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Cigarette Smoke Exposure Exacerbates Lung Inflammation and Compromises Immunity to Bacterial Infection

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The detrimental impact of tobacco on human health is clearly recognized, and despite aggressive efforts to prevent smoking, close to one billion individuals worldwide continue to smoke. People with chronic obstructive pulmonary disease are susceptible to recurrent respiratory infections with pathogens, including nontypeable Haemophilus influenzae (NTHI), yet the reasons for this increased susceptibility are poorly understood. Because mortality rapidly increases with multiple exacerbations, development of protective immunity is critical to improving patient survival. Acute NTHI infection has been studied in the context of cigarette smoke exposure, but this is the first study, to our knowledge, to investigate chronic infection and the generation of adaptive immune responses to NTHI after chronic smoke exposure. After chronic NTHI infection, mice that had previously been exposed to cigarette smoke developed increased lung inflammation and compromised adaptive immunity relative to air-exposed controls. Importantly, NTHI-specific T cells from mice exposed to cigarette smoke produced lower levels of IFN-γ and IL-4, and B cells produced reduced levels of Abs against outer-membrane lipoprotein P6, with impaired IgG1, IgG2a, and IgA class switching. However, production of IL-17, which is associated with neutrophilic inflammation, was enhanced. Interestingly, cigarette smoke–exposed mice exhibited a similar defect in the generation of adaptive immunity after immunization with P6. Our study has conclusively demonstrated that cigarette smoke exposure has a profound suppressive effect on the generation of adaptive immune responses to NTHI and suggests the mechanism by which prior cigarette smoke exposure predisposes chronic obstructive pulmonary disease patients to recurrent infections, leading to exacerbations and contributing to mortality.  

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C hronic obstructive pulmonary disease (COPD) is a disease of the lungs in which the airways become narrow, thereby limiting the airflow and causing dyspnea (1, 2). In contrast with asthma, the limitation of airflow is poorly reversible and usually progressively worsens (3, 4). Chronic bronchitis and emphysema commonly coexist in the lungs of COPD patients. This condition is initiated by respiratory exposure to noxious particles or gas, most commonly tobacco smoke, which triggers an inflammatory response in the lung. Cigarette smoke is recognized as a crucial factor in the development and pathogenesis of COPD (5).

Chronic respiratory disease now ranks as the third most common cause of death in the United States and the fifth worldwide (8). COPD is characterized by intermittent disease exacerbation. Exacerbations account for the majority of hospitalizations for COPD patients and a significant share of direct and indirect economic costs, and they are associated with a significantly increased risk for death (9). Even though the potential contributions of pulmonary infections to the cause, pathogenesis, and clinical course of COPD have been identified, the precise role of bacterial infections to disease outcome has remained a subject of some controversy. Improvements in PCR detection of pathogen gene sequences has confirmed the causal contribution of bacterial infections to exacerbations of COPD, accounting for at least 50% of all exacerbations (10–12). Nontypeable Haemophilus influenzae (NTHI), Moraxella catarrhalis, and Streptococcus pneumoniae are the three most common bacterial pathogens that cause respiratory tract infections in COPD patients (13–15). Besides colonizing the airways when the patient is clinically stable, the acquisition of new NTHI strains is considered an important cause of lower respiratory tract infection and is associated with disease exacerbations. Because mortality rapidly increases with multiple exacerbations (16), developing an efficient immune response after a first exacerbation may be critical to improving patient survival.

