IMPACT OF SMOKE ON MICROBIAL-INDUCED PULMONARY INFLAMMATION

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THE IMPACT OF CIGARETTE SMOKE-EXPOSURE ON PATHWAYS OF MICROBIAL-INDUCED PULMONARY INFLAMMATION

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Abstract

The cellular, molecular, and genetic mechanisms underlying the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) are not well understood. The purpose of this thesis was to address the hypothesis that microbial infection is important for the development and/or progression of COPD through investigation of how cigarette smoke alters the response to a bacterial challenge in a mouse model of cigarette smoke-exposure. To this end, in chapter 2 of this thesis we tested the hypothesis that cigarette smoke-exposure attenuates the ability of alveolar macrophages to sense microbial antigens through innate pattern recognition receptors. The central point of this study was the observation that alveolar macrophages isolated from cigarette smoke-exposed mice had attenuated expression of typical inflammatory cytokines following microbial stimulation. Building on this main observation, in chapter 3 we questioned what the consequences of this would be to an *in vivo* bacterial challenge with nontypeable *Haemophilus influenzae*. We demonstrated that cigarette smoke-exposure resulted in chronic inflammation, this inflammation was exacerbated following bacterial challenge, and perhaps most importantly, the nature of the inflammatory response was altered. Interestingly, an observation from the study in chapter 3 indicated that exacerbated inflammation in cigarette smoke-exposed mice may be beneficial for clearance of the bacteria, but may come at the expense of damage to the lungs. Consequently, in chapter 4 we questioned the strain and dose/frequency stringencies of cigarette smoke-exposure on the observation of accelerated bacterial clearance. We demonstrated a role for antibodies in bacterial clearance. Collectively, this thesis provides insight into our understanding of COPD by demonstrating that cigarette smoke-exposure alters the pulmonary immune/inflammatory response to a microbial challenge, which has a detrimental impact on the lungs.

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Chapter 1:

INTRODUCTION

The fact that cigarette smoke is deleterious to health is well understood. Regardless, an estimated 1.3 billion people, or one sixth of the world's population, are current, active smokers (1). It has been further estimated that while not active smokers, 700 million children, or one half of world's children, are passively exposed to cigarette smoke (2). Approximately half of all smokers, and an unknown percentage of passively exposed individuals, will develop smoking related diseases, most notably the development of Chronic Obstructive Pulmonary Disease (COPD), a disease characterized by progressive, irreversible airway obstruction (3). Based on current estimates of prevalence, COPD is both under-diagnosed and under-treated (4-6). COPD consistently places among the top diseases for total health care expenditures, placing an enormous burden on health services, a burden in large part due to treating periods of symptom exacerbation (7). Exacerbations are acute episodes where the severity of symptoms increases, often as a result of bacterial or viral infection (8).

Despite the understanding that cigarette smoking is by far the dominant etiological factor for COPD, as greater than 90% of COPD patients are current or former smokers (9), the pathogenesis of COPD is not well understood. The purpose of this thesis was to investigate the mechanisms underlying cigarette smoke-induced lung pathology in a mouse model of cigarette smoke-exposure. The underlying hypothesis was that microbial infection(s), leading to periods of symptom exacerbation contribute to cigarette smoke-induced lung pathology.

The remaining part of the first chapter of this thesis is divided into 5 parts:

1. An introduction to COPD.
2. An introduction to experimental modeling of COPD.
3. An introduction to how cigarette smoke elicits inflammation.
4. An introduction to how cigarette smoke alters microbial defense pathways, contributing to infection and exacerbated inflammation.
5. An introduction to the hypothesis and studies completed for chapters 3, 4, and 5 of this thesis.

Chronic Obstructive Pulmonary Disease

COPD is caused by the chronic inhalation of toxic gases and particulate matter, most often in the form of cigarette smoke (3). COPD is not a single disease but rather a syndrome whose functional and clinical hallmark is progressive and largely irreversible airflow limitation (3). Airflow limitation is determined by measuring the maximum volume of air that can be forcibly expired in 1 second (FEV_1) and its ratio to the forced vital capacity (FVC), the maximum volume that can be expired with no time limit. The Global Initiative on Obstructive Lung Disease (GOLD) defined the presence of COPD as an FEV_1:FVC ratio less than 0.7 (3, 10). This initiative further categorized COPD into 4 stages of severity based on FEV_1 (GOLD 1-4): mild COPD, GOLD-1, is present when the FEV_1 is greater than 80% of the predicted normal value; moderate COPD, GOLD-2, is present when the FEV_1 is between 80% and 50% predicted; severe COPD, GOLD-3, is present when the FEV_1 is between 50% and 30% predicted; and finally, very severe COPD, GOLD-4, is present when the FEV_1 is below 30% predicted (3, 10).

In an individual COPD patient, varying degrees of chronic bronchitis, emphysema, and small airway remodeling contribute to airflow limitation (11-13). Chronic bronchitis refers to chronic inflammation of the bronchi, clinically defined as 'a persistent cough that produces sputum and mucus, for at least three months in two consecutive years' (3). Of particular relevance to this thesis, chronic inflammation is present at all GOLD stages and is regarded as a major contributor to COPD pathogenesis, including development of emphysema and small airway remodeling (14).

Emphysema is a histopathological term describing the loss of the functional airspace of the lung. A workshop by the National Heart, Lung and Blood Institute (NHLBI) defined this as 'the abnormal permanent enlargement of air spaces distal to terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis' (15). More specifically, emphysema results in the destruction of the alveolar walls, as well as the loss of the alveolar attachments to the small airways that keep the airways open during expiration, which leads to collapse of the airways (16). As a consequence the elastic recoil of the lung, which is responsible for driving expiratory flow, is reduced. A general hypothesis in COPD is that a protease-antiprotease imbalance, as a result of chronic cigarette smoking and chronic inflammation, drives the development of emphysema (17). This is largely based on the clinical observation that patients with an $\alpha 1$ antitrypsin deficiency developed emphysema (18), and the experimental evidence that instillation of proteases into the lungs of mice led to an emphysema-like phenotype (19).

Small airway remodeling includes deposition of matrix components around the airways and inflammatory cell accumulation and goblet cell metaplasia in the airway wall, with the consequence of narrowing and/or distortion of the airways, as well as obstruction

by mucous (20, 21). An early study by Hogg *et al.* demonstrated that the airways with a diameter of less than 2 mm are the main site of airway obstruction in COPD (20). A further study by Matsuba *et al.* demonstrated that the thickening of the walls and reduction of the lumen of these airways contribute to airflow limitation (22). Importantly, these changes are associated with disease severity as they increase with increasing GOLD stage (21).

In consideration of these processes together, airflow limitation in COPD may be contributed to by small airway remodeling, leading to thickening, narrowing, and obstruction of the airways, as well as emphysema, leading to the loss of the functional airspace. While these process may share the same location, as well as the same cause, chronic inhalation of cigarette smoke, the underlying cellular, molecular, and genetic processes involved in the development and progression of each process as a result of cigarette smoke are not well understood.

A feature of COPD that is well established is that throughout the pathogenesis of COPD patients undergo periods of increased symptom severity. These acute exacerbations are clinically defined as 'a sustained worsening of symptoms from the patient's stable COPD state, beyond normal day-to-day variations, which necessitate a change in medication' (23). Symptoms may include increased dyspnea, wheezing, chest tightness, cough, and changes in the amount, color and/or tenacity of sputum (3). While there is wide variation among patients, the average frequency of exacerbation is typically one to two exacerbations annually, although the frequency may increase with increasing disease severity (24). Similar to stable disease, the cellular and molecular mechanisms underlying clinical exacerbation are not well defined, although it is understood that an intensification of the inflammatory response above stable disease state occurs during exacerbation.

Bacterial and/or viral infections are the main etiological causes of exacerbation, with air pollution and other environmental factors having a minor role (8, 25). Bacterial infections, in particular, are estimated to cause half of all exacerbations, and a number of studies have demonstrated that bacterial infection with nontypeable *Haemophilus influenzae* (NTHI), *Streptococcus pneumoniae*, *Moraxella catarrhalis*, or *Pseudomonas aeruginosa*, result in exacerbations of COPD (26-29). In fact, of all bacterial driven exacerbations, greater than 50% are attributed to acquisition of a new strain of NTHI (28). It is unclear, however, how this agent causes exacerbation, as NTHI is relatively harmless and often found commensally in healthy individuals.

Experimental Modeling of Chronic Obstructive Pulmonary Disease

Experimental models are important tools for investigating pathogenesis of human disease. Furthermore, they are valuable for development and preclinical screening of therapeutic pathways and targets, an especially important point as there are currently very few treatments that may slow the progression of COPD (30). There are a number of experimental approaches that have been used to model COPD in animals, including exposing animals to cigarette smoke or inflammatory stimuli (31-33), instilling proteolytic enzymes into the airways (19, 34), models of induced apoptosis (35, 36), models of starvation or malnourishment (37, 38), and the study of spontaneous gene mutants and knockout strains (39, 40). Typically, these studies have addressed single aspects of COPD, such as inflammation, emphysema, or mucous production, but fail to recapitulate the entirety of the COPD syndrome. This is not overly surprising, however, given both the considerable

complexity observed among individuals, as well as in a single patient's disease profile over time.

Because cigarette smoking is the dominant etiological factor for development of COPD, cigarette smoke-exposure is the logical choice for development of experimental models (9). Indeed, cigarette smoke-exposure models do result in certain aspects of COPD, including chronic inflammation, emphysema, and small airway remodeling (31). Other aspects, including mucous hypersecretion, have proven difficult to recapitulate with mouse models of cigarette smoke-exposure, but may occur in rat models of cigarette smoke-exposure (41). These results, however, often vary considerably among research groups and different groups often report conflicting results. Part of this variability stems from the fact that there is no standard or consistent method for exposure to cigarette smoke, and different research groups utilize different types of exposure (such as whole-body versus nose-only, or main-stream versus side-stream, cigarette smoke-exposure), different frequencies and/or duration of exposure, and different species, strains, and suppliers of animals. Moreover, the disease produced by models of cigarette smoke-exposure is mild compared to human disease, and likely reflects an early or mild form of COPD (21, 33). As these studies never report advanced disease, careful interpretation of these results together would indicate that there is an inherent difference between experimental animals and humans in the sensitivity to cigarette smoke, which may include the duration/frequency of exposure, differences in lung anatomy and physiology, and/or genetic susceptibility. Additionally, there may be other contributing factors that humans are exposed to that are absent in typical experimental models of cigarette smoke-exposure. Along these lines, it has previously been hypothesized

that periods of exacerbation, acute episodes of increased symptom severity and inflammation, are important for disease progression (42-44).

In order to provide understanding of the underlying cellular, molecular, and genetic mechanisms of COPD, addressing the effect of cigarette smoke-exposure on exacerbation-causing bacterial infections in animals may provide significant insight into COPD pathogenesis. To specifically address this, experimental models would have to consider both:

1. How cigarette smoke results in inflammation, and
2. How cigarette smoke alters pathways of microbial host defense.

This would allow for increased understanding of how cigarette smoke alters the baseline state of the lungs, as a result of inducing a chronic inflammatory response, and then to investigate deviations from this new baseline, and from normal, as a result of bacterial infection. Importantly, these two investigations are inherently linked to one another, as the change in the baseline state of the lungs as a result of cigarette smoke and ongoing inflammation may predispose to microbial infection, as well as exacerbated inflammation and damage to the lungs following infection. Furthermore, the exacerbated inflammation and lung damage following infection may contribute to amplifying the chronic inflammation, such as by damaging the lung architecture and innate barriers, or by colonization of the lower airways by microbial agents.

Cigarette smoke & inflammation

Inflammation is an intricate response to infection and tissue damage initiated by stimulation of pattern recognition receptors by either pathogen associated molecular patterns on microbes and viruses or endogenous danger signals. This stimulation leads to the

production of pro-inflammatory mediators that orchestrate a local inflammatory response by increasing the permeability of the endothelium allowing leakage of plasma proteins, clotting factors, and complement into to the tissue, as well as activating endothelial cells to express adhesion molecules to facilitate recruitment of leukocytes, which may then follow a chemotactic gradient to the infected/damaged tissue. As a result of the pronounced physiological changes induced by inflammatory responses, excessive, chronic, or recurrent inflammation can lead to tissue damage (45). Indeed, experimental models of chronic inflammation, as induced by repeated stimulation with microbial antigens (46) or by persistent viral infection (47), result in phenotypes similar to obstructive lung disease independent of cigarette smoke.

Cigarette smoke consists of both vapour and particulate phases, and contains more than 4500 compounds that may have biological effects, including carcinogens, toxins, and oxidants (48,49). On inhalation of cigarette smoke, resident lung cells, including different types of epithelial cells, secretory cells, and immune cells, may be stimulated by components within both the particulate and vapour phase. Stimulation of these different cell populations initiates a sequence of events leading to the recruitment and activation of multiple types of inflammatory cells. Furthermore, components of cigarette smoke may change the function of these cells, as well as either promoting their survival or their death, as discussed below.

With regards to activation of inflammatory pathways, there is evidence that cigarette smoke stimulates cells via a toll-like receptor (TLR) -4, MyD88 dependent pathway (50). TLR-4 is an innate pattern recognition molecule, expressed on many cell types leading to the induction of innate immune responses and the production of pro-inflammatory cytokines and chemokines. Known ligands for TLR-4 include exogenous molecules such as bacterial

LPS (51), as well as endogenous molecules such as heat shock protein (HSP) -70 (52) or high mobility group box -1 protein (HMGB-1) (53). Cigarette smoke does contain appreciable levels of biologically active LPS (54), but cigarette smoke activation of TLR-4 is thought to be largely a result of providing a 'danger signal' and induction of HSP-70 (50). Indeed, there is evidence that both lung epithelial cells (55, 56) and macrophages (57) are activated by cigarette smoke components, leading to the production of pro-inflammatory cytokines and chemokines.

In sputum and bronco-alveolar lavage (BAL) fluid obtained from COPD patients, many cytokines and chemokines are increased. Perhaps most notably are the expression of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 (58). These particular cytokines often work together initiating an acute phase response following tissue injury or infection. TNFα is mainly produced by macrophages, but may be made by a number of other cells, and is a potent inducer of inflammation, endothelial activation, phagocytosis, and fever. Similarly, IL-1β is mainly produced by monocytes, macrophages, and epithelial cells, and contributes to induction of inflammation, endothelial activation, induction of fever, and macrophage and T cell activation. IL-6 is a very pleiotropic cytokine that may either be pro- or anti-inflammatory, and whose main pro-inflammatory functions include fever induction, T and B cell differentiation, and production of acute phase proteins.

Levels of chemokines are also increased in the sputum and BAL fluid of COPD patients (58). Included in this are members of the CC family of chemokines including CCL -2, -3, -4, and -5 (59-62), as well as members of the CXCL family of chemokines including CXCL-1 and CXCL-8 (62, 63). CCL -2, -3, -4, and -5 are important for the recruitment of a number of different cell types, including monocytes, immature dendritic cells, T cells, and

natural killer cells. CXCL-1 and CXCL-8, on the other hand, are somewhat more specific, and are important for the recruitment of neutrophils and fibroblasts, and neutrophils and basophils, respectively. In COPD, the expression of these factors provides a signal recruiting cells into the respiratory tract.

Regulatory cytokines, such as IL-10, act to dampen or suppress immune responses, including the production of pro-inflammatory cytokines and chemokines, such as TNFα and CXCL-8. There is evidence in COPD that there may be decreased levels of IL-10, which may further contribute to the pro-inflammatory mediator environment (64).

These pro-inflammatory cytokines and chemokines, and potentially a lack of regulatory cytokines, are believed to be important for the chronic inflammation observed in patients with COPD, consisting of, among other cells, increased numbers of neutrophils and macrophages. Neutrophil numbers are reported increased in both the lung tissue as well as in sputum of COPD patients (21, 63). This is particularly relevant to the pathogenesis of COPD, as the percentage of airways containing neutrophils increases with increasing GOLD stage (21). Similarly, the number of macrophages is increased in the airways, parenchyma, and lung lumen of patients with COPD, and as with neutrophils increase with increasing GOLD stage (21, 65). Providing further evidence linking inflammation to severity of airflow limitation, Di Stefano *et al.* compared healthy smokers to smokers with airflow limitation and demonstrated that in smokers the severity of airflow limitation is correlated with the severity of airway inflammation, and that severe airflow limitation is associated with an increased number of neutrophils and macrophages in the bronchial mucosa (66).

Inflammation is not only associated with disease severity (21), but is intrinsically linked to tissue damage. In lung biopsies of patients with emphysema macrophages are

localized to sites of alveolar wall destruction (67, 68). Experimental evidence, utilizing both models of cigarette smoke-exposure or instillation of enzymes into the airways, has demonstrated that destruction of the alveolar walls is driven by proteolytic enzymes (69). In the lungs, the dominant sources of proteolytic enzymes are resident alveolar macrophages, as well as recruited neutrophils and macrophages. Both neutrophils and macrophages have the capability of releasing an assortment of proteolytic enzymes, including elastase and members of the matrix metalloproteinases (MMPs) family (70-72).

In bronchial biopsies and BAL fluid of patients with COPD, levels of neutrophil elastase and MMPs -2, -9, and -12 are increased (73, 74). MMP-9, in particular, has been implicated in the pathogenesis of COPD as it is the most abundant proteolytic enzyme secreted by alveolar macrophages from COPD patients, and alveolar macrophages isolated from COPD patients express increased levels of MMP-9 following *ex vivo* stimulation compared to alveolar macrophages isolated from control subjects (75). That being said, other proteolytic enzymes may have important roles as well, as MMP-12 has been experimentally demonstrated to be critical for the development of cigarette smoke-induced emphysema in mice (33). Furthermore, there is evidence that alveolar macrophages isolated from COPD patients express attenuated levels of anti-proteases, enzymes that act to limit the function of proteases, including the expression of tissue inhibitor of metalloproteinase (TIMP) -1 following stimulation (75, 76).

It is the increased expression of proteases, and a lack of anti-proteases, that is thought to contribute to an imbalance leading to tissue destruction. The destructive potential of proteolytic enzymes does not end at the level of cleavage of matrix substrates and lung damage, as breakdown fragments of the matrix have been demonstrated to have chemotactic

properties leading to recruitment of neutrophils and monocytes (77). Specifically, in a mouse model of instillation of proteolytic enzymes into the airways elastin fragments were essential to driving monocyte recruitment and airspace enlargement. Other studies have demonstrated similar chemotactic properties of fragments from collagen (78), fibronectin (79), and laminin (80), *in vitro.* Therefore, inflammatory cells such as neutrophils and macrophages not only have the potential to induce proteolytic damage, but also to promote further inflammation, creating a positive feedback loop.

While cigarette smoke stimulates cells to release inflammatory mediators, the effect of cigarette smoke on survival of cells that are resident or recruited to the lungs is somewhat conflicting. There is evidence that resident macrophages, as well as recruited monocytes, in the lungs of patients with COPD may have increased survival. Particulates in alveolar macrophages as a result of cigarette smoking have been demonstrated to persist for up to 2 years following smoking cessation (81), which likely is the result of particulates being recycled within the alveolar macrophage population. Perhaps contributing to this is the evidence of increased levels of GM-CSF in the airways of COPD patients (82). GM-CSF is an important growth factor not only for the differentiation of stem cells into monocyes/macrophages, but also survival of macrophages. Furthermore, there is evidence of increased alveolar macrophage proliferation in COPD, as a study by Tomica *et al.* demonstrated that alveolar macrophages isolated from smokers had increased cell proliferation as well as reduced apoptosis (83). Collectively, these data may represent the mechanism of increased macrophage accumulation in COPD. These results, however, differ from a number of *in vitro* studies, utilizing cigarette smoke extract (CSE) that have demonstrated that cigarette smoke induces pathways of apoptosis (84-92). CSE or

components of cigarette smoke have been shown to induce apoptosis *in vitro* in fibroblasts (85, 86, 88, 89), endothelial (90, 92) and epithelial cell lines (87, 91), as well as in primary alveolar macrophage cell cultures (84). In COPD there are increased numbers of apoptotic cells (93-95) and there is a deficiency in the ability of macrophages to clear apoptotic cells (96, 97). Building on this, and contributing to the initiation of inflammation, failure to clear dead cells may lead to release of ligands for HMGB-1, and the production of pro-inflammatory cytokines such as TNFα, amplifying the existing inflammatory response (98).

Collectively, these results indicate that stimulation of cells in the lungs via components of cigarette smoke activates pathways of inflammation. Chronic stimulation as a result of continued exposure results in chronic inflammation of the respiratory tract marked by the expression of pro-inflammatory mediators and the accumulation of inflammatory cells. Mechanistically, chronic inflammation is associated with proteolytic damage to the lungs and development of emphysema, as well as airway remodeling. Inflammation is not only a principal factor to consider for development of lung pathology in COPD, but also a factor to consider for the way pathogenic or commensal bacteria are dealt with, as chronic inflammation alters the baseline state of cells in the lungs, due to chronic receptor stimulation, changes to apoptosis and/or survival, and damage to the lung architecture.

Cigarette smoke & host defense against microbial agents

As stated previously, inflammation is prevalent in all stages of COPD and is regarded as a major contributor to COPD pathogenesis. An increase in this chronic inflammation above baseline is observed during periods of exacerbation, largely as a result of viral or bacterial infection (8, 99, 100). The precise role of bacteria in COPD exacerbations has been

controversial, as even during stable disease bacteria are frequently detected in the lower airways of COPD patients, which is a normally sterile compartment (101). Therefore, bacteria isolated during exacerbations may either be from infection or colonization. Recent studies have demonstrated that acquisition of a new strain of bacteria is an important cause of exacerbation (28). Furthermore, defined bacterial exacerbations, as compared to non-bacterial exacerbations, are associated with greater intensity of neutrophilic airway inflammation and systemic inflammation (29). Studies investigating how cigarette smoke modulates the recruitment of cells following infection, and the failure to clear bacteria, should provide insight into the pathogenesis of COPD.

During bacterial infection there are numerous bacterial antigens present, including constituents of bacterial cell walls such as peptidoglycan (PGN) and lipopolysaccharide (LPS), as well as lipoproteins, lipoteichoic acids, flagellum, and microbial toxins. These antigens stimulate pattern recognition receptors of resident innate cells, including TLR and Nod-like receptors (NLR) of epithelial cells and alveolar macrophages, resulting in the production of pro-inflammatory cytokines and chemokines. Indeed, during periods of exacerbation levels of TNFα and IL-6 are increased in the sputum (99, 102), and isolated cells have evidence of increased activation of Nuclear Factor (NF) -κB (103), a pro-inflammatory transcription factor downstream of TLR signaling. Among the numerous effects of TNFα and IL-6 are the induction of acute phase proteins released from the liver, including C reactive protein (CRP) and serum amyloid A (SAA). In COPD serum levels of CRP and SAA have been proposed as useful biomarkers for exacerbation (104). Furthermore, levels of the CC chemokines CCL-11 and CCL-5, as well as the CXC chemokines CXCL-5 and CXCL-8, are also increased during periods of exacerbation (58).

That fact that COPD exacerbations are inflammatory events, and that there is amplification in the level of inflammatory cytokines and chemokines during exacerbation, is especially interesting in light of the evidence demonstrating that cigarette smoke leads to attenuated expression of a number of inflammatory mediators following microbial stimulation. Stimulation of either CSE-treated epithelial (105) or macrophage (106) cell lines *in vitro*, alveolar macrophages isolated from cigarette smoke-exposed mice (107), or alveolar macrophages isolated from smokers (108-111) or COPD patients (112), with bacteria or bacterial antigens results in attenuated levels of pro-inflammatory mediators on both the protein and RNA levels. Of particular relevance, Chen *et al.* reported attenuated expression of TNFα, IL-6, IL-1β, CCL-5, and CXCL-8 following stimulation of alveolar macrophages isolated from smokers with either TLR-2 or TLR-4 agonists (108). Similarly, Berenson *et al.* reported attenuated expression of TNFα, IL-1β, and CXCL-8 following stimulation of alveolar macrophages isolated from patients with COPD with components of NTHI (112). Other studies have reported similar results with respect to levels of TNFα, IL-6, CXCL-8, and other type 1 cytokines (109-111).

There are a number of potential explanations for the observation of increased inflammatory mediators *in vivo* despite attenuated cellular production *ex vivo*, including:

1. The total number of cells producing cytokines: *in vitro* and *ex vivo* studies are controlled for the number of cells between wells. *In vivo*, there may be increased number of cells as a result of the ongoing chronic inflammation, leading to increased total number of cells producing cytokines during infection, overcoming an individual cell's attenuated ability for cytokine production.

2. Absence of other factors in *in vitro* and *ex vivo* studies: generally cell culture models have one cell type present and stimulation with one type of antigen. Multiple cell types, including apoptotic cells, and multiple bacterial antigens may lead to an amplification of the inflammatory response.
3. Differences in bacterial virulence: Heterologous bacteria isolated during exacerbation may have different virulence(s) compared to homologous laboratory strains of bacteria or bacterial antigens used in *in vitro* or *ex vivo* cell culture models.

On further development of the point that exacerbations reflect a balance between host factors, and bacterial virulence, Chin *et al.* tested the hypothesis that exacerbating strains of NTHI induce more inflammation than asymptomatic colonizing strains of NTHI (113). The authors demonstrated that in mice, strains of NTHI associated with exacerbation resulted in greater airway neutrophil recruitment than strains of NTHI associated with asymptomatic colonization. These results indicate that strains of NTHI isolated during episodes of exacerbation may have different virulence compared to those isolated during stable disease, perhaps leading to augmented inflammation.

In addition to the effects of cigarette smoke-exposure on cytokine production, cigarette smoke has been demonstrated to alter other pathways of innate bacterial clearance, including effects on the mechanical and physical barrier functions of epithelial cells (114-117), the bactericidal functions of alveolar macrophages (118, 119), as well as the induction of adaptive immune responses via effects on dendritic cells and cells of the adaptive immune system (120, 121). Given the acute nature of bacterial exacerbations, perhaps the most significant alterations are to the functions of the first line of pulmonary host defense: epithelial cells and resident alveolar macrophages.

