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RESEARCH ARTICLE

Differential lung inflammation and injury with tobacco smoke exposure in Wistar Kyoto and spontaneously hypertensive rats

Alexa K. Pham[a]*, Ching-Wen Wu[a]*, Xing Qiu[b], Jingyi Xu[b], Suzette Smiley-Jewell[a], Dale Uyeminami[a], Priya Upadhyay[a], Dewei Zhao[b] and Kent E. Pinkerton[a]

[a]Center for Health and the Environment, University of California, Davis, CA, USA; [b]Department of Orthopedics, Affiliated Zhongshan Hospital of Dalian University, Dalian, China

ABSTRACT

Objective: Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide and has been associated with periods of intense lung inflammation. The objective of this study was to characterize whether similar rat strains, possessing different genetic predispositions, might play a role in exacerbating the pathophysiology of COPD-like cellular and structural changes with progressive 12-week exposure to tobacco smoke (TS). Normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats were compared.

Materials and methods: WKY and SH rats were exposed to filtered air or to tobacco smoke at a particulate concentration of 80 mg/m^3 for 4, 8, or 12 weeks. Necropsy was performed 24 h after the last exposure to obtain cells by bronchoalveolar lavage for total cell and differential counts. Scoring of lung tissues and immunohistochemical staining for M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages were performed on paraffin-embedded lung sections.

Results and discussion: With progressive exposure, TS-exposed SH rats demonstrated significant airspace enlargement, mucin production, and lung inflammation compared to their FA control and TS-matched WKY rats. Moreover, SH rats also demonstrated increased expression of the M1 marker in alveolar macrophages compared to FA control, as well as the M2 marker compared to controls and TS-exposed WKY rats.

Conclusion: The progressive tobacco smoke exposure contributes to persistent lung injury and inflammation that can be significantly enhanced by rat strain susceptibility in the genesis of COPD.

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KEYWORDS
Tobacco smoke; chronic obstructive pulmonary disease; spontaneously hypertensive rats; Wistar Kyoto rats; macrophage

Introduction

Chronic obstructive pulmonary disease (COPD) is a devastating lung disease and the third leading cause of death worldwide (WHO 2018). The two primary features of COPD are emphysema characterized by destruction of delicate alveolar tissues and chronic bronchitis with persistent mucus production. COPD is characterized by airflow obstruction, inflammation, and remodeling of the airways and pulmonary vasculature (Zhu et al. 2019). Tobacco smoke (TS) is a major driver contributing to the pathogenesis of COPD (80%–90% of all cases) thought to be due to increased inflammatory mediators and immune cells, associated with hypoxic stress and endothelial dysfunction (Szucs et al. 2019).

The classic hallmark of emphysema is the destruction of alveolar tissues leading to airspace enlargement. Loss of alveolar tissue including the capillary structure results in decreased gas exchange, difficulty in breathing and compromised lung function (Suki et al. 2013). The pathogenesis of emphysema can be attributed to airway inflammation that recruits inflammatory cells following tobacco smoke exposure, leading to induced matrix metalloproteinase expression that can degrade alveolar tissues (Churg et al. 2002; Churg et al. 2012). The second classic hallmark of COPD is chronic bronchitis which includes goblet cell metaplasia, mucus hypersecretion, and inflammation of the lining of the bronchus leading to narrowing and restriction of the airways resulting in severe lung dysfunction (Hoang et al. 2016).

COPD patients suffer from persistent airway inflammation. Neutrophils and macrophages are well documented to be present in the lungs of current and former smokers (Gamble et al. 2007; Noguera et al. 2012). Inhalation of cigarette smoke stimulates airway epithelial cells to release chemotactic factors that recruit inflammatory cells to the lung (Che Karlhans et al. 2018). This in turn increases the number of neutrophils and macrophages present in the lung, to further release additional chemotactic factors such as MCP-1 and CXCL-1 to recruit other cell types (Keane and Strieter 2002; Kubo et al. 2005). Neutrophils and macrophages can also release pro-inflammatory cytokines that

CONTACT Dewei Zhao gksys3509@foxmail.com; zhaodewei2016@163.com Department of Orthopedics, Affiliated Zhongshan Hospital of Dalian University, No. 6 Jiefang Street, Zongshan District, Dalian, Liaoning 116001, China; Kent E. Pinkerton kepinkerton@ucdavis.edu School of Medicine; Anatomy, Physiology and Cell Biology, School of Veterinary Medicine; Center for Health and the Environment, University of California, Davis, CA 95616, USA
*These authors contributed equally to the research.

contribute to the persistent inflammatory process and perhaps even hinder the healing process (Kubo et al. 2005; Bozinovski et al. 2015). It has been suggested that the presence of persistent inflammation in some COPD patients is likely attributed to a self-perpetuating mechanism that is triggered following exposure to a number of environmental stimuli including cigarette smoke (Yanbaeva et al. 2006). A number of studies suggest a pathogenic role for low-grade systemic inflammation in the genesis of pulmonary hypertension in COPD patients (Sin and Man 2006), as well as ex-smokers with COPD (Rutgers et al. 2000). It has also been suggested altered inflammatory cell function further contributes to the severity and persistence of COPD pathology (Shaykhiev et al. 2009; Hiemstra 2013).

The severity and persistence of the inflammatory response and structural changes in COPD are most likely influenced by host susceptibility and genetic predisposition, especially given that only 15–20% of smokers will develop COPD (Marsh et al. 2006). There is evidence that genetic predisposition in addition to enhanced airway inflammation can lead to emphysema early in life (Alam et al. 2014; Sorroche et al. 2015). Chronic bronchitis symptom exacerbation over time appears to be associated with host susceptibility in combination with smoke exposure. Current smokers and those suffering from chronic bronchitis often have increased goblet cell number and hyperplasia (Kim et al. 2015) in both large and small airways (Higham et al. 2019). Genetic factors, such as polymorphisms in the CTLA4 and CD86 genes and CFTR mutations (Raju et al. 2014), may contribute to the development of chronic bronchitis. Recent studies have shown that some individuals are more susceptible to environmental factors, such as pollution and cigarette smoke exposure (Hallberg et al. 2008). However, we still do not fully understand how host susceptibility contributes to the development of airway disease.

Various animal models have been used to recapitulate human features of COPD through the use of genetic alterations, protease treatment, and exogenous irritants (Mahadeva and Shapiro 2002; Wright et al. 2008). However, most of these models take months to develop, fail to have persistent and progressive airway inflammation, and do not take into account whether individual susceptibility plays a role in the development and exacerbation of the hallmarks of COPD. Our laboratory has found that the spontaneously hypertensive (SH) rat, derived from the Wistar Kyoto (WKY) rat by phenotypic segregation of the hypertensive trait and inbreeding (Shen et al. 2016), exhibits similar risk factors found in patients with COPD and is a promising human-relevant animal model of airway disease induced by inhaled irritants and tobacco smoke; the SH rat demonstrates increased oxidative stress, protease activity, airway wall thickening, and epithelial cell squamous metaplasia following exposure (Yu et al. 2008; Bolton et al. 2009; Hoang et al. 2016; Shen et al. 2016). In addition, tobacco smoke exposure in SH rats increases leukocyte recruitment by way of the bronchial circulation, lowers apoptotic neutrophils in the lung, and significantly changes in the proximal airway epithelium compared to SH rats exposed only to filtered air (Yu et al. 2008; Bolton et al. 2009; Davis et al. 2012).

Previous work has been done to compare WKY and SH rats following acute and repeated exposure to TS (Yu et al. 2008; Shen et al. 2016). To examine the reproducibility of this COPD animal model, the current study investigated progressive pathological changes in the lungs due to TS exposure. Primary objectives of this study were to examine 1) airway epithelial changes of epithelial volume and mucosubstance abundance, 2) the presence of pulmonary MCP-1 and CXCL-1, 3) the degree and distribution of lung inflammation and histopathology, and 4) M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage phenotype expression with repeated and progressive TS exposure.

