CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Repeated Exposure to *Streptococcus pneumoniae* Exacerbates Chronic Obstructive Pulmonary Disease

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Accepted for publication
May 23, 2019.

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Streptococcus pneumoniae is commonly found in patients with chronic obstructive pulmonary disease (COPD) and is linked to acute exacerbation of COPD. However, current clinical therapy neglects asymptomatic insidious *S. pneumoniae* colonization. We studied the roles of repeated exposure to *S. pneumoniae* in COPD progression using a mouse model. C57BL/6J mice were intranasally inoculated with *S. pneumoniae* ST262 every 4 weeks with or without cigarette smoke (CS) exposure up to 20 weeks to maintain persistent *S. pneumoniae* presence in the lower airways. *Streptococcus pneumoniae* enhanced CS-induced inflammatory cell infiltration at 12 to 20 weeks of exposure. *Streptococcus pneumoniae* also increased CS-induced release of inflammatory cytokines, including IL-1 β , tumor necrosis factor- α , IL-12 (p70), and IL-5 at 20 weeks of exposure. Moreover, a combination of CS and *S. pneumoniae* caused alveolar epithelial injury, a decline in lung function, and an increased expression of platelet-activating factor receptor and bacterial load. Our results suggest that repeated exposure to *S. pneumoniae* in lower airways exacerbates CS-induced COPD. (*Am J Pathol* 2019, 189: 1711–1720; <https://doi.org/10.1016/j.ajpath.2019.05.012>)

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide over the past decade. COPD is characterized by persistent and progressive airflow limitation due to obstructive bronchiolitis and emphysema. Chronic inflammatory response in the airways to cumulative irritants, especially cigarette smoke (CS), plays a key role in the pathogenesis of COPD.¹ Given that bacterial infection triggers acute exacerbation of respiratory symptoms, the use of antibiotics is incorporated into treatments for infectious exacerbation of COPD. However, current clinical therapy neglects asymptomatic insidious bacterial colonization.

The most commonly colonized bacteria in stable-state COPD patients are nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*.^{2,3} Although 30% to 70% of stable COPD patients are colonized by bacteria, as determined by cell culture,^{4,5} the contribution of bacterial colonization in the lower airways to the clinical course of COPD has not been fully elucidated

because of the lack of longitudinal prospective studies. Some studies indicate that bacterial colonization may contribute to the progression of COPD, independent of acute exacerbation. In stable-state COPD, bacterial colonization has been shown to associate with neutrophilic inflammation in airways,^{2,3} and the bacterial load is positively associated with a decline of forced expiratory volume in 1 second.⁶

Several studies use animal models with CS exposure and bacterial infections to address COPD exacerbation and the effects of bacteria on COPD progression. Exposure to CS, followed by challenge with nontypeable *H. influenzae*,

Supported by NIH grants HL135152, AI121591, and GM103648; the Oklahoma Center for Adult Stem Cell Research-A Program of Tobacco Settlement Endowment Trust (TSET); and the Lundberg-Kienlen endowment fund (L.L.).

X.G. and Q.Z. contributed equally to this work.

Disclosures: None declared.

exacerbates innate and adoptive immune response.^{7,8} These studies do not mimic the effects of long-term bacterial presence during stable disease periods. Another study has shown that nontypeable *H. influenzae* exacerbates COPD induced by the exposure to CS.⁹ However, this study used heat-killed nontypeable *H. influenzae*, a relatively short-term CS exposure, and a single time point (2 months).

Herein, we investigated the effects of long-term (20-week) repeated exposure to *S. pneumoniae* in the lower airways on the progression of CS-induced COPD at 4-week intervals. Because a model of repeated exposure to *S. pneumoniae* in the lungs has been successfully developed,¹⁰ *S. pneumoniae* were used in this study. Repeated exposure to *S. pneumoniae* exacerbated the CS-induced COPD, characterized by more severe COPD features, including increased inflammatory cells and mediators along with corresponding changes in lung functions, in comparison with mice exposed only to CS. Moreover, repeated exposure to *S. pneumoniae* increased alveolar epithelial type I cell injury. More important, these changes did not occur in mice with repeated exposure to *S. pneumoniae* that were not exposed to CS. Collectively, these data provide evidence for the role of repeated exposure to *S. pneumoniae* in the progression of CS-induced COPD in mice.

