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SECOND HAND SMOKE AND COPD: LESSONS FROM ANIMAL STUDIES

Topic Editors
Adelheid Kratzer, Laima Taraseviciene-Stewart and Michael Borchers

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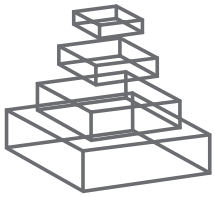

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SECOND HAND SMOKE AND COPD: LESSONS FROM ANIMAL STUDIES

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Cigarette smoke exposure is the key initiator of chronic inflammation, alveolar destruction, and the loss of alveolar blood vessels that lead to the development of chronic obstructive pulmonary disease (COPD) which is comprised of emphysema and chronic bronchitis. Exposure to secondhand smoke (SHS) is the major risk factor for non-smokers to develop emphysema. While the first-hand smoke is directly inhaled by smokers, passive smoking occurs when non-smokers are involuntary exposed to environmental tobacco smoke also known as second hand smoke (SHS). SHS is a mixture of 2 forms of smoke that come from burning tobacco: side stream smoke (smoke that comes from the end of a lit cigarette, pipe, or cigar) and mainstream smoke (smoke that is exhaled by a smoker). These two types of smoke have basically the same composition, however in SHS many toxic components are more concentrated than in first-hand smoke, therefore more hazardous for people's health. Several pathological events have been implicated in the development of SHS-induced COPD, but many aspects of this pathology remain poorly understood halting the development of new advanced treatments for this detrimental disease. In this respect we have welcomed leading investigators in the field to share their research findings and provide their thoughts regarding the mechanisms of the SHS exposure-induced immune responses and inflammatory mechanisms of lung destruction in SHS-induced COPD and related comorbidities.

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EDITORIAL
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Second hand smoke and COPD: lessons from animal studies

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Keywords: COPD, second hand smoke, mouse models, pulmonary inflammation, emphysema

This issue of Frontiers in Physiology presents a collection of manuscripts including original research and comprehensive reviews that examine the effects of second hand smoke (SHS) on the development and progression of chronic obstructive pulmonary disease (COPD). The health effects and costs of SHS are a burgeoning concern as it is estimated that more than 126 million people in the United States are exposed to SHS (Menzies, 2011). Although there is a decrease of smokers in the US, the economic aspects of SHS exposure is substantial and often occurs not only at the workplace, but also at personal homes (Max et al., 2012). As described in the accompanying manuscripts, SHS has been associated with an increased risk of cardiovascular disease, several cancers, stroke, and type 2 diabetes mellitus. Several studies have shown associations between SHS and sudden infant death syndrome, acute respiratory infections, ear infections, and the development of asthma in infants and children. SHS can also contribute to the development of COPD in non-smokers and exacerbate COPD pathogenesis in smokers. Unfortunately, relatively little is known about the cellular and molecular mechanisms whereby SHS contributes to these diseases including COPD and as a result, insight from animal models can be challenging to translate properly into the human disease. The purpose of this collection is to present the newest data and updated reviews on the role of SHS in the pathogenesis of COPD with a focus on mechanistic studies performed in animal models.

The original research articles presented in this collection examine the influence of dietary vitamin D levels on the susceptibility to SHS-induced pathology, the effects of SHS in a model of pre-existing pulmonary inflammation induced by the genetic deletion of a key leukocyte signaling molecule (Arhgef1), and the role of STAT3 (a master transcriptional regulator of pro-inflammatory genes) in the control of inflammation and protease expression in the lung. In the report by Crane-Godreau et al. (2013), the authors utilized a novel mouse model of dietary vitamin D deficiency and SHS exposure to examine the hypothesis that low vitamin D levels contribute to the lower lung function and disease severity in response to cigarette smoke exposure. These studies revealed that vitamin D deficiency exacerbates the alveolar tissue destruction compared to mice receiving a vitamin D supplemented diet following 16 weeks of daily SHS exposure. Furthermore, the authors provide evidence of a significant shift in the protease-antiprotease balance as a consequence of vitamin D depletion that provides mechanistic insight into the pathways affected by vitamin D deficiency. This work establishes a useful model to further study the effects of dietary vitamin D and the development of pulmonary pathology in response to SHS. The work of Harney et al. also utilizes a novel model to examine the effects of sub-chronic SHS exposure on pulmonary inflammation and tissue destruction (Hartney et al., 2012). Utilizing a mouse genetically deficient in the leukocyte signaling molecule Arhgef, the authors were able to demonstrate that SHS has the capacity to significantly enhance underlying pulmonary inflammation and tissue destruction. Importantly, the data revealed that the effects of the SHS were independent of the baseline inflammation in the mutant mouse model. Therefore, this model is extremely useful for extrapolating and defining the mechanisms of SHS-enhanced pathogenesis in the context of pulmonary inflammation unrelated to smoke exposure. Lastly, Geraghty et al. utilize a mouse model deficient in STAT3 to demonstrate the importance of this critical signaling pathway in the pulmonary response to SHS (Geraghty et al., 2013). The authors showed that tobacco smoke activates STAT3—mediated inflammation, proteolysis, and apoptotic responses in the lung. They further provide a link between the activation of STAT3 and the inhibition of pathways that modulate the anti-inflammatory pathways in the lung.

In addition to the original reports, this issue also presents focused reviews on the effects of SHS on airway secretions and mucociliary clearance (Liu and Di, 2012), the generation and physiological consequences of smoke-induced advanced glycation end-products (Robinson et al., 2012), and the current factors that have been reported in animal models to influence SHS-induced COPD pathologies (Birru and Di, 2012). Finally, the issue presents two comprehensive reviews that detail current understanding of the effects of SHS on lung pathogenesis that has been defined by utilizing animal models, which is a challenging aspect as different rodents develop emphysema at different time points. Goldklang et al. provide a provocative and informative discussion of the lessons learned from animal models of smoke exposure including the strengths and weaknesses of the various models (Goldklang et al., 2013). The Taraseviciene-Stewart lab rounds off the issue with the most current and comprehensive review to date of the effects of tobacco smoke induced pathology described in animal models (Leberl et al., 2013). This review thoroughly summarizes the results of 155 studies addressing cigarette smoke exposure in animal models. Parameters including, species, strain, exposure methodologies, exposure duration and dose,

endpoint descriptors, and summaries of the results are thoroughly presented in tables and in discussion formats. In addition to the most common endpoints described in smoke-induced pathologies, this review expands the possibilities and current knowledge of the usefulness of animal studies to investigate small airway remodeling and pulmonary hypertension.

Together, these reports provide insight into the usefulness of animal models of SHS exposure to define potential mechanisms of action in pulmonary pathology as well as timely, thorough updates on our current understanding of the pathologic effects of SHS. Given the tremendous numbers of persons exposed unwillingly to SHS and the likelihood of an increase in COPD in the coming years, it is imperative that the health effects associated with these exposures are carefully and fully examined. The use of animal models for such studies is necessary and, therefore, it is important that the strengths, limitations and relevance to human disease be explored and discussed.

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Sub-chronic exposure to second hand smoke induces airspace leukocyte infiltration and decreased lung elastance

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Exposure to second hand tobacco smoke is associated with the development and/or exacerbation of several different pulmonary diseases in humans. To better understand the possible effects of second hand smoke exposure in humans, we sub-chronically (4 weeks) exposed mice to a mixture of mainstream and sidestream tobacco smoke at concentrations similar to second hand smoke exposure in humans. The inflammatory response to smoke exposures was assessed at the end of this time by enumeration of pulmonary leukocyte infiltration together with measurements of lung elastance and pathology. This response was measured in both healthy wild type (C57BL/6) mice as well as mouse mutants deficient in the expression of Arhgef1 (*Arhgef1*$^{-/-}$) that display constitutive pulmonary inflammation and decreased lung elastance reminiscent of emphysema. The results from this study show that sub-chronic second hand smoke exposure leads to significantly increased numbers of airspace leukocytes in both healthy and mutant animals. While sub-chronic cigarette smoke exposure is not sufficient to induce changes in lung architecture as measured by mean linear intercept, both groups exhibit a significant decrease in lung elastance. Together these data demonstrate that even sub-chronic exposure to second hand smoke is sufficient to induce pulmonary inflammation and decrease lung elastance in both healthy and diseased animals and in the absence of tissue destruction.

Keywords: second hand smoke, inflammation, lung mechanics

INTRODUCTION

Second hand smoke exposure has been associated with a variety of negative health outcomes in a number of epidemiological studies (Centers for Disease Control, 1986; Barnoya and Glantz, 2005; Eisner et al., 2005; Oberg et al., 2011). As association does not prove causation, animal models are often used to determine causational effects. Accordingly, rodent models of tobacco smoke exposure have been used to establish a relationship between associations identified in epidemiological studies. Historically, these types of tobacco smoke exposures have focused on inducing pulmonary pathology such as tissue damage as indicated by airspace enlargement. The findings from these studies have indicated that relatively long term (>4 months) continual daily exposure to tobacco smoke is required to induce pathological changes in otherwise healthy mice (Hautamaki et al., 1997; Guerassimov et al., 2004; Foronjy et al., 2005; Ma et al., 2005). Furthermore, these types of studies have often used concentrations of tobacco smoke similar to what would be experienced by primary cigarette smokers. Less well studied in murine models is the effect of tobacco smoke at concentrations and for periods of time which reflect second hand smoke exposures in human subjects.

In this study we sought to define the consequences to the murine pulmonary compartment of sub-chronic (4 week) exposure to a mixture of mainstream and sidestream tobacco smoke. We evaluated this exposure in both wild type (C57BL/6) and *Arhgef1*$^{-/-}$ mice. Arhgef1 is an intracellular signaling molecule predominantly expressed by leukocytes and that has been shown to contribute to both leukocyte integrin adhesion and migration (Girkontaite et al., 2001; Rubtsov et al., 2005; Francis et al., 2006; Hu et al., 2008). We have reported that Arhgef1-deficient mice spontaneously develop pulmonary features reminiscent of individuals with chronic obstructive pulmonary disease (COPD; Hartney et al., 2010). These pulmonary features of *Arhgef1*$^{-/-}$ animals include chronic inflammation as defined by elevated numbers of pulmonary leukocytes in lung tissue and the airspace compartment, airspace enlargement and loss of elastic recoil in the pulmonary compartment. More recently, we have identified a novel signaling pathway used by pulmonary macrophages to promote inflammation and that is regulated by Arhgef1 (Hartney et al., 2011). In this study we compare cigarette smoke-induced inflammation in wild type and Arhgef1-deficient animals to determine the relationship between the pathways involved in cigarette smoke exposure inflammatory responses and those dependent on the presence of Arhgef1.

MATERIALS AND METHODS

SMOKE EXPOSURE PROTOCOL

C57BL/6 mice were initially obtained from the Jackson Laboratory, Bar Harbor, ME and subsequently bred in our animal facility. Arhgef1-deficient mice were generated and used on

a C57BL/6 genetic background (Rubtsov et al., 2005) and also bred and maintained in our animal colony. All experiments with animals were approved by the Institutional Animal Care and Use Committee. Mice were exposed to cigarette smoke (2RF4 reference cigarettes, University of Kentucky) in TE-10z smoking chambers (Teague Enterprises, Davis, CA) for 6 h per day, 5 days per week (Teague et al., 1994). Smoke exposure is adjusted in this system to generate a mixture of sidestream smoke (89%) and mainstream smoke (11%) by burning five cigarettes simultaneously. Chamber atmosphere was monitored for total suspended particulates and carbon monoxide, with concentrations of 70–80 mg/m^3 and 190 ppm, respectively as previously described (Martin et al., 2006; Tollefson et al., 2010). Animals were sacrificed 1 day after the last smoke exposure and lungs harvested. The 4 week smoke exposure protocol was performed in two separate experiments and cohorts of animals were assessed by leukocyte quantitation, histological examination and lung mechanics on both occasions.

ENUMERATION OF LEUKOCYTES

Lungs were lavaged with Hanks' balanced salt solution with 5 mmol/L EDTA. An aliquot of cells were counted on a Z2 particle count and size analyzer (Beckman Coulter, Fullerton, CA) as previously described (Hartney et al., 2010). Leukocytes were isolated from lavaged lung tissue after treatment with collagenase types II and IV (Sigma-Adrich, St. Louis MO) and dispase II (Roche, Basel, Switzerland), as previously described (Hartney et al., 2010).

FLOW CYTOMETRY

After isolation, cells were stained using standard methods and the following antibodies as previously described (Hartney et al., 2010). Leukocytes were identified using a pan-CD45 antibody (30-F11; eBiosciences, San Diego, CA) and F4/80 (A3-1, Serotec, Raleigh, NC), Gr-1 (RB6-8C5; eBiosciences), B220 (RA3/6B2), or CD3 (2C11) for identification of macrophages, neutrophils, B cells and T cells respectively, and back-gated during analysis to confirm appropriate forward and 90° light scatter. CD4 (GK1.5) and CD8 (53-6.7) staining further differentiated T cell subsets. Data were collected with a FACSCaliber (BD Pharmingen, San Diego, CA) and analyzed with FlowJO 8.8.4 software (Tree Star, Inc., Ashland, OR).

LUNG MECHANICS

Lung mechanics were assessed as previously described (Lovgren et al., 2006) using a Flexivent (Scireq, Montreal, Canada) small animal ventilator. A stepwise inflation up to 1.2 ml of air was applied to the lungs. Pressure-volume graphs were generated with the expiratory phase using the pressure and volume values obtained after a one-second pause in piston movement.

LUNG HISTOLOGY

Lungs were inflated via tracheal cannula to 25 cm of pressure using a tower filled with 4% paraformaldehyde. The trachea was then tied off below the cannula and the lungs removed and immersed in 4% paraformaldehyde for 24 h. Lungs were then imbedded in paraffin and cut into 2–3 μm-thick slices at a random orientation and stained with hematoxylin and eosin. At least twenty-five 20× fields were captured electronically by stratified random sampling as previously described (Subramaniam et al., 2007). Next we used a digital image analysis approach as described by Tschanz and Burri (2002) and subsequently adapted into a macro for ImagePro 4.5 (Media, Cybernetics; Hartney et al., 2010). This process occurs in three automated steps as illustrated in **Figure 1**. First the digital image shown in **Figure 1A** is converted into a binary image (**Figure 1B**). Next this binary image is skeletonized (**Figure 1C**). Then a series of probes are superimposed across the skeletonized image (**Figure 1D**). Points where the skeletonized image intercepts a probe are identified and tallied. The number of intercepts and the length of the probes applied were then reported to a spreadsheet. Mean linear intercept was calculated by the formula:

$$\text{Mean linear intercept} = \frac{\text{Total probe length}}{\text{Total number of intercepts}}$$

STATISTICAL ANALYSIS

JMP® (SAS Institute Inc., Cary NC) was used for all statistical analysis. For data sets including four groups a One-Way ANOVA was performed. If significance was detected with the One-Way ANOVA, a *post hoc* Tukey–Kramer HSD *t* test was performed. To determine whether there was a significant interaction between genotype (B6 vs. *Arhgef1*$^{-/-}$) and exposure [filtered air (FA) vs. second hand smoke (SHS)] a Two-Way ANOVA was performed on all appropriate data sets.

RESULTS

To explore the effects of second hand tobacco smoke exposure on the pulmonary compartment we exposed mice to a mixture

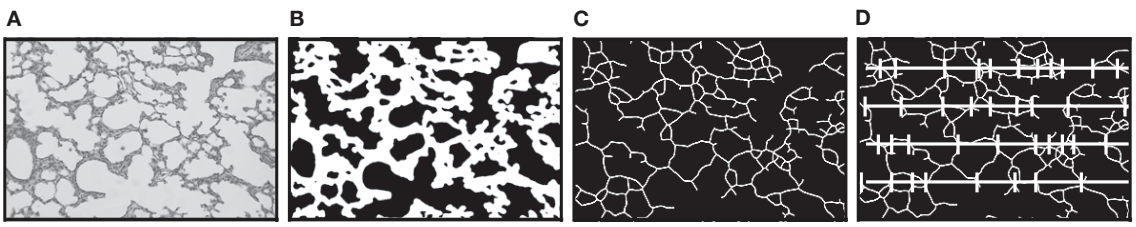

FIGURE 1 | Schema for the process of quantitating mean linear intercept. **(A)** Hematoxylin and eosin stained lung tissue section as an example of the digital images obtained for analysis. **(B)** A binary image of the photomicrograph converted by a macro program. **(C)** The binary alveolar structure is next reduced to a skeletonized structure one pixel in width. **(D)** A series of probe lines are superimposed on the skeletonized image and points where the alveolar structure intercepts the probes are recorded and exported. Mean linear intercept equal total probe length divided by number of intercepts.

of sidestream and mainstream smoke at concentrations similar to what an individual would experience through second hand smoke exposure (Teague et al., 1994; Woodruff et al., 2009). Both naïve wild type C57BL/6 and *Arhgef 1*$^{-/-}$ mutant mice were exposed to either FA or SHS for 4 weeks after which time the number of pulmonary leukocytes in lung tissue and airspace were enumerated and characterized.

LUNG TISSUE LEUKOCYTES

To quantify inflammatory cell infiltration in lavaged lung tissue after FA or SHS exposure we utilized enzymatic digestion of lung tissue followed by cell counting and flow cytometry. The results from these analyses reveal that sub-chronic SHS exposure of wild type mice does not lead to a significant change in the total numbers of CD45^{+} leukocytes in lung tissue (**Figure 2A**). Similarly, the number of total leukocytes in the Arhgef1-deficient lungs did not change following SHS exposure, although there are more pulmonary tissue leukocytes in Arhgef1-deficient lungs compared to the wild type when matched for exposure and as previously reported (**Figure 2A** and Hartney et al., 2010).

Although SHS exposure did not significantly alter the total number of leukocytes in lung tissue from either wild type or mutant mice, the number of pulmonary tissue macrophages in *Arhgef 1*$^{-/-}$ mice was modestly but not significantly increased after 4 weeks of SHS exposure.

In contrast to the increase in macrophages, the neutrophils present in wild type and *Arhgef 1*$^{-/-}$ lung tissues decreases after exposure to SHS. This decrease reaches statistical significance for the Arhgef1-deficient cohort (**Figure 2B**).

AIRSPACE LEUKOCYTES

Despite the modest effect that SHS exposure has on lung tissue leukocyte numbers, we observed a robust and significant increase in the total number of leukocytes recovered in bronchoalveolar lavage (BAL) from both C57BL/6 and *Arhgef 1*$^{-/-}$ animals exposed to SHS (**Figure 3A**). This increase in leukocytes could be largely accounted for by a significant increase in the alveolar macrophages recovered from the BAL of both wild type and mutant lungs (**Figure 3A**). Neutrophils (Gr-1^{+} cells) were also increased in the BAL of SHS exposed animals compared to FA controls for both genotypes, although this increase was only significant in *Arhgef 1*$^{-/-}$ cohort (**Figure 3B**). We performed a Two-Way ANOVA on our BAL leukocyte quantitation and failed to detect a significant interaction between genotype (C57BL/6 vs. *Arhgef 1*$^{-/-}$) and exposure (FA vs. SHS). Because of the increase in leukocytes recovered from SHS exposed animals, we were able to further identify the lymphocyte populations present in these samples (**Figure 3C**). These findings demonstrate that SHS exposed *Arhgef 1*$^{-/-}$ mice harbor significantly more T lymphocytes compared to C57BL/6 mice and is true for both CD4 and CD8 T cell populations (**Figure 3C**).

LUNG ARCHITECTURE

We next evaluated if SHS exposure and the resulting elevated number of airspace leukocytes altered lung architecture. To accomplish this, we inflated and fixed the lungs of a subset of animals and measured airspace structure as determined by mean linear intercept and described in materials and methods. Representative micrographs from each experimental group are

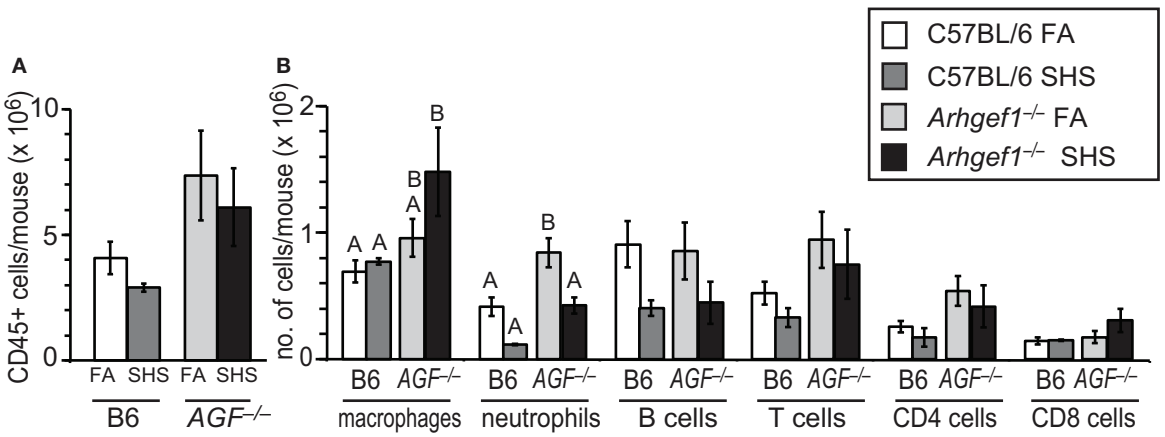

FIGURE 2 | Leukocyte numbers in lung tissue after 4 weeks of second hand smoke exposure. (A) Total number of CD45^{+} leukocytes in enzymatically digested and lavaged lung tissue from C57BL/6 (B6) and *Arhgef 1*$^{-/-}$ (*AGF*$^{-/-}$) mice exposed to filtered air (FA) or second hand smoke (SHS) for 4 weeks. **(B)** Number of leukocytes within different populations from enzymatically digested and lavaged lung tissue. Shown are the number of macrophages (F4/80^{+}), neutrophils (Gr-1^{+}), B lymphocytes (B220^{+}) and T lymphocytes (CD3^{+}), including CD4^{+} and CD8^{+} cells. Three month old C57BL/6 (B6) mice exposed to filtered air (FA) (open bars, $n = 8$), 3 month old C57BL/6 (B6) mice exposed to SHS for 4 weeks prior to harvest (dark gray bars, $n = 4$), 3 month old *Arhgef 1*$^{-/-}$ (*AGF*$^{-/-}$) mice exposed to filtered air (FA) (light gray bars, $n = 7$) and 3 month old *Arhgef 1*$^{-/-}$ (*AGF*$^{-/-}$) mice exposed to SHS for 4 weeks prior to harvest (black bars, $n = 4$). Data represents mean ± SE. A One-Way ANOVA was performed on leukocyte populations. Statistically significant differences between groups were detected only for macrophages and neutrophils. A *post hoc* Tukey–Kramer HSD *t* test was performed on these groups. Groups not sharing the same letter are significantly different, $P < 0.05$. For the macrophages the B6 FA, B6 SHS, and *AGF*$^{-/-}$ FA groups all share the letter A so none of these groups are significantly different from each other. The *AGF*$^{-/-}$ FA and the *AGF*$^{-/-}$ SHS groups share the letter B so these two groups are not significantly different from each other. The significant difference in macrophages occurs between the *AGF*$^{-/-}$ SHS group (black bar) which only has the letter B designation and the B6 FA and B6 SHS groups (open and light gray bars) which only have the letter A designation. Cells were enumerated and analyzed as described in materials and methods.

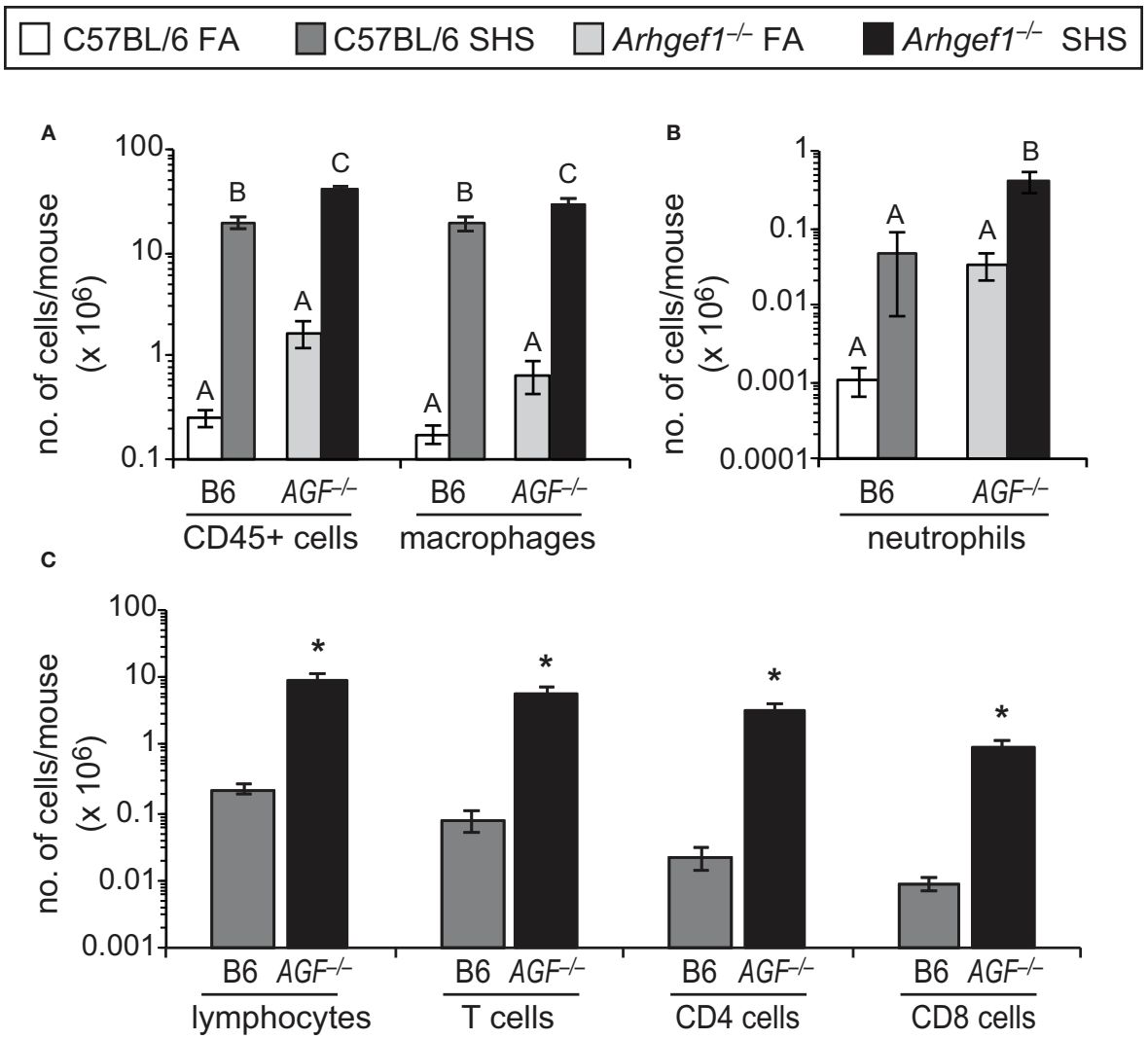

FIGURE 3 | Second hand smoke exposure for 4 weeks leads to increased numbers of pulmonary leukocytes in the airspace. Leukocytes were enumerated and characterized from bronchoalveolar lavage (BAL) as defined in materials and methods. **(A)** Total number of $CD45^+$ leukocytes and macrophages ($F4/80^+$) in BAL from C57BL/6 (B6) and *Arhgef* $1^{-/-}$ (*AGF*$^{-/-}$) mice exposed to filtered air (FA) or second hand smoke (SHS). **(B)** Enumeration of neutrophils ($Gr\text{-}1^+$) from C57BL/6 (B6) and *Arhgef* $1^{-/-}$ (*AGF*$^{-/-}$) samples as in panel **(A)**. **(C)** Quantitation of lymphocytes, T cells ($CD3^+$), CD4 and CD8 cells. Three month old C57BL/6 (B6) mice exposed to FA (open bars, $n = 8$), 3 month old C57BL/6 (B6) mice exposed to SHS for 4 weeks prior to harvest (dark gray bars, $n = 4$), 3 month old *Arhgef* $1^{-/-}$ (*AGF*$^{-/-}$) mice exposed to FA (light gray bars, $n = 7$) and 3 month old *Arhgef* $1^{-/-}$ (*AGF*$^{-/-}$) mice exposed to SHS for 4 weeks prior to harvest (black bars, $n = 4$). Data represents mean ± SE. A One-Way ANOVA was performed for all leukocyte populations. Statistically significant differences between groups were detected in all populations. A *post hoc* Tukey–Kramer HSD *t* test was performed. Groups not sharing the same letter are significantly different, $P < 0.05$. Due to limited number of cells in FA samples quantitation in **C** was only performed on SHS exposed samples. $^*P < 0.05$ Student two-tailed *t* test compared with identically treated C57BL/6 samples.

shown in **Figure 4A**. These data demonstrated that sub-chronic exposure to SHS is not sufficient to alter the airspace structure in the lungs of C57BL/6 mice (**Figure 4B**). When matched for exposure the *Arhgef* $1^{-/-}$ lungs exhibit significant airspace enlargement compared to the C57BL/6 lungs consistent with our previous report (**Figure 4B** and Hartney et al., 2010). Although the Arhgef1-deficient mice exhibited an exaggerated inflammatory response, this increased response was not sufficient to induce an increase in airspace enlargement beyond what is already present in the naïve *Arhgef* $1^{-/-}$ animals (**Figure 4B**).

RESPIRATORY MECHANICS

Prior to leukocyte enumeration and characterization or lung fixation, we assessed lung mechanics in all mice exposed to FA or SHS using a small animal ventilator. Specifically, lung function was assessed by measuring pressure-volume loops (**Figure 5**). As previously shown for naïve unchallenged mice (Hartney et al., 2010), *Arhgef* $1^{-/-}$ mice exposed to filtered air have significantly decreased lung elastance compared to similarly treated wild type mice. Furthermore, a 4 week exposure to SHS significantly decreased lung elastance in both

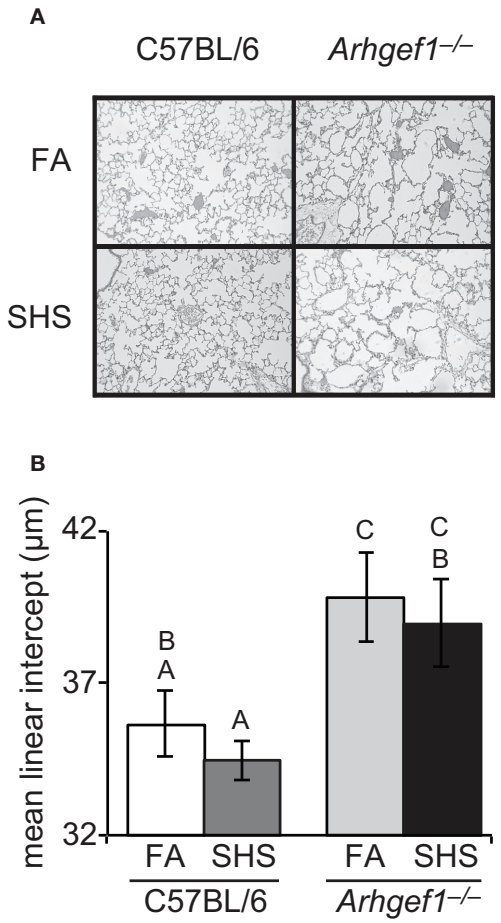

FIGURE 4 | Short term exposure to second hand cigarette smoke does not lead to airspace enlargement. Airspace structure was quantitated by mean linear intercept (μm) of alveolar septae as measured on inflated, hematoxylin and eosin-stained lung sections as described in materials and methods. **(A)** Representative lung tissue sections from C57BL6 animals (left column) or *Arhgef 1*$^{-/-}$ animals (right column) exposed to either filtered air (FA, top row) or second hand smoke (SHS, bottom row). **(B)** Mean linear intercept (μm) of 3 month old C57BL/6 mice exposed to filtered air (FA) (open bar, $n = 7$), 3 month old C57BL/6 mice exposed to second hand smoke (SHS) for 4 weeks prior to harvest (dark gray bar, $n = 8$), 3 month old *Arhgef 1*$^{-/-}$ mice exposed to FA (light gray bar, $n = 9$) and 3 month old *Arhgef 1*$^{-/-}$ mice exposed to SHS for 4 weeks prior to harvest (black bar, $n = 7$). A One-Way ANOVA was performed and significant differences were detected between groups. A *post hoc* Tukey–Kramer HSD *t* test was performed. Groups not sharing the same letter are significantly different, $P < 0.05$.

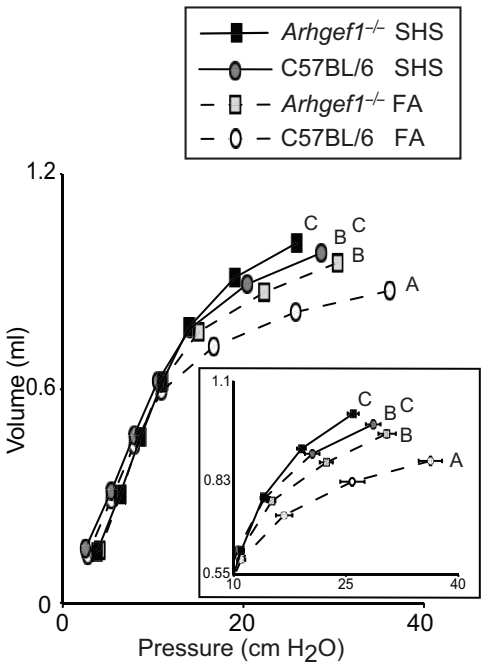

FIGURE 5 | Lung respiratory mechanics of C57BL/6 and *Arhgef 1*$^{-/-}$ mice after 4 weeks of exposure to filtered air (FA) or second hand smoke (SHS). Measurement of quasi-static pressure-volume loops were performed on 3 month old C57BL/6 mice exposed to FA (open circles, dashed line, $n = 8$), 3 month old *Arhgef 1*$^{-/-}$ mice exposed to FA (light gray boxes, dashed line, $n = 12$), 3 month old C57BL/6 mice exposed to SHS for 4 week prior to harvest (dark gray circles, solid line, $n = 7$) and *Arhgef 1*$^{-/-}$ mice exposed to SHS for 4 weeks prior to harvest (black boxes, solid line, $n = 13$). Insert shows expanded view of the upper right portion of pressure-volume loop. Data represents mean ± SE. A One-Way ANOVA was performed and significant differences were detected between groups. A *post hoc* Tukey–Kramer HSD *t* test was performed. Groups not sharing the same letter are significantly different, $P < 0.05$.

wild type and mutant animals (**Figure 5**). Results of a Two-Way ANOVA on these data reveal no interaction between genotype (C57BL/6 vs. *Arhgef 1*$^{-/-}$) and exposure (FA vs. SHS). Therefore, we conclude that the pathways leading to decreased lung elastance in the *Arhgef 1*$^{-/-}$ mice are independent of the pathways leading to decreased lung elastance after SHS exposure. The results from these experiments demonstrate that sub-chronic SHS exposure is sufficient to significantly decrease lung elastance in both mouse strains examined (**Figure 5**).

DISCUSSION

This study documents that 4 week sub-chronic exposure to second hand cigarette smoke does not lead to measureable leukocyte infiltration within lung tissue but does result in airspace inflammation and decreased lung elastance. The inability of sub-chronic smoke exposure to promote lung tissue inflammation or changes in airspace structure is consistent with previous reports (D'Hulst et al., 2005; Rinaldi et al., 2012) and was observed in both C57BL/6 and *Arhgef 1*$^{-/-}$ animals. In contrast to the lack of change in lung tissue inflammation and architecture, SHS exposure elicits a robust increase in all leukocyte populations recovered from BAL as previously reported (Woodruff et al., 2009). Within the SHS exposure cohort, all leukocyte populations examined were increased in *Arhgef 1*$^{-/-}$ airspace compared to C57BL/6. Characterization of lymphocyte subsets recovered from the BAL of SHS exposed animals also reveal a statistically significant increase in the *Arhgef 1*$^{-/-}$ samples compared to identically treated C57BL/6 samples. Together these data demonstrate that even sub-chronic (4 week) exposure to SHS is sufficient to induce significant increases in the number of airspace leukocytes present in either healthy or mutant lungs.

Examination of lung structure in C57BL/6 SHS exposed animals indicates that no morphological changes in lung structure have occurred. This result was not unexpected as it has been previously reported for this mouse strain that at least 3 months of tobacco smoke exposure are required to induce structural changes as measured by an increase in mean linear intercept (Bartalesi et al., 2005). We did hypothesize that the presence of pre-existing inflammation and airspace enlargement in the naïve Arhgef1-deficient mice would decrease the duration of SHS exposure required to induce further pathological changes. However, our current 4 week protocol failed to induce any increase in airspace enlargement in the Arhgef1-deficient animal beyond what is already present in the naïve animals, despite the exaggerated inflammatory response of the *Arhgef1*$^{-/-}$ mice to SHS exposure.

Significant decreases in lung elastance are evident in the C57BL/6 and *Arhgef1*$^{-/-}$ animals when exposed to SHS for a relative short duration (4 weeks). Initially we were surprised to observe a change in lung mechanics with a relative short smoke exposure protocol. A previous report failed to observe any changes in the lung mechanics of C57BL/6 mice exposed to smoke for 6 months (Guerassimov et al., 2004). However close examination of the methods employed to measure lung mechanics reveal an importance difference that may account, at least in part, for the discrepancy between our results and their study. In the previous report the investigators performed a primewave perturbation across a range of positive end expiratory pressures (PEEP) from 3 to 9 cm H_2O in order to generate a P-V loop (Guerassimov et al., 2004). In our study we generated a pressure-volume loop where lung mechanics are measured over a range of pressures from 2 ∼ 30 cm H_2O. Examination of our own pressure and volume measurements between 2 and 10 cm H_2O portion of the loop reveal no discernible difference in the SHS exposure groups. We did perform the primewave perturbation at a PEEP of 3 cm H_2O and consistent with their results do not detect any significant changes in lung elastance in the SHS exposed animals (data not shown). A recent review included both of these measurements performed in the pallid mouse strains (see Figure 4 in Wright et al., 2008). The primewave perturbation yields a modest but significant shift in lung mechanics of the pallid strain (Figure 4A in Wright et al., 2008) while the pressure-volume loop inflating the lungs up to pressures around 30 cm H_2O demonstrate dramatic differences between the healthy and diseased (pallid) lungs (Figure 4B in Wright et al., 2008). Together these data suggest that a P-V loop which inflates the lungs up to higher pressures (∼30 cm H_2O) may be more sensitive to detecting modest changes in lung elastance than measurement performed at lower pressures.

In addition to the differences in methods of measurements it is also worth noting the difference in duration of smoke exposure protocols between the studies, 4 weeks versus 6 months (Guerassimov et al., 2004). Aside from the increased duration of smoke exposure another parameter to consider is the age of the mice at the time of assessment. Our studies performing lung mechanics measurements find a progressive decrease in lung elastance of C57BL/6 mice from 3 months of age to 1 year of age, similar to reports by other investigators (Huang et al., 2007). Comparison of naïve *Arhgef1*$^{-/-}$ mice and C57BL/6 mice across these ages reveal the most pronounced differences between strains occur at 6 months of age (Hartney et al., 2010).

Comparing the lung mechanics and airspace architecture between all four groups suggests that changes in murine lung mechanics can occur in the presence or absence of changes in lung architecture. Note the SHS exposed C57BL/6 mice have lung elastance values lower than the naïve Arhgef1-deficient mice despite the lack of alteration in airspace structure, as measured by mean linear intercept (**Figures 4** and **5**). The lack of a direct correlation between lung mechanics and airspace structure has been noted by several investigators examining the effects of cigarette smoke exposure in mouse models (Guerassimov et al., 2004; Foronjy et al., 2005; Rinaldi et al., 2012). Based on these differences it has been proposed that separate pathways are involved in the development of histological alterations in lung architecture versus physiological changes in lung mechanics (Foronjy et al., 2005). Our study provides another instance of these two pulmonary phenotypes occurring independently and supports their proposed hypothesis.

We have previously described a signaling pathway that operates in pulmonary myeloid cells that leads to the production of pro-inflammatory mediators and is normally inhibited by Arhgef1 (Hartney et al., 2011). To address whether this same Arhgef1-regulated pathway contributes to cigarette smoke-induced inflammation we compared the responses of *Arhgef1*$^{-/-}$ and wild type mice to SHS exposure. Using a Two-Way ANOVA, no interaction is found between genotype and response to cigarette smoke exposure in any of our data sets. Thus, we conclude that these pathways, Arhgef1 and cigarette smoke exposure induced responses appear to occur independently of each other.

