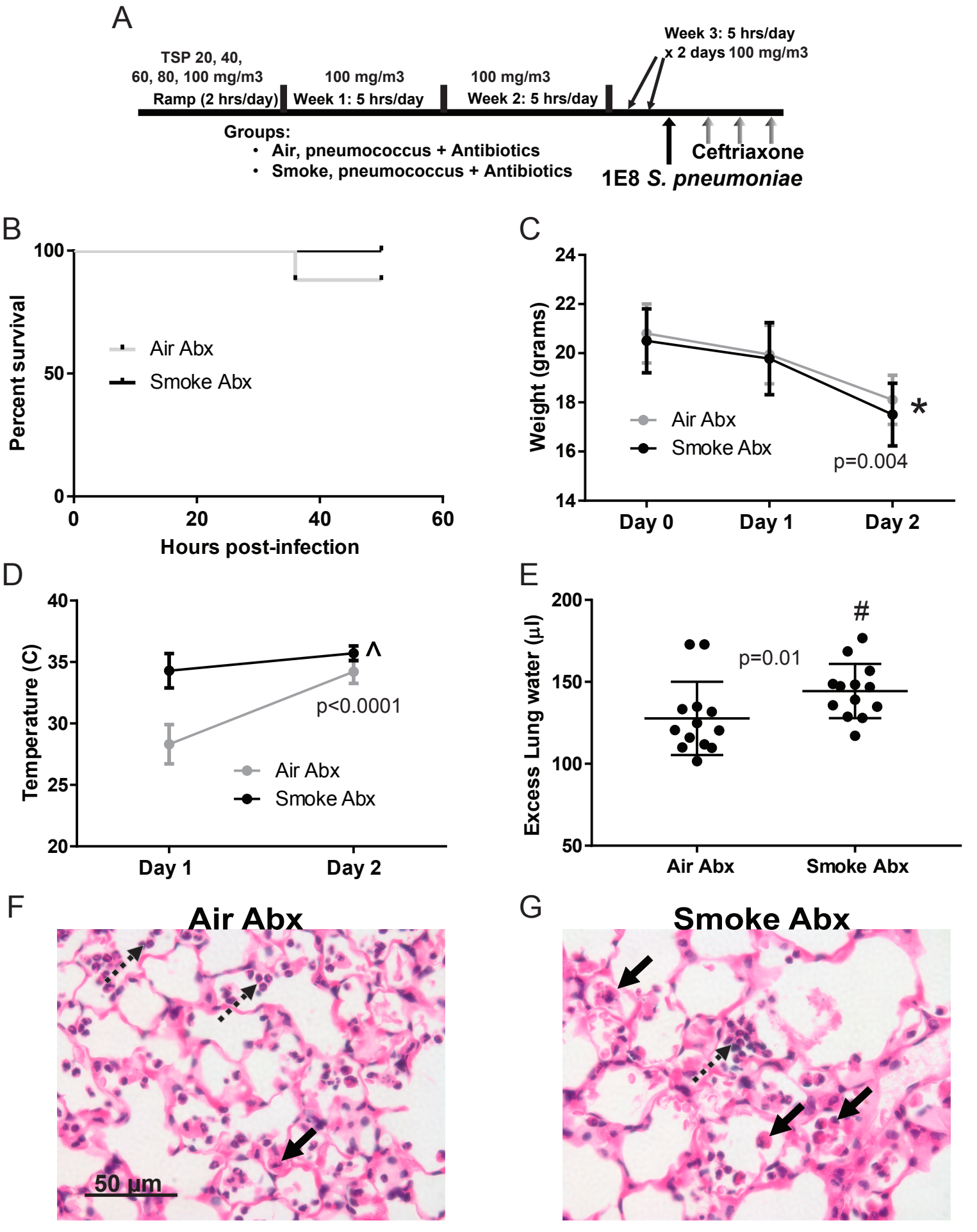
B

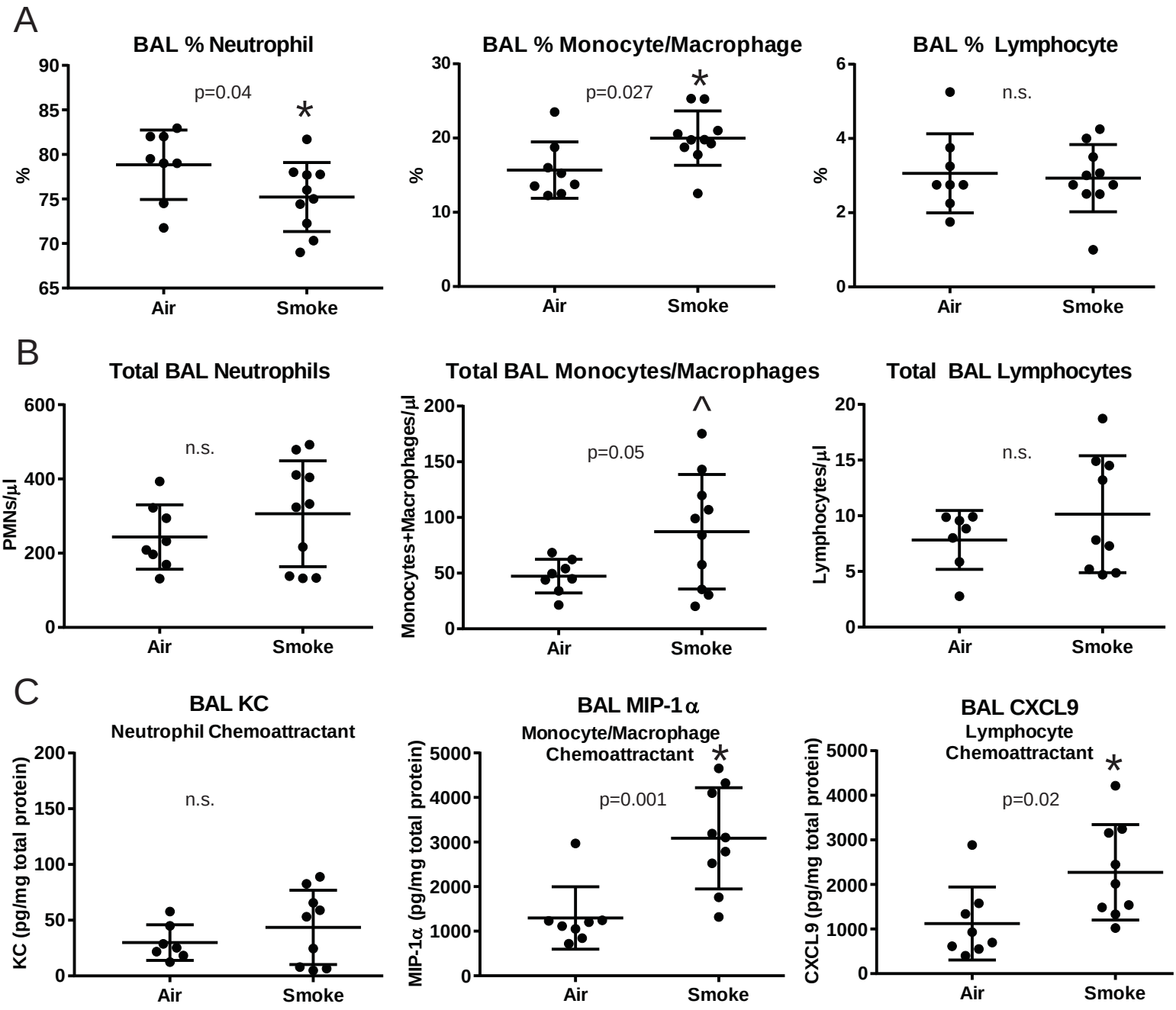

Log (BAL cfu/ml) at 16 hrs
12
10
8
6
4
Air
Smoke

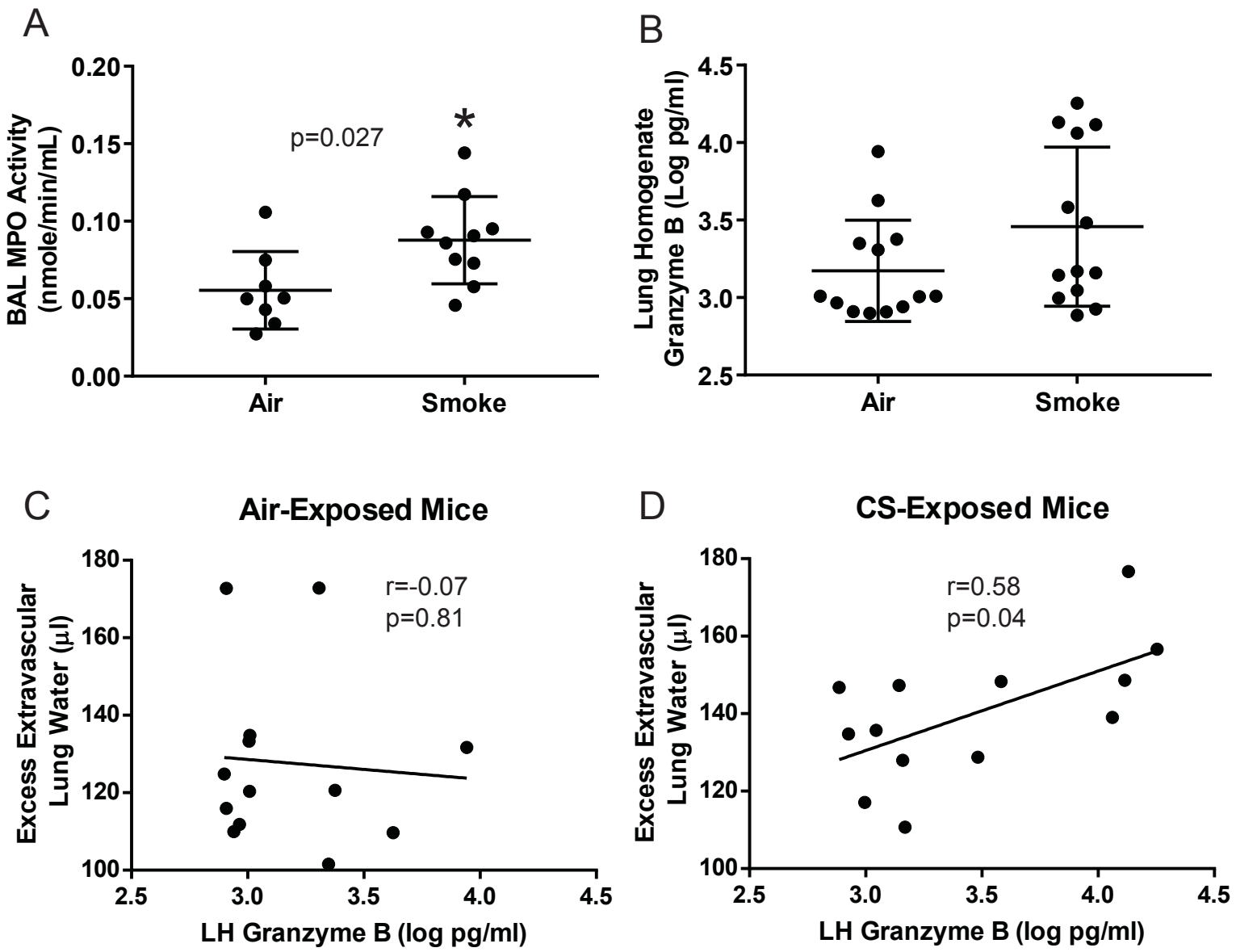
C

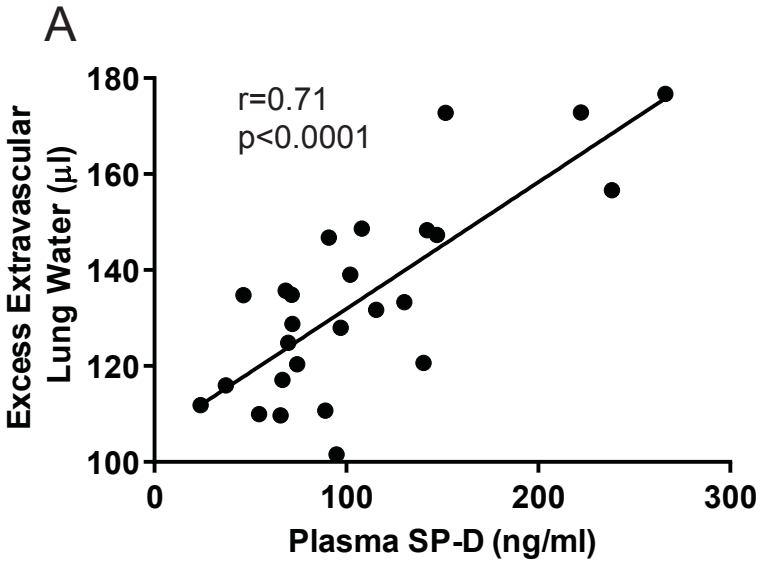

BAL MPO Activity (nmole/min/mL) at 16 hrs
0.30
0.20
0.10
0.00
p<0.0001
^
Air
