FULL TITLE
Mechanisms of Clearance of nontypeable Haemophilus influenzae from Cigarette Smoke-Exposed Mouse Lungs

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ABSTRACT

Inflammation is prevalent in all stages of COPD, and furthermore, individuals undergo periods of exacerbation in which pulmonary inflammation increases, often a result of bacterial infection. In this study, we investigated the in vivo consequences of cigarette smoke exposure on a bacterial challenge with nontypeable Haemophilus influenzae (NTHI).

BALB/c and C57BL/6 mice were exposed to cigarette smoke once- or twice- daily for a total period of 8 weeks. We observed exacerbated inflammation in cigarette smoke compared to room air-exposed mice following challenge with live or heat-inactivated NTHI. Accelerated clearance of live NTHI from cigarette smoke-exposed mice was independent of the establishment of chronic inflammation or direct toxic effects of cigarette smoke components on bacteria. Mechanistically, a cell-free factor in the BAL fluid contributed to accelerated clearance following passive transfer to naive mice. Further investigation demonstrated increased titres of IgA in the BAL, but not the blood, of cigarette smoke-exposed mice, including increased titres of NTHI-specific IgA, while JH−/− B-cell deficient cigarette smoke-exposed mice did not demonstrate decreased bacterial burden following challenge.

These results demonstrate that cigarette smoke exposure results in exacerbated inflammation following challenge with NTHI, as well as increased titres of antibodies that contribute to bacterial clearance.
KEYWORDS:

Bacteria

Chronic Obstructive Pulmonary Disease

Cigarette Smoke

Exacerbation

Inflammation

Mice
INTRODUCTION

Of all the leading causes of death, Chronic Obstructive Pulmonary Disease (COPD) is the only one whose prevalence has been rising for the past 20 years; in both developed and developing countries around the world, COPD is now the 4th leading cause of death [1, 2]. Cigarette smoking is the major etiological factor for the development of COPD, with exposure to pollution, dust or fumes contributing to a much lesser extent [3]. Despite the understanding that cigarette smoke is a causative agent, as greater than 90% of COPD patients are current or former smokers [4], the mechanism by which cigarette smoke leads to COPD is not well understood.

Increasingly, the immunological effects of cigarette smoke are being investigated [5]. Individual experimental studies have demonstrated that cigarette smoke affects innate and adaptive immune mechanisms, including pathways involved in host defense [6]. Indeed, throughout their disease, COPD patients undergo periods of acute exacerbation, where the severity of symptoms and inflammation increases, typically as a result of viral and/or bacterial respiratory infection [7-9]. With regards to bacterial infection, intermittent or chronic infection with nontypeable *Haemophilus influenzae* (NTHI), *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* are the most common causes [10-13]. One hypothesis regarding the pathogenesis of COPD is that that periods of repeated infection are important contributors to the development, and/or progression of COPD [14]. Testing and developing such a hypothesis requires well-defined experimental models investigating cigarette smoke exposure’s impact on bacterial infection. We have previously demonstrated that a pulmonary challenge with NTHI leads to worsened clinical presentation (as assessed by body weight loss), an exacerbated inflammatory response, and evidence of lung damage in a mouse model of cigarette smoke exposure [15]. Of particular interest, this model of cigarette smoke exposure and bacterial challenge demonstrated that the exacerbated inflammation was
associated with accelerated kinetics of bacterial clearance and a skewed inflammatory mediator expression profile.

Given that periods of exacerbation and heightened inflammation, resulting from bacterial infection, may be central to the development and/or progression of the disease, the purpose of this study was to investigate how cigarette smoke exposure alters clearance of NTHI while at the same time leading to an exacerbated inflammatory response. Here we report that clearance of NTHI from cigarette smoke-exposed mice is independent of the establishment of chronic inflammation, direct toxic effects of cigarette smoke components on bacteria viability, and mucus production. We further show increased titres of NTHI-binding antibodies in the bronchoalveolar lavage (BAL), and that accelerated clearance is dependent on B cells. These data support the notion that cigarette smoke alters pulmonary immune responses leading to, among other effects, increased antibodies in the BAL. Taken together, this study suggests that the immunological effects of cigarette smoke on antibody responses are an important consideration for understanding the pathogenesis of COPD.
MATERIALS and METHODS

Animals

Specific pathogen free 6-8 week old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Montreal, PQ, Canada). JH−/− mice [16] on a C57Bl/6 background were a generous gift from Dr. Kathy McCoy (McMaster University, Hamilton, Ontario). Gnotobiotic JH−/− mice were from the McMaster University gnotobiotic facility and housed in the same cages as wild type C57BL/6 mice ordered from Charles River Laboratories for one month prior to being utilized in experiments. All mice were kept in a 12-h light-dark cycle, in autoclaved cages and bedding, with unlimited access to autoclaved food and water. Animals were monitored for weight loss and clinical score throughout each experiment. The McMaster University Animal Research Ethics Board approved all experiments described in this study.