In conclusion the data presented here demonstrate that subchronic SHS exposure is sufficient to induce a significant increase in airspace leukocytes and decrease in lung elastance in both healthy animals and a mutant mouse strain with pre-existing pulmonary inflammation and pathology. This change in lung mechanics appears to occur as a result of processes that can be independent of changes in airspace structure. Further examination of the pathways responsible for SHS induced changes in lung mechanics may identify novel targets for restoring or retaining lung elastance in human subjects exposed to second hand smoke.

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Modeling the influence of vitamin D deficiency on cigarette smoke-induced emphysema

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Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. While the primary risk factor for COPD is cigarette smoke exposure, vitamin D deficiency has been epidemiologically implicated as a factor in the progressive development of COPD-associated emphysema. Because of difficulties inherent to studies involving multiple risk factors in the progression of COPD in humans, we developed a murine model in which to study the separate and combined effects of vitamin D deficiency and cigarette smoke exposure. During a 16-week period, mice were exposed to one of four conditions, control diet breathing room air (CD-NS), control diet with cigarette smoke exposure (CD-CSE), vitamin D deficient diet breathing room air (VDD-NS) or vitamin D deficient diet with cigarette smoke exposure (VDD-CSE). At the end of the exposure period, the lungs were examined by a pathologist and separately by morphometric analysis. In parallel experiments, mice were anesthetized for pulmonary function testing followed by sacrifice and analysis. Emphysema (determined by an increase in alveolar mean linear intercept length) was more severe in the VDD-CSE mice compared to control animals and animals exposed to VDD or CSE alone. The VDD-CSE and the CD-CSE mice had increased total lung capacity and increased static lung compliance. There was also a significant increase in the matrix metalloproteinase-9: tissue inhibitor of metalloproteinases-1 (TIMP-1) ratio in VDD-CSE mice compared with all controls. Alpha-1 antitrypsin (A1AT) expression was reduced in VDD-CSE mice as well. In summary, vitamin D deficiency, when combined with cigarette smoke exposure, seemed to accelerate the appearance of emphysemas, perhaps by virtue of an increased protease-antiprotease ratio in the combined VDD-CSE animals. These results support the value of our mouse model in the study of COPD.

Keywords: emphysema, vitamin D deficiency, second hand cigarette smoke, matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1, alpha-1 antitrypsin, chronic obstructive pulmonary disease, Teague smoke exposure device

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third leading cause of chronic morbidity and mortality in the United States and is projected to rank fifth in 2020 in burden of disease worldwide (Rabe et al., 2007). Smoking, the main risk factor for COPD (Stockley et al., 2009), is a powerful inducer of inflammatory mediators, including oxidants and proteases (Spurzem and Rennard, 2005). The increased activity of proteases is important given that protease-antiprotease imbalance plays a key pathogenic role in the development of emphysema in COPD. In this regard, alpha-1-antitrypsin (A1AT) is the prototypical member of the serine protease inhibitor (serpin) superfamily of proteins, which have a major role in inactivating neutrophil elastase and other proteases to maintain protease-antiprotease balance (Lomas and Mahadeva, 2002). Alpha-1 antitrypsin deficiency (AATD), a genetic disease resulting in low levels of antiproteases, is a risk factor for emphysema, which supports the importance of protease-antiprotease balance in the pathogenesis of emphysema (Sandhaus, 2010). Another family of proteolytic enzymes, the matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), are involved in remodeling the extracellular matrix and in host defense (Elkington and Friedland, 2006) and are also associated with tissue destruction in emphysema (Abboud and Vimalanathan, 2008).

Although smoking and age are major determinants of lung function (as measured by the forced expiratory volume in 1 s;

FEV1), there is an epidemiologic association between low serum concentrations of 25-hydroxy vitamin D and lung function (Black and Scragg, 2005). There is also an association between disease severity and genetic variability in vitamin D pathway genes found in cross-sectional studies of patients with COPD (Janssens et al., 2010). Indeed, there is increasing evidence for diverse roles for vitamin D, such as innate immunity and muscle strength that may contribute to respiratory health (Hughes and Norton, 2009). In a recent single center, double-blind study using high dose vitamin D supplementation, patients who entered the study with severe vitamin D deficiency, experienced fewer COPD exacerbation events after vitamin D supplementation (Lehouck et al., 2012). The results of this interventional study contrast with another study in which there were no differences in baseline vitamin D levels between smokers with rapid vs. slow declines in lung function over approximately 6 years of prospective follow-up (Kunisaki et al., 2011). This non-interventional study was limited—only one vitamin D measurement was made at the start of the study period. Thus, nothing is known about vitamin D intake or levels during the subsequent period of disease progression. Although there is increasing interest in vitamin D and lung disease, two important questions remain unanswered. First, can vitamin D deficiency act as a contributing risk factor for emphysema? Second, what are the mechanisms whereby reduced levels of vitamin D could directly influence the pathogenesis of emphysema? Putative mechanisms include immune modulation and lung tissue remodeling (Janssens et al., 2009).

Given the multitude of confounding factors, it is difficult to address any potential cooperative link between vitamin D deficiency and susceptibility to lung injury by cigarette smoke in humans. Therefore, we developed an animal model to study the separate and combined effects of vitamin D deficiency and cigarette smoke exposure on lung structure and function. We believe that this model provides a useful platform to investigate the individual and joint mechanisms that contribute to complex lung disorders.

MATERIALS AND METHODS

MICE, DIET, AND SMOKE-EXPOSURE

All aspects of the mouse protocol were approved by the Dartmouth Institutional Animal Care and Use Committee and/or the White River Junction VA Institutional Animal Care and Use Committee (*IACUC*). All mice were purchased from the National Cancer Institute at 5–6 weeks of age. Mice were housed with 12-hour dark and light cycles and given free access to food and water. Mice were weighed weekly and monitored for changes in health status. In early experiments, cigarette smoke exposed male mice became aggressive and were excluded from study for that reason. Therefore, we studied female FVB mice, a strain used in cigarette exposure experiments (Keith et al., 2004; Siddens et al., 2012). The animals were fed either a vitamin D deficient diet (VDD) or a vitamin D replete diet as a control (CD) (Harlan-Teklad, Madison, WI, USA; VDD number TD.09498 and CD number TD.09499) beginning at 6 weeks of age. The vitamin D replete diet contained 2.2 i.u vitamin D3/gm of food. Assuming mice consume ∼3 gms of food per day, the control diet animals received near the upper limit of the acceptable daily dose of vitamin D recommended by the Endocrine Society. Because of the effect of vitamin D on lung structure in developing mice (Zosky et al., 2011), we started dietary restriction at six weeks of age when development has progressed beyond the alveolar stage of lung growth (Backstrom et al., 2011). Six weeks after beginning each diet, mice were exposed to a combination of second hand cigarette smoke (89%) and primary cigarette smoke (11%) produced in a Teague-TE10 smoke exposure device (Griffith and Hancock, 1985) (Teague Enterprises, Woodland, CA), using Research Cigarettes 3R4F (University of Kentucky College of Agriculture Reference Cigarette Program, Lexington, KY), for 4 h/day, 5 days/week, for 16 weeks. The average Total Suspended Particle (TSP) content within the chamber was 75 mg/m^3. Control mice breathed filtered air and had handling similar to smoke-exposed mice. Chronic smoke exposures were conducted in four different experiments each with 48 mice randomly divided into the four treatment groups.

Vitamin D levels were confirmed either by Enzyme Immunoassay (EIA) according to the manufacturer's instructions (Immunodiagnostic Systems Ltd., Fountain Hills, AZ) or by outside laboratories (Dartmouth Reference Laboratory, Lebanon NH or ZRT Laboratory, Beaverton, OR). We sent samples to two outside laboratories to confirm our initial findings. Results from the two labs were consistent.

We studied the effects of two interventions, vitamin D deficiency and cigarette smoke exposure. Mice were randomly assigned to one of four treatment groups; control diet-non smoke exposed (CD-NS), control diet-cigarette smoke exposed (CD-CSE), vitamin D deficient-non smoke exposed (VDD-NS) and vitamin D deficient-cigarette smoke exposed (VDD-CSE). The results of this study were analyzed with a Two-Way ANOVA design in which each treatment (cigarette smoke exposure and vitamin D-deficiency) was a between subjects factor. When the ANOVA indicated that significant differences existed among treatment groups, specific pre-planned comparisons were made using *P*-values adjusted by the Bonferroni method. Data were reported as the mean ± the standard error of the mean (SEM).

RESPIRATORY MECHANICS

For pulmonary function testing, each mouse was anesthetized by intraperitoneal (i.p.) injection of Xylazine hydrochloride (10 mg/kg) and pentobarbital sodium (50 mg/kg). Tracheostomy was performed using a 19-gauge cannula and secured with suture ties. Animals were mechanically ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min using a computer-controlled volume ventilator (*flexi*Vent™, SCIREQ, Montreal, PQ) against a positive end-expiratory pressure (PEEP) of 3 cm H_2O. To facilitate measurements of respiratory mechanics, anesthetized animals were paralyzed with pancuronium bromide (0.5 mg/kg i.p.). To ensure adequate anesthesia, heart rate was monitored with a continuous electrocardiogram tracing.

The *flexi*Vent™ system uses a forced oscillation method to create pressure-volume curves in anesthetized animals to measure respiratory system mechanics (Bates and Suki, 2008; Vanoirbeek et al., 2010). The measured variables were fitted to a variety of models depending on the particular stimulus, and the measures

of respiratory mechanics were derived from the parameters in each model. For example, the Snapshot-150 perturbation used a fixed frequency (2.5 Hz) pressure oscillation applied at the trachea, and measurements of respiratory system resistance [R], and compliance [C] were derived from these pressure oscillations using a single compartment model with an equation of motion dependent only on flow and volume. The Quick-prime-3 perturbation consisted of a multiple frequency pressure oscillation used to calculate the input impedance of the respiratory system. From the impedance measurement, estimates of airway resistance [Rn], tissue damping [G], and tissue elasticity [H] were made using a constant phase model. Finally, pressure-volume curves were used to measure static compliance [Cst], total lung capacity [TLC], and hysteresis [Area]. All of the fitted parameters were calculated using *flexi*Vent™ software. The *flexi*Vent™ system has been widely used to make measurements of respiratory mechanics in mice and has been validated in chronic measurements of respiratory mechanics in mice by comparing measurements derived from the *flexi*Vent™ system to more traditional measurements of respiratory mechanics (De Vleeschauwer et al., 2011).

LUNG PATHOLOGY

To preclude the possibility of confounding effects from the measurements of respiratory mechanics, the analysis of lung pathology, including morphometric data and images, came from mice that were not studied by *flexi*Vent™, and alternate groups of mice were selected for analysis of lung pathology or lung mechanics. Mice selected for analysis of lung pathology were euthanized by cardiac puncture to avoid the potential effects of CO_2 asphyxiation on atelectasis. Lungs were inflated at 20 cm H_2O pressure with a 2.5% gluteraldehyde solution. Blocks of fixed tissue were excised, placed in cassettes and set in paraffin. Hematoxylin and eosin stained slides were examined by light microscopy with 4×, 10× and 20× magnification with a standard Olympus microscope by a board certified surgical pathologist.

The pathologist was blinded to the treatment group of each animal. The presence/absence and degree of emphysema, acute neutrophilic inflammation of the tubular airways (bronchi and bronchioles), chronic (mainly lymphocytic) inflammation of the tubular airways, chronic parenchymal inflammation (of spongy alveolar tissue), pigmented macrophages within tubular airways, and pneumocyte cell hyperplasia (without further interpretation as pre-neoplastic or reactive) was recorded for each mouse lung. A four tiered score (0, +, ++, + + +) was recorded for each of the morphological features. Zero, meaning the finding was absent in the lung tissue on the H&E slide, +, meaning that the finding was focal (limited to either one tubular airway or 1 high power field (HPF) of parenchyma), ++, meaning the finding was present in two tubular airways or 2 separate HPF's or parenchyma, and + + +, meaning the finding was present in 3 or more tubular airways or HPFs. Pigmented macrophages, which are commonly seen in human smokers and called "smokers macrophages" are associated with "smokers bronchiolitis" or "respiratory bronchiolitis" and were characterized by the presence of macrophages containing dusty brown non-hemosiderin pigment in tubular airways (Couture and Colby, 2003). Pneumocyte hyperplasia was scared based on an increase in the size and shape of alveolar pneumocytes. The pathological scores ranged from 0 to 2+, and there were few 2+ scores. For that reason, the scores in each mouse were converted to ordinal data: the simple presence or absence of the pathological finding, and the presence (1) or absence (0) of each particular pathological finding was compared among groups using a non-parametric, One-Way ANOVA (Kruskal–Wallis Test).

HISTOMORPHOMETRIC ANALYSIS

Mean Linear Intercept (L_M) is a reflection of the mean air space diameter (Hsia et al., 2010). This variable was chosen to give additional information about alveolar abnormalities, observed as air space enlargement (Heemskerk-Gerritsen et al., 1996; Robbesom et al., 2003; Jacob et al., 2009). Images (JPEG) of histological sections of the glutaraldehyde fixed lungs were taken using a Diagnostic Instruments, Inc., 11.2 Color Mosaic digital camera (at 1600 × 1200 pixel resolution) using the SPOT Basic software, a 40× objective lens, and a 10× eyepiece on an Olympus BX50 microscope. We calculated that 2.764 pixels at this magnification were equal to one micron. From each lung section, ten random pictures were analyzed.

In Matlab® (a high-level technical computing language), each image was automatically separated into two color planes (tissue and white space) using K-means clustering (partitioning algorithm). White spaces with areas of less than 500 pixels were excluded from this analysis so as to avoid measuring vessels and stray white pixels. In parallel horizontal lines 25 microns (69 pixels) apart, transitions from airspace to tissue were automatically counted, as was the total distance measured (28,800 pixels, or 10,420 microns). The mean linear intercept (L_M) in each image was then calculated in microns according to:

$$L_M = \frac{(N \times l)}{\sum I}$$

where N is the number of times the line is placed on the image, l is the length of each line and $\sum I$ is the total number of transitions from airspace to tissue measured.

QUANTITATIVE PCR

For gene expression studies in the lung, we selected genes known to be involved in development of emphysema. Tissues for mRNA analysis were prepared from excised lungs that were placed in RNAlater® (Qiagen, Valencia, CA) and frozen at -80 C. RNA was extracted from each sample using RNeasy® (Qiagen, Valencia, CA). One microgram of RNA was reverse transcribed to cDNA using an iScript™ kit (Bio-Rad, Hercules, CA) and amplified by real time PCR using SYBR green master mix (Bio-Rad, Hercules, CA). Gene expression levels were normalized to mouse housekeeping gene RPL13a (MHK) expression. The primer sets used are shown in **Table 1**. Quantification was determined using the software *Opticon Monitor* software version 3.1.

RESULTS

VITAMIN D LEVELS ARE DECREASED IN VDD MICE

To confirm that the vitamin D deficient diet resulted in low vitamin D levels, we measured vitamin D levels in all mice. All

vitamin D levels were measured at the conclusion of each study at the time of sacrifice. The mean serum level of 25-hydroxyvitamin D3 in CD-NS mice was 18.2 ± 11.2 ng/mL and in CD-CSE it was 19.9 ± 9.8 ng/mL. Mice on the vitamin D deficient diet had levels of <5 ng/mL in both NS and CSE conditions (**Figure 1**). Thus, levels of 25-hydroxyvitamin D in mice fed control diets were significantly greater than 25-hydroxyvitamin D levels in mice fed a vitamin D deficient diet ($p = 0.001$).

Table 1 | Primers used for RT-PCR.

Gene	Primers
TIMP1	F 5-CCGCAGTGAAGAGTTTCTCA-3 R 5-TCACTCTCCAGTTTGCAA GG-3
MHK-RPL13a	F 5-ATGACAAGAAAAAGCGGATG-3 R 5-CTTTTCTGCCTGTTTCCGTA-3
MMP9	F 5-CTCACTCACTGTGGTTGCTG-3 R 5-TGGTTATCCTTCCTGGATCA-3
A1AT	F 5-TTCCAACACCTCCTCCAAAC-3 R 5-CACTTTCTTGGCCTCCTCTG-3

F, forward; R, reverse; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metallopeptidase; A1AT, alpha 1-antitrypsin or α 1-antitrypsin.

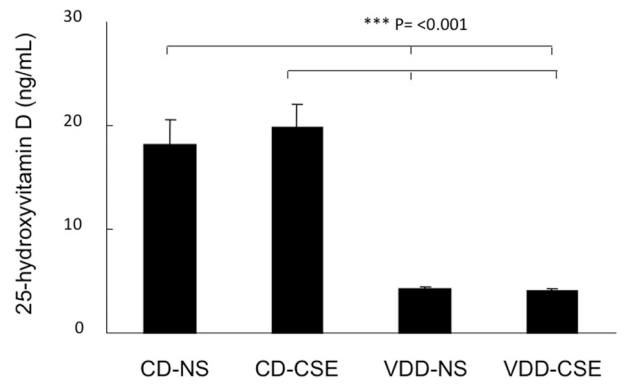

FIGURE 1 | 25-hydroxyvitamin D levels in the four treatment groups at the end of the experimental period. Values shown reflect means ± SEM of 19–23 mice per group. Levels of 25-hydroxyvitamin D in mice fed control diets were significantly different than mice fed a vitamin D deficient diet ($p = 0.001$).

PATHOLOGY

In order to explore the effect of CSE on lung architecture and the frequency of pathological findings within the lung, the histological slides were examined in a blinded fashion. Representative sections are shown in **Figure 2** and summary statistics, based on the presence or absence of specific pathological features in each treatment group, are shown in **Table 2**. Statistically significant differences were noted between treatment groups in the occurrence of pigmented macrophages within small airways in 8 of 11 of the VDD-CSE group and in 5 of 11 of the CD-CSE mice ($p < 0.05$ for both groups compared to control, CD-NS animals). No pigmented macrophages were noted in sections of lung of the mice breathing filtered room air. There were only rare instances of acute neutrophilic inflammation in any of the specimens, but chronic inflammation, indicated by the presence of lymphocytic inflammatory infiltrates, was seen sporadically in all groups of mice. However, none of the other pathological features differed significantly among groups.

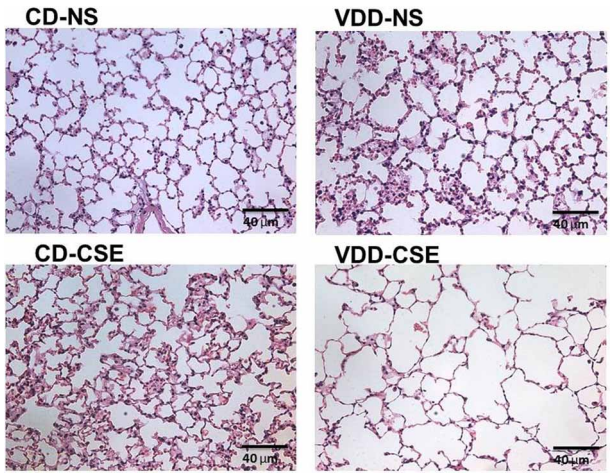

FIGURE 2 | Normal and emphysema-like features in hematoxylin and eosin (H&E) stained sections of lung representative of lung sections from each treatment group. All images are 40× enlargements.

Table 2 | Histological review of mouse lungs.

	Airway inflammation (%)	Parenchymal inflammation (%)	Pigmented macrophages (%)	Pneumocyte hyperplasia (%)
CD-NS	44	78	0	22
VDD-NS	55	73	18	45
CD-CSE	0	67	75*	0
VDD-CSE	36	55	91*	18

*Sections of H&E-stained mouse lung were reviewed for the presence/absence of the four histologic features indicated in **Table 2**. The frequency of occurrence of these features is represented as percentages of animals within each treatment group in which the histological feature was observed. All of the inflammation in these cases was chronic (lymphocytic); there were no cases that showed acute (neutrophil) cells. *Indicates a significant increase in a particular feature compared to control animals (CD-NS) at the p < 0.05 level.*

MORPHOMETRIC ANALYSIS REVEALS ENLARGEMENT OF AIR SPACES IN VDD-CSE MICE

Morphological measurements represent a useful, unbiased method to characterize emphysematous lesions in a semi-quantitative manner (Robbesom et al., 2003). Enlargement of air spaces was evaluated by the Mean Linear Intercept (L_M) measurement technique (Thurlbeck, 1967). Based on analysis of 10 images from each lung section of each of eleven mice in each of the four treatment groups (440 total images analyzed), mice with VDD-CSE treatments had significantly longer mean linear intercept (L_M) distance (36.27μ ± 0.88) compared to animals in the other three groups (**Figure 3**). CD-NS MLI was 32.93 μ (±0.37, $p = 0.0006$). VDD-NS was 33.32 μ (±0.47, $p = 0.0037$) and CD-CSE was 29.08 μ (±0.487, $p = 0.00001$). Thus, VDD-CSE mice had the greatest morphometric air space enlargement, consistent with a contribution of vitamin D deficiency to the development of emphysema.

It is surprising that the CD-CSE animals had, on average, a normal L_M. The analysis was, however, confounded by the co-occurrence of accumulations of macrophages. As shown in **Table 1**, there was a striking increase in the number of animals with increased alveolar macrophages in the airspace among the smoke exposed animals. The measurement of L_M was automated to preclude bias and measured airspace size—not inter-septal distances across alveoli (which is consistent with the ATS Standards for Quantitative Assessment of Lung Structure; (Hsia et al., 2010), and these standardized measurement methods of are specifically meant to exclude changes in septal thickens). Moreover, others have noted that smoke exposure in mice may increase macrophage numbers (Hirama et al., 2007). Thus, increased macrophage accumulation in the alveolar airspace in the CD-CSE animals may have reduced the measured L_M despite the presence of alveolar enlargement shown in some regions of the lung (**Figure 2**, CD-CSE example).

MICE EXPOSED TO CIGARETTE SMOKE HAVE ALTERED RESPIRATORY MECHANICS

Respiratory mechanics are used in COPD and emphysema as an measure of disease severity and progression. To determine if exposure to cigarette smoke and a vitamin D deficient diet influenced lung function, 63 mice were subjected to analysis of respiratory mechanics using a *flexi*Vent™ ventilator. Using the forced oscillation methods described above, static compliance increased significantly in CD-CSE and the VDD-CSE as compared to controls ($P < 0.05$ for both groups; data not shown). However, there was no significant difference in compliance between VDD-CSE mice and CD-CSE mice; compliance was similarly elevated in both groups. Pressure-volume loops were used to estimate Total Lung Capacity (TLC) (Vanoirbeek et al., 2010), an important indicator of the severity and progression of emphysema (Pauls et al., 2010). Consistent with the measurements of compliance, the TLC was also increased in both CD-CSE and VDD-CSE mice compared to the control animals (CD-NS) (**Figure 4**). The TLC and static compliance were elevated in the VDD-CSE mice, and animals in this treatment group had the longest average L_M. Thus, the measurements of respiratory mechanics were consistent with the morphometric analysis of alveolar diameters in VDD-CSE mice. The same was not true of the CD-CSE mice in whom compliance and TLC were significantly increased compared to CD-NS animals, but L_M was not increased. As noted above, the presence

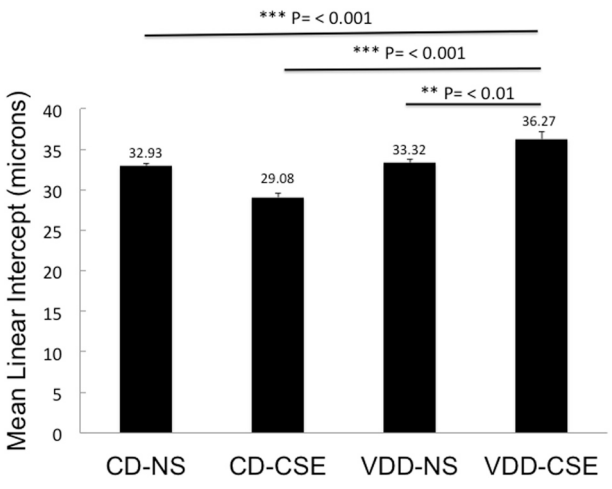

FIGURE 3 | Mean Linear Intercept (L_M) in experimental groups. Levels shown reflect means ± SEM. L_M is significantly longer in VDD-CSE mice compared to all other treatment groups and is consistent with emphysema. Data shown are based on analyses of 10 images from each lung section of each of eleven mice in each of the four treatment groups (440 total images analyzed).

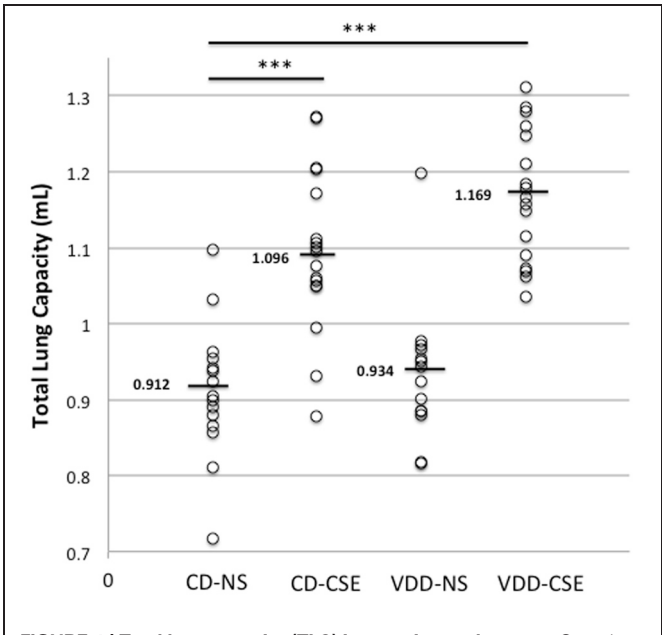

FIGURE 4 | Total lung capacity (TLC) in experimental groups. Over the course of three separate experiments, total lung capacity was determined by *flexi*Vent™ in 63 mice. Each point represents the data from one mouse with 14 to 17 mice per treatment group. Horizontal bars mark medians. A Two-Way ANOVA indicated that the mean TLC in the VDD-NS group and the CD-CSE group was significantly greater than the mean TLC in the control group (CD-NS) (indicated by *** in the figure; $p = 0.001$).

of increased numbers of pigmented macrophages may have confounded the measurements of L_M in the CD-CSE mice, but it is also true that changes in alveolar dimensions may lag changes in respiratory mechanics in cigarette smoke exposed mice (Rinaldi et al., 2012).

mRNA EXPRESSION IN LUNG TISSUES IS ALTERED IN VDD-CSE MICE

Proteases and their inhibitors are involved in tissue damage in emphysema. To begin to address the mechanisms of increased emphysema in the VDD-CSE mice, we looked at genes previously associated with COPD (Kang et al., 2003; Mercer et al., 2005; Tuder et al., 2010). We did quantitative real time PCR analysis of relative mRNA expression in the four treatment groups comparing gene expression of alpha-1 antitrypsin (A1AT), MMP9, and TIMP-1, to the expression of the mouse housekeeping gene RPL13a (MHK). While neither the CD-CSE nor the VDD-NS group mice had a significant change in A1AT, when the two treatments were combined in the VDD-CSE mice, lung expression level of this gene, crucial in protecting the lung against development of emphysema, was significantly decreased (**Figure 5A**). MMPs and their inhibitors, TIMPs, are also important in the pathogenesis of emphysema (Smolonska et al., 2009). While we found a non-significant trend toward increased expression of MMP-9 when comparing CD-NS to VDD-CSE (**Figure 5B**), analyses revealed a significant drop in expression of its cognate inhibitor, TIMP-1 (Mercer et al., 2005) only in the VDD-CSE group (**Figure 5C**). The ratio of MMP-9 to TIMP-1 was significantly different comparing VDD to VDD-CSE ($p = 0.05$) and CD-NS and CD-CSE to VDD-CSE ($p = 0.01$) (**Figure 6**). At the expression level, the increase in MMP-9, the decrease in TIMP-1 and the decrease in A1AT suggest that a significant imbalance in favor of proteolysis existed in the VDD-CSE mice.

DISCUSSION

Lifespan, period of exposure required to develop disease, as well as heterogeneity of lifestyle and co-morbid factors preclude straightforward examination of the contribution of vitamin D deficiency to development of emphysema in humans. For this reason we developed a new, pragmatic model for the study of emphysema by combining Vitamin D deficiency and cigarette smoke exposure in mice. To our knowledge this is the first model to combine dietary vitamin D deficiency and smoke exposure. We demonstrated an effect of vitamin D deficiency and cigarette smoke exposure on the mean linear intercept, the total lung capacity and lung compliance and a shift in the protease/antiprotease balance within the lung that favored proteolysis in this model in the vitamin D deficient animals. Our outcomes are consistent with epidemiological associations between the low Vitamin D levels, cigarette smoke exposure and COPD. These data support a role for vitamin D deficiency in development of emphysema, and our murine

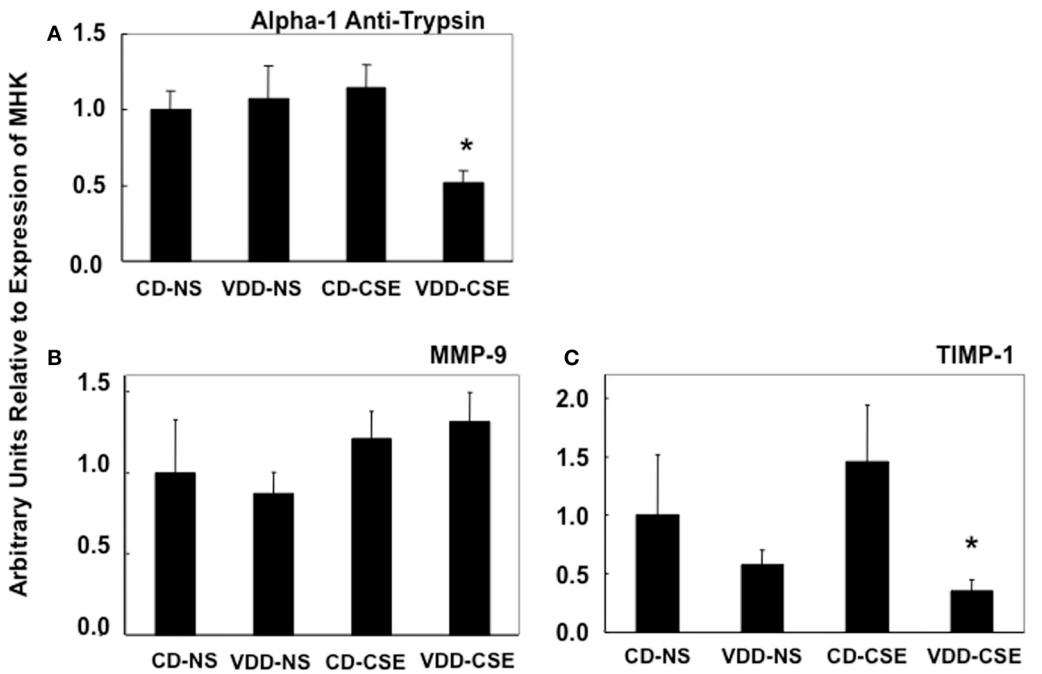

FIGURE 5 | Bars represent the ratio ± SEM of expression of genes to expression of the MHK housekeeping gene from whole lung in 4–8 mice from each treatment group. (A) Expression of A1AT is significantly different in VDD-CSE mice from control mice (CD-NS) ($p = 0.0060$), from CSE ($p = 0.0019$), and from VDD mice ($p = 0.0290$). **(B)** While mean values of MMP-9 expression suggest an increase in CSE and VDD-CSE treated mouse lungs, expression of MMP-9 was not significantly different when comparing various treatment groups. **(C)** Expression of TIMP-1 was significantly different in VDD-CSE mice from CSE group ($p = 0.0448$).

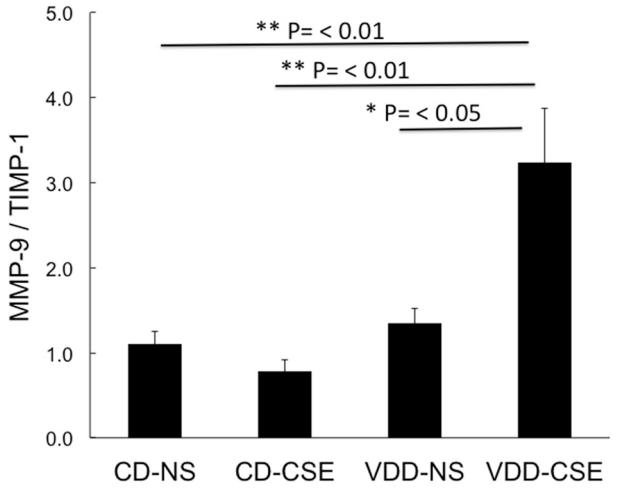

FIGURE 6 | Bars represent the ratio ± SEM of expression of MMP-9 divided by TIMP-1 in each animal studied. Both TIMP-1 and MMP-9 are standardized to expression of the MHK housekeeping gene from whole lung from four to seven mice from each treatment group. Expression of the TIMP-1/MMP-9 ratio was significantly different comparing VDD to VDD-CSE ($p = 0.05$) and CD-NS and CD-CSE to VDD-CSE ($p = 0.01$).

model provides a platform to study the effect of cigarette exposure and vitamin D deficiency on the immunopathogenesis of this common debilitating disease.

The importance of having a model in which to study the relationship of vitamin D deficiency to cigarette smoke induced lung damage is supported by a number of observations. Vitamin D deficiency occurs frequently in COPD, and the severity of vitamin D deficiency correlates with the severity of COPD (Janssens et al., 2009, 2010). However, whether vitamin D deficiency is a consequence of COPD, a comorbid factor in COPD, or a contributing causal risk factor in the pathogenesis of COPD cannot be fully determined from epidemiological data. Vitamin D deficiency could be a consequence of COPD since patients with COPD spend more time indoors when compared to people of the same age and location without COPD (Donaldson et al., 2005). In addition, aging skin (both chronological and from smoking) and glucocorticoids (which are prescribed frequently to patients with COPD and may increase vitamin D metabolism) can compound vitamin D deficiency in COPD patients (Hughes and Norton, 2009). Alternatively, vitamin D deficiency could result in skeletal changes (Franco et al., 2009), muscle weakness (Hopkinson et al., 2008), or propensity to infectious exacerbations (Ginde et al., 2009); all of which are comorbid factors in COPD. Another possibility is that vitamin D deficiency, through its effects on innate and adaptive immunity (Adams and Hewison, 2008; Hewison, 2010; Miller and Gallo, 2010), could serve as a contributing causal, risk factor for COPD, a disease mediated at least in part by inflammatory cell-derived proteases (Churg et al., 2008).

This model of cigarette smoke exposure was designed to address whether low vitamin D levels can cooperate with cigarette smoke exposure in the pathogenesis of emphysema. We demonstrated an increased incidence of smoke-induced lung disease in vitamin D deficient-cigarette smoke exposed mice compared to control diet-cigarette smoke exposed mice. Specifically, morphometric assessment of alveolar dimensions, using the average alveolar size as estimated by the mean linear intercept (L_M), was significantly increased in the VDD-CSE group, which is consistent with the occurrence of emphysema. The L_M distance in the CD-CSE mice was not increased, which was contrary to our expectations. The L_M, as we measured it and as is recommended by the American Thoracic Society, measures the airspace dimensions only. Images of representative lung sections (see **Figure 2**, bottom left) raise the possibility that the relatively smaller L_M in the CD-CSE mice reflects both increased numbers of macrophages (Hirama et al., 2007; Bodas et al., 2011) and less prominent or slower developing emphysema in mice exposed to cigarette smoke (CD-CSE) for only 16 weeks, as compared to the mice with combined vitamin D deficiency and cigarette smoke exposure. The presence of increased macrophages in the airspace of the CD-CSE animals, which is typical of cigarette smoke exposed animals and humans, may have caused us to underestimate the "true" L_M in the CD-CSE mice. This latter explanation is consistent with our *flexi*Vent™ data, which revealed increased TLC and lung compliance in both CD-CSE and VDD-CSE groups. The asymmetrical deposition of macrophages within individual alveoli may not have altered the intrinsic elastic characteristics of the lung, which make a major contribution to the compliance of the respiratory system and to the TLC, but may have diminished the L_M of the alveoli with particularly heavy infiltration of macrophages.

It seems more likely to us, however, that the morphometric changes in lung associated with cigarette smoke exposure simply take longer to develop than the changes in respiratory mechanics (Rinaldi et al., 2012). Emphysematous changes in lung morphology usually occur in mice only after 26 weeks of cigarette smoke exposure (Shapiro et al., 2003). Thus, we found the highest TLC, the highest compliance and the largest L_M after only 16 weeks of cigarette smoke exposure in the vitamin D deficient animals. The next highest average TLC and compliance measurements occurred in the CD-CSE animals, though L_M was not similarly increased. Thus, adding vitamin D deficiency to cigarette smoke exposure may have accelerated the development of the pathological changes typical of emphysema so that the VDD-CSE group had pathological evidence of emphysema after only 16 weeks of cigarette smoke exposure; whereas previous investigators found that pathological evidence of emphysema appeared in non-vitamin D deficient mice only after 26 weeks of cigarette exposure even though changes in lung mechanics occurred after 12 weeks of cigarette smoke exposure (just as we found in the vitamin D replete animals after 16 weeks of cigarette smoke exposure) (Rinaldi et al., 2012). The CD-CSE mice were, in our view, developing emphysema (lung compliance and TLC were increased and there were focal areas of emphysema; see **Figure 2**), but the morphometric hallmark of emphysema (an increased L_M) had not developed after only 16 weeks of cigarette smoke exposure. The possibility of accelerated emphysema formation when vitamin D was deficient is also supported by the greater increase in expression of proteolytic enzymes compared to antiproteolytic factors in the VDD-CSE mice compared to the mice exposed to either vitamin D deficiency or cigarette smoke alone.

Vitamin D is a steroidal hormone produced mainly from precursors within the skin via the action of ultraviolet B and available, to a lesser extent, from dietary sources. To define our model, mice on control diets were fed standard levels of vitamin D found in commercial mouse chow; in contrast the mice on VDD diet had no exogenous source of vitamin D. Vitamin D is recognized as an important hormone that controls genes with a multitude of extra-skeletal functions through its actions as a transcription factor (Bischoff-Ferrari, 2010; Holick, 2010) including control of the expression of a number of genes associated with innate immunity (Adams et al., 2007, 2009). Infectious exacerbations are increasingly frequent in the late stages of COPD in humans and vitamin D insufficiency or deficiency could contribute to COPD pathogenesis by diminishing protection against lung infection. Ongoing experiments are addressing this hypothesis.

To begin to address putative mechanisms whereby vitamin D deficiency might contribute to our observed findings, we looked at enzymes associated with lung remodeling and at TIMP-1, a gene that we noted in early experiments to be altered by cigarette smoke. MMPs have an important role in the breakdown of ECM and may serve as markers of tissue damage in smoking related lung diseases (Elkington and Friedland, 2006; Ilumets et al., 2007; Louhelainen et al., 2009). TIMP-1 is a specific inhibitor of MMP-9; low levels of TIMP-1 relative to MMP-9 suggest a proteinase rich environment that would foster breakdown of the extracellular matrix. The MMP-9:TIMP-1 ratio is increased in sputum of COPD patients (Beeh et al., 2003) and plasma MMP-9 levels are inversely related to vitamin D levels (Timms et al., 2002). It is not surprising then that vitamin D suppresses the production of MMPs and enhances the level of tissue inhibitor of metalloproteinase-1 (TIMP-1) (Anand and Selvaraj, 2009). Thus, our data indicating an increase in MMP-9: TIMP-1 ratio in vitamin D deficient smoke-exposed mice provides a mechanism whereby vitamin D deficiency can influence and accelerate the development of emphysema. In keeping with a role for vitamin D deficiency in protease-antiprotease imbalance, we also found significantly reduced A1AT levels in vitamin D-deficient smoke-exposed mice. Alpha-1 antitrypsin deficiency (AATD) confers susceptibility to emphysema, and polymorphisms in vitamin D-binding protein (VDBP; also known as Gc-globulin) have been linked to AATD (Chishimba et al., 2010).

In this study, designed to address whether low vitamin D levels contribute directly to aspects of COPD and emphysema pathogenesis, we found increased emphysema (increased alveolar L_M, increased static compliance and increased TLC and lung compliance in the VDD-CSE treatment group) and changes in gene expression consistent with enhanced proteolytic activity in the lungs of vitamin D deficient-cigarette smoke exposed mice after a relatively short duration of exposure to cigarette smoke. Moreover, our data suggest that a protease-antiprotease imbalance, which developed only after combined exposure to cigarette smoke and a vitamin D deficient diet, may have accelerated the development of emphysema so that the changes in lung mechanics and lung pathology typical of COPD in mice developed within 16 weeks of cigarette smoke exposure in the vitamin D deficient animals rather than the usual 26 week exposure required to generate all of these changes in previous studies in vitamin D replete, cigarette smoke exposed mice (Shapiro et al., 2003; Rinaldi et al., 2012). In conclusion, this work establishes a potentially useful murine model to study the combined effects of vitamin D deficiency and cigarette smoke exposure in the development of emphysema. Ongoing studies are focusing on the interaction of vitamin D and cigarette smoke in respiratory tract innate immunity.

