Wood Smoke Particle Exposure in Mice Reduces the Severity of Influenza Infection

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Abstract

Elevated ambient temperatures and extreme weather events have increased the incidence of wildfires world-wide resulting in increased wood smoke particle (WSP). Epidemiologic data suggests that WSP exposure associates with exacerbations of respiratory diseases, and with increased respiratory viral infections. To assess the impact of WSP exposure on host response to viral pneumonia, we performed WSP exposures in rodents followed by infection with mouse adapted influenza (HINI-PR8). C57BL/6 male mice aged 6–8 weeks were challenged with WSP or PBS by oropharyngeal aspiration in acute (single dose) or sub-acute exposures (day 1, 3, 5, 7 and 10). Additional groups underwent sub-acute exposure followed by infection by influenza or heat-inactivated (HI) virus. Following exposures/infection, bronchoalveolar lavage (BAL) was performed to assess for total cell counts/differentials, total protein, protein carbonyls and hyaluronan. Lung tissue was assessed for viral counts by real time PCR. When compared to PBS, acute WSP exposure associated with an increase in airspace macrophages. Alternatively, sub-acute exposure resulted in a dose dependent increase in airspace neutrophils. Sub-acute WSP exposure followed by influenza infection was associated with improved respiratory viral outcomes including reduced weight loss and increased blood oxygen saturation, and decreased protein carbonyls and viral titers. Flow cytometry demonstrated dynamic changes in pulmonary macrophage and T cell subsets based on challenge with WSP and influenza. This data suggests that sub-acute WSP exposure can improve host response to acute influenza infection.

Introduction

Wildfire smoke, particularly wood smoke is associated with significant morbidity and mortality and has been estimated to be responsible for 339,000 deaths annually[1]. Emergency visits for asthma, bronchitis and COPD exacerbations and respiratory hospital admissions increase in wildfire smoke affected areas [2–4]. In addition to wildfires, elevated wood smoke particle (WSP) exposures are observed in regions where forests are cleared for use in agriculture and where wood is a component of indoor heating and cooking. The increasing presence of WSPs in the environment and its potential for toxic effects has increased efforts to understand the toxicity and health effects of WSP. Given the large potential exposure to populations, understanding these effects has important public health implications.

WSP exposure has been demonstrated to increase pulmonary inflammation and systemic inflammation. In healthy human volunteers, WSP exposure is associated with an increase in airspace neutrophils [5], peripheral blood IL-1β [6] and lactate dehydrogenase (LDH), without change in bronchoalveolar lavage fluid (BALF) cytokines, total protein/albumin or pulmonary function testing. In rodents, acute WSP exposure (250μg) is associated with airspace neutrophil influx, increased methacholine-induced airway hyperresponsiveness (AHR) and airspace inflammatory cytokines [7]. Alternatively, chronic exposure to WSP (8 weeks, 3 exposures/week of 50μg) increases airspace eosinophils, Th2 cytokines (eotaxin and IL-5) and wood smoke serum IgG. Furthermore, sources of WSP and the extent of combustion have also been shown to impact the ability of WSP to generate lung inflammation [8].

Though data suggests that WSP have the potential to increase pulmonary and systemic inflammation, little is known about how WSP exposure impacts the immune response to viral infection. Work by Rebuli et al. has shown that healthy volunteers challenged with WSP followed by nasal inoculation of live attenuated influenza virus have reduced CXCL10 expression in nasal lavage fluid [9]. Though this suggests a direct effect of WSP on influenza responses, there are no studies to our knowledge that have explored the impact of WSP exposure on influenza responses in the lung. Additionally, previous *in vivo* acute WSP exposure studies have suggested increased severity of bacterial infection and altered macrophage function [10, 11]. The purpose of this study was to develop a rodent model of sub-acute WSP exposure followed by influenza infection to understand the impact of WSP exposure on the severity of pulmonary inflammation to influenza infection. While limited data exists on the role of sub-acute WSP exposure on the pulmonary response to viral challenge, we hypothesized that exposure to WSP would result in increased severity of influenza infection.

Methods

Mice

C57BL/6J 8–10 week old male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Experiments were conducted in accordance with National Institute of Health guidelines and protocols approved by the Animal Care and Use Committee at Duke University.

Wood Smoke Particle and Influenza Exposure Protocol

Previously characterized WSP were obtained as a kind gift from Dr. Andrew Ghio at the National Health and Environmental Effects Research Laboratory (US EPA, Chapel Hill, NC) [12]. The dry particles were suspended in sterile saline (0.9%) to generate a stock solution. Prior to administration to mice, the stock solution was sonicated using a F60 Sonic Dismembrator (Fischer Scientific) on ice for 20 secs. Aliquots were generated of the stock solution and frozen at −20°C. Prior to use, the aliquots were sonicated and vortexed to ensure even distribution of the particles. Mice underwent oropharyngeal aspiration of WSP (100 or 250μg in 50μL of volume) following isoflurane anesthesia. The WSP concentrations used in this study are based on prior WSP exposure studies [7, 8] and is consistent with other exposure studies using different PM sources [13, 14]. WSP concentrations were calculated to approximate high-dose human WSP exposures [7] and according to Kim et al. to approximate exposure conditions experienced by firefighters during wildfires [8]. Acute exposure studies involved a single exposure followed by necropsy at 24 or 48h post exposure. In the sub-acute exposure studies, mice were challenged with WSP on Monday (day 1), Wednesday (day 3), Friday (day 5) of week #1 and then Monday (day 8), and Wednesday (day 10) of week #2. The duration of sub-acute WSP exposures was chosen to model exposures that can occur during prolonged wildfire events. The alternating daily exposure is consistent with other oral aspiration sub-acute or sub-chronic exposures models [7] and was utilized for animal welfare to minimize potential for increased mortality from the daily anesthesia and administration of aspiration volume. At day 12, mice either underwent necropsy to assess the effects of sub-acute WSP exposure or were exposed to murine adapted H1N1 influenza (HINI-PR8 versus heat inactivated HINI-PR8) via oropharyngeal aspiration (Figure 1). Mice were exposed to 100 or 200 PFU of PR8 or heat inactivated virus in 50μL volume. The mice were necropsied at day 15 (3 days post viral exposure) or day 19 (7 days post viral exposure).

Bronchoalveolar lavage fluid (BALF) Protocol

Following euthanasia, mouse tracheas were cannulated with PE-60 tubing (Clay Adams, NJ connected to an infusion set (Terumo, Japan) and phosphate buffered saline infused via the tubing to 20 cm H_2O via connection to syringe on a ring stand until the lung reached total lung capacity. The fluid is then passively drained 3 times and the volume recorded. Cells from the BALF were isolated using centrifugation (1500 rpm, 10 min) and the supernatant used for assessment of total protein, albumin, protein carbonyls and hyaluronan. Cells were counted using a hemocytometer. Following determination of cell counts, cells were immobilized by cytospin, underwent Diff-Quik staining (Fischer Scientific, MA); and counted in a blinded fashion to define cell differentials.

Total Protein/Albumin, Protein Carbonyl Measurements

BAL total protein analysis was performed using Pierce BCA protein Assay kit (Thermo Scientific, Hercules, CA) per manufacturer's protocol. To measure BAL albumin, we used a Mouse Albumin ELISA kit (Immunology Consultants Laboratory, Inc.) per manufacturer's protocol. ELISA BAL samples were diluted in blocking buffer at a 1:1200 dilution. Protein carbonyls were assessed using Phosphatidylcholine Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). BAL fluid was added to colorimetric reaction mix and then absorbance was measured at 585–600nm using a plate reader. This was compared to standard curve generated by kit supplied standard.