Macrophages play a significant role in the immune response in that they have the ability to acquire both inflammatory and defensive roles. M1 and M2 are the two major macrophage phenotypes. The M1 macrophage inhibits cell proliferation and is found in sites of inflammation; in contrast, the M2 macrophage promotes cell proliferation and allows for tissue repair (Mills 2012). To understand how TS contributes to the immune response in the lung, we wished to consider the macrophage phenotype present in the lung induced by TS exposure and whether this change progresses over time with TS exposure. In this study, we hypothesized increased numbers of M1 macrophages would be present in the lung tissue during early TS exposure, showing an active inflammatory process. However, with continued TS exposure we predicted an increased presence of M2 macrophages in the lungs to reflect an ongoing attempt for cellular repair following longer periods of TS exposure, associated with greater development of COPD.

Materials and methods

Animals

Twelve-week-old male normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats were obtained from Charles River Laboratories (Portage, MI). After arrival, all animals were housed in polycarbonate cages under a 12-h light-dark cycle with continuous access to food and water before, during, and after exposures. They were allowed to acclimate for one-week prior to the onset of experimental exposures. Animals were handled according to standards the US Animal Welfare Acts as set forth in the National Institutes of Health guidelines and by the University of California, Davis, Animal Care and Use Committee.

Tobacco smoke (TS) exposure

WKY and SH rats ($n = 4$–6 rats/group) were exposed to filtered air or a mixture of mainstream and side stream tobacco smoke at a particulate concentration of 80 mg/m^3 for 6 h/day, 3 days/week for 4, 8, or 12 weeks. Whole body exposures were performed using a TE10 smoke exposure system that combusts 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, KY).

Cigarettes were combusted using a 35 mL puff volume of 2 seconds duration once each minute for a total of 8 minutes. In order to determine the level of tobacco smoke exposure, measurements were taken for the following known constituents of smoke: carbon monoxide every 30 min, total suspended particulates every 2 h, and nicotine once per day (approximately midway through the exposure period).

Necropsy and sample collection

Eighteen to twenty hours following the final day of exposure, WKY and SH rats were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (65 mg/mL). The trachea was cannulated, the left lung bronchus was tied, and the right lung was used to collect bronchoalveolar lavage fluid (BALF), which was done by lavaging the lung with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS, pH 7.4). The volume of PBS was calculated from an equation adjusted for body weight and right lung weight: 35 ml/kg body weight × 0.6 (right lung being ~60% of total lung weight). Bronchoalveolar lavage was performed with three washes (3× in and 3× out) using the same PBS aliquot. The recovered BALF was collected in tubes and kept on ice before processing. The right lung lobes were frozen in liquid nitrogen and stored at −80 °C until use. For histology, the left lung bronchus was untied and inflated with 4% paraformaldehyde at a hydrostatic pressure of 30 cm water for 1 h that was then followed by immersion fixation.

Histology

Following immersion fixation for a minimum of 24 h, the left lung was sectioned in transverse serial segments beginning immediately above the hilum of the lung and proceeding distally to the caudal portion of the lobe with each level measuring approximately 3 mm. Segments were embedded in paraffin and cut with a microtome (HM 355, Microm, Walldorf, Germany) into five-micron thick sections that were placed on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Sections were stained with hematoxylin and eosin (H&E) and dried overnight at room temperature. Sections were dewaxed in xylene and taken through graded ethanol into water. Lung sections were stained with the following American Master Tech Scientific materials: Harris Hematoxylin, Differentiating Solution, Bluing Solution, and Eosin Y Stain (American Tech Master Scientific Inc., Lodi, CA). Hematoxylin and eosin (H&E) stained lung sections were used for quantitating airspace enlargement and histological scoring of inflammation, as described below.

Quantitation of alveolar airspace enlargement

Airspace enlargement was assessed by measuring the mean linear intercept (Lm) of the parenchyma of sectioned lung tissues stained with H&E. The Lm (μm), representing the distance between the opposing walls of alveoli, was quantified in micrometers using a light microscope (Carl Zeiss Microscopy, Pleasanton, CA) and an image-analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). The Lm of each rat was determined by point intercept counting, where each point on a M42 grid represents an air/tissue intercept, on images of five random fields of one section of lung tissue for each rat taken at 20x magnification.

Quantitation of epithelial mucosubstances and airway epithelial volume

Mucosubstances and airway epithelial volume were evaluated by measuring intracellular mucosubstances and overall airway epithelial volume in sections stained with alcian blue/periodic acid-Schiff (AB/PAS) (American Master Tech Scientific) and H&E, respectively. Four fields were sampled in the main axial airway path with light microscopy (Carl Zeiss Microscopy) and an image-analysis system (Image-Pro Plus). Images were overlaid with a cycloid arc grid in Image J (NIH) software to measure the volume of mucin volume per surface area of epithelial basal lamina. This was done to assess the volume fraction of epithelial AB/PAS staining, which was defined by total points hitting the objectives and total points falling within the reference space. In addition, epithelial volume was assessed using the same images and is expressed as the volume of epithelium per surface area of epithelial basal lamina. The airway epithelial volume fraction was also measured at the level of the proximal region of the main axial airway path. This region constitutes the 2^{nd} and 3^{rd} lobar airway generations of the left lung.

Bronchoalveolar lavage fluid (BALF)

The BALF was centrifuged at 2000 rpm for 10 minutes at 4 °C to separate cells from the supernatant fluid. After centrifugation, the cell pellet was resuspended in Ca^{2+}/Mg^{2+}-free PBS. The cell suspension was assayed for cell viability, as determined by trypan blue exclusion. Total cell number was determined with a hemocytometer. Cytospin slides (Shandon, Pittsburgh, PA) were prepared using aliquots of cell suspensions stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). Cell differentials in BALF were assessed by counting macrophages, neutrophils, lymphocytes, and eosinophils on the cytocentrifuge slides using a light microscope (a total of 500 cells/slide).

Quantification of Pro-Inflammatory mediators

Frozen right accessory lung lobes were thawed, homogenized, and total protein expression was determined according to the manufacturer's instructions (Biorad, Richmond, CA). Lung homogenate was centrifuged for 20 minutes at 14,000 rpm, and the supernatant was analyzed for protein levels of MCP-1 and CXCL-1 via ELISAs, according to the manufacturer's direction (R&D System, Minneapolis, MN). Results were expressed as pg/mL total lung protein.

Histopathology scoring

Semi-quantitative histological assessment for inflammation was done on lung tissues stained with H&E. Histological evaluation included the airways, blood vessels, alveolar space, and pleura of L2 (proximal, airway generation 2–4) and L5 (distal, airway generation 7–9) from serial lung slices, which were graded for the extent and severity of alveolitis, bronchiolitis, vasculitis, and pleural inflammation using a scoring rubric (Table 1). The extent of each pathology was estimated for each lung section as 'none', '<25%', '25%–50%', or '>50%', corresponding to grade 0–3, respectively. Severity was estimated to be 'normal', 'minimal', 'moderate', or 'marked' corresponding to grade 0–3, respectively. The overall assessment of total lung inflammation was determined by the total score of severity multiplied by the total score of extent.