AUTHOR CONTRIBUTIONS

Crane-Godreau—hypotheses, experimental design and organization, mouse smoke exposure, manuscript; Jukowski, Dechen and Ryu—molecular studies, mouse smoke exposure; Giustini and Hoopes—morphometrics; Lee—Vitamin D analysis; Black and Ratcliffe-pathology; Bessette—lung mechanics; Fiering—molecular analysis; Kelly—lung mechanics, design and manuscript, Leiter—statistics, manuscript.

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STAT3 modulates cigarette smoke-induced inflammation and protease expression

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Signal transducer and activator of transcription-3 (STAT3) regulates inflammation, apoptosis, and protease expression, which are critical processes associated with airway injury and lung tissue destruction. However, the precise role of STAT3 in the development of airway diseases such as chronic obstructive pulmonary disease (COPD) has not been established. This study shows that cigarette smoke activates STAT3 in the lungs of mice. Since cigarette smoke activated STAT3 in the lung, we then evaluated how the loss of STAT3 would impact on smoke-mediated lung inflammation, protease expression, and apoptosis. $STAT3^{+/+}$ and $STAT3^{-/-}$ mice were exposed to 8 days of cigarette smoke. Compared to the $STAT3^{+/+}$ mice bronchoalveolar lavage fluid (BALF) cellularity was significantly elevated in the $STAT3^{-/-}$ mice both before and after cigarette smoke exposure, with the increase in cells primarily macrophages. In addition, smoke exposure induced significantly higher BALF protein levels of Interleukin-1α (IL-1α), and monocyte chemotactic protein-1 (MCP-1) and higher tissue expression of keratinocyte chemoattractant (KC) in the $STAT3^{-/-}$ mice. Lung mRNA expression of MMP-12 was increased in $STAT3^{-/-}$ at baseline. However, the smoke-induced increase in MMP-10 expression seen in the $STAT3^{+/+}$ mice was not observed in the $STAT3^{-/-}$ mice. Moreover, lung protein levels of the anti-inflammatory proteins SOCS3 and IL-10 were markedly lower in the $STAT3^{-/-}$ mice compared to the $STAT3^{+/+}$ mice. Lastly, apoptosis, as determined by caspase 3/7 activity assay, was increased in the $STAT3^{-/-}$ at baseline to levels comparable to those observed in the smoke-exposed $STAT3^{+/+}$ mice. Together, these results indicate that the smoke-mediated induction of lung STAT3 activity may play a critical role in maintaining normal lung homeostasis and function.

Keywords: inflammation, cytokines, proteases, apoptosis, signaling, lung, COPD

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is now the third leading cause of death in the United States (Murphy et al., 2012) and it is projected to become the third leading cause worldwide within the next 20 years (Raherison and Girodet, 2009). In contrast to other diseases, the age-adjusted mortality for COPD has increased over the past 30 years (Miller et al., 2000). Current pharmacological therapies improve lung function and slow disease progression but they have not been shown to impact on COPD mortality (Kim and Criner, 2013). Cigarette smoke is the major etiologic factor associated with this disorder and prolonged exposure to cigarette smoke induces damaging inflammatory, apoptotic, and proteolytic responses in the lung (Macnee, 2007). These biological processes lead to dysfunctional matrix remodeling that cause airway obstruction and lung tissue destruction (Cornwell et al., 2010). A better understanding of the varied signaling processes that mediate the loss of normal airway function is needed to more effectively address the underlying mechanisms of this disease.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that mediates IL-6 signaling responses. Upon binding to the gp130 receptor, IL-6 activates Janus kinases (JAKs), which phosphorylate STAT3 thereby inducing its nuclear translocation and transcriptional activation. In addition to IL-6 signaling, STAT3 can be activated by EGFR, PDGFR, and Src kinase. Once activated, STAT3 binds the enhancer element in the promoter region of acute-phase genes, known as the acute-phase response element (Gerhartz et al., 1996) and induces the expression of numerous pro-inflammatory genes in the lung (Saleh et al., 2009). STAT3 was recently shown to be required for the development of allergic inflammation in a mouse model of asthma (Simeone-Penney et al., 2007). Indeed, the loss of STAT3 expression within the airway epithelium decreased the expression of Th2 cytokines, lowered airway inflammation and prevented the development of airway hyperreactivity in an asthma model (Simeone-Penney et al., 2007). Conversely, transgenic expression of STAT3 within the alveolar epithelium of mice markedly induced cytokine expression and lung inflammation (Gobburu et al., 1998). Given these findings, STAT3 activation is believed to be a central factor in the induction of airway inflammatory responses. Nevertheless, the role of STAT3 in airway diseases remains to be determined.

Though STAT3 affects key disease processes, data on the effect of STAT3 in COPD is lacking. Upregulation of STAT3 and induction of genes associated with STAT3 expression have been documented in lung tissue samples from COPD patients (Qu et al., 2009). Since STAT3 expression is increased in COPD, our study examined how cigarette smoke exposure impacted on STAT3 activation in the lungs of mice over the course of 1 year. In addition, we utilized the cigarette smoke exposure model to determine how STAT3 directly affected key biological processes implicated in the development of COPD. To examine this, $STAT3^{+/+}$ and $STAT3^{-/-}$ mice were exposed to cigarette smoke in order to assess how the loss of STAT3 impacted on lung inflammation, apoptosis and protease expression.

METHODS

ANIMAL MODELS

Three-month old 129X1-*Stat*^{3tm1Desi}/J ($STAT3^{-/-}$) and 129X1/SvJ controls ($STAT3^{+/+}$) (Jackson Labs, Bar Harbor, ME) were used for these studies. The 129X1-*Stat*^{3tm1Desi}/J mice have a mutant β isoform of STAT3 and only express the α isoform. C57Bl/6J mice (Jackson Labs) were used to kinetically examine the effects of cigarette exposure (from 1 day to 1 year of smoke exposure) on STAT3 activity in lung protein. All animal experiments were performed with approval from St. Luke's Roosevelt's Hospital Center's Institutional Animal Care and Use Committee approval.

CIGARETTE SMOKE EXPOSURE PROTOCOL

In accordance with our previously published protocol (Geraghty et al., 2013), mice were exposed to cigarette smoke from 3R4F research cigarettes (University of Kentucky, Lexington, KY) in a specially designed whole body smoking apparatus (Teague Enterprises, Davis, CA) for 4 h a day at a total particulate matter concentration of 80 mg/m^3. The mice had access to food and water and were able to move about freely during the period of smoke exposure. The mice were euthanized and bronchoalveolar lavage fluid (BALF) and lung tissue was collected 12 h following the last cigarette smoke exposure.

qPCR

mRNA was isolated from lung tissue of room air and 8-day smoke exposed $STAT3^{+/+}$ and $STAT3^{-/-}$ mice using Trizol reagent (Life Technologies, Grand Island, NY). cDNA was prepared from this mRNA using Superscript (Life Technologies). Lung expression of Cathepsin S, Cathepsin K, matrix metalloproteinase-3 (MMP-3), MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, tumor necrosis factor-α (TNF-α), IL-1β, IL-17, CD68, and KC was assessed by quantitative PCR using Taqman specific probes (Applied Biosystems, Grand Island, NY). Analyzed changes in gene expression in samples were related to another reference sample, usually room air treated $STAT3^{+/+}$ mice where the reference sample is set to 1.

PROTEASE AND CYTOKINE MEASUREMENTS

IL-1α, IL-6, IL-10, IL-13, IL-17, IFN-γ, MCP-1, RANTES (**R**egulated on **A**ctivation, **N**ormal **T** cell **E**xpressed and **S**ecreted), and TNF-α were measured in BALF using a beads assay on the BioRad Bio-Plex 200 system (BioRad, Hercules, CA).

INTRACELLULAR SIGNALING

Lung tissue was subfractionated into cytosolic and nuclear fractions using a commercially available protein compartment kit (Millipore, Billerica, MA). Immunoblots were conducted on the cytosolic protein to assess levels of phospho-B cell lymphoma-2 protein (p-Bcl-2), Bcl-2, suppressor of cytokine signaling3 (SOCS3) and actin (all antibodies from Cell Signaling, Danvers, MA). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking for 1 h with 5% milk protein (BioRad, Hercules, CA), the membranes were incubated for 1 h at room temperature with a 1:1000 dilution of primary antibody (p-Bcl2ser70 #2827, Bcl2 #2876, SOCS3 #2932, Actin #4967) in 2.5% bovine serum albumin (BSA). After washing, the membranes were then incubated with a 1:4000 dilution of horseradish peroxidase linked secondary antibody (#7074) in

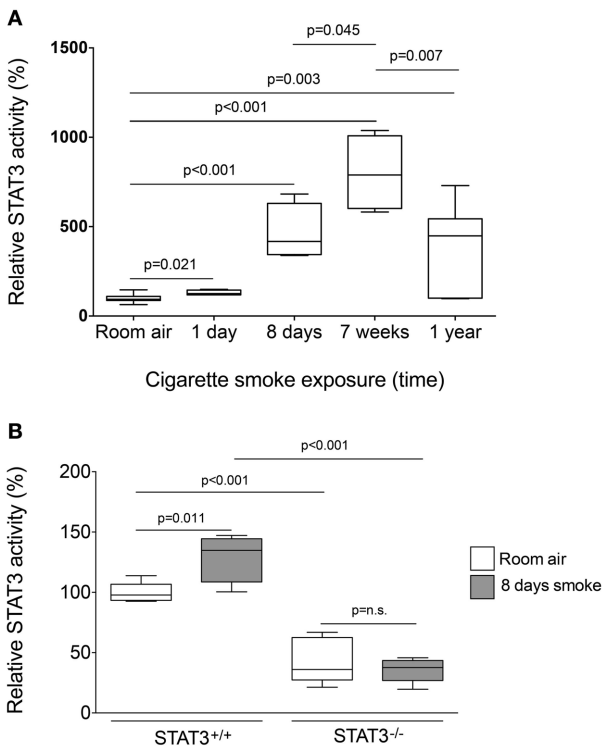

FIGURE 1 | Cigarette smoke exposure induces STAT3 activation in the lungs of exposed mice. (A) Three-month old C57Bl/6J mice were exposed to cigarette smoke for 1 day, 8 days, 7 weeks, and 1 year. Nuclear protein fractions were prepared from the lung tissue and STAT3 activity was measured using a specific oligonucleotide-binding assay. **(B)** Lung nuclear proteins were examined for STAT3 activity from 129X1-*Stat*^{3tm1Desi}/J ($STAT3^{-/-}$) and 129X1/SvJ controls ($STAT3^{+/+}$) mice after 8 days of either room air or cigarette smoke exposure. Data are reported as STAT3 activity relative to air-exposed $STAT3^{+/+}$ mice. Plots show range and average ± S.E.M. $N = 10$ in each group. *p* values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

2.5% BSA for 1 h. After washing, the membranes were incubated with Supersignal West Pico Luminol Enhancer Solution (Thermo Scientific, Rockford, IL) and then imaged on a ChemiDoc XRS Imaging System (BioRad).

STAT3 ACTIVITY

Lung nuclear protein from the lung tissue homogenates of C57Bl/6J mice exposed to 1 day, 8 days, 7 weeks or 1 year of cigarette smoke was used to determine STAT3 activity by conducting an oligonucleotide-binding assay (Active Motif, Carlsbad, CA). Of note, the lung tissue samples had undergone lung lavage prior to measuring STAT3 activity. Results are presented as relative activity compared to controls (%), which is the reference sample (usually room air treated $STAT3^{+/+}$ mice).

CASPASE 3/7 ACTIVITY ASSAY

Caspase 3/7 activity was measured in the lung tissue whole lysates of control and smoke-exposed $STAT3^{+/+}$ and $STAT3^{-/-}$ mice using the Caspase-Glo 3/7 Assay System (Promega, Fitchburg, WI). Data is reported as relative luminescence units (RLU). RLU is determined by analysis of signal relative to background, using a Tecan Genios microplate reader.

STATISTICAL ANALYSES

Data are expressed as means ± S.E.M. We determined statistical significance by one-way analysis of variance for multiple group analysis using GraphPad Prism Software (GraphPad, La Jolla, CA). Student *t*-tests (two tailed) were used throughout the study. All data sets are represented as range and mean ± standard error.

RESULTS

CIGARETTE SMOKE INDUCES STAT3 ACTIVATION IN MOUSE LUNGS

To determine the STAT3 response to cigarette smoke, STAT3 activity was measured in the nuclear fraction of the lungs of C57Bl/6 mice subjected to varying periods of smoke exposure. A 30% increase in STAT3 activation was observed as early as one-day post cigarette smoke exposure (**Figure 1**). Moreover, 8 days exposure to cigarette smoke further activated STAT3 ∼5 fold. Maximal activation occurred after 7 weeks though increased activity was observed up to 1 year of smoke exposure (**Figure 1A**). It is conceivable that down regulation of IL-6 receptors or the

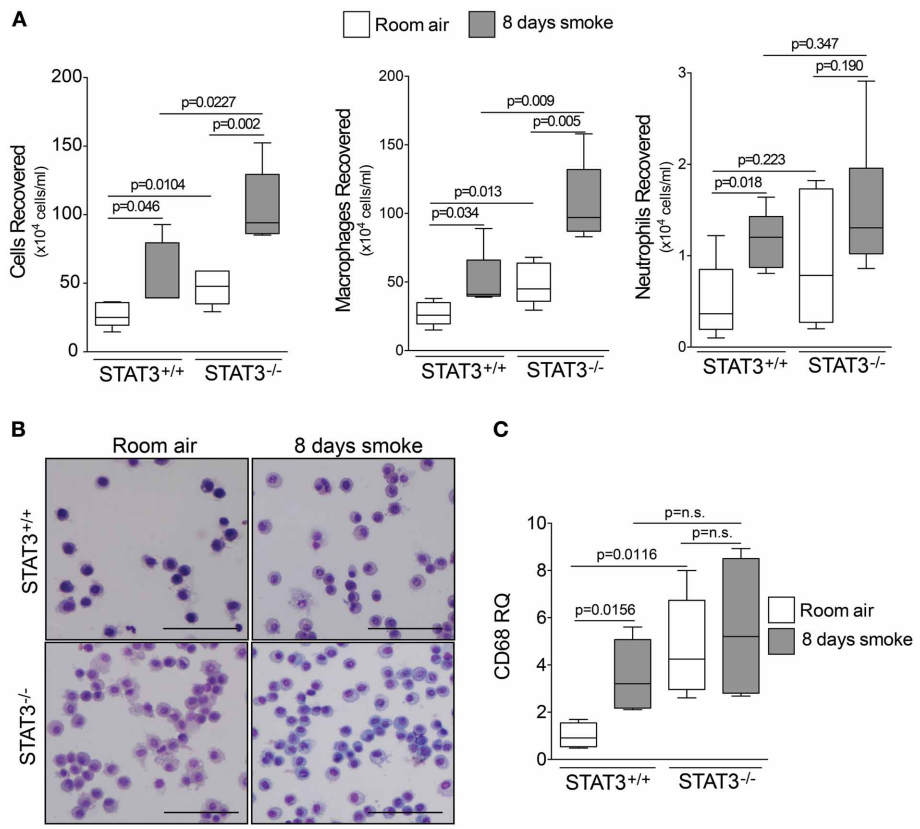

FIGURE 2 | STAT3 expression subdues BALF cellularity. Three-month old $STAT3^{+/+}$ and $STAT3^{-/-}$ mice were exposed to room air (white bars) or cigarette smoke (black bars) for 4 h daily for 8 days. The mice were euthanized 12 h post the last smoke exposure and **(A)** BALF cellularity was measured by bright field microscopy. BALF macrophages and neutrophils were determined by quick diff staining. **(B)** A typical representative staining is shown here (Scale bar = 20 μM). **(C)** qPCR for macrophage marker CD68 also demonstrates higher levels of macrophages within the lung tissue. Data are reported as BALF cells (x 10,000). Plots show range and average ± S.E.M. $N = 10$ in each group. *p* values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

induction of protein inhibitor of activated STAT3 (PIAS3) may have diminished the smoke-mediated induction of STAT3 at the 1-year time point. However, this will need to be explored in future studies. Together, these results show that cigarette smoke causes a rapid and persistent activation of STAT3 in the lung.

INCREASED INFLAMMATORY CELLS IN THE AIRWAYS OF STAT3$^{-/-}$ MICE

Since cigarette smoke potently activated STAT3 in the lungs of mice, we aimed to determine how the loss of STAT3 would impact on smoke-mediated inflammatory responses. To address this question, we exposed 129X1-*Stat*^{3tm1Desi}/J (STAT3$^{-/-}$) and 129X1/SvJ (STAT3$^{+/+}$) mice to 8 days of cigarette smoke exposure. Cigarette smoke increased STAT3 activity levels with the lung of the STAT3$^{+/+}$ after 8 days of smoke exposure (**Figure 1B**). Cigarette smoke significantly increased BALF cellularity in the lungs of the STAT3$^{+/+}$ mice ($26.8 \pm 3.7 \times 10^4$ vs. $53.9 \pm 13.3 \times 10^4$ cells for room air and smoke exposed, respectively; $p < 0.05$) (**Figure 2A**). In comparison, the STAT3$^{-/-}$ mice under room air conditions had significantly higher lung BALF cellularity than STAT3$^{+/+}$ mice ($26.8 \pm 3.7 \times 10^4$ vs. $48.2 \pm 5.8 \times 10^4$ cells for STAT3$^{+/+}$ and STAT3$^{-/-}$, respectively; $p < 0.05$). In fact, the BALF cellularity of room air exposed STAT3$^{-/-}$ mice was comparable to that of STAT3$^{+/+}$ mice that had been exposed to cigarette smoke for 8 days (**Figure 2A**). The increase in BALF cells was primarily macrophages (**Figure 2B**). Consistent with the increase in BALF cells that was measured in the BALF, we found significantly higher expression of the macrophage marker CD68 in the STAT3$^{-/-}$ mice under room air conditions compared to the room air exposed STAT3$^{+/+}$ mice (**Figure 2C**). Cigarette smoke exposure caused a two-fold increase in BALF cellularity in the STAT3$^{-/-}$ mice compared to room air exposed STAT3$^{-/-}$ mice (**Figure 2A**). This smoke-mediated increase was proportional to that experienced by the smoke-exposed STAT3$^{+/+}$ mice though the STAT3$^{+/+}$ had significantly lower levels of inflammatory cells post exposure compared to the STAT3$^{-/-}$ mice. Together, these findings show that the lack of STAT3 activation alters immune cell infiltration in the lung.

STAT3 EXPRESSION IMPACTS ON LUNG CYTOKINE AND PROTEASE INDUCTION

Cigarette smoke induces the expression of pro inflammatory cytokines and destructive proteases in the lung (Foronjy and D'Armiento, 2001; Wright et al., 2002). For this reason, we evaluated how the loss of STAT3 altered lung cytokine and protease expression in smoke-exposed mice. Under room air exposure conditions, TNF-α BALF protein levels were significantly higher in STAT3$^{-/-}$ mice than in STAT3$^{+/+}$ mice (∼2-fold, $p < 0.05$) (**Figure 3**). In response to cigarette smoke, IL-1α BALF protein levels decreased in the STAT3$^{+/+}$ mice ($p < 0.05$), but trended higher in the STAT3$^{-/-}$ mice (**Figure 3**). In contrast, cigarette smoke increased MCP-1 BALF protein levels in the STAT3$^{-/-}$ mice, but not in the STAT3$^{+/+}$ mice (**Figure 3**). STAT3 had no effect on the smoke-mediated increase in IFN-γ and IL-17 BALF protein expression and neither cigarette smoke nor

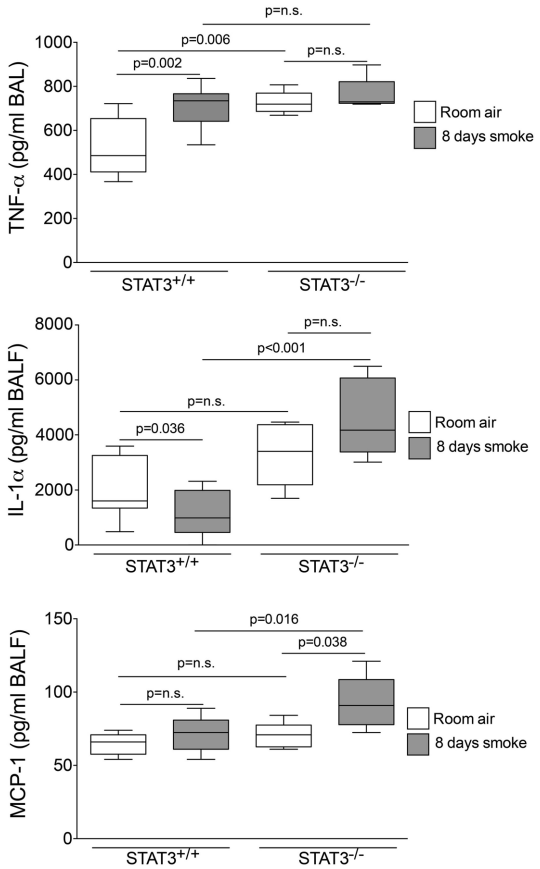

FIGURE 3 | STAT3 expression suppresses TNF-α, IL-1α, and MCP-1 BALF protein levels. Bioplex assays measured BALF cytokines in room air and 8-day smoke-exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice. Data are reported as cytokine level (pg/ml). Plots show range and average ± S.E.M. $N = 10$ in each group. p values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

STAT3 expression altered RANTES or IL-6 BALF protein levels (**Figure 4**). Of note, IL-10 has potent anti-inflammatory effects in the lung (Mays et al., 2013) and BALF protein levels of the anti-inflammatory cytokine IL-10 were significantly lower in the STAT3$^{-/-}$ mice under room air conditions (**Figure 4**). We next conducted quantitative PCR analyses to determine how STAT3 impacted on cytokine and protease expression within the lung tissue of the mice. We found that KC was regulated both by cigarette smoke exposure and STAT3 expression. Compared to STAT3$^{+/+}$ mice, the STAT3$^{-/-}$ mice had a ∼3-fold increase in lung KC expression under room air conditions (**Figure 5**). Furthermore, cigarette smoke induced a 2-fold increase in KC expression in STAT3$^{+/+}$ mice ($p < 0.05$), but a ∼15-fold increase in STAT3$^{-/-}$ mice ($p < 0.05$ vs. room air and vs. smoked wild type; **Figure 5**). In terms of proteases, cigarette smoke induced a ∼10-fold increase in MMP-10 lung mRNA expression ($p < 0.01$) in the STAT3$^{+/+}$ mice but not in the smoke-exposed STAT3$^{-/-}$ mice (**Figure 5**). Importantly, lung MMP-12 mRNA expression was upregulated ∼8-fold in the STAT3$^{-/-}$ mice under room air conditions ($p < 0.02$). However, MMP-12

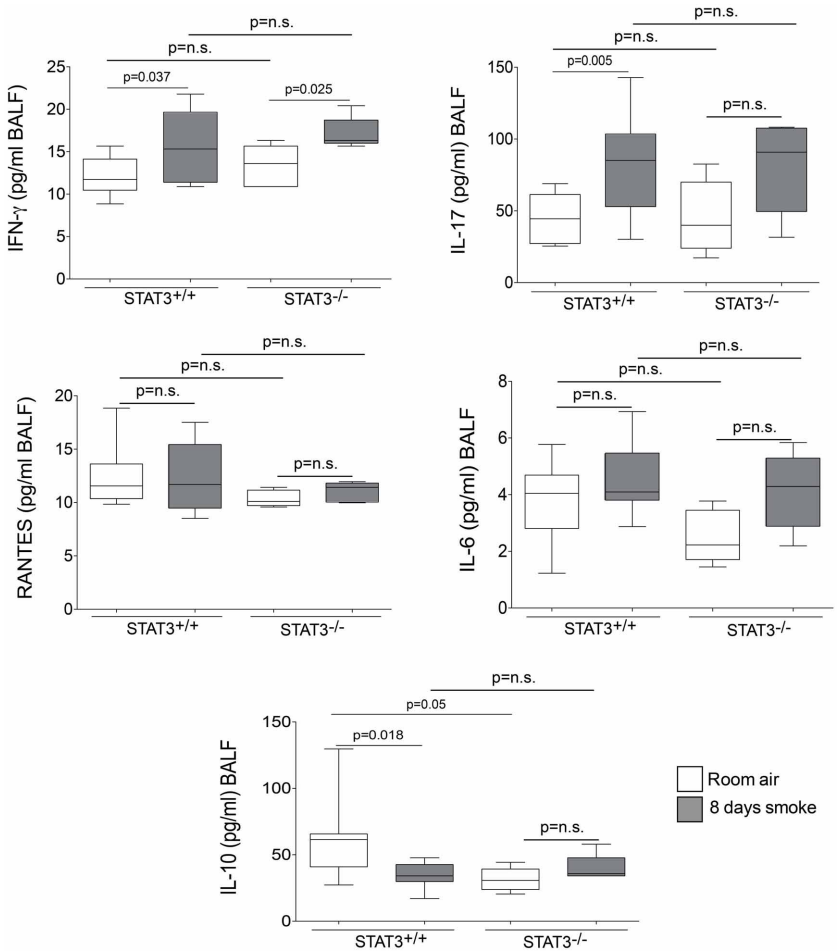

FIGURE 4 | STAT3 expression impacts on IL-10 BALF protein levels. Bioplex assays measured BALF cytokines (IFN-γ, IL-17, RANTES, IL-6, and IL-10) in room air and 8-day smoke-exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice. Data are reported as cytokine level (pg/ml). Plots show range and average ± S.E.M. $N = 10$ in each group. p values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

expression was comparable in STAT3$^{+/+}$ and STAT3$^{-/-}$ mice following cigarette smoke exposure. Similarly, TNF-α, IL-1β, and MCP-1 lung tissue expression were comparable in the STAT3$^{+/+}$ and STAT3$^{-/-}$ mice under smoke exposure conditions (**Figure 6**).

LOSS OF STAT3 IMPACTS ON SOCS3 PROTEIN LEVELS IN THE LUNG

Suppressor of cytokine synthesis 3 (SOCS3) counters inflammation by blocking JAK and their associated receptors to limit the duration and intensity of cytokine signaling (Babon and Nicola, 2012). Compared to STAT3$^{+/+}$ mice, both SOCS3 protein expression was markedly lower in the STAT3$^{-/-}$ mice (**Figure 7**). Thus, the loss of STAT3 expression may enhance lung inflammation by diminishing the anti-inflammatory responses mediated by SOCS3.

CASPASE 3/7 ACTIVITY IS INCREASED IN THE LUNGS OF THE STAT3$^{-/-}$ MICE

Apoptosis is a critical process in the development of emphysema (Tuder et al., 2003) and STAT3 regulates apoptotic responses *in vivo* (Chapman et al., 2000; Abell et al., 2005). Thus, we sought to determine how the loss of STAT3 expression impacted on apoptotic responses in the lungs of smoke-exposed mice. We measured caspase 3/7 activity, an important marker of apoptosis (Albee et al., 2007), in the lungs of room air and 8-day smoke-exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice. Cigarette smoke induced a ∼20 fold increase in caspase 3/7 activity in the smoke-exposed STAT3$^{+/+}$ mice ($p < 0.001$) (**Figure 8**). Compared to the STAT3$^{+/+}$ mice, the STAT3$^{-/-}$ had a ∼10 fold increase in caspase 3/7 activity at baseline ($p < 0.01$). However, the heightened activity did not increase further increase with cigarette smoke exposure in STAT3$^{-/-}$ mice (**Figure 8**). STAT3 can regulate apoptosis by inducing the expression of the apoptotic inhibitor Bcl-2 (Gao and Ward, 2007). Given this, immunoblots for p-Bcl-2 and total Bcl-2 were conducted on lung tissue lysates from control and 8-day smoke-exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice. The expression of both p-Bcl-2 and total Bcl-2 showed a trend toward an increase in STAT3$^{+/+}$ mice exposed to smoke, while the trend was the opposite in the STAT3$^{-/-}$ mice (**Figure 9**). However, the difference in p-Bcl-2 levels in the smoke

exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice did not reach statistical significance.

DISCUSSION

The role of STAT3 in inflammation is not clearly defined, with evidence supporting STAT3 activation having pro-inflammatory and anti-inflammatory roles (El Kasmi et al., 2006; Ruwanpura et al., 2012). These varied effects may be due to disease- and cell-specific mechanisms that determine the ultimate effect that STAT3 exerts in a biological system. The expression of STAT3 and its downstream-related genes is significantly increased in the lung tissue of COPD patients (Qu et al., 2009). This is significant as STAT3 is known to regulate inflammation, protease expression, and apoptosis (Li et al., 2011; Camporeale and Poli, 2012; Du et al., 2012), which are key biological processes in the pathogenesis of COPD (Chung and Adcock, 2008). For this reason, we sought to determine how the loss of STAT3 would impact on these important disease parameters in the cigarette smoke exposure model. This work shows for the first time that STAT3$^{-/-}$ mice have enhanced inflammatory, proteolytic and apoptotic responses in response to cigarette smoke. Furthermore, the loss of STAT3 expression was associated with deficient anti-inflammatory responses in these mice. STAT3 has been viewed as a potential therapeutic target for the treatment of COPD (Gao and Ward, 2007). However, these findings suggest that STAT3 may have protective functions in the lung and inhibiting it could exacerbate the underlying mechanisms involved in cigarette smoke-induced lung diseases.

One of the most remarkable findings in the STAT3$^{-/-}$ mice was the increased numbers of BALF cells, mostly macrophages, under room air or smoke exposure conditions. Macrophages are critical in COPD as these cells release proteases that induce dysfunctional airway remodeling (Shapiro et al., 1991; Shapiro, 1999, 2003). This enhanced macrophage response may have been due to the increased MCP-1 expression that was detected in the STAT3$^{-/-}$ mice. MCP-1 is upregulated in COPD patients (Bracke et al., 2007) and induces mucin production and lung inflammation (de Boer et al., 2000; Monzon et al., 2011) characterized by the influx of macrophages (Hautamaki et al., 1997). It is somewhat surprising that the absence of STAT3

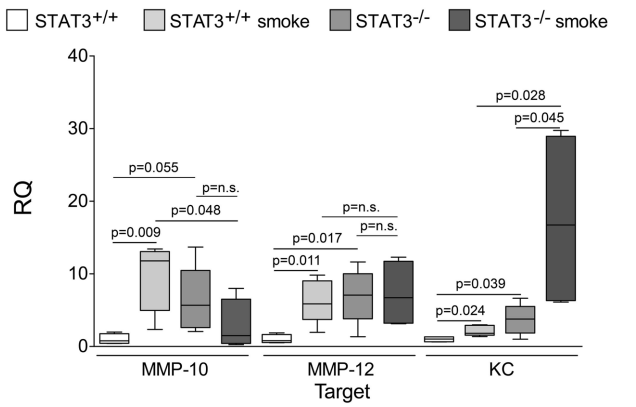

FIGURE 5 | Loss of lung STAT3 activity enhances gene expression of MMP-10 and MMP-12. Quantitative PCR for mouse KC, MMP-10, and MMP-12 was conducted on mRNA isolated from the lung tissue of room air and 8-day smoke exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice using specific Taqman probes. Data are reported as relative expression (RQ) to STAT3$^{+/+}$ in room air conditions. Plots show range and average ± S.E.M. $N = 10$ in each group. p values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

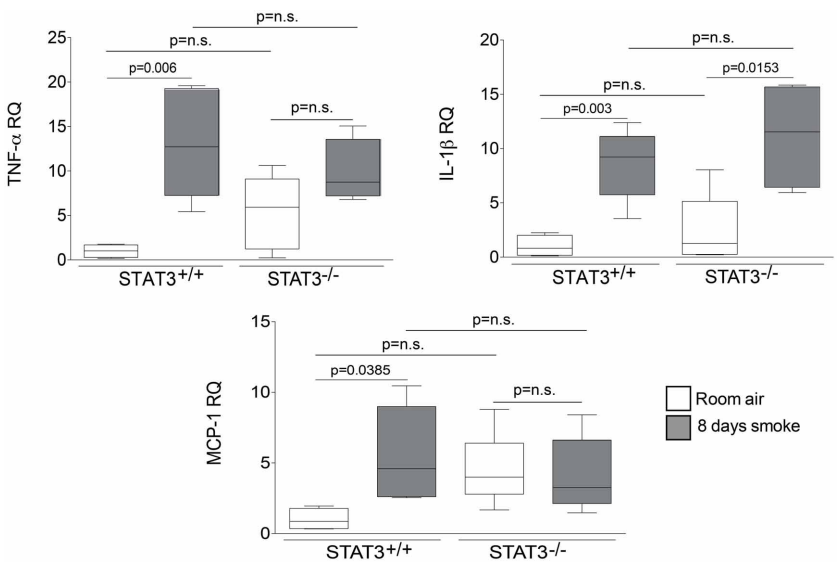

FIGURE 6 | STAT3 does not alter lung expression of TNF-α, IL-1β or MCP-1. Quantitative PCR for mouse TNF-α, IL-1β or MCP-1 was conducted on mRNA isolated from the lung tissue of room air and 8-day smoke exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice using specific Taqman probes. Data are reported as relative expression (RQ) to STAT3$^{+/+}$ in room air conditions. Plots show range and average ± S.E.M. $N = 10$ in each group. p values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

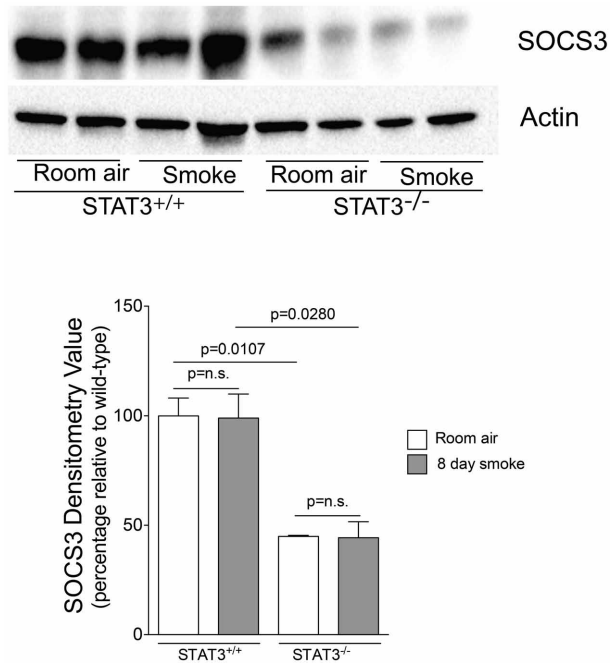

FIGURE 7 | STAT3 activity enhances lung SOCS-3 levels. Lungs from STAT3$^{+/+}$ and STAT3$^{-/-}$ mice exposed to room air or smoke for 8 days were homogenized and SOCS3 and actin were detected by immunoblot. Densitometry analysis was performed and results are represented as SOCS3 protein relative to air-exposed STAT3$^{+/+}$ mice. Actin levels were also used to normalize protein loading and are reflected in graph shown here.

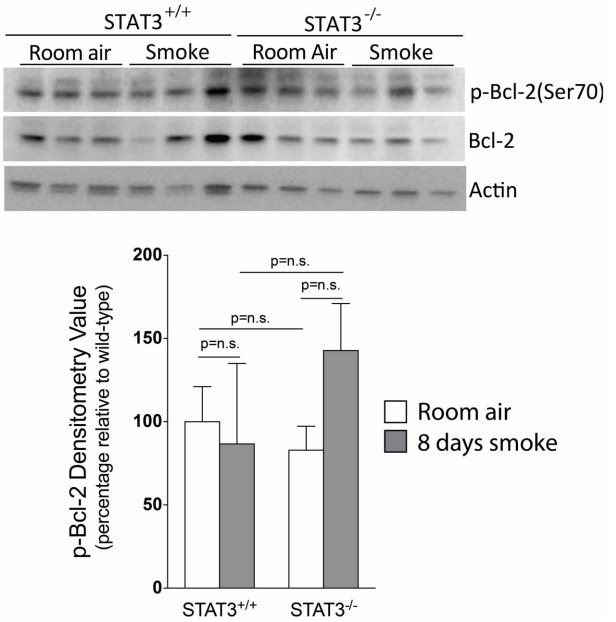

FIGURE 9 | Loss of STAT3 gives a trend increase in smoke induced Bcl-2 phosphorylation in mouse lungs. Lungs from STAT3$^{+/+}$ and STAT3$^{-/-}$ mice exposed to room air or smoke for 8 days were homogenized and p-Bcl-2, Bcl-2, and actin were detected by immunoblot. Each group had replicates of 3. Densitometry analysis was performed and results are represented as p-Bcl-2 protein relative to air-exposed STAT3$^{+/+}$ mice. Bcl-2 levels were also used to normalize protein loading and are reflected in graph shown here.

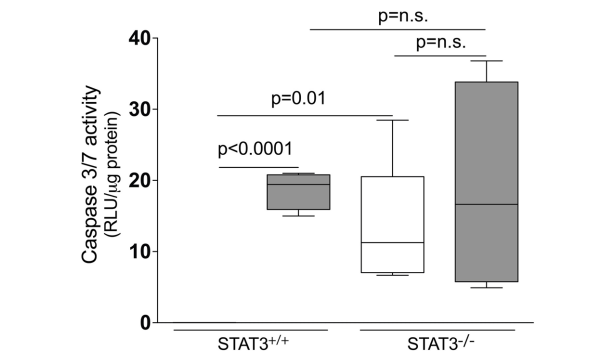

FIGURE 8 | Loss of STAT3 expression increases lung Caspase 3/7 activity. Caspase 3/7 activity was measured from the lung tissue homogenates of control and 8-day smoke exposed STAT3$^{+/+}$ and STAT3$^{-/-}$ mice using a specific luminescence assay (Promega). Data are reported as relative luminescence units (RLU). Plots show range and average ± S.E.M. $N = 10$ in each group. p values shown, comparing both treatments connected by a line. n.s. denotes no significant difference between groups.

increased MCP-1 levels as several studies demonstrated that MCP-1 expression is positively regulated by STAT3 (Burysek et al., 2002; Chatterjee et al., 2009; Zhou et al., 2012). However, the effect of STAT3 is likely influenced by the cell type and physiologic context. Indeed, the loss of STAT3 expression in macrophages enhanced inflammatory responses and increased MCP-1 expression in thioglycollate-challenged mice (Matsukawa et al., 2005).

In addition to the changes in MCP-1 expression, baseline TNF-α levels were significantly increased in the STAT3$^{-/-}$ mice. TNF-α, which is expressed by macrophages and other resident lung cells, induces intracellular signaling events that promote the development of emphysema (Riches et al., 1996; Churg et al., 2004). Thus, our findings indicate that STAT3 expression can impact on the disease by countering TNF-α expression in the lung. Of note, STAT3 regulated BALF TNF-α protein levels but not lung tissue TNF-α expression. This may indicate that STAT3 has a greater role on macrophage TNF-α expression than it does on lung tissue TNF-α expression. In support of this, prior studies have shown that the induction of SOCS3 by STAT3 counters TNF-α expression in macrophages (Berlato et al., 2002; Williams et al., 2004). However, further studies will be needed to definitively evaluate the effect of STAT3 within specific cell types in this model.

In addition to TNF-α, STAT3 also deterred the smoke-mediated induction of IL-1α in mice. This is significant as IL-1α drives the influx of neutrophils in the lungs of COPD patients (Botelho et al., 2011) and neutralizing IL-1α attenuates cigarette smoke-induced inflammation in mice (Pauwels et al., 2011). The enhanced inflammatory response in the STAT3$^{-/-}$ mice was associated with increased MMP-12 expression. The fact that

STAT3 negatively regulated MMP-12 is significant as this protease is upregulated in COPD patients (Demedts et al., 2006; Ilumets et al., 2007) and plays a critical role in smoke-induced emphysema in mice (Hautamaki et al., 1997). While the loss of STAT3 increased MMP-12 expression, STAT3$^{-/-}$ mice had significantly lower lung expression of MMP-10 following cigarette smoke exposure. This is important as MMP-10 was found to decrease inflammation in a mouse model of experimental colitis (Koller et al., 2012). Thus, it is conceivable that the loss of STAT3 expression can enhance lung inflammation by diminishing MMP-10 expression in the lung.