The ciliated respiratory epithelium functions to sweep particles away in the overlying mucous layer, as well as phagocytosing and killing some pathogens, and maintaining a physical barrier via tight junctions to prevent the access of pathogens and foreign material. Furthermore, respiratory epithelial cells have roles in recruiting and activating inflammatory cells. Evidence indicates that cigarette smoke directly compromises the integrity of this physical barrier and increases the permeability of the respiratory epithelium (114, 115). Cigarette smoke also leads to decreased mucocilliary clearance, and *in vitro* studies have demonstrated decreased cilia beat frequency following exposure to CSE (116, 117). As cigarette smoke has been demonstrated to lead to increases in mucous and goblet cell hyperplasia, this may have detrimental effects on clearance of pathogens as a result of increased mucous and inability to clear it via usual pathways.

Alveolar macrophages are critical to pulmonary defense against pathogens and clearance of inhaled particular matter, and for initiating and orchestrating lung innate immune and inflammatory responses. Cigarette smoke has been demonstrated to alter many aspects of alveolar macrophage function, including cytokine and chemokine production following microbial stimulation as stated, but also phagocytosis, and killing of microbial pathogens (118, 119). Consequently, as a result of cigarette smoke-exposure, the ability of the lungs to appropriately handle a bacterial infection is altered.

Along these lines, as early as the 1960s *in vivo* studies demonstrated that bacterial clearance was delayed from the airways of cigarette smoke-exposed animals (122, 123). While this is indicative of cigarette smoke-exposure predisposing to bacterial infection or colonization, the effect of the bacterial challenge on inflammation and the level of exposure was not assessed in these mice. Work previously done in our laboratory with *P. aeruginosa* has

shown that mice exposed to cigarette smoke demonstrate a delayed rate of bacterial clearance as compared to control mice (107). This study demonstrated that delayed *P. aeruginosa* clearance was associated with a change in the nature of inflammatory response: increased *P. aeruginosa* burden was associated with increased airway and tissue inflammation despite evidence of suppressed alveolar macrophage function. Furthermore, increased levels of pro-inflammatory cytokines, chemokines, myeloperoxidase, and proteases were observed, providing evidence that links exacerbation to lung damage.

These results indicate that cigarette smoke impacts a number of different pathways associated with inflammation and microbial host defense. While the underlying mechanisms are not completely understood, studies investigating the mechanisms of airway inflammation and changes as a result of bacterial infection should provide insight into the pathogenesis of COPD. Moreover, they point to the value of modeling different bacteria and environmental stimuli to understand the cellular and molecular mechanisms underlying both exacerbations of COPD as well as the development and/or progression of COPD.

Experimental studies completed for this thesis

The purpose of this thesis was to investigate the mechanisms underlying cigarette smoke-induced lung pathology in a mouse model of cigarette smoke-exposure. The general hypothesis for the studies completed throughout this thesis was that microbial exacerbations contribute to cigarette smoke-induced lung pathology. For each particular study, specific hypotheses were generated and each one built from data generated from the previous study, with the goal of providing increased depth of understanding of the role of bacterial exacerbations in COPD.

In chapter 2 of this thesis we tested the specific hypothesis that cigarette smoke-exposure compromises the ability of alveolar macrophages, to sense microbial antigens through innate pattern recognition receptors. To accomplish this we utilized two different models of cigarette smoke-exposure and stimulation with both TLR and NLR ligands. The central point of this study was the observation that alveolar macrophages isolated from cigarette smoke-exposed mice had attenuated expression of typical inflammatory pathways following TLR or NLR stimulation, including pathways associated with bacterial infection.

Based on the observation that inflammatory cytokine production was attenuated in alveolar macrophages following microbial stimulation *ex vivo*, we examined the consequences of cigarette smoke-exposure on an *in vivo* bacterial challenge with NTHI. This study was of particular interest as exacerbations in COPD are inflammatory events, and we questioned whether the *ex vivo* observation of attenuated cytokine production had relevance to an *in vivo* bacterial challenge. The results of this research are reported in chapter 3 of this thesis.

One of the most interesting results of the previous study was an observation that while exacerbated inflammation in cigarette smoke-exposed mice was associated with decreased bacterial burden, this may occur at the expense of lung damage. In chapter 4 of this thesis we sought to gain further insight into the mechanisms underlying the observation of decreased bacterial burden. More specifically, we examined how the strain of laboratory mice used and how the dose/frequency of cigarette smoke-exposure affected bacterial clearance.

As a final point, at the time of the studies in chapter 2, we wrote a review based on the current literature regarding animal models of COPD exacerbations. The review is presented in its final form in appendix I of this thesis.

Chapter 2

CIGARETTE SMOKE EXPOSURE ATTENUATES CYTOKINE PRODUCTION BY MOUSE ALVEOLAR MACROPHAGES

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A number of studies have indicated that cigarette smoke is detrimental to the innate immune function(s) of alveolar macrophages(112, 118). The underlying mechanisms of these altered pathways, however, were poorly understood. Adding to this, *in vivo, ex vivo, and in vitro* studies often demonstrated conflicting results.

With the discovery and characterization of the TLR family beginning in the late 90s, mechanistic understanding of innate immune pathways was markedly improved. Utilizing this increased characterization we investigated the effect of cigarette smoke-exposure on pro-inflammatory pathways elicited by alveolar macrophages following stimulation with pathogen associated molecular patterns. We utilized a model of *in vivo* cigarette smoke-exposure and isolated alveolar macrophages for *ex vivo* investigation of whether cigarette smoke-exposure might alter the expression or function of innate pattern recognition receptors on alveolar macrophages, including members of the TLR and NLR families.

Dr. Martin Stampfli and myself were responsible for the experimental design(s) and interpretation. I was responsible for generation of data and writing the article, with Dr. Stampfli's involvement in the editing process. Co-authors on the study provided technical assistance, and helpful discussion.

Cigarette Smoke Exposure Attenuates Cytokine Production by Mouse Alveolar Macrophages

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Alveolar macrophages (aMϕs) play a central role in respiratory host defense by sensing microbial antigens and initiating immune-inflammatory responses early in the course of an infection. The purpose of this study was to investigate the effect of cigarette smoke exposure on aMϕs after stimulation of innate pattern recognition receptors (PRRs) in a murine model. To accomplish this, C57BL/6 mice were exposed for 8 weeks using two models of cigarette smoke exposure, nose-only or whole-body exposure, and aMϕs isolated from the bronchoalveolar lavage. After stimulation of aMϕs with pI:C, a mimic of viral replication, and bacterial cell-wall constituent LPS, aMϕs from cigarette smoke–exposed mice produced significantly attenuated levels of the inflammatory cytokines TNF-α and IL-6, and the chemokine RANTES. This attenuation was specific to the aMϕ compartment, and not related to changes in aMϕ viability or expression of Toll-like receptor (TLR)3 or TLR4 between groups. Furthermore, aMϕs from smoke-exposed mice had decreased cytokine RNA as compared with aMϕs from sham-exposed mice. Mechanistically, this was associated with decreased nuclear translocation of the proinflammatory transcription factor NF-κB, and increased activator protein-1 nuclear translocation, in aMϕs from smoke-exposed mice. Attenuated cytokine production was reversible after smoking cessation. Cigarette smoke exposure also attenuated TNF-α production after stimulation with nucleotide-oligomerization domain–like receptor agonists, showing that the effect applies more broadly to other PRR pathways. Our data demonstrate that cigarette smoke exposure attenuates aMϕ responses after innate stimulation, including pathways typically associated with bacterial and viral infections.

Keywords: alveolar macrophage; Toll-like receptor; chronic obstructive pulmonary disease; cigarette smoke; inflammation

Chronic obstructive pulmonary disease (COPD) is reaching epidemic levels (1). Etiologically, COPD is largely associated with cigarette smoking, and epidemiological studies as early as 1982 have shown an increased risk of respiratory infection even in young, asymptomatic smokers (2). Further along in the disease progression of individuals who develop COPD, acute, often self-limiting, bacterial and viral infections are a significant cause of symptom exacerbation, often leading to hospitalization (3, 4). Taken together, this is indicative of cigarette smoke having deleterious effects on innate immunity.

Central to protecting the host early during an infection is the recognition of patterns common to large classes of pathogens by means of pattern-recognition receptors (PRRs). PRRs include members of the prominent Toll-like receptor (TLR) family (5), as well as the nucleotide-oligomerization domain (NOD) family, otherwise called the NOD-like receptor (NLR) family, intracellular receptors recognizing structures from bacterial peptidoglycan (6). TLRs are found on the cell surface and in endosomes of many different cell types. To date there have been approximately 11 TLRs identified in mice and humans with corresponding synthetic or naturally occurring ligands. Included in this are TLR4, which recognizes lipopolysaccharides (LPS) from gram-negative bacteria (7); TLR3, which recognizes double-stranded RNA found during viral infections, or synthetic polyinosinic-polycytidylic acid (pI:C) (8); and TLR9, which recognizes CpG DNA motifs (9). Upon stimulation of TLRs, intracellular signaling pathways are activated, resulting in the nuclear translocation of transcription factors including NF-κB, IRF3, and activator protein (AP)-1 (10). In turn, this leads to the up-regulation and production of cytokines and chemokines important for initiating antibacterial or antiviral immune responses.

CLINICAL RELEVANCE

Given the central role of alveolar macrophages early in the course of an infection, attenuated Toll-like receptor function may predispose smokers to respiratory infections and bacterial colonization.

Due to their strategic positioning in the lumen of the airways, alveolar macrophages (aMϕs) play a central role in innate respiratory host defense (11). Although there is evidence that cigarette smoking may increase the number of aMϕs, further clinical and experimental evidence indicates that these cells may be functionally impaired (12–18). Of particular interest, recently Berenson and coworkers have shown decreased production of inflammatory cytokines upon stimulation of aMϕs from patients with COPD with antigens from *Haemophilus influenzae*—the most commonly isolated bacteria during exacerbations in COPD (19). However, the mechanisms leading to the functional impairment of aMϕs, such as pathways of recognition or PRR expression and function, as well as transcription and translation of cytokines, is less well understood.

The purpose of this study was to investigate the effect of cigarette smoke exposure on PRR-mediated responses by aMϕs in a murine model. To accomplish this, we used two different models of smoke exposure: nose-only and whole-body exposure. We demonstrate that cigarette smoke exposure decreased inflammatory cytokine production by aMϕs upon stimulation with bacterial LPS or muramyl di- and tri- peptides, as wells as pI:C, a mimic of viral replication. To our knowledge, the finding that cigarette smoke exposure attenuates cytokine production after pI:C stimulation is novel and shows that the immunologic effects of cigarette smoke on aMϕs are not limited to bacterial antigens. Mechanistically, we demonstrate that this observation is lung specific, reversible, and is associated with dysregulated activation of transcription factors following TLR stimulation.

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Similar to pI:C and LPS stimulation, we observed decreased cytokine production after stimulation with muramyl di- and tripeptides, ligands for members of the NLR family. Collectively, these data indicate that cigarette smoke has a general impact on aMϕ activation through PRRs, including pathways typically associated with bacterial or viral infections. Some of the results in this study were previously reported in abstract form (20).

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, PQ, Canada) and kept in a 12-hour light-dark cycle with unlimited access to food and water. Cages, food, and bedding were autoclaved, and all animal manipulations were performed in a laminar flow hood by personnel who were gloved, gowned, and masked. The McMaster University Animal Research Ethics Board approved all experiments described in this study.

Cigarette Smoke Exposure

Nose-only exposure. Mice were exposed to two 1R3 reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) daily for 5 days per week using a smoke exposure system that is described in detail elsewhere (21). In an initial lead-up period, animals were accustomed to 1 cigarette in the first and to two cigarettes in the second week. To control for handling, groups of mice were placed into restrainers only and exposed to room air (sham-exposure).

Whole-body exposure. Mice were exposed to the smoke from twelve 2R4F reference cigarettes with the filters removed, twice daily, 5 days per week using an SIU48 (PROMECH LAB AB, Vintrie, Sweden). No lead-up period is required in the whole-body exposure system. In an initial acclimatization period, mice were accustomed to the restrainers over a 3-day period. Specifically, on Day 1 mice were placed into the restrainers for 20 minutes, on Day 2 for 30 minutes, and on Day 3 for 50 minutes. Control animals were exposed to room air only.

Carboxyhemoglobin Measurement

Immediately, or 24 hours, after sham or smoke exposure, blood was drawn in clinitubes (Radiometer, Copenhagen, Denmark) for Carboxyhemoglobin (COHb) measurement by the McMaster University Medical Centre core lab.

Macrophage Isolation and Culture

Mice were anesthetized 18 to 24 hours after their last smoke exposure and killed by exsanguination before excision of the lungs. Tracheas were cannulated and bronchoalveolar lavage (BAL) was collected from sham- and smoke- exposed mice by instilling 0.5 ml of PBS into the lungs through the trachea three times. After each instilment, fluid was collected and pooled. Peritoneal lavage was collected by instilling 5 ml of PBS into the peritoneum via syringe and needle, and fluid was collected with a disposable plastic pipet. Cells were counted by trypan blue dye exclusion. Cytospins for differential cell counts were prepared and stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ). Standard hemocytologic criteria were used to classify mononuclear cells, neutrophils, and eosinophils. At least 500 cells were counted per cytospin. Based on trypan blue dye exclusion and differential cell counts, equal numbers of aMϕs were allowed to adhere for 2 hours at 37°C and 5% CO_2 and washed three times with warm PBS to remove nonadherent cells. Details of aMϕ numbers adhered in the different experiments are provided in the specific method sections below. Adherent cells were cultured in 100 μl of RPMI supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen, Grand Island, NY), and 0.1% β-mercaptoethanol (Invitrogen) (cRPMI) and stimulated with either 1 μg/ml LPS (Invivogen, San Diego, CA) or 10 μg/ml of pI:C or 1–50 μg/ml of CpG (Sigma-Aldrich) for 24 hours. Cells were similarly stimulated with 10 μg/ml muramyl di- or muramyl tri- peptides, generously provided by Anthony Coyle and Jose Lora (Millennium Pharmaceutical, Inc., Cambridge, MA).

Measurement of Cytokines and Nitric Oxide

A quantity of 5×10^4 aMϕs was adhered to a flat-bottom 96-well plate and cultured in 100 μl of cRPMI. After 24 hours of culture, cell supernatants were collected and levels of TNF-α, IL-6, RANTES (regulated on activation, normal T cells expressed and secreted) (R&D, Minneapolis, MN), and IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) were measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturers' instructions and measured on an ELISA plate reader. The limit of detection for the assays were 5.1, 1.6, 2, and 15.6 pg/ml, respectively. Nitric oxide was measured by examining the breakdown products of nitric oxide by Griess reactions as described in detail elsewhere (22).

Macrophage Viabiltiy and Metabolic Activity

For survival cell counts, 1×10^5 aMϕs were adhered to glass slides in flat-bottom 12-well plates. Cells were then counted by trypan dye exclusion counts after 24 hours of culture. For metabolic activity quantification, adherent cells were cultured in 96-well flat-bottom plates in media or stimulated with pI:C and LPS for 24 hours. After this time, MTT activity was assessed by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) kit according to the manufacturers' instructions (Chemicon, Billerica, MA). Briefly, adherent cells were incubated in 100 μl of cRPMI and 10 μl of MTT reagent for 4 hours at 37°C and 5% CO_2. Subsequently isopropanol development solution was added and MTT activity measured at OD570 on an ELISA plate reader.

TaqMan Real-Time RT-PCR

A quantity of 1×10^5 aMϕs was adhered to a flat-bottom 96-well plate. Cells were stimulated with LPS, and RNA was isolated at the indicated time points specified in RESULTS and in the figure legends with the RNeasy mini Kit and the optional DNase step (Qiagen, Mississauga, ON, Canada). RNA was quantified and purity checked using the Agilent 2100 Bio-Analyzer machine operated by the 2100 expert software (Agilent, Palo Alto, CA). Reverse transcription was completed on similar amounts of RNA per group using a RETROscript kit (Ambion, Austin, TX). Mitochondrial ribosomal protein L32 (L32), TLR3, TLR4, TNF-α, IL-6, and RANTES primers and FAM-labeled probes were purchased from Applied Biosystems (Foster City, CA), and PCR was performed in duplicate or triplicate with Universal PCR Master Mix in the ABI PRISM 7900HT Sequence Detection System operated by Sequence Detector Software version 2.2 (Applied Biosystems). PCR was performed in single plex and analysis was completed by first normalizing gene expression to levels of the housekeeping gene L32 in the same sample (ΔCT) and then compared with the control group (ΔΔCT). With this method, RNA extracted from unstimulated aMϕs, isolated from sham-exposed mice, have a relative fold induction (R) defined as 1. Experimental groups are expressed as fold change over this control condition.

Immunofluoresence

For immunofluorescence assays, 2.5×10^4 aMϕs were allowed to adhere per well on glass slides using chamber slide systems (Nalge Nunc Int., Rochester, NY) for 2 hours. Cells were then washed twice with warm PBS to remove nonadherent cells. Slides were placed in pre-chilled acetone at −20°C for 20 minutes and stored at −70°C. To minimize nonspecific binding, cells were incubated with 20% normal goat and normal donkey serum for 1 hour at room temperature. Slides were stained with TLR3 (Imgenex Corp., San Diego, CA) and TLR4 (eBiosciences, San Diego, CA) primary antibodies for 1 hour. Slides were washed with PBST (0.05% Tween20) and stained with Alexa-flour 633– and Alexa-fluor 488–conjugated secondary antibodies (Molecular Probes, Invitrogen) for TLR3 and TLR4, respectively. Control slides were incubated with either primary or secondary antibody alone. Slides were washed and nuclei were stained with SYTO3 in the mounting media (Vector, Burlingame, CA). Fluorescent pictures were taken by confocal microscopy (Leica, Richmond Hill, ON, Canada) with the LSM 510 software. Quantification was performed by analyzing the sum of the TLR stain divided by the sum of the nuclear stain for individual pictures with Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

Nuclear Isolation and NF-κB and AP-1 ELISAs

A quantity of 2×10^6 aMϕs was adhered to flat-bottom 12-well plates. After 2 hours of adherence and stimulation with LPS for the indicated

times, nuclear isolation was preformed with a nuclear isolation kit (Active Motif, Carlsbad, CA) as per the manufacturer's instructions. Extracts were re-suspended in a final volume of 20 μl. Nuclear extracts were used in a TransAM NF-κB ELISA and TransAM c-JUN ELISA (Active Motif) and run according to the manufacturer's instructions. Analysis shown is the sample OD540 divided by the positive control OD540, multiplied by 100, thereby giving percent of positive control signal. The positive control, stimulated Jurkat cell extracts, was provided with the kit, and 2 μg of extract was loaded per well.

Data Analysis

Data are expressed as mean ± SD or SEM as indicated in the figure legends. Statistical analysis was performed using Student's *t* test unless otherwise stated. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Nose-Only Cigarette Smoke Exposure

C57BL/6 mice were exposed to the smoke from two cigarettes a day, 5 days per week for 8 weeks. We observe carboxyhemoglobin (COHb) levels immediately following smoke exposure of 13.58% ± 2.47%, compared with 3.77% ± 0.93% in sham-exposed mice (Table 1).

In the BAL from smoke-exposed mice there is a moderate, but significant, increase in the total cell number, as compared with sham-exposed mice (Figure 1A). Using standard hemacytologic criteria for differential cells counts, isolated cells from the BAL of nose-only smoke- or sham-exposed mice are greater than 95% mononuclear cells (sham: 99.13 ± 0.50; smoke: 97.90 ± 0.57; $n = 6$ with 5 animals pooled per experiment). The remaining cells were neutrophils with no eosinophils present. No difference in cellular composition in the BAL was observed between the groups (Figure 1B).

TNF-α, IFN-β, Nitric Oxide, IL-6, and RANTES Production by aMϕs after pI:C, LPS, or CpG Stimulation

To characterize the impact of cigarette smoke exposure on cytokine production by aMϕs after stimulation with TLR ligands, aMϕs were isolated from the BAL and cultured in medium alone or stimulated with the TLR3 ligand pI:C, the TLR4 ligand LPS, or the TLR9 ligand CpG. After 24 hours, cell supernatants were collected and levels of TNF-α measured by ELISA. As demonstrated in Figure 1C, aMϕs isolated from smoke-exposed mice produce significantly less TNF-α after stimulation with either pI:C or LPS. After stimulation with either pI:C or LPS, we were unable to measure any appreciable levels of IFN-β in the supernatants by ELISA, up-regulation of iNOs by TaqMan, or the byproducts of nitric oxide (NO) breakdown by Griess reaction at the time points measured (data not shown).

To further characterize the specificity of the effect of cigarette smoke on aMϕ cytokine production after TLR stimulation, we measured the levels of the cytokine IL-6 and the chemokine RANTES in supernatants from stimulated aMϕ isolated from sham- or smoke-exposed mice. As shown in Figures 1E and 1F, the levels of IL-6 and RANTES produced by aMϕs from smoke-exposed mice is decreased compared with aMϕs from sham-exposed mice. Thus, the decreased response from aMϕ isolated from smoke-exposed mice is not limited to TNF-α, but includes IL-6 and RANTES.

We did not observe production of TNF-α, IL-6, IFN-β, NO, RANTES, or up-regulation of iNOs from any group after stimulation with the TLR9 ligand CpG (Figures 1C, 1E, 1F, and data not shown).

TABLE 1. CARBOXYHEMOGLOBIN LEVELS IN SHAM- AND SMOKE-EXPOSED MICE

Condition	% Carboxyhemoglobin in Serum
Sham nose-only exposure	3.77 ± 0.93
Smoke nose-only exposure	13.58 ± 2.47*
Sham whole-body exposure	3.67 ± 0.58
Smoke whole-body exposure	8.67 ± 1.55*

Data represent mean ± SD, $n = 13$ for sham nose-only exposure, 12 for smoke nose-only exposure, 3 for sham whole-body exposure, and 3 for smoke whole-body exposure.

* Statistical analysis was performed with Student's *t* test, $P < 0.05$.

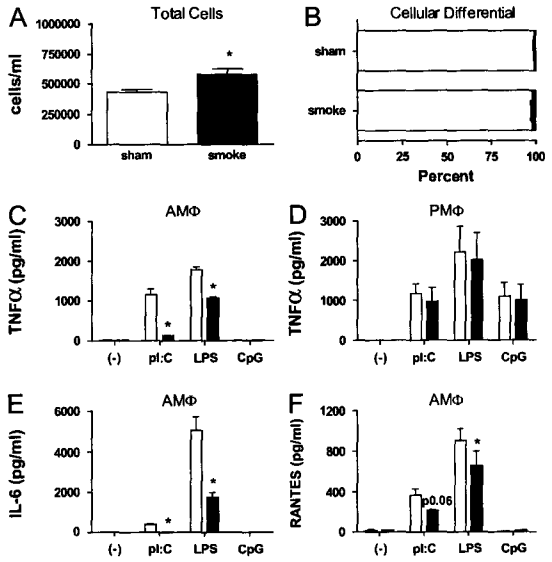

Figure 1. Bronchoalveolar lavage (BAL) cellular profile and cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. *A* and *B* represent the BAL total cell number and cellular differential. (*C–F*) aMϕs and pMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml pI:C, 1 μg/ml LPS, or 10 μg/ml CpG for 24 hours and levels of cytokines measured in cell supernatants by ELISA. (*C* and *D*) TNF-α production by aMϕs and pMϕs, respectively. *E* and *F* represent IL-6 and RANTES production from aMϕs. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. (*A* and *B*) Data represent mean ± SEM of six independent experiments, with five mice pooled per group in each experiment. (*C–F*) AMϕs and pMϕs were isolated in three to five separate experiments, with five animals per experimental group. Data shown is mean ± SEM. Statistical analysis was performed with Student's *t* test *$P < 0.05$.

TNF-α Production by Peritoneal Macrophages after pI:C, LPS, or CpG Stimulation

To investigate whether the effect of cigarette smoke exposure on cytokine production after TLR stimulation was specific to aMϕs, peritoneal macrophages (pMϕs) were isolated in parallel to aMϕs. Similar to aMϕs, pMϕs were cultured in media alone or stimulated with pI:C, LPS, or CpG, supernatants collected after 24 hours of culture, and levels of TNF-α measured by ELISA. As demonstrated in Figure 1D, in contrast to the significant decrease observed in aMϕs from smoke-exposed mice, smoke exposure did not affect the production of TNF-α from pMϕs. In contrast to aMϕs, pMϕs produce TNF-α after CpG stimulation.

aMϕ Survival and Viability in Culture

One explanation for the observed decrease in cytokine production by smoke-exposed aMϕs may be decreased aMϕ viability

as a result of smoke exposure, and consequently a result of culturing fewer live, viable cells. To address this, aMϕs were isolated and cultured as previously for cell viability and metabolic activity measurements. As shown in Table 2, aMϕs from sham- and smoke-exposed mice have similar cell numbers after 24 hours of culture, as determined by trypan blue dye exclusion counts. Similarly, on stimulation with pI:C or LPS, aMϕs from sham- and smoke-exposed mice have similar MTT activity after 24 hours of culture. Together, these observations indicate that decreased cytokine production from aMϕs isolated from smoke-exposed mice after stimulation with TLR agonists likely was not a result of increased death or decreased viability in culture.

aMϕ Expression of TLR3 and TLR4

A further avenue to investigate regarding decreased cytokine production in smoke-exposed aMϕs was to assess whether deceased cytokine production by aMϕs after pI:C and LPS stimulation was related to decreased TLR expression. To this end, aMϕs were isolated from sham- and smoke-exposed mice and RNA extracted after 2 hours of adherence in culture. Levels of TLR3 and TLR4 expression were assessed by real-time quantitative PCR. Levels of the target genes were first normalized to the housekeeping gene L32 (ΔCT) and expressed as relative fold induction to sham-exposed aMϕs (ΔΔCT). As shown in Figure 2A, we observe similar levels of TLR3 and TLR4 RNA in aMϕs from smoke- or sham-exposed mice.

In further experiments, aMϕs were allowed to adhere to glass slides and expression of TLR3 and TLR4 was determined by immunofluoresence. Adherent cells were either stained with TLR3 or TLR4 primary antibodies, as well as a nuclear stain. Control slides were stained with primary antibody, or secondary antibody alone. Immunofluorescent stains were assessed by confocal microscopy. Representative immunofluorescent (IF) pictures from sham- and smoke-exposed mice are shown in Figure 2B. Quantification of fluorescence, as measured by Northern Eclipse software, demonstrated no difference between groups (Figure 2C). No fluorescence signal was detected on control slides stained with either primary or secondary antibody alone.