Immunohistochemistry - Macrophage phenotypes

Briefly, sections were deparaffinized in xylene and taken through graded ethanol into water. Antigen retrieval was performed according to the manufacturer's instruction (DAKO, Carpinteria, CA). Sections were incubated with primary antibodies against the M1 marker (CD86; sc-9092; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:30 (200 µg/ml) and M2 marker (CD206; sc-48758; Santa Cruz Biotechnology) at a dilution of 1:3000 (200 µg/ml) to determine macrophage phenotype. Bound antibodies were detected using Envision polymer technology followed with chromogen staining using diaminobenzidine (DAB) according to the manufacturer's instructions (DAKO). Images of ten random fields of each lung section were acquired, and M1 and M2 positively stained macrophages were counted.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) with Tukey HSD tests using Prism software (GraphPad Prism, San Diego, CA) to determine statistical significance between exposure groups. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was considered at a p value of < 0.05.

Results

TS constituent concentrations

The average smoke chamber concentrations for total suspended particulate (TSP), nicotine, and carbon monoxide are shown in Table 2 following 4-, 8- and 12-weeks of exposure. The range of TSP concentrations during this 12-week experiment would be relevant to concentrations encountered by a moderate smoker (Wu et al. 2020). To ensure the reproducibility of this COPD animal model, TSP concentrations used in this study were similar to those levels used in previous studies comparing WKY and SH rats following acute and repeated TS exposure (Yu et al. 2008; Shen et al. 2016).

Alveolar airspace enlargement

Tobacco smoke (TS)-exposed SH rats had evidence of emphysema that was characterized by significant alveolar destruction. The Lm (µm) of TS-exposed SH rats was significantly ($p < 0.05$) greater than their filtered air (FA) controls at 8 weeks (Lm, 79.18 ± 4.83 vs. 55.84 ± 1.86, respectively) and 12 weeks (Lm, 92.71 ± 5.49 vs. 74.05 ± 2.17, respectively) of TS exposure (Figure 1). Moreover, TS-exposed SH rats had significantly ($p < 0.05$) increased Lm

Table 2. Tobacco smoke characteristics.

Exposure	Total suspended particulate (mg/m^3)	Nicotine (mg/m^3)	Carbon monoxide (ppm)
4 weeks	68.1 ± 4.0	10.7 ± 2.0	237 ± 14
8 weeks	75.2 ± 2.2	10.3 ± 1.1	256 ± 7
12 weeks	86.7 ± 1.2	7.0 ± 0.7	194 ± 3

Note. All measurements were taken directly in the inhalation chambers during the entire exposure period (6 h/day, 3 days/week for 4, 8, or 12 weeks). Data are expressed as mean ± SEM.

Table 1. Semi-quantitative histopathology inflammation scoring rubric.

Score	Severity	Extent
0	Normal. Thin alveolar walls, with very few free macrophages in the lumen. No inflammatory cells. Normal respiratory epithelium, 1 cell-layer thick. Normal smooth muscle and submucosal layers. Normal vascular endothelium. Little/no cells at the pleura.	None of the lung is involved.
1	Similar to 0 score with more free macrophages and/or monocytes in the alveolar lumen, airway submucosa, perivascular and/or pleura region. No polymorphonuclear cells (PMNs). Nearly all the connective tissue in the perivascular region is still visible.	< 25% involvement
2	Slightly thickened airway due to moderate influx of PMNs and/or phagocytes such as neutrophils, eosinophils, or macrophages, into the submucosa. Moderately increased cellularity in the alveolar, pleural, and/or perivascular region. Much of the connective tissue still visible in the perivascular region.	25%–50% involvement
3	Marked influx of mixed cells (phagocytes and/or PMNs) into the alveolar lumen, submucosal layer, pleural, and/or perivascular region forming large cellular agglomerates. Thickened alveolar walls and/or airway. Much of the connective tissue is obscured in the perivascular region. A high percentage of PMNs may be present with foamy macrophages.	> 50% involvement
overall score = severity × extent		

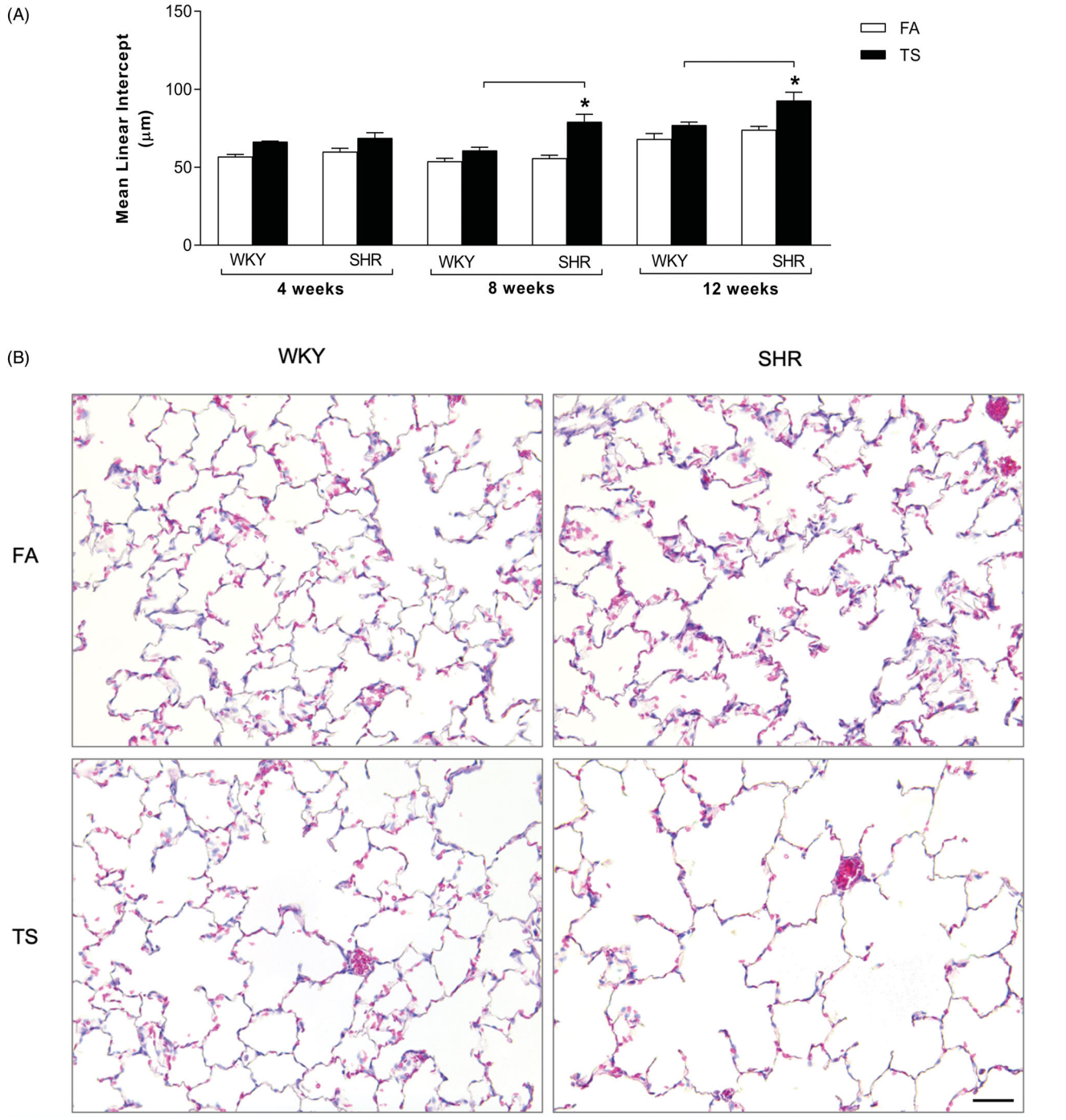

Figure 1. Airspace enlargement analysis of filtered air (FA) and tobacco smoke (TS) exposed WKY rats and SH rats (SHR) for 4, 8, and 12 weeks. (A) The mean linear intercept (Lm), a measurement of distance between the opposing walls in the alveoli, were quantified in lungs sections stained with hematoxylin and eosin (H&E) using a light microscopy and image analysis system. The Lm of TS-exposed SH rats showed significant increases compared to both their FA control and TS-exposed WKY rats at 8 and 12 weeks of exposure. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR. (B) H&E stained lung sections of SHR reflect significant airspace enlargement after 8 weeks of progressive TS exposure. Scale bar = 50 μm.

measurements compared to TS-exposed WKY rats (8 weeks of TS: Lm, 79.18 ± 4.83 vs. 60.82 ± 2.03; 12 weeks of TS: Lm, 92.71 ± 5.49 vs. 77.05 ± 1.93, respectively) (Figure 1).