The loss of STAT3 expression greatly impaired innate anti-inflammatory responses in the lung. SOCS3 expression is positively regulated by STAT3 and, once activated, SOCS3 binds gp130 and inhibits Janus activated kinase (JAK) activity. Through this mechanism, SOCS3 then modulates immune responses by down regulating IL-1 and IL-6 signaling (Croker et al., 2003; Frobose et al., 2006) and deterring MAPK activation (Puhr et al., 2010). Thus, SOCS blocks key factors in the pathogenesis of COPD (Mercer et al., 2004; Botelho et al., 2011). The loss of SOCS3 expression in macrophages causes a polarization into an M1 or pro inflammatory phenotype (Qin et al., 2012a) and can enhance cell death and apoptosis (Ruan et al., 2010; Qin et al., 2012b). This may explain the increased macrophage levels and heightened apoptotic responses observed in our study. Aside from SOCS3, basal levels of the anti-inflammatory cytokine IL-10 were significantly lower in the STAT3$^{-/-}$ mice. IL-10 is positively regulated by STAT3 (Benkhart et al., 2000) and IL-10 deficiency renders the lung more sensitive to pro inflammatory stimuli (Penttila et al., 2008). Thus, we assert that the loss of STAT3 expression enhanced susceptibility to smoke-induced inflammation by decreasing IL-10 levels in the lung. Lastly, the STAT3$^{-/-}$ mice did not increase MMP-10 expression in response to cigarette smoke exposure. While most MMPs are believed to promote lung inflammation, MMP-10 exerts anti-inflammatory effects and promotes repair responses following tissue injury (Rodriguez et al., 2008; Koller et al., 2012). Together, these results show that the loss of STAT3 expression causes deficient anti-inflammatory and repair processes in mice.

There are several study limitations that merit discussion. For one, constitutive STAT3$^{-/-}$ mice were utilized. STAT3 plays an important role in myeloid cell maturation and differentiation (Smithgall et al., 2000). Thus, the lack of STAT3 activation may have altered the development of the immune system to render the STAT3$^{-/-}$ mice more responsive to cigarette smoke exposure. Moreover, since a whole body knockout was used the study cannot address the effect of STAT3 loss specifically in the lung. Lastly, though the loss of STAT3 exacerbated inflammation, protease expression and apoptosis, it is uncertain whether these effects would have increased lung tissue destruction in the smoke-exposed mice. Long-term smoke exposure studies and complete morphological analyses are needed to address this question.

In summary, our findings show that STAT3 is activated by cigarette smoke exposure, which may regulate key inflammatory, proteolytic and apoptotic responses in the lung. STAT3 mediates these effects at least in part by modulating anti-inflammatory responses such as SOCS3 and IL-10 expression in the lung. Future studies are needed to address the role of STAT3 in the disease to determine whether targeting STAT3 activity could be used as an approach to counter the injurious effects of cigarette smoke exposure in the lung.

AUTHOR CONTRIBUTIONS

Patrick Geraghty, Anya Wyman, Itsaso Garcia-Arcos, Abdoulaye J. Dabo and Sonya Gadhvi conducted the animal studies and interpreted experimental results. Patrick Geraghty and Itsaso Garcia-Arcos wrote the paper. Robert Foronjy led the project, interpreted the data and wrote the paper.

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RAGE and tobacco smoke: insights into modeling chronic obstructive pulmonary disease

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Chronic obstructive pulmonary disease (COPD) is a progressive condition characterized by chronic airway inflammation and airspace remodeling, leading to airflow limitation that is not completely reversible. Smoking is the leading risk factor for compromised lung function stemming from COPD pathogenesis. First- and second-hand cigarette smoke contain thousands of constituents, including several carcinogens and cytotoxic chemicals that orchestrate chronic lung inflammation and destructive alveolar remodeling. Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors primarily expressed by diverse lung cells. RAGE expression increases following cigarette smoke exposure and expression is elevated in the lungs of patients with COPD. RAGE is responsible in part for inducing pro-inflammatory signaling pathways that culminate in expression and secretion of several cytokines, chemokines, enzymes, and other mediators. In the current review, new transgenic mouse models that conditionally over-express RAGE in pulmonary epithelium are discussed. When RAGE is over-expressed throughout embryogenesis, apoptosis in the peripheral lung causes severe lung hypoplasia. Interestingly, apoptosis in RAGE transgenic mice occurs via conserved apoptotic pathways also known to function in advanced stages of COPD. RAGE over-expression in the adult lung models features of COPD including pronounced inflammation and loss of parenchymal tissue. Understanding the biological contributions of RAGE during cigarette smoke-induced inflammation may provide critically important insight into the pathology of COPD.

Keywords: RAGE, COPD, tobacco, mouse model

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is defined by airflow obstruction that is not fully reversible (Carp and Janoff, 1978). In particular, COPD involves chronic airway inflammation and pulmonary emphysema, which is defined anatomically via pathology samples as an abnormal permanent enlargement of airspaces distal to the terminal bronchioles accompanied by destruction of their walls without obvious fibrosis (Pauwels et al., 2001). COPD morbidity and mortality continue to rise as physician diagnoses of COPD increased from approximately 7 million in 1980 to approximately 13.1 million in 2004 (Adams and Barnes, 2006). COPD was responsible for 8 million outpatient visits, 1.5 million emergency room visits, and 672,000 hospitalizations in the U.S. in 2006 (US Department of Health and Human Services, 2009) and compared to 1980, deaths in 2007 increased 74% to over 124,000 people (American Lung Association COPD Fact Sheet, 2011). While as recent as 2010 the cost associated with COPD was $49.9 billion (Dalal et al., 2010), the precise pathobiochemical basis of COPD exacerbated by voluntary or involuntary tobacco smoke exposure remains enigmatic.

Cigarette smoking is currently the most considerable risk factor for the development of COPD, consisting of emphysema and chronic obstructive bronchitis (Anderson et al., 1964; Fletcher and Peto, 1977; Thun et al., 2000; Hogg, 2004). Notwithstanding, only one quarter of cigarette smokers develop clinically detectible airflow limitation and other symptoms of COPD, suggesting an important role for genetic susceptibility (Sethi and Rochester, 2000; Stockley et al., 2009). Although most people that develop COPD currently smoke cigarettes or have smoked in the past, COPD also develops in individuals that have never smoked (Higgins, 1991). This harmful outcome is due in part to exposure to second-hand smoke (Janson, 2004; Wakefield et al., 2005; Eisner et al., 2006). Furthermore, because some former smokers still live with active smokers and are observed to develop COPD later in life, passive smoke exposure is likely to contribute to disease progression.

First- and second-hand smokers diagnosed with moderate COPD have altered expression of several genes, including transcription factors, growth factors, and extracellular matrix proteins (Ning et al., 2004). These and other gene products likely function to stimulate the recruitment of inflammatory cells, cytokine secretion, cell death, and elevated protease production observed after prolonged cigarette smoke exposure (Carp and Janoff, 1978; Wright and Churg, 1990; Kuschner et al., 1996; Hautamaki et al., 1997; Sopori, 2002). As such, it is critical to examine how genes influence disease presentation so that precise mechanisms through which passive and active cigarette smoke contribute to COPD/emphysema can be identified.

GENERAL MECHANISMS OF COPD PATHOGENESIS

Numerous reviews that address COPD pathogenesis, its impact, and plausible therapies have been composed (Bridevaux and Rochat, 2011; Budinger and Mutlu, 2011; Caramori et al., 2011; Lugade et al., 2011; Rooney and Sethi, 2011). The intent of the current work is to concisely provide a foundational summary of conserved COPD modalities and discuss the plausible influence of receptors for advanced glycation end-products (RAGE) signaling. The prevailing pathogenic concept states that COPD is associated with chronic inflammation, imbalances between proteases/antiproteases, oxidative stress, and an elevated apoptotic index. Inflammation arising predominantly from neutrophilic contributions has been proposed due to enhanced neutrophil abundance in bronchoalveolar lavage (BAL) and sputum from COPD patients (Thompson et al., 1989; Stanescu et al., 1996; O'Donnell et al., 2004). Levels of chemoattractants that recruit neutrophils and other potent inflammatory mediators are also elevated in COPD, including leukotriene B4 (Beeh et al., 2003), CXCL2 and 8 (Keatings et al., 1996; Tanino et al., 2002; Beeh et al., 2003), CXCL1 (Keatings et al., 1996), CXCL5 (Tanino et al., 2002), IFN-γ (Hodge et al., 2007), IL-1β (Thacker, 2006; Churg et al., 2009), and TNF-α (Barnes and Karin, 1997). Matrix metalloproteinases (MMPs) produced by macrophages and neutrophils are also misregulated in COPD (Shapiro, 1994). In particular, levels of MMP-1, MMP-2, MMP-7, MMP-9, and MMP-12 are all upregulated in pulmonary tissue, BAL, and/or sputum of patients with COPD (Shapiro et al., 1993; Hautamaki et al., 1997; Ohnishi et al., 1998; Pratico et al., 1998; Shaykhiev et al., 2009), however because smoke exposed MMP-9 knockout mice are protected from emphysema, MMP-9 may require cooperation with other proteases during adverse lung remodeling (Atkinson et al., 2011) The chemical assessment of tobacco smoke reveals that it contains high levels of reactive oxygen species (ROS) that are in excess of intrinsic antioxidant defense mechanisms (Pauwels et al., 2001; Barnes et al., 2003). Generated in the airways, oxidants lead to cell dysfunction and/or death and also influence inflammatory signaling and protease augmentation via NF-κB-mediated mechanisms (Moodie et al., 2004). During the last decade, enhanced apoptosis stemming from diverse signaling pathways has also been implicated in alveolar septal cell loss observed in COPD patients (Kasahara et al., 2000, 2001; Tuder et al., 2003; Petrache et al., 2006). As a programmed event of removing unwanted cells and debris, apoptosis occurs via extrinsic signaling processes (Degterev et al., 2003), and intrinsic mitochondria or endoplasmic reticulum-mediated processes (Darmon et al., 1995; Slee et al., 1999). In summary, COPD is characterized by progressive destruction of the distal lung and small airway obstruction resulting from chronic inflammation and elevated cell death.

CONSTITUENTS OF TOBACCO SMOKE

Tobacco smoke is a toxic and carcinogenic mixture of more than 5000 chemicals (Talhout et al., 2011). Of these, around 400 have been quantified, at least 200 are toxic to humans and/or experimental animals, and over 50 have been identified as known, probable, or possible human carcinogens (Kirsti, 2004). Studies indicate that compared with mainstream smoke collected under standard FTC/ISO smoking parameters, sidestream smoke has higher levels of PAHs (Grimmer et al., 1987; Evans et al., 1993), nitrosamines (Brunnemann et al., 1977, 1980; Hoffmann et al., 1979a; Ruhl et al., 1980), aza-arenes (Dong et al., 1978; Grimmer et al., 1987), aromatic amines (Patrianakos and Hoffmann, 1979), carbon monoxide (Hoffmann et al., 1979b; Rickert et al., 1984), nicotine (Rickert et al., 1984; Pakhale and Maru, 1998), ammonia (Brunnemann and Hoffmann, 1975), pyridine (Johnson et al., 1973; Brunnemann and Hoffmann, 1978), and the gas phase components 1,3-butadiene, acrolein, isoprene, benzene, and toluene (Brunnemann et al., 1990). In addition to these deleterious compounds, other factors such as the type of tobacco, the chemicals added to the tobacco, the way the tobacco product is smoked, and, for cigarettes and cigars, the material in which the tobacco is wrapped can also affect second-hand smoke chemical composition (International Agency for Research on Cancer, 2002; National Toxicology Program, 2005; US Department of Health and Human Services, 2006).

Cigarette smoke is also an important exogenous source of reactive glycation products capable of promoting formation of AGEs, advanced glycation end-products, which are irreversibly glycated proteins that efficiently bind RAGE (Cerami et al., 1997). Studies have shown that both aqueous extracts of tobacco and cigarette smoke contain glycotoxins, highly reactive glycation products that can rapidly induce AGE formation on proteins *in vitro* and *in vivo* (Nicholl and Bucala, 1998; Nicholl et al., 1998). These activities can be eliminated by passing the samples through a dry packed column of aminoguanidine, a potent and specific inhibitor of AGE formation. Additional studies have shown that serum AGEs and apolipoprotein B-linked AGE levels are significantly elevated in cigarette smokers relative to non-smokers and AGEs or immunochemically related molecules are present at higher levels in the tissues of smokers compared to non-smokers, regardless of the presence of diabetes (Nicholl et al., 1998).

RECEPTOR FOR ADVANCED GLYCATION END-PRODUCTS

RAGE are cell-surface receptors of the immunoglobulin superfamily expressed in many cell types including endothelial and vascular smooth muscle cells, fibroblasts, macrophages/monocytes, and epithelium (Brett et al., 1993). RAGE expression is most abundant in the lung, from which it was initially isolated, and is selectively localized to well-differentiated alveolar type I (ATI) epithelial cells (Schmidt, 2001). Identification in respiratory epithelium (Dahlin et al., 2004; Koslowski et al., 2004) and studies that document RAGE-mediated adherence to collagen IV (Demling et al., 2006) have led to the implication of RAGE in important developmental processes such as the spreading, thinning, and adherence that characterize the transitioning of ATII cells to squamous ATI cells. RAGE was first described as a progression factor in cellular responses induced by AGEs that accumulate in hyperglycemia and oxidant stress. Subsequent studies have distinguished RAGE as a pattern recognition receptor that also binds S100/calgranulins, amyloid-β-peptide, and HMGB-1 (or amphoterin), to influence gene expression via divergent signal transduction pathways (Reddy et al., 2006; Hudson et al., 2008; Kim et al., 2008; Toure et al., 2008). Because RAGE expression can also increase when ligands accumulate (Schmidt, 2001), RAGE-ligand interaction may contribute to chronic pathological states

where ligands are common including diabetic complications, neurodegenerative disorders, atherosclerosis, and inflammation (Hofmann et al., 1999; Taguchi et al., 2000). Specifically, a host of pro-inflammatory responses such as those coordinated by MAP kinases (ERK, JNK, and p38), NF-κB, ROS, and other pro-inflammatory mediators such as TNF and IL-1 (Bianchi et al., 2010) result from RAGE-ligand interactions (**Figure 1**). In contrast to short-lived cellular activation mediated by LPS, engagement of RAGE by its ligands results in prolonged inflammation (Lin et al., 2009). If left unchecked, such chronic inflammation results in severe tissue injury.

The full length membrane bound form of RAGE (mRAGE) contains an extracellular variable V-region-like immunoglobulin domain crucial for ligand binding and two constant C-region-like immunoglobulin domains, a single-pass hydrophobic transmembrane domain and a short, 43 amino acid, highly charged cytoplasmic domain essential for intracellular signaling (Buckley and Ehrhardt, 2010). The cytoplasmic domain of RAGE contains four possible phosphorylation sites, S391, S399, S400, and T401, of which only S391 is conserved among humans, mice, guinea pigs, rats, rabbits, dogs, and cats (Sakaguchi et al., 2011). Replacement of S391 to alanine was sufficient to abrogate PKCζ-dependent phosphorylation and subsequent signal transduction *in vitro* (Sakaguchi et al., 2011). Although not explicitly stated, RAGE behaves similarly to a receptor tyrosine kinase (RTK) cell surface receptor, requiring homodimerization to effectively potentiate intracellular signaling cascades (Zong et al., 2010). Distinct alternative isoforms also exist for the receptor due to differential splicing variants of the RAGE message. Dominant negative RAGE (dn-RAGE) is a membrane anchored splice variant of RAGE capable of ligand binding but lacking the intracellular domain necessary for signal transduction. Endogenous secreted RAGE (esRAGE) is generated via alternative splicing at exon 9 yielding the same V and C-regions of the full length-RAGE but lacks both the hydrophobic transmembrane and the intracellular domains (Buckley and Ehrhardt, 2010). Additionally, full-length RAGE can be cleaved by MMPs to render sRAGE, a non-splice variant of RAGE closely resembling esRAGE in structure and

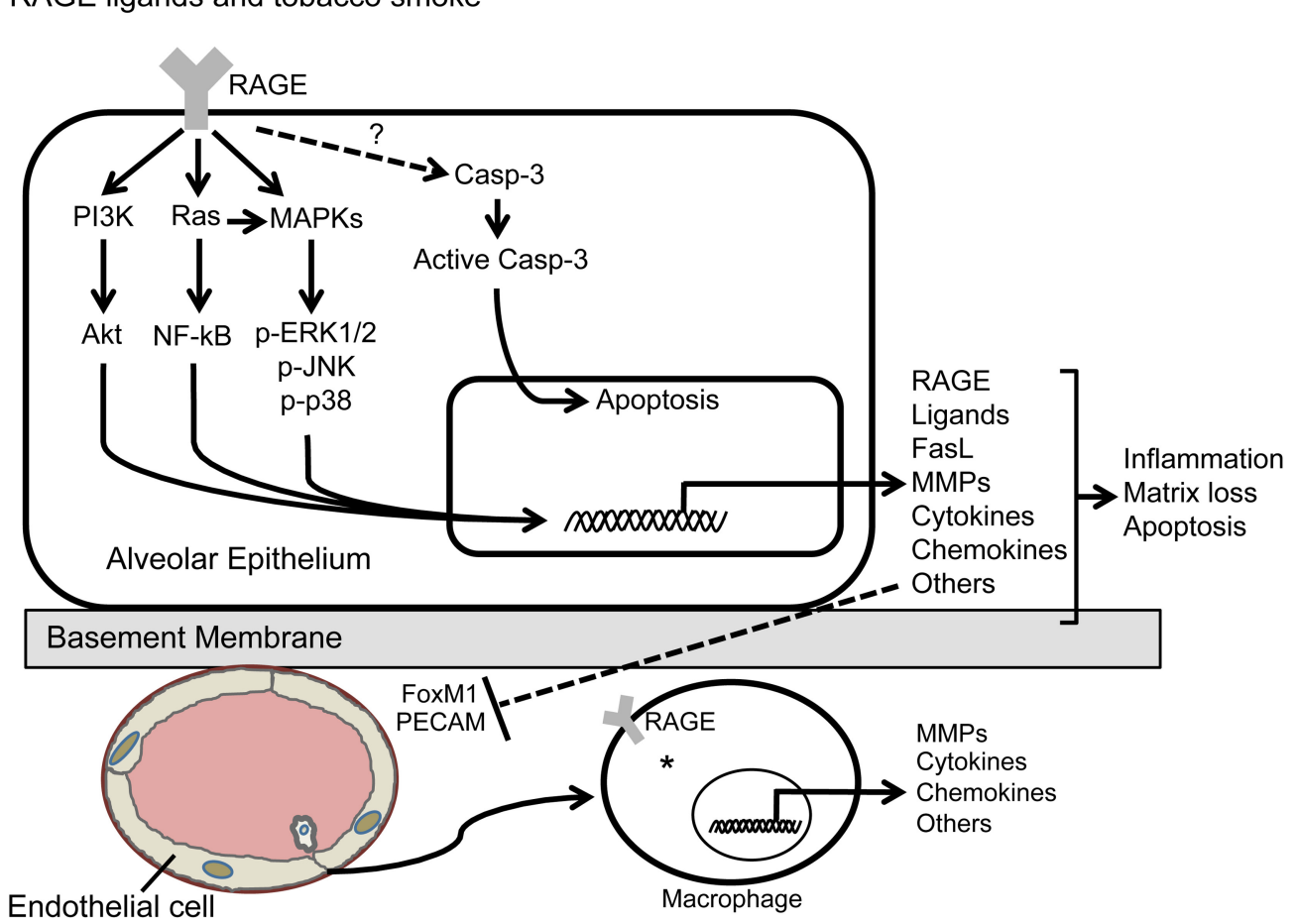

FIGURE 1 | Deleterious effects characteristic of COPD are elicited via several pro-inflammatory signaling pathways observed in RAGE-expressing alveolar epithelial cells and resident alveolar macrophages (∗). Direct stimulation of RAGE by tobacco smoke, *de novo* AGE generation in a tobacco smoke environment, or genetic up-regulation of RAGE in the lungs of conditional bi-transgenic mice results in characteristics of COPD including inflammation, matrix destabilization, and apoptosis.

function (Yamakawa et al., 2011). These altered variants of RAGE incapable of transducing signals are thought to function as decoy receptors that prevent the interaction of mRAGE with its ligands.

The pro-inflammatory role of RAGE in cardiovascular diseases is well documented (Yan et al., 2009). Furthermore, several studies strongly suggest that RAGE signaling is a key regulator of inflammation in major pulmonary diseases. A study demonstrated that abrogation of RAGE signaling (using RAGE null mice) attenuated pulmonary ischemia and reperfusion injury associated with decreased NF-κB activation and IL-8 production (Sternberg et al., 2008). Another important role for RAGE signaling in lung disease shows that RAGE-deficient mice under hyperoxic conditions survived longer than wild type controls and the mice had less airway cellularity and diminished alveolar damage compared to wild type controls (Reynolds et al., 2010). RAGE has been implicated in the fibrotic process in a number of tissues, including the peritoneum, kidney, and liver (Li et al., 2004; De Vriese et al., 2006; Xia et al., 2008), where it has been shown to promote fibrosis. In the lung, evidence continues to accumulate suggesting an important role for RAGE in pulmonary fibrosis, yet conflicting data portray RAGE as having both protective and destabilizing functions. Acute lung injury (ALI) and a more severe condition known as acute respiratory distress syndrome (ARDS) are characterized by deterioration of the alveolar-capillary barrier and impaired alveolar fluid clearance (Lucas et al., 2009). ALI and ARDS are associated with damage to ATI cells, a population of cells with significant RAGE expression, and several different animal models of ALI express increased RAGE levels in BALF (Uchida et al., 2006; Su et al., 2007, 2009; Zhang et al., 2008). A published study from our laboratory considered the effects of smoke exposure on RAGE expression both in lung cells and mice (Reynolds et al., 2008). The research revealed that RAGE and its ligands were up-regulated in lung epithelial cells cultured with cigarette smoke extract (CSE) and that mice exposed to cigarette smoke for 6 months had elevated RAGE expression in pulmonary epithelium (Reynolds et al., 2008). While the full extent of RAGE function in smoke-induced COPD has not been sufficiently examined, these studies demonstrate that RAGE may play a role in COPD pathogenesis.

CONTRIBUTIONS OF RAGE TO COPD PROGRESSION

RAGE and two of its ligands S100A12 and HMGB-1 were up-regulated in a rat alveolar type I-like cell line (R3/1), a human alveolar type 1I-like epithelial cell line (A549), and a macrophage-like murine cell line (RAW 264.7) following exposure to CSE (Reynolds et al., 2008). S100A12 is a calcium-binding pro-inflammatory modulator and HMGB-1 is a non-histone nuclear protein that acts as a potent pro-inflammatory mediator when secreted. In human lungs with smoke-related lesions, widespread RAGE expression has been documented in bronchiolar epithelia, small respiratory airways, reactive ATI cells, and alveolar macrophages (AMs; Morbini et al., 2006). The same study identified elevated S100A12 in polymorphonuclear granulocytes and in extracellular fluid and the number and intensity of carboxymethyl-lysine positive cells (cells that stain for AGEs) were measurably enhanced in epithelial and inflammatory cells of the lungs of smokers (Morbini et al., 2006).

Another factor highly expressed in the lungs of smokers with COPD is early growth response gene 1 (Egr-1), a zinc finger-containing, hypoxia-inducible transcription factor (Ning et al., 2004). Egr-1 expression significantly increased in lung cell lines following CSE exposure *in vitro* and it activated the RAGE promoter (Reynolds et al., 2006, 2008). Because the RAGE gene also contains NF-κB and SP-1 promoter response elements (Li and Schmidt, 1997) and is transcriptionally regulated by cis-acting Egr-1 (Reynolds et al., 2006), a possible auto-inflammatory loop may be triggered suggesting cooperation between Egr-1 and RAGE in chronic smoke-related inflammatory disease states. More recently, it was discovered that Ras, a small GTPase that functions as a molecular switch in the control of diverse signaling cascades, was induced in R3/1 cells following exposure to CSE, resulting in up-regulation of NF-κB-mediated secretion of TNF-α, IL-1β, and IL-8 (**Figure 1**; Reynolds et al., 2011a).

Our lab has recently expanded research into the biology of smoke-exposed primary mouse AMs also known to express RAGE. Studies document that low levels of RAGE are expressed by mouse primary macrophages during normal conditions and that RAGE overexpression by these primary macrophages is associated with inflammation and the coordination of lung damage (Morbini et al., 2006). Our studies indicate that acute exposure of mice to CSE via nasal instillation resulted in diminished BAL cellularity and fewer AMs in RAGE null mice compared to controls. Additionally, AMs isolated from wild type mice exposed to CSE significantly increased RAGE expression (Robinson et al., 2012). This recently published work also demonstrated for the first time that RAGE null AMs exposed to CSE experienced reduced Ras and p38 MAPK activation, less NF-κB translocation, and diminished expression of TNF-α and IL-1β when compared to CSE exposed wild type AMs (**Figure 1**). Evidence suggests that primary AMs coordinate CSE-induced inflammation, at least in part, via RAGE-mediated mechanisms and that cooperation with alveolar epithelium in coordinated inflammatory responses is likely.

USE OF RAGE TRANSGENIC MICE IN MODELING CHARACTERISTICS OF COPD

Several animal models that seek to recapitulate various aspects of COPD have been presented within the past decade. These models include mouse IL-1β over-expressers (Lappalainen et al., 2005), rat VEGF signaling nulls (VEGF or VEGFR2 blockers: Kasahara et al., 2000), intratracheal administration of active caspase-3 (Aoshiba et al., 2003) and several others that aim to elucidate inflammatory and other destructive mechanisms during smoke-less and smoke-exposed disease progression (Petrache et al., 2005; Giordano et al., 2008; Kang et al., 2012). The vast majority of these models present emphysema-like anatomical characteristics and inflammatory indexes in the presence of room-air and notable exacerbation in the presence of cigarette smoke. Although RAGE has been shown to be a marker for many inflammatory diseases including COPD, a genetic mouse model for COPD had not been previously examined.

We generated a bi-transgenic *in vivo* mouse model that utilizes two transgenes to conditionally up-regulate RAGE (**Figure 2**).

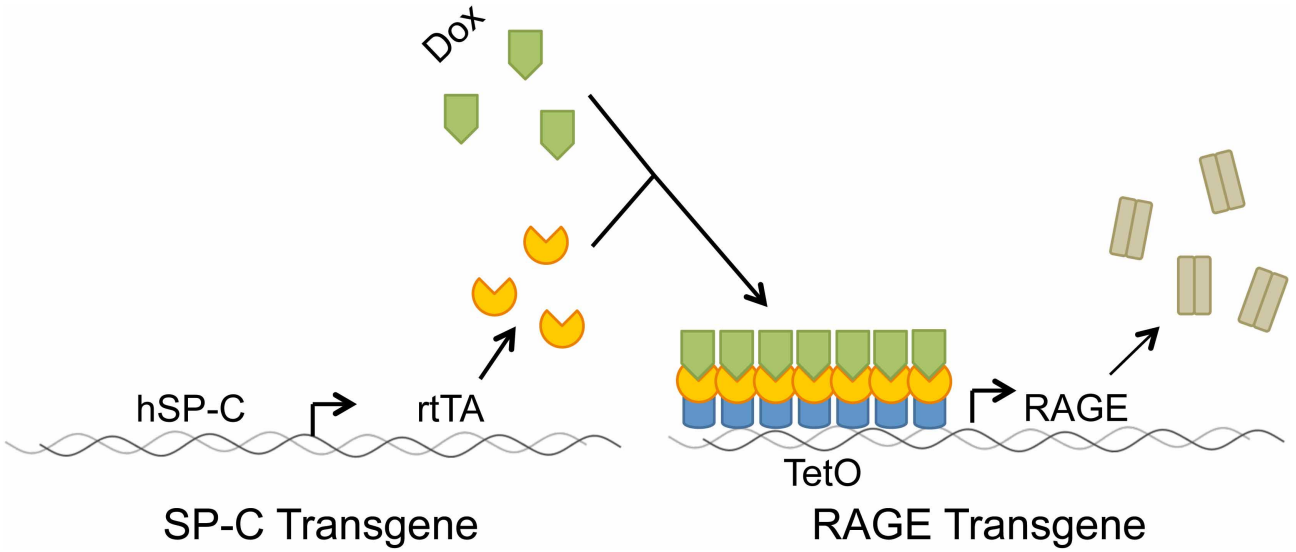

FIGURE 2 | Full length RAGE was over-expressed in alveolar type (AT) II cells by obtaining progeny from two transgenic lines of mice. The reverse tetracycline transactivator (rtTA) was produced under the control of the human surfactant protein C (hSP-C) promoter in distal respiratory epithelium. Following the administration of doxycycline (dox), a dox-rtTA complex activates the TetO promoter, thereby expressing RAGE.

One transgenic mouse line employs surfactant protein C (SP-C) to drive expression of rtTA (reverse tetracycline transactivator) and another transgenic line contains binding sites for a complex between rtTA and doxycycline (dox; Reynolds et al., 2011b). Although COPD is an adult lung disease, we initially sought to characterize RAGE bi-transgenic mice during development with the realization that aspects of COPD may be detected during organogenesis. Our model was thought to compliment research that centers on bronchopulmonary dysplasia (BPD), an embryonic disease highly correlated with emphysema in terms of oxidative stress, pulmonary inflammation, increased apoptosis, protease/antiprotease imbalance and altered microvasculature (Hargitai et al., 2001; Danan et al., 2002; Saugstad, 2003; Ekekezie et al., 2004; Speer, 2006). While COPD is characterized by sustained inflammation and alveolar destruction, remarkably similar mechanisms are implicated in the altered branching and impaired alveolarization observed in BPD (Bourbon et al., 2009).

EMBRYONIC RAGE BI-TRANSGENIC MICE HAVE PERTURBED DISTAL EPITHELIUM

Complete perinatal lethality was observed when dox was supplied to RAGE bi-transgenic mice throughout embryogenesis. At embryonic day (E) 18.5, pulmonary tissues were severely hypoplastic and only minimal respiratory surface area near the visceral pleura remained. Several immunohistochemical and flow cytometric experiments demonstrated diminished abundance of differentiated distal lung cell types, most notably ATI and ATII cells (Reynolds et al., 2011b).

Altered cellular differentiation has not sufficiently been characterized in the distal lung of COPD patients; however, new research has emerged demonstrating that human ciliated cells can respond to cigarette smoke by promoting GDF15, a factor capable of driving Muc5A expression in goblet cells (Wu et al., 2011). RAGE and RAGE ligands have been implicated in altered cellular differentiation of several cell types including smooth muscle cells, skeletal myocytes and developing neural tissue (Suga et al., 2011; Kim et al., 2012; Riuzzi et al., 2012). Thyroid transcription factor 1 (TTF-1; also known as Nkx2.1) is a key regulator of pulmonary development and present in distal lung epithelium that can negatively regulate RAGE expression (Reynolds et al., 2008) and SP-1 positively regulates the active promoter region of TTF-1 in surfactant producing cells (Das et al., 2011). Because NF-κB (a crucial intermediate of RAGE signaling) can interfere with SP-1 binding (Benjamin et al., 2010), RAGE may play a role in inhibited surfactant synthesis observed when ATII cells are abnormally regulated.

EMBRYONIC RAGE BI-TRANSGENIC MICE HAVE ABNORMAL DISTAL PULMONARY ENDOTHELIAL CELL GROWTH

In addition to the decreased cellularity of the lungs, RAGE overproduction disturbed capillary growth and maintenance through the inhibition of FoxM1 (a critical transcription factor necessary for endothelial expansion) and PECAM (a marker for endothelial cells) expression (Geyer et al., 2011). Endothelial cell apoptosis has been observed in COPD patients using TUNEL, immunohistochemistry and DNA ligation techniques that coincided with the reduction of endothelial markers including VEGF and VEGFR2 (Kasahara et al., 2001). Additionally Dinh-Xuan et al. and Peinado et al. both showed that resected lung samples from COPD patients had extensive endothelial dysfunction, which they proposed to contribute to hypertension (Dinh-Xuan et al., 1991; Peinado et al., 1998). It is hypothesized that vascular tone in the lung can be regulated by direct stimulation of the vascular compartment by cigarette smoke and indirect

stimulation stemming from smoke-exposed epithelial cells. Our discoveries relating to pulmonary endothelium in the RAGE bi-transgenic mouse correlate with numerous studies that demonstrate RAGE signaling in cases of depressed endothelial function and increased barrier disruption (Sun et al., 2009; Pollreisz et al., 2010; Wolfson et al., 2011; Chen et al., 2012; Huang et al., 2012).

EMBRYONIC RAGE BI-TRANSGENIC MICE HAVE EXTRACELLULAR MATRIX ABNORMALITIES

We also demonstrated that MMP-9 secretion is increased, coincident with diminished collagen IV (a principle component of the alveolar basement membrane) deposition and production (Bukey et al., 2011). COPD is characterized by an increase in several MMPs including MMP-1, 2, 9, and 12 (Ohnishi et al., 1998; Geraghty et al., 2011). Other research groups have also demonstrated AGE-RAGE dependent mechanisms in MMP-9 production (Ishibashi et al., 2010; Zhang et al., 2010; Zhu et al., 2012). While not yet evaluated in our embryonic RAGE bi-transgenic mouse model, MMPs 1 and 2 have been implicated as RAGE targets (Kamioka et al., 2011; Du et al., 2012; Yu et al., 2012). Interestingly, MMP-1 has been shown to be up-regulated not only in the lungs of COPD patients but in osteoarthritis as well, a chronic inflammatory disease affecting articular cartilage (Steenvoorden et al., 2006). Ongoing research seeks to test hypotheses related to matrix-targeting protease imbalances such as those that involve α1-antitrypsin.

EMBRYONIC RAGE BI-TRANSGENIC MICE HAVE ELEVATED PARENCHYMAL CELL APOPTOSIS

Thorough evaluations of apoptosis were performed in order to ascertain causes for the hypoplastic lung phenotype in the embryonic RAGE bi-transgenic mouse. RAGE over-expressing lungs detrimentally declined during the canalicular phase, a period identified by terminal bronchiole branching, initial alveolarization, and microvascular organization. The abrupt loss of tissue was observed in tandem with a significant increase in pro-apoptotic Fas ligand (FasL), a decrease in the anti-apoptotic factor Bcl-2, elevated cleaved active caspase-3 (a critical mediator of cell death), and quantifiable apoptosis by TUNEL assessment (Stogsdill et al., 2012). Electron microscopy also confirmed apoptosis via the detection of numerous bleb-like structures within cells that were physically separated from the underlying basement membrane. Importantly, cellular proliferation was not changed, suggesting there was no feedback mechanism to compensate for elevated cell death. Evidence is mounting that demonstrates active apoptosis of epithelial and endothelial cells in human COPD patients (Segura-Valdez et al., 2000; Kasahara et al., 2001; Majo et al., 2001; Yokohori et al., 2004; Hodge et al., 2005; Imai et al., 2005). Lending support for FasL-mediated apoptosis observed in RAGE bi-transgenic mice was research by Mahali et al. that demonstrated FasL elaboration is a direct product of AGE-RAGE ligation (Mahali et al., 2011). Furthermore, RAGE and its ligands have been shown to promote apoptosis in other tissue types, including myocytes (Tsoporis et al., 2010), endothelial cells (Chen et al., 2010), neuronal cells (Kim et al., 2011), epithelial cells (Jin et al., 2011), and pancreatic β-cells (Lee et al., 2010). Our studies have shown for the first time that increased expression of RAGE using transgenic mouse technology directly activates apoptosis in lung parenchyma. In fact, sustained RAGE expression during development is capable of modeling disorders characterized by cell loss including BPD. Furthermore, these data reveal important RAGE-mediated mechanisms that control cell quantity possibly introduced at the initiation of smoke-induced COPD pathogenesis.

ADULT RAGE OVER-EXPRESSION YIELDS AN EMPHYSEMATOUS LUNG

Conditional up-regulation of RAGE for 2 to 3 months in the adult bi-transgenic mouse lung lead to incremental dilation of alveolar spaces, assessed by standard H&E staining (Stogsdill et al., 2011). Quantification of the mean chord length of the airspace revealed progressive dilation of alveolar spaces as RAGE over-expression persisted (unpublished data). The adult RAGE bi-transgenic mice had increased MMP-9 and decreased elastin expression consistent with other COPD models. Furthermore, RAGE bi-transgenic mice manifested significant inflammation measured by elevated BALF protein, leukocyte infiltration, and secreted cytokines (MIP-2, IFN-γ; Stogsdill et al., 2011). These data support the concept that innovative transgenic mice that over-express RAGE may model pulmonary inflammation and alveolar destabilization independent of tobacco smoke. Furthermore, it validates RAGE signaling as a target pathway in the prevention or attenuation of smoke-related inflammatory lung diseases.

CONCLUSIONS

Despite the progression in the field of RAGE biology in the context of lung disease, the full extent of RAGE localization, the molecular mechanisms that control its expression and its downstream effects should remain topics of focused investigation. While a great deal is known about COPD, relatively little is known about factors that perpetuate inflammation or modalities that sustain them. Our research has shown that mechanisms of COPD progression including chronic inflammation, imbalances involving proteases, oxidative stress, and elevated apoptosis may be mediated by RAGE. Several endogenous (S100/calgranulins, HMGB-1, AGEs) and exogenous ligands (cigarette smoke) may be responsible for the sustained activation of RAGE leading to disease progression (**Figure 1**). As such, it remains possible that targeting RAGE may, at least in part, provide successful opportunities in the therapeutic alleviation of debilitating inflammatory lung disease exacerbated by tobacco smoke.

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Second hand smoke and COPD: lessons from animal studies

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Exposure to second hand smoke is a major cause of chronic obstructive pulmonary disease (COPD) in the non-smoker. In this review we explore the use of animal smoke exposure models and their insight into disease pathogenesis. The methods of smoke exposure, including exposure delivery systems, are described. Key findings from the acute and chronic smoke exposure models are outlined, including descriptions of the inflammation processes, proteases involved, oxidative stress, and apoptosis. Finally, alternatives to rodent models of lung disease are presented.

Keywords: emphysema, COPD, animal models, cigarette smoke, inflammation, apoptosis, proteases

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of disability and death worldwide, with a prevalence estimated at 10% of the general population and up to 50% in heavy smokers (Rennard and Vestbo, 2006). As the incidence of COPD rises, associated health care costs will increase as well. Exposure to tobacco smoke is a key etiologic agent of this disease, which is characterized by progressive airflow limitation with an abnormal inflammatory response in the small airways and alveoli. The inflammation induced by cigarette smoke leads to an increase in protease production, a major contributor to the lung destruction seen in emphysema (Churg et al., 2008).

For the past 50 years emphysematous lung destruction was believed to arise from an imbalance between proteases and antiproteases. This theory was originally formulated after it was noted that smokers with a congenital deficiency of alpha-1-antitrypsin (α_1-AT), leading to unopposed neutrophil elastase activity, had an increased incidence of emphysema (Laurell and Erickson, 1963). Imbalances between matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), have been identified in emphysema tissues (Finlay et al., 1997; Hautamaki et al., 1997; Ohnishi et al., 1998; Imai et al., 2001; Houghton et al., 2006; Lee et al., 2009; Silverman and Sandhaus, 2009). MMPs are a family of structurally related zinc-dependent endopeptidases involved in the degradation and remodeling of the extracellular matrix during a variety of important physiological and pathological processes (Visse and Nagase, 2003) including embryological development, angiogenesis, and wound healing (Chang and Werb, 2001). Studies in our laboratory, and others, have shown that cigarette smoke directly induces expression of MMPs in resident lung cells (Mercer et al., 2004; Seagrave et al., 2004; Valenca et al., 2004).

Exposure to second hand tobacco smoke is a major risk factor for the development of COPD in non-smokers. Based upon plasma cotinine levels, a non-smoker living with a smoker has a smoke exposure of approximately 1% of that from an individual actively smoking 20 cigarettes a day (Thompson et al., 1990; Law and Hackshaw, 1996). Despite the low level of exposure, those exposed to second hand smoke exhibit increased levels of elastin degradation products in the serum (Slowik et al., 2011); these degradation products are chemotactic for macrophages in the lung, promoting ongoing inflammation, and lung degradation (Houghton et al., 2006). Genetic factors influence the development of COPD in smokers (Silverman, 2006) and likely impact the inflammatory response to second hand smoke. It should be noted that in the developing world, the burning of biomass fuels for cooking and heating has been implicated in the development of COPD in non-smoking adults (Da Silva et al., 2012).

COPD is manifested by several different pathologies: emphysema, small airway remodeling, chronic bronchitis, and pulmonary hypertension (Wright et al., 2008). Frequent COPD exacerbations lead to further decline in lung function in patients with moderate to severe disease (Donaldson et al., 2002). With variation in pathologies and clinical manifestations, no single animal model can serve as a surrogate to the human disease. The structural and mechanical changes in animal models of COPD can be quite mild, but information regarding second hand smoke exposure can be gained through examination of the appropriate susceptible species and mouse strains. It is essential that researchers understand the differences in smoke-exposure animal models in order to better understand the effects of second hand smoke in humans.

METHODS OF SMOKE EXPOSURE IN ANIMAL MODELS OF EMPHYSEMA

There are two main systems used for smoke exposure of rodents, nose-only smoke exposure machines and whole body exposure

systems (Coggins, 1998). Nose-only smoke systems offer the advantages of more controlled dosage and exposure as well as smoke delivery only to the respiratory system. However, this method of nasal smoke exposure also involves prolonged whole-body restraint, a known stressor for mice (van Eijl et al., 2006). The metal nosepieces utilized in the restraining system have also been associated with severe hypothermia (van Eijl et al., 2006).