Real Time PCR for Viral Titers and Viral Genes

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instruction. Every 100ng–500ng of RNA per sample was reversely transcribed into cDNA using first strand cDNA synthesis kit (Bio-Rad, Hercules, CA) and amplified for OPN mRNA expression with iTaq SYBR green master mix (Bio-Rad, Hercules, CA) in ABI 7300 real time PCR system (Applied Biosciences, Carlsbad, CA). Housekeeping gene 18s mRNA expression was used as internal control. RTPCR standard was generated by isolating total RNA from H1N1 stock of 70 million PFU and converted to cDNA as described above. cDNA was serially diluted 10-fold × 7 times, assuming 100% efficiency. Each dilution was assigned a PFU number and assumed as standard to create standard curve. Total RNA was isolated and converted to cDNA from pre-weighed lung-tissue from as described above with subsequent RTPCR. H1N1 forward primer: CAT CCT GTT GAT TAT GAG GCC CAT. H1N1 Reverse primer: GGA CTG CAG CGT AGA CGC TT. H1N1 primers for this study were generated by Charles River Laboratory (North Franklin, CT). Lung viral PFU was calculated by using standard curve generated from serial dilution by Ct value. Final H1N1 viral load was normalized from calculated PFU by measured lung weight (grams, PFU/gram).

Viral gene assay was performed using RT^2 PCR Array Mouse Antiviral Response (Qiagen, Geneglobe ID-PAMM-122ZA-2) by manufacturer's instruction.

Sphk1 and Sgp1 assessment performed by method described above. Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instruction. Every 100ng-500ng of RNA per sample was reversely transcribed into cDNA using first strand cDNA synthesis kit (Bio-Rad, Hercules, CA) and amplified for OPN mRNA expression with iTaq SYBR green master mix (Bio-Rad, Hercules, CA) in ABI 7300 real time PCR system (Applied Biosciences, Carlsbad, CA). Housekeeping gene 18s mRNA expression was used as internal control. Sphk1 forward primer: GCT GTC AGG CTG GTG TTA TG, Sphk1 reverse primer: ATA TGC TTG CCC TTC TGC AT. Sgpl1 forward primer: AAC TCT GCC TGC TCA GGG TA, Sgpl1 reverse primer: CTC CTG AGG CTT TCC CTT CT (Integrated DNA Technologies, Coralville, IA).

Whole Lung Flow Cytometry

The lungs were perfused with HBSS to remove residual RBCs from the pulmonary circulation. Finely minced tissues were then digested for 40 min at 37°C in HBSS with 1

mg/ml collagenase A (Roche, Indianapolis, IN) and 0.2 mg/ml DNase 1 (Sigma-Aldrich, St. Louis, MO). The digestion solution was passed through a 70 μm mesh strainer and centrifuged at 800 rpm for 10 min at 4°C. After RBC lysis, cells were suspended in PBS with 3% FBS, and 10 mM EDTA. To reduce non-specific binding, the cells were blocked with 1% FcBlock (BD Pharmingen, Franklin Lakes, NJ), 5% normal rat and mouse serum for 10 minutes at 4°C and then labeled for 30 minutes at 4°C. Two panels (macrophage and T cells) were utilized with the following antibody combinations: Macrophages: IA/IE – FITC (1:2000), Ly6-G – PE (1:200), CD11c – PE-Cy5.5 (1:800), Gr-1 – APC (1:250), CD11b – APC-Cy7 (1:200) and CD45 – PE-Cy7 (1:200) (from BD Pharmingen); Lymphocytes: CD3 – FITC (1:200), CD44 – PE (1:200), CD4 – PE-Cy5.5 (1:200), CD62L – APC (1:200), CD8 – APC-Cy7 (1:200) and CD19 – PE-Cy7 (1:200). After staining, cells were washed and analyzed on a BD Canto II flow cytometer. Data analysis was performed using FlowJo software (version 8.8.6, Ashland, OR).

Statistics

Data are expressed as mean + standard error. The statistical difference between groups was assessed by one-way ANOVA followed by Tukey test for multiple comparisons. A p value of < 0.05 was considered statistically significant. All statistics were performed using GraphPad Prism version 9.0.

Results

Acute exposure to wood smoke particles (WSP) increases airspace inflammation in C57BL/6J Mice

To define the impact of wood smoke particle exposure on pulmonary inflammation, we utilized previously characterized WSPs [12] in an acute exposure model. We first defined a dose-response relationship of the WSP *in vivo*. Our initial doses were based on prior work by Sussan et al. [7] and Kim et al. [8] C57BL/6J mice were exposed by oropharyngeal aspiration to either 10ug, or 100ug WSP. Neither dose resulted in enhanced airspace inflammation at either 24 or 48h post-exposure (data not shown). Additional exposures were performed with 250ug of WSP. C57BL/6J mice were necropsied at 24 and 48h post-exposure and underwent BAL to assess airspace inflammation. Compared to PBS control mice, BAL total cell counts were significantly elevated after acute WSP exposure at both the 24 and 48h time points (Figure 2A). While BAL neutrophils were only increased at 48 hours, macrophage count increased significantly at both 24 and 48h after WSP exposure (Figure 2B, C). The effects of increased airspace inflammation were more prominent at 48h post-exposure.

Sub-acute WSP exposure causes neutrophil-predominant inflammation in C57J/BL6 mice

Following characterization of acute response to WSP, we then characterized the impact of sub-acute WSP exposure to model more prolonged exposures observed during wildfires. To investigate this impact, we developed a sub-acute exposure model where C57J/BL6 male mice were exposed to either 100ug and 250ug of WSP via oropharyngeal aspiration on days 1, 3, 5, 7, 10. At day 12, 48h following final exposure, the mice were assessed for airspace inflammation (Figure 1). We observed an increase in total BAL cellularity and BAL

neutrophil count with 250ug WSP exposure (Figure 3A, C). BAL macrophages were also increased, though this did not reach statistical significance (Figure 3B). Airspace neutrophils were increased in the 100ug WSP exposure group to a lesser degree than in the 250μg exposure group (Figure 3C). Furthermore, though there were trends towards increased BAL total cells and macrophages in the 100ug WSP exposure group, these were not statistically different from PBS groups. These results support that sub-acute WSP exposure resulted in a dose dependent increase in airspace inflammation.

Sub-acute exposure to WSP reduces airspace inflammation and viral replication following influenza (H1N1-PR8) infection

To understand the impact of WSP exposure on host immune response to viral respiratory infection, we developed a model of sub-acute WSP exposure followed by challenge with influenza (HINI-PR8) and then defined effects on inflammation and viral titer level C57BL/6J male mice were exposed to 100μg or 250μg of WSP on days 1, 3, 5, 7, and 10 to model sub-acute exposure followed by 100 pfu of HINI-PR8 or HI-HINI-PR8 on day 12 to model acute viral infection. Mice were necropsied at day 15 (3d post exposure). BAL was performed to assess airspace inflammation and lung tissue was harvested to define viral titers. When compared to PBS control, mice sub-acutely exposed to 100ug WSP prior to viral infection demonstrated suppression of total BAL cellularity, macrophage and neutrophil counts (Figure 4A–C), while BAL total protein remained unchanged (Figure 4D). Similarly, PR8 viral titers were also suppressed (Figure 4E). Alternatively, mice exposed to 250ug WSP and then virally infected did not have evidence of a reduction in BAL cells or macrophages, though there was a trend for a reduction in neutrophils (Figure 4A–C). Consistent with the response at the 100μg exposure, viral titers were suppressed after 250ug WSP exposure (Figure 4E), though this suppression was less than that seen in the 100μg exposure group. This suggests that sub-acute WSP exposure reduced respiratory viral infection.