Airway intracellular mucosubstance volume

TS-exposed SH rats had significantly ($p < 0.05$) more mucosubstances compared to their FA control at 4, 8, and 12 weeks of TS exposure (Figure 2(A)). At 8 weeks of TS-exposed, SH rats had significantly ($p < 0.05$) more mucin positive staining than WKY rats (Figure 2(A,B)). TS-exposed WKY rats only significantly ($p < 0.05$) differed from their FA controls at 8 weeks (Figure 2(A,B)). Epithelial volume was significantly ($p < 0.05$) increased in SH and WKY rats following 8 and 12 weeks of TS exposure (Figure 2(C)), with SH rats having significantly ($p < 0.05$) more epithelial volume than WKY rats at 8 weeks of exposure (Figure 2(C)).

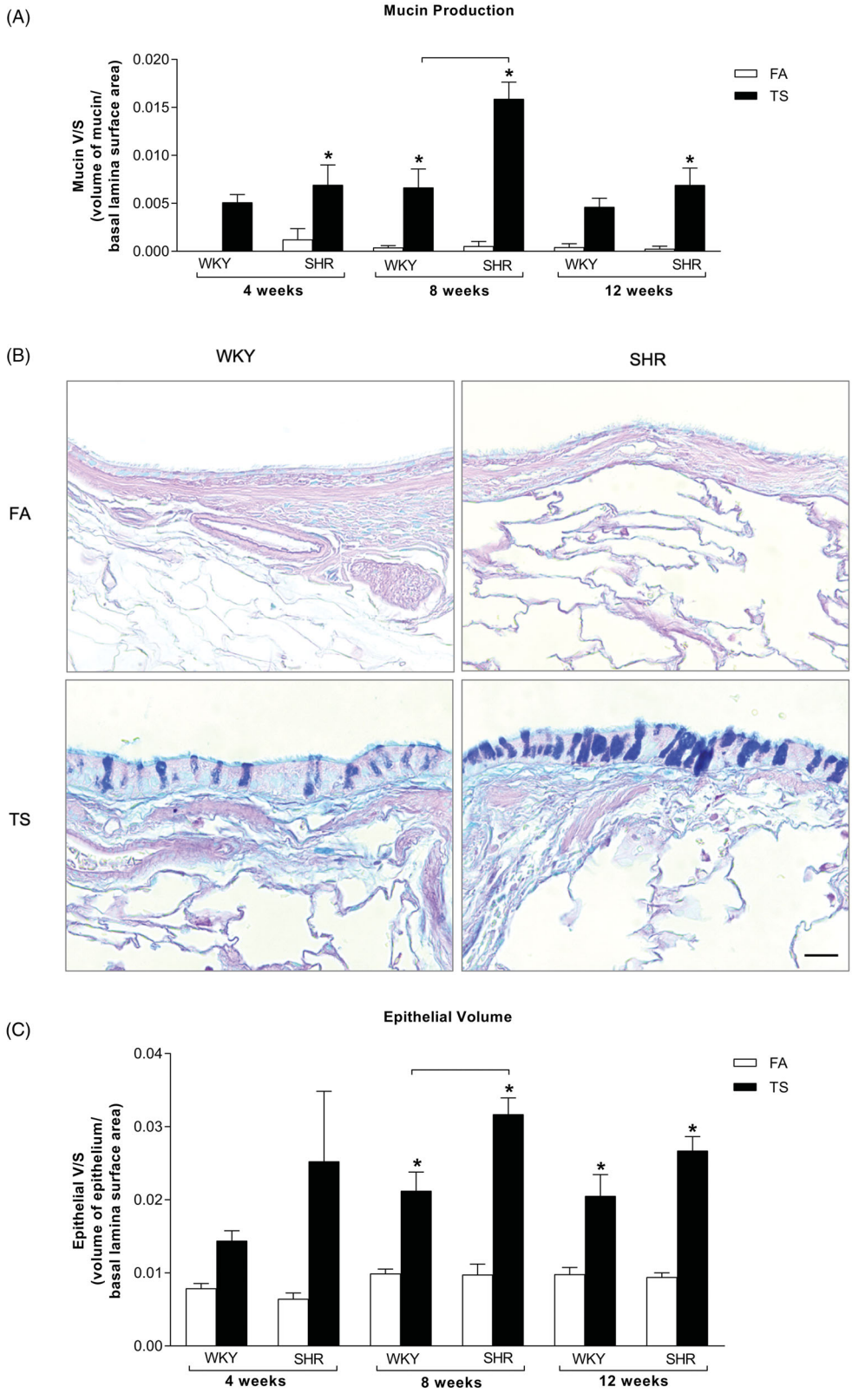

Figure 2. Morphometric analysis of mucin production and airway epithelial volume. (A) Intraepithelial mucosubstances were quantified and compared between tobacco smoke (TS) and filtered air (FA) treated WKY rats and SH rats (SHR) at 4, 8, and 12 weeks of exposure. The mucin content of TS-exposed SH rats increased significantly compared to both their FA-treated rats and TS-exposed WKY rats at 8 weeks of exposure. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR. (B) Alcian blue/periodic acid-Schiff (AB/PAS) stained lung sections showing epithelial mucin content in the lungs of TS-exposed WKY and SHR at 8 weeks of exposure. Scale bar = 50 μm. (C) Epithelial volume of TS exposed WKY and SHR was analyzed and compared to their FA control rats at 4, 8, and 12 weeks of exposure. TS-exposed SH rats had significantly increased epithelial volume compared to the FA control and TS-exposed WKY rats at 8 weeks. * $p < 0.05$ compared with respective FA control. Brackets, $p < 0.05$ between WKY and SHR.