Materials and Methods

S. pneumoniae Culture

The *S. pneumoniae* strain ST262 was purchased from ATCC (number 49619; Manassas, VA). Bacteria were cultured on blood agar plates (Thermo Scientific, Waltham, MA) or in Todd Hewitt Broth (Sigma-Aldrich, St. Louis, MO). To construct a linear regression curve of OD versus colony-forming units, bacteria cultured in Todd Hewitt Broth were serially diluted and the culture OD600 values were determined. Bacteria were then plated onto blood agar plates and incubated at 37°C overnight. The colonies on plates were counted and correlated with the OD value. To prepare *S. pneumoniae* for mouse studies, bacteria were inoculated in Todd Hewitt Broth, and the culture was incubated with shaking at 37°C to an OD600 value of 0.2 to 0.8. The bacteria concentration was deduced from the pre-determined regression equation and then adjusted to a density of 5×10^6 colony-forming units/mL by suspending bacteria in phosphate-buffered saline (PBS; Gibco, Carlsbad, CA). Mice were instilled via both nares with 30 μ L of *S. pneumoniae* or PBS every 4 weeks.

Cigarette Smoke Exposure and Repeated Exposure to *S. pneumoniae*

Female 12-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were divided into the following four groups: room air (RA), cigarette smoke (CS), *S. pneumoniae*, and combined CS and *S. pneumoniae*. Each group contained six

Figure 1 Body weight changes in *Streptococcus pneumoniae*— and cigarette smoke (CS)—exposed mice. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. The weight changes are presented as a percentage of the initial mouse weight. The initial weights in the RA, CS, *S. pneumoniae*, and combined CS and *S. pneumoniae* groups were 19.1, 19.3, 19.2, and 19.0 g, respectively. Data are expressed as means $\pm$ SEM. At each time point from the 3rd to 20th weeks, $n = 30$, $n = 30$, $n = 30$, $n = 24$, $n = 18$, $n = 12$, and $n = 6$, respectively, in the RA group; $n = 40$, $n = 40$, $n = 40$, $n = 34$, $n = 21$, $n = 14$, and $n = 8$, respectively, in the *S. pneumoniae* group; $n = 30$, $n = 30$, $n = 24$, $n = 18$, $n = 12$, and $n = 6$, respectively, in the CS group; and $n = 48$, $n = 48$, $n = 48$, $n = 39$, $n = 28$, $n = 17$, and $n = 10$, respectively, in the combined CS and *S. pneumoniae* group. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus the RA group in the corresponding week (two-way analysis of variance, followed by Tukey multiple-comparisons test); ††† $P < 0.001$, †††† $P < 0.0001$ versus the CS group in the corresponding week (two-way analysis of variance, followed by Tukey multiple-comparisons test).

mice at each time point. Mice were exposed to CS generated from 3R4F research cigarettes (University of Kentucky, Lexington, KY; 11% mainstream and 89% sidestream; total suspended particulate, 140 μ g/L) or RA for 5 hours/day and 5 days/week up to 20 weeks using a Teague 10E whole body exposure chamber.¹¹ Mice were intranasally inoculated with *S. pneumoniae* (1×10^6 colony-forming units) monthly starting on the fourth week. Mice were monitored for changes in body weight and lung functions every 4 weeks. The left lungs were then lavaged with 0.8 mL of PBS twice, and the collected lung tissue was homogenized in 0.8 mL of PBS. The right lungs were fixed in 10% formalin (Thermo Scientific) and then immersed in fixative solution for histologic analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Stillwater, OK).

Lung Function Measurements

Lung function was measured using a flexiVent system (Scireq, Montreal, QC, Canada). Mice were intraperitoneally injected with 0.004 to 0.006 mL/g body weight of an anesthetic cocktail containing 25 mg/mL ketamine and 2.5 mg/mL xylazine. The trachea was cannulated and connected to a computer-controlled ventilator. Five parameters were automatically measured by the software, including the ratio of forced expiratory volume in 0.1 seconds/forced vital

Figure 2 Repeated exposure to *Streptococcus pneumoniae* increases cigarette smoke (CS)—induced infiltration of inflammatory cells. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. Inflammatory cells were determined every 4 weeks. Total cells (A), macrophages (B), neutrophils (C), and lymphocytes (D) are shown. Data are expressed as means $\pm$ SEM. $n = 6$ animals (A–D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus the RA group in the corresponding week (one-way analysis of variance, followed by Tukey multiple-comparisons test); † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.0001$ versus the CS group in the corresponding week (one-way analysis of variance, followed by Tukey multiple-comparisons test).

capacity (FVC), inspiratory capacity, compliance, FVC, and pressure-volume (PV) loop area.