Cigarette smoke exposure

Mice were exposed to the smoke generated from twelve 2R4F reference cigarettes (University of Kentucky, Lexington, USA) with the filters removed for 50 minutes once- or twice- daily as indicated in the individual Results sections and Figure Legends, 5 days per week using an SIU48 exposure system (PROMECH LAB AB, Vintrie, Sweden). In an initial 3-day acclimatization period, mice were placed in restrainers only for 20 minutes on day 1, 30 minutes on day 2 and 50 minutes on day 3. Control animals were exposed to room air only.

To control for the level of exposure, immediately, or 24 hours, following cigarette smoke exposure, mixed arterial-venous blood was drawn in clinitubes (Radiometer, Copenhagen, Denmark) for determination of Carboxyhemoglobin (COHb) saturation by spectrophotometry (Hamilton Regional Laboratory Medicine Program, McMaster University Medical Centre, Hamilton, Ontario, Canada). Cotinine levels were measured by ELISA (Bio-Quant, San Diego CA) in serum obtained by
incubating whole blood for 30 min at 37°C, followed by centrifugation. With this model system of exposure levels of COHb were reported in reference [17] and levels of continine reported in reference [15]. Total particulates in the exposure chamber were measured twice weekly during the exposure periods for these experiments, and the mean levels of total particulates was 984.95 ± 240.63 μg/l, n=47.

Preparation of NTHI

Nontypeable Haemophilus influenzae 11P6 was used in all experiments [18, 19]. This is a clinical strain of NTHI isolated from sputum of a patient with COPD experiencing an acute exacerbation (kindly provided by Sanjay Sethi, Department of Medicine, University at Buffalo, State University of New York, Buffalo, NY, USA). Demonstration of specific immune response and an inflammatory response to this strain establish it as causative of exacerbation [19, 20]. NTHI was grown on chocolate blood agar supplemented with 1% Isovitalex or to log phase in brain-heart infusion (BHI) broth (DIFCO, Fisher Scientific, Ottawa, Ontario, Canada) supplemented with 0.01 mg/ml hemin and 7 μg/ml nicotinamide adenine dinucleotide (NAD) (SIGMA, Oakville, Ontario, Canada). To grow NTHI to log phase, colonies from a fresh plate were inoculated into 10ml of BHI broth supplemented with Hemin+NAD, and the culture was incubated with rotary shaking at 37°C until an OD600 value of 0.7-0.8 units was obtained. Bacterial titres were verified by plating serial dilutions of broth cultures onto chocolate agar plates. For heat-inactivation of NTHI, aliquots were heated at 56°C for 10 minutes. For intranasal delivery, the broth was washed three times with phosphate buffered saline (PBS). Isoflurane-anesthetized mice were inoculated intranasally with 10⁶ cfu of NTHI in a total volume of 35 μL of PBS. Animals were inoculated at least 4 hours after the last cigarette smoke exposure.
Collection of specimens

Broncho-alveolar lavage (BAL), lungs, and blood, were collected at the time of killing. BAL was performed as previously described \[15, 21\]. Total cell counts in the BAL were determined using a haemocytometer. BAL cytospins were prepared by for differential cell counts were prepared and stained with Hema 3 as per the manufacturer’s instructions (Biochemical Sciences Inc., Swedesboro, New Jersey, USA), and at least 500 leukocytes were counted per cytospin and classified according to standard hemocytologic criteria as neutrophils, eosinophils, or mononuclear cells. Blood was collected by retroorbital bleeding, total cell counts were determined using a haemocytometer, and blood smears for differential cell counts were prepared, stained with Hema 3 as per the manufacturer’s instructions and at least 200 leukocytes were counted per smear and classified according to standard hemocytologic criteria as neutrophils, eosinophils, or mononuclear cells. Serum was obtained and stored at –20°C. For histological assessment the left lung was inflated with 10% neutral buffered formalin at a constant pressure of 20 cm H₂O, and then fixed in 10% neutral buffered formalin for 48 to 72 hours.

Preparation of lung tissue homogenate and measurement of NTHI burden

For bacterial burden assessments, the left lung was tied off before BAL and placed in 2 ml of PBS on ice. Lungs were homogenized and NTHI burden was assessed in the homogenized sample by plating serial dilutions onto chocolate agar plates in duplicate. Burden was expressed as the number of colony forming units (CFU) per milliliter (ml) of lung tissue.

Measurement of MUC5AC, goblet cell hyperplasia and airway morphometric analysis

To assess MUC5AC expression, RNA was isolated from whole lung tissue that had been preserved in RNA later (Ambion, Austin, TX) using an RNeasy Mini Kit with the optional DNase step
(Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s protocol. RNA was quantified using the Agilent 2100 Bio-Analyzer (Agilent, Santa Clara, CA). Total RNA (150 ng) was reverse transcribed with 100 U Superscript II (Invitrogen) in a total reaction volume of 20 µl. A random hexamer primer was used to synthesize cDNA at 42°C for 50 min, followed by 15 min incubation at 70°C. Real-time quantitative PCR was performed in triplicate in a total volume of 25 µl using Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers for MUC5AC and GAPDH along with FAM-labeled probes were purchased from Applied Biosystems. PCR was run in the ABI PRISM 7900HT Sequence Detection System using the Sequence Detector Software version 2.2 (Applied Biosystems). Data were analyzed using the delta, delta Ct (ΔCt) method. Gene expression was normalized to the housekeeping gene (GAPDH) and expressed as fold change over a control animal (control, vehicle treated mouse).