TNF-α, IL-6, or RANTES RNA Expression in aMϕs after LPS Stimulation

To investigate if decreased cytokine production by aMϕs from smoke-exposed mice was upstream of protein translation, aMϕs from sham- and smoke-exposed mice were isolated as previously and cultured with media alone or stimulated with LPS. After 2, 6, or 24 hours of culture, RNA was extracted from adherent cells. Levels of TNF-α, IL-6, and RANTES were measured by real-time quantitative PCR. As shown in Figure 3, TNF-α, IL-6, and RANTES mRNA from smoke-exposed mice were reduced compared with the levels observed in sham-exposed mice. This is indicative of aMϕs from smoke-exposed mice having decreased ability to up-regulate transcriptionally expression of TNF-α, IL-6, and RANTES RNA after stimulation.

TABLE 2. *EX VIVO* VIABILITY AND METABOLIC ACTIVITY OF ALVEOLAR MACROPHAGES

	Treatment	Sham	Smoke
Viability (cells/ml *10^4)	Media	2.34 ± 0.23	2.6 ± 0.46
Metabolic activity	Media	0.253 ± 0.014	0.264 ± 0.042
(MTT OD570)	pI:C	0.195 ± 0.023	0.232 ± 0.033
	LPS	0.245 ± 0.010	0.252 ± 0.017

Data represent mean ± SD of the mean, $n = 10$ for survival cell counts, $n = 5$ per group for viability, 1 shown of 2 separate experiments.

* Statistical analysis was performed with Student's t test, $P < 0.05$.

NF-κB and AP-1 Nuclear Translocation in aMϕs after LPS Stimulation

To further investigate the transcriptional dependence of the effect of smoke exposure on aMϕ cytokine production, we investigated levels of transcription factors entering the nucleus after LPS stimulation. aMϕs from sham- and smoke-exposed mice were isolated as previously and stimulated with LPS for 30, 60, or 90 minutes and nuclear extracts isolated from each group. Extracts were quantified and equal amounts of protein were loaded per well in TransAm ELISA plates. The activated NF-κB or AP-1 contained in nuclear extracts specifically binds to oligonucleotide coated on the solid phase. Using antibody specific for p65 or c-Jun, the NF-κB or AP-1 complex bound to the oligonucleotide is detected. As demonstrated in Figure 4A, we observe decreased nuclear associated NF-κB subunit p65 in aMϕs isolated from smoke-exposed mice. In contrast to p65, we observed increased nuclear translocation of the AP-1 subunit c-Jun after stimulation with LPS (Figure 4B).

TNF-α Production by aMϕs after Muramyl-Dipeptide and Muramyl-Tripeptide Stimulation

To further investigate the effects of cigarette smoke exposure on PRR pathways leading to cytokine production by aMϕs, we stimulated aMϕs with ligands for members of the NLR family. Specifically, aMϕs were isolated similar to previously and stimulated with agonists for NOD1, muramyl-dipeptides, and NOD2, muramyl-tripeptides. After 24 hours of culture, supernatants were collected and levels of TNF-α measured by ELISA. Similar to responses observed for TLR signaling pathways, we observed decreased production of TNF-α after stimulation with either NOD ligand (Figure 5). Thus, the decreased response of aMϕs isolated from smoke-exposed mice is not restricted to TLR agonists, but includes NLR agonists as well.

Whole-Body Cigarette Smoke Exposure

To investigate whether the effects of cigarette smoke observed with the nose-only exposure system were limited to that exposure system, or were consistent with other models of smoke exposure, mice were cigarette smoke exposed with a whole-body exposure system. With this model of exposure, C57BL/6 mice were exposed twice daily to the cigarette smoke from 12 cigarettes, 5 days a week for 8 weeks. We observe COHb levels of 8.67% ± 1.55%, compared with 3.67% ± 1.55% in sham-exposed mice (Table 1).

In BAL from whole-body smoke-exposed mice, there is a significant increase in the total cell number, as compared with sham-exposed mice (Figure 6A). Isolated cells from the BAL of whole-body smoke- or sham-exposed mice are greater than 95% mononuclear cells (sham: 99.88 ± 0.29; smoke: 96.96 ± 4.82; $n = 5$ per group). Similar to nose-only exposure, the balance of the remaining cells was neutrophils, and no difference in cellular composition in the BAL was observed between groups (Figure 6B).

TNF-α Production by aMϕs and pMϕs after pI:C, LPS, and CpG Stimulation

To characterize the impact of whole-body cigarette smoke exposure on cytokine production by aMϕs after stimulation with TLR ligands, aMϕs were isolated from the BAL and cultured in medium alone or stimulated with pI:C, LPS, or CpG. After 24 hours, cell supernatants were collected and levels of TNF-α measured by ELISA. As demonstrated in Figure 6C, aMϕs isolated from smoke-exposed mice produce significantly less TNF-α after stimulation with either pI:C or LPS.

Similar to aMϕs, pMϕs were cultured in media alone or stimulated with pI:C, LPS, or CpG, supernatants collected after

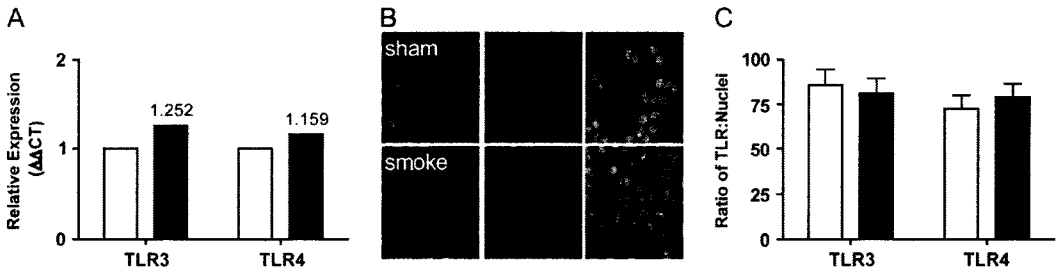

Figure 2. TLR3 and TLR4 expression in alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. (*A*) RNA was isolated from aMϕs and levels of TLR3 and TLR4 RNA expression was measured by real-time quantitative PCR (TaqMan). Expression of TLR3 and TLR4 in sham-exposed mice were defined as 1 and levels in smoke-exposed mice were expressed relative to this. (*B*) BAL cells were adhered to glass slides, and TLR3, TLR4, and nuclei were stained. The *top* and *bottom panels* show aMϕs from sham- and smoke-exposed mice, respectively. Nuclear stains are shown in the *left panels*, TLR3 in the *middle panels*, and TLR4 in the *right panels*. (*C*) Immunofluoresencent picures were quantified using Northern Eclipse. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively.

24 hours of culture, and levels of TNF-α measured by ELISA. As demonstrated in Figure 6D, in contrast to the significant decrease observed in aMϕs from smoke-exposed mice, whole-body smoke exposure did not affect the production of TNF-α from pMϕs.

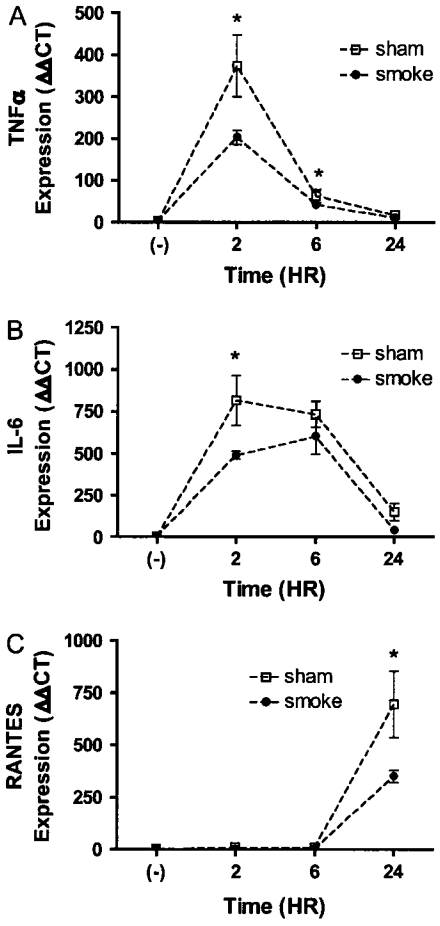

Figure 3. *Ex vivo* alveolar macrophage TNF-α, IL-,6 and RANTES RNA expression. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. AMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS for 2, 6, or 24 hours. RNA was isolated and levels of (*A*) TNF-α RNA, (*B*) IL-6 RNA, and (*C*) RANTES expression was measured using real-time quantitative PCR (TaqMan). Data represent mean ± SEM, $n = 10$ (*A*), $n = 5$ (*B* and *C*). Statistical analysis was performed with Student's *t* test, $^*P < 0.05$. For all panels, aMϕs from sham-exposed mice are *open squares* and aMϕs from smoke-exposed mice are *solid circles*.

Time Course and Reversibility of the Cigarette Smoke Exposure on aMϕ Function

Next we investigated the minimal duration of cigarette smoke exposure necessary to observe attenuated cytokine production by aMϕs. Mice were sham- or smoke-exposed for 1, 2, 4, or 8 weeks. At each time point, aMϕs were isolated from the BAL and stimulated with LPS for 24 hours. Levels of TNF-α were measured in cell supernatants. As shown in Figure 7A, we observed no difference in TNF-α production between aMϕs isolated from sham- and smoke-exposed mice after 1 or 2 weeks of smoke exposure ($P = 0.91$ and $P = 0.22$, respectively). After 4 and 8 weeks, we observed significantly decreased TNF-α in the cell supernatants of aMϕs isolated from smoke-exposed mice ($P = 0.004$ and $P = 0.018$, respectively).

To characterize the reversibility of the effects of cigarette smoke exposure on aMϕs, mice were sham- or smoke-exposed for 8 weeks. AMϕs were isolated from the BAL and rested for 1 week in media alone. aMϕs were then stimulated with LPS for 24 hours and levels of TNF-α measured in cell supernatants. We

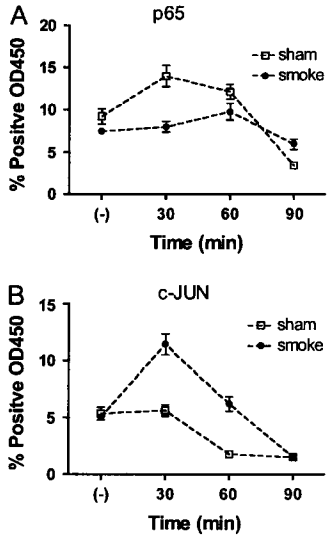

Figure 4. NF-κB and AP-1 nuclear translocation in alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. aMϕs were cultured in media alone or stimulated with 1 μg/ml LPS for 30, 60, or 90 minutes and nuclear extracts were isolated. Levels of (*A*) p65 and (*B*) c-Jun nuclear translocation were measured by ELISA. Shown is one representative of two experiments. Data represent mean ± SD (technical error), with 10 mice pooled per experimental group. For all panels, aMϕs from sham-exposed mice are *open squares* and aMϕs from smoke-exposed mice are *solid circles*.

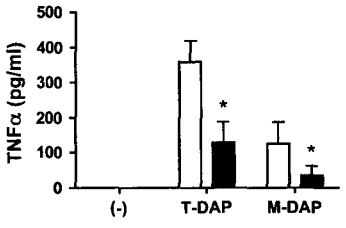

Figure 5. *Ex-vivo* TNF-α production by alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. aMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml M-DAP or 10 μg/ml T-DAP for 24 hours and levels of TNF-α measured in cell supernatants by ELISA. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. Data represent mean ± SEM, $n = 2$; in each experiment, five animals were pooled per group. Statistical analysis was performed with Student's *t* test, $^*P < 0.05$.

observed significantly decreased TNF-α production by aMϕs isolated from smoke- compared with sham-exposed mice (Figure 7B), suggesting that aMϕs maintain their attenuated phenotype *in vitro*.

To investigate whether the effects of cigarette smoke exposure on aMϕs are reversible *in vivo*, mice were sham- or smoke-exposed for 8 weeks. Subsequently, smoke-exposed mice were divided into two groups: the first continued their smoke-exposure regime (smoke), while the second were exposed to room air (cessation). After 2 or 4 weeks of smoking cessation, aMϕs were isolated and cultured in medium alone or stimulated with LPS. After 24 hours of culture, cell supernatants were collected and levels of TNF-α measured by ELISA. After 2 weeks of smoking cessation, we observe significantly decreased TNF-α in both the smoke-exposed and the cessation group, as compared with the sham-exposed group (Figure 7B). No difference was observed between the smoke and cessation groups. After 4 weeks of smoking cessation aMϕs regained normal function; we observed similar levels of TNF-α in supernatants of aMϕs from the cessation group as compared with the sham group. Together, these findings indicate that smoking cessation may reverse attenuated aMϕ function, but only after a sufficient period of time.

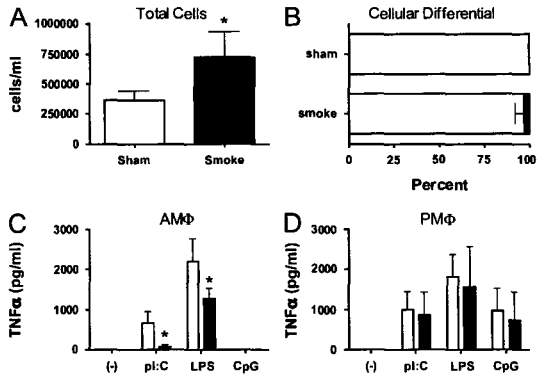

Figure 6. BAL cellular profile and cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. *A* and *B* represent the BAL total cell number and cellular differential. (C and *D*) aMϕs and pMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml pI:C, 1 μg/ml LPS, or 10 μg/ml CpG for 24 hours and levels of cytokines measured in cell supernatants by ELISA. *C* and *D* represent TNF-α production by aMϕs and pMϕs, respectively. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. Data represent mean ± SEM, $n = 5$ per experiment. Statistical analysis was performed with Student's *t* test $^*P < 0.05$.

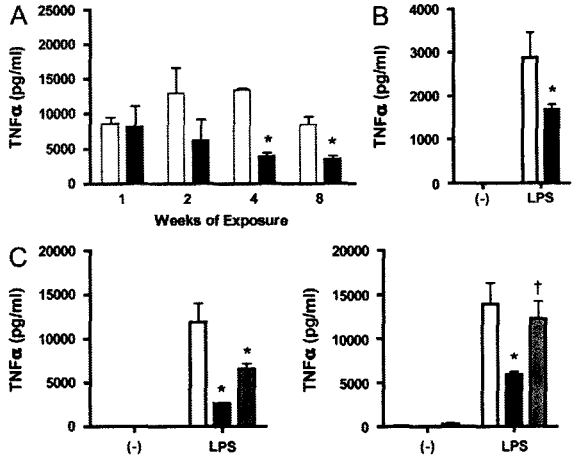

Figure 7. Cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 1, 2, 4, 8, 10, and 12 weeks. aMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS for 24 hours and levels of TNF-α measured in cell supernatants by ELISA. (*A*) TNF-α production by aMϕs after 1, 2, 4, and 8 weeks of smoke-exposure. (*B*) Mice were sham or smoke exposed for 8 weeks and aMϕs were isolated and cultured for 1 week before stimulation with 1 μg/ml LPS for 24 hours. Levels of TNF-α were measured in cell supernatants by ELISA. In *A* and *B*, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. (*C*) Mice were sham- and smoke-exposed for 8 weeks, and after this time mice continued their exposure regime (*open bars*, sham exposure; *solid bars*, smoke exposure) or ceased smoke exposure (*shaded bars*, cessation). After 2 or 4 weeks aMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS, and levels of TNF-α were measured in cell supernatants by ELISA (*C*). Data represent mean ± SEM, $n = 3$–5 per experiment. Statistical analysis was performed with Student's *t* test $^*P < 0.05$ (*A*, *C*) or one-way ANOVA (*B*.) $^*P < 0.05$ compared with sham, $^\dagger P < 0.05$ compared with smoke.

DISCUSSION

aMϕs are fundamental to respiratory host defense by sensing microbial agents early in the course of an infection and initiating immune inflammatory responses. In this study, we investigated the effect of cigarette smoke exposure on the production of inflammatory cytokines after *ex vivo* stimulation of aMϕs. To this end, mice were exposed to mainstream cigarette smoke using two distinct smoke-exposure systems. The first, a nose-only exposure system, is widely used to study smoke-induced emphysema in small rodents. With this system Hautamaki and coworkers demonstrated the requirement of macrophage elastase for cigarette smoke–induced emphysema (21). The second system, a whole-body exposure system, has been developed more recently.

Similar to human smokers (23), mice exposed to smoke in either of these model systems have an increase in the number of cells isolated from the BAL, with aMϕs representing greater than 95% of cells. Importantly, based on carboxyhemoglobin blood measures, levels of smoke exposure are similar to that reported clinically (24). Although we observed increased number of aMϕs in the BAL of smoke-exposed mice, *ex vivo* cytokine production after stimulation was attenuated. More specifically, we observed significantly decreased production of the inflammatory cytokines TNF-α and IL-6, and the chemokine RANTES, after stimulation with the TLR ligands pI:C or LPS, as well as the NLR ligands muramyl di- or tripeptides. Our findings are in line with previous clinical and experimental observations, including a recent report in which aMϕs from patients with COPD were stimulated with bacterial antigens from *H. influenzae* (19). That cigarette smoke

exposure also attenuates cytokine production after NLR stimulation, as well as pI:C stimulation, is novel and shows the effect of cigarette smoke is not limited to bacterial antigens. Therefore, cigarette smoke likely has a general impact on immune activation through PRRs, including pathways typically associated with viral infections. Along these lines, the fact that RANTES is decreased is of particular interest, as it indicates that cigarette smoke impairs not only the MyD88-dependent pathway of TLR4 signaling, but likely also the MyD88 independent pathway—a pathway associated with the production of type 1 interferons (25).

Despite the production of RANTES, we failed to measure any appreciable level of IFN-β production by aMϕs. As demonstrated in peritoneal macrophages, downstream events from IRF3 activation (via TLR3 or TLR4 stimulation) include the production of the type 1 interferon IFN-β. IFN-β then has paracrine and autocrine function through STAT1 to up-regulate the expression of inducible nitric oxide (iNOs) and ultimately the production of NO (26, 27). Consistent with the lack of IFN-β production by aMϕ, we observed neither induction of iNOs or the production of NO, after LPS or pI:C stimulation (data not shown). This observation is in agreement with a previous report by Punturieri and colleagues, in which the authors demonstrated that aMϕs do not produce IFN or NO in response to TLR3 or TLR4 ligands alone (28). Notably, this unresponsiveness is maintained in aMϕs isolated from smoke-exposed animals.

While we observed robust cytokine production by aMϕs after pI:C or LPS stimulation, we were unable to demonstrate any response to CpG. This observation is consistent with the lack of TLR9 expression by aMϕs in either sham- or smoke-exposed mice (data not shown). In agreement with this, a previous study has demonstrated that aMϕs, unlike peritoneal macrophages, do not respond to CpG stimulation (29), likely indicating the specialized function of aMϕs in respiratory host defense.

The impact of cigarette smoke was lung specific, as no differences were observed between peritoneal macrophages isolated from sham- and smoke-exposed mice in their production of TNF-α. Similarly, a recent clinical study indicates that cytokine production from aMϕs stimulated with *H. influenzae* antigens from individuals with COPD are impaired, but not peripheral blood monocytes (19).

On further characterization of the observed attenuated effect, we sought to control for cell viability in culture, as smoke exposure may be detrimental to aMϕ survival, leading to stimulation of less cells. Our observations indicate that decreased cytokine production by aMϕs from smoke-exposed mice is not a result of increased cell death due to cellular toxicity. In agreement with this, numerous clinical studies have shown that aMϕs from smokers may have an increased life span, despite exposure to toxic components contained within cigarette smoke (30–33). Therefore, mechanisms relating to decreased aMϕ production of cytokines are likely unrelated to direct toxic effects on cell survival by cigarette smoke.

Of the potential mechanisms leading to decreased cytokine production in response to pI:C and LPS, decreased surface expression of TLR4 and endosome expression of TLR3 as a result of smoke exposure may be hypothesized. However, with these experimental approaches we did not observe any changes in TLR3 or TLR4 expression between sham- and smoke-exposed mice. Specifically, no difference was seen on the RNA level as assessed by TaqMan or the protein level as assessed by immunofluorescent microscopy. Together these data indicate that the decrease in cytokine production by aMϕs from smoke-exposed mice is likely downstream of TLR3 and TLR4 expression. Indeed, while numerous studies have shown impaired function of aMϕs from smokers or patients with COPD, a previous report demonstrated no difference in TLR4 expression on aMϕs (34).

pI:C may stimulate aMϕs via alternate pathways than TLR3, including cytoplasmic RNA helicases, such as retinoic acid–inducible gene I (RIG-I) (35). Therefore, while attenuated cytokine production after LPS, T-DAP, or M-DAP stimulation can be attributed to effects on TLR4, NOD1, and NOD2 pathways, respectively, attenuated cytokine production after pI:C stimulation may involve multiple pathways.

TLR stimulation leads to the activation of intracellular signaling pathways, resulting in the nuclear translocation of the transcription factors NF-κB, IRF3, and AP-1 (10). The functional unit of NF-κB, usually a heterodimer of the p65 and p50 subunits, is held in its inactive form in the cytoplasm by the inhibitory molecule, IkB (36). Upon activation, the IkB molecule is degraded, allowing free NF-κB to enter the nucleus and initiate pro-inflammatory gene transcription (37). In our hands, we observe decreased levels of p65 after stimulation with LPS in nuclear extracts isolated from aMϕs from smoke-exposed mice. Furthermore, this is associated with decreased TNF-α, IL-6, and RANTES RNA after stimulation at a later time point, indicating effects of smoke exposure at the transcriptional level. However, we acknowledge that TNF-α expression is regulated at the level of transcription as well as translation. Our data does not exclude the possibility that attenuated TNF-α production may be, in part, due to the effect of cigarette smoke on TNF-α protein translation. Together these data intimate that decreased production of cytokines by aMϕs may be due to attenuated activation of NF-κB. Clinically this is of particular relevance, as recently it has been demonstrated that rhinovirues, an important cause of exacerbations in COPD, activate aMϕs in an NF-κB–dependent manner (38). While there are likely critical differences between antigens and replicating pathogens, this indicates the importance of understanding the effect of cigarette smoke on attenuated signaling pathways in aMϕs for bacterial and viral infections.

AP-1 proteins play a large role in the expression of many of the genes involved in proliferation and cell cycle progression, and include a mixture of heterodimeric complexes of proteins from the Fos and Jun families. In contrast to decreased p65 nuclear translocation, we observed increased nuclear associated c-Jun in aMϕs isolated form smoke-exposed mice. This observation is at variance to a recent report by Laan and colleagues (39). The authors showed that impaired production of cytokines in an epithelial cell line after culture in cigarette smoke–conditioned media was associated with decreased nuclear translocation of the AP-1 (39), while NF-κB nuclear translocation was not affected. These findings demonstrate that cigarette smoke may differentially impact aMϕs and epithelial cells. However, this may also be accounted for by differences in the experimental approach, primary cells versus a cell line or human versus murine cells. Expression and production of RANTES in macrophages is generally believed to be dependent on IRF3 activation (26). That we observed decreased RANTES expression in aMϕs isolated from smoke-exposed animals suggest that cigarette smoke also attenuates IRF 3 activation.

Overall, we show that aMϕs from smoke-exposed mice have a basal restraint on cytokine production after TLR stimulation, associated with dysregulated activation of transcription factors. Mechanistically there are several possible explanations. Cigarette smoke has been to shown to contain oxygen free radicals that may directly damage signaling pathways. Alternatively, cigarette smoke has been shown to contain significant levels of biologically active LPS (40), and repeated LPS stimulation is associated with the induction of negative regulators of TLR signaling and LPS tolerance (41). Therefore, smoking may be associated with the expression of negative regulators of TLR activation. On the other hand, several components of cigarette

smoke have been shown to exert direct immunosuppressive activity on aMϕs, including acrolein and NKK (42, 43).

Importantly, attenuated aMϕ function was reversible, as aMϕs regained normal function 4 weeks after smoking cessation. This may either be a consequence of individual aMϕs regaining function over time, or that the attenuated aMϕs were replaced by new macrophages via natural turnover. *Ex vivo*, aMϕs did not regain normal function when rested in culture medium for 1 week, indicating the attenuated phenotype persisted. Attempts to rest the cells longer (2 or 4 wk) resulted in markedly decreased TNF-α production in either group (data not shown); hence, we cannot rule out either hypothesis.

Despite evidence for attenuated aMϕ cytokine production, we have previously observed increased inflammation and cytokine expression in smoke-exposed animals after *in vivo* infection with either replication-competent influenza virus (44) or live replicating *Pseudomonas aeruginosa* (45). Increased *in vivo* inflammation and cytokine expression appears to be at variance with the *ex vivo* observations reported in the current manuscript. A complex and multilayered defense system protects the host against microbial agents, through a combination of physical barriers, and innate and adaptive immune mechanisms. Based our own findings and studies from other labs (reviewed in Ref. 46), we postulate that cigarette smoke suppresses resident respiratory host defense mechanisms, including aMϕs. As a consequence, respiratory pathogens are dealt with inefficiently, necessitating the recruitment of immune-inflammatory cells from the circulation to compensate for this local deficiency. This may explain the increased inflammation observed in our *in vivo* studies.

In summary, we show that cigarette smoke exposure compromises the ability of aMϕs to produce inflammatory cytokines in response to TLR and NLR stimulation. Attenuated production of inflammatory cytokines is associated with dysregulated activation of the transcription factors NF-κB and AP-1. Together, our findings indicate that aMϕs from smoke-exposed mice have decreased ability to transcriptionally up-regulate inflammatory mediators and initiate innate immune inflammatory responses.

Conflict of Interest Statement: M.L. has been employed by AstraZeneca R&D Lund since 2001. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Chapter 3

BACTERIA CHALLENGE IN SMOKE EXPOSED MICE EXACERBATES INFLAMMATION AND SKEWS THE INFLAMMATORY PROFILE

The following article appeared in the *American Journal of Respiratory and Critical Care Medicine*, 2009 Jan 29 (125)

In the study reported in this chapter we questioned whether cigarette smoke-exposure may alter responses to a bacterial challenge, leading to, or contributing to exacerbated inflammation and damage to the lung. This was of particular interest, as the studies reported in chapter 2 indicated a suppressed or attenuated ability of alveolar macrophages, to produce typical pro-inflammatory cytokines, which was difficult to reconcile how COPD exacerbations are characterized by heightened inflammation. To this end, we superimposed a bacterial challenge with nontypeable *Haemophilus influenzae* on a mouse model of whole-body cigarette smoke-exposure. The experimental design we utilized is included in appendix II of this thesis, the Online Data Supplement that accompanied the study reported in this chapter.