Smoke

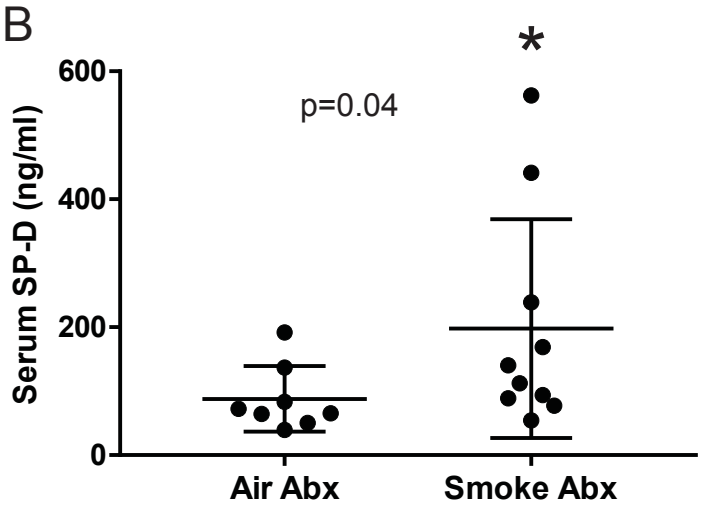

A
Saline or ceftriaxone 150 mg/kg i.p.
1E8 S. pneumoniae
B
Weight (grams)
No Abx
Abx
Day 0
Day 1
Day 2
*
C
Temperature (C)
No Abx
Abx
Day 1
Day 2
^
D
Log (BAL CFU/ml)
p=0.0003
#
No Abx
Abx
E
BAL MPO Activity (nmole/min/mL)
%
No Abx
Abx
Uninfected
F
No Abx
Abx
Uninfected
50 µm


A
TSP 20, 40,
60, 80, 100 mg/m3
Ramp (2 hrs/day)
100 mg/m3
Week 1: 5 hrs/day
100 mg/m3
Week 2: 5 hrs/day
Week 3: 5 hrs/day
x 2 days 100 mg/m3
Groups:
• Air, pneumococcus + Antibiotics
• Smoke, pneumococcus + Antibiotics
Ceftriaxone
1E8 S. pneumoniae
B
Percent survival
100
50
0
Air Abx
Smoke Abx
0
20
40
60
Hours post-infection
C
Weight (grams)
24
22
20
18
16
14
Air Abx
Smoke Abx
p=0.004
*
Day 0
Day 1
Day 2
D
Temperature (C)
40
35
30
25
20
^
p<0.0001
Air Abx
Smoke Abx
Day 1
Day 2
E
Excess Lung water (µl)
200
150
100
50
p=0.01
#
Air Abx
Smoke Abx
F
Air Abx
50 µm
G
Smoke Abx