Efforts to reduce the disease burden caused by repeat NTHI infections have focused on the major outer-membrane proteins and lipooligosaccharide as potential candidate vaccine Ags (17–19). However, antigenic heterogeneity of these molecules in many of the NTHI strains suggests that a highly conserved, immunogenic molecule is required for formulation of an effective vaccine.
It comprises <1% of the total outer membrane protein, the minor outer membrane lipoprotein P6 (P6) is highly conserved at the nucleotide and amino acid level among all tested strains of NTHI because of its integral function as an anchor between the outer membrane and the bacterial cell wall (20). Importantly, in consideration of vaccine development, P6 expresses epitopes on the outer membrane accessible for Ab binding and contains an immunodominant T cell epitope for assessing generation of cellular immunity (21–23). We have previously demonstrated that T cell responses to P6 are associated with relative protection against NTHI infection in adults with COPD (24). The immunogenic nature of this highly conserved lipoprotein makes P6 a promising vaccine candidate for NTHI (25, 26). The expectation would be that vaccine-induced immunity would minimize NTHI-mediated lung damage during COPD exacerbations.

Although previous studies have provided good evidence that cigarette smoke may be immunosuppressive (6, 27–30), no reports have described the impact of cigarette smoke exposure on the development of adaptive immune responses to respiratory pathogens. Cigarette smoke is itself an inflammatory mediator and induces pulmonary inflammation by damaging the respiratory epithelial barrier, thereby facilitating repeated infections (31). Inflammatory mediators generated in response to these infections further accentuate a milieu of chronic inflammation in the lungs of smokers. Most models of respiratory inflammation simply evaluate the impact of either smoke exposure or infection alone, neglecting that the combination of several inflammatory mediators creates a unique microenvironment that may have an additive effect. To better understand the connections among chronic smoking, chronic pulmonary infection, chronic inflammation, and changes in adaptive immunity, we developed a mouse model of these events. We have studied how chronic cigarette smoke exposure affects the generation of adaptive immune responses to respiratory pathogens. Cigarette smoke is itself an inflammatory mediator and induces pulmonary inflammation by damaging the respiratory epithelial barrier, thereby facilitating repeated infections (31). Inflammatory mediators generated in response to these infections further accentuate a milieu of chronic inflammation in the lungs of smokers. Most models of respiratory inflammation simply evaluate the impact of either smoke exposure or infection alone, neglecting that the combination of several inflammatory mediators creates a unique microenvironment that may have an additive effect. To better understand the connections among chronic smoking, chronic pulmonary infection, chronic inflammation, and changes in adaptive immunity, we developed a mouse model of these events. We have studied how chronic cigarette smoke exposure affects the generation of adaptive immune responses to chronic smoke exposure to NTHI. In addition, we have evaluated the vaccination efficacy of systemic P6 immunization to determine whether this treatment modality has the potential to alleviate respiratory inflammation and minimize lung damage resulting from combined cigarette smoke and NTHI exposure.

Materials and Methods

Mice
Six-week-old female C57BL/6J mice (Jackson Laboratory) were used in all experiments. Mice were maintained under specific pathogen-free conditions. Number of animals used in each experiment is provided in the figure legends. All procedures performed on animals were Institutional Animal Care and Use Committee approved and complied with all state, federal, and National Institutes of Health regulations.

Cigarette smoke exposure

Mice were housed in the Inhalation Core Facility at the University of Rochester and were exposed to mainstream cigarette smoke as previously described (28, 32, 33). Mice were placed in individual compartments of a wire cage, which was placed inside a closed plastic box connected to the smoke source. 3R4F research cigarettes (University of Kentucky College of Agriculture Reference Cigarette Program) were smoked according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35-ml puff). A 3% concentration of cigarette smoke exposure chamber. The smoke exposure (total volume) in a Jaeger-Baumgartner CSM2072i cigarette smoking machine (CH Technologies). Mainstream cigarette smoke was diluted with filtered air and directed into the exposure chamber. The smoke exposure (total particulate matter per cubic meter of air) was monitored by gravimetric sampling. The smoke concentration was set at a nominal value of 250 mg/m³ total particulate matter per cubic meter of air by adjusting the flow rate of the dilution air. The average actual exposure for these experiments was 259 ± 47 mg/m³. Mice were exposed for 5 h per day, 5 d per week, for 4 wk.