To assess the presence of mucus and the extent of goblet cell hyperplasia, left lungs were inflated with 10% formalin at a constant pressure of 20 cm H₂O, and fixed in 10% formalin for 48 to 72 hours. After formalin fixation, tissues were embedded in paraffin, and 3-µm-thick cross-sections of the left lung were cut and stained with alcian blue-periodic acid-Schiff (AB/PAS). Images for morphometric analysis were taken under polarized light and captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada) as previously described [22]. For analysis, images were colour-inverted and image analysis was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada).

**Immunoglobulin measurements by ELISA**

Levels of total IgG, IgA, and IgM in the BAL and serum were measured by sandwich ELISA, using kits from Bethyl laboratories (Montgomery, Texas, USA) according to the manufacturer’s
instructions. The limits of detection for the IgM, IgA, and IgG ELISAs were 15.625 ng/ml, 15.625 ng/ml, and 7.8 ng/ml, respectively. Serial ten-fold dilutions of BAL and serum samples were prepared for measurements.

NTHI-specific antibodies were detected in the BAL or serum using a minor modification of the procedure previously described [23]. Briefly, MaxiSorp plates (Nalge NUNC International, USA) were coated overnight at 4°C with a lysate from log-phase NTHI. Coated wells were blocked with 1% casein in PBS for 2 h at room temperature. After washing, BAL samples serially diluted in PBS were incubated overnight at 4°C, washed, and developed with biotin-labeled, anti-mouse IgG, IgM, or IgA (Southern Biotechnology Associates, Birmingham, AL). Plates were washed and incubated with alkaline-phosphatase streptavidin for 1 h at room temperature. The color reaction was developed with p-Nitrophenyl phosphate tablets (Sigma) in diethanolamine buffer.

Isolation of BALF and challenge transfer of naïve of mice with BALF and NTHI

BAL was isolated at the time of killing as described in the collection of specimens section. BAL fluid (BALF) was isolated by removing the cellular fraction by centrifugation at 329 g for 10 minutes, followed by aspiration of the supernatant. For assessment of direct killing or impairment of growth of NTHI by BALF, BALF was incubated with serial dilutions of NTHI for 30 minutes at 37°C. Following this time, total bacteria CFU was assessed in the sample by plating serial dilutions onto chocolate agar. For in vivo transfer experiments, BALF and NTHI were incubated at concentrations of 10⁶ CFU per 35µl of BALF for a period of 10 minutes at 37°C. Isoflurane-anesthetized mice were inoculated intranasally with the BALF + NTHI inoculums in a total volume of 35 µl of PBS. Animals were inoculated at least 4 hours after the last cigarette smoke exposure.

Data analysis
Data are expressed as means ± SEMs as indicated in the Figure Legends. Statistical analysis was performed with the general linear model (GLM) with SPSS statistical software version 16.2 (Chicago, IL, USA). Tests used were T test for 2-group comparison, or one- two- or three- way ANOVAs with the LSD post hoc test for multiple group comparison, as specified by the individual Figure Legends. Differences were considered statistically significant when p<0.05.
RESULTS

Impact of frequency of cigarette smoke exposure on the bacterial burden and cellular profile following pulmonary challenge with NTHI

We have previously reported that cigarette smoke exposure results in the establishment of chronic inflammation, and that cigarette smoke-exposed mice clear a pulmonary NTHI challenge more rapidly than do control mice, associated with an exacerbated inflammatory response [15]. Given the prominent role of neutrophils in pulmonary host defense, we questioned whether the chronic neutrophilia as a result of cigarette smoke exposure might drive accelerated bacterial clearance. To this end, we exposed BALB/c and C57BL/6 mice to cigarette smoke (smoke) or room air (control), either once- or twice- daily, 5 days per week, for a total exposure period of 8 weeks. Neither strain of mice had increased total cell number in the bronchoalveolar lavage (BAL) (Figure 1A and 1E), nor evidence of increased number of neutrophils (Figure 1C and 1G), as result of once-daily cigarette smoke exposure. Following twice-daily cigarette smoke exposure, both BALB/c and C57BL/6 had significantly increased numbers of neutrophils, although the total number of neutrophils was higher in BALB/c mice (Figure 1C and 1G). Furthermore, both BALB/c and C57BL/6 mice had significantly increased number of mononuclear cells following twice-daily cigarette smoke exposure (Figure 1B and 1F).