Sub-acute WSP exposure reduces the severity of influenza infection

In addition to the observation of reduced airspace inflammation and viral titers, we evaluated the impact of WSP on the immune response to influenza infection. We focused on the 100μg WSP sub-acute exposure as this had the improved suppressive effect in the prior studies, while generating less inflammation in the HI groups. Furthermore, to model severe viral pneumonia, an increased dose of HINI-PR8 (250 pfu) was administered and the outcomes were assessed post infection (p.i.) at time points focused on initiation and peak inflammation based on prior studies [15]. Groups of C57BL/6J mice were exposed to 100ug WSP or PBS on days 1, 3, 5, 8 and 10. Then on day 12 mice were infected with 250 pfu of HINI-PR8 or HI-HINI-PR8. BAL was performed and lung tissue was harvested at day 15 (3 days p.i.) or day 19 (7 days p.i.). At day 15, we observed a slight increase in BAL total cells and macrophages in the WSP-HINI-HI versus PBS-HINI-HI groups. When comparing the PBS-HINI-PR8 and WSP-HINI-PR8 groups, we noted increased BAL cells, macrophages and neutrophils (Figure 5A) in the WSP-HINI-PR8 group. At day 19, no difference in the PBS-HINI-HI or the WSP-HINI-HI groups was seen. In the PBS-HINI-PR8 and WSP-HINI-PR8 groups, we noted similar levels of BAL cellularity, macrophage, and neutrophil counts (Figure 5B). Assessing viral titers from whole lung tissue, we observed no change in viral

titers in the HI control groups (Figure 5C). In the H1N1-PR8 exposed groups, we observed an increase in viral titers at day 15 without differences between the PBS or the WSP exposure groups. Alternatively, at day 19, the WSP-H1N1-PR8 group had significantly lower viral titers when compared to the PBS-PR8 group (Figure 5C). These data suggest that prior exposure to 100ug WSP exposure results in lower viral titers with a 250 pfu dose of H1N1-PR8.

To clarify the consequences of WSP exposure on the severity of PR8 infection we measured the impact on physiologic, injury and oxidant stress measures. Weight loss is associated with severe rodent PR8 infection and is a relevant measure of severity [16–19]. Therefore, weight was measured at time of PR8 virus exposure on day 12, then daily through day 19 and then expressed as a percent change from baseline. The HI-H1N1-PR8 exposure groups did not have evidence of weight loss but rather demonstrated a slight weight gain through day 19 (Figure 6A). Alternatively, the H1N1-PR8 exposed groups demonstrated weight loss consistent with response to viral infection. The PBS-H1N1-PR8 group exhibited weight loss starting at days 16–19. Though the WSP-H1N1-PR8 group developed weight loss, this was only present at day 19 and was reduced compared to the PBS-H1N1-PR8 exposure group (Figure 6A). To assess the impact on oxygen level as a measure of hypoxemia from lung injury, mouse oxygen saturation was assessed prior to necropsy at day 19. Again the heat-inactive groups did not experience a reduction in oxygen level (Figure 6B). The H1N1-PR8 infected groups did have evidence of reduced oxygen saturation compared to their HI controls. However, the reduction in saturation was more severe in the PBS-H1N1-PR8 group as opposed to the WSP-H1N1-PR8 exposed mice. Protein carbonyl formation, a measure of oxidant stress, was also assessed. Protein carbonyls were not changed in the PBS or WSP exposed groups prior to H1N1-PR8 infection (Figure 6C, Day 12). Additionally, there was no difference in the protein carbonyls in the heat inactive or H1N1-PR8 infected groups at day 15. At day 19, during peak inflammation, protein carbonyls were increased in the PBS-H1N1-PR8 exposed mice when compared to their heat inactive control group. Interestingly the WSP-H1N1-PR8 exposed mice were not different than their heat inactive control group and was reduced compared to PBS-H1N1-PR8 exposed mice (Figure 6C, Day 19) suggesting that the WSP exposure prior to infection reduces a measure of oxidant stress following influenza infection. BAL albumin was assessed as a measure of alveolar-capillary permeability. BAL albumin levels were not different in the groups prior to H1N1-PR8 infection (Figure 6D, Day 12) or 3 days post infection (Figure 6D, Day 15). Though the BAL albumin levels were increased at day 19 in the PR8 exposed groups, there was not a difference between the PBS or WSP groups (Figure 6D, Day 19). A similar observation was noted for BAL hyaluronan, a known extracellular matrix component upregulated in lung injury and influenza infection [20, 21], with increased BAL levels at day 19 but without difference between the PBS and WSP H1N1-PR8 infected groups (Supplemental Figure 1). Overall, these findings support decreased severity of H1N1-PR8 viral infection among mice who had previously exposed to sub-acute WSP.

Whole lung flow cytometry reveals increased Gr-1+ macrophages in WSP exposed mice

To define potential mechanisms underlying the reduction in the immune response to influenza infection following sub-acute WSP exposure, we performed whole lung flow

cytometry to assess differences in immune cell populations. We focused on macrophages and T cells due to their known roles in influenza responses [22, 23]. Whole lung T cells subsets were assessed for CD4 and CD8 cells as well as naïve, activated and central memory and effector memory subsets based on a previously published panel [24]. T cell subsets were reported as a percentage of total cells from prior to HINI-PR8 infection on day 12, and at days 15 and 19. T cell subsets were not largely different between the PBS or WSP exposed groups (Supplemental Figure 3). We observed changes in T cell subsets based on comparisons between the HI-H1N1-PR8 and HINI-PR8 infections, but we did not observe a difference in T-cell subsets between the PBS-H1N1-PR8 and WSP-H1N1-PR8 exposure groups, suggesting that T-cells subtypes did not drive the difference in influenza severity. However, we did observe differences in macrophage subsets. Using a previously published flow cytometry panel for macrophages [25, 26], we defined alveolar macrophages and exudative macrophages from whole lung tissue following WSP exposure and H1N1-PR8 infection. Following WSP exposure, when compared to PBS exposure, we identified alveolar macrophages (singlet, CD45$^+$, Ly6G$^-$, CD11c$^+$, CD11b$^-$) but also observed a subset of alveolar macrophages with enhanced Gr-1 cell surface expression (Supplemental Figure 2). The presence of alveolar macrophages, Gr-1+ macrophages and exudative macrophages (singlet, CD45$^+$, Ly6G$^-$, CD11c$^+$, CD11b$^+$) were quantified and expressed as a percentage of total cells at day 12, 15 and 19. Alveolar macrophages proportions were largely unchanged between the PBS or WSP exposure groups and the heat inactive versus H1N1-PR8 infection with the exception of day 19, where the alveolar macrophage proportions were reduced compared to the respective heat-inactive groups (Figure 7). Gr-1$^+$ macrophages as a proportion of total cells were increased following WSP exposure. This increase persisted in the WSP-HINI-PR8 exposed mice at day 15 (Figure 7B) but then were not different between the PBS-H1N1-PR8 exposed mice at day 19 (Figure 7C). Exudative macrophages were only increased at day 19 in the H1N1-PR8 infected groups and were largely the same between the PBS-HINI-PR8 and WSP-HINI-PR8 groups. This data suggests that macrophage subsets are altered following WSP exposure with an increase in Gr-1$^+$ macrophages.

WSP exposure alters mRNA transcription of genes related to anti-viral response

Whole lung RNA transcriptome analyses were performed to define potential genetic pathways regulating the immune response to influenza following WSP exposure using a PCR profiler array targeting various transcription products relevant to anti-viral activity (Supplemental Table 1). We observed genes that were both increased and decreased in the WSP exposed mice as opposed to the PBS control mice. mRNA transcripts increased following WSP exposure included Card9, Casp1, Ccl3, Chuck, Cxcl9, and Cxcl10; though none met statistical significance by paired t-test. mRNA transcripts decreased following WSP exposure included Ccl5, CD40, Cxcl11, Fos, Ifih1, Ifna2, Ifnb1, IL12a. Of these, only the decrease in Fos (p=0.0019) reached statistical significance.