Control mice were exposed to filtered air in an identical chamber according to the same schedule. Following the final smoke exposure, the mice were transported to Roswell Park Cancer Institute for infection and vaccination experiments.
ELISPOTs

Frequency of P6-specific T cells was evaluated by ELISPOTs. Multiscreen HA plates (Millipore) were coated with 3 μg/ml anti–IFN-γ (clone AN-18), anti–IL-4 (clone 11B11), or anti–IL-17 (clone eBio17CK15A5). Lymphocytes were cocultured with APCs pulsed with 1 μM P641-55 peptide (Genscript) (21). After 18 h, the plates were washed and cytokines were detected with biotinylated Abs (anti–IFN-γ clone R4-6A2; anti–IL-4 clone BV6D-24G2; anti–IL-17 clone eBio17B7) followed by addition of streptavidin-HRP (all reagents from eBioscience). Spots were developed with TMB substrate (Vector Labs) and enumerated microscopically. Frequency of P6-specific, Ab-secreting cells in bone marrow and spleen was enumerated on ELISPOT plates coated with 3 μg/ml P6; after 18 h, bound anti-P6 Ab was detected with HRP-conjugated secondary Abs to mouse IgG1 and IgG2a (Southern Biotech). Spots were developed with TMB.

NTHI clearance

Lungs were excised from mice and mechanically disrupted with a tissue homogenizer in 1 ml PBS on ice. Serial dilutions of the lung homogenate were cultured on chocolate-agar plates at 37˚C to enumerate CFU per gram of lung tissue.

Statistical analysis

Testing for differences between mean values was determined using either Student’s t test or two-way ANOVA with posttest comparisons. The area under the curve for the anti-P6 Ab responses from week 0 to week 16 was estimated per animal using the standard trapezoidal approach. Direct comparisons were made between the two exposure groups using an exact-permutation Kruskal–Wallis rank test and tested at level α = 0.05 (two-sided).

Results

Cigarette smoke exposure exacerbates NTHI-mediated chronic respiratory inflammation

We first examined how two important inflammatory mediators in the lung, cigarette smoke and bacterial infection, impact pulmonary inflammation. C57BL/6 mice were exposed to cigarette smoke or air for 4 wk, followed by 8 wk of chronic NTHI exposure (Fig. 1A). Histopathological examination of the lungs revealed that characteristic lymphocytic accumulation surrounding airways and bronchovascularature was observed in both groups (Fig. 1B); however, the extent and severity of immune cell infiltration was greatly increased in cigarette smoke–exposed mice compared with control air-exposed mice. Lungs of mice exposed to cigarette smoke only (i.e., no NTHI) exhibit mild inflammatory changes but completely lack marked lymphocytic infiltration or accumulations of immune cells (Supplemental Fig. 1 and data not shown) (33, 34). Pulmonary inflammation was scored using a blinded semiquantitative system by a pathologist to determine whether any differences existed between air and smoke exposure groups (Fig. 1C). Bronchovascular inflammation in air-exposed mice was scored primarily as moderate, whereas inflammation in cigarette smoke–exposed mice was scored as marked. The extent of pleural and interstitial inflammation was equivalent in both groups. Thus, although the localization pattern of infiltrating cells was similar between air- and cigarette smoke–exposed mice, the extent of infiltration was substantially greater after cigarette smoke exposure, indicating that this insult plays an important role in the subsequent respiratory response to bacterial infection.