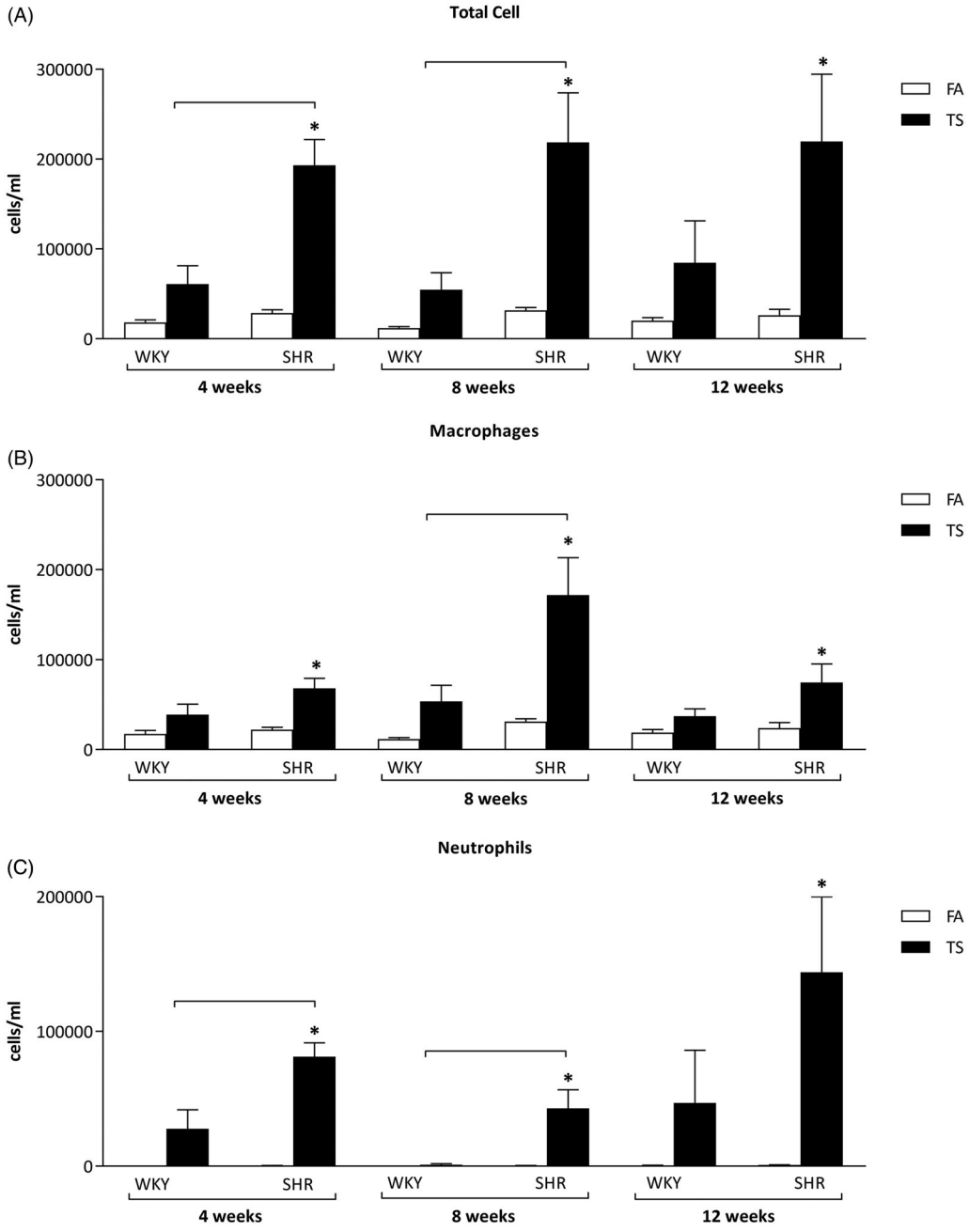

Figure 3. Total and differential cell counts in bronchoalveolar lavage of filtered air (FA) and tobacco smoke (TS) exposed WKY rats and SH rats (SHR) following 4, 8, and 12 weeks of exposure. (A) The total cell, (B) macrophages, and (C) neutrophils were analyzed by counting the number of cells collected from the bronchoalveolar lavage fluid (BALF) of rats. The total cells, macrophages, and neutrophils counts were significantly higher in TS-exposed SH rats compared to their FA control and TS-exposed WKY rats at 8 weeks of TS exposure. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR.

Bronchoalveolar lavage

TS-exposed SH rats had significantly ($p < 0.05$) higher total cell count, macrophages, and neutrophils compared to their FA control at 4, 8, and 12 weeks of TS exposure (Figure 3(A–C)). Compared to WKY rats exposed to TS, SH rats had significantly ($p < 0.05$) higher number of total cells and neutrophils at 4 weeks of TS exposure (Figure 3(A,C)) and significantly ($p < 0.05$) higher number of total cells, macrophages, and neutrophils at 8 weeks of TS exposure (Figures 3(A–C)).

Lung chemokine protein expression

Protein levels of monocyte chemoattractant protein-1 (MCP-1), which regulates the migration and infiltration of macrophages, and chemokine (C-X-C motif) ligand 1 (CXCL-1), which attracts neutrophils, were assessed. TS-exposed SH rats had significantly ($p < 0.05$) higher levels of MCP-1 compared to their FA control at 8 and 12 weeks of TS exposure, but not at 4 weeks (Figure 4(A)). Furthermore, compared to WKY rats exposed to TS, SH rats had

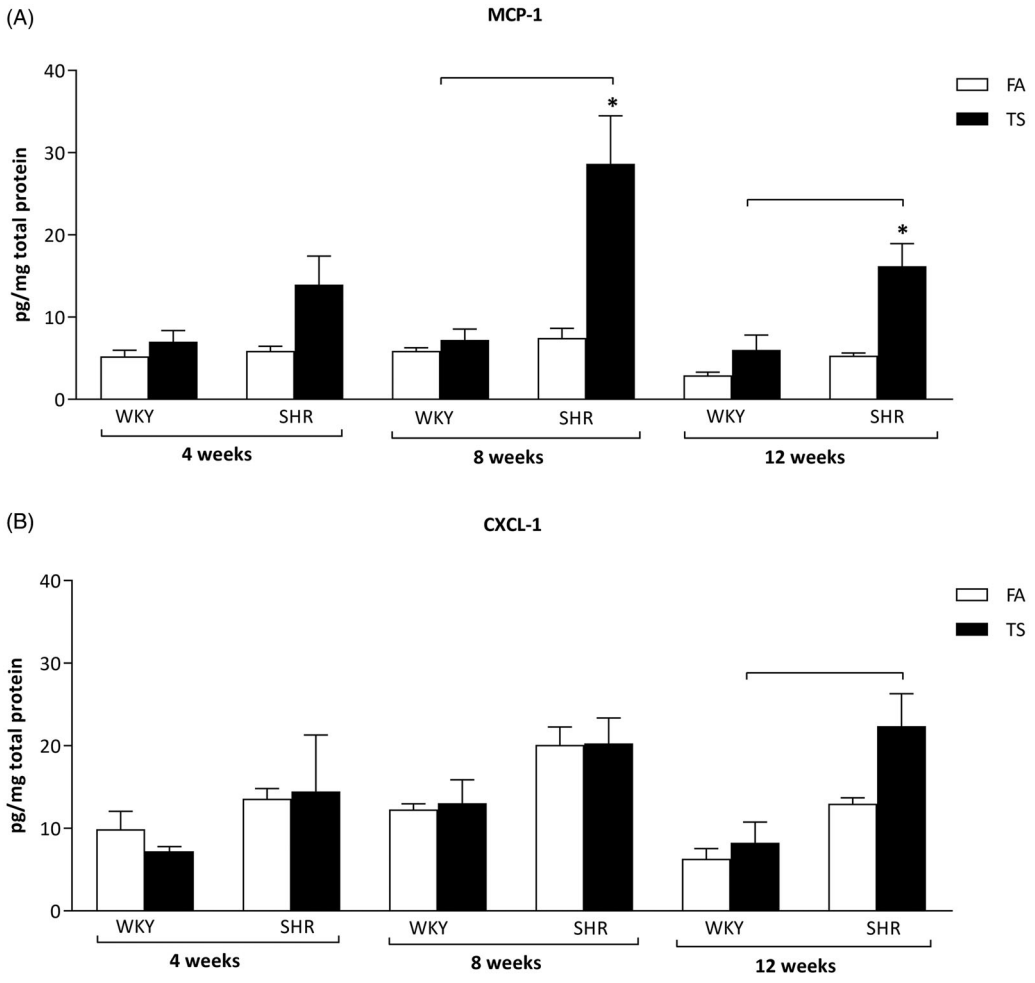

Figure 4. Lung chemokine expression of filtered air (FA) and tobacco smoke (TS) exposed WKY rats and SH rats (SHR) following 4, 8, and 12 weeks of exposure. (A) The protein expression of MCP-1 and (B) CXCL-1 was assessed by ELISA. SH rats exposed to TS had significantly higher levels of MCP-1 and CXCL-1 at 12 weeks compared to WKY rats exposed to TS. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR.

significantly ($p < 0.05$) higher levels of MCP-1 at 8 and 12 weeks (Figure 4(A)) and CXCL-1 at 12 weeks (Figure 4(B)).