I was responsible for design, execution, and writing of this study. Marko Skrtic and Cale Zavitz provided technical assistance and discussion. Per-Ola Onnervik and Maria Lindhal provided technical assistance. Tim Murphy and Sanjay Sethi provided technical advice, reagents, and discussion. Dr. Martin Stampfli supervised the study, assisted in interpretation of the study and editing of the manuscript.

Cigarette Smoke Exposure Attenuates Cytokine Production by Mouse Alveolar Macrophages

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Alveolar macrophages (aMϕs) play a central role in respiratory host defense by sensing microbial antigens and initiating immune-inflammatory responses early in the course of an infection. The purpose of this study was to investigate the effect of cigarette smoke exposure on aMϕs after stimulation of innate pattern recognition receptors (PRRs) in a murine model. To accomplish this, C57BL/6 mice were exposed for 8 weeks using two models of cigarette smoke exposure, nose-only or whole-body exposure, and aMϕs isolated from the bronchoalveolar lavage. After stimulation of aMϕs with pI:C, a mimic of viral replication, and bacterial cell-wall constituent LPS, aMϕs from cigarette smoke–exposed mice produced significantly attenuated levels of the inflammatory cytokines TNF-α and IL-6, and the chemokine RANTES. This attenuation was specific to the aMϕ compartment, and not related to changes in aMϕ viability or expression of Toll-like receptor (TLR)3 or TLR4 between groups. Furthermore, aMϕs from smoke-exposed mice had decreased cytokine RNA as compared with aMϕs from sham-exposed mice. Mechanistically, this was associated with decreased nuclear translocation of the proinflammatory transcription factor NF-κB, and increased activator protein-1 nuclear translocation, in aMϕs from smoke-exposed mice. Attenuated cytokine production was reversible after smoking cessation. Cigarette smoke exposure also attenuated TNF-α production after stimulation with nucleotide-oligomerization domain–like receptor agonists, showing that the effect applies more broadly to other PRR pathways. Our data demonstrate that cigarette smoke exposure attenuates aMϕ responses after innate stimulation, including pathways typically associated with bacterial and viral infections.

Keywords: alveolar macrophage; Toll-like receptor; chronic obstructive pulmonary disease; cigarette smoke; inflammation

Chronic obstructive pulmonary disease (COPD) is reaching epidemic levels (1). Etiologically, COPD is largely associated with cigarette smoking, and epidemiological studies as early as 1982 have shown an increased risk of respiratory infection even in young, asymptomatic smokers (2). Further along in the disease progression of individuals who develop COPD, acute, often self-limiting, bacterial and viral infections are a significant cause of symptom exacerbation, often leading to hospitalization (3, 4). Taken together, this is indicative of cigarette smoke having deleterious effects on innate immunity.

Central to protecting the host early during an infection is the recognition of patterns common to large classes of pathogens by means of pattern-recognition receptors (PRRs). PRRs include members of the prominent Toll-like receptor (TLR) family (5), as well as the nucleotide-oligomerization domain (NOD) family, otherwise called the NOD-like receptor (NLR) family, intracellular receptors recognizing structures from bacterial peptidoglycan (6). TLRs are found on the cell surface and in endosomes of many different cell types. To date there have been approximately 11 TLRs identified in mice and humans with corresponding synthetic or naturally occurring ligands. Included in this are TLR4, which recognizes lipopolysaccharides (LPS) from gram-negative bacteria (7); TLR3, which recognizes double-stranded RNA found during viral infections, or synthetic polyinosinic-polycytidylic acid (pI:C) (8); and TLR9, which recognizes CpG DNA motifs (9). Upon stimulation of TLRs, intracellular signaling pathways are activated, resulting in the nuclear translocation of transcription factors including NF-κB, IRF3, and activator protein (AP)-1 (10). In turn, this leads to the up-regulation and production of cytokines and chemokines important for initiating antibacterial or antiviral immune responses.

CLINICAL RELEVANCE

Given the central role of alveolar macrophages early in the course of an infection, attenuated Toll-like receptor function may predispose smokers to respiratory infections and bacterial colonization.

Due to their strategic positioning in the lumen of the airways, alveolar macrophages (aMϕs) play a central role in innate respiratory host defense (11). Although there is evidence that cigarette smoking may increase the number of aMϕs, further clinical and experimental evidence indicates that these cells may be functionally impaired (12–18). Of particular interest, recently Berenson and coworkers have shown decreased production of inflammatory cytokines upon stimulation of aMϕs from patients with COPD with antigens from *Haemophilus influenzae*—the most commonly isolated bacteria during exacerbations in COPD (19). However, the mechanisms leading to the functional impairment of aMϕs, such as pathways of recognition or PRR expression and function, as well as transcription and translation of cytokines, is less well understood.

The purpose of this study was to investigate the effect of cigarette smoke exposure on PRR-mediated responses by aMϕs in a murine model. To accomplish this, we used two different models of smoke exposure: nose-only and whole-body exposure. We demonstrate that cigarette smoke exposure decreased inflammatory cytokine production by aMϕs upon stimulation with bacterial LPS or muramyl di- and tri- peptides, as wells as pI:C, a mimic of viral replication. To our knowledge, the finding that cigarette smoke exposure attenuates cytokine production after pI:C stimulation is novel and shows that the immunologic effects of cigarette smoke on aMϕs are not limited to bacterial antigens. Mechanistically, we demonstrate that this observation is lung specific, reversible, and is associated with dysregulated activation of transcription factors following TLR stimulation.

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Similar to pI:C and LPS stimulation, we observed decreased cytokine production after stimulation with muramyl di- and tripeptides, ligands for members of the NLR family. Collectively, these data indicate that cigarette smoke has a general impact on aMϕ activation through PRRs, including pathways typically associated with bacterial or viral infections. Some of the results in this study were previously reported in abstract form (20).

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, PQ, Canada) and kept in a 12-hour light-dark cycle with unlimited access to food and water. Cages, food, and bedding were autoclaved, and all animal manipulations were performed in a laminar flow hood by personnel who were gloved, gowned, and masked. The McMaster University Animal Research Ethics Board approved all experiments described in this study.

Cigarette Smoke Exposure

Nose-only exposure. Mice were exposed to two 1R3 reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) daily for 5 days per week using a smoke exposure system that is described in detail elsewhere (21). In an initial lead-up period, animals were accustomed to 1 cigarette in the first and to two cigarettes in the second week. To control for handling, groups of mice were placed into restrainers only and exposed to room air (sham-exposure).

Whole-body exposure. Mice were exposed to the smoke from twelve 2R4F reference cigarettes with the filters removed, twice daily, 5 days per week using an SIU48 (PROMECH LAB AB, Vintrie, Sweden). No lead-up period is required in the whole-body exposure system. In an initial acclimatization period, mice were accustomed to the restrainers over a 3-day period. Specifically, on Day 1 mice were placed into the restrainers for 20 minutes, on Day 2 for 30 minutes, and on Day 3 for 50 minutes. Control animals were exposed to room air only.

Carboxyhemoglobin Measurement

Immediately, or 24 hours, after sham or smoke exposure, blood was drawn in clinitubes (Radiometer, Copenhagen, Denmark) for Carboxyhemoglobin (COHb) measurement by the McMaster University Medical Centre core lab.

Macrophage Isolation and Culture

Mice were anesthetized 18 to 24 hours after their last smoke exposure and killed by exsanguination before excision of the lungs. Tracheas were cannulated and bronchoalveolar lavage (BAL) was collected from sham- and smoke- exposed mice by instilling 0.5 ml of PBS into the lungs through the trachea three times. After each instilment, fluid was collected and pooled. Peritoneal lavage was collected by instilling 5 ml of PBS into the peritoneum via syringe and needle, and fluid was collected with a disposable plastic pipet. Cells were counted by trypan blue dye exclusion. Cytospins for differential cell counts were prepared and stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ). Standard hemocytologic criteria were used to classify mononuclear cells, neutrophils, and eosinophils. At least 500 cells were counted per cytospin. Based on trypan blue dye exclusion and differential cell counts, equal numbers of aMϕs were allowed to adhere for 2 hours at 37°C and 5% CO_2 and washed three times with warm PBS to remove nonadherent cells. Details of aMϕ numbers adhered in the different experiments are provided in the specific method sections below. Adherent cells were cultured in 100 μl of RPMI supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen, Grand Island, NY), and 0.1% β-mercaptoethanol (Invitrogen) (cRPMI) and stimulated with either 1 μg/ml LPS (Invivogen, San Diego, CA) or 10 μg/ml of pI:C or 1–50 μg/ml of CpG (Sigma-Aldrich) for 24 hours. Cells were similarly stimulated with 10 μg/ml muramyl di- or muramyl tri- peptides, generously provided by Anthony Coyle and Jose Lora (Millennium Pharmaceutical, Inc., Cambridge, MA).

Measurement of Cytokines and Nitric Oxide

A quantity of 5×10^4 aMϕs was adhered to a flat-bottom 96-well plate and cultured in 100 μl of cRPMI. After 24 hours of culture, cell supernatants were collected and levels of TNF-α, IL-6, RANTES (regulated on activation, normal T cells expressed and secreted) (R&D, Minneapolis, MN), and IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) were measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturers' instructions and measured on an ELISA plate reader. The limit of detection for the assays were 5.1, 1.6, 2, and 15.6 pg/ml, respectively. Nitric oxide was measured by examining the breakdown products of nitric oxide by Griess reactions as described in detail elsewhere (22).

Macrophage Viabiltiy and Metabolic Activity

For survival cell counts, 1×10^5 aMϕs were adhered to glass slides in flat-bottom 12-well plates. Cells were then counted by trypan dye exclusion counts after 24 hours of culture. For metabolic activity quantification, adherent cells were cultured in 96-well flat-bottom plates in media or stimulated with pI:C and LPS for 24 hours. After this time, MTT activity was assessed by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) kit according to the manufacturers' instructions (Chemicon, Billerica, MA). Briefly, adherent cells were incubated in 100 μl of cRPMI and 10 μl of MTT reagent for 4 hours at 37°C and 5% CO_2. Subsequently isopropanol development solution was added and MTT activity measured at OD570 on an ELISA plate reader.

TaqMan Real-Time RT-PCR

A quantity of 1×10^5 aMϕs was adhered to a flat-bottom 96-well plate. Cells were stimulated with LPS, and RNA was isolated at the indicated time points specified in RESULTS and in the figure legends with the RNeasy mini Kit and the optional DNase step (Qiagen, Mississauga, ON, Canada). RNA was quantified and purity checked using the Agilent 2100 Bio-Analyzer machine operated by the 2100 expert software (Agilent, Palo Alto, CA). Reverse transcription was completed on similar amounts of RNA per group using a RETROscript kit (Ambion, Austin, TX). Mitochondrial ribosomal protein L32 (L32), TLR3, TLR4, TNF-α, IL-6, and RANTES primers and FAM-labeled probes were purchased from Applied Biosystems (Foster City, CA), and PCR was performed in duplicate or triplicate with Universal PCR Master Mix in the ABI PRISM 7900HT Sequence Detection System operated by Sequence Detector Software version 2.2 (Applied Biosystems). PCR was performed in single plex and analysis was completed by first normalizing gene expression to levels of the housekeeping gene L32 in the same sample (ΔCT) and then compared with the control group (ΔΔCT). With this method, RNA extracted from unstimulated aMϕs, isolated from sham-exposed mice, have a relative fold induction (R) defined as 1. Experimental groups are expressed as fold change over this control condition.

Immunofluoresence

For immunofluorescence assays, 2.5×10^4 aMϕs were allowed to adhere per well on glass slides using chamber slide systems (Nalge Nunc Int., Rochester, NY) for 2 hours. Cells were then washed twice with warm PBS to remove nonadherent cells. Slides were placed in pre-chilled acetone at −20°C for 20 minutes and stored at −70°C. To minimize nonspecific binding, cells were incubated with 20% normal goat and normal donkey serum for 1 hour at room temperature. Slides were stained with TLR3 (Imgenex Corp., San Diego, CA) and TLR4 (eBiosciences, San Diego, CA) primary antibodies for 1 hour. Slides were washed with PBST (0.05% Tween20) and stained with Alexa-flour 633– and Alexa-fluor 488–conjugated secondary antibodies (Molecular Probes, Invitrogen) for TLR3 and TLR4, respectively. Control slides were incubated with either primary or secondary antibody alone. Slides were washed and nuclei were stained with SYTO3 in the mounting media (Vector, Burlingame, CA). Fluorescent pictures were taken by confocal microscopy (Leica, Richmond Hill, ON, Canada) with the LSM 510 software. Quantification was performed by analyzing the sum of the TLR stain divided by the sum of the nuclear stain for individual pictures with Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

Nuclear Isolation and NF-κB and AP-1 ELISAs

A quantity of 2×10^6 aMϕs was adhered to flat-bottom 12-well plates. After 2 hours of adherence and stimulation with LPS for the indicated

times, nuclear isolation was preformed with a nuclear isolation kit (Active Motif, Carlsbad, CA) as per the manufacturer's instructions. Extracts were re-suspended in a final volume of 20 μl. Nuclear extracts were used in a TransAM NF-κB ELISA and TransAM c-JUN ELISA (Active Motif) and run according to the manufacturer's instructions. Analysis shown is the sample OD540 divided by the positive control OD540, multiplied by 100, thereby giving percent of positive control signal. The positive control, stimulated Jurkat cell extracts, was provided with the kit, and 2 μg of extract was loaded per well.

Data Analysis

Data are expressed as mean ± SD or SEM as indicated in the figure legends. Statistical analysis was performed using Student's *t* test unless otherwise stated. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Nose-Only Cigarette Smoke Exposure

C57BL/6 mice were exposed to the smoke from two cigarettes a day, 5 days per week for 8 weeks. We observe carboxyhemoglobin (COHb) levels immediately following smoke exposure of 13.58% ± 2.47%, compared with 3.77% ± 0.93% in sham-exposed mice (Table 1).

In the BAL from smoke-exposed mice there is a moderate, but significant, increase in the total cell number, as compared with sham-exposed mice (Figure 1A). Using standard hemacytologic criteria for differential cells counts, isolated cells from the BAL of nose-only smoke- or sham-exposed mice are greater than 95% mononuclear cells (sham: 99.13 ± 0.50; smoke: 97.90 ± 0.57; $n = 6$ with 5 animals pooled per experiment). The remaining cells were neutrophils with no eosinophils present. No difference in cellular composition in the BAL was observed between the groups (Figure 1B).

TNF-α, IFN-β, Nitric Oxide, IL-6, and RANTES Production by aMϕs after pI:C, LPS, or CpG Stimulation

To characterize the impact of cigarette smoke exposure on cytokine production by aMϕs after stimulation with TLR ligands, aMϕs were isolated from the BAL and cultured in medium alone or stimulated with the TLR3 ligand pI:C, the TLR4 ligand LPS, or the TLR9 ligand CpG. After 24 hours, cell supernatants were collected and levels of TNF-α measured by ELISA. As demonstrated in Figure 1C, aMϕs isolated from smoke-exposed mice produce significantly less TNF-α after stimulation with either pI:C or LPS. After stimulation with either pI:C or LPS, we were unable to measure any appreciable levels of IFN-β in the supernatants by ELISA, up-regulation of iNOs by TaqMan, or the byproducts of nitric oxide (NO) breakdown by Griess reaction at the time points measured (data not shown).

To further characterize the specificity of the effect of cigarette smoke on aMϕ cytokine production after TLR stimulation, we measured the levels of the cytokine IL-6 and the chemokine RANTES in supernatants from stimulated aMϕ isolated from sham- or smoke-exposed mice. As shown in Figures 1E and 1F, the levels of IL-6 and RANTES produced by aMϕs from smoke-exposed mice is decreased compared with aMϕs from sham-exposed mice. Thus, the decreased response from aMϕ isolated from smoke-exposed mice is not limited to TNF-α, but includes IL-6 and RANTES.

We did not observe production of TNF-α, IL-6, IFN-β, NO, RANTES, or up-regulation of iNOs from any group after stimulation with the TLR9 ligand CpG (Figures 1C, 1E, 1F, and data not shown).

TABLE 1. CARBOXYHEMOGLOBIN LEVELS IN SHAM- AND SMOKE-EXPOSED MICE

Condition	% Carboxyhemoglobin in Serum
Sham nose-only exposure	3.77 ± 0.93
Smoke nose-only exposure	13.58 ± 2.47*
Sham whole-body exposure	3.67 ± 0.58
Smoke whole-body exposure	8.67 ± 1.55*

Data represent mean ± SD, $n = 13$ for sham nose-only exposure, 12 for smoke nose-only exposure, 3 for sham whole-body exposure, and 3 for smoke whole-body exposure.

* Statistical analysis was performed with Student's *t* test, $P < 0.05$.

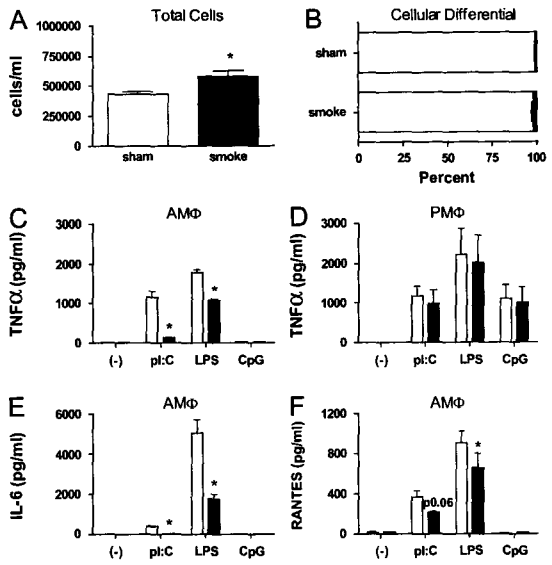

Figure 1. Bronchoalveolar lavage (BAL) cellular profile and cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. *A* and *B* represent the BAL total cell number and cellular differential. (*C–F*) aMϕs and pMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml pI:C, 1 μg/ml LPS, or 10 μg/ml CpG for 24 hours and levels of cytokines measured in cell supernatants by ELISA. (*C* and *D*) TNF-α production by aMϕs and pMϕs, respectively. *E* and *F* represent IL-6 and RANTES production from aMϕs. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. (*A* and *B*) Data represent mean ± SEM of six independent experiments, with five mice pooled per group in each experiment. (*C–F*) AMϕs and pMϕs were isolated in three to five separate experiments, with five animals per experimental group. Data shown is mean ± SEM. Statistical analysis was performed with Student's *t* test *$P < 0.05$.

TNF-α Production by Peritoneal Macrophages after pI:C, LPS, or CpG Stimulation

To investigate whether the effect of cigarette smoke exposure on cytokine production after TLR stimulation was specific to aMϕs, peritoneal macrophages (pMϕs) were isolated in parallel to aMϕs. Similar to aMϕs, pMϕs were cultured in media alone or stimulated with pI:C, LPS, or CpG, supernatants collected after 24 hours of culture, and levels of TNF-α measured by ELISA. As demonstrated in Figure 1D, in contrast to the significant decrease observed in aMϕs from smoke-exposed mice, smoke exposure did not affect the production of TNF-α from pMϕs. In contrast to aMϕs, pMϕs produce TNF-α after CpG stimulation.

aMϕ Survival and Viability in Culture

One explanation for the observed decrease in cytokine production by smoke-exposed aMϕs may be decreased aMϕ viability

as a result of smoke exposure, and consequently a result of culturing fewer live, viable cells. To address this, aMϕs were isolated and cultured as previously for cell viability and metabolic activity measurements. As shown in Table 2, aMϕs from sham- and smoke-exposed mice have similar cell numbers after 24 hours of culture, as determined by trypan blue dye exclusion counts. Similarly, on stimulation with pI:C or LPS, aMϕs from sham- and smoke-exposed mice have similar MTT activity after 24 hours of culture. Together, these observations indicate that decreased cytokine production from aMϕs isolated from smoke-exposed mice after stimulation with TLR agonists likely was not a result of increased death or decreased viability in culture.

aMϕ Expression of TLR3 and TLR4

A further avenue to investigate regarding decreased cytokine production in smoke-exposed aMϕs was to assess whether deceased cytokine production by aMϕs after pI:C and LPS stimulation was related to decreased TLR expression. To this end, aMϕs were isolated from sham- and smoke-exposed mice and RNA extracted after 2 hours of adherence in culture. Levels of TLR3 and TLR4 expression were assessed by real-time quantitative PCR. Levels of the target genes were first normalized to the housekeeping gene L32 (ΔCT) and expressed as relative fold induction to sham-exposed aMϕs (ΔΔCT). As shown in Figure 2A, we observe similar levels of TLR3 and TLR4 RNA in aMϕs from smoke- or sham-exposed mice.

In further experiments, aMϕs were allowed to adhere to glass slides and expression of TLR3 and TLR4 was determined by immunofluoresence. Adherent cells were either stained with TLR3 or TLR4 primary antibodies, as well as a nuclear stain. Control slides were stained with primary antibody, or secondary antibody alone. Immunofluorescent stains were assessed by confocal microscopy. Representative immunofluorescent (IF) pictures from sham- and smoke-exposed mice are shown in Figure 2B. Quantification of fluorescence, as measured by Northern Eclipse software, demonstrated no difference between groups (Figure 2C). No fluorescence signal was detected on control slides stained with either primary or secondary antibody alone.

TNF-α, IL-6, or RANTES RNA Expression in aMϕs after LPS Stimulation

To investigate if decreased cytokine production by aMϕs from smoke-exposed mice was upstream of protein translation, aMϕs from sham- and smoke-exposed mice were isolated as previously and cultured with media alone or stimulated with LPS. After 2, 6, or 24 hours of culture, RNA was extracted from adherent cells. Levels of TNF-α, IL-6, and RANTES were measured by real-time quantitative PCR. As shown in Figure 3, TNF-α, IL-6, and RANTES mRNA from smoke-exposed mice were reduced compared with the levels observed in sham-exposed mice. This is indicative of aMϕs from smoke-exposed mice having decreased ability to up-regulate transcriptionally expression of TNF-α, IL-6, and RANTES RNA after stimulation.

TABLE 2. *EX VIVO* VIABILITY AND METABOLIC ACTIVITY OF ALVEOLAR MACROPHAGES

	Treatment	Sham	Smoke
Viability (cells/ml *10^4)	Media	2.34 ± 0.23	2.6 ± 0.46
Metabolic activity	Media	0.253 ± 0.014	0.264 ± 0.042
(MTT OD570)	pI:C	0.195 ± 0.023	0.232 ± 0.033
	LPS	0.245 ± 0.010	0.252 ± 0.017

Data represent mean ± SD of the mean, $n = 10$ for survival cell counts, $n = 5$ per group for viability, 1 shown of 2 separate experiments.

* Statistical analysis was performed with Student's *t* test, $P < 0.05$.

NF-κB and AP-1 Nuclear Translocation in aMϕs after LPS Stimulation

To further investigate the transcriptional dependence of the effect of smoke exposure on aMϕ cytokine production, we investigated levels of transcription factors entering the nucleus after LPS stimulation. aMϕs from sham- and smoke-exposed mice were isolated as previously and stimulated with LPS for 30, 60, or 90 minutes and nuclear extracts isolated from each group. Extracts were quantified and equal amounts of protein were loaded per well in TransAm ELISA plates. The activated NF-κB or AP-1 contained in nuclear extracts specifically binds to oligonucleotide coated on the solid phase. Using antibody specific for p65 or c-Jun, the NF-κB or AP-1 complex bound to the oligonucleotide is detected. As demonstrated in Figure 4A, we observe decreased nuclear associated NF-κB subunit p65 in aMϕs isolated from smoke-exposed mice. In contrast to p65, we observed increased nuclear translocation of the AP-1 subunit c-Jun after stimulation with LPS (Figure 4B).

TNF-α Production by aMϕs after Muramyl-Dipeptide and Muramyl-Tripeptide Stimulation

To further investigate the effects of cigarette smoke exposure on PRR pathways leading to cytokine production by aMϕs, we stimulated aMϕs with ligands for members of the NLR family. Specifically, aMϕs were isolated similar to previously and stimulated with agonists for NOD1, muramyl-dipeptides, and NOD2, muramyl-tripeptides. After 24 hours of culture, supernatants were collected and levels of TNF-α measured by ELISA. Similar to responses observed for TLR signaling pathways, we observed decreased production of TNF-α after stimulation with either NOD ligand (Figure 5). Thus, the decreased response of aMϕs isolated from smoke-exposed mice is not restricted to TLR agonists, but includes NLR agonists as well.

Whole-Body Cigarette Smoke Exposure

To investigate whether the effects of cigarette smoke observed with the nose-only exposure system were limited to that exposure system, or were consistent with other models of smoke exposure, mice were cigarette smoke exposed with a whole-body exposure system. With this model of exposure, C57BL/6 mice were exposed twice daily to the cigarette smoke from 12 cigarettes, 5 days a week for 8 weeks. We observe COHb levels of 8.67% ± 1.55%, compared with 3.67% ± 1.55% in sham-exposed mice (Table 1).

In BAL from whole-body smoke-exposed mice, there is a significant increase in the total cell number, as compared with sham-exposed mice (Figure 6A). Isolated cells from the BAL of whole-body smoke- or sham-exposed mice are greater than 95% mononuclear cells (sham: 99.88 ± 0.29; smoke: 96.96 ± 4.82; $n = 5$ per group). Similar to nose-only exposure, the balance of the remaining cells was neutrophils, and no difference in cellular composition in the BAL was observed between groups (Figure 6B).

TNF-α Production by aMϕs and pMϕs after pI:C, LPS, and CpG Stimulation

To characterize the impact of whole-body cigarette smoke exposure on cytokine production by aMϕs after stimulation with TLR ligands, aMϕs were isolated from the BAL and cultured in medium alone or stimulated with pI:C, LPS, or CpG. After 24 hours, cell supernatants were collected and levels of TNF-α measured by ELISA. As demonstrated in Figure 6C, aMϕs isolated from smoke-exposed mice produce significantly less TNF-α after stimulation with either pI:C or LPS.