Inflammatory Cell Counts

Bronchoalveolar lavage (BAL) was centrifuged at $1150 \times g$ for 10 minutes, and the supernatant [BAL fluid (BALF)] was stored at -80°C for further analysis. The cell pellet was suspended in 200 μL of PBS, and the total cell number was counted using an automated cell counter (Bio-Rad Laboratories, Hercules, CA). The suspension was spun at $55 \times g$ for 5 minutes in a cytocentrifuge (Beckman Coulter, Brea, CA) and stained with Wright-Giemsa Stain (Electron Microscopy Sciences, Hatfield, PA) to enumerate the differentiated cell populations, including macrophages, neutrophils, and lymphocytes.

Bacterial Load

BAL and lung tissue homogenates were inoculated on blood agar plates and incubated overnight at 37°C . The bacterial colonies were counted and depicted as the number of

colony-forming units per milliliter of BAL or lung tissue homogenate.

Histologic Analysis

The fixed lungs were dehydrated and embedded in paraffin. Tissue sections (4 μm thick) were cut and stained with hematoxylin and eosin to quantify emphysema with the mean linear intercept, as previously described.¹² Briefly, five lines were drawn on each image; two lines connected opposite vertices, two bisected the opposite vertices, and one was placed at a random position. The mean linear intercept was calculated by dividing the length of the line by the total number of alveolar intercepts for that line to yield the mean length between two alveolar interfaces. Twenty images of representative fields per mouse were obtained for quantification.

T1 α and Cytokine Measurements

The levels of T1 α in BALF were measured by enzyme-linked immunosorbent assay using a Mouse Podoplanin

Table 1 Effects of *Streptococcus pneumoniae* and CS on Cytokine Levels in BALFs

Cytokine	Concentration, pg/mL			
	RA	<i>S. pneumoniae</i>	CS	Combined CS and <i>S. pneumoniae</i>
IL-1 α	8.89 $\pm$ 2.57	5.68 $\pm$ 3.73	0.95 $\pm$ 0.12 [§]	2.35 $\pm$ 0.17
IL-1 β	0	3.00 $\pm$ 2.95 [§]	0	14.42 $\pm$ 1.98 ^{**,††,‡,§}
IL-2	3.19 $\pm$ 1.22 [§]	2.24 $\pm$ 2.17 [§]	0.03 $\pm$ 0.03 [§]	0.04 $\pm$ 0.03 [§]
IL-3	0	0.19 $\pm$ 0.19 [§]	0.36 $\pm$ 0.35 [§]	0.58 $\pm$ 0.11 [§]
IL-4	2.35 $\pm$ 0.03 [§]	2.66 $\pm$ 0.07 [§]	2.90 $\pm$ 0.34 [§]	3.10 $\pm$ 0.04 [§]
IL-5	0.25 $\pm$ 0.05 [§]	1.07 $\pm$ 0.38 [§]	0.92 $\pm$ 0.11 [§]	2.99 $\pm$ 0.15 ^{***,†††,‡†}
IL-6	0.19 $\pm$ 0.10 [§]	0.34 $\pm$ 0.22 [§]	0.39 $\pm$ 0.17 [§]	1.08 $\pm$ 0.12 ^{*,†}
IL-9	0	0	0	0
IL-10	0	0.61 $\pm$ 0.61 [§]	0.69 $\pm$ 0.29 [§]	3.11 $\pm$ 0.23 ^{**,††,‡†,§}
IL-12 (p40)	1.28 $\pm$ 0.11 [§]	14.61 $\pm$ 5.22	28.31 $\pm$ 2.45	13.61 $\pm$ 0.48 ^{***,††,‡†}
IL-12 (p70)	0	1.73 $\pm$ 1.73 [§]	0	9.20 $\pm$ 1.15 ^{***,†††,‡†,§}
IL-13	41.06 $\pm$ 6.00	21.88 $\pm$ 19.33 [§]	1.65 $\pm$ 1.07 [§]	12.50 $\pm$ 2.06
IL-17 α	0.80 $\pm$ 0.04 [§]	2.42 $\pm$ 0.51 [§]	3.61 $\pm$ 0.50 ^{**}	4.85 $\pm$ 0.23 ^{***,††}
Eotaxin	0	0	0	0
G-CSF	1.07 $\pm$ 0.04 [§]	1.45 $\pm$ 0.14 [§]	5.22 $\pm$ 0.73 [§]	1.77 $\pm$ 0.07 ^{***,††,§}
GM-CSF	0	0	0	0
IFN- γ	0.13 $\pm$ 0.10 [§]	0.36 $\pm$ 0.35 [§]	0.13 $\pm$ 0.10 [§]	0.57 $\pm$ 0.36 [§]
KC	0.53 $\pm$ 0.27 [§]	2.17 $\pm$ 1.03 [§]	8.93 $\pm$ 1.08 ^{***,†††}	4.22 $\pm$ 0.25 [*]
MCP-1	0	38.38 $\pm$ 38.38 [§]	7.05 $\pm$ 4.15 [§]	29.57 $\pm$ 2.30 [§]
MIP-1 α	0	0	0	0
MIP-1 β	3.29 $\pm$ 1.08	4.24 $\pm$ 1.17	2.45 $\pm$ 0.58	6.52 $\pm$ 0.61 [†]
RANTES	0.24 $\pm$ 0.08 [§]	0.23 $\pm$ 0.17 [§]	0	0.22 $\pm$ 0.06 [§]
TNF- α	5.48 $\pm$ 3.49 [§]	13.70 $\pm$ 7.22 [§]	1.20 $\pm$ 0.93 [§]	24.10 $\pm$ 3.52 ^{†,§}