12 hours post-challenge with NTHI, once-daily cigarette smoke-exposed BALB/c and C57BL/6 mice had increased total cell numbers in the BAL compared to control mice challenged with NTHI (Figure 1A and 1E). This largely consisted of significantly increased number of neutrophils (Figure 1C and 1G), but not mononuclear cells (Figure 1B and 1F). 12 hours post challenge with NTHI, both twice-daily cigarette smoke-exposed BALB/c and C57BL/6 mice had an increased total cell number in the BAL compared to control mice challenged with NTHI (Figure 1A and 1E). In BALB/c mice this consisted of significantly increased numbers of both mononuclear
cells and neutrophils (Figure 1B and 1C), whereas in C57BL/6 mice this consisted of significantly increased number of neutrophils but not mononuclear cells (Figure 1F and 1G).

Despite the lack of a pre-existing chronic neutrophilia in once-daily cigarette smoke-exposed mice, both BALB/c and C57BL/6 mice demonstrated reduced bacterial burden 12 hours post NTHI challenge, compared to control mice (Figure 1D and 1H). Furthermore, equivalent bacterial burdens were observed between once- and twice-daily cigarette smoke-exposed mice.

Collectively, these data suggest that the frequency of cigarette smoke exposure was important for the changes we observed regarding both the establishment of cigarette smoke-induced inflammation, as well as the exacerbated inflammatory response following NTHI challenge.

Measurements of mucus and goblet cell hyperplasia in cigarette smoke-exposed mice challenged with NTHI

Mucus is an important innate immune mechanism contributing to bacterial host defense. In patients with COPD, and certain experimental models, mucus and goblet cell hyperplasia is often reported as one of the characteristic pathologies [24, 25]. Given the observation of decreased bacterial burden in cigarette smoke-exposed mice, we next measured MUC5AC RNA expression in total lung tissues. As a positive control, we utilized lung tissue from a mouse chronically exposed to house dust mite (HDM), a model of allergic airway inflammation, which is known to induce mucus production and goblet cell hyperplasia [26]. We observed marked induction in the lung tissue of a HDM exposed mouse, while MUC5AC expression in response to cigarette smoke exposure, NTHI challenge, or the combination of cigarette smoke and NTHI was marginal (Figure 2A). We next assessed the presence of mucus and the extent of goblet cell hyperplasia by staining tissue sections with alcian blue/periodic acid-Schiff base (AB/PAS). Light microscopic examination followed by morphometric quantification revealed that in this model of exposure, neither control nor cigarette smoke-exposed mice produced appreciable levels of mucus, or demonstrated evidence of goblet cell
hyperplasia (Figure 2B). Similarly, bacterial challenge with NTHI alone and the combination of cigarette smoke exposure and NTHI challenge did not induce the production of mucus. In agreement with the MUC5A RNA expression, we observed marked mucus production and goblet cell hyperplasia in tissues of mice chronically exposed to house dust mite [26]. Representative images of the mucus measurements are shown in Figures 2A-D, the image of the positive control is shown in Figure 2E, and the morphometric quantification is presented in Figure 2F.

Impact of the broncho-alveolar lavage fluid from cigarette smoke-exposed mice on growth and clearance of NTHI

We next questioned whether elements within the BAL fluid (BALF) of cigarette smoke-exposed mice could directly interfere with NTHI growth, leading to the observation of decreased bacterial burden in cigarette smoke-exposed mice following NTHI challenge. To address this hypothesis, BALB/c and C57BL/6 mice were cigarette smoke-exposed twice daily for a period of 8 weeks. Following this time, mice were killed, BAL isolated, and the cellular fraction was removed by centrifugation. Incubation of NTHI with BALF from cigarette smoke-exposed mice for 30 minutes, with subsequent plating in BALF on chocolate agar for 2 days demonstrated no difference in the concentration of NTHI, as compared to BALF from control mice incubated with NTHI (control BALF + NTHI: 384.8 ± 63.9 CFU/ml, smoke BALF + NTHI: 318.8 ± 50.7 CFU/ml, p=0.29). Thus, BALF from cigarette smoke-exposed mice was not directly toxic to the growth of NTHI.

To further investigate whether cell-free components within the BALF of cigarette smoke-exposed mice may contribute to clearance of NTHI, we incubated NTHI with BALF isolated from either cigarette smoke-exposed or control BALB/c mice and inoculated the NTHI + BALF suspension into naïve BALB/c mice. Twelve (12) hours post-challenge we observed significantly decreased NTHI burden in the lungs of the mice that received the inoculum of NTHI + BALF from cigarette smoke-exposed mice, as compared to the mice that received the inoculum of NTHI.
+ BALF from control mice (Figure 3A). Taken together, these data suggested that the decreased NTHI burden in cigarette smoke-exposed mice may be contributed to, in part, by a cell-free factor in the BAL of cigarette smoke-exposed mice.