Similar to aMϕs, pMϕs were cultured in media alone or stimulated with pI:C, LPS, or CpG, supernatants collected after

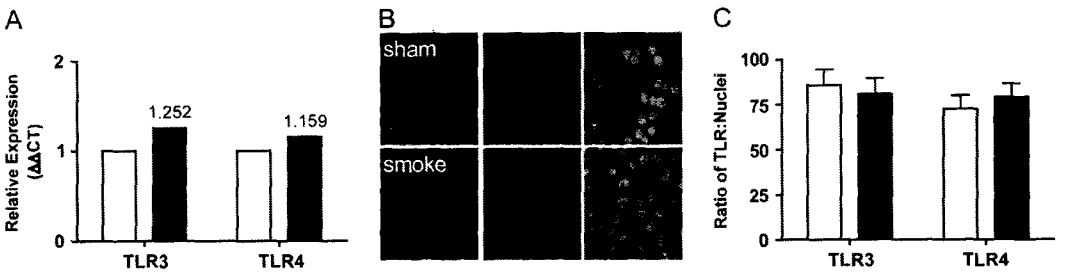

Figure 2. TLR3 and TLR4 expression in alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. (*A*) RNA was isolated from aMϕs and levels of TLR3 and TLR4 RNA expression was measured by real-time quantitative PCR (TaqMan). Expression of TLR3 and TLR4 in sham-exposed mice were defined as 1 and levels in smoke-exposed mice were expressed relative to this. (*B*) BAL cells were adhered to glass slides, and TLR3, TLR4, and nuclei were stained. The *top* and *bottom panels* show aMϕs from sham- and smoke-exposed mice, respectively. Nuclear stains are shown in the *left panels*, TLR3 in the *middle panels*, and TLR4 in the *right panels*. (*C*) Immunofluoresencent picures were quantified using Northern Eclipse. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively.

24 hours of culture, and levels of TNF-α measured by ELISA. As demonstrated in Figure 6D, in contrast to the significant decrease observed in aMϕs from smoke-exposed mice, whole-body smoke exposure did not affect the production of TNF-α from pMϕs.

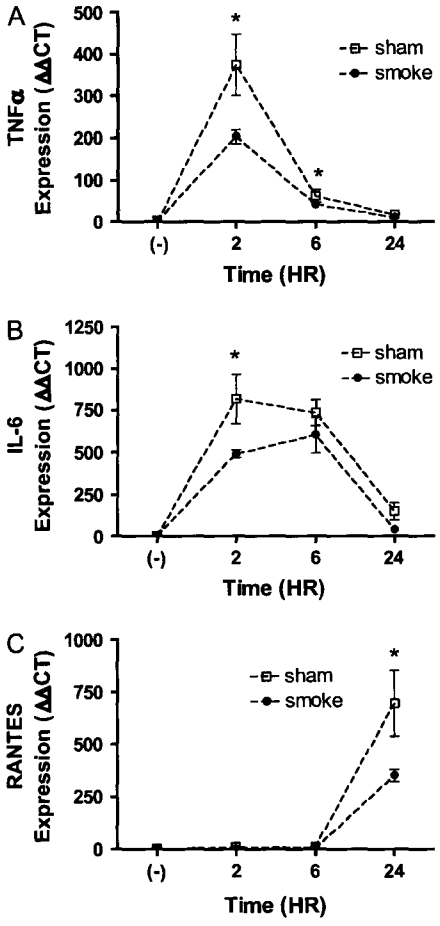

Figure 3. *Ex vivo* alveolar macrophage TNF-α, IL-,6 and RANTES RNA expression. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. AMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS for 2, 6, or 24 hours. RNA was isolated and levels of (*A*) TNF-α RNA, (*B*) IL-6 RNA, and (*C*) RANTES expression was measured using real-time quantitative PCR (TaqMan). Data represent mean ± SEM, n = 10 (*A*), n = 5 (*B* and *C*). Statistical analysis was performed with Student's t test, $^*P < 0.05$. For all panels, aMϕs from sham-exposed mice are *open squares* and aMϕs from smoke-exposed mice are *solid circles*.

Time Course and Reversibility of the Cigarette Smoke Exposure on aMϕ Function

Next we investigated the minimal duration of cigarette smoke exposure necessary to observe attenuated cytokine production by aMϕs. Mice were sham- or smoke-exposed for 1, 2, 4, or 8 weeks. At each time point, aMϕs were isolated from the BAL and stimulated with LPS for 24 hours. Levels of TNF-α were measured in cell supernatants. As shown in Figure 7A, we observed no difference in TNF-α production between aMϕs isolated from sham- and smoke-exposed mice after 1 or 2 weeks of smoke exposure (P = 0.91 and P = 0.22, respectively). After 4 and 8 weeks, we observed significantly decreased TNF-α in the cell supernatants of aMϕs isolated from smoke-exposed mice (P = 0.004 and P = 0.018, respectively).

To characterize the reversibility of the effects of cigarette smoke exposure on aMϕs, mice were sham- or smoke-exposed for 8 weeks. AMϕs were isolated from the BAL and rested for 1 week in media alone. aMϕs were then stimulated with LPS for 24 hours and levels of TNF-α measured in cell supernatants. We

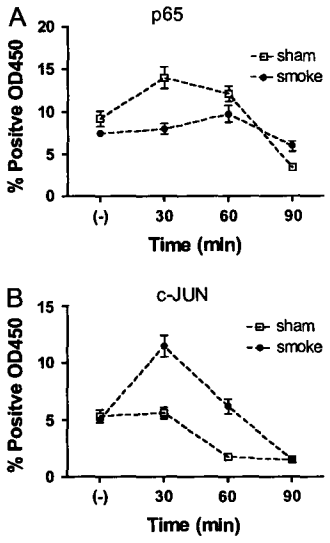

Figure 4. NF-κB and AP-1 nuclear translocation in alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 weeks. aMϕs were cultured in media alone or stimulated with 1 μg/ml LPS for 30, 60, or 90 minutes and nuclear extracts were isolated. Levels of (*A*) p65 and (*B*) c-Jun nuclear translocation were measured by ELISA. Shown is one representative of two experiments. Data represent mean ± SD (technical error), with 10 mice pooled per experimental group. For all panels, aMϕs from sham-exposed mice are *open squares* and aMϕs from smoke-exposed mice are *solid circles*.

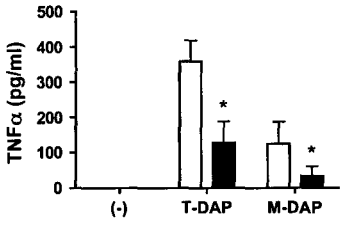

Figure 5. *Ex-vivo* TNF-α production by alveolar macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. aMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml M-DAP or 10 μg/ml T-DAP for 24 hours and levels of TNF-α measured in cell supernatants by ELISA. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. Data represent mean ± SEM, $n = 2$; in each experiment, five animals were pooled per group. Statistical analysis was performed with Student's *t* test, $^*P < 0.05$.

observed significantly decreased TNF-α production by aMϕs isolated from smoke- compared with sham-exposed mice (Figure 7B), suggesting that aMϕs maintain their attenuated phenotype *in vitro*.

To investigate whether the effects of cigarette smoke exposure on aMϕs are reversible *in vivo*, mice were sham- or smoke-exposed for 8 weeks. Subsequently, smoke-exposed mice were divided into two groups: the first continued their smoke-exposure regime (smoke), while the second were exposed to room air (cessation). After 2 or 4 weeks of smoking cessation, aMϕs were isolated and cultured in medium alone or stimulated with LPS. After 24 hours of culture, cell supernatants were collected and levels of TNF-α measured by ELISA. After 2 weeks of smoking cessation, we observe significantly decreased TNF-α in both the smoke-exposed and the cessation group, as compared with the sham-exposed group (Figure 7B). No difference was observed between the smoke and cessation groups. After 4 weeks of smoking cessation aMϕs regained normal function; we observed similar levels of TNF-α in supernatants of aMϕs from the cessation group as compared with the sham group. Together, these findings indicate that smoking cessation may reverse attenuated aMϕ function, but only after a sufficient period of time.

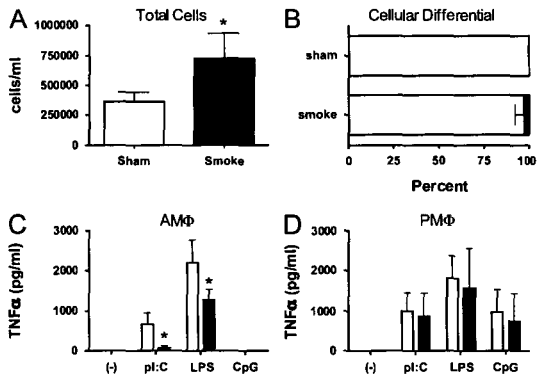

Figure 6. BAL cellular profile and cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 8 to 10 weeks. *A* and *B* represent the BAL total cell number and cellular differential. (*C* and *D*) aMϕs and pMϕs were isolated and cultured in media alone or stimulated with 10 μg/ml pI:C, 1 μg/ml LPS, or 10 μg/ml CpG for 24 hours and levels of cytokines measured in cell supernatants by ELISA. *C* and *D* represent TNF-α production by aMϕs and pMϕs, respectively. In all panels, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. Data represent mean ± SEM, $n = 5$ per experiment. Statistical analysis was performed with Student's *t* test $^*P < 0.05$.

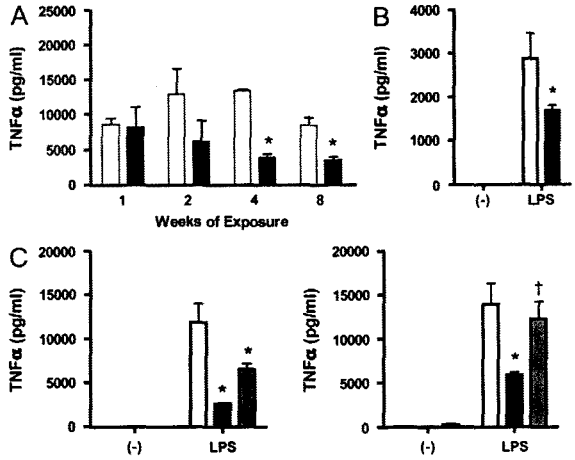

Figure 7. Cytokine production by alveolar and peritoneal macrophages. C57BL/6 mice were sham- and smoke-exposed for 1, 2, 4, 8, 10, and 12 weeks. aMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS for 24 hours and levels of TNF-α measured in cell supernatants by ELISA. (*A*) TNF-α production by aMϕs after 1, 2, 4, and 8 weeks of smoke-exposure. (*B*) Mice were sham or smoke exposed for 8 weeks and aMϕs were isolated and cultured for 1 week before stimulation with 1 μg/ml LPS for 24 hours. Levels of TNF-α were measured in cell supernatants by ELISA. In *A* and *B*, aMϕs from smoke- or sham-exposed mice are shown in *solid* or *open bars*, respectively. (*C*) Mice were sham- and smoke-exposed for 8 weeks, and after this time mice continued their exposure regime (*open bars*, sham exposure; *solid bars*, smoke exposure) or ceased smoke exposure (*shaded bars*, cessation). After 2 or 4 weeks aMϕs were isolated and cultured in media alone or stimulated with 1 μg/ml LPS, and levels of TNF-α were measured in cell supernatants by ELISA (*C*). Data represent mean ± SEM, $n = 3$–5 per experiment. Statistical analysis was performed with Student's *t* test $^*P < 0.05$ (*A*, *C*) or one-way ANOVA (*B*.) $^*P < 0.05$ compared with sham, $^\dagger P < 0.05$ compared with smoke.

DISCUSSION

aMϕs are fundamental to respiratory host defense by sensing microbial agents early in the course of an infection and initiating immune inflammatory responses. In this study, we investigated the effect of cigarette smoke exposure on the production of inflammatory cytokines after *ex vivo* stimulation of aMϕs. To this end, mice were exposed to mainstream cigarette smoke using two distinct smoke-exposure systems. The first, a nose-only exposure system, is widely used to study smoke-induced emphysema in small rodents. With this system Hautamaki and coworkers demonstrated the requirement of macrophage elastase for cigarette smoke–induced emphysema (21). The second system, a whole-body exposure system, has been developed more recently.

Similar to human smokers (23), mice exposed to smoke in either of these model systems have an increase in the number of cells isolated from the BAL, with aMϕs representing greater than 95% of cells. Importantly, based on carboxyhemoglobin blood measures, levels of smoke exposure are similar to that reported clinically (24). Although we observed increased number of aMϕs in the BAL of smoke-exposed mice, *ex vivo* cytokine production after stimulation was attenuated. More specifically, we observed significantly decreased production of the inflammatory cytokines TNF-α and IL-6, and the chemokine RANTES, after stimulation with the TLR ligands pI:C or LPS, as well as the NLR ligands muramyl di- or tripeptides. Our findings are in line with previous clinical and experimental observations, including a recent report in which aMϕs from patients with COPD were stimulated with bacterial antigens from *H. influenzae* (19). That cigarette smoke

exposure also attenuates cytokine production after NLR stimulation, as well as pI:C stimulation, is novel and shows the effect of cigarette smoke is not limited to bacterial antigens. Therefore, cigarette smoke likely has a general impact on immune activation through PRRs, including pathways typically associated with viral infections. Along these lines, the fact that RANTES is decreased is of particular interest, as it indicates that cigarette smoke impairs not only the MyD88-dependent pathway of TLR4 signaling, but likely also the MyD88 independent pathway—a pathway associated with the production of type 1 interferons (25).

Despite the production of RANTES, we failed to measure any appreciable level of IFN-β production by aMϕs. As demonstrated in peritoneal macrophages, downstream events from IRF3 activation (via TLR3 or TLR4 stimulation) include the production of the type 1 interferon IFN-β. IFN-β then has paracrine and autocrine function through STAT1 to up-regulate the expression of inducible nitric oxide (iNOs) and ultimately the production of NO (26, 27). Consistent with the lack of IFN-β production by aMϕ, we observed neither induction of iNOs or the production of NO, after LPS or pI:C stimulation (data not shown). This observation is in agreement with a previous report by Punturieri and colleagues, in which the authors demonstrated that aMϕs do not produce IFN or NO in response to TLR3 or TLR4 ligands alone (28). Notably, this unresponsiveness is maintained in aMϕs isolated from smoke-exposed animals.

While we observed robust cytokine production by aMϕs after pI:C or LPS stimulation, we were unable to demonstrate any response to CpG. This observation is consistent with the lack of TLR9 expression by aMϕs in either sham- or smoke-exposed mice (data not shown). In agreement with this, a previous study has demonstrated that aMϕs, unlike peritoneal macrophages, do not respond to CpG stimulation (29), likely indicating the specialized function of aMϕs in respiratory host defense.

The impact of cigarette smoke was lung specific, as no differences were observed between peritoneal macrophages isolated from sham- and smoke-exposed mice in their production of TNF-α. Similarly, a recent clinical study indicates that cytokine production from aMϕs stimulated with *H. influenzae* antigens from individuals with COPD are impaired, but not peripheral blood monocytes (19).

On further characterization of the observed attenuated effect, we sought to control for cell viability in culture, as smoke exposure may be detrimental to aMϕ survival, leading to stimulation of less cells. Our observations indicate that decreased cytokine production by aMϕs from smoke-exposed mice is not a result of increased cell death due to cellular toxicity. In agreement with this, numerous clinical studies have shown that aMϕs from smokers may have an increased life span, despite exposure to toxic components contained within cigarette smoke (30–33). Therefore, mechanisms relating to decreased aMϕ production of cytokines are likely unrelated to direct toxic effects on cell survival by cigarette smoke.

Of the potential mechanisms leading to decreased cytokine production in response to pI:C and LPS, decreased surface expression of TLR4 and endosome expression of TLR3 as a result of smoke exposure may be hypothesized. However, with these experimental approaches we did not observe any changes in TLR3 or TLR4 expression between sham- and smoke-exposed mice. Specifically, no difference was seen on the RNA level as assessed by TaqMan or the protein level as assessed by immunofluorescent microscopy. Together these data indicate that the decrease in cytokine production by aMϕs from smoke-exposed mice is likely downstream of TLR3 and TLR4 expression. Indeed, while numerous studies have shown impaired function of aMϕs from smokers or patients with COPD, a previous report demonstrated no difference in TLR4 expression on aMϕs (34).

pI:C may stimulate aMϕs via alternate pathways than TLR3, including cytoplasmic RNA helicases, such as retinoic acid–inducible gene I (RIG-I) (35). Therefore, while attenuated cytokine production after LPS, T-DAP, or M-DAP stimulation can be attributed to effects on TLR4, NOD1, and NOD2 pathways, respectively, attenuated cytokine production after pI:C stimulation may involve multiple pathways.

TLR stimulation leads to the activation of intracellular signaling pathways, resulting in the nuclear translocation of the transcription factors NF-κB, IRF3, and AP-1 (10). The functional unit of NF-κB, usually a heterodimer of the p65 and p50 subunits, is held in its inactive form in the cytoplasm by the inhibitory molecule, IkB (36). Upon activation, the IkB molecule is degraded, allowing free NF-κB to enter the nucleus and initiate pro-inflammatory gene transcription (37). In our hands, we observe decreased levels of p65 after stimulation with LPS in nuclear extracts isolated from aMϕs from smoke-exposed mice. Furthermore, this is associated with decreased TNF-α, IL-6, and RANTES RNA after stimulation at a later time point, indicating effects of smoke exposure at the transcriptional level. However, we acknowledge that TNF-α expression is regulated at the level of transcription as well as translation. Our data does not exclude the possibility that attenuated TNF-α production may be, in part, due to the effect of cigarette smoke on TNF-α protein translation. Together these data intimate that decreased production of cytokines by aMϕs may be due to attenuated activation of NF-κB. Clinically this is of particular relevance, as recently it has been demonstrated that rhinovirues, an important cause of exacerbations in COPD, activate aMϕs in an NF-κB–dependent manner (38). While there are likely critical differences between antigens and replicating pathogens, this indicates the importance of understanding the effect of cigarette smoke on attenuated signaling pathways in aMϕs for bacterial and viral infections.

AP-1 proteins play a large role in the expression of many of the genes involved in proliferation and cell cycle progression, and include a mixture of heterodimeric complexes of proteins from the Fos and Jun families. In contrast to decreased p65 nuclear translocation, we observed increased nuclear associated c-Jun in aMϕs isolated form smoke-exposed mice. This observation is at variance to a recent report by Laan and colleagues (39). The authors showed that impaired production of cytokines in an epithelial cell line after culture in cigarette smoke–conditioned media was associated with decreased nuclear translocation of the AP-1 (39), while NF-κB nuclear translocation was not affected. These findings demonstrate that cigarette smoke may differentially impact aMϕs and epithelial cells. However, this may also be accounted for by differences in the experimental approach, primary cells versus a cell line or human versus murine cells. Expression and production of RANTES in macrophages is generally believed to be dependent on IRF3 activation (26). That we observed decreased RANTES expression in aMϕs isolated from smoke-exposed animals suggest that cigarette smoke also attenuates IRF 3 activation.

Overall, we show that aMϕs from smoke-exposed mice have a basal restraint on cytokine production after TLR stimulation, associated with dysregulated activation of transcription factors. Mechanistically there are several possible explanations. Cigarette smoke has been to shown to contain oxygen free radicals that may directly damage signaling pathways. Alternatively, cigarette smoke has been shown to contain significant levels of biologically active LPS (40), and repeated LPS stimulation is associated with the induction of negative regulators of TLR signaling and LPS tolerance (41). Therefore, smoking may be associated with the expression of negative regulators of TLR activation. On the other hand, several components of cigarette

smoke have been shown to exert direct immunosuppressive activity on aMϕs, including acrolein and NKK (42, 43).

Importantly, attenuated aMϕ function was reversible, as aMϕs regained normal function 4 weeks after smoking cessation. This may either be a consequence of individual aMϕs regaining function over time, or that the attenuated aMϕs were replaced by new macrophages via natural turnover. *Ex vivo*, aMϕs did not regain normal function when rested in culture medium for 1 week, indicating the attenuated phenotype persisted. Attempts to rest the cells longer (2 or 4 wk) resulted in markedly decreased TNF-α production in either group (data not shown); hence, we cannot rule out either hypothesis.

Despite evidence for attenuated aMϕ cytokine production, we have previously observed increased inflammation and cytokine expression in smoke-exposed animals after *in vivo* infection with either replication-competent influenza virus (44) or live replicating *Pseudomonas aeruginosa* (45). Increased *in vivo* inflammation and cytokine expression appears to be at variance with the *ex vivo* observations reported in the current manuscript. A complex and multilayered defense system protects the host against microbial agents, through a combination of physical barriers, and innate and adaptive immune mechanisms. Based our own findings and studies from other labs (reviewed in Ref. 46), we postulate that cigarette smoke suppresses resident respiratory host defense mechanisms, including aMϕs. As a consequence, respiratory pathogens are dealt with inefficiently, necessitating the recruitment of immune-inflammatory cells from the circulation to compensate for this local deficiency. This may explain the increased inflammation observed in our *in vivo* studies.

In summary, we show that cigarette smoke exposure compromises the ability of aMϕs to produce inflammatory cytokines in response to TLR and NLR stimulation. Attenuated production of inflammatory cytokines is associated with dysregulated activation of the transcription factors NF-κB and AP-1. Together, our findings indicate that aMϕs from smoke-exposed mice have decreased ability to transcriptionally up-regulate inflammatory mediators and initiate innate immune inflammatory responses.

Conflict of Interest Statement: M.L. has been employed by AstraZeneca R&D Lund since 2001. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Chapter 4

MECHANISMS OF CLEARANCE OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE* FROM CIGARETTE SMOKE EXPOSED MICE LUNGS

The following study is submitted

An intriguing finding of the previous study was the observation that clearance of NTHI infection in cigarette smoke-exposed mice, while more effective in terms of reducing CFU, may be at the expense of lung damage. Our interpretation of the data was that the exacerbated inflammatory profile we observed in the BAL and lung tissue, consisting of increased number of mononuclear cells and neutrophils, were contributing to increased clearance of the bacteria from the cigarette smoke-exposed lung. Given the importance of this central point, we thought this observation deserved further attention and investigation to address the specific mechanism of decreased bacterial burden.

The study presented in this chapter was the result of our investigation into the specific mechanisms involved in clearance of NTHI from the cigarette smoke-exposed mouse lungs.

I was responsible for design, execution, and writing of this study. Cale Zavitz provided technical assistance, discussion, and editing of the manuscript. Dr. Martin Stampfli supervised the study, assisted in interpretation of the data, and editing of the manuscript.

FULL TITLE

Mechanisms of Clearance of nontypeable *Haemophilus influenzae* from Cigarette Smoke-Exposed Mouse Lungs[1]

1. This study was funded by the Canadian Institute for Health Research (CIHR)

RUNNING TITLE

Mechanism of clearance of NTHI from smoke-exposed mice

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ABSTRACT

Inflammation is prevalent in all stages of COPD, and furthermore, individuals undergo periods of exacerbation where the 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 nontypeabale *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 and subsequently challenged with NTHI. We observed increased pulmonary inflammation as a result of twice-daily cigarette smoke-exposure, largely consisting of increased number of neutrophils. Following bacterial challenge we observed exacerbated inflammation associated with accelerated kinetics of bacterial clearance in both once- and twice- daily cigarette smoke-exposed mice. Mechanistically, we do not observe evidence of mucous production in any experimental group, and while the broncho-alveolar lavage (BAL) fluid of cigarette smoke-exposed mice is not directly toxic to NTHI, there is a cell-free factor in the BAL fluid that contributes to accelerated clearance following passive transfer of the BAL fluid to naive mice. Further investigation demonstrated increased titres of IgA in the BAL, but not the blood, of cigarette smoke-exposed mice, and $JH^{-/-}$ 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, but also to increased titres of antibodies that contribute to clearance of the bacteria.

INTRODUCTION

Of all the leading causes of death, Chronic Obstructive Pulmonary Disease (COPD) is the only one that has been rising for the past 20 years, and in both developed and developing countries COPD is now the 4th leading cause of death in the world (1, 2). Cigarette smoking is the major etiological factor for development of COPD with exposure to pollution or occupational exposure to 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.

Airflow limitation in COPD is associated with chronic inflammation of the respiratory tract (5, 6). Contributing to this inflammation are increased number of neutrophils and macrophages, which are understood to contribute to a protease/antiprotease imbalance and consequently, proteolytic damage to the lungs (7). Lymphoid follicles consisting of B and T cells are also observed in the lungs of patients with COPD, and both CD4+ and CD8+ T cell subsets are thought to contribute to disease pathogenesis (5, 8). Increased numbers of B cells, as well as titres of autoantibodies, have been reported in COPD, leading to speculation that COPD may have an autoimmune component (9, 10).

Increasingly, the immunological effects of cigarette smoke are being investigated (11). Individual experimental studies have demonstrated that cigarette smoke affects components of innate and adaptive immune mechanisms, including pathways involved in host defense (12). 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 (13-15). With regards to bacterial infection, intermittent or chronic infection with nontypeable *Haemophilus influenzae* (NTHI), *Moraxella catarrhalis*, *Pseudomonsa aeruginosa,* and *Streptoccocus pneumoniae* are the most common causes (16-19). 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 (20). 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 (21). 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.

Given that periods of exacerbation and heightened inflammation, as a result of 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 mucous production. We further show increased titre of antibodies in the broncho-alveolar lavage (BAL), accelerated clearance is dependent on B cells, and the protective agent within the BAL can be transferred to naïve mice. 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 supports that the immunological effects of cigarette smoke on antibody responses are an important consideration for understanding the pathogenesis of COPD.

MATERIALS and METHODS

Animals

6-8 week old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Montreal, PQ, Canada). $JH^{-/-}$ mice (22) on a C57Bl/6 background were a generous gift from Dr. Kathy McCoy (McMaster University, Hamilton, Ontario). $JH^{-/-}$ 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 (23) and levels of continine reported in reference (21). 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 Ug/L, n=47.

Preparation of NTHI

Nontypeable *Haemophilus influenzae* 11P6 was used in all experiments (24, 25). 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 (25, 26). 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 hemin and 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+Hemin+NAD broth, and the culture was incubated with rotary shaking at 37°C until an OD_{600} 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 10^6 cfu of NTHI in a total volume of 35 μL of PBS. Animals were inoculated at least 4 hours after the last smoke-exposure.