Data are expressed as means $\pm$ SEM. Mice were exposed to CS or RA and/or inoculated every 4 weeks with *S. pneumoniae* (*S. pneumoniae* and combined CS and *S. pneumoniae* groups). Cytokines in BALFs were determined at the 20th week. One-way analysis of variance, followed by Tukey multiple-comparisons test, was performed. $n = 3$ RA; $n = 4$ *S. pneumoniae*, CS, and combined CS and *S. pneumoniae*.

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus RA.

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus CS.

‡ $P < 0.05$, ‡† $P < 0.01$, and ‡†† $P < 0.001$ versus *S. pneumoniae*.

§At least two values are out of range from standard curves.

0, no reading; BALF, bronchoalveolar lavage fluid; CS, cigarette smoke; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; KC, keratinocyte chemoattractant; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RA, room air; RANTES, regulated on activation normal T cell expressed and secreted; TNF, tumor necrosis factor.

ELISA Kit (MyBioSource, San Diego, CA), according to the manufacturer's instructions. Briefly, the samples were placed onto T1 α antibody-coated plates and incubated with horseradish peroxidase-conjugated secondary antibodies for 60 minutes at 37°C. After washing, the reaction was developed with chromogen reagent for 15 minutes at 37°C. The absorbance was read at 450 nm. A standard curve was prepared using serial dilutions of the standard (1000, 500, 250, 125, 62.5, 31.2, and 0 pg/mL). The protein levels of T1 α were calculated from the linear standard curve of best fit.

Cytokines in BALF were detected using the Mouse Cytokine Group 1 Panel 23-Plex kit on a Bio-Plex multiplex system (Bio-Rad Laboratories), which allows simultaneous detection of multiple cytokines from a single well of a 96-well microplate. Capture antibodies (50 μ L) covalently coupled to magnetic beads were added to each well. After two washes, 100 μ L of each sample was loaded in duplicate onto the plates, and the plates were incubated with shaking

for 30 minutes at room temperature in the dark. After a series of washes, 25 μ L of a biotinylated detection antibody was added to generate a sandwich complex. Finally, the complex was developed with the addition of 50 μ L of streptavidin-phycoerythrin conjugate and phycoerythrin, which acted as a fluorescent reporter. The cytokine concentration was determined using Bio-Plex Manager software version 6.1 (Bio-Rad Laboratories).