The role and contribution of cigarette smoke to the immune cell composition (Fig. 2A) and cytokine production (Fig. 2B) from BAL fluid were also assessed. Air-exposed mice exhibited a combination of high lymphocytic accumulation and low neutrophil presence observed in the BAL after NTHI exposure, which is characteristic of a chronic inflammatory environment. In contrast, the frequency and number of neutrophils were elevated in the airways of cigarette smoke–exposed mice compared with air-exposed mice, and lymphocyte levels were significantly decreased (Fig. 2A). Levels of IL-1β, IL-6, and TNF-α in BAL fluid were evaluated as hallmarks of an inflammatory response to bacterial challenge in the lung. IFN-γ, IL-4, and IL-17 were evaluated to determine the relative levels of Th1/Th2/Th17 cytokine production in response to chronic NTHI infection. Levels of proinflammatory IL-1β, IL-6, and TNF-α were increased in cigarette smoke–exposed mice compared with air-exposed mice (Fig. 2B), corroborating the histopathological phenotype of enhanced pulmonary inflammation. In addition, prior smoke exposure modulated the levels of IFN-γ and IL-17, cytokines that are associated with Th1 and Th17 inflammatory responses. Whereas IFN-γ was present at lower levels in BAL fluid from cigarette smoke–exposed mice, IL-17 levels were increased (Fig. 2B). Collectively, these findings demonstrate that the milieu in the lungs of cigarette smoke–exposed mice promotes sustained chronic inflammation and hinders the accumulation of immune mediators and lymphocytes designed to combat bacterial infection.

FIGURE 1. Cigarette smoke exposure exacerbates NTHI-mediated chronic respiratory inflammation. (A) C57BL/6J female mice were exposed for 4 wk to cigarette smoke or air, followed by 8 wk of chronic NTHI exposure (n = 10 air + NTHI; n = 8 cigarette smoke + NTHI). (B) H&E-stained lung sections prepared after combined inflammatory insult were evaluated for the extent and severity of inflammation. Lymphocytic cuffs are present adjacent to vasculature (arrows) and airways (arrowheads). Scale bars, 100 μm. (C) Consensus scores from two blinded nonconsecutive sessions evaluating respiratory inflammation in peribronchial, pleural, and interstitial regions of the lung.

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Prior cigarette smoke exposure suppresses the adaptive immune response

Because cigarette smoke exposure increased NTHI-mediated chronic respiratory inflammation, we also assessed whether this inflammatory insult impacted the generation and function of NTHI-specific adaptive immune responses. The P6 of NTHI is highly conserved and immunogenic, and has an immunodominant T cell epitope. We used the P641–55 peptide to measure the frequency of cytokine-secreting, P6-specific T cells, the level of cytokines produced from lung-infiltrating lymphocytes (Fig. 3A), and the spleen (Fig. 3B) of air- and cigarette smoke–exposed mice. T cells isolated from the lungs and spleen exhibited lower levels of IFN-γ and IL-4 secretion but higher levels of IL-17 (Fig. 3A, 3B). Importantly, the profile of Th1, Th2, and Th17 P6-specific T cells in the lungs of cigarette smoke–exposed mice (Fig. 3A) resembled that of the BAL cytokine profile (Fig. 2B). The frequency of Th17 cells and levels of IL-17 production were significantly increased in lung lymphocytes from cigarette smoke–exposed mice compared with air-exposed mice. IFN-γ– and IL-4–producing T cells from the lungs and the spleen were decreased (Fig. 3A, 3B), demonstrating that antibacterial T cell immunity and development of helper cells for anti-NTHI Ab responses are dysregulated by prior cigarette smoke exposure.

T cell–derived IL-4 is required for Ab class switching to IgG1, whereas IFN-γ is required for class switching to IgG2a. Because the number of T cells secreting these cytokines was reduced in cigarette smoke–exposed mice (Fig. 3A, 3B), we also evaluated B cell responses. The frequency of P6-specific IgG1- and IgG2a-secreting B cells from the bone marrow and spleen was measured by B cell ELISPOT (Fig. 4). We observed a significant decrease in the frequency of anti-P6 Ig–secreting cells for both IgG subclasses in cigarette smoke–exposed mice compared with control mice. Although the presence of both IgG1 and IgG2a subclasses indicates that class switching did occur, it was clearly less efficient in mice previously exposed to cigarette smoke.