Whole body smoke exposure systems offer several advantages. Rodents do not require restraint for smoking, but rather are housed in their usual cages and bedding. They have free access to food and water for the duration of smoke exposure. In addition, the setup of most whole body smoke exposure systems allows for a large number of rodents and small animals to undergo simultaneous smoke exposure and therefore it is less labor intensive than nose-only systems. On the other hand, the whole body smoke exposure system is less controlled than the nose-only system; though, measurement of total particulate matter can aid in ensuring steady smoke exposure during animal experiments. In addition, in whole body smoke exposure systems animals develop deposits of nicotine and tar on the fur, which can be ingested through usual grooming behaviors (Mauderly et al., 1989; Wright et al., 2008). Regardless of the chosen method of smoke exposure, the quality of smoke exposure can be measured through carboxyhemoglobin and serum cotinine levels (Wright et al., 2008).

Cigarette smoke, delivered either by nose-only or whole body exposure systems, is composed of both mainstream and side stream smoke. Mainstream smoke is derived directly from the cigarettes during the inhalation phase or puff of the smoke machine. Side stream smoke refers to the smoke generated from the lit end of a cigarette in between puffs, and is the main component of second hand smoke. In an evaluation of unpublished work from Phillip Morris in the 1980's, fresh side stream smoke is approximately four times more toxic per gram total particulate matter than mainstream smoke (Schick and Glantz, 2005). Aged side stream smoke (over 10 s old) increases the toxicity fourfold as compared to fresh side stream smoke (Schick and Glantz, 2006). All of these findings are particularly relevant to animal smoke exposure systems, which utilize both aged side stream and mainstream smoke.

LESSONS FROM CHRONIC AND ACUTE SMOKE EXPOSURE STUDIES

In order to develop structural and physiological changes in the lung consistent with emphysema, investigators undertake long-term smoke exposure studies. Variation between investigators exists, but it is generally accepted that in the mouse model, at least 6 months of smoke exposure is required to produce structural and physiological changes consistent with emphysema (Churg et al., 2008). The exception to this is the highly susceptible A/J strain, which can develop emphysematous changes, as measured by mean linear intercept, after only 4 months of smoke exposure (Braber et al., 2011). The literature describing studies on acute smoke exposure are much more variable with regards to the exact duration of exposure, ranging from hours to weeks. Common to all smoke exposure studies in animals is the development of pulmonary inflammation. However, the inflammatory cell characteristics vary by the chosen method of exposure, species, and within mice based on strains (van der Vaart et al., 2004).

In both the chronic and acute smoke exposure models, an increase in the number of alveolar macrophages is induced by smoke in the bronchoalveolar lavage (BAL) fluid and lung tissue (van der Vaart et al., 2004); increases in neutrophil counts are more variable. Increased alveolar macrophages in BAL fluid can be identified immediately following smoke exposure (Ortega et al., 1992) and persist throughout the duration of the exposure. Despite the increase in alveolar macrophage numbers, alveolar macrophages from 8-week smoke-exposed mice demonstrate an attenuation of the inflammatory signaling pathways associated with bacterial and viral infections, including TNF-α and IL-6 (Gaschler et al., 2008) and exhibit an impaired phagocytic activity immediately following smoke exposure (Ortega et al., 1992).

Early chronic smoke exposure studies in mice focused mainly on the carcinogenic properties of tobacco smoke. Initial studies of non-malignant pulmonary abnormalities caused by chronic smoke exposure focused on alterations in the inflammatory response. Findings included peribronchiolar and perivascular accumulations of lymphocytes and macrophages (Matulionis, 1984), with increased macrophage size due to lysosomal abnormalities (Matulionis and Simmerman, 1985). Chronic smoke exposure was also found to reduce antigen-specific T-cell proliferative response in pulmonary associated lymph nodes (Chang et al., 1990) as well as the phagocytic index of macrophages (Ortega et al., 1992). Although structural changes in the mouse lung occur after long-term smoke exposure, it is important to note that the changes in mice following chronic smoke exposure are still only mild as measured by mean linear intercept or lung compliance (Foronjy et al., 2005, 2006; Churg et al., 2008).

With the development of genetically modified animals, researchers have been able to better define the contribution of isolated pathways in emphysema pathogenesis, including the interstitial collagenase MMP-1 (D'Armiento et al., 1992). The strength of these animal studies was extended when follow-up studies demonstrated that indeed MMP-1 expression was increased in the lung parenchyma of patients with emphysema (Imai et al., 2001) and that cigarette smoke directly induced expression of MMP-1 in pulmonary small airway epithelial cells. Building upon the long known association between emphysema development in smokers and congenital deficiency of α_1-AT (Laurell and Erickson, 1963), Shapiro and colleagues established the critical role of MMP-12 (macrophage elastase) in emphysema development (Hautamaki et al., 1997). Following the development of the MMP-12 knockout mouse (Shipley et al., 1996), the group demonstrated that smoke-exposed mice deficient in MMP-12 do not develop parenchymal changes consistent with emphysema (Hautamaki et al., 1997). In addition to the role of MMP-12 in chronic smoke exposure, MMP-12 is also involved in the acute inflammatory response by augmenting TNF-alpha release from macrophages, causing neutrophil influx and endothelial activation (Churg et al., 2003). Elastin

degradation products, by-products of MMP-12 activity, promote inflammation and ongoing lung degradation (Houghton et al., 2006).

Other laboratories have built upon the MMP-12 work, documenting the importance of additional MMPs in emphysema pathogenesis (Shapiro et al., 2003; Seagrave et al., 2004; March et al., 2006; Valenca et al., 2006; Foronjy et al., 2008; Vecchio et al., 2010). Our laboratory has demonstrated that the transgenic overexpression of MMP-9 in macrophages causes spontaneous adult-onset emphysema due to elastin degradation (Foronjy et al., 2008). In contrast, Atkinson and colleagues have shown that the MMP-9 deficient mouse still develops smoke-induced emphysema (Atkinson et al., 2011). It is likely that in MMP-9 deficient mice, the presence of other proteases, including MMP-12, are sufficient to destroy the lung; yet, MMP-9 potentially plays a role in the destructive process in combination with other proteases.

Beyond the protease/antiprotease hypothesis, transgenic mice have shed light into additional pathways critical in emphysema pathogenesis. A substantial quantity of work has been performed examining the effect of cigarette smoke on inflammatory pathways. TNF-alpha is a key pro-inflammatory factor implicated in emphysema pathogenesis. TNF-alpha receptor 2 plays a critical role in this proinflammatory pathway, as mice deficient in the receptor have both attenuated pulmonary inflammation and emphysema development in the setting of long-term smoke exposure (D'Hulst et al., 2006). The pro-inflammatory cytokine IFN-gamma is a potent stimulator of MMP-9 and CCR5 ligands, the expression of which ultimately results in DNA damage, apoptosis, and emphysema (Ma et al., 2005). The inflammatory cell surface receptor CX3CR1 is upregulated by cigarette smoke exposure; expression of CX3CR1 results in the recruitment of other CX3CR1 positive cells, mainly macrophages and T lymphocytes, amplifying the inflammatory response to cigarette smoke exposure (McComb et al., 2008). Other pathways implicated in modulation of the inflammatory response in smoke exposure include protein phosphatase 2A (PP2A) (Wallace et al., 2012), forkhead box class O 3a (FOXO3) (Hwang et al., 2011), CCR6 (Bracke et al., 2006), and endothelial monocyte-activating protein 2 (EMAPII) (Clauss et al., 2011).

Oxidative stress is another key mediator in emphysema pathogenesis and the pathways of lung injury (Yao and Rahman, 2011; Rahman, 2012). The acute smoke exposure model is well-suited to examine cellular oxidative stress and mechanisms leading to increased inflammation (Valenca et al., 2008; Richens et al., 2009; Gould et al., 2010; Geraghty et al., 2011b; Lu et al., 2011). Within 6 h of smoke exposure, evidence of oxidative stress can be detected within the pulmonary vascular endothelium (Lu et al., 2011). Many different pathways alter the levels of oxidative stress within the lung following smoke exposure. Superoxide dismutase (SOD) is an enzyme that catalyzes the breakdown of superoxide radicals into oxygen and hydrogen peroxide. Our laboratory first demonstrated a protective role of antioxidants when transgenic mice expressing elevated levels of SOD in the lung were shown to be protected from the development of emphysema through attenuation of the inflammatory response, particularly neutrophil inflammation after smoke exposure and intratracheal elastase administration (Foronjy et al., 2006). The loss of antioxidants were shown by Rahman and colleagues to lead to impaired lung function and exercise capacity following chronic cigarette smoke exposure in mice lacking SOD3 (Yao et al., 2010). Moreover, overexpression of SOD3 as well as exogenous administration of SOD analogues attenuated emphysema development (Yao et al., 2010) in smoke exposure models. SOD appears to provide protective benefits against oxidative stress mediated fragmentation of the extracellular matrix (Yao et al., 2010). Another interesting area of research in emphysema relates to endoplasmic reticulum (ER) stress. In the setting of acute smoke exposure, inflammatory cells, predominately macrophages, develop ER stress through CHOP induction by cigarette smoke, but during *in vivo* long-term smoke exposure, the ER stress response is reduced (Geraghty et al., 2011b). The finding of decreased ER stress in long-term smoke exposed mice is analogous to the human disease, where in human emphysematous lung tissue, there is a decrease in markers of ER stress (Korfei et al., 2008).

Other critical pathways modulating oxidative stress that were shown to be important in the smoke exposure model include the mTOR pathway, where smoke exposure upregulates the mTOR inhibitor and stress related protein Rtp801, amplifying oxidative stress, NF-kappa-B activation and subsequent alveolar inflammation and alveolar septal apoptosis (Yoshida et al., 2010). Nrf2, a regulator of the antioxidant pathway, also plays a major role in determining susceptibility to smoke-induced emphysema by upregulating antioxidants and thereby decreasing inflammation (Rangasamy et al., 2004, 2009; Iizuka et al., 2005; Gebel et al., 2010). Additionally, the TGF-β signaling pathway influences oxidative stress in the setting of smoke exposure; antagonism of this pathway with the angiotensin receptor type 1 blocker losartan decreased oxidative stress, inflammation and extracellular matrix remodeling (Podowski et al., 2012). Furthermore, the class III histone/protein deacetylase SIRT1 regulates cigarette smoke-exposure mediated proinflammatory signaling through NF-kappa-B and levels are decreased in rats following smoke exposure (Yang et al., 2007). SIRT1 levels are also decreased in both macrophages and the lung tissue of smokers and patients with COPD (Rajendrasozhan et al., 2008). Activation of the SIRT1 pathway *in vitro* with resveratrol decreases proinflammatory cytokine release (Yang et al., 2007; Arunachalam et al., 2010), and both genetic overexpression and pharmacologic activation of SIRT1 protects against smoke-induced emphysema via a FOXO3-mediated reduction of cellular senescence independent of the inflammatory effects (Yao et al., 2012).

The pattern recognition receptor toll-like receptor-4 (TLR4), mediated through the NADPH oxidase (NOX) pathway, is another important component in the oxidative stress pathway critical in emphysema development. Lee and colleagues have shown that deficiency of TLR4 leads to upregulation of NOX2 in lungs and endothelial cells, causing increased oxidant generation and elastolytic activity within the parenchyma (Zhang et al., 2006). TLR4 deficiency also promotes apoptosis and autophagy in the setting of smoke exposure, with increased airspace enlargement as compared to smoked wild-type mice (An et al., 2012), suggesting a protective benefit to TLR4 expression in the lung. However, TLR4 activation by cigarette smoke also has negative effects within the lung. Lipopolysaccharide (LPS) is a component

of cigarette smoke as well as a known TLR4 ligand; activation of TLR4 leads to IL-1 production, IL-1 receptor 1 signaling, and downstream inflammation (Doz et al., 2008). Our group has demonstrated that TLR4 is upregulated within the lungs of both smoke-exposed mice and rabbits, and have documented increased signaling through TLR4 in human emphysematous lung tissue (Geraghty et al., 2011a). When MMP-1 is present, as is seen in both human emphysema as well as in the smoke-exposed rabbit, TLR4 activation by cigarette smoke ultimately leads to expression of the collagenase MMP-1 (Geraghty et al., 2011a). In addition to the smoking model of emphysema, hyperlipidemia alone, as is seen in the ApoE knockout mouse fed a high fat diet, is sufficient to cause peribronchial and perivascular inflammation through TLR4 activation leading to emphysema development (Goldklang et al., 2012), suggesting a new mechanism linking emphysema and cardiovascular disease. The role that this specific pathway plays in smoke exposure induced emphysema remains to be determined in future studies.

Finally, apoptosis is an additional critical process in emphysema pathogenesis that has been investigated in the smoke exposure model. Apoptosis is a mechanism of programmed cell death that occurs during normal lung morphogenesis, but can also be triggered by multiple stimuli that are pertinent in emphysema development, including loss of contact with the extracellular matrix, reduction in VEGF signaling, induction by immune cells, and various stresses including oxidative stress (Morissette et al., 2009). As mentioned above, the TLR4 pathway modulates both cellular apoptosis and autophagy in response to chronic cigarette smoke exposure (An et al., 2012). The ceramide pathway, modulated by neutral sphingomyelinase 2 (nSMase2) also modulates cigarette smoke related epithelial cell apoptosis. nSMase is upregulated in the lung following smoke exposure, while knockdown of nSMase with a heterozygous mouse decreases smoke related ceramide production and apoptosis (Filosto et al., 2011). Other implicated pathways in smoke related emphysema include the TGF-β signaling pathway via SMAD-3 (Farkas et al., 2011), cytokine IL-6 (Ruwanpura et al., 2011), VEGF (Kasahara et al., 2000; Tuder et al., 2003b), and autophagy via early growth response-1 (Egr-1) and the protein microtubule-associated protein 1 light chain-3B (LC3B) (Chen et al., 2008, 2010). In summary, there are numerous pathways involved in emphysema pathogenesis. Utilizing transgenically modified mouse models and pharmacological interventions, there have been significant advances in the understanding of human disease pathogenesis and treatment.

THE DEVELOPMENT OF EMPHYSEMA IS MOUSE STRAIN DEPENDENT

One of the most important considerations in smoke exposure studies is understanding that the development of emphysema in a chronic smoke exposure model, as measured by morphometry and lung compliance, is mouse strain dependent (Guerassimov et al., 2004; Foronjy et al., 2005). In a comprehensive study performed by Guerassimov and colleagues, NZWLac/J, C57BL6/J, A/J, SJ/L, and AKR/J strains underwent 6 months of cigarette smoke exposure. In morphometric analysis of emphysema, the most impressive increase in mean linear intercept was observed in the AKR/J strain (38% increase as compared to control mice), with more modest increases seen in the A/J, SJ/L and C57BL6/J mice (Guerassimov et al., 2004). The NZWLac/J mice did not develop any increase in mean linear intercept (11% decrease as compared to control mice) (Guerassimov et al., 2004). Furthermore, despite detectible, albeit modest, alterations in lung morphometry, there is not necessarily a correlation with alterations in lung mechanics (Guerassimov et al., 2004; Foronjy et al., 2005).

A difference in the inflammatory response explains at least some of the variations between mouse strains in response to chronic smoke exposure. Again, the AKR/J mice demonstrate smoke-induced increases in macrophages, PMNs, and T cells with a Th1 cytokine predominance; increased alveolar macrophages were also noted in the C57BL6/J and NZWLac/J mice (Foronjy et al., 2005). *In vitro* analysis of alveolar macrophages from susceptible (C57BL6/J) and resistant (ICR) strains demonstrates that alveolar macrophages from susceptible mice release increased proinflammatory cytokines and MMPs when exposed to cigarette smoke, and have a higher baseline production of reactive oxygen species as compared to those obtained from resistant mice (Vecchio et al., 2010). The pro-inflammatory early growth response gene-1 (Egr-1) may provide some insight into strain variation. Egr-1 induction by cigarette smoke varies by strain, with marked increase in the emphysema-susceptible AKR/J mice as compared to the relatively resistant NZWLac/J strain (Reynolds et al., 2006).

It is important to understand that dissimilar to what is seen in the human disease, in the mouse model of smoke-induced emphysema, smoking cessation is accompanied by a reparative response (Braber et al., 2011). Within 1 week of smoking cessation, there is a partial normalization of MMP-2 and MMP-9 levels in BAL fluid (Seagrave et al., 2004), though lymphocyte abnormalities including CD8 T cell oligoclonal expansions persist (Seagrave et al., 2004; Motz et al., 2008). It is likely that the combination of altered inflammatory response and cytokine profiles are responsible for the variations between strains in structural and functional measures of emphysema as well as for the reparative response seen upon smoking cessation. However, understanding the limitations of the mouse models has led our group and others to investigate alternative animal models of emphysema.

SMOKE EXPOSURE USING ALTERNATIVE ANIMAL SPECIES

While mice are the most widely utilized animal species for smoke exposure studies, the mouse model of emphysema has several limitations. Mice do not express the same proteolytic repertoire upon smoke exposure as humans. Mice do not even have the gene for MMP-1, a key protease implicated in human emphysema pathogenesis (D'Armiento et al., 1992). As stated above, mice develop only mild structural and mechanical changes to the lung following smoke exposure (Foronjy et al., 2005). In addition, both mice and rats undergo alveolar development entirely post-natally (Massaro and Massaro, 2007). For these reasons and others, investigators have long been interested in identifying alternative animal models of emphysema that more closely mimic the human disease process.

As opposed to rodents, the guinea pig model offers several advantages that aid in the study of the effects of smoke exposure. Guinea pigs begin lung alveolarization at birth, and exhibit a progressive increase in air space size until 18 months of age (Wright and Churg, 1990). The emphysema that guinea pigs develop is easily recognizable (Golovatch et al., 2009), with noted small airway remodeling and goblet cell metaplasia that is absent in mouse exposure models (Wright and Sun, 1994; Wright et al., 2007). Guinea pigs develop airway inflammation, with increased alveolar macrophages as well as activation of the MAP kinases ERK and JNK, as is seen in the human disease state (Golovatch et al., 2009). In addition, as opposed to mice, guinea pigs can develop significant pulmonary arterial hypertension after only 1 month of smoke exposure (Wright et al., 2006). Most interestingly, in guinea pigs the development of emphysema is cathepsin K dependent, as opposed to a metalloproteinase or elastase driven lung destructive process, and while extracellular matrix changes similar to those in the human disease were observed in the guinea pig smoke exposure model, there were no noted alterations in collagenolytic MMPs (Golovatch et al., 2009).

Furthermore, guinea pigs differ from mice in their reparative responses following smoke exposure. While all-trans retinoic acid treatment has proven to be useful in reversal of emphysematous changes of alveolar structures in mice and rats (Massaro and Massaro, 1997; Belloni et al., 2000), it does not produce the same protective effects in smoke-exposed guinea pigs (Meshi et al., 2002). Another major reparative response following smoke exposure is the increase in matrix and structural cells in the small airways and intrapulmonary arteries, but not in the alveolar wall. In both mice and guinea pigs, increased deposition of collagen in the airway has been noted after chronic smoke exposure (Churg et al., 2006, 2007). It is theorized that an increase in apoptosis in emphysematous lungs could explain this failure to repair in certain strains of mice, rats (Kasahara et al., 2000; Tuder et al., 2003a), and humans (Kasahara et al., 2001). However, our group has shown that certain strains of mice and guinea pigs do not develop increased apoptosis with smoke exposure and emphysema (Foronjy et al., 2005; Golovatch et al., 2009).

The rabbit smoke exposure model is another alternative to the rodent models. Rabbits express MMP-1 and therefore the macrophages express a protease repertoire similar to humans (Fukuda et al., 2000). Studies have not only demonstrated the presence of MMP-1 in the rabbit, but specifically MMP-1 expression during lung development is similar to human lung development (Fukuda et al., 2000). Our group has recently demonstrated that the smoke-exposed rabbit model exhibits the activation of similar mechanistic pathways following smoke exposure as is seen in the human disease (Geraghty et al., 2011a). Since it is well-documented that MMP-1 is fundamental to emphysema pathogenesis, the rabbit is the ideal species to examine when exploring the MMP-1 regulated lung destruction and determining if pharmacological inhibition of MMP-1 is a feasible approach to the treatment of emphysema.

Rabbits, similar to guinea pigs, differ from mice in their reparative response to smoke exposure. In a study of rabbits exposed to pine wood smoke, Thorning and colleagues document reparative responses that include intact epithelial basal lamina acting as a substratum for proliferating reparative epithelial cells (Thorning et al., 1982). However, more recent studies have shown that as opposed to mice the smoke-exposed rabbit does not exhibit reversal of emphysematous changes after all-trans-retinoic acid treatment (Nishi et al., 2003).

Both the rabbit and guinea pig models offer the specific advantages over the rodent model as outlined above, but the disadvantages cannot go without mention. The large size and lack of available reagents in rabbits and guinea pigs limit the ease of experimentation. The high costs associated with purchase, housing, and smoke exposure in rabbits are practical deterrents to their use. Furthermore, the lung destruction seen in the smoke-exposed guinea pig model is cathepsin dependent, but cathepsins do not play as significant a role in emphysema pathogenesis in humans as MMPs (Golovatch et al., 2009).

Other alternative animal models for smoke exposure include lambs and primates. In young lambs morphological changes in the lung following second hand smoke exposure can appear after a short period of time (<1 month) (Stecenko et al., 1986) making it a useful model for smoke exposure studies. In addition, the lungs of lambs are still developing after birth similar to humans, and therefore the model is potentially useful for research into the effects of second hand smoke on infant lung development. Aside from the effects of cigarette smoke on the lung, lambs have also been used to study the effect of passive smoke on laryngeal chemoreflexes (St-Hilaire et al., 2010).

Non-human primate smoking models have been utilized to demonstrate the effects of environmental smoke exposure with studies focusing on infants, lung development, and pre vs. postnatal effects. This model is particularly useful for studying the effects of maternal smoke exposure during pregnancy (United States. Public Health Service. Office of the Surgeon General, 2006) as well as the carcinogenic effects of cigarette smoke (Coggins, 2001). Investigators have used a variety of smoke exposure methods with the primates including training non-human primates to puff cigarettes (Ando and Yanagita, 1981). Unfortunately the primate model is difficult to work with secondary to regularity issues and enormous costs.

The use of large animals including lambs and non-human primates offer the advantage of more closely mimicking human exposure, especially in prenatal and neonatal environments. However, the disadvantages of all of the large animal models include cost, size, and feasibility of smoke exposure studies, as well as a lack of available reagents.

CONCLUSION

In conclusion, there is a substantial amount of information pertinent to second hand smoke exposure that can be gained from an understanding of animal models of disease. It is important to understand the limitations of each model of smoke exposure. Rodents, while widely used, have several limitations, including strain differences in disease susceptibility, and relatively mild forms of emphysema. However, there is significant information that can be gained from the use of mouse models, especially in an era of transgenic manipulation. The most attractive alternative smoke exposure animal models are guinea pigs and rabbits,

both developing clear evidence of inflammation and emphysema. A better understanding of the benefits and drawbacks of these models will assist researchers in best choosing the appropriate species and exposure model prior to conducting smoke exposure experiments.

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Tobacco smoke induced COPD/emphysema in the animal model—are we all on the same page?

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Chronic Obstructive Pulmonary Disease (COPD) is one of the foremost causes of death worldwide. It is primarily caused by tobacco smoke, making it an easily preventable disease, but facilitated by genetic α-1 antitrypsin deficiency. In addition to active smokers, health problems also occur in people involuntarily exposed to second hand smoke (SHS). Currently, the relationship between SHS and COPD is not well established. Knowledge of pathogenic mechanisms is limited, thereby halting the advancement of new treatments for this socially and economically detrimental disease. Here, we attempt to summarize tobacco smoke studies undertaken in animal models, applying both mainstream (direct, nose only) and side stream (indirect, whole body) smoke exposures. This overview of 155 studies compares cellular and molecular mechanisms as well as proteolytic, inflammatory, and vasoreactive responses underlying COPD development. This is a difficult task, as listing of exposure parameters is limited for most experiments. We show that both mainstream and SHS studies largely present similar inflammatory cell populations dominated by macrophages as well as elevated chemokine/cytokine levels, such as TNF-α. Additionally, SHS, like mainstream smoke, has been shown to cause vascular remodeling and neutrophil elastase-mediated proteolytic matrix breakdown with failure to repair. Disease mechanisms and therapeutic interventions appear to coincide in both exposure scenarios. One of the more widely applied interventions, the anti-oxidant therapy, is successful for both mainstream and SHS. The comparison of direct with indirect smoke exposure studies in this review emphasizes that, even though there are many overlapping pathways, it is not conclusive that SHS is using exactly the same mechanisms as direct smoke in COPD pathogenesis, but should be considered a preventable health risk. Some characteristics and therapeutic alternatives uniquely exist in SHS-related COPD.

Keywords: second hand cigarette smoke, COPD, emphysema, inflammation, animal model, chamber, pulmonary hypertension, matrix degradation

INTRODUCTION

Cigarette smoke is the main preventable cause for chronic obstructive pulmonary disease (COPD), resulting in progressive proteolytic, inflammatory, and vasoactive responses that lead to emphysema, small airway obstruction, and pulmonary hypertension. COPD in itself is a serious burden throughout the world, both economically and socially, costing $193 billion in the United States alone (CDC, 2008). The disease is the third largest cause of death in the United States and the fourth worldwide (Pauwels et al., 2001; Minino, 2010). An estimated 95% of COPD cases are attributed to smoking (Barnes et al., 2003), while only a relatively small margin of smokers is susceptible (Fletcher and Peto, 1977). The United States Centers for Disease Control and Prevention considers tobacco smoke to be "the single most important preventable risk to human health in developed countries and an important cause of premature death worldwide," not only to smokers, but also to those involuntary exposed to second hand smoke (SHS or environmental tobacco smoke, known as ETS) (Oberg et al., 2011).

There are three kinds of smoke that humans are exposed to: mainstream or first hand smoke that is directly inhaled through a person's mouth after taking a puff on a lit cigarette; Side stream smoke, which goes into the air directly from a burning *cigarette*, *cigar*, or *smoking pipe*; and SHS, which is a combination of both, side stream smoke being the main component of SHS, also known as ETS. Cigarette smoke thereby not only affects smokers, but also contributes to health problems in non-smokers. While a smoker voluntarily inhales the first hand smoke, the non-smoker is inadvertently exposed to SHS that comes from the burning end of a cigarette and the smoke exhaled by the smoker. SHS is therefore also a potential risk factor for COPD and entails symptomatic disease in individuals who are not actually smokers. At present, the dose-response relationship between SHS exposure and COPD is not well established and there is limited understanding of the mechanisms responsible for its pathogenesis, thus halting the development of new advanced treatments for this detrimental disease.

This review is an attempt to sort out the cigarette smoke exposure studies according to levels (low/high) and manner (mainstream or whole body) of the exposure, focusing largely on newer studies applying the rodent model.

SECOND HAND CIGARETTE SMOKE EXPOSURE

While the qualitative composition of the components is nearly identical in mainstream smoke, side stream smoke, and SHS, the quantitative composition of each is different. In the enclosed environment, due to relatively low ventilation rates (Lofroth, 1989; Jinot and Bayard, 1994), some compounds are emitted at levels up to more than 10 times greater in side stream smoke and SHS when compared with mainstream smoke (Moritsugu, 2007). Side stream smoking has therefore been classified as a Class A *carcinogen* by the *US Environmental Protection Agency*. Still, data regarding the biological evidence linking SHS exposure and COPD are scarce. A 2006 US Surgeon General Report on the health consequences of SHS concluded that the evidence linking SHS and COPD is suggestive (www.surgeongeneral.gov/library/secondhandsmoke), but not sufficient, to infer a causal relationship. The conclusion of the report was drawn primarily from dated epidemiologic evidence that did not establish a biological link (Hirayama, 1981; Kalandidi et al., 1987; Sandler et al., 1989; Robbins et al., 1993; Dayal et al., 1994; Leuenberger et al., 1994; Piitulainen et al., 1998; Berglund et al., 1999). A very recent retrospective analysis of data from 192 countries was published in 2011 and shows that worldwide 40% of children, 33% of male non-smokers, and 35% of female non-smokers are exposed to SHS (Oberg et al., 2011). In 2004 alone, 603,000 deaths were attributable to SHS, accounting for approximately 1% of worldwide mortality. For this reason, the US Surgeon General Report's conclusion should be challenged by providing evidence that SHS exposure can in fact cause COPD by utilizing and comparing animal models as well as assessing patient exposure to SHS.

THE ANIMAL MODEL AND EXPOSURE SYSTEM

In order to gain insight into the mechanisms of disease development during cigarette smoke exposure, both mainstream and second hand, animal models have been of exceptional use. Animals are exposed to cigarette smoke in a smoking apparatus for either mainstream (nose or head only) or side stream (whole body) applications. In addition, smoke can either be filtered or not, which either depends on the cigarette used (with a filter or without) or the set-up of the apparatus. Another diversity factor is the selection of different animal species with varying susceptibilities, which can be particular to each strain within a species. Early studies of SHS used self-constructed chambers for laboratory animals, allowing environmental smoke to diffuse within a confined space in which the animals were kept. These chambers, unique to each group, did not have the capabilities to measure the parameters essential for assessing cigarette smoke dose and composition. Also, the number and type of cigarettes as well as the length of exposure varied in each study as much as they do today, adding to the difficulty of comparing results. Generally, standardized research-grade cigarettes should be used to easily define a specified dose of total suspended particles (TSP) or total particulate matter (TPM), including nicotine and carbon monoxide levels. Standardized cigarettes became available for worldwide use in 1969 (Roemer et al., 2012) and are most commonly from the University of Kentucky (http://www.ca.uky.edu/refcig/), although there are still often publications using non-reference cigarettes. The introduction of the Teague chamber in 1994 (Teague et al., 1994) has revolutionized the field by allowing maintenance of consistent levels of TSP/TPM that can be set at a variety of concentrations for the exposure of animals to SHS that can mimic human exposures.

In human exposure studies, only the distribution of fine particles ($PM_{2.5}$, which are under 2.5 μm in size and are able to reach the alveoli of the lung) is measured. This accounts for only a small fraction (about 0.1%) of the TSP measurements that are usually reported in animal studies. Data from 66 US casinos with smoking in California, Delaware, Nevada, New Jersey, and Pennsylvania, developed $PM_{2.5}$ frequency distributions, comparing them with three non-smoking casinos. Geometric means for $PM_{2.5}$ were 53.8 $\mu g/m^3$ (range 18.5–205 $\mu g/m^3$) inside smoking casinos, 4.3 $\mu g/m^3$ (range 0.26–29.7 $\mu g/m^3$) outside those casinos, and 3.1 $\mu g/m^3$ (range 0.6–9 $\mu g/m^3$) inside the three non-smoking casinos (Jiang et al., 2010; Lu et al., 2011; Repace et al., 2011; Cochran et al., 2012).

Most commonly for SHS studies listed in **Table 2**, the Teague chamber was set to exposures between 70 and 150 mg/m^3. Concurrently, the mainstream smoke exposures listed in **Table 1** were as low as 75 mg/m^3 and as high as 600 mg/m^3 TSP/TPM (Hodge-Bell et al., 2007). Most of the mainstream smoke studies were performed at 140 mg/m^3 TSP/TPM levels (**Table 1**). Ideally, more recent protocols should thereby produce results that can compare the assessed proteolytic, inflammatory, and vasoactive reaction based on similar exposure methods, duration, and cigarette content. This can only be achieved if researchers pertain to the standard procedures available today, such as the use of reference cigarettes in a chamber with defined settings for exposure. These conformities are essential, since a majority of studies are performed in rodents (mice, rats, and guinea pigs), where there is an overwhelming assortment of strains with varying susceptibility, especially when considering mice.

Today, rodents are the most commonly used models. While mice are surely favored for their wide variety of applicable gene expression manipulations, it remains difficult to standardize measurements of pulmonary function to assess disease parameters. The guinea pig model is also occasionally applied, mainly by one group of investigators (Simani et al., 1974; Wright and Churg, 1990, 2002; Wright and Sun, 1994, 1999; Wright et al., 2002, 2011) though increased inflammatory cells and muscularization of pulmonary vessels was recently documented (Dominguez-Fandos et al., 2012). The rat is a favorable model, since measurable emphysematous changes which further progress can be detected after only 2 months of smoke exposure (Kratzer et al., 2013).

Mainstream smoke and SHS exposure studies are summarized in **Tables 1**, **2**, respectively. Animal studies with an unidentified smoke exposure are presented in **Table 3**. The cigarette brands used are listed in **Table 4**.

Table 1 | Mainstream cigarette smoke exposure modeled in animals.

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	Copd/emphysema	Remarks	References
Guinea pig	**X**	2 × 20 ml puffs/min, 8–9 min/cig, 10 min rest, 10/day; 1–60 days	**X**	**X**	**X**			Simani et al., 1974
Sprague-Dawley rat	**X**	12 s puffs, 4 s rest, 2/day, 5 day/week; 25 days	**X**	1.5 mg/cig	**X**		Mainstream presumed	Pittilo et al., 1982
Wistar rat nicorandil p.o.	**Seven star** unfiltered	30 cig, 2 s puff, 15 puffs/min, 8 min	**X**	1.88%	**X**		Mainstream presumed	Gomita et al., 1990
Rat	**X**	8 cig	**X**	1.5 mg/cig	**X**		"Whole smoke"; mainstream presumed; exposure as in Pittilo et al. (1982)	Pittilo et al., 1990
Guinea pig	Commer-cial unfiltered	10/day, 5 day/week; 1–12 month	**X**	**X**	**X**	Emphysema (age and exposure dependent)	mainstream presumed; exposure as in Simani et al. (1974)	Wright and Churg, 1990
Wistar rat	**Long peace**	2 × 20 min, 15 puffs/min; 21 days	**X**	2 mg/cig	**X**		Hamburg II; exposure as in Gomita et al. (1990)	Suemaru et al., 1992
Sprague-dawley rat	Commercial unfiltered	7/day; 1–7 days	**X**	**X**	**X**	PH	"Whole smoke"; exposure as in Simani et al. (1974)	Sekhon et al., 1994
Guinea pig	**X**	10/day, 5 day/week; 4–8 months	**X**	**X**	5% CHG	Emphysema and arteriole muscularization	Exposure as in Wright and Churg (1990)	Wright and Sun, 1994
C57Bl/129 MMP-12 KO with i.t. MCP-1	**KY** unfiltered	2/day, 6 days/week; 6 months	**X**	**X**	10–14% CHG	100% protected	Mainstream presumed; exposure as in Wright and Churg (1990)	Hautamaki et al., 1997
C57Bl/129 MMP-12 KO	**KY** unfiltered	2/day, 6 days/week; 6 month	**X**	**X**	10–14% CHG	100% protected	Mainstream presumed; exposure as in Wright and Churg (1990)	Hautamaki et al., 1997
Sprague-Dawley rat	**X**	20 ml/10 min, 7/day, 5 days/week; 2–12 months	**X**	**X**	4% CHG		Exposure as in Wright and Churg (1990)	Wright et al., 1997
Cam hartley guinea pig	Commercial unfiltered	7/day, 5 days/week; 6 months	**X**	**X**	**X**	COPD and PH	Exposure as in Wright and Churg (1990)	Yamato et al., 1997
Sprague-Dawley rat	Unfiltered	10/day, 5 day/week; 1–6 months	**X**	**X**	10.1 ± 1.5% CHG	Emphysema	Exposure as in Simani et al. (1974)	Ofulue et al., 1998
Sprague-Dawley rat PMN Ab	Unfiltered	10/day, 7 days/week; 2 months	**X**	**X**	**X**	Emphysema		Ofulue and Ko, 1999
Sprague-Dawley rat MoMac Ab	Unfiltered	10/day, 7 day/week; 2 months	**X**	**X**	**X**	Protected		Ofulue and Ko, 1999
Sprague-Dawley rat	**X**	10/day; 24 h	**X**	1.1 mg/cig	11 mg/cig	Small airway constriction	Exposure as in Wright et al. (1997)	Wright et al., 1999
Guinea pig	**Canada Tobacco** unfiltered	7/day, 5 day/week; 24 h-4 months	**X**	1.1 mg/cig	11 mg/cig	Pulmonary arteriole musculariza-tion/hyperplasia	"Whole smoke"	Wright and Sun, 1999
C57Bl/6 × DBA/2 hexavalent chromium i.p.	Commercial **Arda-Bulgar-tabac** filtered	50 ml/cig, 10 min × 9/day; 5 days	533	1.6 mg/cig	**X**		"Whole body mainstream"	Balansky et al., 2000
C57Bl/6	**KY** 2R1	2/day or 1–3/day; 6–48 h	**X**	**X**	**X**	Emphysema via neutrophil elastase	"Whole smoke"; exposure as in Sekhon et al. (1994)	Dhami et al., 2000
C57Bl/6 PMN Ab	**KY** 2R1	2/day or 1–3/day; 6–48 h	**X**	**X**	**X**	Reduced emphysema	"Whole smoke"; exposure as in Sekhon et al. (1994)	Dhami et al., 2000
C57Bl/6 A1AT i.p.	**KY** 2R1	2/day or 1–3/day; 6–48 h	**X**	**X**	**X**	Protected	"Whole smoke"; exposure as in Sekhon et al. (1994)	Dhami et al., 2000

(Continued)

Table 1 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	Copd/emphysema	Remarks	References
C57Bl/129 TNFR KO and 129J	**KY** 2R1	4/day; 24 h	**X**	**X**	**X**	Protected	"Whole smoke"; exposure as in Dhami et al. (2000)	Churg et al., 2002a
C57Bl/129 metallo-protease inhibitor RS113456	**KY** 2R1	4/day; 24 h	**X**	**X**	**X**	Protected	"Whole smoke"; exposure as in Sekhon et al. (1994)	Churg et al., 2002b
C57Bl/129 MME Tg	**KY** 2R1	4/day; 24 h	**X**	**X**	**X**	Emphysema	"Whole smoke"; exposure as in Sekhon et al. (1994)	Churg et al., 2002b
C57Bl/129 MME KO	**KY** 2R1	4/day; 24 h	**X**	**X**	**X**	Protected	"Whole smoke"; exposure as in Sekhon et al. (1994)	Churg et al., 2002b
Hartley guinea pig p.o. serine elastase inhibitor ZD0892	**KY** 2R1	20 ml/1.5 min, 5/day, 5 day/week; 1 day- 6 months	**X**	**X**	**X**	45% protected	"Whole smoke"; exposure as in Wright and Sun (1999)	Wright et al., 2002
Hartley guinea pig	**Canada Tobacco** unfiltered	20 puff/cig, 10/day, 5 day/week; 4–8 months	**X**	1.1 mg/cig	11 mg/cig	Partial recovery after cessation	Exposure as in Wright and Sun (1994)	Wright and Churg, 2002
C57Bl/129 MMP-12 KO	**KY** 2R1	4 in 1 h; 2–24 h (harvest)	**X**	**X**	**X**	No emphysema	"Whole smoke"	Churg et al., 2003a
C57Bl/6 CD-1 α_1 antitrypsin (prolastin)	**KY** 2R1	2/day, 5 day/week; 6 months	**X**	**X**	**X**	67% protected	"Whole smoke"; exposure as in Sekhon et al. (1994)	Churg et al., 2003b
C57Bl/6 NE KO	**KY** unfiltered	2/day, 6 day/week; 6 months	**X**	**X**	10% CHG	59% protected	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Shapiro et al., 2003
C57Bl/6 MMP-12 KO	**KY** unfiltered	2/day, 6 day/week; 6 months	**X**	**X**	10% CHG	100% protected	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Shapiro et al., 2003
C57Bl/129 TNFR KO	**KY** 2R1	4/day, 5 day/week; 6 months	**X**	**X**	**X**	71% protected	"Whole smoke"; exposure as in Churg et al. (2002b)	Churg et al., 2004
SHR and Wistar-Kyoto rat	**Long Peace** filtered	23 (5 rat), 26, 30/day (10 rat) 20 min/day, 5 day/week; 8–14 weeks	**X**	1.9 mg/cig	**X**		Hamburg II; exposure as in Suemaru et al. (1992)	Tanaka et al., 2004
Balb/C SCID	**KY** 2R4F unfiltered	5 cig × 4/day, 30 min rest, 5 day/week (1. week 1/day); 5 weeks-6 months	**X**	**X**	8.3 ± 1.4 CHG	Emphysema	Exposure as in D'Hulst et al. (2005b)	D'Hulst et al., 2005a
C57Bl/6	**KY** 1R3	5 cig × 4/day, 30 min rest, 5 day/week; 1 day-24 weeks	**X**	**X**	**X**	Inflammatory cells progressively accumulate	Mainstream presumed (Kobayashi chamber)	D'Hulst et al., 2005b
Sprague-Dawley rat Simvastatin	**Eighty Eight Lights** South Korea	10/day; 16 weeks	**X**	**X**	**X**	100% protected	"Whole smoke"; mainstream presumed; exposure as in Pittilo et al. (1990)	Lee et al., 2005
Balb/C ovalbumin i.p. d0 and d7	**KY** 1R3	5 cig × 4/day, 5 day/week; 10 days	**X**	**X**	**X**	Airway inflammation		Moerloose et al., 2005
C57Bl/6J	**KY** 2R1	2 × 2/day, 10 puffs each, 5 day/week; 2–6 months	**X**	**X**	**X**	Progressive emphysema		van der Strate et al., 2006
Hartley guinea pig MMP-9/-12 inhibitor AZ11557272	**KY** 2R1	7/day, 5 day/week; 1–6 months	**X**	**X**	**X**	68% protected (70% against SAR)	Mainstream presumed; exposure as in Wright and Churg (1990)	Churg et al., 2007a
C57Bl/6 and ICR	**KY** 2R4F	2 h/day, 5 day/week; 6 months	75, 250, 600	**X**	**X**	Mild emphysema		Hodge-Bell et al., 2007
C57Bl/6 IL-18Ra KO	**KY** 2R4 unfiltered	2 × 2/day, 5 day/week; 6 months	**X**	**X**	**X**	51% protected	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Kang et al., 2007