Immunostaining

Paraffin-embedded mouse lung sections were deparaffinized. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) by heating in a microwave for 10 minutes. Sections were blocked with 10% goat serum and 1% bovine serum albumin in PBS for 2 hours at room temperature and incubated with monoclonal anti-platelet-activating factor receptor (PAFr) antibodies (Cayman Chemical, Ann Arbor, MI; catalog number 160600; 1:50

Figure 3 Cigarette smoke (CS) exposure enhances bacterial burden in bronchoalveolar lavage (BAL) and lung tissue. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *Streptococcus pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. Bacterial loads in BAL (A) and lung tissue (B) were measured every 4 weeks before each *S. pneumoniae* inoculation. Data are expressed as means $\pm$ SEM. $n = 6$ (A and B). * $P < 0.05$ versus the *S. pneumoniae* group (t -test). CFU, colony-forming unit.

dilution) at 4°C for 16 hours. Sections were then incubated with horseradish peroxidase–conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch Inc., West Grove, PA; 1:200 dilution) at room temperature for 60 minutes. Sections were developed with DAB HRP Substrate Kit (Vector Laboratories, Burlingame, CA). PAFr expression in the alveolar region was quantified as the percentage of the PAFr-positive cells over total cells.

Statistical Analysis

The data are presented as the means $\pm$ SEM. A t -test was used to compare data between two groups, and one- or two-way analysis of variance, followed by Tukey multiple-comparisons test, was used to compare data for multiple groups. $P < 0.05$ was considered statistically significant.

Results

Body Weight Changes

To determine the effects of long-term repeated exposure to *S. pneumoniae* on the progression of COPD, mice were exposed to RA or CS for 20 weeks and *S. pneumoniae* were intranasally administered to mice every 4 weeks in room

air (*S. pneumoniae*) and CS (combined CS and *S. pneumoniae*). The body weights were monitored. CS induced body weight reductions from the beginning of exposure. Although mice recovered from the fourth week of CS exposure, the average body weight remained significantly lower than that in the RA group. During the early stage of exposure, the combination of *S. pneumoniae* and CS did not make a difference in body weight. However, after 16 weeks of exposure, *S. pneumoniae* induced a significant decrease of body weight in CS-exposed mice, which indicated that CS-induced body weight loss was enhanced by repeated exposure to *S. pneumoniae* (Figure 1).

Repeated Exposure to *S. pneumoniae* Exacerbates CS-Induced Inflammation

Inflammatory cells contribute to the progression of COPD by secreting a spectrum of chemotactic factors and proinflammatory cytokines.¹³ To assess whether long-term repeated exposure to *S. pneumoniae* in the lower airways exacerbates inflammation induced by CS exposure, the total cells and differentiated cell populations were enumerated. Mice exposed to CS showed significantly higher counts of total inflammatory cells than those in the RA groups beginning at the fourth week of CS exposure. After 12 weeks of exposure, the total cells were further increased in the presence of *S. pneumoniae* compared with mice only exposed to CS. However, mice only inoculated with *S. pneumoniae* did not show significantly increased inflammatory cells (Figure 2A). Among the total inflammatory cells, macrophages play an important role in the pathogenesis of COPD. A markedly increased number of macrophages was elicited by CS and further enhanced by repeated exposure to *S. pneumoniae* (Figure 2B). Both neutrophils and lymphocytes were significantly higher in the lower airways of CS-exposed mice. In the presence of *S. pneumoniae*, these two populations were significantly increased compared with CS-only mice beginning at the eighth week. However, mice inoculated with *S. pneumoniae* also demonstrated increased neutrophil and lymphocyte populations with 8- and 12-week CS exposures (Figure 2, C and D).

Using a Bio-Plex multiplex system, cytokine levels were also measured in BALF at the 20th week. Significant increases were observed in the levels of inflammatory cytokines, IL-1 β , IL-5, IL-10, IL-12 (p70), macrophage inflammatory protein 1 β , and tumor necrosis factor- α in BALF from mice exposed to CS with repeated exposure to *S. pneumoniae* compared with the CS group (Table 1).