In addition to these genes, previous work suggested that sphingosine kinases and lyases are critical to anti-viral influenza responses in mice [27, 28]. Therefore, we defined gene expression for sphingosine kinase (SphK1) and sphingosine lyase (Sglp-1) by Real Time PCR following sub-acute WSP (Figure 8A, day 12) exposure and at day 15 and day 19

following H1N1-PR8 infection. We observed that both SphK-1 and Sglp-1 gene expression was decreased in the WSP compared to PBS exposed mice at the d12 time point (Figure 8B). Interestingly, SphK1 gene expression was largely unchanged amongst the groups at day 15 but then was decreased in the WSP-H1N1-PR8 infected mice at day 19 (Figure 8C), which associated with the reduction in viral titers. This data is consistent with prior data suggesting that decreased SphK-1 activity enhanced anti-influenza activity [27]. Alternatively, Sglp-1 mRNA was largely unchanged amongst the PBS and WSP groups at either day 15 or 19.

Discussion

In the current study, we examined the effect of WSP exposure on the host pulmonary immune response to influenza infection. We developed a novel model to study the effect of sub-acute WSP exposure on viral inflammation. Counter to our initial hypothesis, at a WSP exposure dose that did not exhibit significant airspace inflammation, we observed that sub-acute WSP exposure decreased viral titers following infection with influenza and associated with a reduction in body weight loss, improved oxygen saturation and a reduction in oxidant stress. This reduction in viral titers and improvement in influenza severity was associated with an increase in a Gr-1$^+$ macrophage subset and a reduction in the gene expression of sphingosine kinase-1 suggesting that WSP exposure alters immune cell composition and expression of pathways that may regulate influenza propagation. Overall, this data suggests, in a murine model of sub-acute WSP exposure followed by influenza infection, that WSP exposure reduces overall severity of viral infection.

Though increased WSP exposures clearly associate in epidemiologic studies with exacerbations of chronic lung disease and with the presence of systemic inflammation, the effects of WSP on influenza infection and its impact on the pulmonary immune response to viral infection have not been well characterized. Furthermore, based on our review of the present literature, WSP exposure and influenza infection have not been modeled in rodents. To address this, we performed acute and sub-acute WSP exposure studies to define pulmonary effects of the WSP and then assess the effect on influenza responses. We used previously characterized WSP obtained from investigators at the US EPA. In the acute exposure model, consistent with prior acute human and rodent exposure studies [7, 10, 29], we observed an increase in airspace total macrophages and neutrophils in a time and dose-dependent manner. As most exposures to WSP are not a single acute exposure, we also performed sub-acute exposures to model exposures that could occur with wildfire events. These events are becoming a more regular source of human air pollution as wildfires are occurring more frequently as a consequence of increased extreme drought [30] and urbanization [31]. These sub-acute WSP exposures did not result in significant airspace inflammation. To model a subsequent influenza infection, the sub-acute WSP exposed mice were then infected with HINI-PR8. Interestingly, we observed reduction in viral titers and an improvement in the severity of physiologic responses to infection. These findings were contrary to our initial hypothesis and the prior data suggesting that WSP exposure associated with worsening of chronic lung diseases and reduced anti-viral responses [2–4, 9]. For example, our findings are in contrast to work done by Reubli et. al. where a single acute WSP exposure in human subjects followed by live attenuated viral infection resulted in a

reduction in the anti-viral CXCL10 in the WSP exposed group. This data suggested that viral responses could be enhanced by the WSP exposure. A possible explanation is that sub-acute WSP exposure responses may be distinct from acute exposure viral responses. Alternatively, the differences could be a result of sex as they observed sex-dependent effects. Our study only performed exposures in male mice and therefore did not consider sex as an effect modifier. Regardless, our data suggests a more complicated effect of WSP exposure to viral infection than observed in prior controlled exposure and epidemiologic studies supporting that further evaluation is required.

To explore potential mechanisms underlying the WSP influenza responses we considered alterations in immune cell profiles and in genetic pathways associated with anti-viral responses. We focused on macrophage and T cell subsets due to their known modulation of influenza infections [22, 23]. Interestingly, though we did not identify effects of WSP exposure on T cell subsets, we did observe changes in macrophage subsets. In particular, we observed an increase in alveolar macrophages with elevated Gr-1 cell surface expression following WSP exposure. These, Gr-1^{+} macrophages are consistent with our prior description of Gr-1^{+} macrophages that upregulated genes associated with detoxification, and reduced ozone-induced acute lung injury and airway hyperresponsiveness [22, 23]. In non-pulmonary tissues, Gr-1^{+} macrophages have roles in infectious parasite responses in gut tissue [32], anti-viral activity in murine cardiac tissue [33], and the enhancement of phagocytic activity following hemorrhagic stroke [34]. This suggests that beyond their effects on ozone exposure responses, Gr-1^{+} may also have a role in promoting viral clearance or reducing the severity of viral infection. Future studies could explore the direct effects of Gr-1^{+} macrophages on clearance of influenza virus and/or by clearance of viral intermediates to limit the severity of acute lung injury associated with infection.

In addition to alterations in immune cell profiles, we observed effects on gene expression following WSP exposure. We first defined gene expression in a panel of genes associated with anti-viral responses. This demonstrated differential expression of genes of varying pathways important for anti-viral response, only one of which (*Fos*) achieved statistical significance (Supplemental Table 1). *Fos* has been identified as part of the IFNAR-MAPK-Fos-CHD6 pathway and has been shown to be important in IFN-K mediated suppression of influenza a replication[35]. As our gene profiling data did not implicate a consistent anti-viral pathway modified by WS exposure, we then focused on the activities of sphingosine kinase and lyase. This is based on previous work that demonstrated inhibition of sphingosine kinase (SphK-1) results in protection against influenza A infection in mice while sphingosine 1-phosphate lyase (Sgpl-1) worsens influenza infection. Mechanistically, SphK-1 expression is increased by the influenza virus which then regulates viral RNA synthesis and nuclear export of viral ribonucleoprotein complexes. When inhibited, there is suppression of NF-kB activation and reduction in synthesis of viral RNA and proteins[27]. We identified that SphK-1 mRNA demonstrated a trend towards reduction in groups of mice that were exposed to WSP prior to H1N1-PR8 infection. At day 19 (7d p.i.), where viral titers were reduced, there was also a reduction in SphK-1 mRNA. As there was associated reductions in viral titers at this time point, it could suggest that reduction of SphK-1 could be a downstream effect of WSP exposure resulting in anti-viral activity and improvement in influenza severity. However, this effect was not directly confirmed in these studies and will

be a subject of future studies. Alternatively, Sgpl-1 promotes immune responses to influenza A infection[28]. Sgpl-1 is a positive regulator of IKKe, whose activity is enhanced in IFN-mediated immune responses to influenza A. Inhibition of Sgpl-1 activity results in reduction of anti-influenza activity. Our data demonstrate an initial suppression of Sgpl-1 mRNA following WSP exposure when compared to PBS. However, there was no difference between the PBS-HINI-PR8 and WSP-H1N1-PR8 groups at day 15 and 19. This data could suggest that WSP exposure may result in improved anti-viral signaling through SphK-1. Future studies will need to clarify the mechanism of reduced SphK-1 in the WSP-H1N1-PR8 infection.