Anti-P6 Abs in BAL fluid and sera were also measured to evaluate whether alterations in B cell frequency affected Ab responses. Total anti-P6 Ab levels were significantly lower in the BAL and sera of cigarette–smoke exposed mice (Fig. 5A, 5B). In both groups, BAL fluid contained only IgA and IgG2b anti-P6 Abs, and once again, these levels were significantly lower in
cigarette smoke–exposed mice (Fig. 5C). Serum levels of IgG1 and IgG2a anti-P6 Abs were also significantly reduced with prior smoke exposure (Fig. 5D). Collectively, the diminished anti-NTHI T cell and B cell responses observed present a complete profile of the dysfunctional adaptive immune response to a chronic pathogen infection after cigarette smoke exposure.

Efficacy of immunization is reduced after cigarette smoke exposure

The altered immune response elicited to NTHI in smoke-exposed mice prompted us to evaluate whether smoke exposure also impacted the immune response to a candidate vaccine Ag. Control and cigarette smoke–exposed mice were immunized with purified P6 Ag; vaccination efficacy was measured by anti-P6 Ab titers (Fig. 6A). Not only were the kinetics of anti-P6 Ab appearance slower in cigarette smoke–exposed mice, the magnitude of the Ab titers in the sera was substantially lower in comparison with control mice (Fig. 6B). Ab titers were also significantly reduced in the BAL fluid of cigarette smoke–exposed mice after 16 wk (Fig. 6C). Serum Abs were predominantly of the IgG1 and IgG2a subclasses in both groups of mice, but, consistent with total Ab titers, cigarette smoke exposure reduced the levels of these anti-P6 isotypes as well (Fig. 6D). Anti-P6 of IgA isotype was present in the BAL but at significantly lower levels in cigarette smoke–exposed mice compared with air-exposed mice (Fig. 6E).

Because Ab titers were reduced after immunization of cigarette smoke–exposed mice, we determined the frequency of P6-specific B cells and T cells by ELISPOT. The frequency of P6-specific, Ab-secreting cells was lower in the bone marrow and spleen of P6-immunized cigarette smoke–exposed mice compared with control mice (Supplemental Fig. 2). The frequency of P6-specific T cells secreting IFN-γ or IL-4 was also significantly reduced in cigarette smoke–exposed mice, confirming that prior smoke exposure impairs Th1 and Th2 responses and class switching (Supplemental Fig. 3). Interestingly, the number of IL-17–expressing T cells was unaffected by prior smoke exposure. Thus, in cigarette smoke–exposed mice, the inability to elicit robust Th1 and Th2 immunity after vaccination likely hinders the generation of protective responses against pathogen infection.

We reasoned that although the immunization efficacy was substantially weaker in cigarette smoke–exposed mice, this level of protection might still be effective against an acute NTHI challenge. To test this, we immunized air- and cigarette smoke–exposed mice with P6 and challenged them with live bacteria 16 wk.
later. Sham-immunized air- and cigarette smoke–exposed mice served as controls to evaluate the effectiveness of P6 immunization on mitigating the hallmarks of acute respiratory inflammation 4 and 24 h after NTHI challenge (Fig. 7A). The kinetics of NTHI clearance from the lungs was more rapid in P6-immunized mice (Fig. 7B, black circles, gray triangles) compared with sham-immunized (Fig. 7B, open circles, open triangles) mice regardless of cigarette smoke exposure, establishing that the attenuated immune response elicited in cigarette smoke–exposed mice was still effective in clearing bacteria from the lungs. However, the beneficial effect of immunization was clearly demonstrated in that bacterial clearance rates in P6-immunized, cigarette smoke–exposed mice were similar to those in sham-immunized, air-exposed mice. Thus, immunization was able to mitigate the negative impact of cigarette smoke exposure.