(Continued)

Table 1 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	Copd/emphysema	Remarks	References
C57Bl/6 CD8 KO	**KY** unfiltered	2/day, 6 day/week; 6 months	**X**	**X**	10% CHG	100% protected	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Maeno et al., 2007
C57Bl/6 CD4 KO	**KY** unfiltered	2/day, 6 day/week; 6 months	**X**	**X**	10% CHG	Emphysema	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Maeno et al., 2007
FVB MrpI/Mdrla/Ib KO	**KY** 2R1	2 cig × 2/day, 10 puffs each, 5 day/week; 6 months	**X**	**X**	**X**	No emphysema or inflammation		van der Deen et al., 2007
C57Bl/6J influenza or viral PAMP	**KY** 2R4 unfiltered	1. week 0.5 cig × 2/day 2. week 1 cig × 3/day	**X**	**X**	**X**	Accelerated emphysema	Mainstream presumed; exposure as in Hautamaki et al. (1997)	Kang et al., 2008
C57Bl/6	**KY** 1R3	2 or 4 cig 5 day/week; 2–6 months	**X**	**X**	**X**	T and B lymphocyte response	Exposure as in Simani et al. (1974); Hautamaki et al. (1997)	Zavitz et al., 2008
C57Bl/6 i.p. caspase inhibitor	**KY** 2R1	4/day acute or 3/day, 5 day/week; 24 h	**X**	**X**	**X**	100% protected	"Whole smoke"; mainstream presumed	Churg et al., 2009a
C57Bl/6 TNFR KO	**KY** 2R1	4/day acute or 3/day, 5d/week; 6 months	**X**	**X**	**X**	83% protected (100% against SAR)	"Whole smoke"; mainstream presumed	Churg et al., 2009b
C57Bl/6 IL-1R KO	**KY** 2R1	4/day acute or 3/day, 5 day/week; 24 h-6 months	**X**	**X**	**X**	65% protected (100% against SAR)	"Whole smoke"; mainstream presumed	Churg et al., 2009b
C57Bl/6	**KY** 2R1	4/day acute or 3/day, 5 day/week; 2 h-6 months	**X**	**X**	**X**	Emphysema	"Whole smoke"; mainstream presumed	Churg et al., 2009b
C57Bl/6 clarithro-mycin p.o.	**KY** unfiltered	2/day, 6 day/week; 6 months	**X**	**X**	**X**	Reduced emphysema	Mainstream presumed; exposure as in Hautamaki et al. (1997); Shapiro et al. (2003)	Nakanishi et al., 2009
C57Bl/6J curcumin p.o.	Commercial filtered **Marlboro**	12 puffs/min, 60 min/day, 10 day or 5 day/week; 10 day-12 weeks	971 ± 98.3 in 5% CS	1 mg/cig 104.5 ± 49.3 ng/ml cotinin	**X**	Reduced emphysema		Suzuki et al., 2009
C57Bl/6 MMP-9 KO	**KY** 3R4F unfiltered	4/day, 6 day/week; 6 months	**X**	**X**	**X**	Emphysema	Exposure as in Hautamaki et al. (1997)	Atkinson et al., 2010
C57Bl/6 adipo-nectin KO	**KY** 2R4F	35 ml puff/25 s, 5 min/cig, 2/day, 5 day/week; 6 months	173 ± 5.3 (100–250)	2.45 mg/cig	**X**	Reduced emphysema and inflammation		Miller et al., 2010
C57Bl/6J SOD3 Tg	**KY** 3R4F	2 × 1 h/day; 3 day-6 months	300 (3 day) or 100 (month)	**X**	**X**	Reduced emphysema		Yao et al., 2010
C57Bl/6J iNOS KO	**KY** 3R4F	6 h/day, 5 day/week; 8 months	140	**X**	**X**	Protected emphysema and PH		Seimetz et al., 2011
C57Bl/6J eNOS KO	**KY** 3R4F	6 h/day, 5 day/week; 8 months	140	**X**	**X**	Emphysema and PH		Seimetz et al., 2011
C57Bl/6J iNOS inhibitor L-NIL	**KY** 3R4F	6 h/day, 5 day/week; 8 months	140	**X**	**X**	Protected emphysema and PH		Seimetz et al., 2011
Hartley guinea pig AZD9668 NE inhibitor p.o.	**KY** R21	2 h/day, 5 day/week; 24 weeks	**X**	**X**	**X**	Protected emphysema and SAR	Exposure as in Churg et al. (2007a)	Stevens et al., 2011
Hartley guinea pig simvastatin	**KY** 2R1 and 2R4F	5/day, 5 day/week; 6 months	**X**	**X**	**X**	Protected PH and emphysema, *not* SAR	Exposure as in Barnes (1990) (review)	Wright et al., 2011
Hartley guinea pig MPO inhibitor AZ1	**KY** 2R1	5/day, 5 day/week; 6 months	**X**	**X**	**X**	Reduced emphysema, SAR, PH	Mainstream presumed; exposure as in Simani et al. (1974)	Churg et al., 2012

(Continued)

Table 1 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	Copd/emphysema	Remarks	References
Hartley guinea pig	**KY** 1R3F unfiltered	7/day, 5 day/week; 3–6 months	X	X	X	COPD		Dominguez-Fandos et al., 2012
C57Bl/6	**KY** 2R1	4/day; 24 h	X	X	X		Exposure as in Churg et al. (2003b)	Preobrazhenska et al., 2012
C57Bl/6	**Marlboro** 100	4/day 4–5 min, 5 day/week; 5 s puff, 10 min rest; 4 months	X	X	X	Emphysema	"Active smoke"	Shan et al., 2012
C57Bl/6 IL17a KO	**Marlboro** 100	4/day 4–5 min, 5 day/week; 5 s puff, 10 min rest; 4 months	X	X	X	Reduced emphysema	"Active smoke"	Shan et al., 2012
C57Bl/6 IL17a Tg	**Marlboro** 100	4/day 4–5 min, 5 day/week; 5 s puff, 10 min rest; 4 months	X	X	X	Exacerbated emphysema	"Active smoke"	Shan et al., 2012
C57Bl/6 Tcrd KO	**Marlboro** 100	4/day 4–5 min, 5 day/week; 5 s puff, 10 min rest; 4 months	X	X	X	Exacerbated emphysema	"Active smoke"	Shan et al., 2012
C57Bl/6 Spp1 KO	**Marlboro** 100	4/day 4–5 min, 5 day/week; 5 s puff, 10 min rest; 6 months	X	X	X	Reduced emphysema	"Active smoke"	Shan et al., 2012
Mouse eNOS KO	**KY** 2R1 and 2R4F	5/day, 5 day/week; 6 months	X	X	X	Pulmonary hypertension, COPD	Exposure as in Barnes (1990) (review)	Wright et al., 2012
Balb/C	**Marlboro red**	9 cig/day, 10 s CS and 50 s fresh air; 4 days	X	1 mg/cig	X			Nemmar et al., 2013

CHG, carboxyhemoglobin; PH, pulmonary hypertension; SAR, small airway remodeling. Cigarette brand names are in bold.

CHRONIC CIGARETTE SMOKE EXPOSURE

It is important to differentiate acute versus chronic smoke exposure in respect to lung structure and function (Martin and Tamaoka, 2006). Though both models induce airway narrowing to a certain extent, the inflammation seen initially diminishes during the time-course of exposure, along the lines of early repair and late-stage failure to repair (Churg and Wright, 2009; Kratzer et al., 2013). Mechanisms of repair seem to be sufficient for the first month of cigarette smoke exposure, but since lesions change over the exposure time, it is predicted that the pathological mechanisms differ in acute and chronic cases. Acute models can be as short as 24 h or last up to as many as 2 weeks, while chronic models should be standardized to 6 months (24 weeks) of exposure to induce the anatomical changes characterizing COPD (Churg and Wright, 2009). Both acute and chronic cigarette smoke exposure lead to oxidant stress, which pathologically affects airway cells to promote remodeling (Martin and Tamaoka, 2006).

Since COPD can only manifest the pathological characteristics seen in humans after long-term cigarette smoke exposure, animals are often utilized in chronic studies to relate to the human disease. Emphysema, airway remodeling and pulmonary hypertension, which progress over time, can only be induced in chronic models where structural alterations occur. Irreversible matrix destruction, fibrosis, airway wall thickening, and hyperplasia of smooth muscle and goblet cells as well as fibroblasts can only be seen in chronic cigarette exposure. Since COPD in smoking patients is typically GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage III or IV and less commonly I and II (Retamales et al., 2001), animal models must attempt to mimic this by developing emphysema, small airway remodeling (SAR), or pulmonary hypertension. The excess mucus production that defines chronic bronchitis is also thought to be important in the pathogenesis of acute exacerbations of COPD (Burge and Wedzicha, 2003). This was previously considered difficult to reproduce in animal models due to the fact that, in contrast to humans, the anatomical localization of bronchial glands in rats and mice are concentrated in the proximal trachea (Churg et al., 2008). Recently, the mucus secretion was demonstrated in two rat models (Nie et al., 2012). Authors showed significantly increased mucus production in epithelium of trachea, bronchi, and bronchiole in a 6 week cigarette smoke model and in a chronic bronchitis model with 6 weeks cigarette smoke exposure combined with a single intratracheal injection of LPS on day 39. There was also a significant increase in MUC5A protein levels in broncheoalveolar lavage in both models (Nie et al., 2012).

THE ANATOMICAL MANIFESTATIONS OF COPD

For chronic cigarette smoke exposure studies, many months are required to model the lung pathology, as acute models do not have this effect on lung structure and therefore do not allow the prediction of chronic outcomes. In order to evaluate, for example, drug efficacies, a chronic exposure most reliably modeling the human disease is essential. Only chronic models can present the lesions of COPD defined as emphysema, SAR, and pulmonary hypertension, though still more mildly than in human COPD (Wright and Churg, 2008; Churg and Wright, 2009). This

Table 2 | Second hand cigarette smoke exposure modeled in animals.

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/emphysema	Remarks	References
Wistar rat	Commercial **Virginia**	3/day in 90 min, 5 day/week; 3 months	**X**	1 g/cig	35	Emphysema	Nicotine levels correct?	Escolar et al., 1995
A/J	**KY** 1R4F	6 h/day, 5 day/week; 6 months	4.1 ± 0.4	1.011 ± 0.289	17 ± 2			Witschi et al., 1995
Guinea pig	Commercial unfiltered	20/day, 5 day/week; 1–8 weeks	**X**	**X**	16.3 ± 4.9% CHG	Emphysema	"Whole smoke"	Selman et al., 1996
A/J	**KY** 1R4F	6 h/day, 5 day/week; 5 month (harvest 9 months)	78.5 ± 12.4	13.4 ± 3.3	211 ± 24		"Whole smoke"; exposure as in Witschi et al. (1995)	Witschi et al., 1997
A/J	**KY** 1R4F	6 h/day, 5 day/week; 5 month (harvest 9 months)	0.1 ± 0.2	3.1 ± 2	113 ± 23		"Filtered smoke"	Witschi et al., 1997
Sprague-Dawley rat hexavalent chromium i.t.	**KY** 2R1 humidified	10 cig × 2/day, 3 h rest, 23 mm butt; 18 days	120	2.45 mg/cig	**X**		"Whole body environmental"	Balansky et al., 2000
C57Bl/6, DBA/2, ICR	Commercial **Virginia**	33 ml/min, 3/day, 5 day/week; 7 months		0.9 mg/cig	**X**	ICR resistant to emphysema (anti-oxidants)	"Macrolon cages"; exposure as in Escolar et al. (1995)	Cavarra et al., 2001
Pallid mouse α_1 proteinase inhibtor deficiency	Commercial **Virginia**	33 ml/min, 3/day, 5 day/week; 4 months		0.9 mg/cig	**X**	More severe emphysema	"Macrolon cages"; exposure as in Escolar et al. (1995)	Cavarra et al., 2001
NZ White rabbit	**Ultratech Corp** research cigarettes	6 h/day, 48/day, 5 day/week; 10 weeks	24.08 ± 3.79	339 ± 74.6 nmol/L plasma	44.91 ± 1.81			Sun et al., 2001
SH rat catalytic anti-oxidant i.t. (AEOL 10150)	**KY** 1R4F humidified	35 ml 2 s puff/min, 6 h/day, 3 day/week; 2 day-8 weeks	68.6 ± 11.7	5.7 ± 2.0	275 ± 39	Reduced lung injury		Smith et al., 2002
Balb/C a-tocopherol	Commercial filtered	5/day over 60 min; 10 weeks	**X**	**X**	**X**			Koul et al., 2003
ICR Nrf2 KO	**KY** 2R4F	35 m^3 2 s puff × 8, 1 min rest, 7 h/day, 7 day/week; 6 months	90	2.45 mg/cig	350	More severe emphysema	Teague chamber; exposure as in Witschi et al. (1997)	Rangasamy et al., 2004
C57Bl/6J	Commercial **Virginia** filtered	3/day, 5 day/week; 1–10 months	**X**	0.9 mg/cig	**X**	Disseminated emphysema	"Macrolon cages"; exposure as in Cavarra et al. (2001)	Bartalesi et al., 2005
DBA/2	Commercial **Virginia** filtered	3/day, 5 day/week; 1–10 months	**X**	0.9 mg/cig	**X**	Uniform emphysema	"Macrolon cages"; exposure as in Cavarra et al. (2001)	Bartalesi et al., 2005
C57Bl/CBA and A/J	**X**	6 h/day, 5 day/week; C57Bl/CBA 12 month; A/J 6 months	250	**X**	10–12% CHG	Emphysema (A/J w/o SAR)	Teague chamber	Foronjy et al., 2005
B6C3F and A/J	**KY** 1R3, unfiltered	6 h/day, 5 day/week; 15 weeks	1. week 100–125, then 250	**X**	**X**	A/J 51% greater mean linear intercept, B6C3F 38%		March et al., 2005
B6C3F all trans-retinoic acid inhalation	**KY** 1R3, unfiltered	6 h/day, 5 day/week; 32 weeks	1. week 100–125, then 250	**X**	**X**	No emphysema reversal		March et al., 2005
A/J all trans-retinoic acid i.p. and inhalation	**KY** 1R3, unfiltered	6 h/day, 5 day/week; 15 weeks	1. week 100–125, then 250	**X**	**X**	No emphysema reversal		March et al., 2005
C57Bl/6 p.o. Rofluminlast (PDE4 inhibitor)	Commercial **Virginia** filtered	5 in 20 min or 3/day, 5 day/week; 4 h-7 months	**X**	0.9 mg/cig	**X**	100% protected	"Macrolon cages"; side stream presumed; exposure as in Cavarra et al. (2001)	Martorana et al., 2005

(Continued)

Table 2 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/emphysema	Remarks	References
SH rat sEH inhibitor s.c.	**KY** 2R4F humidified	35 ml 2 s puff/min, 6 h/day; 3 days	76.4 ± 16.0	6.8 ± 0.2	234 ± 2	Attenuated inflammation		Smith et al., 2005
Sprague-Dawley rat CXCR2 antagonist SB-332235	**KY** 2R4F filtered	2–5 cig/day, 50 ml/30 s over 32 min; 24 h 3–4 days (unclear)	**X**	**X**	**X**	Reduced COPD (time and dose dependent)	No mention of number of cigarettes; exposure period unclear	Stevenson et al., 2005
C57Bl/6 CCR6 KO	**KY** 2R4F unfiltered	5 cig × 4/day, 30 min rest, 5 day/week; 4 week-6 months	**X**	**X**	8.3 ± 1.4 CHG	67% protected (none against SAR)	Exposure as in D'Hulst et al. (2005b)	Bracke et al., 2006
C57Bl/6 TNFR KO	**KY** 2R4F unfiltered	5 cig × 4/day, 30 min rest, 5 day/week; 3–6 months	**X**	**X**	8.3 ± 1.4 CHG	100% proteced	Side stream presumed; exposure as in D'Hulst et al. (2005b)	D'Hulst et al., 2006
C57Bl/CBA SOD Tg	**KY** 2R4F	2 × 70 ml puffs/min, 6 h/day, 5 day/week; 12 months	250	**X**	10% CHG	Prevents emphysema	Teague chamber	Foronjy et al., 2006
A/J	**KY** 1R3 unfiltered	10–22 week (+39 weeks harvest)	1. week 100, then 250	**X**	**X**	Emphysema (concentration and duration dependent)	"Whole body mainstream"	March et al., 2006
A/J female	**KY** 1R3 unfiltered	10–22 weeks	1. week 100, then 250	**X**	**X**	Emphysema less severe (concentration and duration dependent)	"Whole body mainstream"	March et al., 2006
A/J female p.o. EGCG anti-oxidant	**KY** 1R3 unfiltered	16 weeks	1. week 100, then 250	**X**	**X**	Emphysema (concentration and duration dependent)	"Whole body mainstream"	March et al., 2006
A/J female NAC anti-oxidant p.o.	**KY** 2R4F filtered	10 weeks	1. week 100, then 250	**X**	**X**	Emphysema (concentration and duration dependent)	"Whole body mainstream"	March et al., 2006
A/J female	**KY** 2R4F filtered	10 weeks	1. week 100, then 250	**X**	**X**	Emphysema (concentration and duration dependent)	"Whole body mainstream"	March et al., 2006
C57Bl/6 CCR5 KO	**KY** 2R4F unfiltered	5 cig × 4/day, 30 min rest, 5 day/week; 4 week-6 months	**X**	**X**	8.3 ± 1.4 CHG	25% protected (none against SAR)	Exposure as in D'Hulst et al. (2005b)	Bracke et al., 2007
C57Bl/6, A/J, AKR, CD-1 (ICR), 129Sv	**KY** 2R4F	35 ml 2 s puff/min; 3 days (2 and 24 h harvest)	80: 6 h/day or 300: 2 × 1 h/day	0.85 mg/cig	297 (300 TPM); 79.4 (80 TPM)	C57Bl/6 highly susceptible to inflammatory and oxidative response; A/J, AKR, CD-1 (ICR) less susceptible; 129Sv resistant		Yao et al., 2008
C57Bl/6, Balb/C, A/J, 129Sv	**KY** 1R3F	40 ml puffs/min; 2, 3, 4, or 5 cig/day 1 h/day; 3 days	481	**X**	**X**	Neutrophilia dose- and time-dependent	Exposure as in Stevenson et al. (2005)	Morris et al., 2008
C57Bl/6 and Balb/C PKF242-484 MMP inhibitor p.o. and i.n.	**KY** 1R3F	40 ml puffs/min; 2, 3, 4, or 5 cig/day 1 h/day; 3 days	481	**X**	**X**	Strain-dependent inhibition of neutrophil inflammation	Exposure as in Stevenson et al. (2005)	Morris et al., 2008
Hartley guinea pig	**KY** 2R4F	2 × 70 ml puff/min, 4 h/day, 5 day/week; 1–12 weeks	250	**X**	**X**	Inflammation (4 week), emphysema (12 week)	Teague chamber	Golovatch et al., 2009
Balb/C	**KY** 1R4F	6 h/day, 5 day/week; 12 weeks	30 ± 1	4.8 ± 0.5	**X**	CS augments inflammatory cell recruitment in COPD		Rao et al., 2009

(Continued)

Table 2 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/emphysema	Remarks	References
C57Bl/6J 10^6 apoptotic thymocytes i.t.	**KY** 3R4F	5 h; 1 day (harvest and i.t. d0–5)	25 or 100	**X**	**X**	Reversible and cell type independent impaired efferocytosis in COPD	Teague chamber	Richens et al., 2009
C57Bl/6J 10^6 apoptotic thymocytes i.t.	**KY** 3R4F	5 h/day; 5 day (harvest +1 and 4 weeks)	100	**X**	**X**	Reversible impaired efferocytosis in COPD	Teague chamber	Richens et al., 2009
FVB/N apoptotic neutrophils	**KY** 3R4F	5 h/day, 5 day/week; 22 week (harvest + 20 weeks)	1. week 100, then 250	**X**	**X**	Non-reversible impaired efferocytosis and termnal bronchiolitis in COPD	Teague chamber	Richens et al., 2009
ICR oxidant resistant	**KY** 3R4F	5 h; 1 day (harvest d0–2)	100	**X**	**X**	No COPD	Teague chamber	Richens et al., 2009
C57Bl/6 i.p. MnTBAP	**KY** 3R4F	5 h; 1 day (harvest d0–2)	100	**X**	**X**	Anti-oxidant treatment clears apoptotic cells and inhibits RhoA	Teague chamber	Richens et al., 2009
ecSOD Tg	**KY** 3R4F	5 h; 1 day (harvest d0–2)	100	**X**	**X**	Anti-oxidant treatment clears apoptotic cells and inhibits RhoA	Teague chamber	Richens et al., 2009
C57Bl/129 TNFR KO	**KY** 3R4F	5 h; 1 day (harvest d0–2)	100	**X**	**X**	CS inhibition of efferocytosis is TNF-α dependent	Teague chamber	Richens et al., 2009
C57Bl/6J	**KY** 2R4F humidified	35 ml 2 s puff/min, 8 puff/cig, 6 h/day; 3 months	Med 69; high 131	2.5–6.8 mg/cig	Med 238; high 394	Inflammatory COPD	Teague chamber	Woodruff et al., 2009
A/J	**KY** 2R4F	1. week 125 4 h/day, 5 day/week; 20 week (harvest 20 or 28 weeks)	750	40 μg/l	800	Smoking cessation stops emphysema progression and reduces inflammation	Performed with maintream and side stream	Braber et al., 2010
Balb/C and C57Bl/6	**KY** 2R4F filtered	12 cig × 2/day 1. day 20 min 2. day 30 min, then 50 min, 5 day/week; 4 day-24 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	Inflammation adaptive (T regulatory lymphocytes)		Botelho et al., 2010
C57Bl/6 $P2Y_2R$ KO	Commercial **Virginia** filtered	3/day, 5 day/week; 3 day-7 months	**X**	0.9 mg/cig	**X**	Protected	"Whole smoke"; side stream presumed; exposure as in Cavarra et al. (2001)	Cicko et al., 2010
Sprague-Dawley rat	**KY** 2R4F	4/day, 5 day/week; 3 day-6 months	27.1 ± 0.8/cig	2.66 ± 0.12 μM cotinin	42 ± 4 μM CHG	Emphysema w/o apoptosis		Marwick et al., 2010
Balb/C Rag KO with CS CD3+ T lymphocyte	**KY** 3R4F	4 h/day, 5 day/week; 6 month (+13 week post T cell transfer)	150 ± 15	**X**	400 ± 30	Emphysema	Teague chamber	Motz et al., 2010a
C57Bl/6J	**KY** 3R4F	4 h/day, 5 day/week; 2–24 weeks	150 ± 15	**X**	**X**	NK cells activate innate immune system in COPD	Teague chamber	Motz et al., 2010b
Sprague-Dawley rat celecoxib i.g.	Commercial **Eighty Eight Lights**	10/day, 2 h/day, 5 day/week; 20 weeks	**X**	**X**	**X**	Reduced emphysema		Roh et al., 2010
C57Bl/6J SOD3 Tg	**KY** 3R4F	5 h/day, 5 day/week; 2–6 months	100	**X**	**X**	Protected	Teague chamber	Yao et al., 2010

(Continued)

Table 2 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/emphysema	Remarks	References
C57Bl/129 Rtp801 KO	**KY** 2R4F unfiltered	4/day, 5 h/day; 6 months	**X**	2.45 mg/cig	**X**	Protected	Teague chamber; exposure as in Rangasamy et al. (2004)	Yoshida et al., 2010
A/J	**KY** 2R4F, whole body	4 h/day, 5 day/week; 20 week (harvest 20 or 28 weeks)	2. week 125, then 750	40 μg/l	800	Emphysema	"Whole body mainstream"	Braber et al., 2011
C57Bl/6 IL-1R1 KO	**KY** 2R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	IL-1R1/IL-1α dependent inflammation in COPD	Exposure as in Botelho et al. (2010)	Botelho et al., 2011a
C57Bl/6 IL-1α KO; Balb/C IL-1α Ab i.p.	**KY** 2R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	IL-1R1/IL-1α dependent inflammation in COPD	Exposure as in Botelho et al. (2010)	Botelho et al., 2011a
C57Bl/6 IL-1β KO; Balb/C IL-1β Ab i.p.	**KY** 2R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	IL-1β independent inflammation in COPD	Exposure as in Botelho et al. (2010)	Botelho et al., 2011a
Balb/C GM-CSF and GM-CSFR Ab i.p.	**KY** 2R4F unfiltered	12 cig × 2/day 1. day 20 min 2. day 30 min then 50 min; 4 days	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	Reduced inflammatory response	Exposure as in Botelho et al. (2010)	Botelho et al., 2011b
DBA/2J hyaluronan	**KY** 2R4F filtered	35 ml 2 s puff/min, 3 h/day, 5 day/week; 2–10 months	**X**	**X**	**X**	Reduced emphysema	Teague chamber	Cantor et al., 2011
DBA/2 caspase-3 inhibition	**KY**	33 ml/min, 3/day, 5 day/week; 6 months	90	**X**	350	Reduced emphysema	Teague chamber; exposure as in Cavarra et al. (2001)	Clauss et al., 2011
SH rat sEH inhibitor s.c. or p.o.	**KY** 3R4F	6 h/day; 3 days	80–90	**X**	**X**	sEH anti-inflammatory effect independent of leukocyte recruitment	Teague chamber	Davis et al., 2011
C57Bl/6 P2X7 receptor KO	**KY** 3R4F unfiltered	250, 500, 750 ml/min, 50 min × 2/day; 3 days	**X**	**X**	**X**	Inflammation through P2X7 pathway	Teague chamber	Eltom et al., 2011
C57Bl/129 Smad3 KO	**KY** 2R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	Accelerated emphysema	Exposure as in Botelho et al. (2010)	Farkas et al., 2011
C57Bl/6 and DR4 Tg M. tuberculosis or influenza A i.n.	**KY** 1R4F	2 × 120 min/day (2 h rest) 5 day/week; 6 weeks	80	**X**	**X**		Teague chamber	Feng et al., 2011
C57Bl/CBA	**KY** 2R4F	6 h/day, 5 day/week; 4 week-12 months	**X**	**X**	**X**		Teague chamber; exposure as in Foronjy et al. (2005, 2006), Golovatch et al. (2009)	Geraghty et al., 2011
Hartley Guinea pig	**KY** 2R4F	6 h/day, 5 day/week; 12 weeks	**X**	**X**	**X**		Teague chamber; exposure as in Foronjy et al. (2005, 2006); Golovatch et al. (2009)	Geraghty et al., 2011
C57Bl/6 and DBA/2J adipose stem cell treatment	**KY** 3R4F	4, 6 months	90	**X**	350	Reduced emphysema	Teague chamber	Schweitzer et al., 2011
Balb/C AZD9668 NE inhibitor p.o.	**KY** 1R3F	12 cig 2 × 50 min/day; 4 days	**X**	**X**	**X**	Reduced emphysema and SAR		Stevens et al., 2011

(Continued)

Table 2 | Continued

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/emphysema	Remarks	References
C57Bl/6 IL-1R1 and IL-1α KO	**KY** 3R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90	377–503.2 ng/ml cotinin	10–15% CHG	DC accumulation and activation is IL-1R1/IL-1α dependent	Exposure as in Botelho et al. (2010)	Botelho et al., 2012
C57Bl/6 TLR4 KO	**KY** 3R4F unfiltered	12 cig × 2/day 50 min, 5 day/week; 4 day-8 weeks	622 ± 90 μg/l TPM	377–503.2 ng/ml cotinin	10–15% CHG	DC accumulation and activation is TLR4-independent	Exposure as in Botelho et al. (2010)	Botelho et al., 2012
Balb/C IL-1α Ab i.p.	**KY** 3R4F unfiltered	12 cig × 2/day 50 min; 4 days	622 ± 90 μg/l TPM	377–503.2 ng/ml cotinin	10–15% CHG	DC accumulation and activation is IL-1α dependent	Exposure as in Botelho et al. (2010)	Botelho et al., 2012
Balb/C IL-1β Ab i.p.	**KY** 3R4F unfiltered	12 cig × 2/day 50 min; 4 days	622 ± 90 μg/l TPM	377–503.2 ng/ml cotinin	10–15% CHG	DC accumulation and activation is IL-1β-independent	Exposure as in Botelho et al. (2010)	Botelho et al., 2012
SH rat	**KY** 3R4F	35 ml 2 s puff, 6 h/day, 3 day/week; 3 day-12 weeks	80–90	X	X	Leukocytes from bronchial circulation in COPD	Nicotine and CO levels measured daily, but not listed; Teague chamber	Davis et al., 2012
Sprague-Dawley rat	**Ye Shu** unfiltered	1. day 3/h each; 2. day: 7/h each; 3–5. day: 12/3 min; 6. day-end: 12/h; 4–6 weeks	70–110	1.2 mg/cig	310–380	COPD	Chamber uniquely described; side stream presumed	Nie et al., 2012
SH rat sEH inhibitor and Rolipram p.o.	**KY** 3R4F	35 ml 2 s puff/min, 6 h/day, 3 day/week; 4 weeks	80–90	X	X	Reduced emphysema	Teague chamber	Wang et al., 2012
Mouse Apo-E KO C. pneumoniae	**KY** 3R4F	35 ml 2 s puff/min, 6 h/day, 5 day/week; 8 weeks	X	X	X	Enhanced artherosclerosis		Zhao et al., 2012
Balb/C and C57Bl/6	**KY** 3R4F	4 h/day, 5 day/week; 6 months	150 ± 15	X	400 ± 30	Emphysema CD4+ and CD8+ T lymphocyte dependent (Ag-specific response)	Teague chamber	Eppert et al., 2013
Sprague-Dawley rat	**KY** 1R3F	5 cig/9 min 6 h/day; 2–4 months	100–120 $\mu g/m^3$	X	X	Emphysema	Teague chamber	Kratzer et al., 2013
C57Bl/6 IKK-2 KO	**KY** 3R4F unfiltered	500 ml/min, 50 min; 3–14 days	X	X	X	Unaltered inflammation	Teague chamber;exposure as in Eltom et al. (2011)	Rastrick et al., 2013

CHG, carboxyhemoglobin; SAR, small airway remodeling. Cigarette brand names are in bold.

is especially important when noting that, because rodents develop a mild form of COPD, drug tests should be performed as late as possible to mimic a treatment versus a prophylaxis (Churg et al., 2011).

EMPHYSEMA

The pathogenesis of emphysema is studied by inducing the disease with various methods not necessarily restricted to cigarette smoke. The protease/anti-protease hypothesis has been the main focus in elucidating the cause of emphysema. In addition, oxidative stress has been shown to cause lung cell apoptosis in an emphysema model using a vascular endothelial growth factor (VEGF) receptor blocker (Kasahara et al., 2000; Tuder et al., 2003). Cigarette smoke induces both oxidative stress and the infiltration of inflammatory cells, which release proteases that overwhelm the anti-proteolytic defenses. Inflammatory cell and consequently endothelial cell proteases lead to the breakdown of pulmonary matrix and alveolar walls following particle inhalation, creating airspace enlargement and minimizing surface area for gas exchange (March et al., 2000). This protease theory is based on the 1963 finding that patients deficient in α-1 antitrypsin, a major neutrophil elastase inhibitor, have accelerated emphysema development (Laurell and Eriksson, 1963). The capability of proteases to cause emphysema was then verified by instilling them intratracheally (Gross et al., 1965; Janoff et al., 1977; Snider et al., 1984). Protease inhibition has since been tested in the animal model with varying efficacies, depending on the protease depleted. Serine proteases from neutrophils (59% protection in neutrophil elastase deficient mice) (Shapiro et al., 2003) as well as macrophage metalloproteases (Hautamaki et al., 1997) have been considered most important in a cigarette smoke induced disease model, but the more broad application of anti-inflammatory

Table 3 | Animal studies where the exposure (mainstream or second hand smoke) is not known.

Species/strain	Cigarette type	Dose and exposure	TSP/TPM (mg/m^3)	Nicotine (mg/m^3)	CO (ppm)	COPD/Emphysema	Remarks	References
C57Bl/6J A1AT low pallid mouse	**KY** 2R1 unfiltered	2–6 months	**X**	**X**	10–12% CHG	Emphysema	No exposure method	Takubo et al., 2002
C57Bl/6, NZW, A/J, SJ/L, AKR/J	**KY** 2R1 unfiltered	2/day, 5 day/week; 6 months	**X**	**X**	10–12% CHG	Strain-dependent susceptibility to emphysema	No exposure method	Guerassimov et al., 2004
C57Bl/6 and Balb/C INF-g Tg	**X**	6 months	**X**	**X**	**X**	Enhanced emphysema	No exposure method	Ma et al., 2005
C57Bl/6 CCR5 KO	**X**	6 months	**X**	**X**	**X**	100% protected	No exposure method	Ma et al., 2005
C57Bl/6	**KY** 2R1	4/day once or 3/day, 5 day/week; 2 h-6 months	**X**	**X**	**X**	SAR	No exposure method	Churg et al., 2006
C57Bl/6 A1AT i.p.	**KY** 2R1	4 in 2 h	**X**	**X**	**X**	No emphysema	No exposure method	Churg et al., 2007b
C57Bl/6	**KY** 2R1	20 ml × 2 cig/day 15 min, 6 day/week; 6 months	**X**	**X**	**X**	Emphysema	No exposure method	Adair-Kirk et al., 2008

CHG, carboxyhemoglobin; SAR, small airway remodeling. Cigarette brand names are in bold.

Table 4 | Cigarette types used for the summarized experiments.

Cigarette brand	Type	Year	TSP/TPM (mg/cig)	Nicotine (mg/cig)	CO (mg/cig)	References
Kentucky	1R3	1974	27.1 and 23.6	1.46 and 1.23	17.1 and 14.7	Davis et al., 1983
Kentucky	1R3F	1974	18.1	1.16	17.2	Davis et al., 1983
Kentucky	1R4F	1983	10.3*	0.8*	11.6*	Davis et al., 1983
Kentucky	2R1	1974	14.6 and 38.8	2.45 and 2.19	25.1 and 22.2	Davis et al., 1983
Kentucky	2R1F	1974	28.6	1.74	22	Davis et al., 1983
Kentucky	2R4F	2001	11.7	0.9	13	Roemer et al., 2012
Kentucky	3R4F	2008	11	0.7	12	Roemer et al., 2012
Virginia	F			0.9	35 (Escolar et al., 1995)	Escolar et al., 1995; Cavarra et al., 2001; Bartalesi et al., 2005; Martorana et al., 2005; Cicko et al., 2010
Seven Star	UF			1.88%		Gomita et al., 1990
Long Peace	F			1.9–2		Suemaru et al., 1992; Tanaka et al., 2004
Ultratech Corp	n.a.		24.08 ± 3.79	339 ± 74.6 nmol/L	44.91 ± 1.81	Sun et al., 2001
Ye Shu	UF		70–110	1.2	310–380	Nie et al., 2012
Marlboro	100F		971 ± 98.3 in 5%	1	12	Suzuki et al., 2009; Shan et al., 2012
Eighty Eight Lights	n.a.			0.72	9	Lee et al., 2005; Roh et al., 2010
Arda-Bulgartabac	F		533	1.6 mg/cig		Balansky et al., 2000
Canada Tobacco	UF			1.1	11	Wright and Churg, 1990

*F, filtered (KY 30 mm butt); UF, unfiltered; KY values, 23 and 30 mm butt; *, 35 mm butt.*

interventions has proven useful in smoked rodents as well (Churg et al., 2008). This is especially of interest, as sampling of human COPD specimens noted T lymphocytes in alveolar destruction and airflow obstruction, though smoking history was not assessed in the patient population (Hogg et al., 2004). This has shed light on the participation of auto-immunity contributing to the overall pathogenesis of emphysema through anti-endothelial cell antibodies and pathogenic CD4+ T lymphocytes (Taraseviciene-Stewart et al., 2005). Because emphysema was initially believed to occur through the same mechanism as SAR and to be the underlying cause of pulmonary hypertension, both in COPD as well as following cigarette smoke exposure, many studies have focused only on the emphysematous pathology, especially on the proteolytic aspect, neglecting further complex contributing factors.

SMALL AIRWAY REMODELING (SAR)

Emphysema is not the only manifestation of cigarette smoke induced COPD. SAR is also a major contributor to the disease as it limits airflow (Hogg et al., 2004) and is characterized by continuous repair for the duration of smoke exposure (Churg and Wright, 2009). While emphysema is the breakdown of extracellular matrix in the lung parenchyma, SAR pathogenesis involves a fibrotic process leading to airway

narrowing due to airway wall thickening. Therefore, it is not surprising that SAR appears to develop through a mechanism independent of the one that drives emphysema. This correlates well with the fact that methods for emphysema prevention cannot be uniformly applied to treat SAR. Only TNF-α and IL-1 receptor knockouts (Churg et al., 2009b) as well as mice treated with an MMP-9/MMP-12 inhibitor (Churg et al., 2007a) were protected against the development of both emphysema and SAR when exposed to cigarette smoke, while other interventions such as chemokine receptor 5 (CCR5) (Bracke et al., 2007) and CCR6 (Bracke et al., 2006) knockouts are merely protected from emphysema following cigarette smoke.

PULMONARY HYPERTENSION

COPD morbidity and mortality are significantly increased by the not uncommon occurrence of pulmonary hypertension associated with cigarette smoke (Chaouat et al., 2008; Elwing and Panos, 2008), which correlates with a poor prognosis (Weitzenblum et al., 1981). Originally, it was believed that the increase in pulmonary artery pressure is secondary to the lung pathology associated with COPD, as a result of hypoxia, emphysema, and loss of the vascular bed (Wright et al., 2005). With the realization that smoke and its mediators effect vasculature to induce remodeling, and that pulmonary hypertension is an early symptom that manifests long before airflow obstruction, emphysema, and SAR, the independent mechanism became evident (Wright et al., 2005). It has recently been shown that inducible nitric oxide synthase (iNOS) is important in the development of pulmonary hypertension following cigarette smoke exposure (Gielis et al., 2011; Kratzer et al., 2013). Mice lacking iNOS or treated with an iNOS inhibitor are protected against the cigarette smoke induced development of emphysema and pulmonary hypertension (Nathan, 2011; Seimetz et al., 2011). Both prostacyclin and endothelial NOS are protective against pulmonary hypertension induced by hypoxia, while endothelin-1 and VEGF contribute to the pathogenesis (Wright et al., 2004, 2005; Voelkel, 2008; Wright and Churg, 2008). The oxidative damage to the vasculature results from reactive nitrogen species that are not of endothelial source, but more likely contained in the cigarette smoke itself (Wright et al., 2012). The result is altered vasoconstriction and vasodilation as well as vascular cell proliferation leading to the pathological thickening of vessels. In the intima, elastin and collagen are deposited, while smooth muscle cells proliferate (muscularization), and adventitia harbors increased numbers of CD8+ T lymphocytes (Wright et al., 1983, 1992; Barbera et al., 2003).

Smoking is not only the main risk factor for COPD and pulmonary hypertension, but also for coronary artery disease summarized in a recent review (Ghoorah et al., 2012).