There are limitations to this study. First, environmental wood smoke is a complex substance made up of particulate matter and gaseous elements whose composition is variable based on the underlying burning material [36, 37]. Therefore, our data may not reflect responses to wood smoke particles derived from different burning materials or the type of combustion. Second, we focused on male mice for this study. This is despite well-established data suggesting that influenza responses in mice exhibit sex-dependence [38]. It is possible that WSP-HINI-PR8 responses might differ in female mice as opposed to the male mice used in these studies. Third, though heat inactivation is a standard design for providing a viral infection control, it is possible that HI samples may interact with the WSP to alter inflammation. This, in addition to known variations in cell counts and differentials between individual experiments, could be the reason some of the intra-experimental variation in these measures observed in this study. Fourth, while we attempted to address transcription level changes following WSP exposure, we focused on those known to be responsible for anti-viral responses. However, there are likely other genetic pathways that are not anti-viral pathways that could be regulated by WSP exposure and could alter the severity of influenza infection. Further studies will consider additional downstream effects of WSP exposure that could lead to altered influenza responses.

In summary, in this study, we demonstrate that murine WSP exposure prior to H1N1-PR8 viral infection confers protective benefit, reducing both pulmonary viral titers and physiologic parameters of influenza severity. This effect was associated with an increase in Gr-1^{+} macrophages and reductions in SphK-1 gene expression. This data suggests that sub-acute WSP exposure has a protective effect against the severity of influenza infection and may need to be considered as we determine the relationship between ever increasing wildfires and the incidence and severity of respiratory viral infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

1. Johnston FH, et al., Estimated global mortality attributable to smoke from landscape fires. Environ Health Perspect, 2012. 120(5): p. 695–701. [PubMed: 22456494]

2. Liu JC, et al., A systematic review of the physical health impacts from non-occupational exposure to wildfire smoke. Environ Res, 2015. 136: p. 120–32. [PubMed: 25460628]

3. Tinling MA, et al., Repeating cardiopulmonary health effects in rural North Carolina population during a second large peat wildfire. Environ Health, 2016. 15: p. 12. [PubMed: 26818940]

4. Youssouf H, et al., Non-accidental health impacts of wildfire smoke. Int J Environ Res Public Health, 2014. 11(11): p. 11772–804. [PubMed: 25405597]

5. Tan WC, et al., The human bone marrow response to acute air pollution caused by forest fires. Am J Respir Crit Care Med, 2000. 161(4 Pt 1): p. 1213–7. [PubMed: 10764314]

6. van Eeden SF, et al., Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM(10)). Am J Respir Crit Care Med, 2001. 164(5): p. 826–30. [PubMed: 11549540]

7. Sussan TE, et al., Source of biomass cooking fuel determines pulmonary response to household air pollution. Am J Respir Cell Mol Biol, 2014. 50(3): p. 538–48. [PubMed: 24102120]

8. Kim YH, et al., Mutagenicity and Lung Toxicity of Smoldering vs. Flaming Emissions from Various Biomass Fuels: Implications for Health Effects from Wildland Fires. Environ Health Perspect, 2018. 126(1): p. 017011. [PubMed: 29373863]

9. Rebuli ME, et al., Wood Smoke Exposure Alters Human Inflammatory Responses to Viral Infection in a Sex-Specific Manner. A Randomized, Placebo-controlled Study. Am J Respir Crit Care Med, 2019. 199(8): p. 996–1007. [PubMed: 30360637]

10. Ghio AJ, Particle exposures and infections. Infection, 2014. 42(3): p. 459–67. [PubMed: 24488331]

11. Migliaccio CT, et al., Adverse effects of wood smoke PM(2.5) exposure on macrophage functions. Inhal Toxicol, 2013. 25(2): p. 67–76. [PubMed: 23363038]

12. Ghio AJ, et al., Exposure to wood smoke particles produces inflammation in healthy volunteers. Occup Environ Med, 2012. 69(3): p. 170–5. [PubMed: 21719562]

13. Gilmour MI, et al., Comparative toxicity of size-fractionated airborne particulate matter obtained from different cities in the United States. Inhal Toxicol, 2007. 19 Suppl 1: p. 7–16. [PubMed: 17886044]

14. Kim YH, et al., Comparative lung toxicity of engineered nanomaterials utilizing in vitro, ex vivo and in vivo approaches. J Nanobiotechnology, 2014. 12: p. 47. [PubMed: 25424549]

15. Lin KL, et al., CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. J Immunol, 2008. 180(4): p. 2562–72. [PubMed: 18250467]

16. Milner JJ, et al., Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice. J Immunol, 2015. 194(10): p. 4846–59. [PubMed: 25862817]

17. Sadasivan S, et al., Induction of microglia activation after infection with the non-neurotropic A/CA/04/2009 H1N1 influenza virus. PLoS One, 2015. 10(4): p. e0124047. [PubMed: 25861024]

18. Gonzalez AJ, et al., Attenuation of Influenza A Virus Disease Severity by Viral Coinfection in a Mouse Model. J Virol, 2018. 92(23).

19. Trammell RA and Toth LA, Markers for predicting death as an outcome for mice used in infectious disease research. Comp Med, 2011. 61(6): p. 492–8. [PubMed: 22330575]

20. Bell TJ, et al., Defective lung function following influenza virus is due to prolonged, reversible hyaluronan synthesis. Matrix Biol, 2019. 80: p. 14–28. [PubMed: 29933044]

21. Tighe RM and Garantziotis S, Hyaluronan interactions with innate immunity in lung biology. Matrix Biol, 2019. 78–79: p. 84–99.

22. Hufford MM, et al., The effector T cell response to influenza infection. Curr Top Microbiol Immunol, 2015. 386: p. 423–55. [PubMed: 25033753]

23. Tate MD, et al., Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice. J Virol, 2010. 84(15): p. 7569–80. [PubMed: 20504924]

24. Pastva AM, et al., Lung effector memory and activated CD4+ T cells display enhanced proliferation in surfactant protein A-deficient mice during allergen-mediated inflammation. J Immunol, 2011. 186(5): p. 2842–9. [PubMed: 21257967]

25. Tighe RM, et al., Ozone inhalation promotes CX3CR1-dependent maturation of resident lung macrophages that limit oxidative stress and inflammation. J Immunol, 2011. 187(9): p. 4800–8. [PubMed: 21930959]

26. Tighe RM, et al., Recruited exudative macrophages selectively produce CXCL10 after noninfectious lung injury. Am J Respir Cell Mol Biol, 2011. 45(4): p. 781–8. [PubMed: 21330464]

27. Seo YJ, et al., Sphingosine kinase 1 serves as a pro-viral factor by regulating viral RNA synthesis and nuclear export of viral ribonucleoprotein complex upon influenza virus infection. PLoS One, 2013. 8(8): p. e75005. [PubMed: 24137500]

28. Vijayan M, et al., Sphingosine 1-Phosphate Lyase Enhances the Activation of IKKepsilon To Promote Type I IFN-Mediated Innate Immune Responses to Influenza A Virus Infection. J Immunol, 2017. 199(2): p. 677–687. [PubMed: 28600291]

29. Hargrove MM, et al., Smoldering and flaming biomass wood smoke inhibit respiratory responses in mice. Inhal Toxicol, 2019. 31(6): p. 236–247. [PubMed: 31431109]

30. Littell JS, et al., A review of the relationships between drought and forest fire in the United States. Glob Chang Biol, 2016. 22(7): p. 2353–69. [PubMed: 27090489]

31. Radeloff VC, et al., Rapid growth of the US wildland-urban interface raises wildfire risk. Proc Natl Acad Sci U S A, 2018. 115(13): p. 3314–3319. [PubMed: 29531054]

32. Dunay IR, Fuchs A, and Sibley LD, Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. Infect Immun, 2010. 78(4): p. 1564–70. [PubMed: 20145099]

33. Xu D, et al., Gr-1+ Cells Other Than Ly6G+ Neutrophils Limit Virus Replication and Promote Myocardial Inflammation and Fibrosis Following Coxsackievirus B3 Infection of Mice. Front Cell Infect Microbiol, 2018. 8: p. 157. [PubMed: 29868513]

34. Hammond MD, Ai Y, and Sansing LH, Gr1+ Macrophages and Dendritic Cells Dominate the Inflammatory Infiltrate 12 Hours After Experimental Intracerebral Hemorrhage. Transl Stroke Res, 2012. 3(1): p. s125–s131. [PubMed: 23259009]

35. He Y, et al., IFN-kappa suppresses the replication of influenza A viruses through the IFNAR-MAPK-Fos-CHD6 axis. Sci Signal, 2020. 13(626).