Collection of specimens

Brocho-alveolar lavage (BAL), lungs, and blood, were collected at the time of killing. BAL was performed as previously described (21, 27). 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 (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. Peripheral blood was collected by retroorbital bleeding and total cell counts were determined using a haemocytometer. Blood smears for differential cell counts were prepared, stained with Hema 3 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 bacterial burden assessments, the left lung was tied off before BAL and placed in PBS. For histological assessment the left lung was inflated with 10% neutral buffered formalin at a constant pressure of 20 cm H_2O, and then fixed in 10% neutral buffered formalin for 48 to 72 hours.

Preparation of lung tissue homogenate and measurement of NTHI burden

At the time of killing, lung lobes were placed into phosphate buffered saline (PBS) on ice. Lungs were then 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 mucous, goblet cell hyperplasia and airway morphometric analysis

The left lung was inflated with 10% formalin at a constant pressure of 20 cm H_2O, 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 captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). 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 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. Serial ten-fold dilutions of BAL and serum were prepared for measurements.

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 specimen's section. BAL fluid (BALF) was isolated by removing the cellular fraction by centrifugation at 1200 RPM 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 10^6 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 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 (21). 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 developed a model of once-daily cigarette smoke-exposure, rather than our general model of exposure that consists of twice-daily exposure. Specifically, we exposed both 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. In contrast to twice-daily smoke-exposure, neither strain of mice had increased total number of cells in the broncho-alveolar lavage (BAL) (Figure 1A and 1E), or evidence of increased number of neutrophils (Figure 1C and 1G), as result of once-daily cigarette smoke-exposure, while C57BL/6 but not BALB/c mice had increased number of mononuclear cells (Figure 1B and 1F). Following twice-daily cigarette smoke-exposure, both BALB/c and C57BL/6 had significantly increased numbers of neutrophils, however 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, both once-daily cigarette smoke-exposed BALB/c and C57BL/6 mice had increased total number of cells 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 had 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 number 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 indicate that while 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 inflammation following NTHI challenge, the presence of neutrophils in the BAL, as a result of once- or twice- daily cigarette smoke-exposure, was not sufficient to explain the observation of decreased bacterial burden in cigarette smoke-exposed mice.

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

Mucous is an important innate immune mechanism contributing to bacterial host defense. In patients with COPD, and certain experimental models, mucous and goblet cell hyperplasia is often reported as one of the characteristic pathologies (28, 29). Given the observation of decreased bacterial burden in cigarette smoke-exposed mice we next measured mucous production and the extent of goblet cell hyperplasia in the lung tissues of control mice, cigarette smoke-exposed mice, NTHI-challenged mice, and cigarette smoke-exposed NTHI-challenged mice, for extent of positive staining with alcian blue/periodic acid-Schiff (AB/PAS). Light microscopic examination followed by

morphometric quantification revealed that in this model of exposure, neither control nor cigarette smoke-exposed mice produce appreciable levels of mucous, or demonstrate evidence of goblet cell hyperplasia. Similarly, bacterial challenge with NTHI alone did not lead to mucous production; and finally, the combination of cigarette smoke-exposure and NTHI challenge was still not sufficient to induce the production of mucous. As a positive control for the morphometric measurement of mucous production and goblet cell hyperplasia we utilized lung tissue from a mouse chronically exposed to house dust mite, a model of allergic airway inflammation, which is known to induce mucous production and goblet cell hyperplasia (30). Representative images of the mucous 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 were cigarette smoke-exposed twice-daily for a period of 8 weeks. Following this time, mice were killed, BAL isolated, and the BALF was isolated by centrifugation to remove the cellular fraction. Incubation of BALF from cigarette smoke-exposed mice with NTHI and subsequent plating 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 be contributing to clearance of NTHI, we again incubated NTHI with BALF isolated from either cigarette smoke-exposed or control BALB/c mice, but then inoculated the NTHI + BALF suspension into naïve BALB/c mice. 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 suggest 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 inflammation. 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 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 observation of decreased bacterial burden and the initiation of an 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. Similar to previous, 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 number of total cells (Figure 4A), consisting of significantly increased numbers of neutrophils (Figure 4C). These data indicate that the exacerbated inflammatory profile that we observe 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 contain increased titres of antibodies, 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 mice of both mouse strains (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 (22). 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. Similar to previous, cigarette smoke-exposed WT C57BL/6 mice had decreased bacterial compared to control WT mice.

As a control for the 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).

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 BAL, consisting of increased number of mononuclear cells (Figure 7C) and neutrophils (Figure 7D), similar to the case seen in WT controls (Figure 1).

DISCUSSION

The purpose of this study was to investigate how cigarette smoke-exposure alters the bacterial host response following a pulmonary NTHI challenge. To this end, we utilized a model of experimental cigarette smoke-exposure that results in chronic inflammation of the respiratory tract (21), and the dominant cause of bacterial exacerbation in COPD patients, NTHI (18).

Experimentally, there are many means by which to expose mice to cigarette smoke (31). One of the strengths of this study is the investigation into the requirements for cigarette smoke-induced inflammation in mice. Our general model of cigarette smoke-exposure consists of twice-daily smoke-exposure for an 8-week period, which leads to the presence of a sustained neutrophilia in the BAL, similar to that often reported from clinical studies (32). In contrast, once-daily cigarette smoke-exposure did not result in the presence of neutrophils in the BAL, indicative of a dose response of cigarette smoke-induced pulmonary inflammation in mice. Additionally, we also note a genetic difference in susceptibility to cigarette smoke-exposure. BALB/c mice appear more susceptible to cigarette smoke-induced inflammation than C57BL/6 mice, as BALB/c mice present with greater number of neutrophils following cigarette smoke-exposure. A number of studies have investigated the sensitivity for cigarette smoke-induced pulmonary inflammation in various strains of mice, and there is considerable variability between research groups (33-36). For example, similar to these results Vlahos *et al.* demonstrated that BALB/c have greater pulmonary inflammation following cigarette smoke-exposure than C57BL/6 mice (35). In difference, Yao *et al.* demonstrated the opposite result - that C57BL/6 mice were the most susceptible to cigarette smoke-induced inflammation, although in this study BALB/c mice were not included (36). While the most likely explanation for differences between groups are differences in exposure model, the precise genetic differences between strains that may give rise to the observed discrepancies is more difficult to ascertain. However, given the importance of macrophages in the pathogenesis of COPD (37), and

that one of the key differences between BALB/c and C57BL/6 mice is the M1/M2 polarization of macrophages (38), this may be a key mechanism. Providing further support to this idea, Woodruff *et al.* have demonstrated that alveolar macrophages isolated either from COPD patients or from cigarette smoke-exposed mice have distinctive genetic profiles (39).

Although there are strain-specific differences, cigarette smoke-exposed mice of both C57BL/6 and BALB/c backgrounds have an exacerbated inflammatory response following bacterial challenge. Based upon the results of this study it seems that with this model of cigarette smoke-exposure and bacterial challenge clearance of the bacteria and the initiation of an inflammatory response are independent events. The evidence for this is twofold: First, the differences between once- versus twice- daily cigarette smoke-exposed mice indicate that decreased bacterial burden is observed in either group, however we observe a dose-response effect with regards to inflammation. Second, the decreased bacterial burden is contributed to by a factor within the fluid component of cigarette smoke-exposed mouse lungs, which the B-cell deficient studies indicate is likely antibodies, which may be passively transferred to naïve mice; while exacerbated inflammation is contributed to by a cellular or resident component within the cigarette smoke-exposed mouse lung that is not transferred.

On further development of this point, the exacerbated inflammatory profile is observed following challenge with either live, or heat-killed bacteria. This demonstrates that it is the components of the bacteria, including components that are not heat labile, that are important for the exacerbated response. Such components include bacterial cell walls such as LOS and outer membrane proteins such as P2 and P6, which stimulate innate pattern recognition receptors on resident innate cells, such as TLR2 and TLR4 on epithelial cells, alveolar macrophages, or neutrophils, leading to the production of pro-inflammatory cytokines (40, 41). This has important consequences as challenge with NTHI lysate alone can lead to aspects of COPD (42). These data

demonstrate that at the same time of recruiting inflammatory cells, compensatory innate clearance mechanisms are working to clear the bacteria from the lungs. As we do not observe a difference in the leukocyte number in the blood of cigarette smoke-exposed mice (unpublished observations), this indicates that the exacerbated pulmonary inflammation post- challenge is driven by specific recruitment of cells into the lungs, rather than a global increase in circulating neutrophils.

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 (37). We have demonstrated that the skewed profile of inflammatory mediators which alveolar macrophages isolated from cigarette smoke-exposed mice express may contribute to the exacerbated response. Specifically, alveolar macrophages from cigarette smoke-exposed mice produce a different subset of pro-inflammatory cytokines than those that would result in an inflammatory response in control mice (21). Importantly, these effects are reversible, as smoking cessation reversed the effects on alveolar macrophages, likely a result of turnover of the alveolar macrophage population (23).

An alternate explanation may include that B cells are important contributors to exacerbated inflammation in cigarette smoke-exposed, NTHI challenged mice. This hypothesis deserves particular attention, as lymphoid follicles are observed in patients with COPD, consisting of, among other cells, increased number of B cells (5, 8). Similarly, lymphoid follicles may be observed in models of chronic cigarette smoke-exposure (43, 44). Furthermore, Lund *et al.* demonstrated that B cells may be an important cell type for the induction of inflammation following pulmonary infection with *Pneumocystis (P.) carini* (45). The data presented here would argue against this hypothesis as a contributing mechanism for exacerbated inflammation in cigarette smoke-exposed mice, as the exacerbated inflammatory profile observed was similarly observed in the absence of B cells.

The question of effects on B cells deserves further attention, specifically the mechanism underlying the increased antibody titres we observed in the BAL. Given the evidence demonstrating that cigarette smoke may disrupt both epithelial (46, 47) and endothelial permeability (48, 49), we first questioned whether the observations were a result of vascular leakage into the lung lumen. While it is indirect evidence, we did not observe increases in serum IgM, IgG, or IgA levels in BALB/c or C57BL/6 mice, following either once- or twice- daily cigarette smoke-exposure. That being said, increases in total antibodies may not be observed even in cases where there are clear increases in antigen-specific antibodies as they represent such a small amount of the total. Given that NTHI is an obligate human pathogen, it is unlikely the mice would have had the contact with NTHI that would be necessary for the induction of protective antigen specific antibodies. This leads to the inference that the cigarette smoke-induced antibodies are likely antigen non-specific 'natural' antibodies. Along these lines, Lund *et al.* demonstrated that mice are protected from *P. carini* in a B cell and antibody-dependent, but antigen-specific antibody-independent, manner (45). Therefore, the chronic inflammatory stimulus leading to the specific transport of antibodies into the lung lumen is likely responsible for the increased titre of antibodies in the lung lumen. Interestingly, the threshold for increased transport of IgA into the lumen demonstrates a lower threshold of cigarette smoke-exposure (once-daily) than the threshold required for recruitment of neutrophils into the lung lumen (twice-daily).

Similar to these results, there is clinical evidence that the absolute titres of IgA in the BAL of patients with chronic bronchitis may be increased (50, 51). Although, this is a controversial result as other studies demonstrated decreased titres of IgA in the BALF (52). Furthermore, when corrected for total albumin, the antibody to albumin ration is decreased (50). In difference, and similar to these results, in an experimental model investigating broncho-associated lymphoid tissue (BALT) in a model of cigarette smoke-exposure, Demoor *et al.* demonstrated increased titres of IgM and IgA in

BAL fluid (53). Our results here indirectly demonstrate that vascular leakage is likely not the mechanism leading to increased antibody titre as we do not observe increased in IgM in the BAL.

Other experimental models of cigarette smoke-exposure have demonstrated that the burden of bacteria may be increased following challenge, rather than decreased (54, 55). 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 may 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 (56, 57).

These data 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. Clinically, ongoing trials are investigating whether intermittent treatment of antibiotics may be effective for prevention of exacerbations. The data presented here would argue that while prevention of exacerbation are an important strategy for reducing exacerbation-induced hospitalization or death, strategies aimed at treating existing exacerbations of COPD may be designed to specifically target the inflammatory pathways. Along these lines, investigating the anti-inflammatory action of antibiotics may be the most beneficial in treatment of exacerbation. As a final point, these data would further argue that a lung resident cell 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.

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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 10^6 CFU of NTHI and killed 12 hours post challenge. A-C), and E-G) Differential cell analysis showing the number of total cells, mononuclear cells (MNCs), and neutrophils (NEUs) in the BAL. Inserts in panels C) and G) are the data for PBS challenge shown on a smaller scale (10^5 compared to 10^6). 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 mucous 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. Panels in A-D show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue stained with alcian blue/periodic acid-Schiff (AB/PAS) indicating mucous production by epithelial goblet cells obtained 12 hours post challenge. Panel D) shows a picture of a mouse chronically exposed to house dust mite as a positive control. F) Morphometric analysis of lung histology; data represent 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 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, mononuclear cells (MNCs) and neutrophils (NEUs) 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 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 cells (A), mononuclear cells (MNCs) (B), and neutrophils (NEUs) (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 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 intranasaly with sterile vehicle (PBS) or 10^6 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 (B), mononuclear cells (MNCs) (C) and neutrophils

(NEUs) (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.

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FIGURE 1

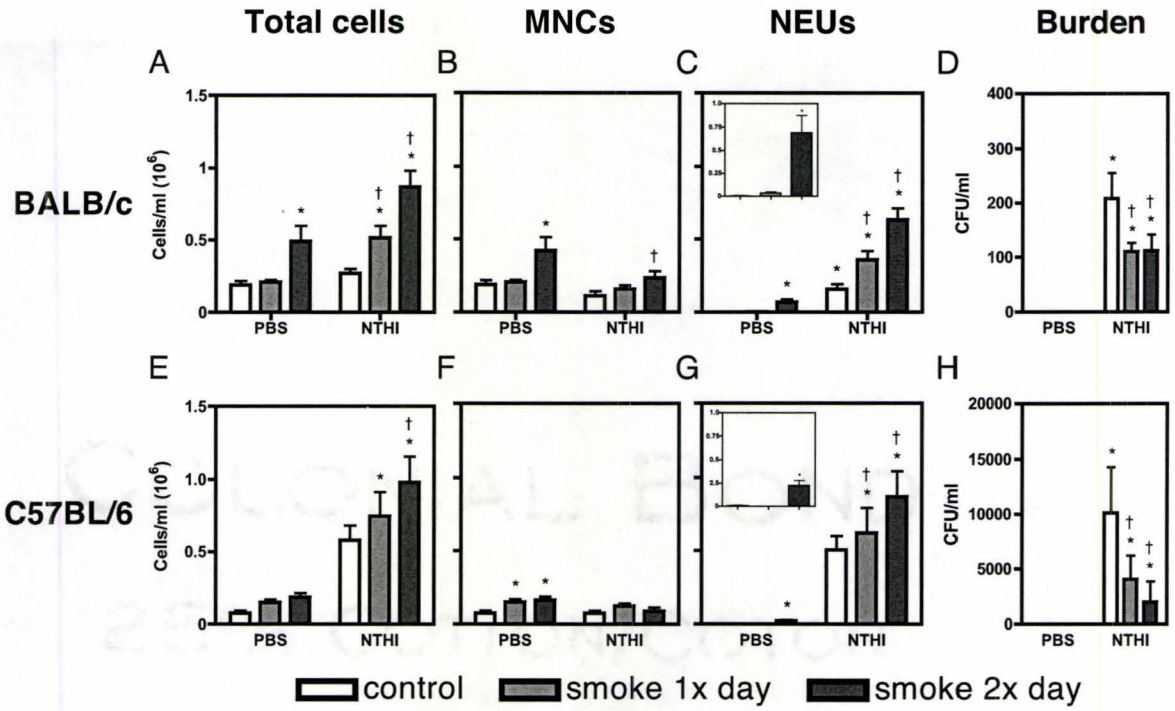

Total cells
MNCs
NEUs
Burden
A
B
C
D
E
F
G
H
BALB/c
C57BL/6
Cells/ml (10^6)
CFU/ml
PBS
NTHI
control
smoke 1x day
smoke 2x day

FIGURE 2

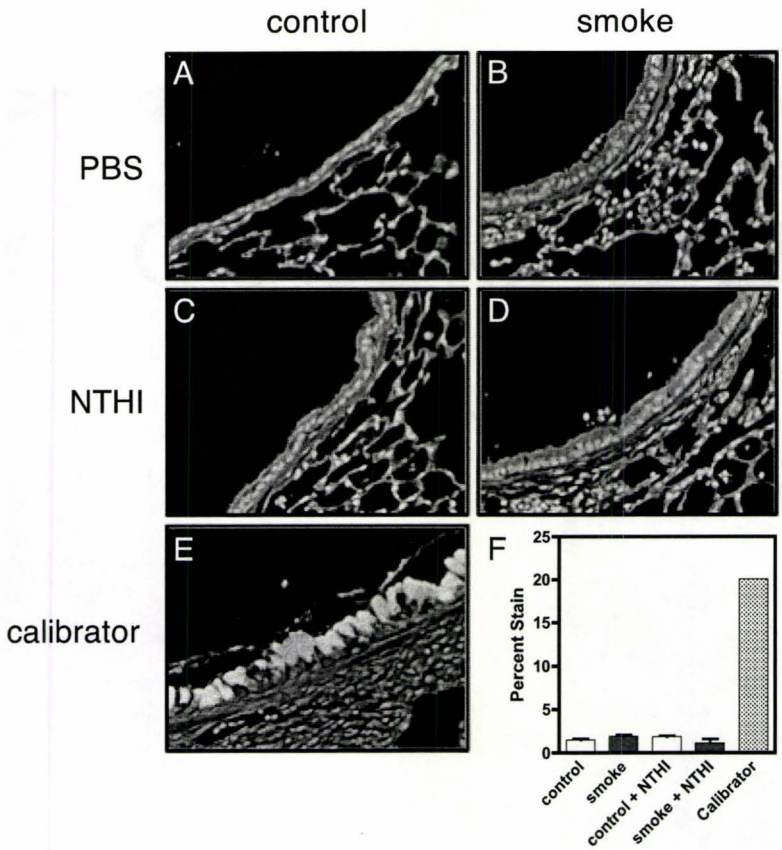

control
smoke
A
B
PBS
C
D
NTHI
E
calibrator
F
Percent Stain
25
20
15
10
5
0
control
smoke
control + NTHI
smoke + NTHI
Calibrator

FIGURE 3

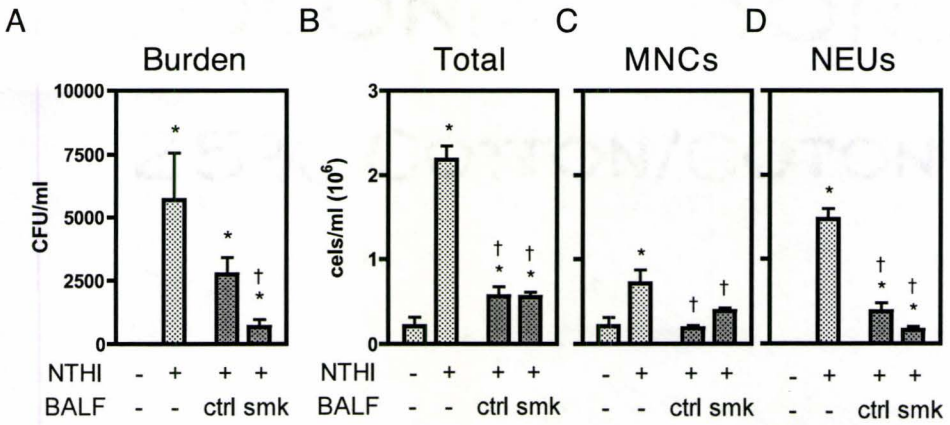

A
Burden
B
Total
C
MNCs
D
NEUs
CFU/ml
10000
7500
5000
2500
0
cels/ml (10^6)
3
2
1
0
NTHI - + + +
BALF - - ctrl smk
NTHI - + + +
BALF - - ctrl smk
- + + +
- - ctrl smk
- + + +
- - ctrl smk

FIGURE 4

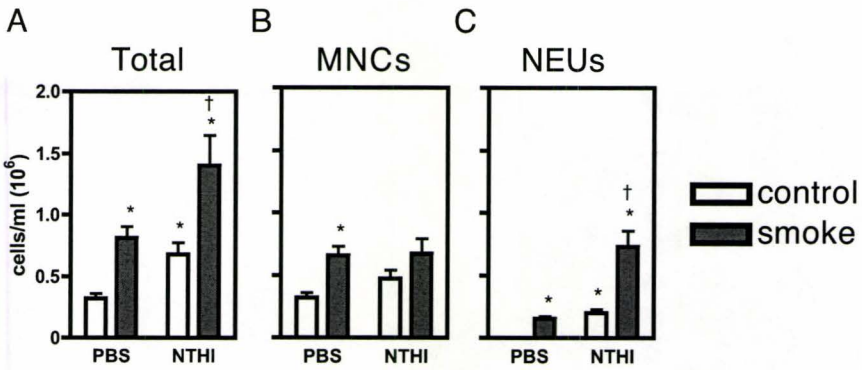

A
Total
B
MNCs
C
NEUs
cells/ml (10^6)
2.0
1.5
1.0
0.5
0
PBS
NTHI
PBS
NTHI
PBS
NTHI
control
smoke

FIGURE 5

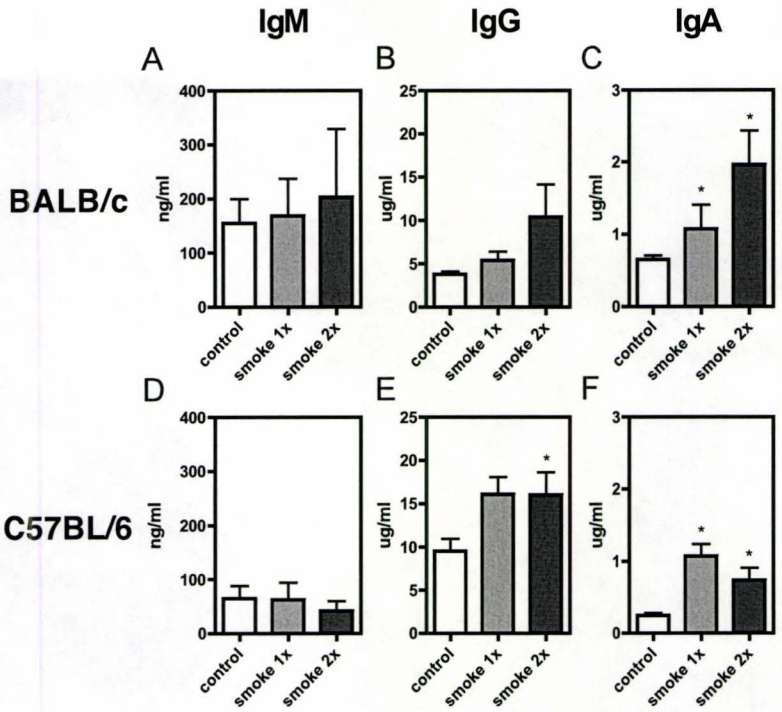

IgM
IgG
IgA
A
B
C
D
E
F
BALB/c
C57BL/6
ng/ml
ug/ml
control
smoke 1x
smoke 2x
*

FIGURE 6

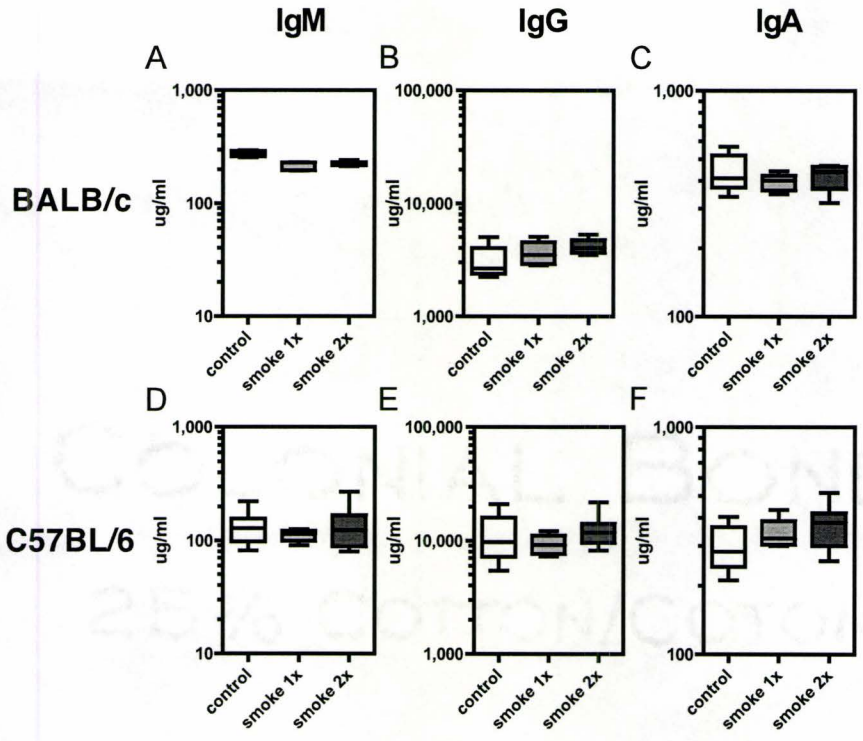
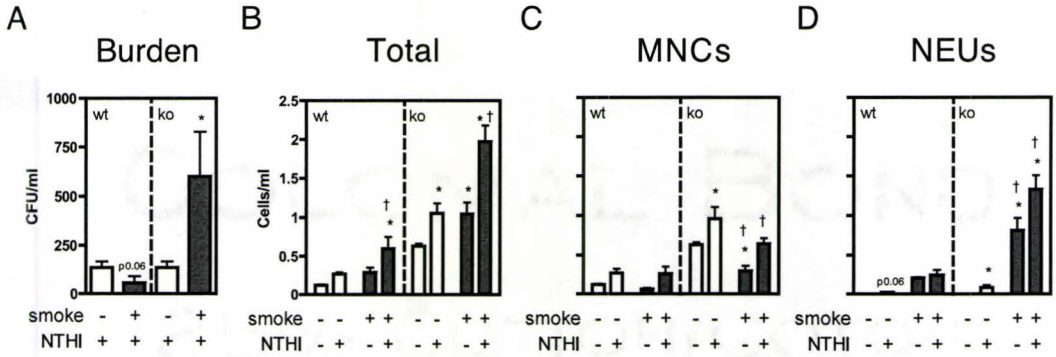

IgM
IgG
IgA
A
B
C
D
E
F
BALB/c
C57BL/6
ug/ml
1,000
100
10
100,000
10,000
1,000
control
smoke 1x
smoke 2x

FIGURE 7


A
Burden
B
Total
C
MNCs
D
NEUs
CFU/ml
Cells/ml
wt
ko
p0.06
smoke
NTHI

Chapter 5

DISCUSSION

The cellular, molecular, and genetic mechanisms underlying the pathogenesis of COPD are not well understood. The purpose of this thesis was to provide insight into these mechanisms through investigation of how cigarette smoke alters the response to a bacterial challenge in a mouse model of cigarette smoke-exposure. The hypothesis being that microbial infection(s), leading to periods of symptom exacerbation contribute to cigarette smoke-induced lung pathology, and therefore to the development and/or progression of COPD.