THE PROTEOLYTIC AND INFLAMMATORY RESPONSE IN COPD

COPD, like asthma, is characterized by chronic and abnormal inflammation of the distal airways resulting in airflow limitation, but differs from asthma in that it is progressive and largely irreversible. This chronic disease encompasses bronchiolitis and fibrosis with obstruction of small airways, vascular remodeling leading to pulmonary hypertension, destruction of lung parenchyma with loss of the alveolar wall and subsequent airspace enlargement defined as emphysema, as well as loss of lung elasticity. The underlying mechanisms involve protease/anti-protease imbalance following inflammation, with subsequent proteolytic matrix destruction (Churg et al., 2008), oxidant damage that leads to apoptosis of resident lung structure cells (Yoshida and Tuder, 2007; Churg et al., 2011). accelerated aging (Csiszar et al., 2009), a failure to repair, and the contribution of autoimmunity (Taraseviciene-Stewart et al., 2006; Maeno et al., 2007; Stampfli and Anderson, 2009). The most prominent and frequent etiology of COPD remains cigarette smoke and its onset is mid-life. For COPD, the inflammatory response is slowly progressive and leads to the actual destruction of lung parenchyma where alveolar walls disappear and the distal airspaces become pathologically and permanently enlarged, resulting in emphysema (Barnes, 2004). The contributing inflammatory cells in COPD are neutrophilic granulocytes, alveolar macrophages, and CD8+ T lymphocytes (Taraseviciene-Stewart and Voelkel, 2008; Stampfli and Anderson, 2009). The accumulation of both T and B lymphocytes following apoptosis of resident lung cells has been described to create follicles within the lung parenchyma, adjacent to airways, in COPD (Hogg et al., 2004; Taraseviciene-Stewart et al., 2006). In a rat model, neutrophil depletion did not prevent smoke induced emphysema, while treatment with anti-monocyte/macrophage antibody did. These findings implicate macrophages rather than neutrophils as the critical pathogenic factor in cigarette smoke induced emphysema (Ofulue and Ko, 1999). Macrophages are known to secrete the cytokines interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) as well as leukotriene B4 (LTB4), all found to be increased in COPD patients (Keatings et al., 1996). Additionally, macrophages also generate reactive oxygen species (ROS), monocyte chemotactic protein 1 (MCP-1) and elastolytic enzymes such as matrix metalloproteinase (MMP)-2, MMP-9, MMP-12, and cathepsins K, L, and S thus contributing to the lung destruction (Barnes et al., 2003).

Neutrophils are found to be increased in bronchial biopsies and sputum of patients during COPD, correlating with severity (Keatings et al., 1996; Di Stefano et al., 1998; Pesci et al., 1998; Retamales et al., 2001). Neutrophils apparently contribute to the disease pathogenesis by secreting serine proteases (neutrophil elastase, cathepsin G, proteinase) and metalloelastases MMP-8 and MMP-9 (Barnes et al., 2003).

The major pathogenetic factor of neutrophil and macrophage accumulation in emphysema is their secretion of proteases and inflammatory mediators. Cleaved fragments of elastin, collagen, and fibronectin are believed to possibly act as auto-antibodies, which would explain the accumulation of T and B lymphocytes in COPD patients (Hogg et al., 2004; Taraseviciene-Stewart and Voelkel, 2008), It has been shown that antibodies against endothelial cells correlate with alveolar septal cell apoptosis as well as the activation of MMP-2 and MMP-9, thereby initiating the proteolytic cascade and inducing emphysema independent of cigarette smoke (Taraseviciene-Stewart et al., 2005). This specific mechanism of anti-endothelial cell auto-immunity is dependent on CD4+ T lymphocytes believed to be pathogenic

(Voelkel and Taraseviciene-Stewart, 2005; Taraseviciene-Stewart et al., 2007). Anti-endothelial cell antibodies were also detected in a rat model of cigarette induced emphysema (Taraseviciene-Stewart, unpublished observation).

GENETIC PREDISPOSITIONS FOR COPD DEVELOPMENT

Interestingly, only 15–20% of smokers are susceptible to developing COPD, underlining the contribution of genetic factors (Fletcher and Peto, 1977). This susceptibility is mirrored in the animal model, where the development of cigarette smoke induced emphysema is strain-dependent (Guerassimov et al., 2004; Bartalesi et al., 2005). To this date, though, the only human genetic predisposition identified is the rare hereditary deficiency in α-1 antitrypsin (Laurell and Eriksson, 1963). Transgenic mice over-expressing genes of interest or knockout mice lacking a specific gene are exposed to mainstream or SHS elucidating how a specific gene modifies the pathogenesis of this disease. Though mice are so small that the assessment of disease progression and COPD symptoms is hindered, the application of transgenic and knockout mice to define the effects of a gene insertion or expression increase as well as that of a gene deletion, can answer many questions. Additionally, 80% of the mouse genome contains genes that have direct orthologues in the human genome and less than 300 genes (1%) are unique to the murine species (Waterston et al., 2002; Pennacchio, 2003). Interestingly, 89–90% of rat genes contain single orthologues in the human genome and 76% of well-characterized human disease genes are found in the rat genome (Gibbs et al., 2004). Both rats (Pauwels et al., 1985; Martin et al., 1992) and mice (Guerassimov et al., 2004; Bartalesi et al., 2005) have strain-dependent susceptibilities to excessive airway inflammation and smoke-induced emphysema, respectively, just as humans have genetic susceptibility to developing COPD.

PREVENTATIVE THERAPIES

The most prominent interventions studied for COPD are anti-proteolytic, anti-inflammatory, as well as anti-oxidant and are directed against the development of emphysema, often not even being tested for SAR or pulmonary hypertension efficacies (Churg et al., 2011). Proteases are released by both accumulating inflammatory as well as resident cells, causing matrix destruction in the alveolar wall and therefore emphysema. This hypothesis is summarized in the term protease/anti-protease imbalance, because both an excessive protease release as well as a hindered anti-protease activity is necessary to contribute to the pathogenesis. The inhibition of various proteases during cigarette smoke exposure has been tested: serine proteases (neutrophil elastase) (Cavarra et al., 2001; Takubo et al., 2002; Wright et al., 2002; Churg et al., 2003b; Shapiro et al., 2003; Pemberton et al., 2006), different metalloproteases (MMP-9 and MMP-12) (Hautamaki et al., 1997; Selman et al., 2003; Mahadeva and Shapiro, 2005; Pemberton et al., 2005; Churg et al., 2007a), and cysteine proteases (cathepsins B and S) (Kang et al., 2007). The preventative success depends on the specific protease inhibited.

The immune reaction to chronic smoke exposure has been investigated by blocking certain inflammatory responses using knockout mice or simply applying an anti-inflammatory treatment. Both anti-TNF-α receptor (Churg et al., 2004; D'Hulst et al., 2006) and anti-PDE4 (Rofluminlast) (Martorana et al., 2005) therapies are protective anti-inflammatory measures in the animal model, though these results could not be as successfully reproduced for the chronic human disease (Barnes, 2007). Significant protection against SHS induced emphysema in the animal model was also achieved using a hyaluronan aerosol (Cantor et al., 2005) and by blocking interferon γ (INF-γ) or CCR5 (Ma et al., 2005; Bracke et al., 2007) or CCR6 (Bracke et al., 2006), After it was shown that SCID (severe combined immunodeficient) and Rag (recombinase activating gene) knockout mice, which both lack functional T and B lymphocytes, are not protected from mainstream smoke induced emphysema (D'Hulst et al., 2005a), CD4+ and CD8+ T lymphocyte-deficient mice were tested individually. While CD4+ T lymphocyte-defecient mice are not at all protected, CD8+ T lymphocyte-deficient mice are 100% protected against emphysema following mainstream exposure (Maeno et al., 2007). Toll-like receptor 4 (TLR4) knockout mice were also not protected (Maes et al., 2006). Statins, however, have proven to be protective against cigarette smoke induced emphysema in rats (Lee et al., 2005) and appear to be promising therapy in humans as well (Soyseth et al., 2007). Simvastatin additionally is effective against pulmonary hypertension, but unfortunately does not prevent SAR (Wright et al., 2011), unless administered before disease onset (Lee et al., 2005; Ou et al., 2009).

ROS are both contained in cigarette smoke and released by the resultant infiltrating cells, such as neutrophils and macrophages (Pryor and Stone, 1993; Rahman and MacNee, 1996; MacNee, 2001). This, again, leads to an imbalance, that of oxidants and anti-oxidants, so that the lung is exposed to oxidative stress (Petrache et al., 2006). Susceptibility to oxidative stress is, like the genetic predisposition for cigarette smoke induced emphysema, specific to a certain strain of mice (Cavarra et al., 2001; Bartalesi et al., 2005). Oxidant sensitive mice show less anti-oxidant capacity when exposed to cigarette smoke, developing emphysema. This effect can be reduced when applying an oral anti-oxidant (Koul et al., 2003), while elimination of the redox sensitive transcription gene Nuclear factor 2 (Nrf2) enhanced the cigarette smoke induced oxidative stress and emphysema in the otherwise resistant ICR mouse strain (Rangasamy et al., 2004).

Concisely, emphysema induced by cigarette smoke is a multi-factorial disease and therapeutic approaches should be undertaken to repair the lung structure by preventing marix degradation by reducing proteases, inflammation, oxidative stress, cell death, and/or autoimmune-mediated destruction.

STUDY PARAMETERS THUS FAR

Here we have compiled 114 publications containing 155 studies addressing cigarette smoke exposure in the animal model. Approximately half of the studies (71 experiments) are mainstream exposure (**Table 1**). Side stream smoke exposures (**Table 2**) make up 77 experiments of the studies listed herein and a small portion (7 studies) is unclear about the exposure method utilized (**Table 3**). Of the studies listed here, a large portion (115 studies) utilizes quite a strain diversity of mice (C57Bl/6, DBA/2, Balb/C, A/J, ICR, FVB, or a strain not described), although

C57Bl/6 mice are dominant (83 experiments). C57Bl/6 mice are a common strain for knockout studies and generally a popular model for cigarette smoke induced emphysema (Takubo et al., 2002; Guerassimov et al., 2004; Bartalesi et al., 2005; Yao et al., 2008; Botelho et al., 2010) due to their deficiency in anti-elastase (Gardi et al., 1994; Cavarra et al., 2001). Balb/C mice with higher levels of the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) (Morris et al., 2008; Botelho et al., 2010, 2011b) and the oxidant sensitive strain DBA/2 (Cavarra et al., 2001; Bartalesi et al., 2005) have also been shown to have severe emphysema following cigarette smoke exposure. ICR mice are considered oxidant resistant and are of interest when attempting to induce disease (Cavarra et al., 2001; Rangasamy et al., 2004; Hodge-Bell et al., 2007; Yao et al., 2008). A/J express less severe pulmonary complications (Guerassimov et al., 2004; Yao et al., 2008; Braber et al., 2010) and have found use in drug tests, but have been used as a model for chronic cigarette smoke exposure as well (Foronjy et al., 2005; March et al., 2005, 2006). Apart from mice, one study addresses rabbits, 15 utilize guinea pigs (either Hartley strain or not named), while 24 studies gravitate toward using rats. These are almost exclusively Sprague-Dawley, while spontaneously hypertensive rats (SH) as well as Wistar Kyoto are also popular.

Originally, only studies from 1997 onwards were added to **Tables 1–3**, but with such consistency of certain researchers to repeatedly reference earlier publications, the original publishers of certain exposure procedures were included as far back as 1974. A number of authors stumble when it comes to actually stating clearly whether their exposure method was mainstream or side stream. This is most likely due to the assumption that this would be known according to the exposure system more or less detailed in the Materials and Methods section or merely mentioned in the abstract. The Materials and Methods section, though, frequently references a publication that, in turn, references the original design. Such a basic fact as mainstream or side stream exposure must be pointed out, as must the fact that there was a cigarette smoke exposure at all, also something that can be quite hidden in a less specific manuscript. Fortunately, many publications do announce a head/nose only or a whole body exposure in the Materials and Methods section, if mainstream or side stream (environmental or second hand) has not already been mentioned in the abstract or introduction. There are some that allow you to look up their chamber of choice, although this leads to lengthy product guidelines tediously researched online (i.e., the long outdated Hamburg II chamber). There are only a few groups that generally use the same procedure and one can at least assume that they are utilizing the same method, simply using their latest publication as the current reference (which then must be referenced back to the original procedure). It is a relatively safe assumption that the method always remains mainstream or side stream within the same research group, unless otherwise noted, but it is left for the reader to assume, nonetheless. In this summary, only two research groups resort to this form of documentation.

Seven publications segregated in **Table 3** leave it completely unknown what exposure they have used, and one uses a "whole body mainstream" exposure, apparently employing a high concentration of cigarette particulates into a chamber to mimic mainstream smoking (Braber et al., 2011), but that is, again, left to be assumed. Generally, one can still assess the exposure method in most cases, it becomes less clear at the level of nicotine, carbon monoxide, and particulate levels. Some researches are very diligent here, while others may not have measured these parameters and therefore do not address them. There is also somewhat variability to whether nicotine and TSP/TPM are measured as mg/m^3 or $\mu g/l$ (which can easily be calculated to mg/m^3). Some quote mg/cig for the nicotine levels (Pittilo et al., 1982, 1990; Suemaru et al., 1992; Escolar et al., 1995; Wright and Sun, 1999; Wright et al., 1999; Balansky et al., 2000; Cavarra et al., 2001; Wright and Churg, 2002; Rangasamy et al., 2004; Tanaka et al., 2004; Bartalesi et al., 2005; Martorana et al., 2005; Yao et al., 2008; Woodruff et al., 2009; Cicko et al., 2010; Miller et al., 2010; Yoshida et al., 2010; Nie et al., 2012; Nemmar et al., 2013), meaning this was likely looked up through the manufacturer. The same is true for carbon monoxide, which is either measured in ppm or percentage of carboxyhemoglobin in the serum. It is clear that the vast majority of studies has, at bare minimum, one of these parameters missing, as only 24 (15.5%) exemplary studies listed all three. Usually, the levels named are an average kept during the entire exposure period, which is of importance when considering SHS exposure. The TSP/TPM levels in SHS exposures is as low as $24\ mg/m^3$ using a unique rabbit model (Sun et al., 2001), but as high as $250\ mg/m^3$ in standard Teague chamber mouse models using Kentucky reference cigarettes (Foronjy et al., 2005, 2006; Golovatch et al., 2009; Richens et al., 2009). The Stampfli lab (Botelho et al., 2010, 2011a,b, 2012; Farkas et al., 2011) and one other group (Braber et al., 2010) go beyond $700\ mg/m^3$ for their mouse models that do not utilize the Teague chamber.

There is still diversity in the use of cigarettes (**Table 4**), despite the fact that reference cigarettes, specifically designed for this type of research, have been available for approximately four decades now. The vast majority of studies (111 studies making up 71.6%) do use the reference cigarettes provided by the University of Kentucky's Tobacco Research and Development Center (formerly the Tobacco and Health Research Institute). Two groups have listed all necessary parameters with the unique cigarettes (Ultratech Corp and Ye Shu) they have chosen (Sun et al., 2001; Nie et al., 2012). Unfortunately, it appears that commercial Virginia cigarettes are popular, without specifying which kind, though apparently generally a type with 0.9 mg nicotine per cigarette, except for one study that claims to use Virginia cigarettes with 1 g nicotine per cigarette, presumably a typo (Escolar et al., 1995). A total of 17 studies do not list the cigarettes used (either commercial unfiltered, simply unfiltered, or no reference at all), while five publications compromising eight studies do opt for Kentucky reference cigarettes, but then neglected to list which kind, let alone the parameters (Hautamaki et al., 1997; Shapiro et al., 2003; Maeno et al., 2007; Nakanishi et al., 2009; Clauss et al., 2011).

Other cigarette brands listed in **Tables 1–3** stated only some or none of the parameters for TSP/TPM, nicotine, and carbon monoxide levels. The eight remaining brands of cigarettes are Marlboro 100 and Marlboro red (for Marlboro Medium sort, the nicotine level is 1 mg/cig and carbon monoxide is

12 mg/cig), Long Peace and Seven Star (Japan), Eighty Eight Lights (South Korea), Arda-Bulgartabac (Bulgaria), Canada Tobacco, and Virginia brand. For these cigarettes, the parameters could not be found to supplement the information available in the original publications themselves, as the actual type of cigarette was not stated in any one of them. Therefore, data regarding reference cigarettes used in the studies summarized here have been left incomplete in **Table 4**.

Kentucky reference cigarettes, on the other hand, are very well documented, but sometimes it is unclear in a publication whether a mix of two types was used for smoke exposure. At times, the filtered version (named with an "F" by the manufacturer) is used, but the filter is removed. There are also those who list using 2R4, which presumably means that the filter was removed for the study. The first line of reference cigarettes from the University of Kentucky were from 1974 and included 1R3, 1R3F, and 2R1, of which the filtered kind (1R3F) had 30 mm butts and the unfiltered cigarettes (1R3 and 2R1) could be purchased with either 23 or 30 mm butt lengths. In 1983, the new generation of reference cigarettes was 1R4F, only available in a 35 mm butt length. In 2001, the next generation, named 2R4F, was launched and as of 2008 3R4F has been produced. Studies presumably reflect the reference cigarette of the time and the well-documented levels of Kentucky reference cigarettes can be found in their entirety in **Table 4**.

COMPARING MAINSTREAM AND SECOND HAND EXPOSURES

Although parameters appear to rarely be listed to the extent they should be, we have attempted to compare the direct exposure of smoke in these animal studies to those of animals exposed via a whole body method, mimicking SHS. One must remember that while mainstream smoke is inhaled into the lungs from the cigarette directly, SHS is the inhalation of suspended particles from the smoker's exhale and the burning end of the cigarette, thereby concentrations of particles and individual components are not necessarily reduced, as one would hope. What becomes clear is, that SHS exposure does indeed lead to emphysema and other COPD symptoms in animal models. A multitude of studies elucidate this fact and they are summarized in **Table 2**, which also highlights what effects (whether emphysema, SAR, or pulmonary hypertension) were assessed.

Studies to unravel the pathogenic mechanisms of cigarette smoke exposure were undertaken in the knockout models, where the effect can easily be seen based on only one factor. The central role of TNF-α has been documented for both exposure applications. This was made evident by inhibition of TNF-α using a receptor knockout model (TNFR KO), which attenuated the development of emphysema by 71–83% and of SAR by 100% in mainstream cigarette smoke exposure (Churg et al., 2002a, 2004, 2009b). TNFR KO were 100% protected against emphysema using side stream smoke (D'Hulst et al., 2006). Additionally, inhibition of efferocytosis is TNF-α dependent in the side stream knockout model (Richens et al., 2009).

C–C CCR5 knockout mice were 100% protected against emphysema in an exposure method that was not defined, where INF-γ over-expression enhanced emphysema (Ma et al., 2005). In a side stream exposure study (Bracke et al., 2007), the effect of CCR5 knockout was only protective to 25%, whereas CCR6 knockout was more effective with 67% protection against emphysema (Bracke et al., 2006). It is possible that the same manipulation would be more protective in a mainstream versus a second hand exposure, although the exposure in the first study, which assessed a number of pathways leading to apoptosis, is not known.

Another well-documented knockout model for the mechanism of smoke induced pathology is that of macrophage elastases, specifically MMP-12 (Hautamaki et al., 1997; Churg et al., 2002a, 2003a; Shapiro et al., 2003) and MMP-9 (Atkinson et al., 2010). The elastinolytic activity central to matrix breakdown and alveolar enlargement had already been attributed to macrophages (Ofulue et al., 1998; Ofulue and Ko, 1999) and these two enzymes were pinpointed via inhibition (Churg et al., 2007a). Here, MMP-12 (also termed macrophage metalloelastase MME) has been identified to be of foremost importance in lung destruction, since MMP-12 knockout mice are resistant to smoke induced emphysema, while emphysema can occur independent of MMP-9 (Atkinson et al., 2010). Mice deficient in monocytes and macrophages do not develop enlarged airspaces upon cigarette smoke exposure, while those deficient in polymorphonuclear cells (PMN) do (Ofulue et al., 1998; Ofulue and Ko, 1999; Dhami et al., 2000). Unfortunately, all studies regarding MMP contributions, which are considerable, have solely been tested in the mainstream smoke model.

The neutrophil-derived serine elastase has been implicated in both a mainstream and a side stream exposure model (Dhami et al., 2000). In a direct smoke exposure model, the neutrophil elastase knockout mouse is 59% protected against emphysema development (Shapiro et al., 2003), while specific neutrophil elastase inhibitors are similarly protective in the guinea pig model (Wright et al., 2002; Stevens et al., 2011). In a SHS exposure model of the mouse, one group (Stevens et al., 2011) was also able to detect a marked decrease in SAR development in both their mainstream and side stream exposure models upon treatment with neutrophil elastase inhibitor AZD9668.

The only thus far defined true genetic factor described in man predisposing to COPD is α-1 antitrypsin deficiency. This deficiency leads to accelerated development of emphysema following cigarette smoke exposure. Although the importance of α-1 antitrypsin has been documented in the animal model of direct cigarette smoke (Dhami et al., 2000; Churg et al., 2003b), there are two studies where it is not clear whether an indirect exposure was utilized (Takubo et al., 2002; Churg et al., 2007b). Nevertheless, α-1 antitrypsin is indeed protective against emphysema in a dose-dependent manner, indicating that this is likely also true for indirect cigarette smoke exposure (Dhami et al., 2000; Churg et al., 2003b).

In addition to genetic manipulations elucidating molecular mechanisms of cigarette smoke pathogenesis, there are also models for drug testing, more specific to an intervention protocol. These go beyond simply attenuating the proteolytic breakdown via inhibition of just one protease and encompass mainly statins and anti-oxidants. Simvastatin, as mentioned above, has a protective effect against emphysema and pulmonary

hypertension, but not SAR, and has been tested in both the guinea pig (Wright et al., 2011) and the rat (Lee et al., 2005), but only using a mainstream exposure method. Therefore, a homologous mechanism for side stream smoke is yet to be described.

Beyond being influenced by mouse strain (Guerassimov et al., 2004; Bartalesi et al., 2005; Foronjy et al., 2005), emphysema development is also dependent on the airspace content of anti-oxidants. This was demonstrated by comparing bronchoalveolar lavage (BAL) levels of endogenous anti-oxidants from the oxidant resistant mouse strain ICR with that from other strains (Cavarra et al., 2001; Richens et al., 2009). Successful anti-oxidant therapy is seen for mainstream (March et al., 2006; Suzuki et al., 2009; Churg et al., 2012) and side stream exposure models (Smith et al., 2002; Richens et al., 2009), as well as in a Rtp801 knockout model for side stream smoke (Yoshida et al., 2010). This is reflected in the success of superoxide dismutase (SOD) transgenic mice (Foronjy et al., 2006; Richens et al., 2009), specifically those with enhanced SOD3 expression (Yao et al., 2010), in protecting against emphysema in both exposure scenarios.

WHAT IS MISSING

Unfortunately, even with chronic exposure studies, the animal model has limitations to how well it can mimic COPD, as seen by the inadequacy of interventions that protect mice against cigarette smoke induced emphysema, but not humans (Barnes, 2007). A procedure that is applied in the same way for each study could greatly contribute to our understanding of pathogenesis and how the animal model relates to the human disease. For example, with a standard 6-month exposure period for mouse models, research groups have been able to establish a disease time frame that is utilized for most chronic studies. In rats, this chronic period starts at 2 months of exposure (Ofulue et al., 1998; Kratzer et al., 2013) and in guinea pigs after 3 months of exposure (Wright et al., 2002). In order to truly compare results, though, it is of importance to standardize exposure methods and levels as well.

Generally, there is favoritism toward the somewhat more recent application of chambers, which was greatly improved so as to be utilized as a standard method for SHS exposure in 1994 (Teague et al., 1994). A chamber may contain any number of animals and is often applied to produce a cigarette smoke mixture consisting of 89% side stream and 11% mainstream smoke, exposing animals to environmental smoke versus attaching a nose or head piece to introduce smoke first hand. Often, though, even mainstream or side stream smoke exposure are neglected to be pointed out, as it is assumed that using a chamber method should suffice as information, even though it can require extensive back-referencing to discover the actual original procedure. Beyond the smoke chamber, which wasn't developed until 1994 (Teague et al., 1994), few standardized procedures were possible. Since then, though, there is still very little homogeneity in the applications used for research in this field. We could greatly improve our understanding of the consequences of inhaled cigarette smoke if we were to apply a similar and comparable model in every case. This would require selecting conform research cigarettes, whether filtered or not, preferably from the same source, so that contents and levels of important toxins and mediators can be compared.

If a variety of cigarettes are necessary to accommodate research across the globe, it is imperative that at least cigarette parameters are assessed and noted. Reference cigarettes were specifically designed from 1969 onwards for the purpose homogenous and comparable studies around the world. To this day, commercial cigarettes with varying or unknown parameters are applied.

There is still an abundance of publications that do not mention or may even not determine nicotine or carbon monoxide amounts or the contributing particles in cigarettes (whether as TSP or TPM), though it could easily be evaluated from online supplements for major cigarettes in use. For randomly selected commercial cigarettes, of course, there is not even this option, as only nicotine and in very few cases carbon monoxide levels are available. Some studies do not even list the cigarette type or brand used, so there is practically no information concerning the exposure. These factors complicate matters even further, as there are already numerous ways that cigarette smoke is administered, using as few as two cigarettes a day in mice (Hautamaki et al., 1997; Shapiro et al., 2003) or as many as 12 cigarettes a day in rats (Nie et al., 2012).

Additionally, many studies use an initial acclimation period for their cigarette smoke exposure, employing an increasing number of cigarettes or longer smoke periods after beginning the study, thereby also allowing variability, but acclimation is usually necessary. Inconsistencies can be avoided by assessing the most important parameters, such as nicotine, carbon monoxide, and particle levels. If the scientific community could agree on a protocol to establish similar exposures both for mainstream and side stream cigarette smoke, results would be much more comparable. At a bare minimum, simply correctly documenting the method used would add a great deal to the knowledge that could have already been gained. In this way, cigarette smoke researchers all around the world would be able to collectively assess and compare their data.

CONCLUSION

Cigarette smoke exposure is the most important risk factor for developing COPD, both in smokers and in non-smokers who are involuntarily exposed to the toxic and carcinogenic contents of environmental cigarette smoke. Studies of long-term cigarette smoke exposure effects are very costly, time-consuming, and require an abundance of resources. For this reason, tests should be optimized so that experimental models are comparable and provide as much knowledge as possible. It is evident from the literature that there is, to this day, enormous unnecessary diversity in study designs despite improvements to our experimental exposure methods (chambers and reference cigarettes). There is a great need for a standard protocol defining parameters to be evaluated and exposure procedures to be followed. With this quite simple documentation of easily assessed facts and we would be able to gain much more knowledge with each study. It should be possible to determine molecular mechanisms, oxidative stress, protease participation, inflammatory cytokine levels, and cellular death following mainstream and side stream cigarette

smoke exposure, comparing them. These insights would greatly improve and expand today's comprehension of cellular and molecular mechanisms implicated in the pathology of COPD. A more defining knowledge of the pathogenesis of mainstream and second hand cigarette smoke induced emphysema, SAR, and pulmonary hypertension could have been achieved with the undertaken studies if a unified procedural method had been applied.

Despite the shortcomings in terms of documentation in a large amount of experiments, they have nonetheless led to great advances through gene knockout models or transgenic mice as well as inhibitory tactics and drug treatments. When combining these animal studies in one overview, it becomes highly evident that second hand cigarette smoke has many of the same mechanisms and detrimental effects that mainstream smoke does. For this reason, we wish to point out that the US Surgeon General's assessment of a missing link should be revisited, so that the public becomes more aware of the health risks to innocent bystanders, where SHS exposure causes COPD/emphysema in non-smokers.

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Pathogenic mechanism of second hand smoke induced inflammation and COPD

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Second hand smoke (SHS) introduces thousands of toxic chemicals into the lung, including carcinogens and oxidants, which cause direct airway epithelium tissue destruction. It can also illicit indirect damage through its effect on signaling pathways related to tissue cell repair and by the abnormal induction of inflammation into the lung. After repeated exposure to SHS, these symptoms can lead to the development of pulmonary inflammatory disorders, including chronic obstructive pulmonary disease (COPD). COPD is a severe pulmonary disease characterized by chronic inflammation and irreversible tissue destruction. There is no causal cure, as the mechanism behind the development and progression of the disease is still unknown. Recent discoveries implicate genetic predisposition associated with inflammatory response contributed to the development of COPD, linked to irregular innate and adaptive immunity, as well as a risk factor for cancer. The use of animal models for both cigarette smoke (CS) and SHS associated *in vivo* experiments has been crucial in elucidating the pathogenic mechanisms and genetic components involved in inflammation-related development of COPD.

Keywords: second hand smoke, inflammation, COPD, immunity, cancer

CLASSIFICATION OF SHS

Second hand smoke (SHS) is classified as exposure to sidestream smoke, produced directly by tobacco containing products (cigarettes, cigars, and pipes), or mainstream smoke, exhaled by smokers. The US Environmental Protection Agency determined in 1986 that SHS is a cause of lung cancer development, responsible for approximately 3000 lung cancer deaths annually (Jinot and Bayard, 1994; IARC, 2004; Talhout et al., 2011). In 2010, there were 10.3% adult smokers worldwide, or 45.3 million people, so the frequency of SHS exposure can be extensive (King et al., 2011). Around 10–25% of lung cancers are diagnosed in nonsmokers, who are considered to have smoked less than 100 cigarettes in their lifetime (Couraud et al., 2012). Evidence for the toxicity of SHS was found in non-smoking individuals with spouses who smoked cigarettes, who displayed elevated risks for lung cancer, heart disease, and respiratory disorders (HHS, 2006; Sebelius, 2011).

The burning tip of a cigarette is hot enough to allow for the release of tobacco smoke (TS) components into a gas and particulate vapor that is easily absorbed into the lung (Pappas, 2011; Talhout et al., 2011). This vapor rapidly enters the lower airways of the human lung, and eventually the circulatory and lymphatic systems (IARC, 2004; Baker, 2006). While tobacco is comprised of more than 5000 constituents, TS contains roughly 2800 molecules not found in tobacco, including reactive oxygen species (ROS) and nitric oxides (Baker, 2006). This indicates that the combustion, pyrolysis, and prosynthetic reactions during the flaming of the tobacco product are what create the components of TS (Baker, 2006). Approximately 250 carcinogenic and noxious chemicals have been measured in both sidestream and mainstream smoke (HHS, 2006). Mainstream smoke is generated at high temperatures in the presence of oxygen drawn through the column of a smoking apparatus, resulting in larger particles than sidestream smoke (HHS, 2006). Sidestream smoke is generated at lower temperatures in an oxygen-poor environment, with higher concentrations of ammonia, nitric oxides, and carcinogens (HHS, 2006). While all forms of environmental TS exposure have been shown to cause genetic damage, the detriments of SHS to a person vary based on proximity to source of smoke, time, and environment.

SHS-INDUCED INFLAMMATION AND COPD

In response to SHS exposure, there is enhanced recruitment of inflammatory cells to the lung, particularly neutrophils and macrophages (Rennard et al., 2006). Short-term exposure to SHS does not result in a notable difference in inflammation in humans, though endothelial function deteriorates (Bonetti et al., 2011). Long-term TS exposure in mice leads to significantly increased inflammation, as measured by the influx of alveolar macrophages, neutrophils, and antioxidant enzymes (Bezerra et al., 2011). TS can also directly bind to DNA to effect the expression of genes related to inflammation. Sekhon et al. determined that nicotine can enter the placenta and directly interact with nicotine receptors on non-neuronal cells of the fetus (Sekhon et al., 1999). They also found that nicotine exposure leads to the enhancement of elastin and collagen type I and III mRNA expression, as well as airway wall expansion in the fetal lung (Sekhon et al., 2002).

SHS increases the incidence and severity of respiratory infections and disorders in humans (Jinot and Bayard, 1994; HHS,

2006; Sebelius, 2011). Exposure to TS introduces thousands of xenobiotics to the lung, and can lead to a persistent inflammatory response in the small airways and alveoli. This is the foundation for the development of pulmonary inflammatory disorders, such as COPD. COPD is a progressive and irreversible airflow obstructive disease of the lung and the third leading cause of death in the US. Of patients who are diagnosed with lung cancer, 40–70% of patients have COPD (Young et al., 2009). Chronic bronchitis, characterized by a consistent cough with mucus secretion, and emphysema, characterized by the destruction of airway epithelium and thickening of airway walls, is the distinct phenotypes that define COPD, though they can occur concurrently. Manifestation of COPD is a result of an interaction of TS exposure with other toxic environmental exposures, genetic factors, and unresolved childhood respiratory infections (Decramer et al., 2012). While TS is the main risk factor for COPD, only 20% of smokers develop COPD, suggesting a genetic predisposition (Young et al., 2009). Evidence for this includes the discovery of the genetic variants and mutations associated with TS-induced inflammation and COPD (Gwilt et al., 2007; Guo et al., 2012; Hunt and Tuder, 2012). These polymorphisms and mutations may be responsible for the exacerbation of inflammatory symptoms, resulting in COPD and lung cancer development (Young et al., 2009).

One of the most damaging effects of TS is oxidative damage, which promotes COPD development (Decramer et al., 2012). SHS contains $>10^{16}$ free radicals per cigarette (Barcelo et al., 2008), comprising of ROS and peroxides (Baker, 2006). When introduced to the lung, an imbalance of oxidant and antioxidants, which protect against free radicals, occurs and results in oxidative stress (HHS, 2006). Oxidative stress induces direct airway epithelial damage, as well as indirect damage by altering signaling pathways. These pathways are related to cell proliferation, differentiation, and proinflammatory cytokines and chemokines through the upregulation of the transcription factors nuclear factor-κB (NF-κB) and activator-protein 1 (AP-1) (MacNee, 2001; HHS, 2006). Oxidative stress also leads to the oxidation of DNA, lipids, and proteins, resulting in lung injury and the production of secondary ROS (MacNee, 2001). Additionally, it can prevent repair processes in the damaged epithelium through inhibition or damage to surfactant and antiproteases, which leads to the development of fibrosis (MacNee, 2001; Decramer et al., 2012). Howard et al. developed a short-term SHS rat model and found considerable DNA damage in several tissues, measured by the presence of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a major product of DNA oxidation (Howard et al., 1998). Chiang et al. found that 8-OHdG levels in human plasma increases with SHS exposure in a dose-dependent manor (Chiang et al., 2012).

Both the innate and adaptive immune responses play a role in the pathogenesis of COPD. In response to SHS, the innate immune response is triggered, resulting in inflammatory cell infiltration, mainly neutrophils and macrophages, to the mucosa and submucosa glands of the airway epithelium (van Antwerpen et al., 1995; MacNee, 2001; Decramer et al., 2012). Neutrophils and macrophages release neutrophil elastase and macrophage metalloproteases, respectively, along with pro-apoptotic factors to combat toxins and prevent the spread of injury. Accumulation of activated inflammatory cells from repeated SHS exposure reduces their usefulness, resulting in tissue damage and oxidative stress (Bosken et al., 1991; Rennard et al., 2006). This exacerbates TS-induced airway destruction, fibrosis, and remodeling, which are the basis for the development of inflammatory disorders (Bosken et al., 1991; Rennard et al., 2006).

TS can enhance the damaging phenotype of inflammatory cells. In study participants exposed to 3 hours of sidestream smoke, there was an average of 71% more reactive oxidants released by neutrophils (Anderson et al., 1991). Furthermore, activated polymorphonuclear cells are delayed in the lung microvessels by TS, allowing for enhanced tissue destruction (Klut et al., 1993). A positive correlation has been found with higher numbers of neutrophils in the circulating blood and reduced airway function, measured by spirometric levels (FEV1), in smoker lungs (van Antwerpen et al., 1995).

Dendritic cells are the link between the innate and adaptive immunity. If the innate immune response is unable to control the damage by TS, the recruited inflammatory cells, cytokines, chemokines, antigens, and other factors can induce dendritic cells to migrate to the lymphnodes for activation and differentiation (Cosio, 2004). Dendritic cells interact with T-cells and B-cells to instigate and shape the adaptive immune response. Naïve T-cells differentiate into several subsets, including T-helper 1 (Th1), T-Helper 2 (Th2), T-helper 17 (Th17), and regulatory T cells (Treg). These are distinct in the T-cell factors and cytokines they activate. The differentiation is largely dependent on the local inflammatory environment and the strength of the T cell receptor with the antigen (Zhou et al., 2009). The characteristic of the T-cells in disease manifestation and progression is important to consider, because the imbalance of T-cell populations can lead to irregular and severe inflammatory responses. Further analysis into the inflammatory microenvironment of the COPD lung has led to the discovery that the Th1 (Grumelli et al., 2004; Lee et al., 2007) and Th17 (Vargas-Rojas et al., 2011) subsets are particularly high in the COPD lung, with Th17 cells conceivably mediating the Th1 activity (Alcorn et al., 2010; Vanaudenaerde et al., 2011). Chen et al. exposed wild-type and IL-17Ra deficient mice to sidestream smoke for 6 months and found that the deficient mice developed significantly less tissue emphysema and airspace enlargement (Chen et al., 2011).

In addition, Tregs are absent in the bronchoalveolar lavage (BAL) fluid and blood of COPD patients (Lee et al., 2007; Barcelo et al., 2008), while smokers without COPD show an upregulation of this subtype (Barcelo et al., 2008). Tregs are critical in containing the immune response and maintaining tolerance to self-antigens. Therefore, without Treg regulation, continual exposure to TS can lead to an overpowering pro-inflammatory response mediated by Th1 and Th17 lymphocytes, resulting in the severe airway damage characterized by COPD.

B-cells have also been found to be upregulated in TS-driven emphysema patients. B-cell follicles were found in the bronchial walls and parenchyma of these patients and increased over time, which correlated with progressive airspace enlargement (van der Strate et al., 2006). While exposure to TS illicits airway damage and subsequent release of antigens by the innate immune system

in all lungs, not all people react to the antigens and have resulting B- and T-cell differentiation, which explains why only a percentage of smokers develop COPD (Cosio et al., 2009). There is also a variation in the degree in which people react to the antigens, which explains the deviation in severity of the disease (Cosio et al., 2009).

Of growing interest is the hypothesis that COPD is linked to autoimmunity. Reduced levels of Tregs are an indication of autoimmunity (Shevach, 2000). Also, in order for T-cells to migrate to the lung, they must be activated by antigens (self or modified-self) (Cosio, 2004). Lee et al. discovered that antibodies toward elastin, a self-antigen, were significantly increased in emphysema patients (Lee et al., 2007). They propose TS-exposure leads to proteolytic-induced cleavage of elastin, resulting in fragments that generate T- and B-cell immunity against elastin (Lee et al., 2007). Kirkham et al. propose that chronic oxidative stress in COPD induces carbonyl-modification of self-proteins, creating neoantigens that are targeted by the immune system. In support of this hypothesis, they found increased antibody titer against carbonyl-modified self-proteins in COPD patients versus control subjects (Kirkham et al., 2011). Additionally, the persistence of COPD symptoms after smoking cessation indicates that T- and B-cells are recruited in response to self-antigens (Motz et al., 2008; Cosio et al., 2009).

INFLAMMATION AND CANCER

The enhanced inflammatory cell environment of the lung from exposure to TS can promote the development of mutated cells into malignant cells, eventually resulting in tumor formation and progression. While acute inflammation inhibits tumor growth, long-term inflammation promotes tumor enlargement and metastasis. Because TS can compromise alveolar repair mechanisms, such as chemotaxis, apoptosis, and matrix restoration, these malignant cells can develop into tumors and metastasize (Rennard et al., 2006). Jinushi et al. generated genetically modified mice study the relationship of chronic inflammation and lung cancer by simulating defects in apoptotic cell clearance, autoreactive Th17, and increased vulnerability to infection (Jinushi et al., 2007). These mice developed chronic pulmonary inflammation and lung adenocarcinomas, as well as increased mortality (Jinushi et al., 2007).

The main link between chronic inflammation and oncogenesis is considered to be TNF-α mediated upregulation of NF-κB, which induces anti-apoptotic and proliferative effects. TNF-α has a seemingly contradictory role of stimulating apoptosis through activation of caspase 8, while simultaneously activating NF-κB, which protects cells from pro-apoptotic stimuli. NF-κB is a transcription factor which plays an integral role in the immune response to infection. It is activated by cellular signals resulting from stimuli such as necrotic cells, cytokines, and ROS. Once activated, NF-κB translocates into the nucleus to bind to DNA, activating hundreds of different genes encoding proteins related to the immune response, inflammation, and cell growth. In an environment of pre-malignant cells due to environmental exposures like SHS, continual NF-κB activation will support tumor development and progression by inhibiting apoptosis while activating cell proliferation, metastasis, and survival through the products of genes it regulates (Karin et al., 2002; Luo et al., 2004; Philip et al., 2004; Karin, 2006).

In addition to TNF-α, there are other molecular pathways implicated in upregulating NF-κB expression and other transcription factors (TFs). Zhao et al. used protein and DNA arrays to examine potential upstream signaling pathways responsible for TS-induced TF activation. By exposing cells to TS, they examined 244 different TFs. TS significantly regulates at least 20 TFs including NF-κB, which may be involved in tumorigenesis and cell cycle regulation, activated primarily by MAPK signaling pathways (Zhao et al., 2007). These results indicate that MAPK signaling is also essential in TS-induced NF-κB activation and subsequent inflammatory gene expression.