Although clearance rates of NTHI were compromised in cigarette smoke–exposed mice, the influx of neutrophils was elevated compared with air-exposed mice regardless of immunization status (Fig. 7C). Despite the increased neutrophil infiltration in the lungs, clearance of NTHI was not improved, suggesting that the phagocytic function of the neutrophils was compromised by prolonged cigarette smoke exposure. The combination of decreased bacterial clearance and increased neutrophil influx resulted in increased lung epithelial damage, which was quantified by measuring BAL fluid albumin leak as a surrogate marker of lung damage. P6- and sham-immunized cigarette smoke–exposed mice had higher levels of albumin in the BAL compared with air-exposed mice (Fig. 7D). Evaluation of proinflammatory cytokines in the BAL fluid revealed that increased levels of IL-1β, IL-6, and TNF-α were not sustained in P6-immunized mice after NTHI challenge, whereas their concentration remained increased in sham-immunized mice (Fig. 7E–G). Immunization of cigarette smoke–exposed mice reduced the level of proinflammatory cytokines to below that of sham-immunized mice, but not to the level of air-exposed, immunized mice. Thus, although prior smoke exposure promotes increased inflammation and impairs the immune response, P6 immunization mitigated the negative impact of cigarette smoke exposure on bacterial clearance, lung inflammation, and the adaptive immune response.

Discussion

COPD patients suffer from chronic inflammation resulting from prolonged cigarette smoke exposure combined with repeated bouts of respiratory infections. We have established a novel model of chronic smoke exposure followed by chronic infection to address this knowledge gap and to determine how cigarette smoke exposure impacts the generation of adaptive immunity after exposure to the bacteria and vaccination against a key Ag. A previous study by Gaschler et al. (35) evaluated the inflammatory response in the lungs of female C57BL/6 and BALB/c mice that had been ex-

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**FIGURE 4.** Frequency of Ab-secreting, NTHI-specific cells is decreased in cigarette smoke–exposed mice. B cell ELISPOTs were performed to quantify frequency of P6-specific IgG1- and IgG2a-secreting cells from bone marrow (top panels) and spleens (bottom panels) of air- or cigarette smoke–exposed mice. Line represents mean. *p < 0.05, **p < 0.01, two-tailed unpaired t-test.

**FIGURE 5.** Cigarette smoke exposure modulates accumulation of anti-P6 Ig in airways and serum. Levels of anti-P6 Ig were measured in (A) BAL fluid and (B) serum from air- or cigarette smoke–exposed mice receiving 8 wk of chronic NTHI exposure. OD values at 405 nm for (C) BAL fluid dilutions at $10^{-2.6}$ (1:400) and (D) sera dilutions at $10^{-3.2}$ (1:1600) were analyzed for levels of P6-specific IgA, IgG1, IgG2a, and IgG2b. Line represents mean. *p < 0.05, **p < 0.01, two-tailed unpaired t-test.
FIGURE 6. Immunization efficacy is compromised in cigarette smoke–exposed mice. (A) C57BL/6 female mice were immunized i.p. with 40 μg lipoprotein P6 from NTHI outer membrane after receiving 4 wk of air or cigarette smoke exposure (n = 10 air + NTHI; n = 9 cigarette smoke + NTHI). Vaccination efficacy was measured 16 wk after i.p. immunization. Levels of anti-P6 Ig were measured from (B) weekly serum samples and (C) BAL fluid obtained at termination. (D) OD values at 405 nm for sera were determined for levels of P6-specific IgG1 and IgG2a. (E) OD values at 405 nm for BAL fluid were analyzed for levels of P6-specific IgA and IgG2b. Mean ± SD. Line represents mean. *p < 0.05, area under the curve Kruskal–Wallis rank test. †p < 0.05, two-tailed unpaired t test.

posed to air or cigarette smoke, and subsequently infected with a single NTHI challenge. As such, their study design queried only the innate immune response, primarily mediated by neutrophils, monocytes, and NK cells. They demonstrated that cigarette smoke exposure led to decreased bacterial clearance and increased acute (24 h) inflammation. In contrast, by using multiple repeated NTHI challenges over 8 wk, this study queries the adaptive immune response (B and T cells) in addition to the innate response. As a result, our study has conclusively demonstrated for the first time, to our knowledge, that cigarette smoke exposure has a negative effect on the generation of adaptive immune responses to NTHI, a respiratory pathogen known to colonize the lungs of smokers with COPD. In addition, NTHI-mediated lung inflammation was worsened in cigarette smoke–exposed mice, a pathological feature frequently observed in COPD patients who have experienced recurrent bacterial respiratory infections. Importantly, this deficiency in adaptive immunity could be rescued by immunization. Although immunization only raised the immune response of smoke-exposed mice to that of nonexposed, nonimmunized mice, that level of immunity may be sufficient to protect COPD patients from the detrimental effects of repeated exacerbation.