Perhaps most interestingly, while BALF isolated from cigarette smoke-exposed mice was able to passively transfer the effect of decreased bacterial burden, the same was not true for the effect of exacerbated inflammation post-challenge. As shown in Figure 3B-D, we observed similar number of total cells, MNCs, and neutrophils between naïve mice challenged with control BALF + NTHI and mice challenged with smoke BALF + NTHI. Thus, while the decreased bacterial burden observed in the lungs of cigarette smoke-exposed mice may be contributed to by a cell-free factor, exacerbated inflammation may be dependent on a cellular component resident to the cigarette smoke-exposed lungs. Notably, transfer of BALF isolated from control mice to naïve mice demonstrated a protective effect, as the total number of cells, mononuclear cells, and neutrophils were significantly decreased 12 hours post-challenge.

**Impact of cigarette smoke exposure on cellular profile of mice challenged with heat-inactivated NTHI**

The evidence from the transfer experiment indicated that the mechanisms underlying the decreased bacterial burden and exacerbated inflammatory profile may be independent events following challenge. As such, we next investigated whether live bacteria was required for our observation of an exacerbated inflammatory response following challenge. Similarly to previous experiments, BALB/c mice were cigarette smoke-exposed twice-daily for a total period of 8 weeks. Following this time mice were challenged with $10^6$ CFU equivalents of heat-inactivated NTHI. Plating of heat-inactivated NTHI onto chocolate agar verified that the bacteria had been inactivated (data not shown).
Mice that were cigarette smoke-exposed and challenged with heat-inactivated NTHI had a heightened inflammatory profile compared to control mice challenged with heat-inactivated NTHI (Figure 4A-C). Specifically, cigarette smoke-exposed mice challenged with heat-inactivated NTHI had significantly increased numbers of total cells (Figure 4A), consisting of significantly increased numbers of neutrophils (Figure 4C). These data indicate that the exacerbated inflammatory profile observed following challenge in cigarette smoke-exposed mice is not dependent on live NTHI.

Measurement of antibody titres in cigarette smoke-exposed mice

Immunoglobulins (Igs) are found in the fluid phase of the BAL, and have important in vivo antibacterial functions, but often rely on cells and other host factors to mediate a number of effector functions, limiting their in vitro antibacterial activity. Therefore, we next questioned whether the cell-free factor in the BALF of cigarette smoke-exposed mice might be NTHI-binding immunoglobulin, which would be consistent with the observation that BALF from cigarette smoke-exposed mice is not directly toxic to NTHI, but does contribute to accelerated NTHI clearance in vivo. We first measured antibody titres of IgM, IgG, and IgA, in the BAL of once- or twice-daily cigarette smoke-exposed BALB/c or C57BL/6 mice. Levels of IgM were similar between control, and once- or twice-daily cigarette smoke-exposed BALB/c or C57BL/6 mice (Figure 5A and 5D). In contrast, levels of IgG were increased only in twice-daily cigarette smoke-exposed C57BL/6 mice, and not in BALB/c mice under either condition (Figure 5B and 5E). Levels of IgA were increased in both once- or twice-daily cigarette smoke-exposed BALB/c or C57BL/6 mice (Figure 5C and 5F).

To test for the possibility that cigarette smoke-exposure may have led to increased systemic production of antibody, we measured the level of circulating immunoglobulin in the serum of BALB/c or C57BL/6 mice, and observed no difference in the levels of IgM (Figure 6A and 6D),
IgG (Figure 6B and 6E), or IgA (Figure 6C and 6F), between cigarette smoke-exposed and control mice.

**Impact of B cell deficiency on the bacterial burden and cellular profile of cigarette smoke-exposed mice challenged with NTHI**

As we observed increased titres of antibodies in the BAL of cigarette smoke-exposed mice, we next investigated how the absence of antibodies would affect our observation of decreased bacterial burden in the lungs following challenge with NTHI. To this end, we utilized JH⁻/⁻ mice that are deficient in B cells [16]. Wild type (WT) C57BL/6 or knockout (KO) JH⁻/⁻ mice were exposed twice-daily to cigarette smoke for a period of 8 weeks, subsequently challenged intranasally with NTHI, and killed 12 hours post challenge. As demonstrated in Figure 7A, in contrast to WT mice, cigarette smoke-exposed JH⁻/⁻ mice had increased bacteria burden as compared to control JH⁻/⁻ mice. To verify B cell deficiency, levels of IgM, IgG, and IgA in the serum and BAL were measured and found to be below the limit of detection for all isotypes (data not shown). Similar to previous, cigarette smoke-exposed WT C57BL/6 mice had decreased bacterial compared to control WT mice.

12 hours post-challenge with NTHI, we observed increased total cells, mononuclear cells, and neutrophils in the BAL of cigarette smoke-exposed, NTHI-challenged JH⁻/⁻ mice, as compared to control NTHI-challenged JH⁻/⁻ mice (Figure 7B-D). Cigarette smoke exposure alone in JH⁻/⁻ mice was associated with an increased cell number in the BALs, consisting of increased number of mononuclear cells (Figure 7C) and neutrophils (Figure 7D), similar to the case seen in WT controls (Figure 1).