CS Exposure Enhances Bacterial Burden in BAL and Lung Tissue

Monthly inoculation of *S. pneumoniae* maintained persistent presence of viable bacteria in the lower airways. The amount of bacteria was similar in BAL and lung tissue homogenates. Similar amounts of bacteria were recovered

Figure 4 Repeated exposure to *Streptococcus pneumoniae* exacerbates cigarette smoke (CS)—induced alterations of lung function. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. Lung function was assessed every 4 weeks using the flexiVent System. Forced expiratory volume in 0.1 seconds (FEV_{0.1})/forced vital capacity (FVC) ratio (A), compliance (Cst; B), inspiratory capacity (IC; C), FVC (D), and pressure-volume (PV) loop (E) are shown. Data are expressed as means $\pm$ SD. $n = 6$ (A–E). * $P < 0.05$ versus the RA group in the corresponding week (one-way analysis of variance, followed by Tukey multiple-comparisons test); † $P < 0.05$ versus the CS group in the corresponding week (one-way analysis of variance, followed by Tukey multiple-comparisons test).

from the *S. pneumoniae* and combined CS and *S. pneumoniae* groups before the 16th week. Afterward, mice that were inoculated with *S. pneumoniae* and also exposed to CS demonstrated a significantly higher bacterial burden in the lungs than did the *S. pneumoniae* group (Figure 3).

Repeated Exposure to *S. pneumoniae* Exacerbates CS-Induced Alterations in Lung Function

In comparison with mice from the RA or *S. pneumoniae* groups, CS exposure induced progressive alterations in lung function. The alterations were more dramatic in the combined CS and *S. pneumoniae* group. From the eighth week of exposure, the forced expiratory volume in 0.1 seconds/FVC ratio gradually declined; and at the 16th week, the forced expiratory volume in 0.1 seconds/FVC ratio in the combined CS and *S. pneumoniae* group was 65.5%, which was less than the diagnostic standard for COPD in the clinic, 70%. At the 20th week, the forced expiratory volume in 0.1 seconds/FVC ratio values in CS-exposed mice either with or

without *S. pneumoniae* were 68.4% and 62.0%, respectively (Figure 4A). Progressive increases were also observed in compliance beginning at the eighth week of exposure, and these increases were significantly augmented by repeated exposure to *S. pneumoniae* at the 20th week (Figure 4B). In addition, the inspiratory capacity was increased with CS exposure. Repeated exposure to *S. pneumoniae* furthered the increase observed beginning at the 16th week (Figure 4C). Because of the high air volume trapped in the lungs, a gradual increase was observed in FVC and the pressure-volume loop area induced by CS exposure. Repeated exposure to *S. pneumoniae* significantly enhanced the alterations in FVC and the pressure-volume loop area in mice after 20 weeks of CS exposure (Figure 4, D and E).

Repeated Exposure to *S. pneumoniae* Has No Effects on CS-Induced Alveolar Airspace Enlargement

CS-induced alveolar epithelium injury resulted in the loss of alveolar attachments to the small airway and eventual

Figure 5 Effect of repeated exposure to *Streptococcus pneumoniae* on cigarette smoke (CS)-induced emphysema. Mice were exposed to CS or room air (RA) for 20 weeks and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. **A:** Hematoxylin and eosin staining. Representative images of each group are presented. **B:** The airspace enlargement was quantified with the mean linear intercept (MLI). $n = 6$ (**B**). ** $P < 0.01$ versus the RA group (one-way analysis of variance, followed by Tukey multiple-comparisons test). Scale bars = 100 μm (**A**).

rupture of the alveoli. At the 20th week, significantly enlarged airspaces were observed in both the CS and combined CS and *S. pneumoniae* groups, whereas RA-exposed or *S. pneumoniae*-challenged mice showed baseline airspace size (Figure 5). However, the CS-induced alveolar airspace enlargement was not enhanced by repeated exposure to *S. pneumoniae*.

Repeated Exposure to *S. pneumoniae* Exacerbates CS-Induced Alveolar Epithelial Type I Cell Damage

To detect injury to alveolar epithelial type I cells induced by CS exposure, T1 α released into BALF was measured by enzyme-linked immunosorbent assay. At the fourth week, the levels of T1 α were not changed by CS exposure. After 8 weeks, the T1 α concentration was significantly higher in the CS group than in the RA group. After 16 weeks, the CS and combined CS and *S. pneumoniae* groups showed significantly higher T1 α concentrations than mice exposed to RA.

After 20 weeks of exposure, *S. pneumoniae* significantly accelerated the T1 α levels induced by CS (Figure 6).