Although it was previously reported that smoke exposure impaired acute clearance of a single bacterial infection, in this article, we have provided convincing evidence that increased pulmonary inflammation arising from chronic cigarette smoke exposure and bacterial infection is compounded by the host’s inability to mount an effective immune response against the bacteria. Although we did not measure bacterial clearance in the initial experiment, we note that bacterial clearance was impaired in the immunization study in mice that were exposed to smoke and received sham immunization (Fig. 7). Because this challenge was 16 wk after smoke exposure, we believe it probable that clearance was also impaired in this experiment 8 wk after exposure. In this way, our results are consistent with but also extend those of Gaschler et al. (35), because we have demonstrated defects not only in clearance, but also in B and T cell function. Intriguingly, these deficits in adaptive immune function persisted up to 16 wk after the final smoke exposure, demonstrating that tobacco smoke exposure has dramatic and long-term effects on immune cell function even after smoking cessation.

Cigarette smoke is known to be immunosuppressive, although the exact mechanisms are not clearly understood (6, 27, 28, 30, 36). In this article, we show that chronic cigarette smoke exposure followed by chronic infection results in decreased levels of Th1 and Th2 cytokines and decreased T cell and B cell function, but an increased IL-17 response; IL-17 is associated with maintenance of chronic inflammation (37, 38). Thus, cigarette smoke exposure skews the T cell response away from productive responses that could clear bacterial infections and toward a response that sustains chronic inflammation. Exaggerated IL-17 production in the lung hastens bronchoconstriction and asthmatic symptoms (39). Although little is known about the role of Th17 cells in COPD, these cells have been identified in lung biopsies from COPD patients and IL-17 has been detected in the sputum from patients during disease exacerbations (40). Chronic cigarette smoke exposure can tip the balance of Th17 and regulatory T cells by decreasing Foxp3 and IL-10 expression while simultaneously increasing ROR-γT and IL-17 expression (41). In this article, we also show that prior smoke exposure increased production of IL-6, a cytokine that favors Th17 cells (35). One mechanistic explanation for the altered immune response may involve the aryl hydrocarbon receptor (AhR). Cigarette smoke contains multiple AhR ligands and activates AhR-dependent transcription (42), whereas Chen and colleagues have shown that AhR-deficient mice are incapable of generating Th17 cells (43). Thus, AhR ligands found in cigarette smoke may play a role in driving Th17 differentiation. Taken together, these findings point to the IL-17 signaling axis as a key player in the sustenance of chronic inflammation in COPD and demonstrates that cigarette smoke exposure skews T cell responses.
Prior smoke exposure significantly reduced levels of IFN-γ in BAL, splenic and lung P6-specific T cells, and IL-4 in splenic and lung T cells but not BAL. Although BAL fluid levels of IL-4 were similar in air- and cigarette smoke–exposed mice, P6-specific T cell production of this cytokine is reduced in cigarette smoke–exposed mice. IL-4 measured in BAL fluid by ELISA does not allow us to elucidate the source of the cytokine; thus, it could have been produced by lymphocytes and possibly alveolar macrophages (44). Because this cytokine is primarily required for B cell activation, it is likely that IL-4 is required in lymphoid organs rather than at the site of inflammation. The failure of P6-specific T cells to produce IL-4 is thus implicated as one of the causes for the deficit in Ab production, because P6-specific T cells are unable to provide appropriate helper signals to NTHI-specific B cells at the level of the immunological synapse.