The data presented here provides insights into the cellular changes that result from cigarette smoke-exposure alone, and how this is altered during times of microbial infection. Furthermore, this work identifies how cigarette smoke-exposure alters the expression of a number of inflammatory mediators and pathways, both prior to, and following microbial challenge, which may be examined to address molecular aspects of development or progression of COPD. While the specific genes underlying susceptibility to airflow limitation have not yet been identified, these data provide some insight into strain differences in consequences of cigarette smoke-exposure, and provide data on mediators/pathways potentially related to genetic susceptibility for developing disease.

It has been previously stated that one of the reasons for 'the current paucity of data regarding mechanisms of COPD and exacerbations is the lack of a valid model' (126). The lack of treatments for COPD, at least in part, may be contributed to by this lack of quality experimental models. This may not be a failure of the models themselves, but likely reflects the inherent difficulty in modeling COPD, due to the considerable heterogeneity observed

among individuals with COPD, within individuals over time, and the further difficulty in superimposing an exacerbating stimulus, such as infection with a bacteria or virus. In fact, given the heterogeneity in human smoking behavior, data derived from each model likely represents features of the overall disease. Therefore, while it is unlikely that there will ever be one perfect, standardized method across different research groups for exposing experimental animals to cigarette smoke, there is considerable insight to be gained from comparing multiple models, and to carefully interpret what state and what type of the disease each may be replicating. Given the central role of experimental models for understanding of disease pathogenesis, and for designing and screening of therapeutic interventions, understanding the strengths, limitations, and stringencies of each model will certainly contribute to better understanding of the complex clinical pathophysiology.

A necessary factor to consider for interpreting data from experimental models of cigarette smoke-exposure is the duration, frequency, and relative amount or dose of exposure. In the three papers completed for this thesis, we were careful to include specific details on our method of cigarette smoke-exposure, at times with further detail on the method of exposure and detailed diagram of our timeline of exposure included in an online data supplement (appendix II). Furthermore, one of the strengths of these papers is the inclusion of markers for amount or dose of exposure. While not standard, including markers of exposure are important for interested researchers to be able to interpret and compare our model of exposure to others. Included in this is measurement of levels of serum carboxyhemoglobin (COHb), a measurement of the saturation of hemoglobin with carbon monoxide, levels of urine or serum cotinine, a byproduct of nicotine metabolism, and total particular matter in our exposure chamber as a result of our method of exposure. Essential

to the interpretation of these studies, based on clinical reports the mice cigarette smoke-exposed during these studies would be considered within the range of that reported for a 'heavy' human smoker (127, 128).

In addition to the detail on our method of exposure, while the general research question for this thesis was regarding the effect of cigarette smoke on bacterial inflammatory pathways, with the central readouts being cellular profile, inflammatory mediator production, and bacterial burden following bacterial stimulation or challenge, we were careful to always include the appropriate control groups for the cigarette smoke-exposure as well as the bacterial challenge. The importance of designing the studies with these control groups is it permits us to simultaneously investigate the effect of cigarette smoke-exposure alone on inflammatory/immune processes in the lung prior to bacterial challenge, and subsequently to investigate deviations from this new baseline, or from the normal host response, following bacterial challenge.

Having detail on method and dose of exposure, and the appropriate control groups, allows for the interpretation of the three studies completed for this thesis together. The remainder of this chapter is divided into my interpretation of the alterations to immune/inflammatory pathways prior to, and following, bacterial challenge as a result of this model of cigarette smoke-exposure, and a discussion of the underlying mechanisms and implications.

General discussion of cigarette smoke-induced inflammation

As outlined in the introduction, there have been a number of studies investigating the means by which cigarette smoke induces inflammation. Included in this are *in vitro* cell

culture models with CSE and individual components of cigarette smoke, *ex vivo* cell cultures from exposed animals, human smokers, and COPD patients, and *in vivo* measurements from exposed animals and COPD patients providing detail on ongoing inflammatory processes. The purpose of this section is to develop the pattern of inflammation observed in the studies reported in this thesis. The importance of this is not solely for the purpose of providing depth to the understanding of cigarette smoke-induced inflammation, but it is fundamental for understanding how cigarette smoke-exposure alters the baseline state of the lungs, which allows for interpretation of changes following bacterial stimulation or challenge.

The general method of exposure utilized for these studies is a whole-body cigarette smoke-exposure system, where mice are exposed twice-daily, to the cigarette smoke generated from 12 research cigarettes, for a period of 8 weeks (appendix II). That being said, these studies were completed with a number of different factor variables deviating from this general method. Included in this are 2 strains of laboratory mice (BALB/c and C57BL/6), 2 different systems of cigarette smoke-exposure (nose-only and whole-body), the dose/frequency of cigarette smoke-exposure (once- or twice- daily), and the duration of exposure (1 to 8 weeks of exposure). Furthermore, while largely an *in vivo* investigation, a number of the studies were completed *ex vivo* with primary cell cultures, to further provide insight into mechanism(s). These different variations provide insight into the mechanisms and stringencies for cigarette smoke-induced inflammation in mice.

Whole-body cigarette smoke-exposure systems are often utilized in experimental models due to the capacity for exposure of many animals simultaneously, whereas nose-only exposure systems have a technical limitation in number of animals that may be exposed but are thought to represent a more relevant method of exposure. Because of this whole-body

cigarette smoke-exposure systems suffer the criticism of not being entirely representative of cigarette smoking, as cigarette smoke-exposed animals may ingest components of cigarette smoke while cleaning their fur, during and for a period of time after exposure (129). In our study in chapter 2, we demonstrated similar total numbers of cells in the BAL following 8 weeks of nose-only or whole-body cigarette smoke-exposure. As a reference for the level of exposure, COHb was measured following either whole-body or nose-only cigarette smoke-exposure and found to be quite similar. Furthermore, we reported that both alveolar macrophages and peritoneal macrophages isolated from mice in either model behaved similarly. These results indicate that for the outcomes measured for the experiments in these studies the method of cigarette smoke-exposure did not matter.

We observe significantly increased total numbers of cells in the BAL of both BALB/c and C57BL/6 mice following 8 weeks of exposure. Increased numbers of cells are largely composed of increased number of neutrophils, and to a lesser extent increased number of mononuclear cells. Increased percentages of neutrophils are similarly observed in lung digests of cigarette smoke-exposed mice. An increase in neutrophil number is observed as early as one week following cigarette smoke-exposure, and is maintained up to the last time point included in these studies. While the number of neutophils are significantly increased at all time points, the peak level is reached following one week of exposure, and is maintained at a lower level for the remaining time of exposure.

Associated with this chronic inflammation was increased level of soluble hydroxyproline in the BAL of cigarette smoke-exposed mice. Measurement of soluble hydroxyproline in lung tissues digests is often used as a measurement of fibrosis, as it measures the extent of collagen turnover, whereas measurement in the BAL may be

considered as a measurement of lung damage, as it reflects the leakage of collagen into the airspaces/airway lumen. In addition to hydroxyproline, we measured the extent of collagen surrounding the airways and found no differences as a result of cigarette-smoke exposure. This particular observation was in contrast to a study by Churg *et al.,* who demonstrated increased collagen deposition in the airways of cigarette smoke-exposed mice (130). Given that the mice in the study by Churg *et al.* were cigarette smoke-exposed for a period of 6 months, these results indicate that while damage to the lung may be observed at 8 weeks of exposure, a longer exposure period is necessary to observe fibrotic changes of the airways. The fibrotic changes may be incipient, however, as the data reported in chapter 3 indicated that cigarette smoke-exposure led to increased expression of a number of tissue remodeling factors, including FGF-basic, which have been implicated in airway remodeling in COPD (131, 132).

While both BALB/c and C57BL/6 mice demonstrate neutrophilic inflammation as a result of cigarette smoke-exposure, we do note a genetic difference in susceptibility, as BALB/c mice are more sensitive to cigarette smoke-induced inflammation than C57BL/6 mice. The sensitivity of various strains of mice to cigarette smoke-induced inflammation has been investigated by a number of studies and there is considerable variability among different groups of investigators (133-136). For example, similar to these results Vlahos *et al.* demonstrated that BALB/c mice had the greatest pulmonary inflammation following cigarette smoke-exposure, whereas C57BL/6 mice had the least (135). In contrast, Yao *et al.* demonstrated the opposite result that C57BL/6 mice were the most sensitive to cigarette smoke-induced inflammation compared to a number of other strains (136). The most likely explanation for differences between groups is differences in exposure models. Alternatively,

and less likely, is the supplier of mice utilized. The precise genetic differences between these strains is more difficult to ascertain, but given the importance of macrophages in the pathogenesis of COPD (137), and that one of the key differences between BALB/c and C57BL/6 mice is the M1/M2 polarization of macrophages following stimulation, this may be a key mechanism. Indeed, Mills *et al.* reported that macrophages isolated from C57BL/6 mice demonstrate an M1 phenotype and influence Th1 polarized responses following LPS stimulation, whereas macrophages isolated from BALB/c mice demonstrate an M2 phenotype and influence Th2 polarized responses (138). Therefore, the macrophage phenotype present in different strains of mice influences the nature of the inflammatory response, and may behave differently when exposed to cigarette smoke.

Interestingly, in contrast to twice-daily cigarette smoke-exposure, neither BALB/c nor C57BL/6 mice demonstrate a neutrophilic inflammation following once-daily cigarette smoke-exposure. Because these experiments were completed following only 8 weeks of exposure, we cannot rule out that once-daily cigarette smoke-exposure may have induced an inflammatory response that resolved. Nevertheless, twice-daily cigarette smoke-exposure was a requirement for a sustained neutrophilia at 8 weeks. These results must be interpreted carefully, as the increased frequency of exposure, or the increased dose, may be driving the inflammation in twice-daily cigarette smoke-exposure. While not addressed in these studies, a natural extension to these experiments would be to cigarette smoke-expose mice twice-daily for half the amount of time, or once-daily for twice as long.

These experiments demonstrate that cigarette smoke-exposure induces pulmonary inflammation, consisting of increased numbers of macrophages and neutrophils. Data from our laboratory indicate that inflammation begins as early as one day of cigarette smoke-

exposure (Submitted Manuscript), reaches its peak at one week of exposure, and is sustained at a lower level for at least 8 weeks. This occurs in both C57BL/6 and BALB/c mice, although the extent of neutrophilic inflammation is higher in BALB/c mice, and is dependent on frequency of exposure. This chronic or sustained inflammatory state has significant implications for understanding how cigarette smoke-exposure changes the 'baseline' state of the lungs and how this new baseline alters the response to a bacterial challenge.

General discussion of cigarette smoke-exposure and pathways of microbial-induced inflammation

The consequences of a pulmonary bacterial challenge in normal mice and the ensuing inflammatory response have been demonstrated in a number of studies. Onofrio *et al.*, for example, demonstrated that challenge with a bacterial dose less than 10^6 CFU/ml of *Staphylococcus aureus* led to bacterial clearance, whereas challenge with higher doses led to an inflammatory host response (139). Of particular relevance for this thesis, inflammatory responses following challenge with NTHI are characterized by the expression of a number of pro-inflammatory cytokines, most notably TNFα, IL-6, and IL-1β, as well as number of neutrophil chemoattractants, most notably CXCL-1 and CXCL-8 (140). The purpose of this section is to provide insight on the consequences of a pulmonary bacterial challenge with NTHI in the context of cigarette smoke-exposure. Because we have the appropriate control groups of cigarette smoke-exposure alone and bacteria challenge alone, we can investigate both the normal pathways of host response to bacteria and can investigate alterations as a result of cigarette smoke-exposure.

One of the central observations of these studies is the effect of cigarette smoke-exposure on inflammatory cytokine production from alveolar macrophages following stimulation of microbial pathways. Alveolar macrophages isolated from cigarette smoke-exposed mice, either with nose-only or whole-body exposure, express lower levels of a number of pro-inflammatory cytokines, including TNFα and IL-6, following microbial stimulation. Attenuated cytokine production was observed following stimulation with either TLR or NLR ligands, as well as live or heat-killed bacteria. In addition, the attenuated cytokine production was observed both on the level of total protein as well as messenger RNA, demonstrating that decreased cytokine production was transcriptionally regulated. Associated with this were alterations to transcription factors, where nuclear translocation of NFκB was attenuated following stimulation, while nuclear translocation of AP-1 was augmented. These data are of significance as they indicate that the mechanism by which cigarette smoke-exposure leads to attenuated cytokine production following stimulation is contributed to, at least in part, to events that occur early in the signaling pathways leading to the initiation of pro-inflammatory gene expression.

An especially interesting result from these studies was that despite attenuated levels of typical pro-inflammatory mediators in *ex vivo* cell cultures of alveolar macrophages, bacterial challenge in cigarette smoke-exposed mice is characterized by an exacerbated inflammatory state *in vivo*. Specifically, challenge with NTHI lead to increased number of neutrophils and macrophages in both the BAL and lung tissue of cigarette smoke-exposed mice, as compared to either cigarette smoke-exposed mice alone or control NTHI challenged mice. Furthermore, this was associated with increased lung damage as assessed by hydroxyproline analysis.

As outlined in the introduction there are a number of potential explanations for the observation of exacerbated inflammation despite attenuated cytokine production, including the total number of cells producing cytokine, the absence of other factors in *in vitro* and *ex vivo* studies, or differences in bacterial virulence. The data presented in chapter 3, however, indicate that it may not be any of these explanations, as levels of TNFα, IL-6, and a number of other mediators were similarly attenuated *in vivo*. These data would argue that other inflammatory mediators are driving the exacerbated inflammation in bacterial challenged cigarette smoke-exposed mice.

Mechanistically, we sought to investigate the factors responsible for, or contributing to the exacerbated inflammatory cellular profile. To this end, for the experiments in chapter 3 we did an extended bead-array based protein characterization of ~70 rodent metabolites as a screen. Similar to other reported bacterial challenge models (140), in our study pulmonary challenge with NTHI in control mice is predominantly marked by increases in early pro-inflammatory cytokines such as TNFα, IL-6, IL-1β, and chemokines such as CXCL-1, CXCL-8. Pulmonary challenge with NTHI in cigarette smoke-exposed mice, on the other hand, is marked by a prominent up-regulation of a different subset of inflammatory mediators, most notably the CC chemokines CCL-2, CCL-7, and CCL-12. Therefore, exacerbated inflammation and recruitment of cells in cigarette smoke-exposed mice following bacterial challenge is contributed to by a change in the profile of the inflammatory mediators.

The change in mediator expression was likely not due to an overwhelming bacterial load and increased pattern recognition receptor stimulation in cigarette smoke-exposed mice due to delayed clearance of NTHI, as we actually observed decreased bacterial burden. Nor

was it due decreased pattern recognition receptor stimulation as a result of delivery of fewer bacteria in cigarette smoke-exposed mice, as we observed similar bacterial burden between control and cigarette smoke-exposed mice immediately post challenge. Of particular interest, the data in chapter 4 indicated that decreased burden of NTHI was independent of the chronic inflammation, direct toxic effects of cigarette smoke components, and mucous production. We did, however, observe increased titres of IgA antibodies in the BAL of cigarette smoke-exposed mice, and furthermore, accelerated bacterial clearance was dependent on B cells. As additional evidence, the factor in the BAL contributing to the accelerated clearance was passively transferred to naïve mice.

These experiments demonstrate that cigarette smoke-exposure results in exacerbated pulmonary inflammation in response to a bacterial challenge. Exacerbated inflammation is characterized by increased numbers of neutrophils in the BAL and lung tissue, and is associated with lung damage. Bacterial burden, with this model of exposure and NTHI as the bacterial challenge, is decreased in cigarette smoke-exposed mice, likely due to increased titres of antibodies in the BAL of cigarette smoke-exposed mice. Furthermore, as a result of the cigarette smoke-exposure typical pathways of cytokine production are effectively 'turned off' leading to attenuated levels of pro-inflammatory cytokines following stimulation of alveolar macrophages *ex vivo,* or challenge with NTHI *in vivo.* This attenuation does not apply to all inflammatory mediators, however, as other pathways are induced in the context of cigarette smoke-exposure, leading to the expression of a different inflammatory mediator profile following challenge.

Discussion of underlying pathways

This thesis demonstrates that cigarette smoke-exposure in mice results in chronic inflammation, and this inflammation is exacerbated following bacterial challenge. Perhaps the most important result for providing insight into the pathophysiology of COPD is not necessarily that bacterial challenge in cigarette smoke-exposed mice results in exacerbated inflammation, but that the nature of the inflammatory response is altered. Specifically, typical pro-inflammatory mediators, such as TNFα and IL-6, are attenuated in cigarette smoke-exposed mice following NTHI challenge, while atypical mediators, such as CCL -2, -7, and -12, among others, are induced.

Utilizing passive transfer of BAL fluid from cigarette smoke-exposed or control mice to naïve mice, the data presented in Chapter 4 indicated that it is the cells that are present in the lungs of cigarette smoke-exposed mice at the time of challenge that are central in driving this altered microbial responsiveness. The data reported in chapters 2 and 3 provide further insight into the underlying cellular mechanism, that while we cannot rule out changes to epithelial cells or other cells resident or recruited to the lungs at time of challenge, alveolar macrophages have altered responsiveness to microbial stimulation. In comparing the characterization of inflammatory mediators *in vivo* in the BAL following challenge and *ex vivo* in alveolar macrophages cultures following stimulation, the mediator expression profiles are quite similar. Mechanistically, the data from chapter 2 further indicate that alterations are not only observed at the level of protein, but also on the level of the message, associated with alterations to transcription factors. With all of this in mind, the central question for this thesis is what are the underlying molecular mechanisms leading to this altered cellular responsiveness? Or more specifically, what are the underlying molecular mechanisms that

lead to the induction of atypical inflammatory mediators following bacterial challenge/stimulation and the attenuation of typical inflammatory mediators?

The key point in addressing this question is that as a result of the cigarette smoke-exposure the baseline state of the lungs is altered, and therefore the bacterial challenge is occurring at a time on ongoing or 'chronic' inflammation. Due to the pronounced physiological changes induced by inflammatory responses, to avoid tissue damage there are several regulatory mechanisms in place to limit the development of excessive, chronic, or recurrent inflammation (141-148). For example, within the TLR signaling pathway there are multiple mechanisms that act to regulate steps in this pathway, including the functions of the molecules IRAK-M (142), SOCS1 (145), MyD88s (147), SIGIRR (143), ST2 (144), A20 (141), SHIP (146), as well as receptor internalization following stimulation (148). The net effect of these molecules is attenuated nuclear translocation of transcription factors and as a result, attenuated expression of pro-inflammatory cytokines.

Repeated exposure to non-lethal doses of LPS leads to the expression of these regulatory molecules, and furthermore leads to animals and cells becoming resistant to challenge with a lethal dose of LPS, a phenomenon termed LPS tolerance (149). In addition to LPS, repeated exposure to other molecules that stimulate TLR pathways, such as bacterial lipopeptides and lipoteichoic acids, lead to a similar hypo-responsive state, although it is still referred to as LPS tolerance (150). Of particular relevance, macrophages have been demonstrated to be directly responsible for the induction and maintenance of LPS tolerance (151). Mechanistically, following initial treatment with LPS, macrophages become unresponsive to subsequent stimulation with LPS, as indicated by decreased NFκB activation

and nuclear translocation and expression of pro-inflammatory mediators such as TNFα, IL-6, and IL-1β.

The data presented here are consistent with the described phenomenon of LPS tolerance. When compared to control mice, in cigarette smoke-exposed mice we observed decreased expression of TNFα and IL-6 following bacterial challenge with NTHI, which is known to stimulate cells in a TLR -2 and -4 dependent manner (140, 152). Decreased TNFα and IL-6 protein, and mRNA, were observed following LPS stimulation of alveolar macrophages isolated from cigarette smoke-exposed mice, and this was associated with attenuated NFκB nuclear translocation. Chen *et al.* reported similar results in a study investigating stimulation of cultures of alveolar macrophages isolated from human smokers with TLR agonists (108). This study went further and demonstrated attenuated activation of IRAK-1, a key signaling molecule in the TLR signaling pathway (153). While expression of regulatory molecules has not been addressed in any study to date, given that cigarette smoke-exposure is known to stimulate cells via a TLR dependent pathway (50), together these data are indicative of cigarette smoke inducing a state of LPS tolerance, or a state similar to LPS tolerance, in resident cells of the lungs.

These data beg the question, however, that if macrophages are in a 'tolerant' state what leads to the production of the different subset of molecules that we observed to be increased? A similar question was asked by Foster *et al.* in trying to understand the basis of LPS tolerance (154). The authors surmised that since TLR stimulation leads to the up-regulation of a large number of gene products with different functions, including pro-inflammatory cytokines and chemokines, antimicrobial proteins, tissue repair factors, metabolic regulators, and controllers of adaptive immunity, that in a state of LPS tolerance

not all TLR-induced gene products would have attenuated expression following stimulation to protect the host from a lethal dose of LPS. Because of this, they hypothesized that since these different genes are induced by the same receptor, TLR-4, in the same cell type, the macrophage, the regulation of gene expression following stimulation must be partially gene-specific.(154)

To test this hypothesis they utilized a model of LPS tolerance in bone marrow-derived macrophages and identified two categories of TLR-induced genes: *Tolerizeable genes,* gene products which are not inducible in tolerant macrophages following LPS stimulation, and *Non-tolerizeable genes*, gene products which are inducible in tolerant macrophages following LPS stimulation (154). Tolerizable genes include pro-inflammatory mediators, such as TNFα, which do not directly contribute to bacterial clearance but have a high potential to cause lethality, whereas nontolerizeable genes include antimicrobial effectors, pathogen recognition receptors, and most relevant to this discussion, certain chemokines, that may directly contribute to bacterial clearance but have a low potential to cause lethality. Therefore, in their model of LPS tolerance in bone marrow-derived macrophages, regulation of gene expression following stimulation is partially gene-specific. As a further *in vivo* line of evidence for this, recently Natarajan *et al.* reported the observation that while induction of acute pulmonary LPS tolerance resulted in attenuated level of TNFα, expression of chemokines and recruitment of neutrophils was not impaired (155).

Gene specific regulation of gene expression is partially regulated by modifications to chromatin structure within a cell. Chromatin modifying enzymes include histone modifying enzymes that work by placing covalent marks on histone tails to modify the chromatin structure (156). Acetylation of histones is correlated with activation of transcription and is

therefore a positive mark associated with open, active chromatin (157). Histone deacetylation, on the other hand, is correlated with transcriptional repression, and is therefore associated with condensed, silent chromatin (158). Given that we observed attenuated TNFα and IL-6 following challenge, and the evidence that suppression of TNFα in LPS-tolerant cells is due to chromatin modifications at the promoters of these genes (159, 160), this may be a mechanism for the altered microbial responsiveness that we observe.

The natural extension from this line of reasoning, is asking whether there is evidence for histone/chromatin changes in COPD. Recent research by Ito *et al.* demonstrated histone modifications in both lung tissue biopsies and alveolar macrophages of patients with COPD (161). Specifically, they noted decreased expression of histone deacetylase -2, -5, and -8 mRNA, as well as decreased histone deacetylase -2 protein. Associated with this reduction was increased expression of CXCL-8 and increased acetylation of histones associated with the CXCL-8 promoter. Alterations to other promoters, such as that for CCL-2, -7, and -12, were not addressed in this study, but given our results would be of significant interest to understanding the molecular events that underlying COPD exacerbations. Additionally, in order to address whether histone acetylase activity is a mechanism contributing to decreased pro-inflammatory expression of such mediators as TNFα and IL-6, changes to the histones associated with the promoters for these genes would need to be determined.

While there is evidence for chromatin/histone modifications in COPD (161), and histone modifications are associated with LPS tolerance (159, 160), the data does not perfectly match with observations from our laboratory or Chen's (108, 124). For example, our studies and Chen's report attenuated expression of CCL-5 following stimulation, whereas Foster *et al.* report CCL-5 to be a nontolerizable gene (154). There are a number of potential

explanations for this, however the most likely explanation is due to the cell type utilized. The study by Foster *et al.* utilized bone marrow derived macrophages, whereas the latter two studies utilized isolated alveolar macrophages, which have a basal restraint on inflammatory pathways compared to other macrophage populations (162), and this may be reflected in the expression of CCL-5.

Regardless, there is strong evidence for LPS tolerance and histone modifications in COPD, and these studies represent a very exciting avenue for future work investigating inflammatory pathways in COPD to pursue. Understanding the consequences of cigarette smoke-induced LPS tolerance on genes that are induced or suppressed during bacterial infection, for example, may lead to the identification of novel immune targets for treatment of periods of COPD exacerbation. Furthermore, understanding of the histone alterations in COPD may lead to treatment with molecules that reverse those alterations, which may similarly lead to new therapeutic approaches. At the very least, pursuing these avenues will shed light into the pathogenesis of COPD.

Summary & future directions

In experimental research it is imperative to carefully interpret how the model, and data gained from investigation with that model, relate to aspects of human disease. With this in mind, given the pathology reported for COPD and the pathology observed in this model of cigarette smoke-exposure, this study relates considerably more closely to events that occur early in the development and progression of COPD, and to a much lesser extent to severe disease.

These data indicate that inflammation and lung damage following bacterial challenge are exacerbated *in vivo*, and the profile of inflammatory mediators is altered both *in vivo* following bacterial challenge and *ex vivo* following bacterial stimulation, in cigarette smoke-exposed mice compared to control mice. It is important to note that these observations were made following a single bacterial challenge *in vivo* or a single bacterial stimulation *ex vivo*. Given the considerable length of time over which the disease develops in COPD patients, and the numerous microbial infections and symptom exacerbations, repeated infection may be central to driving disease development and/or progression.