Lung inflammation

Inflammation was evident in the bronchioles, perivascular cuff, as well as in the parenchyma with TS exposure (Figure 5(A)). When sectioned lung tissues were scored for the presence of bronchiolitis, vasculitis, alveolitis, and pleural inflammation, TS-exposed SH rats had significantly ($p < 0.05$) higher overall inflammatory scores compared to their FA control at 4, 8, and 12 weeks of TS exposure (Figure 5(B)). Moreover, TS-exposed SH rats had significantly ($p < 0.05$) higher inflammatory score compared to TS-exposed WKY rats exposed at 4 and 8 weeks (Figure 5(B)).

Semi-quantitative immunohistochemistry of lung M1 and M2 macrophage phenotypes

To better understand the role of macrophages following progressive TS exposure, lung tissues were stained for M1 (CD86) pro-inflammatory and the M2 (CD206) anti-inflammatory macrophage markers and quantified through random field image capture. Compared to FA controls, TS-exposed SH and WKY rats demonstrated a significantly ($p < 0.05$) higher number of M1 and M2 expressing macrophages at 8 weeks of exposure (Figure 6(A–C)). M2 expression was still significantly ($p < 0.05$) greater than FA controls in both species after 12 weeks of TS exposure (Figure 6(B)). Whereas M1 staining peaked at 8 weeks of TS exposure (Figure 6(A)), M2 staining peaked at 12 weeks and TS-exposed SH rats had significantly ($p < 0.05$) more M2 protein staining than WKY rats (Figure 6(B)).

Discussion

The objective of this study was to further characterize if strain differences, and genetic predisposition, play a significant role to exacerbate the pathophysiology of COPD-like cellular and structural changes with long-term progressive exposure to tobacco smoke (TS). Previous work in our laboratory demonstrated acute TS exposure induced a greater inflammatory response associated with lower numbers of apoptotic neutrophils in lung airways and recovered

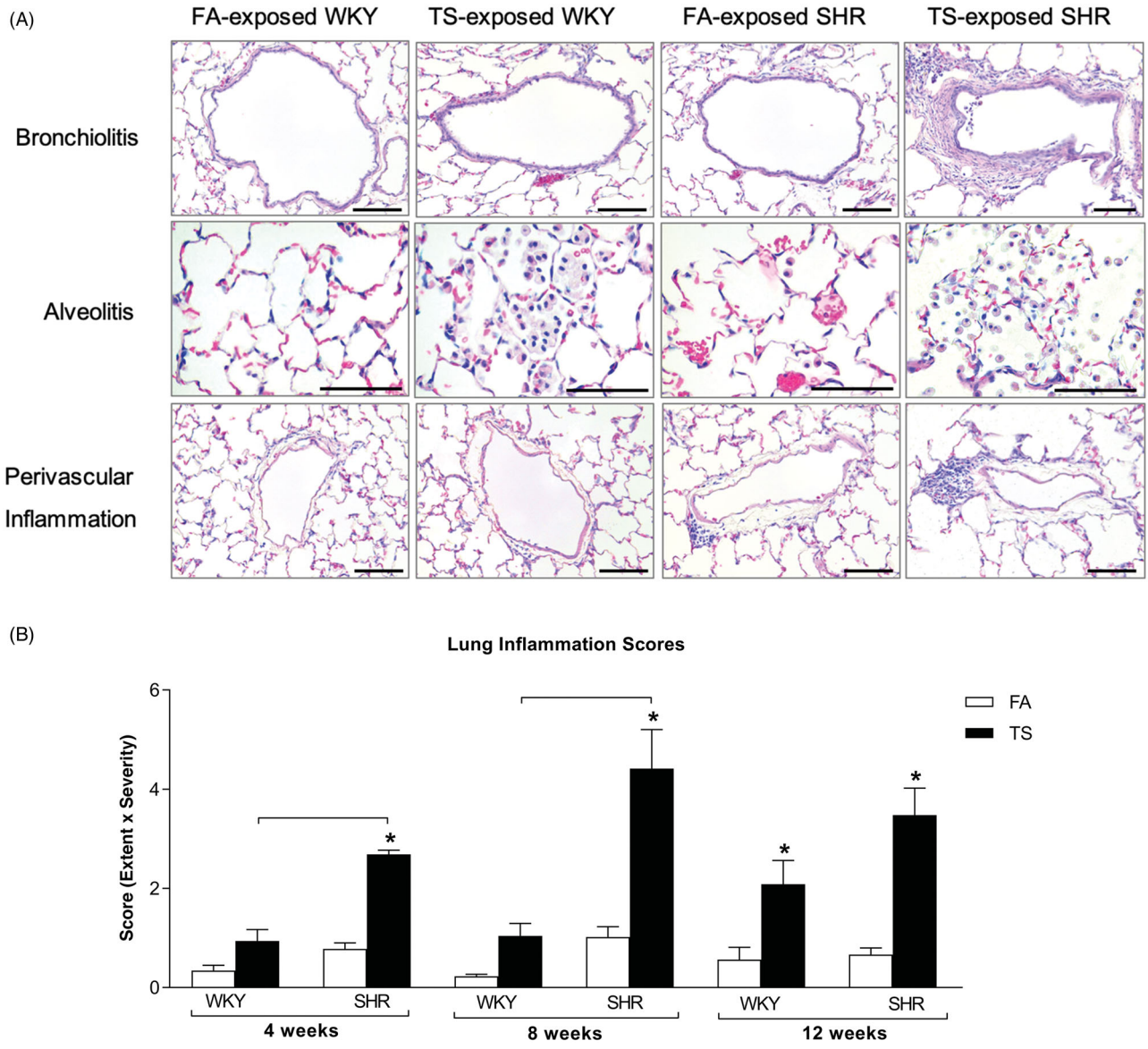

Figure 5. Histopathological scoring of lung inflammation for filtered air (FA) and tobacco smoke (TS) exposed WKY rats and SH rats (SHR) following 4, 8, and 12 weeks of exposure. (A) The micrographs of hematoxylin and eosin (H&E) stained lung sections show increased lung inflammation of bronchioles, alveolar and perivascular regions in TS-exposed WKY and SH rats compared to the FA control at 8 weeks of exposure. Scale bars = 100 μm. (B) Inflammatory cells in the bronchioles, perivascular cuff, as well as the parenchyma of the lung were evaluated by a semiquantitative scoring method by H&E. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR.

in bronchoalveolar lavage fluid (BALF) in the spontaneously hypertensive (SH) rat compared to the normotensive Wistar Kyoto (WKY) rat (Yu et al. 2008). We wished to determine if the SH rat would also develop greater chronic pulmonary inflammation with prolonged exposure to TS, as seen in COPD, compared to the WKY rat. To this end, our laboratory found significant differences in inflammation (BALF), cytokine levels (tumor necrosis factor-α and heme oxygenase-1), as well as airspace enlargement in the male SH rat compared to the female SH rat as well as the WKY rat exposed to TS for 4 and 12 weeks (Shen et al. 2016). In the present study, we expanded our comparative study of both male WKY and SH rats by investigating progressive TS exposure for 4, 8, and 12 weeks. This repeated study further demonstrated a high degree of reproducibility with the SH rat showing significantly greater susceptibility than the WKY rat to TS-induced chronic lung inflammation and injury. These differences included the following: 1) increased airspace enlargement of the alveoli, 2) mucus hypersecretion of the major airway, 3) elevated chemokines (MCP-1 and CXCL-1) in the lung involved in the recruitment and retention of macrophages and neutrophils found in the BALF, and 4) a significantly greater degree of inflammatory cells infiltration surrounding bronchioles, the vasculature, and parenchyma in TS-exposed SH rats significantly above those found in TS-exposed WKY rats, as well as filtered air (FA)-exposed SH controls.