**Measurement of NTHI-specific antibody titres in cigarette smoke-exposed mice**
To investigate the specificity of antibodies in cigarette smoke-exposed mice, we measured the level of NTHI specific immunoglobulin in the BAL of BALB/c and C57BL/6 mice prior to NTHI challenge. We observed increased levels of IgA (Figure 8C and 8F) in the BAL of twice-daily cigarette smoke-exposed BALB/c and C57BL/6 mice as compared to control mice. No difference was observed with respect to IgM (Figure 8A and 8D), and IgG (Figure 8B and 8E), between cigarette smoke-exposed and control mice.
DISCUSSION

The purpose of this study was to investigate how cigarette smoke exposure alters the host-defense response to a pulmonary bacterial challenge. To this end, we utilized a model of experimental cigarette smoke exposure that results in chronic inflammation of the respiratory tract [15], and NTHI - the dominant cause of bacterial exacerbation in COPD patients [12].

COPD is a heterogeneous and multifactorial disease, and the experimental systems used to study it are similarly diverse [27]. Environmental factors (principally cigarette smoke) and genetics both play a key role. With respect to the former, our general model of cigarette smoke exposure consists of twice-daily smoke exposure for an 8-week period, which leads to a sustained neutrophilia in the BAL, similar to that often reported from clinical studies [28]. In contrast, once-daily cigarette smoke exposure did not result in BAL neutrophilia, indicative of a dose-response of cigarette smoke-induced pulmonary inflammation in mice. Importantly, inflammation observed in both cigarette smoke-exposed C57BL/6 and BALB/c mice was further exacerbated following bacterial challenge, and significantly increased compared to sham-exposed NTHI-challenged animals.

While mucus hypersecretion is common in COPD patients and may increase during periods of acute exacerbations [24, 29], neither cigarette smoke nor NTHI induced mucus metaplasia. The latter observation is in agreement with studies by Moghaddam et al [30], demonstrating robust inflammation following administration of an NTHI lysate, but no airway mucin staining. With regard to cigarette smoke-induced mucus production, there appears to be clear differences between species as mucus hypersecretion is a common pathologic feature of cigarette smoke-exposed rats [25].

Of note, cigarette smoke-exposed animals cleared the NTHI from their lungs more rapidly than did control mice. The present study suggests that this accelerated bacterial clearance is not dependent on pre-existing neutrophilic inflammation. The evidence for this is twofold: firstly, a
decreased bacterial burden is observed in both once- and twice-daily exposed mice, while pre-existing neutrophilia is observed only in twice-daily exposed mice. Second, by transferring cell-free BAL fluid from cigarette smoke-exposed, NTHI-naïve mice to control mice, we were also able to transfer the accelerated bacterial clearance. That B-cell deficient, smoke-exposed mice did not clear a pulmonary NTHI challenge any more quickly than did unexposed B-cell deficient controls provides evidence that the cell-free mediator of protection in these mice is in fact an immunoglobulin. With respect to the mechanism underlying this phenomenon, we observed increased total IgA, and increased NTHI-binding IgA, but not IgG or IgM in the BAL of smoke-exposed, NTHI-naïve mice. All together, our findings suggest that the protective factor is an immunoglobulin, most likely IgA. This mode of protection appears to be distinct to the recently-described protection conferred by bacterial lysate, which was mediated by epithelial cells’ innate antimicrobial defenses pathways [31]. Mechanisms of accelerated clearance via NTHI-specific IgA likely involve opsonization of bacteria and subsequent phagocytosis. The relative contribution of macrophages and neutrophils to bacterial clearance remains to be determined.

There is clinical evidence that the absolute titers of IgA in the BAL of patients with chronic bronchitis may be increased [32, 33], although other studies demonstrated decreased titers of IgA in the BALF [34]. To our knowledge however, no study has yet addressed the consequences of smoking on an in vivo bacterial challenge in humans, likely due to ethical considerations. It is important to note that our model recapitulates the clinical finding of exacerbated inflammation following challenge.

Experimentally, Demoor et al. demonstrated increased titres of IgM and IgA in BAL of cigarette smoke-exposed mice [35], an observation that concurs with our findings. The origin of the NTHI-specific IgA in the BAL of cigarette smoke exposed mice remains to be determined. NTHI is an obligate human pathogen, so it is unlikely that mice were exposed to NTHI prior to bacterial
challenge, a necessary step for the induction of antigen-specific antibodies. This implies that the cigarette smoke-induced antibodies are instead natural antibodies against conserved bacterial targets, as have been shown to protect against nasal colonization with *Haemophilus influenzae* in mice [36]. Similarly, Lund *et al.* demonstrated that mice are protected from *P. carini* in a B cell and antibody-dependent, but antigen-specific antibody-independent, manner [37]. Of note, accumulation of IgA in the airway lumen required a lower threshold of cigarette smoke exposure (once-daily) than the threshold necessary for recruitment of neutrophils into the lung lumen (twice-daily).