36. Bolling AK, et al., Wood smoke particles from different combustion phases induce similar pro-inflammatory effects in a co-culture of monocyte and pneumocyte cell lines. Part Fibre Toxicol, 2012. 9: p. 45. [PubMed: 23176191]

37. Naeher LP, et al., Woodsmoke health effects: a review. Inhal Toxicol, 2007. 19(1): p. 67–106.

38. Klein SL and Flanagan KL, Sex differences in immune responses. Nat Rev Immunol, 2016. 16(10): p. 626–38. [PubMed: 27546235]

Highlights

- Sub-acute wood smoke particle (WSP) exposure in mice increases lung inflammation

- WSP exposure and subsequent influenza infection reduces influenza severity

- Improved severity associated with altered lung macrophages and viral gene expression

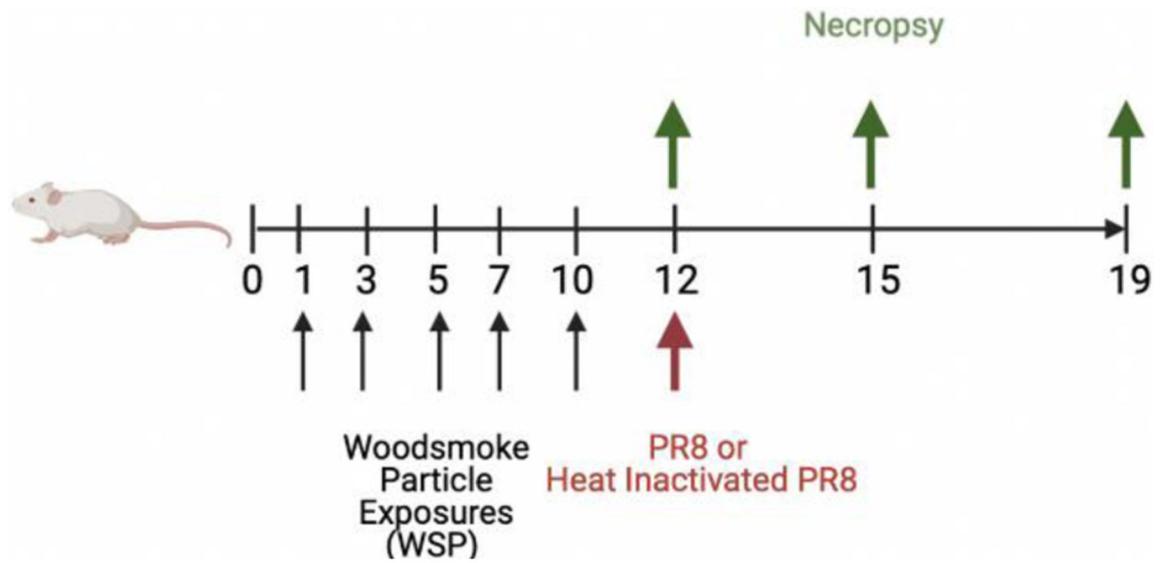- This supports that WSP exposure reduces the severity of viral infection in mice

Figure 1-

Overview of experimental design for sub-acute wood smoke particle (WSP) exposure followed by H1N1-PR8 infection. C57BL/6 male mice underwent exposure to WSP on alternating days. At day 12, mice were either necropsied to define sub-acute WSP effects or underwent infection with heat-inactivated (HI) or live H1N1-PR8. The mice were then monitored post-infection with assessments at day 15 and 19 to define influenza responses.

Figure 2 –.
Acute wood smoke particle (WSP) exposure increased airspace inflammation. C57BL6 male mice were exposed to 250 μg WSP by oropharyngeal aspiration and then underwent bronchoalveolar lavage (BAL) at 24h and 48h post exposure. BAL total cell counts (A), macrophages (B) and neutrophils (PMN, C) were assessed. N=4 mice per group, *p<0.05
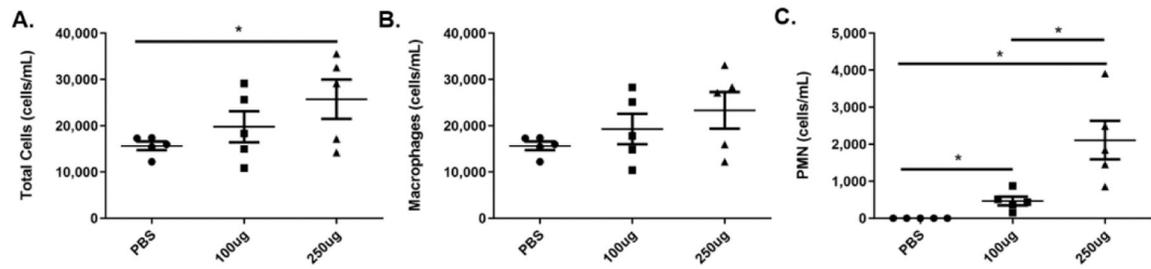
Figure 3 –.
Sub-acute wood smoke particle (WSP) exposure increased airspace neutrophils in a dose dependent fashion. C57BL6 male mice were exposed to PBS, 100μg or 250μg WSP by oropharyngeal aspiration at day 1, 3, 5, 8 and 10 and then underwent bronchoalveolar lavage (BAL) 48h post exposure (day 12). BAL total cell counts (A), macrophages (B) and neutrophils (PMN, C) were assessed. N=5 mice per group, *p<0.05
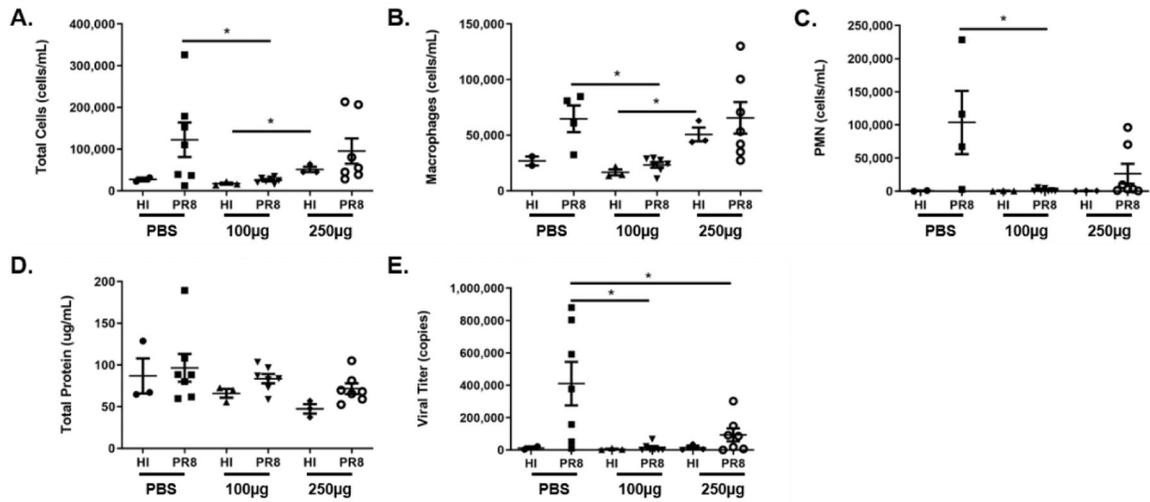
Figure 4 –.