The long-term use of anti-inflammatory agents have been linked to decreased cancer incidence, indicating inflammation as a contributor to cancer development (Dougan et al., 2011). Witschi et al. exposed mice both mainstream and sidestream smoke for 5 months, followed by a 4 month recovery period (Witschi et al., 2005). When mice were introduced to dexamethasone, an anti-inflammatory and immunosuppressant glucocorticoid steroid drug, for 4 months, the lung tumor multiplicity decreased by 64% compared to control mice (Witschi et al., 2005).

COPD is believed to be an independent risk factor for lung cancer. Prevalence of COPD in lung cancer cases is six-fold higher than in smokers without lung cancer (Young et al., 2009). Because chronic airway inflammation is a risk factor for COPD and is related to the increase of human cancers, it is hypothesized that COPD and lung cancer may share chronic inflammation as a common pathogenic mechanism (Young et al., 2009).

USE OF ANIMAL MODELS

Animal models continue to be crucial in determining the genetic factors underlying SHS and COPD (**Table 1**). When considering mouse models for experimental use, the choice of strain is critical in studies related to both SHS and COPD as there are strain-related differences in the metabolism of TS as well as the inflammatory cell composition and magnitude. Vecchio et al. examined this issue by comparing C57BL/6J and Institute of Cancer Research (ICR) mice post-cigarette smoke extract exposure (Vecchio et al., 2010). They found that alveolar macrophages from C57BL/6J mice produced higher levels of ROS, NF-κB, and proinflammatory cytokines (Vecchio et al., 2010). They hypothesize that the higher pro-inflammatory response in C57BL/6J versus ISC mice is due to higher oxidative stress in this strain, leading to increased activation of NF-κB. This may describe the differences in susceptibility of the different strains of mice (Vecchio et al., 2010). Cavarra et al. found that after acute TS exposure, DBA/2 and C57BL/6J mice had decreased antioxidant defenses, measured in bronchoalveolar lavage fluid, while ICR mice had increased antioxidants (Cavarra et al., 2001). After chronic exposure to TS for 7 months, they found that DBA/2 and C57BL/6J mice are more likely to develop emphysema and decreased lung elastin levels, while ICR mice did not develop these phenotypes (Cavarra et al., 2001). Tsuji et al. compared CS-exposure in AKR/J and C57BL/6J mice and found that C57BL/6J mice inhaled higher

Table 1 | Factors that influence SHS-induced COPD development and progression.

Exposure	Factor	Role in COPD development	Animal model	Strain background	References
Lipopolysacharide	Acetylcholine	Airway remodeling and emphysema	Guinea pig	Dunkin Hartley	Pera et al., 2011
Cigarette smoke Extract	Akt serine/threonine protein kinase (Akt)	Reduces cytotoxicity of TS, TS-exposure causes ubiquination of Akt	Rat	Lewis	Kim et al., 2011
Cigarette smoke	Caspase 1 (Casp1)	Inflammatory cell influx through IL-1β/IL-18	Mouse	C57BL/6	Churg et al., 2009
Cigarette smoke	C-Jun/Activator protein 1 (AP-1)	Regulates inflammation after long-term SHS exposure, restrains emphysema symptoms	Mouse	C57BL/6 × 129SVJ	Reddy et al., 2012
Cigarette smoke	Clara cell 10 kDa (Ccsp)	Protects the airway epithelium, TS exposure causes metaplasia of clara cells	Mouse	BALB/c	Cuzic et al., 2012
Cigarette smoke	Chemokine (C-X3-C) receptor 1 (Cx3cr1)	Required for IL-6 and TNF-α production by phagocytes; development of emphysema phenotype	Mouse	C57BL/6	Xiong et al., 2011
Cigarette smoke	Early growth response-1 (Egr-1)	Promotes autophagy and apoptosis in early stages of COPD	Mouse	C57BL/6	Chen et al., 2008
Cigarette smoke	endothelial monocyte-activating protein 2 (EmapII)	Inducing apoptosis through caspase 3, macrophage influx, emphysema phenotype	Mouse	C57BL/6	Clauss et al., 2011
Cigarette smoke	Extracellular signal-regulated kinase 1/2 (Erk 1/2)	Airway mucus hypersecretion	Rat	Sprague-Dawley	Xiao et al., 2011
Cigarette smoke	Extracellular superoxide dismutase (Ecsod)	Reduces TS-induced oxidative stress	Mouse	C57BL/6	Tollefson et al., 2010
Cigarette smoke	Forkhead box O3 (Foxo3)	Regulates inflammation, antioxidant genes; downregulated in COPD	Mouse	FVBx129S6	Hwang et al., 2011
Cigarette smoke	Granulocyte/Macrophage colony–stimulating factor (Gm-CSf)	Initiation of inflammatory cell influx	Mouse	BALB/c	Vlahos et al., 2010
Cigarette smoke	IFN regulatory factor (Irf7)	Inhibited in COPD lung, dampens proinflammatory cytokines in lung dendritic cells	Mouse	C57BL/6	Shan et al., 2012
Overexpression of IL-11 through transgenic mouse model	Interleukin 11 (Il-11)	Emphysema phenotype, airway remodeling and fibrosis	Mouse	Not reported	Kuhn et al., 2000
Cigarette smoke extract	Interleukin 17 Receptor A (Il-17RA)	Induces matrix metalloproteinase -12 (MMP-12) and CCl2, required for emphysema development	Mouse	C57BL/6	Chen et al., 2011
Cigarette smoke	Interleukin 1 Receptor, Type 1 (Il1R1)	Critical in initiation of neutrophilic inflammatory response to short-term TS	Mouse	C57BL/6	Doz et al., 2008
Cigarette smoke	Interleukin 1 alpha (Il-1α)	Critical in the initiation of the neutrophilic inflammatory response to TS	Mouse	BALB/c	Botelho et al., 2011
Overexpression of IL1-β through transgenic mouse model	Interleukin 1 beta (Il1-β)	Macrophage and neutrophil influx, emphysema phenotype	Mouse	Not reported	Lappalainen et al., 2005

(continued)

Table 1 | Continued

Exposure	Factor	Role in COPD development	Animal model	Strain background	References
Overexpression of IL-6 through transgenic mouse model	Interleukin 6 (Il-6)	Emphysema phenotype, airway remodeling and fibrosis	Mouse	Not reported	Kuhn et al., 2000
Cigarette smoke	Mucin-5ac (Muc5ac)	Mucus secretion in response to pro-inflammatory cytokines	Rat	Sprague-Dawley	Xiao et al., 2011
Cigarette smoke	NADPH oxidase (Nox)	Highly expressed in neutrophils, source of oxidative stress	Mouse	C57BL/6	Tollefson et al., 2010
Cigarette smoke	Osteopontin (Opn)	Th17 differentiation, emphysema phenotype	Mouse	C57BL/6	Shan et al., 2012
Cigarette smoke	P2X7	Neutrophil influx, caspase 1 activity, IL-1β	Mouse	C57BL/6	Eltom et al., 2011
Lipopolysacharide and cigarette smoke	Peroxisome proliferator-activated receptor-gamma (Pparγ)/PPARγ coactivator-1α (Pgc-1α)	Relieves oxidative stress; expression decreases with progression of COPD	Rat	Sprague-Dawley	Li et al., 2010
Cigarette smoke	Transforming growth factor beta (Tgf-β)	Airway remodeling, emphysema phenotype	Mouse	AKR/J, C57BL/6	Podowski et al., 2012
Cigarette smoke	Toll-like receptor 4 (Tlr4)	Critical in the initiation of the neutrophilic inflammatory response to TS short-term (61, 63); Role is diminished in chronic TS-exposure model (61)	Mouse	C3H/HeJ (Maes), C57BL/6J (Doz)	Maes et al., 2006; Doz et al., 2008
Cigarette smoke	Toll-like receptor 4 (Tlr4)	Induces Metalloproteinase-1 (MMP-1), required for emphysema development	Mouse, Rabbit	C57BL/CBA, New Zealand White	Geraghty et al., 2011
Cigarette smoke	Tumor necrosis factor-alpha (Tnf-α)	Inflammatory cell influx, chronic inflammation, emphysema phenotype	Mouse	C57BL/6	Churg et al., 2004
Knock-out genetic model	Vascular endothelial growth factor (Vegf)	Preventative role in emphysema	Mouse	C57BL/6	Tang et al., 2004

Listed in alphabetical order are proteins, receptors, and other factors which have been discovered in animal models that influence SHS-induced COPD. Their degree of influence in vivo could vary based on the experimental design; therefore the exposure models and mouse strains are also included in the table.

amounts of smoke and more severe respiratory lesions, while AKR/J mice had higher inflammatory cytokine levels (Tsuji et al., 2011).

Our lab developed a mouse model to simulate the development of COPD and SHS (Birru et al., 2012). We separated mice into four treatment groups: filtered-air control, lipopolysacharide (LPS) to stimulate inflammation, CS, and LPS combined with CS. Mice were sacrificed after 6 months of weekly LPS and daily CS exposure. The inflammatory response, alveolar space enlargement, and lung tumor incidence were assessed. In the LPS only group, mice displayed increased inflammation, but no alveolar space enlargement (**Figure 1**). In the LPS and CS group, mice displayed enhanced inflammation and alveolar space enlargement compared to the CS only group (**Figure 1**). No groups developed tumors in this exposure model at the duration examined. Our results indicate that chronic inflammation enhances emphysema-like alveolar space enlargement.

We also developed an animal exposure paradigm using components of TS to determine the role of inflammation in the development and progression of tumor formation (Keohavong et al., 2011). We used LPS to incite inflammation and nicotine-derived nitrosamine (NNK) for tumorigenesis (14). Mice were assigned into four different treatment groups: saline control, LPS only, NNK only, and LPS combined with NNK for 4 months. The saline and LPS only groups had no tumor development, but there was a six-fold increase in tumor numbers in the LPS and NNK group compared to the NNK only group. The LPS only and LPS with NNK groups displayed significantly elevated inflammation compared to the saline and NNK only groups. Our results indicate that repeated exposure to inflammation enhances the progression of TS carcinogen-induced lung tumorigenesis.

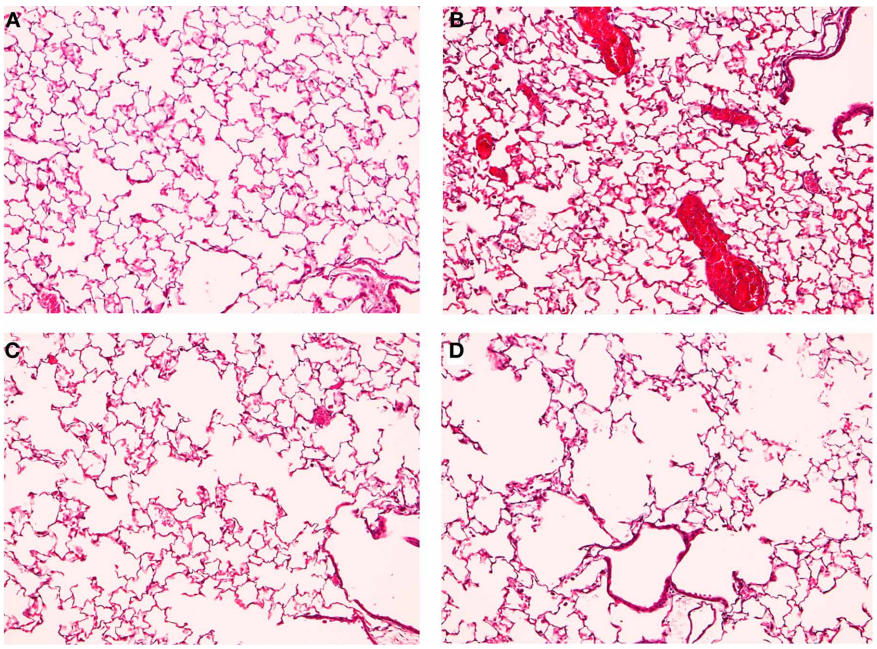

FIGURE 1 | TS and LPS treated mice have enhanced inflammation and alveolar space enlargement. Mice were exposed to filtered air **(A,B)** or TS **(C,D)** generated by Kentucky Research Cigarettes for 6 months. Inflammation was further induced by intranasal LPS instillation **(B,D)**, with saline as a control **(A,C)**. Lung histology was analyzed by staining lung sections with hematoxylin and eosin. Results shown are representative images for each treatment at 10X magnification. TS-exposed mice displayed alveolar space enlargement **(C)** compared to filtered air exposure **(A)**. LPS stimulated inflammatory cell influx **(B)** and enhanced the alveolar space enlargement induced by TS **(D)** relative to TS-only exposure **(C)**.

CONCLUSIONS

SHS exposure is detrimental to the lung, resulting in lung destruction through the introduction of toxic chemicals to the lung and oxidants, as well as the inhibiting the repair pathways of the lung. Continued SHS exposure can also lead to the development of inflammation, which worsen COPD, due to the abnormal polarization of T- and B-cell differentiation. The enhanced inflammatory environment of the lung can also promote tumor initiation and progression of malignant cells through the activation of transcription factors that promote cell proliferation and inhibit apoptosis. Both environmental factors and genetic components underlying COPD continue to be uncovered, and will be crucial in developing useful treatments for the disease. Animal models for SHS-driven COPD studies will continue to play an essential role for this objective.

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Effects of second hand smoke on airway secretion and mucociliary clearance

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The airway acts as the first defense against inhaled pathogens and particulate matter from the environment. One major way for the airway to clear inhaled foreign objects is through mucociliary clearance (MCC), an important component of the respiratory innate immune defense against lung disease. MCC is characterized by the upward movement of mucus by ciliary motion that requires a balance between the volume and composition of the mucus, adequate periciliary liquid (PCL) volume, and normal ciliary beat frequency (CBF). Airway surface fluid (ASL) is a thin layer liquid that consists of the highly viscous mucus upper "gel" layer, and the watery lubricating lower "sol" layer. Mucus production, secretion and clearance are considered to play a critical role in maintenance of airway health because it maintains hydration in the airway and traps particulates, bacteria, and viruses. Different types of epithelial cells, including secretory cells, and ciliated cells, contribute to the MCC function. Cigarette smoke (CS) contains chemicals and particulates that significantly affect airway secretion. Active and passive CS-induced chronic obstructive pulmonary disease (COPD) is frequently associated with hyperplasia of goblet cells and submucosal glands (SMGs), thus increasing the secretory capacity of the airways that impairs MCC.

Keywords: epithelium, mucus, mucociliary clearance, smoke

CIGARETTE SMOKE AND SECOND HAND SMOKE (SHS)

Cigarette smoking (CS) is the single largest cause of preventable disease, disability, and death globally. Tobacco use is a major risk factor for heart attacks, strokes, chronic obstructive pulmonary disease (COPD), and cancer. Approximately 90% of lung cancer cases in the USA are attributed to cigarette smoking. Similarly, the United States Centers for Disease Control and Prevention describes tobacco use as "the single most important preventable risk to human health in developed countries and an important cause of premature death worldwide." CS aerosols are highly concentrated mixtures of particles and gases. This "gas/particle partitioning" is the same type of physical, gas/liquid distribution process by which an atmospheric gas, such as nitrogen, dissolves in blood. When a CS aerosol is drawn into the respiratory tract (RT), a portion of each compound of interest (e.g., nicotine) will initially be in the gas phase, and a portion will be in the particle phase. Both of these phases can serve as pathways for the delivery of the compound to RT tissues include pharyngeal, bronchial, or alveolar regions (Pankow, 2001). There are an estimated 5000 different chemicals in tobacco smoke (Borgerding and Klus, 2005; Thielen et al., 2008). Many of them are the most significant source of toxic chemicals of chemically mediated disease in humans (Ezzati and Lopez, 2003; Fowles and Dybing, 2003). Most of these compounds are not present in the tobacco plant but are formed when the cigarette burns. Many studies provide evidence of the relationship between the amount of exposure to tar or CS and the risk for head and neck, lung and skin cancers. The most known CS carcinogens include polynuclear aromatic hydrocarbons (PAH), acrolein, and nitrosamines. The gas phase of CS contains various gases including ammonia, carbon dioxide, carbon monoxide, hydrogen cyanide, and nitric oxide. The particle phase contains important constituents of CS, such as tar, nicotine, aromatic hydrocarbons, phenol, and cresol. The mean size of CS particles is 0.1–0.5 μm, so they are capable of reaching small airways (Domagala-Kulawik, 2008). In addition, radioactive carcinogens such as lead-210 (210Pb) and polonium-210 (210Po) linger in second-hand smoke (SHS), which is deeper and longer than when inhaling cigarettes especially in an indoors environment.

SHS is the inhalation of smoke, called passive smoking, or environmental tobacco smoke (ETS), by persons other than the intended "active" smoker. It occurs when CS permeates any environment, causing its inhalation by people within that environment. SHS contains largely the same components as mainstream smoke, but with varying concentrations. SHS has been shown to produce more particulate-matter (PM) pollution than an idling low-emission diesel engine and it contains not only the gas phase of exhaled smoke, but also the products of combustion of a cigarette. Therefore, persons exposed to SHS are exposed to up to 50 times higher concentration of some chemicals than smokers themselves (Domagala-Kulawik, 2008). Additionally, the smoke can also enter the body through the skin, adding an additional source of exposure.

HEALTH EFFECT OF SHS

Similar to active smoking, exposure to SHS is also a significant health problem concern worldwide. SHS exposure increases nonsmokers' risk of cardiovascular disease and stroke by inducing

several proatherosclerotic changes, including endothelial damage related to oxidative stress and inflammation, increased platelet aggregation, and increased arterial stiffness (Venn and Britton, 2007; Jefferis et al., 2010). SHS is also linked to several diseases of the lung, including cancer (Brownson et al., 2002; Hecht, 2006), and COPD (Eisner et al., 2006, 2009), asthma (Butz et al., 2011). Furthermore, SHS is associated with an increased risk of type 2 diabetes mellitus (Houston, 2006; Kowall et al., 2010) and with neurocellular changes (Slotkin et al., 2006) in infants with prenatal SHS exposure. Several studies show that causal associations exist between SHS and sudden infant death syndrome, acute respiratory infections, middle ear disease, asthma in children, and coronary heart disease as well as lung and sinus cancers in adults. Evidence is suggestive but not conclusive regarding a causal relationship between SHS and many other diseases. SHS is a major public health problem because more than 126 million people in the United States are exposed to SHS. Comprehensive bans on smoking in public lead to a reduction in overall exposure to SHS for both adults and children (Menzies, 2011), but the incidence of SHS exposure is still worrisome. SHS also contributes to the development of COPD in non-smokers and significantly impairs their airway secretion and mucociliary clearance (MCC).

MUCOCILIARY CLEARANCE

MCC is a self-clearing mechanism of the airway to remove inhaled pathogens and particulates. The mechanisms of bronchial secretion and MCC are critical components of lung innate immunity. Bronchial secretions are mainly produced by goblet cells and submucosal glands (SMGs), but also include small amounts of surfactant from Clara cells and other fluids that are part of the airway epithelium fluid. Cilium is present on respiratory surface epithelium, bearing hair-shaped structures on its surface (cilia). Cilia beat within a PCL layer with low viscosity for which PCL is the "sol" layer with a height that approximates the length of the outstretched cilia (about 7 μm) and keeps mucus at an optimal distance from the underlying epithelia, consequently affecting the clearance of mucus (Knowles and Boucher, 2002; Tarran, 2004). The airway epithelium fluid contains many antibacterial proteins and peptides including lysozyme, lactoferrin, and β-defensins. On the top of "sol" layer is a second viscous film of mucus "gel" that traps foreign particles and microorganisms. Acute inflammatory or toxic stimuli can promote hypersecretion of mucus mediated by a large variety of cytokines and chemokines. Within the thin fluid film of mucus, the cilia act out movements coordinated in a direction toward the pharynx. Therefore the viscous film of mucus that includes its freight is transported off in the direction of the mouth, where it is either swallowed or expelled via coughing. The "gel" layer of the airway epithelium fluid is formed mainly by water, mucins (MUC), and free proteins. Mucins are highly glycosylated macromolecules. To date, more than 21 different MUC-genes have been described, at least eight of which are expressed in the RT, although MUC5AC and MUC5B are the two main gel-forming mucins secreted in the airway. Also important for good MCC are the number, structure, activity, and coordinated movement of cilia. Function of ciliary cell is essential for MCC and ciliary cells may suspend their transport function after a short time under the adverse condition of insufficient temperature and humidity, which subsequently facilitate bacterial germinal colonization. Inhaled SHS contains PM and toxic chemicals that negatively impact MCC by increasing mucus secretion and decreasing ciliated cell numbers and ciliary beat frequency (CBF). Pulmonary infections and injury to the lung tissues in COPD patients may arise when the MCC function is compromised.

LUNG EPITHELIAL CELLS AND MCC

In addition to acting as a physical barrier, airway epithelium regulates fluid balance, modulates metabolism and clearance of inhaled agents, and secretes numerous mediators (Knight and Holgate, 2003). The MCC function is a coordinated action of lung epithelial cells that include a variety of cell types such as mucous, serous, goblet, ciliated, Clara, and basal cells (**Figure 1**). Secretory cells located in airway epithelium and SMGs are an important component of MCC mechanisms in the normal lung, and alterations in the phenotype of these cells are associated with the pathogenesis of several lung diseases. In human large airways, goblet cells of the surface epithelium, as well as serous and mucous cells of the glands, are the principal secretory cell types. Serous and mucous cells may control the properties of the mucus gel by regulating the ratio between secreted proteoglycans and mucins. Airway glandular epithelium forms an integrated structure with functionally appropriate proportion of ciliated cells, goblet cells, and basal cells. A proper ciliary movement improves valid host defense and is necessary in the cleansing processes of the airway epithelium. A brief description of major types of epithelial cells is provided in the following paragraph and summarized in **Table 1**. Effects of SHS on these cells and MCC will then be discussed further.

Mucous cells are defined by electron-lucent acidic-mucin granules that secrete mucus into the airway to trap foreign objects such as pathogens and dust particles (Jeffery, 1983). The mucous layer present in the airway from the level of the trachea to the bronchioles consists of a mixture of highly glycosylated mucin proteins. In the normal airways, there is a fine equilibrium between mucous production and clearance. However, in chronic airway inflammatory diseases, such as chronic bronchitis and asthma, mucous cell hyperplasia and metaplasia occur, which lead to excessive sputum production.

Goblet cells are simple columnar epithelial cells that share multiple characteristics with mucous cells but are scattered on the surface epithelium instead of located in the secretory glands. The term goblet refers to these cells' unique "goblet-like" shape. The apical portion is shaped like a cup, as it is distended by abundant mucinogen granules; its basal portion is shaped like a stem, as it is narrow for lack of these granules. Goblet cell hyperplasia is a prominent feature in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation (Wongsurakiat et al., 1998).

Serous cells are the most abundant secretory cells in human airway glands (estimated serous/mucous cell volume ratio is 60%:40%) with variable numbers of electron-dense granules that contain large quantities of enzymes. They have irregularly shaped basally oriented nuclei, a perinuclear zone containing

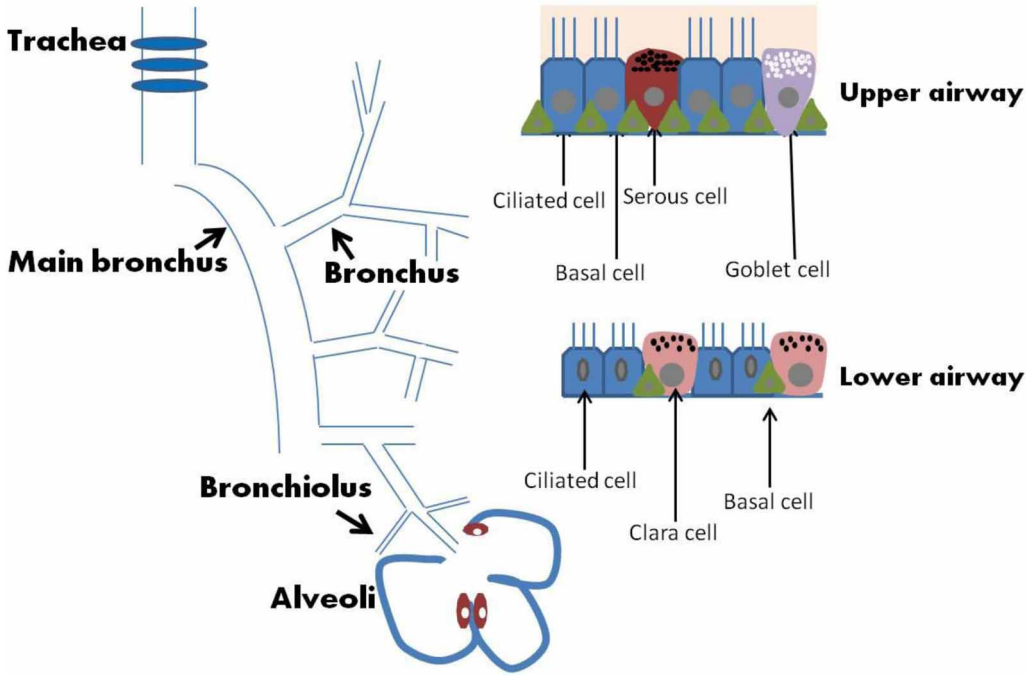

FIGURE 1 | Airway and epithelium.

Table 1 | Major cell types of respiratory tract epithelium.

Cell type	Feature(s)	Function(s)	Location	Marker(s)
Mucous cells	Columnar mucus-secreting cells; contain mucous electron-lucent, acidic-mucin granules	Secret mucus	Trachea, bronchi and bronchioles	MUC5AC, MUC5B
Goblet cells	Columnar mucus-secreting cells; contain mucous electron-lucent granules, which discharges apically. More abundant in human airway epithelium compared to the mouse	Contribute to airway mucus	Bronchi; small numbers in bronchioles Present in acinar region of SMGs	MUC5AC, MUC5B, SPDEF
Serous cells	Contain variable number of electron-dense granules concentrated in apical cytoplasm	Produce secretion of lower viscosity than that from mucous cells	Trachea, bronchi, present in acinar region of SMGs	Lysozyme, Lactoferrin, ZAG, SPLUNC1
Clara cells	Columnar nonciliated bronchiolar cells projecting in lumen; protuberant apical cytoplasm with large, round electron-dense secretory granules; comprise the majority of nonciliated bronchiolar cells	Secretory functions contributing to the mucous pool and maintaining extracellular lining fluid; progenitor for other bronchiolar cells	Predominantly in bronchioles	CCSP SP-A, SP-B, SP-D
Basal cells	Short cells with relatively little cytoplasm; oriented along the basement membrane; stem cells of the pseudostrafified airway epithelium	Regenerate the epithelium after loss of luminal cells and maintain it during homeostasis	Bronchi; rare in bronchioles	TP63, KRT5, PDPN and NGFR
Ciliated epithelial cells	Columnar, cuboidal, ciliated bronchial lining cells; each cell has approximately 250–300 cilia at the apical surface, and each cilium is approximately 6–7 μm long	Proximal transport of mucous stream (mucociliary escalator)	Bronchi and bronchioles	FOXJ1

large quantities of rough endoplasmic reticulum (RER) required for protein (enzyme) manufacture. Serous cells are the primary defensive cells of the mucosa because they discharge bactericidal compounds that deal efficiently with pathogens (Basbaum and Jany, 1990; Joo et al., 2004). Serous cells secrete various antibacterial proteins including lysozyme, lactoferrin, secretory immunoglobin A, peroxidase, and protease inhibitors, many of which are cationic in nature.

Clara cells contain electron-dense granules and are found mostly in the small airways in humans (Knight and Holgate, 2003). To regulate bronchiolar epithelial integrity and immunity, Clara cells have been shown to produce bronchiolar surfactants and specific antiproteases, such as secretory leukocyte protease inhibitor. In addition, Clara cells can produce p450 monooxygenases (Dewater et al., 1986), which are able to metabolize xenobiotic compounds such as aromatic hydrocarbons, which are found in cigarette smoke (CS). Clara cells produce Clara cell 10-kDa protein, Clara cell 55-kDa protein, Clara cell tryptase, galactoside-binding lectin, possibly a specific phospholipase, and surfactant proteins A, B, and D.

Basal cells are highly abundant in both the upper and lower airways (Boers et al., 1998). Basal cells have been demonstrated to possess stem cell-like properties in that they can self-renew and give rise to secretory and ciliated epithelial cells in response to epithelial injuries (Hong et al., 2004). Thus, basal cells are thought to be the progenitor cells of lung epithelium and are potentially capable of differentiating and proliferating in many pathological circumstances.

Ciliated epithelial cells are columnar epithelial cells with specialized ciliary modifications and are terminally differentiated cells that originate in either basal or secretory cells (Ayers and Jeffery, 1988). Usually, there are 250–300 cilia per cell and a large number of mitochondria provide energy to the cilia for MCC via proper ciliary beating. In general, cilia are sensory organelles and motile cilia in the airway epithelium are the engine for MCC. Ciliary beats provide the required physical force to move mucus and the trapped foreign particles while the CBF determines the flow speed of the movement. SHS decreases not only ciliated cells and ciliogenesis but also CBF.

EFFECT OF SHS ON MCC

COPD is characterized by chronic obstruction of expiratory flow affecting peripheral airways, associated with chronic bronchitis (mucus hypersecretion with goblet cell and SMG hyperplasia) and emphysema (destruction of pulmonary parenchyma), together with fibrosis and tissue damage, and inflammation of the small airways (Chung, 2001; Reid and Sallenave, 2003; Hogg et al., 2004). Most COPD is caused by long-term active smoking or SHS and it has been documented that smoke significantly compromises MCC. Multiple toxins in SHS, including PM, oxidative chemicals, and organic compounds induce mucin production (Deshmukh et al., 2008) and excessive airway mucus is a hallmark feature of COPD. Airflow obstruction in COPD correlates with changes in mucin gene expression, increases in goblet-cell number and size (Innes et al., 2006), the occlusion of small airways with mucus (Sheehan et al., 1995), and expansion of the SMGs (Hogg, 2004; Hogg et al., 2004). CS-induced MCC dysfunction is complex and incompletely understood, but it involves adverse effects on the structure and function of cilia (Verra et al., 1995; Leopold et al., 2009; Tamashiro et al., 2009), activation of ErbB receptors, and proinflammatory effects that increase mucin production while decreasing mucus hydration and clearance. It is suggested that a CS-decreased MCC in COPD may lead to airway infection, which subsequently leads to inflammation and fibrosis.

One major characteristics of airway remodeling in COPD is airway goblet cells metaplasia and hyperplasia, which results in airway mucus hypersecretion. Airway goblet cell hyperplasia is a prominent pathophysiological feature of COPD and is an often-used end point in animal models of respiratory disease (Rogers, 1997). The cellular composition of the airway epithelium can be altered both by cell division and by differentiation of one cell into another (Ayers and Jeffery, 1988). In terms of goblet cell hyperplasia, differentiation is the major pathway for producing of new goblet cells, and cell division is the major carcinogenic pathway. The basal, serous and Clara cells are considered the primary progenitor cells, because they have the capacity to undergo division, followed by differentiation into "mature" ciliated or goblet cells. In specific experimental conditions such as exposure to SHS, goblet cell division contributes in part to the hyperplasia. However, differentiation of nongranulated airway epithelial cells is a major route for production of new goblet cells (Rogers, 1994; Nadel and Burgel, 2001). In experimental animals, production of goblet cells is usually at the "expense" of the progenitor cells, most notably serous and Clara cells, which decrease in number as goblet cell numbers increase. Serous-like cells and Clara cells are found in macroscopically normal bronchioles in human lung (Rogers et al., 1993). The decreased numbers of serous and Clara cell has pathophysiological importance because these cells produce a large number of anti-inflammatory, immunomodulatory, and antibacterial molecules that are vital to host defense (Basbaum et al., 1990; Singh and Katyal, 2000). Therefore, in CS-related respiratory diseases associated with airway mucus hypersecretion and impaired MCC, it seems that not only is there goblet cell hyperplasia, with associated mucus hypersecretion, but also a reduction in serous and Clara cells, with concomitant impaired potential for host defense.

It has been supported by epidemiological data that mucus hypersecretion is significantly associated with a more rapid decline in FEV1 and increased hospitalization of patients with COPD. Among all identified mucins, MUC5AC appears to be the predominant mucin in healthy human airway epithelial cells, and its expression is augmented in smokers and COPD patients (Peter Di et al., 2012). CS induces mucin expression in cultured human airway epithelial cells (Phillips et al., 2005; Cortijo et al., 2011) and MUC5AC secretion can be regulated by several inflammatory cytokines including TNF-α and IL-13. The expression level of MUC5B is approximately 20% of that seen for MUC5AC in airway epithelial cell and it is secreted mainly by mucous cells in SMGs and partially by goblet cells. Several mechanisms have been reported to be responsible for CS-induced mucus hypersecretion. First, CS could cause mucin production via epidermal growth factor receptor (EGFR) activation both *in vitro* and *in vivo* (Takeyama et al., 2001).

Then, TNF-α-converting enzyme (TACE)-proligand-EGFR signal pathway could also be involved in CS-induced mucin overproduction (Shao et al., 2004). Furthermore, CS could promote goblet cell metaplasia indirectly through activation and recruitment of neutrophils and subsequent epithelial cell oxidative stress (Fischer and Voynow, 2002). *In vivo*, neutrophils and neutrophil products can cause the upregulation and release of mucin from surface epithelial cells (Takeyama et al., 1998; Voynow et al., 2004). EGF increased MUC5AC gene expression via ERK/MAP kinase but not p38/MAP kinase in NCI-H292 cells (Takeyama et al., 2000). Extracellular signal-regulated Kinase1/2 (ERK 1/2) plays an important role in airway mucus hypersecretion induced by CS in rats (Xiao et al., 2011).

In general, animal models have been essential to the development of every drug and therapeutic used to treat of COPD. One of important reasons for using animal models to tissues and cells isolated from COPD patients is that animals provide experimental settings that allow a clearer understanding of how an active immune system interacts with whole respiratory system. There are a large number of experimental researches on drugs and therapies of COPD induced by CS in laboratory animals. These include celecoxib (Roh et al., 2010), simvastatin (Lee et al., 2005), roflumilast (Martorana et al., 2005), neutrophil elastase inhibitors or neutrophil elastase knockout (Wright et al., 2002; Shapiro et al., 2003), matrix metalloprotease inhibitors (Pemberton et al., 2005), α1-antitrypsin (Churg et al., 2003; Pemberton et al., 2006), curcumin (Suzuki et al., 2009), and overexpression of CuZnSOD (Foronjy et al., 2006), which all prevent CS-induced inflammation and emphysema. However, not all of the useful treatments in animal models have been used in humans. Actually, no one animal species can accurately model all features of human disease situations and humans normally get treated when they have more severe and sophisticated COPD than used experimentally in animal models. Current COPD animal models concentrate on emphysema rather than small airway remodeling, the key cause of airflow obstruction. It should be noted that small airway remodeling and emphysema are independent symptoms of CS that could co-exist in COPD patients.

CONCLUSIONS

CS/SHS induces airway mucus hypersecretion and inflammation through various chemical components and signaling pathways. The significantly increased mucus production and secretion result in excess sputum that may promote airflow obstruction and inflammation. CS/SHS exposure also results in composition changes in airway secretion and impairment of cilia beating that are critical to a normal MCC. Increased mucin production, decreased luminal liquid, and impaired cilia beating motion in COPD have deleterious consequences for airway health, including mucus stasis and airway infection. CS/SHS-impaired MCC results in the increased infection rate and disease severity that could be associated with COPD exacerbations.

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A history of second hand smoke exposure: are we asking the right questions?

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This commentary is written to accompany a special research topic, *Second Hand Smoke and COPD: lessons from animal studies*, hosted by *Frontiers in Respiratory Physiology.*

Model systems allow researchers to study disease, to tease out cause and effect and mechanisms of action, and to conduct the preliminary studies of the safety and efficacy of treatments. The underlying objective of the research is, of course, to improve healthcare outcomes. Pointing out the difficulty of studying a disease that takes decades to develop in humans, this collection of articles focuses on what has been learned from studies using models of COPD. It also presents an opportunity to circle back to the implications of the research and to ask a most basic question: do healthcare providers recognize the impact of second hand smoke (SHS) exposure on health and, if they do, are they asking the right questions?

The 2006 Surgeon General's Report, "Health Consequences of Involuntary Exposure to Tobacco Smoke" (Surgeon General, 2006) documents the health implications of exposure to SHS, including firm evidence that SHS contributes to coronary and lung disease, lung cancer, premature death in adults, slow lung development, SIDS, asthma, and ear infections in children, as well as suggestive evidence that implicate SHS in COPD, asthma, breast cancer, and nasal sinus cancer in adults, and leukemia, lymphoma, and brain tumors in children. The report indicates that there is no risk-free level of SHS.

Despite evidence that SHS is a risk factor for disease, most healthcare organizations and many physicians fail to ask patients about their history of SHS exposure. The implications of that failure are considerable because knowledge of a patient's history of SHS exposure enables providers to make better-informed decisions about what to include in each patient's examination and lab tests, and how to conduct long-term monitoring, as well as alerting the patient to the need for measures to help them avoid further smoke exposure.

An example of a medical history of SHS exposure on health status can be seen in the development of lung disease in flight attendants, a group who historically worked in SHS filled aircraft. Recent research has revealed decreased exercise tolerance, decreased diffusing capacity with decreased pulmonary capillary recruitment as well as air trapping and airway obstruction in never-smoking flight attendants who were exposed prior to the ban on smoking in aircraft (Arjomandi et al., 2009, 2012). US aircraft became smoke-free by 1990 for flights less than 6 h, and by 2000 all US carriers were required to be smoke-free. These flight attendants, despite never smoking, have evidence of lung disease 10–20 years after their SHS exposure. For patients with a history of SHS exposure, failure of providers to include the right questions may leave risks hidden and opportunities for early intervention lost.

The American Academy of Pediatrics (AAP) has pioneered efforts to encourage physicians to ask the right questions regarding the exposure of children to SHS. Recognizing that SHS is a major contributor to childhood morbidity and mortality, the AAP has made asking about SHS exposure an essential part of regular pediatric care. This practice of asking the right questions could serve as a model for healthcare providers worldwide.

Prevention of disease progression due to SHS of course has clear implications for patients' suffering and their ability to continue to function in society. SHS exposure also has significant financial implications for healthcare payers. As the health care system in the US moves from a fee-for-service to a fee-for-results structure, prevention of disease and promotion of wellness become much more important. Early recognition of the presence of risk factors for the development of tobacco smoke-related disease enables intervention before more severe conditions arise; from the most pragmatic perspective, advanced disease is simply more costly to treat.

The importance of asking about SHS exposure is not limited to clinical care. Researchers too can improve study design and outcomes when they recognize the impact of SHS exposure. In human studies that look for risk factors for disease, the failure to ask about an individual's history of SHS exposure may lead to confusing or less significant results. For instance, when looking for the impact of smoking on cancer incidence, osteoporosis, or lung disease, placing those who have been exposed to SHS in the category "non-smoker," as many studies have done, simply makes no sense.

Additional reason for asking about a history of SHS exposure is suggested by the links between Vitamin D deficiency and the development of COPD. Our own laboratory has recently focused on the impact of Vitamin D deficiency on the development and progression of SHS-related emphysema in a mouse model. Epidemiologic studies by Janssens and colleagues demonstrated low serum levels of 25-hydroxyvitamin D in advanced stage COPD patients, as well as an increased incidence of COPD in individuals who carry a mutation in one or more of the genes involved in vitamin D metabolism (Janssens et al., 2009a, 2011). Our model of cigarette smoke exposure with vitamin D deficiency supports the hypothesis that low levels of 25-hydroxyvitamin D may contribute to the severity of SHS-related

emphysema. Given the multiple roles of vitamin D in infection, cardiovascular disease, and osteoporosis (all comorbidities of COPD), it seems prudent for health care providers to screen for vitamin D levels in at-risk patients (Holick, 2004; Lee et al., 2008; Janssens et al., 2009b). Without knowing a patient's history of SHS exposure, vitamin D screening might not be considered.

COPD is the third leading cause of death worldwide. While lung damage from COPD is irreversible, recognition of the early stages of the disease allows providers to intervene so as to slow or arrest disease progression. While smoking is widely acknowledged as a risk factor, the danger of SHS exposure is significantly underrecognized. Since the initial symptoms of COPD are hard to detect, increased awareness of SHS exposure as a risk factor should alert caregivers to screen for early signs of disease. This would give them an earlier indicator of increased disease risk and provide crucial additional time in which to mitigate the onset and progression of disease. Counseling to avoid smoke exposure, monitoring for early evidence of changes in lung function, vitamin D monitoring, and early inclusion of interventions such as exercise, respiratory training, and mind-body practices such as Qigong or Tai Chi, can all be part of a strategy to control the disease process.

There is an ongoing emphasis on improving outcomes and lowering costs of health care. The AAP has taken the lead in encouraging a simple and inexpensive approach to lowering the impact of SHS exposure: simply talking to patients about it and taking it into account when considering the patient's overall health. It's a winning strategy; asking about the history of SHS exposure as part of the course of regular care would give providers greater opportunities for early intervention and patients opportunities for improved long-term health.

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