Evidence suggests that B cells may be an important cell for the induction of inflammation following pulmonary infection with *Pneumocystis (P.) carini* [37]. The data presented here does not support a role for B cells as a contributing mechanism for exacerbated inflammation, since the inflammatory profile observed in B cell deficient mice was similar to wild type control animals. Building on previous observations in our lab and others, it is tempting to speculate that alterations to the alveolar macrophage population contribute to the exacerbated inflammation observed in cigarette smoke-exposed, NTHI challenged mice [38]. We have demonstrated that alveolar macrophages from cigarette smoke-exposed mice produce a different subset of pro-inflammatory cytokines than do those from control mice, and this may contribute to the exacerbated response [15]. Importantly, these effects are reversible, as smoking cessation reversed the effects on alveolar macrophages, likely a result of turnover of the alveolar macrophage population [17].

Other experimental models of cigarette smoke exposure have demonstrated that the burden of bacteria may be increased following challenge, rather than decreased [39, 40]. These studies, however, did not report levels of antibodies in the BAL fluid and consequently it is difficult to directly compare these different studies. It is entirely possible that different models of cigarette smoke exposure result in conflicting data on bacterial clearance, especially following challenge with different types of bacteria. This certainly reflects the complexity of modeling COPD, which itself is
quite heterogeneous, and demonstrates the need to understand as many models, and variation of those models, including dose, time, frequency, and exacerbating stimulus, in order to fully appreciate the pathological process(es) underlying COPD development and/or progression. Further adding to the complexity, the initial stimulus may be altered from models using cigarette smoke exposure, to models using different initial stimuli, such as that seen with models of elastase-induced emphysema, and a streptococcus bacteria as the exacerbating stimulus [41, 42].

Data presented in this study demonstrate that cigarette smoke exposure leads to exacerbated inflammation following bacterial challenge and strongly suggest that antibodies are important for clearance of bacteria from a cigarette smoke-exposed mouse lung. These data would further argue that a lung resident cell, rather than bacterial burden, is central to driving the exacerbated inflammatory profile, and should be an important consideration for therapeutic approaches aimed at interfering with mechanisms underlying the inflammation. Given the enormous burden of disease, the heterogeneity between subjects and models, efforts into understanding the exacerbated inflammatory profile are certainly warranted.
ACKNOWLEDGEMENTS

This study was funded by the Canadian Institutes for Health Research (CIHR). MRS holds a Canadian Institutes for Health Research New Investigator award. The authors gratefully acknowledge the expert technical support of Joanna Kasinska, Sussan Kianpour, Calvin Yeh, and the secretarial assistance of Marie Colbert.
FIGURE LEGENDS

FIGURE 1: Impact of once- or twice-daily cigarette smoke exposure on the bacterial burden and cellular profile of mice challenged with NTHI. BALB/c [Top panels] and C57BL/6 mice [Bottom panels] were exposed to room air (open bars), once-daily cigarette smoke (grey bars), or twice-daily cigarette smoke (black bars), 5 days a week, for 8 weeks. In the eighth week of exposure mice were challenged intranasaly with sterile vehicle (PBS) or 10⁶ CFU of NTHI and killed 12 hours post challenge. A-C), and E-G) Differential cell analysis showing the number of total cells (total), mononuclear cells (MNC), and polymorphonuclear cells (PMN) in the BAL. Inserts in panels C) and G) are the data for PBS challenge shown on a smaller scale (10⁵ compared to 10⁶). D) and H) Concentration of NTHI in the lung homogenates. Data are representative of 2 independent experiments, n=5/group, and are expressed as means ± SEMs, n=10/group. Statistical analysis was completed by 2-way ANOVA, * and † denote p values less than 0.05 compared to control + PBS or control + NTHI, respectively.
FIGURE 2: Impact of cigarette smoke exposure and NTHI challenge on mucus production and goblet cell hyperplasia. BALB/c mice were exposed to cigarette smoke (smoke) or room air (control) twice daily, 5 days a week, for 8 weeks. In the eighth week of exposure mice were challenged intranasaly with sterile vehicle (PBS) or $10^6$ CFU of NTHI. A) Expression of Muc5AC, assessed by quantitative RT-PCR in lung tissue collected 12 hours post-challenge. B) Representative light photomicrographs and quantification of paraffin-embedded cross-sections of lung tissue stained with alcian blue/periodic acid-Schiff (AB/PAS) indicating mucus production by epithelial goblet cells obtained 12 hours post-challenge. The calibrator sample was obtained from mouse chronically exposed to house dust mite as a positive control. Morphometric analysis indicates the percentage of the area of interest that is
stained with AB/PAS. Pictures were taken at 20x original magnification. Data are representative of 2 independent experiments, n=5/group, and are expressed as means ± SEMs, n=10/group. Statistical analysis was completed by 2-way ANOVA.