In the current study, the use of 12-week old rats is based on previous studies using the same age for WKY and SH rats at the beginning of both acute and repeated exposure to TS (Yu et al. 2008; Shen et al. 2016). This age was chosen for two reasons: 1) to examine the reproducibility of this COPD animal model and 2) to allow SH rats to acclimate to attain constancy in their level of vascular hypertension,

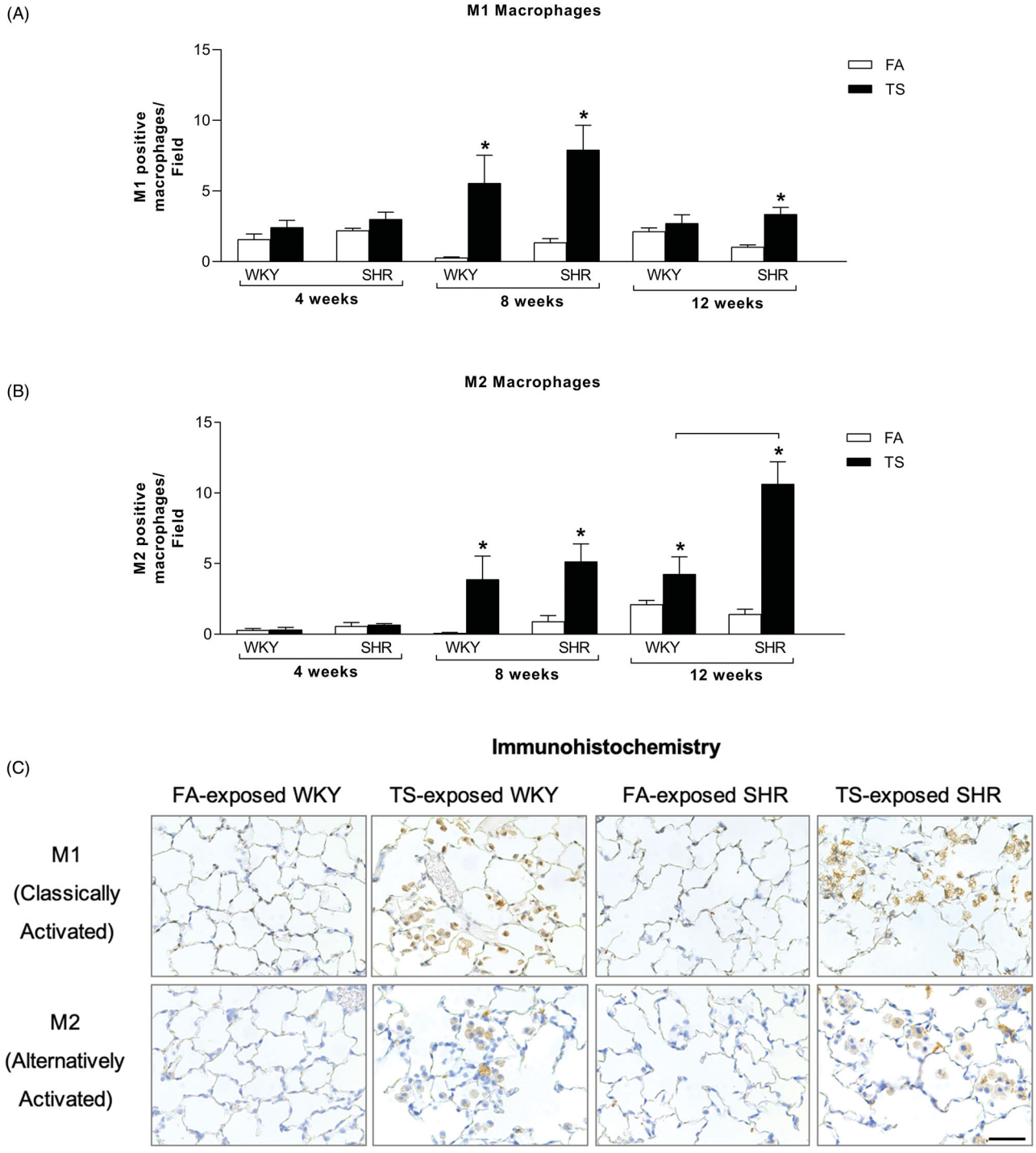

Figure 6. Immunohistochemical staining and quantification of M1 (CD86) and M2 (CD206) expressing alveolar macrophages of filtered air (FA) and tobacco smoke (TS) exposed WKY rats and SH rats (SHR) at 4, 8, and 12 weeks. (A) The numbers of M1 and (B) M2 expressing macrophages were quantified using (C) immunohistochemical stained sections of rat lungs. TS-exposed WKY and SH rats showed more M1 and M2 expressing macrophages in the lungs compared to their FA controls at 8 weeks. * Significant difference ($p < 0.05$) compared with respective FA control. Brackets, significant differences ($p < 0.05$) between WKY and SHR. Scale bar = 50 μm.

achieved at 12 to 13 weeks of age, based on information from the commercial vendor (Charles River Laboratories, Portage, MI).

COPD is a complex multifactorial disease consisting of many pathological processes. Although smoking elicits airway inflammation in all smokers, the presence of persistent inflammation that leads to the development of COPD suggests an abnormal inflammatory response in susceptible individuals. We have chosen to study TS exposure in the SH rat because this species responds in similar manner as humans with increases in oxidative stress, protease activity, airway wall thickening and epithelial cell squamous metaplasia (Yu et al. 2008; Bolton et al. 2009). In addition, SH rats have some of the same genetic polymorphisms found in humans that increase the risk of COPD, including the subunit of NADPH oxidase and CD36 (Zalba et al. 2001;

Noguera et al. 2012; Santamaria et al. 2014), and a tendency to develop hypertension. Certain polymorphisms found in COPD patients are implicated in increased risks for other types of diseases, including cancer and cardiovascular disease (Kaur-Knudsen et al. 2011). The current study was done because few animal studies focus on the outcome of having an enhanced risk of developing airway disease and being exposed to TS long-term.

The reported prevalence of pulmonary hypertension in moderate to severe COPD patients ranges from 10% to 91% and increases with the severity of COPD, with the majority of patients having mild to moderate pulmonary hypertension (Smith and Wrobel 2014). COPD significantly drives pulmonary hypertension through endothelial dysfunction, inflammation and impaired lung function, thus leading to pulmonary vascular remodeling and severe capillary loss in cases of severe emphysema and concomitant increases in pulmonary arterial pressure (Brassington et al. 2019). In patients with COPD, the prevalence of systemic hypertension ranges between 35% and 55% (Smith and Wrobel 2014). It has been reported COPD and impaired lung function are independently associated with an increased risk of developing systemic hypertension (Kim et al. 2017). With the frequent presence of systemic hypertension in COPD patients, such a condition may compound the risk of cardiovascular disease (Finks et al. 2020).