Dose response of sub-acute wood smoke particle (WSP) exposures followed by PR8 or heat inactivated virus (100 pfu). C57BL6 male mice were exposed to PBS, 100μg or 250μg WSP by oropharyngeal aspiration at day 1, 3, 5, 8 and 10 and then underwent PR8 or heat-inactivated (HI) exposure (day 12). Mice were then harvested 3 days post exposure (day 15) for bronchoalveolar lavage (BAL) and lung tissue harvest. BAL total cell counts (A), macrophages (B) and neutrophils (PMN, C) and total protein (D) were assessed. Lung tissue was processed for PCR to define viral copy number (E). N=3–7 mice per group, *p<0.05
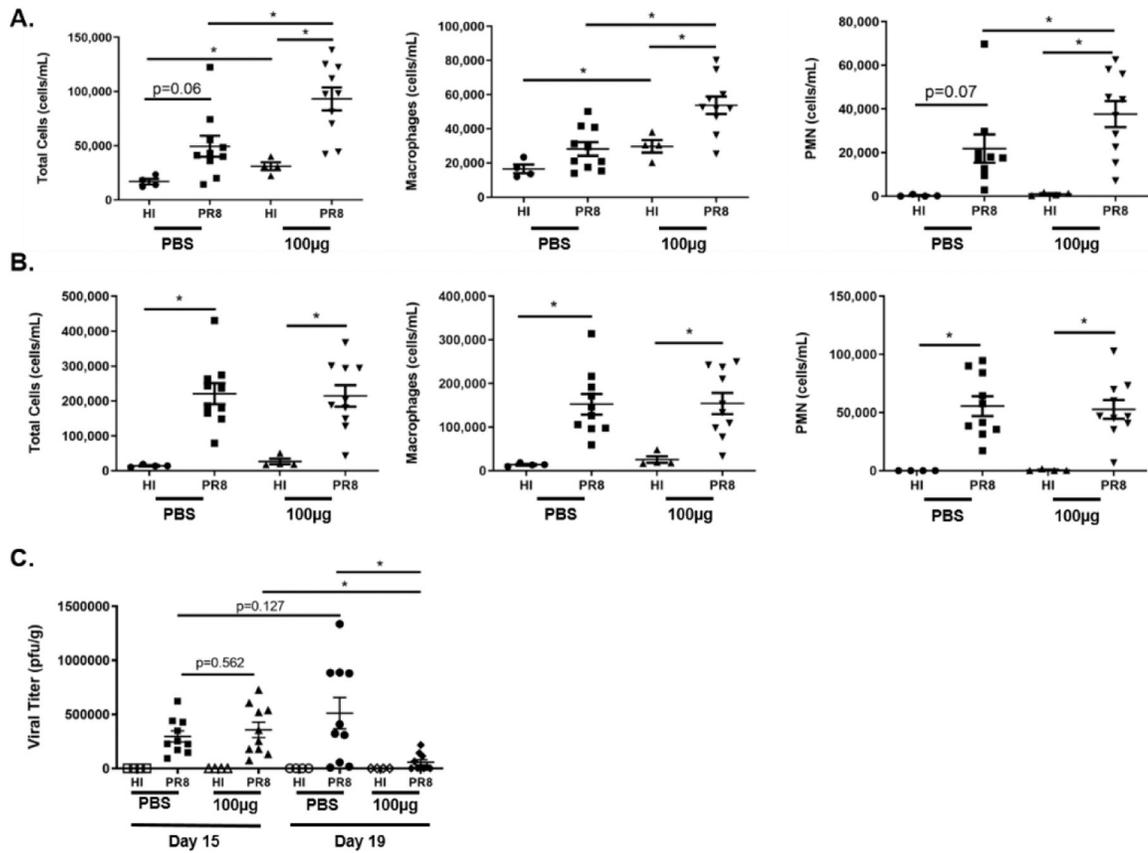
Figure 5 –.

Bronchoalveolar lavage airspace inflammation and viral titers in sub-acute wood smoke particle (WSP) exposure mice with PR8 infection. C57BL6 male mice were exposed to PBS, or 100μg WSP by oropharyngeal aspiration at day 1, 3, 5, 8 and 10 and then underwent PR8 (250 pfu) or heat-inactivated (HI) exposure (day 12). Mice were then harvested 3 days (day 15) or 7 days (day 19) post exposure for bronchoalveolar lavage (BAL) and lung tissue harvest. BAL total cell counts and differentials for macrophages and neutrophils were assessed at day 15 (A) and day 19 (B). Lung tissue was processed for PCR to define viral copy number at day 15 and 19 (C). N=4–10 mice per group, *p<0.05
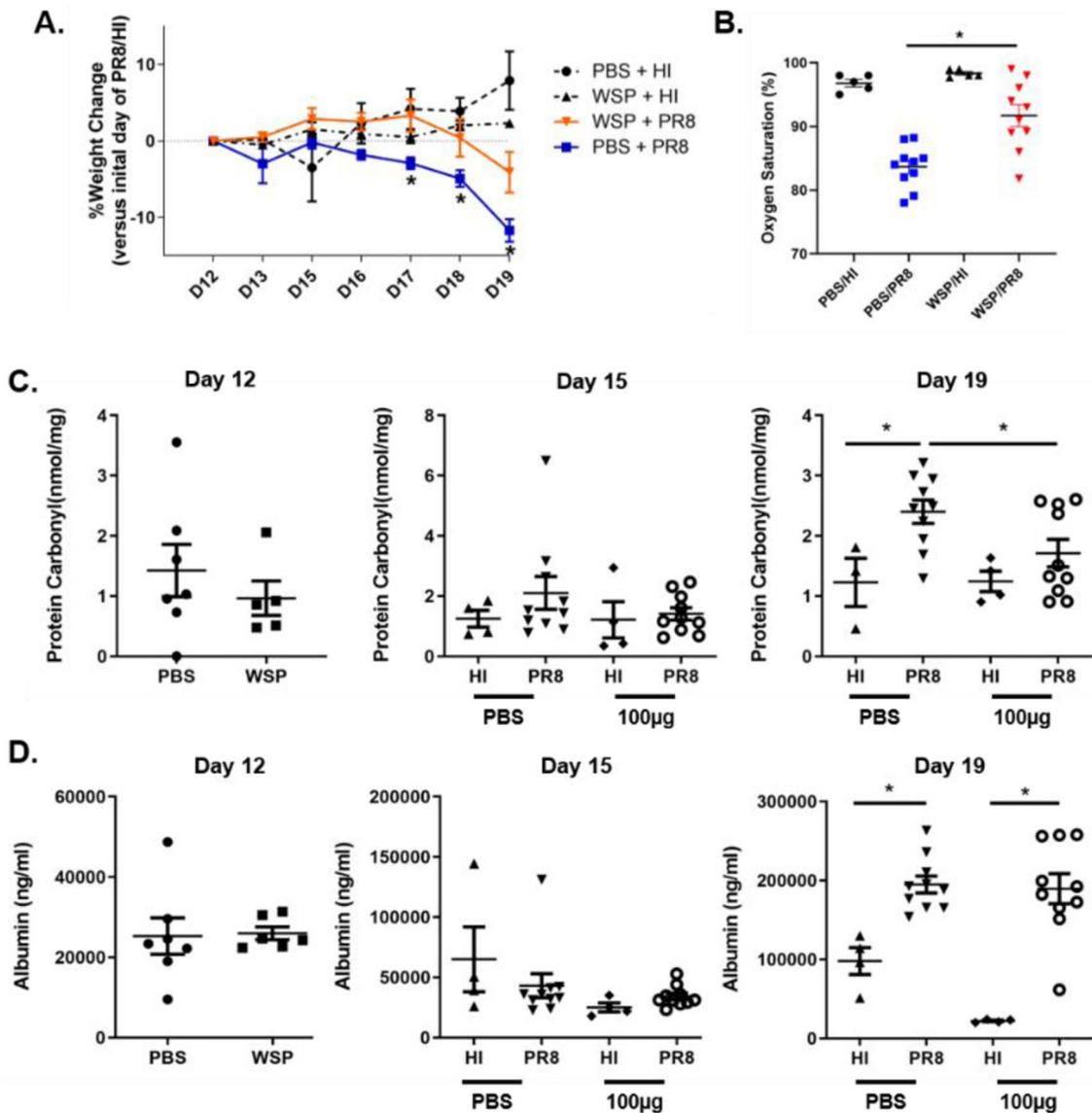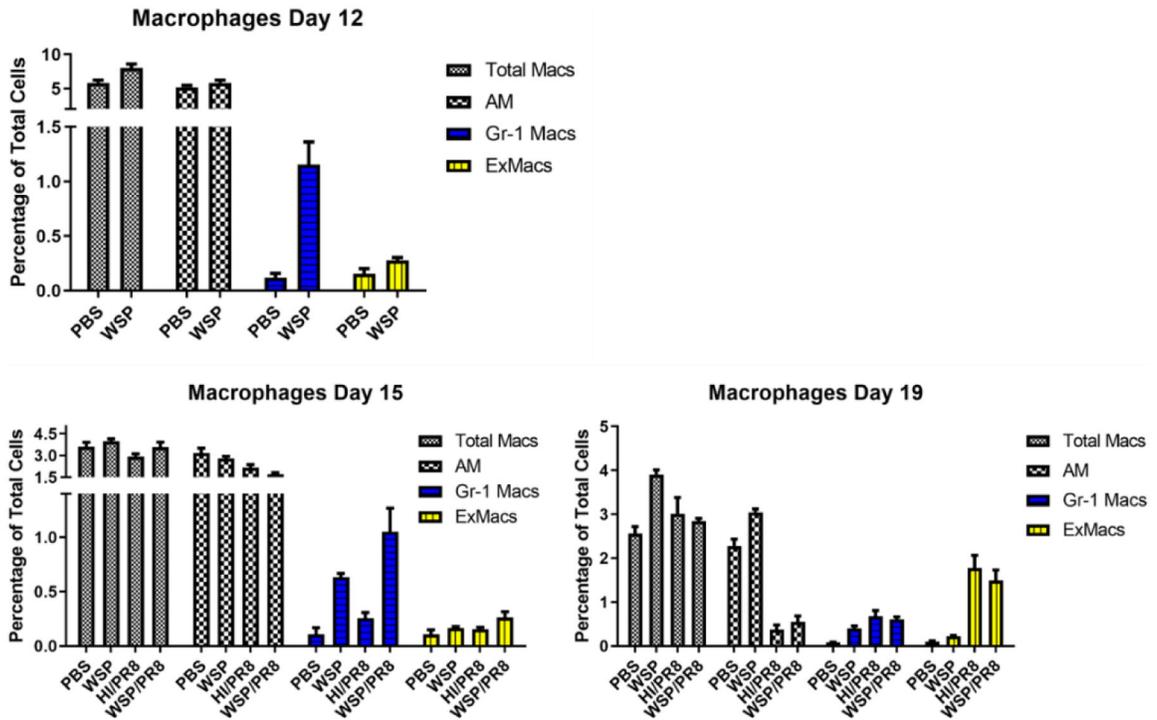
Figure 6 –.