FIGURE 2

FIGURE 3: Impact of transfer of BALF isolated from cigarette smoke-exposed mice on bacterial burden and cellular profile following challenge of naïve mice with NTHI. BALB/c mice were exposed to cigarette smoke (smk) or room air (ctrl) twice daily, 5 days a week, for 8 weeks. In the eighth week of exposure, mice were killed, BAL isolated, and the cellular fraction removed to obtain the BALF. BALF from
control or smoke-exposed mice was mixed with $10^6$ CFU of NTHI, and naïve mice were challenged with the BAL fluid + NTHI inoculum. 12 hours post challenge, mice were killed and lung homogenates plated onto chocolate agar for assessment of bacterial burden (A). B-D) Differential cell analysis showing the number of total cells (total), mononuclear cells (MNC) and polymorphonuclear cells (PMN) in the BAL of the naïve mice 12 hours post-challenge with the BAL fluid NTHI mixture. Data are representative of 2 independent experiments, n=5/group, and are expressed as means ± SEMs, n=10/group. Statistical analysis was completed by 2-way ANOVA, * and † denote p values less than 0.05 compared to naïve + PBS or naïve + NTHI, respectively.

FIGURE 3

FIGURE 4: Impact of cigarette smoke exposure on the cellular profile of mice challenged with heat-inactivated NTHI. BALB/c mice were exposed to cigarette smoke (smoke) or room air (control) twice daily, 5 days a week, for 8 weeks. In the eighth week of exposure mice were challenged intranasaly with sterile vehicle (PBS) or $10^6$ CFU of heat-inactivated NTHI and killed 12 hours post challenge. A-C)
Differential cell analysis showing the number of total cell (total) (A), mononuclear cells (MNC) (B), and polymorphonuclear cells (PMN) (C), in the BAL. Data are representative of 2 independent experiments, n=5/group, and are expressed as means ± SEMs, n=10/group. Statistical analysis was completed by 2-way ANOVA, * and † denote p values less than 0.05 compared to control + PBS or control + NTHI, respectively.

FIGURE 4

FIGURE 5: Level of IgM, IgG, IgA, in the BAL of cigarette smoke-exposed mice. BALB/c [Top panels] and C57BL/6 mice [Bottom panels] were exposed to room air (open bars), once-daily cigarette smoke (grey bars), or twice-daily cigarette smoke (black bars), 5 days a week, for 8 weeks. In the eighth week of exposure, mice were killed and BAL isolated. Panels A and D) show total levels of IgM; B and E) total levels of IgG; C and F) total levels of IgA. Data are representative of 2 independent experiments, n=4-5/group, and are expressed as means ± SEMs, n=8-10/group. Statistical analysis was completed by one-way ANOVA, * denotes p values less than 0.05 compared to control + PBS.
FIGURE 6: Level of IgM, IgG, IgA, in the serum of cigarette smoke-exposed mice. BALB/c [Top panels] and C57BL/6 mice [Bottom panels] were exposed to room air (open bars), once-daily cigarette smoke (grey bars), or twice-daily cigarette smoke (black bars), 5 days a week, for 8 weeks. In the eighth week of exposure, mice were killed and serum isolated. Panels A and D) show total levels of IgM; B and E) total levels of IgG; C and F) total levels of IgA. Data are representative of 2 independent experiments, n=5/group, and are expressed as means ± SEMs, n=10/group. Statistical analysis was completed by one way ANOVA, * denotes p values less than 0.05 compared to control + PBS.
FIGURE 7: Impact of B cell deficiency on the bacterial burden and cellular profile of cigarette smoke-exposed mice challenged with NTHI. C57BL/6 mice (WT) and JH⁻/⁻ B-cell deficient mice (KO) were exposed to room air (open bars) or cigarette smoke (closed bars) twice daily, 5 days a week, for 8 weeks. In the eighth week of exposure mice were challenged intranasally with sterile vehicle (PBS) or 10⁶ CFU of NTHI and killed 12 hours post challenge. A) NTHI burden in lung homogenates. B-D) Differential cell analysis showing the number of total cells (total) (B), mononuclear cells (MNC) (C) and polymorphonuclear cells (PMN) (D) in the BAL. Data are expressed as means ± SEMs, n=5/group. Statistical analysis was completed by 3-way ANOVA, * and † denote p values less than 0.05 compared to control PBS or smoke PBS, respectively.
FIGURE 8: Level of NTHI-specific IgM, IgG, IgA, in the BAL of cigarette smoke-exposed mice. BALB/c [Top panels] and C57BL/6 mice [Bottom panels] were exposed to room air (open bars), once-daily cigarette smoke (grey bars), or twice-daily cigarette smoke (black bars), 5 days a week, for 8 weeks. In the eighth week of exposure, mice were killed and BAL isolated. Panels A and D) show total levels of NTHI specific IgM; B and E) total levels of NTHI specific IgG; C and F) total levels of NTHI specific IgA. Data are expressed as means ± SEMs, n=2-5/group.
**Figure 8**

A. IgM for BALB/c

B. IgG for BALB/c

C. IgA for BALB/c

D. IgM for C57BL/6

E. IgG for C57BL/6

F. IgA for C57BL/6

- ■ control
- ○ smoke

OD$_{405nm}$ vs. Dilution
REFERENCES