Repeated Exposure to *S. pneumoniae* Enhances CS-Induced PAFr Expression

PAFr is a key adhesion receptor for *S. pneumoniae* in airway cells.¹⁴ The effects of CS on PAFr expression were examined in the lung using immunostaining. *Streptococcus pneumoniae* or CS increased the PAFr expression in the large airway epithelial cells, which was further enhanced by a combination of CS and *S. pneumoniae* (Figure 7A). Similar observations were found in alveolar regions (Figure 7B). Moreover, the PAFr-positive cells in alveoli were increased by CS, which was again further enhanced by combined CS and *S. pneumoniae* (Figure 7C).

Discussion

Asymptomatic bacteria may have a substantial influence on stable-state COPD patients in the absence of acute exacerbation. However, antibiotic therapy is not recommended in stable-state COPD patients because the contribution of these bacteria to the progression of COPD and the underlying mechanisms are not well understood. In this study, long-term bacterial colonization was mimicked by inoculating mice with *S. pneumoniae* every 4 weeks and COPD progression by exposing mice to CS daily. The inflammatory profile, structural changes, and functional impairment at regular intervals were monitored during disease progression over 20 weeks. A significant association between long-term repeated exposure to *S. pneumoniae* was demonstrated in the lower airways and progression of CS-induced COPD.

The upper airways of the normal human respiratory track are colonized by commensal and opportunistic

Figure 6 A combination of *Streptococcus pneumoniae* and cigarette smoke (CS) increases T1 α release into alveoli. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. The protein levels of T1 α in bronchoalveolar lavage were detected using enzyme-linked immunosorbent assay. $n = 6$. * $P < 0.05$ versus the RA group (one-way analysis of variance, followed by Tukey multiple-comparisons test); † $P < 0.05$ versus the CS group (one-way analysis of variance, followed by Tukey multiple-comparisons test).

Figure 7 Repeated exposure to *Streptococcus pneumoniae* enhances cigarette smoke (CS)-induced platelet-activating factor receptor (PAFr) expression. Mice were exposed to CS or room air (RA) and/or inoculated every 4 weeks with *S. pneumoniae* [*S. pneumoniae* (SP) and combined CS and *S. pneumoniae* (CSSP) groups]. PAFr expression was determined by immunostaining. **A** and **B**: Representative images of each group are presented. **C**: Quantification of PAFr-positive cells in alveoli. $n = 6$ (**C**). $*P < 0.05$, $***P < 0.001$ versus the RA group (one-way analysis of variance, followed by Tukey multiple-comparisons test). Scale bars = 50 μm (**A** and **B**).

microorganisms, whereas the lower airways are sterile. However, in both stable-state and exacerbation-phase COPD patients, bacteria are found in the lower airways, particularly nontypeable *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*.^{2,3} The percentage of stable-state COPD patients with bacteria found in the lower airways varies, depending on the techniques for sampling and detection. Using bacterial culture, 38% to 74% were positive for potentially pathogenic microorganisms in sputum samples, which are likely contaminated with upper airway microorganisms; lower numbers, 23% to 31% and 33% to 43%, were found in the samples from bronchoscopic protected specimen brush and bronchial or bronchoalveolar lavage, respectively.^{4,5} Using a more sensitive PCR technique and 18S sequencing, higher bacterial load and more species were found in sputum of stable COPD patients.^{15–17} Using BAL or sputum, bacterial counts were positively associated with neutrophil counts, IL-8 level, and matrix metalloproteinase 9 level,^{2,3} and a decline in lung functions,^{6,15} in stable COPD patients.

Innate and adaptive immunity in the airway lumen is one of the major contributors to the pathogenesis of COPD, whereas the role of airway wall inflammation is debatable.^{18,19} In the current study, CS-exposed mice exhibited a chronic inflammatory profile in BAL, marked by high accumulation of inflammatory cells, including macrophages, neutrophils, and lymphocytes, compared with mice exposed to RA. In contrast, mice only inoculated with *S. pneumoniae* maintained a low level of inflammatory cells. Of importance, the CS-induced inflammatory cell infiltration was further enhanced by repeated exposure to *S. pneumoniae* from the 12th to the 20th week. However, this enhancement was not observed in the mice with exposure of ≤ 8 weeks. These results were consistent with a previous study in which mice were infected with respiratory syncytial virus and exposed to CS for 24 weeks, although only one time point (24 weeks) was analyzed.²⁰ In another study, mice were