A
BAL % Neutrophil
p=0.04
*
%
90
85
80
75
70
65
Air
Smoke
BAL % Monocyte/Macrophage
p=0.027
*
%
30
20
10
0
Air
Smoke
BAL % Lymphocyte
n.s.
%
6
4
2
0
Air
Smoke
B
Total BAL Neutrophils
n.s.
PMNs/µl
600
400
200
0
Air
Smoke
Total BAL Monocytes/Macrophages
p=0.05
^
Monocytes+Macrophages/µl
200
150
100
50
0
Air
Smoke
Total BAL Lymphocytes
n.s.
Lymphocytes/µl
20
15
10
5
0
Air
Smoke
C
BAL KC
Neutrophil Chemoattractant
n.s.
KC (pg/mg total protein)
200
150
100
50
0
Air
Smoke
BAL MIP-1α
Monocyte/Macrophage
Chemoattractant
p=0.001
*
MIP-1α (pg/mg total protein)
5000
4000
3000
2000
1000
0
Air
Smoke
BAL CXCL9
Lymphocyte
Chemoattractant
p=0.02
*
CXCL9 (pg/mg total protein)
5000
4000
3000
2000
1000
0
Air
Smoke


A
BAL MPO Activity (nmole/min/mL)
0.20
0.15
0.10
0.05
0.00
p=0.027
*
Air
Smoke
B
Lung Homogenate Granzyme B (Log pg/ml)
4.5
4.0
3.5
3.0
2.5
Air
Smoke
C
Air-Exposed Mice
Excess Extravascular Lung Water (µl)
180
160
140
120
100
r=-0.07
p=0.81
2.5
3.0
3.5
4.0
4.5
LH Granzyme B (log pg/ml)
D
CS-Exposed Mice
Excess Extravascular Lung Water (µl)
180
160
140
120
100
r=0.58
p=0.04
2.5
3.0
3.5
4.0
4.5
LH Granzyme B (log pg/ml)


A
r=0.71
p<0.0001
Excess Extravascular Lung Water (µl)
180
160
140
120
100
0
100
200
300
Plasma SP-D (ng/ml)


B
p=0.04
*
Serum SP-D (ng/ml)
600
400
200
0
Air Abx
Smoke Abx

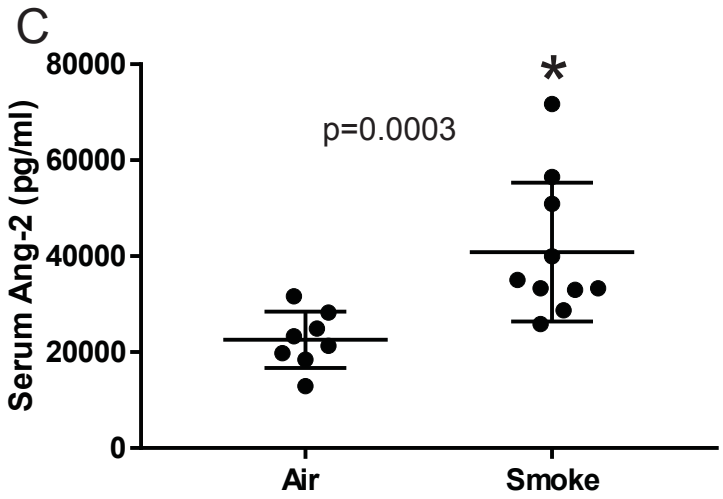

C
p=0.0003
*
Serum Ang-2 (pg/ml)
80000
60000
40000
20000
0
Air
Smoke

Table 1: CS-exposure increases monocyte and lymphocyte chemokines measured in BAL