In our model, mice previously exposed to cigarette smoke displayed increased levels of neutrophils after chronic infection, compared with air-exposed mice. Our observation of the persistence of neutrophils in the lungs of the cigarette smoke–exposed mice compared with air-exposed mice is a novel finding, because neutrophils are typically short-lived and have a high turnover rate. A potential reason for their continued presence may be because of impaired cell death of the neutrophils. Cigarette smoke hinders spontaneous neutrophil cell death by blocking Akt deactivation via suppression of diposphoinositol pentakisphosphate production (45); thus, dysregulation of neutrophil cell death may account for their persistence in the lungs and contribute to the chronic inflammation mediated by cigarette smoke.

Cigarette smoke has additional immunosuppressive effects. For example, cigarette smoke inhibits bacterial phagocytosis by alveolar macrophages (46) and reduces macrophage expression of TLR2 (47). We have previously shown that TLR2 is essential for the generation of NTHI-specific adaptive immune responses (48). Thus, altering the ability of phagocytic cells to sense and clear bacteria represents a second immunosuppressive mechanism of prior chronic smoke exposure. Our results show that mice exposed to chronic infection develop heightened inflammation and are unable to efficiently generate an adaptive immune response. This likely explains the susceptibility of COPD patients to exacerbations.

Repeated infections from newly acquired strains of NTHI further exacerbate the poor lung function in COPD patients; thus, it is critical to induce robust, long-lived immune responses that prevent bacterial colonization and further respiratory inflammation. In this study, we evaluated a candidate vaccine Ag selected based on our previous work describing the immunogenic and protective potential of the lipoprotein P6 (26). Although anti-P6 responses were diminished in the cigarette smoke–exposed mice, the vaccine was still protective and conferred immunity similar to that of unimmunized mice never exposed to smoke. An interesting aspect

**FIGURE 7.** P6 immunization in cigarette smoke–exposed mice marginally mitigates acute NTHI-mediated inflammation. (A) Hallmarks of acute inflammation were measured in air- or cigarette smoke–exposed mice 16 wk after P6 immunization or sham immunization. Kinetics of acute inflammation was evaluated at baseline and 4 or 24 h after NTHI challenge. (B) Rate of NTHI clearance in the lung was measured by colony-plating assay. (C) Accumulation of neutrophils in BAL determined by Wright–Giemsa differential staining. (D–G) Concentration of albumin and proinflammatory cytokines in BAL fluid determined by ELISA. *p < 0.05. Bonferroni posttest comparison of air versus cigarette smoke.
of this immunization was the decreased amount of P6-specific IgA in the lungs of cigarette smoke–exposed mice, suggesting that the chronic cigarette smoke exposure may have altered the bioavailability or transport of this Ab in the respiratory mucosa. Because the presence of specific IgA in the lungs is critical for minimizing pathogen colonization and biofilm formation, this represents a third mechanism by which chronic cigarette smoke exposure impairs effective immune responses to lung infection.

Corticosteroid treatment is used in COPD patients to reduce exacerbations and improve health outcomes (49). Unfortunately, this treatment option is immunosuppressive and is associated with increased bacterial-mediated pneumonia (50). New strategies are needed to prevent COPD exacerbations and associated increased mortality. Our results demonstrate that immunization with an appropriate Ag can overcome some of the immune defects associated with chronic cigarette smoke exposure, not only restoring T cell and B cell responses, but also moderating tissue inflammation. However, in light of the clinical use of corticosteroids in this patient population, future studies will need to investigate the impact of corticosteroids on anti-NTHI immunization efficacy.

Overall, our study shows that the altered inflammatory profile induced by cigarette smoke has profound long-term negative consequences on the host’s ability to respond to respiratory pathogens and provides an understanding of the persistent and progressive nature of COPD. It also lays the groundwork for the design of therapeutic interventions in COPD patients that should take into consideration its impact on adaptive immunity against NTHI, a main cause of devastating COPD exacerbations.

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Disclosures
The authors have no financial conflicts of interest.

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