exposed to heat-killed nontypeable *H. influenzae* and CS for 8 weeks at the same time, and an increase in inflammatory cells was observed in the nontypeable *H. influenzae* and CS group compared with the CS group.⁸ The discrepancy is likely due to the use of different strains of bacteria and/or conditions for CS exposure. In two other studies that mimicked the exacerbation of COPD, mice were first exposed to CS for 8 or 4 weeks and then to nontypeable *H. influenzae* for 12 hours or 8 weeks, respectively; in both cases, an increased inflammatory response was observed.^{8,16} In another study, mice were exposed to CS for 3 or 7 months, followed by intranasal inoculation with nontypeable *H. influenzae* or *S. pneumoniae* for 24 hours or 7 days, respectively. CS increased the presence of both bacteria in upper airways with enhanced inflammation. Furthermore, an increased translocation of *S. pneumoniae* into the lung was observed.²¹ These results support a role of repeated bacterial exposure in the lower airways in chronic airway inflammation.

Proinflammatory mediator levels in BAL, including IL-1 β , tumor necrosis factor- α , and macrophage inflammatory protein 1 β , were significantly higher in the combined CS and *S. pneumoniae* group than in the other groups at the 20th week. In addition, increased IL-12, which is known to induce CD4⁺ T-cell differentiation,²² was observed in BAL from the combined CS and *S. pneumoniae* group. Likewise, a high presence of type 2 helper T-cell-derived cytokines and IL-5 was observed in the same group. This up-regulation of inflammatory mediators is consistent with the influx of inflammatory cells to the alveoli.

The combination of CS with repeated exposure to *S. pneumoniae* in mice also led to the full profile of lung dysfunction in mice, reflecting the chronic progression of this disease. Furthermore, CS-exposed mice displayed a significantly enlarged airspace in comparison with those exposed only to RA or challenged by *S. pneumoniae*. However, the combination with *S. pneumoniae* did not

enhance the CS-induced airspace enlargement. At the later stage (20th week), alveolar epithelial damage was evident, as demonstrated by an increase in T1 α , an alveolar epithelial type I cell marker, in BAL of the combined CS and *S. pneumoniae* group. These data indicate that long-term repeated exposure to *S. pneumoniae* in lower airways exacerbates COPD.

Streptococcus pneumoniae was consistently recovered from BAL and lung tissue homogenates in bacteria-treated mice. Although the levels of macrophages and neutrophils were increased, the bacterial burden showed a steep incline in CS-exposed mice with *S. pneumoniae* challenge at late stages of exposure (16th and 20th weeks). One possible explanation is that CS impairs bacterial clearance. CS exposure diminishes the normal function of inflammatory cells that protect against *S. pneumoniae*.²³ Another explanation is that CS increases bacterial adhesion receptor expression in the airway epithelium. PAFr is a G-protein-coupled PAF receptor that has been shown to be a key receptor for respiratory bacteria, including *S. pneumoniae*.¹⁴ CS increases the adhesion of *S. pneumoniae* and nontypeable *H. influenzae* to lower airway cells in a PAFr-dependent manner *in vitro*.^{24,25} Furthermore, increased PAFr expression has been observed in airway cells of COPD.²⁶ In the current study, increased PAFr protein expression in small airway epithelial cells and an increased percentage of PAFr-positive cells in alveolar epithelium in the CCSP group were observed, suggesting that increased bacterial loads in the combined CS and *S. pneumoniae* group are likely due to increased adhesion of *S. pneumoniae* to airway epithelium.

In addition to luminal innate immune activation, there is increased evidence suggesting that gene reprogramming in airway epithelium occurs in COPD, which results in fibrosis and malignancy, such as epithelial-to-mesenchymal transition.^{27–29} Cellular senescence and oxidative stress are two other contributing factors to the pathophysiology of COPD.¹ Whether CS and repeated exposure to *S. pneumoniae* affect these biological processes and associated molecular and signaling pathways is interesting and remains to be determined.

In conclusion, this study manifests a striking acceleration of the development of the COPD phenotype in CS-exposed mice with repeated exposure to *S. pneumoniae*. This study emphasizes the important role of bacterial colonization in stable-state COPD. Clinical follow-up is needed to determine whether bacterial colonization is an independent risk factor for prognosis.

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