Cigarette smoke exposure along with genetic contribution can lead to emphysema with significant remodeling of the alveolar bed characterized by airspace enlargement due in part to inflammation and anti-protease/protease imbalance. For example, α-1 antitrypsin deficiency and other polymorphisms in MMPs, SERPINE2, and the HHIP loci have been associated with susceptibility to COPD in humans (Kukkonen et al. 2011; Sorroche et al. 2015; Ragland et al. 2019). Previous study has shown abnormalities in the lungs of SH rats after long-term TS exposure, including signs of developing emphysema, which are different from the lung changes in other strains of rats induced by TS (Wu et al. 2020). This suggests variations to be strain dependent and a genetic component in the development of emphysema, which is important for predicting the disease onset in humans. The TS-exposed SH rats in this study had greater alveolar destruction and airspace enlargement as quantitated by mean linear intercept (Lm) than TS-exposed WKY and filtered air (FA)-exposed SH rats at 8 and 12 weeks of TS exposure (Figure 1). These results indicate that alveolar enlargement progresses with time and that SH rats are more susceptible to alveolar destruction from TS exposure than WKY rats, which may be a more appropriate animal model to represent COPD patients with extensive parenchymal damage and history of symptom exacerbations.

Chronic bronchitis is a classic feature of COPD resulting from mucus hypersecretion and goblet cell hyperplasia, which can lead to thickening of the airway walls, airway obstruction and decreased lung function (Rigden et al. 2016). Overproduction of mucus can be caused from exposure to cigarette smoke or other bronchial irritants, such as particulate matter or dust. If an irritant damages ciliated cells of the airways, normal mucocilliary clearance of mucus, dust, and microbes from the lung is reduced. Over time, the excess mucus can cause COPD patients to become vulnerable to viral and bacterial infections. There are various animal models of chronic bronchitis induced by exposure to particulate matter, sulfur dioxide, and lipopolysaccharide that demonstrate changes in the airway epithelium resulting in mucus hypersecretion and goblet cell hyperplasia; however, many of the studies with these models are short term and result in only mild mucus secretion (Kodavanti et al. 2000; Wagner et al. 2006; Ghorani et al. 2017). Furthermore, the changes are reversible. Most cases of chronic bronchitis in humans occur after long-term cigarette use, and there are few animal studies of whether prolonged exposure to TS can permanently change the airway epithelium to develop persistent bronchitis-like features. Here, we show that TS-exposed SH rats had increased mucin and epithelial volume compared to TS-exposed WKY rats at 8 weeks of exposure (Figure 2). These results suggest that SH rats respond to TS by producing more mucosubstances than WKY rats. This data demonstrates that with prolonged TS exposure, goblet cell hypertrophy can take place resulting in mucous hypersecretion that can permanently change the airway epithelium to induce a chronic bronchitis-like phenotype consistent with human COPD.

Our results demonstrated inflammatory markers, MCP-1, a chemoattractant responsible for monocyte and macrophage recruitment, and CXCL-1, a neutrophil chemoattractant, were significantly elevated in SH rats following exposure to TS compared to TS-exposed WKY rats and FA-controls (Figure 4). Associated with the increased lung chemokine expression, significant increases in the numbers of macrophages and neutrophils were also found in the BALF of SH rats compared to TS-exposed WKY rats (Figure 3). These results show that SH rats have a more robust inflammatory response than WKY rats when exposed to TS, with macrophages prominent following 8 weeks of exposure. Furthermore, TS-exposed SH rats had significantly higher overall lung inflammation based on the extent and severity of bronchiolitis, vasculitis, alveolitis, and pleural inflammation than TS-exposed WKY rats within a few weeks of TS exposure (Figure 5). The accumulation and persistence of macrophages and neutrophils that may lead to induction of adaptive immunity, resulting in the recruitment of dendritic cells, CD8+ and CD4+ T-lymphocytes, which have been shown to play a key role in the persistent inflammation of COPD by releasing cytokines that further contribute to the inflammatory response (Barcelo et al. 2006).

It has been suggested macrophage polarization and unique phenotypic expression may play a significant role in the pathogenesis of COPD (Shaykhiev et al. 2009). Macrophages have the potential to release various mediators, including proteases, such as MMP-9 and MMP-12 that play a role in emphysema, contribute to persistent inflammation by producing pro-inflammatory cytokines and chemokines, such as IL-6 and TNF-alpha, and be involved in the repair and remodeling of lung structures (Shaykhiev et al. 2009; Hiemstra 2013). IL-6 mRNA expression has been found to

be elevated in SH male rats after 12 weeks of TS exposure (Shen et al. 2016). M1 (classically activated, pro-inflammatory) and M2 (alternatively activated, anti-inflammatory) macrophage markers are known to be involved in the pro-inflammatory and repair mechanisms of immune responses, respectively. While in this study, BALF macrophage numbers were significantly elevated in TS-exposed animals (Figure 3(B)), the lungs of TS-exposed SH rats had clusters of macrophages that were highly prevalent in the parenchyma of these animals (Figure 5(A)).

In COPD patients, macrophages are prominent throughout the lungs, contributing to the inflammatory process. A previous study reported the number of M1 macrophage markers is reduced in smokers with COPD compared to smokers without COPD (Hodge et al. 2011). This observation supports the potential for smoking to alter the macrophage activation state, particularly in those with disease (Brown et al. 2016). To better understand the role of macrophages in our model, we characterized the phenotypes of alveolar macrophages by performing immunohistochemistry for M1 and M2. Both markers were found increased in TS-exposed WKY and SH rats; however, M1 expression remained significantly elevated in SH rats following 12 weeks of TS exposure, whereas M1 expression went back to control levels in WKY rats (Figure 6(A)). Furthermore, M2 expression was significantly higher in TS-exposed SH compared to TS-exposed WKY rats at 12 weeks (Figure 6(B)). This demonstrates that SH rats had not only persistent inflammation, but they also may undergo more wound healing and repair. Previous studies demonstrate that COPD patients have a smoking-dependent reprograming of alveolar macrophage polarization, and M1 and M2 markers, such as CD163 and CD206, are overexpressed in the alveolar macrophages of patients with severe COPD (Shaykhiev et al. 2009; Kaku et al. 2014).

In this study, significant inflammatory changes in the lungs were noted for SH rats compared to WKY rats at a time point as early as at 4 weeks of TS exposure. However, greater morphological changes, in combination with elevated chemokines and changes in macrophage phenotype became significant in SH rats following 8 and 12 weeks, compared to 4 weeks of TS exposure. In general, SH rats regardless of the length of TS exposure, were found to demonstrate a more prominent response to TS compared to WKY rats, in particular following 8 and 12 weeks of exposure. These findings strongly suggest the SH rat strain serves as an ideal model to study the genesis and pathophysiology of COPD-like changes induced by TS. A recent study reported pulmonary changes, including mucous and macrophage cell abundance and alveolar airspace enlargement in male SH rats induced by long-term TS exposure (9 months). These changes did not attenuate with a period of smoking cessation (8–9 months), resulting in irreversible damage similar to COPD (Wu et al. 2020). These observations further emphasize the relevance of the SH rat and the irreversible nature of COPD-like changes even after as little as 12 weeks of TS exposure.

In conclusion, The SH rat strain demonstrates many of the hallmarks of human COPD with long-term TS exposure, such as airspace enlargement, increased mucin production and the presence of persistent lung inflammation characterized by accumulation of inflammatory cells, macrophages and neutrophils. We believe that the SH rat has the potential to help investigators understand the pathophysiology of COPD-like cellular and structural changes with long-term progressive exposure to TS, and this knowledge can be used to further therapeutic interventions for those who currently smoke and wish to stop.

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Author contributions

A.K.P. performed the study, and prepared the manuscript and preliminary figures; C-W.W. finalized the manuscript and all figures; X.Q. assisted in the study and data analysis; J.X. designed the original study; S.S-J. assisted in manuscript preparation and editing; D.U. oversaw the experimental exposures; D.Z. designed, guided and supported the experiments; K.E.P. oversaw the experimental design, final analysis and guidance in manuscript preparation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Ching-Wen Wu http://orcid.org/0000-0003-4302-9295
Kent E. Pinkerton http://orcid.org/0000-0001-9047-0353

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