Ligand	Cell*	Air (n=8)	Smoke (n=9-10)	Ratio† (S/A)	P‡
KC	N	25	53	2.1	0.61
IL-6	N	198	180	0.9	0.24
MIP-1α	M	1159	3103	**2.7**	0.001
MCP-1	M	837	1340	**2.4**	0.008
MCP-2	M	747	1159	**1.6**	0.04
MCP-3	M	1155	2400	2.1	0.21
CXCL9	L	814	2013	**2.5**	0.02
CXCL12	L	38072	64603	**1.7**	0.009

Median values at 48 hours post-infection are expressed in pg/mg BAL protein; *predominant target cell for each cytokine (N=neutrophil, M=monocyte/ macrophage, L=lymphocyte); †Smoke to air ratio, bolded values significant by ‡uncorrected p value <=0.05; IL-6, interleukin-6; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

Table 2: Antibiotic treatment in CS-exposed mice induces significant changes in BAL cytokine profile

Ligand	- Abx (n=3-5)	+ Abx (n=8-9)	Ratio* -/+	P†
IL-1α	442	152	**2.9**	0.004
IL-1β	196	129	**1.5**	0.001
TNF-α	1924	438	**4.4**	0.001
IL-17	5.3	1.5	**3.5**	0.03
KC	6940	98	**70.8**	0.001
IL-6	5439	599	**9.1**	0.02
MIP-1α	44925	11737	**3.8**	0.03
MCP-1	5564	6036	0.9	>0.99
MCP-2	6260	5378	1.2	0.71
MCP-3	2275	3603	0.6	0.10
CXCL9	9475	8769	1.1	0.44
CXCL12	148956	254114	0.6	0.09

Median values at 48 hours post-infection are expressed in pg/ml; *Ratio of –Abx/+Abx, bolded values significant by †uncorrected p value <=0.05; IL-6, interleukin-6.

Table 3: Blood biomarkers of CS-exposure related lung injury in antibiotic-treated pneumonia

Ligand	Air (n=8)	Smoke (n=9-10)	Ratio* (S/A)	P†
MMP-9	**13008**	**49834**	**3.8**	**0.0085**
MIP-1α	**73.2**	**138.2**	**1.9**	**0.03**
VEGF	**1.6**	**3.0**	**1.9**	**0.007**
SP-D	**68692**	**126446**	**1.8**	**0.04**
GM-CSF	5.5	8.5	1.5	0.12
CXCL9	**130.4**	**200.3**	**1.5**	**0.045**
Ang-2	**22314**	**34147**	**1.5**	**0.0003**
IL-17	**7.8**	**10.5**	**1.4**	**0.02**
MMP-8	**309065**	**445069**	**1.4**	**0.009**
FGF-2	**124**	**159.7**	**1.3**	**0.006**
IL-6	31.2	41.6	1.3	0.20
IFN-γ	19.4	23.2	1.2	0.50
IL-1β	94.7	105.3	1.1	0.15
IL-2	6.5	7.1	1.1	0.47
MCP-3	1209	1299	1.1	0.76
IL-5	14.9	15.0	1.0	>0.99
ICAM-1	54245	54956	1.0	0.68
CXCL12	13254	12845	1.0	0.67
IL-12	28.2	24.3	0.9	0.09
MCP-1	72.7	63.7	0.9	0.85
TNFR1	1394	1259	0.9	0.46
TNF-α	13.1	10.8	0.8	0.85
CXCL10	306.7	238.5	0.8	0.57
KC	1328	732.2	0.6	0.24
IL-10	Too low to measure			
IL-13	Too low to measure			
IL-4	Too low to measure			

Median serum values at 48 hours post-infection are expressed in pg/ml; *Ratio of CS-exposed to air-exposed, bolded values significant by †uncorrected p value <=0.05; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; SP-D, surfactant protein D; GM-CSF, granulocyte macrophage-colony stimulating factor; Ang-2, angiopoietin-2; FGF, fibroblast growth factor; IFN, interferon; ICAM, intercellular adhesion molecule; TNFR, tumor necrosis factor receptor.