Assessments of physiologic, oxidant stress and lung injury in wood smoke particle (WSP) exposure and PR8 infected mice. C57BL6 male mice were exposed to PBS, or 100μg WSP by oropharyngeal aspiration at day 1, 3, 5, 8 and 10 and then were harvested at day 12 or underwent PR8 or heat-inactivated (HI) exposure (250 pfu). Mice weights were assessed daily till harvest and expressed as a percent change from baseline (A). Additionally, mouse oxygen saturation was assessed at day 19 prior to harvest (B). Mouse groups were harvested 3 days (day 15) or 7 days (day 19) post exposure for bronchoalveolar lavage (BAL) and lung tissue harvest. BAL fluid was assessed for protein carbonyls (C) and albumin N=4–10 mice per group, *p<0.05

Figure 7-

Macrophage subset phenotypes following sub-acute wood smoke particle (WSP) exposure and PR8 infection. C57BL6 male mice were exposed to PBS, or 100μg WSP by oropharyngeal aspiration at day 1, 3, 5, 8 and 10 and then were harvested at day 12 or underwent heat-inactivated (HI) exposure or PR8 (250 pfu) infection and were harvested at day 15 and 19. Whole lung tissue was digested to obtain a single cell suspension and then stained for flow cytometry. Macrophage groups were defined by flow cytometry and reported as a percentage of total cells. N = 4 mice per group, blue shaded = Gr-1 positive macrophages, yellow shaded = Exudative macrophages (ExMacs)
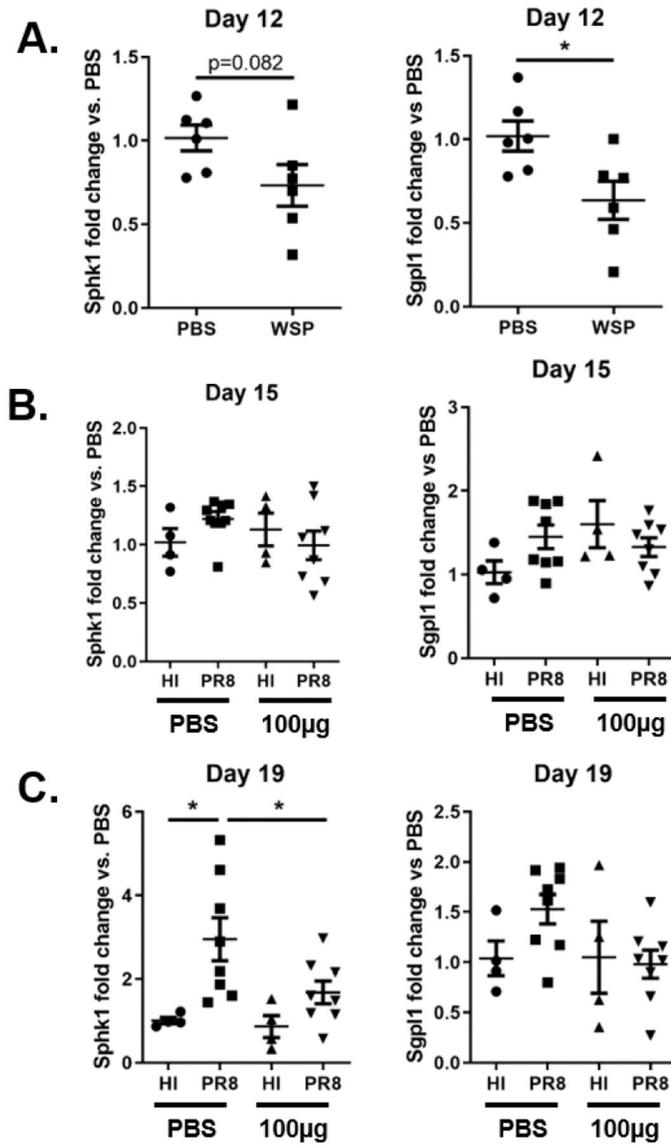
Figure 8 –.

Sub-acute wood smoke particle (WSP) exposure regulation of Sgpl1 and SphK1 expression. SphK1 and Sgpl1 were assessed by real time PCR following sub-acute WSP exposure (A) and then following HI or PR8 infection at d15 (B) and d19 (C). Data expressed as fold change compared to PBS-HI group. N=4–10 mice per group, *p<0.05