If this hypothesis is correct, over time repeated microbial challenges should accelerate the development of the pathological changes in the lungs. To address this, a very interesting and relevant study would be to investigate the effect of repeated bacterial challenge in cigarette smoke-exposed mice, with either heterologous strains of bacteria, such as different strains of NTHI, or heterologous species of bacteria, such as NTHI and *Streptococcus pneumonia*. This would be of particular interest in investigating the development of airspace enlargement and bacterial colonization in COPD, as first, cigarette smoke-exposure in mice generally results in mild to moderate airspace enlargement (33), and repeated challenge may accelerate this process; Secondly, studies have indicated that damage to the lung architecture may influence bacterial clearance (163, 164), and as such bacterial colonization may be a consequence of lung damage.

A natural progression from the work completed for this thesis would be to investigate histone/chromatin modifications. Specifically, the effects of cigarette smoke-exposure on histone acetylase and histone deacetylase expression in lung tissues of cigarette smoke-exposed mice, as well as in alveolar macrophages isolated from exposed mice,

particularly around the promoters of genes we observe to be have altered expression. Moreover, a screening profile could be utilized to investigate genetic changes as a result of cigarette smoke-exposure, bacterial challenge, or both. In particular, 'tolerizeable' or 'non-tolerizeable' genes could be identified, in line with the report from Foster *et al.* (154).

This model of cigarette smoke-exposure and bacterial challenge provides a platform for further examination of the impact of cigarette smoke-exposure on inflammatory responses in the lung. Potential applications include to first, increase our understanding the role of bacterial exacerbations in the pathogenesis of COPD, and second, to further develop and characterize these experimental models of exposure and bacterial challenge for the progression and development of new models of human disease.

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Appendix I:

ANIMAL MODELS OF COPD EXACERBATION

The following article appeared as a book chapter in *Contributions to Microbiology.* 2007;14:126-41(42)

Experimental models are important tools for elucidating mechanisms of disease pathogenesis, for identifying novel therapeutic targets, and for preclinical screening of intervention strategies. Various approaches have been used to model the histo-pathological features of COPD. These have included exposing animals to cigarette smoke or inflammatory stimuli, instilling proteolytic enzymes into the airways, and the study of spontaneous gene mutants and knockout strains. Typically, these studies have addressed single aspects of COPD, such as inflammation, emphysema, or mucous production, but fail to recapitulate the entirety of the COPD syndrome.

Our lab and others have hypothesized that periods of exacerbation of COPD, episodes of increased symptom severity and inflammation, are important for COPD pathogenesis. In the following article we discuss methods of experimentally modeling exacerbations of COPD, with particular attention to how to incorporate the causative agents of exacerbation – bacteria and viruses, alone or together, into experimental models of COPD.

Dr. Martin Stampfli, Carla Bauer, Caleb Zavitz, and myself were responsible for conceptualization, design, and writing of the manuscript.

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Animal Models of Chronic Obstructive Pulmonary Disease Exacerbations

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Abstract

Modeling acute exacerbations of chronic obstructive pulmonary disease (AECOPD) in animals has proven challenging due to the clinical and pathological complexity of the underlying disease. This has hindered the progress in understanding the cellular and molecular mechanisms that lie beneath AECOPD. In this chapter, we will address modeling possibilities of AECOPD that may be drawn from the current knowledge of factors that cause exacerbations. Importantly, since it is widely accepted that the most common causes of AECOPD are viral and bacterial infections, animal models of AECOPD should incorporate both the causative agents of exacerbation: viruses and bacteria. However, other factors that are also believed to determine both progression of COPD, as well as the frequency and severity of AECOPD, such as proteolytic enzymes, cigarette smoke or other noxious stimuli must also be considered. Such animal models will provide mechanistic insight into the etiology of AECOPD and will prove invaluable in furthering our understanding of key events in disease pathogenesis.

Introduction

Animal models are important tools for elucidating mechanisms of disease pathogenesis, for identifying novel therapeutic targets, and for preclinical screening of intervention strategies. Modeling acute exacerbations of chronic obstructive pulmonary disease (AECOPD) in animals has proven challenging due to the clinical and pathological complexity of the underlying disease. This has hindered the progress in understanding the cellular and molecular mechanisms underlying AECOPD. In this chapter, we will address modeling possibilities

that may be drawn from the current knowledge of factors that cause exacerbations, and determinants of exacerbation frequency and severity.

COPD Exacerbation

Clinically, AECOPD is viewed as a sustained worsening of symptoms from the patient's stable COPD state, beyond normal day-to-day variations, which necessitate a change in medication [1]. Symptoms may include increased dyspnea, wheezing, chest tightness, cough, and changes in the level, color and/or tenacity of sputum [2]. Unfortunately, this clinical definition of AECOPD is of limited guidance for the development of animal models.

COPD is not a single disease but rather a syndrome whose functional and clinical hallmark is progressive and largely irreversible airflow limitation [2]. In an individual patient, varying degrees of chronic bronchitis, bronchiolitis, and emphysema contribute to this chronic airflow limitation [3–5]. Various approaches have been used to model the histopathological features of COPD. These have included exposing animals to cigarette smoke or inflammatory stimuli, instilling proteolytic enzymes into the airways, and the study of spontaneous gene mutants and knockout strains [reviewed in 6–10]. Typically, these studies have addressed single aspects of COPD, such as inflammation, emphysema, or mucus production, but have failed to recapitulate the entirety of the COPD syndrome. Because we lack a comprehensive model of COPD, it is impossible to model AECOPD as a worsening of COPD. As a solution to this inherent problem, we have to consider the individual factors that are associated with COPD and AECOPD, and subsequently incorporate them either independently or in combination into animal models.

It is widely accepted that the most common causes of AECOPD are viral and bacterial infections [11–14]. Hence, animal models of AECOPD should incorporate the causative agents of exacerbation, namely viruses and bacteria. In addition other factors that may both be determinants for the progression of stable disease, and in some cases probably also of AECOPD, such as noxious stimuli, including cigarette smoke and susceptibility factors, such as genetic perturbation of the protease-antiprotease balance, need also to be included.

Determinants of Exacerbation Frequency and Severity

COPD is found almost exclusively in smokers; over 80% of all cases are attributable to active smoking, and an additional 10% are believed to be due to exposure to environmental tobacco smoke [15]. Notably, patients with COPD,

especially in the early stages of disease, typically continue to smoke. These patients often suffer from greater sputum production and increased episodes of coughing than do patients who cease smoking [16], which is understood to be associated with more frequent exacerbations. In view of this, cigarette smoking is an important component of AECOPD models, not only as an etiologic factor for COPD itself, but also as a determinant of exacerbation frequency and severity. Addressing the effect of cigarette smoke exposure on exacerbation-causing bacterial and viral infections in animals may provide insight into the mechanisms underlying AECOPD and may lead to the development of more complete models.

In addition to the association between smoking and increased risk of viral and bacterial infection, clinical evidence shows a relationship between lower airway bacterial colonization and exacerbation frequency [17]. Why microbial agents colonize the lower respiratory tract, normally a sterile compartment, is unclear and will be discussed in more detail later in this chapter. Similarly, bacterial colonization may impact the susceptibility to secondary viral and bacterial infections, or alter the ensuing immune-inflammatory responses, through mechanisms that are still poorly understood.

The severity of the stable COPD state is an important factor of exacerbation frequency. It has been postulated that changes within the diseased lung such as increased mucus production, thickening of the epithelium, or changes in epithelial cell integrity, and parenchymal damage, all of which become more marked with disease progression, may predispose COPD patients to microbial infection [18]. Similarly, the lung function in patients suffering from COPD may have deteriorated to an extent that relatively minor changes in lung function brought on by microbial infections may be clinically manifested as exacerbations. Complicating these issues is the fact that relatively little is known about how viral and bacterial infections affect lung function. Thus, understanding the underlying pathology of viral and bacterial infections and their consequences to lung function both in healthy and diseased lungs becomes increasingly important to the study of mechanisms leading to AECOPD.

Modeling Bacterial Exacerbations

Bacterial infections have been estimated to cause half of all AECOPD [12]. The most commonly isolated bacteria during episodes of AECOPD are nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, while in patients with more severe disease progression, invasive bacteria such as *Pseudomonas aeruginosa* or *Chlamydia pneumoniae* may be isolated [12]. As previously mentioned, even during stable disease bacteria are

frequently detected in the lungs of COPD patients [19–21]. This has made the precise role of bacterial infections in AECOPD controversial, as bacteria isolated during episodes of AECOPD may either be from infection or colonization. However, recent studies have elegantly shown that AECOPD in colonized individuals is often associated with the acquisition of a new strain of bacteria [22], which is also linked to the appearance of antibodies specifically directed against the new bacterial strain [23]. Animal models of bacterial infection or colonization in AECOPD should therefore include not only drivers of COPD such as cigarette smoke exposure and persistent bacterial colonization, but also strains of bacteria that are capable of causing exacerbation.

Cigarette Smoke and Bacterial Agents

Cigarette smoke has been demonstrated to compromise various aspects of the immune system. For example, cigarette smoke can cause impaired mucociliary clearance [24], decreased levels of both the phospholipid fraction and surfactant-associated proteins A and D of the surfactant system [25, 26], impaired alveolar macrophage phagocytosis, killing and cytokine induction [27–36], and disruption of the epithelial cell barrier [37, 38]. All of these consequences of exposure to smoke may contribute to an impaired antibacterial immune response in smokers, and by extension, to bacterial exacerbation of COPD.

Numerous studies have examined the effect of cigarette smoke exposure on responses to bacterial antigens. More specifically, mediators of the adaptive immune response such as the ex vivo production of Th1-type cytokines have been shown to be attenuated by smoke [34, 35]. In contrast the inflammatory response to an innate stimuli such as lipopolysaccharide may be heightened in vivo [39]. These studies serve to identify some of the pathways affected by cigarette smoke, elucidate mechanisms contributing to chronic inflammation, and provide a basic scientific framework to develop and test therapeutic targets. The limitation of these experimental approaches, however, is that they ignore the intricacy associated with infection by live, replication-competent bacteria. During bacterial infections numerous bacterial antigens are present, including constituents of bacterial cell walls such as peptidoglycan and lipopolysaccharide, as well as lipoproteins, lipoteichoic acids, flagella, and toxins. These antigens stimulate pattern recognition receptors of resident innate cells, including Toll-like and Nod-like receptors, resulting in the production of proinflammatory cytokines and chemokines. In turn, neutrophils and monocytes/ macrophages are recruited from the circulation and activated, resulting in local inflammation. Accordingly, the immunosuppressive effects of cigarette smoke may in fact propagate inflammatory processes by delaying bacterial clearance, driving tissue pathology and progressive airway obstruction.

As early as the 1960s, in vivo studies demonstrated that bacterial clearance was delayed from the airways of cigarette smoke-exposed animals [40–43]. This is indicative of cigarette smoke predisposing to bacterial infection or colonization. Work previously done in our lab with *P. aeruginosa* has shown that mice exposed to cigarette smoke demonstrate a delayed rate of bacterial clearance as compared to sham-exposed mice [44]. This study also demonstrated that delayed clearance was associated with a skewing of the innate inflammatory response: the increased bacterial burden was associated with *increased* airway and tissue inflammation *despite* evidence of suppressed alveolar macrophage function. Furthermore, we observed increased levels of proinflammatory cytokines and chemokines, myeloperoxidase, proteases, and perhaps most importantly, these changes were associated with a significant deterioration of the animals' health status. In this model, exposure to cigarette smoke alone did not result in changes in the health status of mice or cause overt lung inflammation, indicating that the observed differences were due to the combined effects of bacterial infection and cigarette smoke exposure. Furthermore, the exacerbated inflammatory response was only observed when mice were inoculated with live bacteria; similar responses were observed between sham- and smoke-exposed mice following inoculation of inactivated bacteria. While this model lacks many of the clinical hallmarks of COPD, including chronic inflammation and airspace enlargement, it does demonstrate the complexity of the effects of cigarette smoke on innate antibacterial responses and the importance of using replicating infectious agents to model AECOPD.

Proteases and Bacterial Agents

Proteolytic enzymes, such as macrophage or neutrophil elastase, are important components of the antibacterial defense and are induced during immune responses. Proteolysis is further involved in cell recruitment and repair during an active infection or during the resolution of an infection [45]. Although proteolytic enzymes are essential for normal lung biology and defense, dysregulated expression may lead to tissue remodeling/destruction and disease [46]. This is of particular interest in conditions such as emphysema and COPD, where an imbalance between proteases and antiproteases has been implicated as an important factor leading to disease [47].

The most commonly used experimental model for the study of emphysema is achieved by instilment of elastase into the airways [48]. In this model, airspace enlargement has been shown to be dependent both on the enzyme's proteolytic activity and the inflammation that ensues as a result of the chemoattractive properties of degraded elastin fragments [49, 50]. There are few studies investigating the effect of elastase-induced emphysema on the antibacterial immune response as a model for AECOPD. However, recently, Inoue et al. [51] demonstrated that

while control mice survived, mice instilled with elastase before inoculation with *S. pneumoniae* died in a dose-dependent manner, likely as a result both of excessive pulmonary and of systemic inflammation. These results indicate that under experimental conditions resembling emphysema, acute bacterial defense is impaired, resulting in excessive inflammation. Given that one of the clinical hallmarks of COPD is airspace enlargement, especially in late stage disease, this may contribute to the observed increases in bacterial infections and inflammation, as well as to the development of persistent bacterial colonization.

Inflammation has been suggested to be one of the driving forces underlying airspace enlargement. The combination of impaired antibacterial immune responses and a dysregulation of the protease/antiprotease balance may further contribute to tissue damage and disease progression. Along these lines, we observed greater proteolytic activity in the lungs of cigarette smoke- compared to sham-exposed mice following inoculation with *P. aeruginosa* [44]. The increased proteolytic burden was likely a reflection of the increased inflammation observed in these mice, as it was not observed in mice exposed to cigarette smoke only. Collectively, these findings indicate that cigarette smoke and bacterial infection have synergistic effects on airway damage, and that the damage may be mediated through effects on proteolytic activity. Such findings further underscore the importance of understanding the concurrent effect of cigarette smoke, protease regulation, and infection on mechanisms leading to AECOPD.

Bacterial Colonization

Bacterial colonization is believed to contribute to the pathogenesis of COPD by inducing a chronic inflammatory state thereby driving a progressive airway obstruction. Moreover, it has been shown to attenuate subsequent immune responses, which might perpetuate colonization or lead to a permanent inability to clear the pathogen [35]. Although an important hallmark of disease, bacterial colonization has been an understudied aspect of COPD in animal models, primarily because it is difficult to select relevant microbial species; pathogens which will colonize experimental animals often lack clinical applicability, while the clinically relevant pathogens do not often colonize animals.

When used to inoculate mice, pathogens and serotypes associated with AECOPD including nontypeable *H. influenzae* [52], *M. catarrhalis* [53], and *P. aeruginosa* [44] are cleared within a few days at most, and the elimination does not require the induction of major adaptive immune responses for clearance. *S. pneumoniae* has been shown to persist in some models [54], but not in others [55].

Several strategies have been pursued to prolong the persistence of these bacteria in the airways, including coupling them to agarose beads or another

suitable medium [56], and the implantation of bacterium-coated tubing into the airways [57]. Although these methods prolong the pathogen's persistence in the airways, they do not obviate the fact that the bacterium is not actually colonizing the host. Alternatively, some murine respiratory pathogens, or human pathogens that have been adapted to murine systems, have been employed in models, which may shed light on AECOPD. Bacteria including *Mycoplasma pulmonis* [58], *Neisseria meningitidis* [59], *Bordetella parapertussis* and *B. bronchiseptica* [60, 61], and some isolates of *S. pneumoniae* [54] are all capable of colonizing the murine airway for significant periods of time under experimental conditions, and may therefore become useful as models for studying the impact of cigarette smoke on colonization and the consequences of colonization to respiratory host defense.

Alternatively, bacteria which would otherwise be noncolonizing can become colonizing agents in immunocompromised hosts [62]. The use of transgenic animals or immunodepleted hosts is an intriguing avenue for AECOPD research, as COPD patients may, in some respects, be viewed as immunocompromised. However, although useful for studying aspects of disease, this approach requires a detailed understanding not only of all the effects of a given gene deletion, but also of the immune status of COPD patients in order to correctly interpret data generated.

As previously mentioned, the bacteria most frequently associated with AECOPD is *H. influenzae*, an exclusively human bacterium found commensally in a large proportion of the population [12]. While modeling and comparing different strains of *H. influenzae* in animals are challenging because of the lack of infectivity in species other than humans [52], Chin et al. [53] recently tested the hypothesis that strains of *H. influenzae* associated with AECOPD induce more inflammation in mice than strains associated with asymptomatic colonization. Strains of *H. influenzae* associated with AECOPD or with colonization were inoculated into C57BL/6 mice in an in vivo model of airway infection. In this model, AECOPD-associated strains resulted in increased airway neutrophil recruitment that based on further in vitro experiments was likely mediated via increased induction of IL-8 and activation of the NF-κB and MAPK signaling pathways. These results indicate that strains of *H. influenzae* isolated during episodes of AECOPD may have different virulence compared to those isolated during stable disease, leading to augmented inflammation upon infection. Although this study did not address the consequences of cigarette smoke on inflammatory processes following inoculation with these bacterial strains, we speculate that cigarette smoke exposure would further exacerbate the inflammatory response.

While the antigens and virulence factors that lead to these differences have not been identified, studies such as these provide insight into mechanisms of

airway inflammation observed during AECOPD. Moreover, they point to the importance of modeling different bacteria and environmental stimuli to understand the cellular and molecular mechanisms underlying both progression of stable COPD and exacerbations of this disease.

Modeling Viral Exacerbations

Several current studies have shown that as many as 40–60% of AECOPD are associated with respiratory virus infections [14, 63, 64]. In one study, a respiratory virus was detected in 56% of patients with COPD admitted to a hospital in Germany [65]. The major viruses that cause upper and lower respiratory tract infections and that are implicated in AECOPD include rhinovirus, respiratory syncytial virus, coronavirus, adenovirus, influenza A and B, and parainfluenza [14, 63, 64]. Among these, rhinovirus, the cause of common cold, is currently considered to be the most important trigger of COPD exacerbations [66]. As many as half of all colds during the peak fall cold season are estimated to be a result of rhinovirus infection [67]. Unfortunately, developing animal models to study rhinovirus infection has been met with some difficulties; rhinovirus is highly specific and does not recognize ICAM-1 from species other than humans [68]. Since this virus has significant implications in AECOPD, further work is warranted to develop animal models for the study of this virus.

Similar to the antibacterial host defense, cigarette smoke has been shown to compromise antiviral host defense mechanisms and increase the risk of respiratory viral infection. The incidence of influenza virus infection is increased in a smoking compared to a nonsmoking and nonimmunized population [69]. In addition, in vitro experiments from the 1980s suggested that the innate immune response, specifically that mediated by type I interferon is impaired upon exposure to cigarette smoke [70–72]. Taken together, these studies suggest that cigarette smoke affects innate antiviral immune defense mechanisms required to combat viral pathogens; hence, incorporating cigarette smoke into models of viral infections is essential for the understanding of virally induced AECOPD.

Adenoviruses have also been detected in AECOPD. DNA coding for the adenoviral E1A protein has been found in excess amounts in the lungs of patients with COPD when compared to controls matched for age, sex, and smoking history [73]. Adenoviral DNA has been shown to persist after acute infection, and viral E1A protein is expressed in lung epithelial cells long after the virus has stopped replicating [73, 74]. Retamales et al. [75] have shown that the excess inflammation observed in the lungs of smokers with severe emphysema is associated with increased numbers of alveolar epithelial cells expressing E1A. Importantly, Meshi et al. [76] showed a marked amplification of the

cigarette smoke-induced inflammatory response and an increase in emphysematous lesion formation in guinea pigs with latent adenoviral infection when compared to noninfected controls. In mice, we have shown that cigarette smoke exposure was associated with attenuated T cell activation and decreased production of neutralizing antibodies following administration of a replication-deficient adenoviral construct [77]. This study may suggest that tobacco smoke-induced impairment of adaptive antiviral immunity contributes to the persistence of E1A protein in patients with COPD, although additional studies are required to provide direct evidence.

Recently, Behzad et al. [78] have determined the role of adenovirus infection in inducing epithelial-mesenchymal transformation, an important feature of tissue remodeling. Remodeling processes contribute to (or cause) the obstruction of the small airways. This process is associated with a gradual decline in forced expiratory volume in 1 s, an important feature of COPD that is also linked to the progressive nature of the disease [3]. The authors demonstrated that adenovirus E1A is the key in epithelial-mesenchymal transformation of primary lung epithelial cells derived from guinea pigs [78]. Taken together, these data suggest a role for adenoviruses in COPD pathogenesis and illustrate the need for further research of these models, specifically examining the underlying molecular implications cigarette smoke may have on virus-mediated AECOPD.

Influenza A and B viruses have also been shown to be important respiratory viral infections leading to AECOPD. In one study of a nonimmunized population between the ages of 60 and 90, rates of influenza infection in smokers were approximately 23% as compared to 6% in nonsmokers [69]. The relative contribution of this virus to AECOPD is thought to be less serious in regions that now offer influenza immunizations to patients with lung disease; however, it is still an important consideration as exacerbations may occur at times of influenza epidemics. In addition, recent data suggest that the level of protection by current influenza vaccines may be lower than what has been believed [79, 80]. Understanding the nature of viral infections in a healthy compared to cigarette smoke-exposed lung becomes important to understanding the role of viruses in AECOPD. Since cigarette smoking has been implicated in the changes observed in the COPD lung (as discussed above), superimposing a viral infection over the background of cigarette smoke adds a new element to animal modeling.

In a murine model of influenza virus infection, we have recently demonstrated that while viral clearance in sham- and smoke-exposed animals was similar, smoke exposure worsened inflammatory outcome and health status of animals [81]. The heightened inflammatory response observed in smoke-exposed animals was associated with increased expression of TNF-α, IL-6 and

type I interferons in the airway, as well as with increased mortality [81]. In addition, we have shown that smoke exposure does not compromise the development of an influenza-specific memory response [81]. We postulate that the exaggerated airway inflammatory response to viral agents contributes to the inflammation observed in smokers and to the deterioration in clinical status associated with AECOPD.

Modeling Coinfection

Current disease models generally study interactions between a single pathogen and the infected/colonized host. While it is necessary to understand the basic science of disease, this 'single-agent:single-host' paradigm contrasts starkly with the situation in the clinic, where patients frequently present with multiple simultaneous infections, and where at the very least they present with a *history* of other infections. Moreover, as molecular biological techniques such as PCR have succeeded traditional clinical virological methods, it has become increasingly apparent that many COPD exacerbations are typically viral in nature [82]. Concomitantly, it has been suggested that viral infections may predispose a patient to bacterial infections, leading to exacerbations driven not by virus or bacteria alone, but by a bacterial-viral coinfection. Both from human studies and from experiments in animal models, it has become clear that the hosts' response to a pathogen is at least partially dictated by their immune system's history. In this regard, experimental models utilizing animal models and a single infection may not be predictive of an immunologically experienced COPD patient's response.

There is debate about the importance of heterologous viral-bacterial infections in the context of COPD; some studies have found correlations between these agents, while others have found no such relationships in patients with chronic bronchitis [83, 84]. However, these studies were performed without the aid of modern PCR methods, and therefore likely underestimated the true prevalence of viral-bacterial coinfections. More recently, a number of papers using PCR techniques have indicated that bacterial-viral coinfections are a major issue in clinical COPD management. Work by Papi et al. [85] found that approximately one quarter of all COPD exacerbations requiring hospitalization were of a mixed bacterial and viral etiology. Similarly, work by Cameron et al. [86] found that nearly one quarter of infectious exacerbations requiring ventilation were associated with simultaneous bacterial and viral infections. Wilkinson et al. [87] reported that exacerbations of putatively coinfectious etiology are marked by increased airflow limitation and symptomatology when compared to single-agent exacerbations. In addition to the epidemiological evidence, there is

also ample experimental evidence to support the notion that infection with one agent predisposes to infection with another. Experimental influenza infection in humans increases the likelihood of natural colonization with *S. pneumoniae* [88]. Similarly, mice are more susceptible to *S. pneumoniae* following influenza A infection, and mount exacerbated inflammatory responses to the bacterium [89, 90].

Although the observation that two pathogens are more harmful to a host than one may not be surprising, investigating the mechanisms underlying this phenomenon is not trivial. In fact a number of mechanisms by which infection with one pathogen can modify the immune system's response to another pathogen have been proposed. Viral infection can effectively alter every stage of a bacterium's interaction with a host, by promoting bacterial attachment to epithelial cells [91, 92], impairing innate cell function [93, 94], and modulating the adaptive immune response [95]. Similarly, bacterial infections may alter preexisting antiviral immunity [96], and predispose individuals to subsequent viral infection [97].

Given that colonization of the airway is one of the defining characteristics of stable COPD, and that bacterial and viral exacerbations are a hallmark of acute exacerbation, modeling viral and bacterial infection over the background of colonization is likely to yield novel and exciting insight into AECOPD. Although complicated, such a model has recently been described by Seki et al. [98], in which *P. aeruginosa* colonization was achieved by implantation of bacterium-coated tubing into the airway, and animals were subsequently challenged with *S. pneumoniae* and influenza A virus. In this study, animals exposed to all three pathogens showed exacerbated cytokine responses, poor viral clearance, and reduced myeloperoxidase activity and lysozyme secretion compared to animals receiving only one or two of the pathogens. This and other developing models of colonization and coinfection must now be studied in detail, then overlaid on a background of other factors involved in the pathogenesis of COPD, such as cigarette smoke exposure, to realize their potential as models of AECOPD.

The current understanding is that infections are of critical importance to the pathogenesis of COPD. Given that a COPD patient's immune system is far from immunologically naïve, the need for animal models that examine heterologous infections in the context of COPD is apparent.

Conclusions

To date, there are no complete models of AECOPD. Instead, models of individual disease facets must be interpreted not only in the context of the

specific aspect of human disease they mimic, but also in light of the effects other aspects may play. Ultimately, the most appropriate approach to modeling AECOPD is an inclusive one, in which we model various aspects of COPD, including cigarette smoke exposure, protease regulation, bacterial colonization, and mucus production, and then superimpose a relevant exacerbating stimulus. As such, we believe animal models will provide detailed mechanistic insight into the etiology of AECOPD and will prove invaluable in furthering our understanding of key events in disease pathogenesis.

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Appendix II:

BACTERIA CHALLENGE IN SMOKE EXPOSED MICE EXACERBATES INFLAMMATION AND SKEWS THE INFLAMMATORY PROFILE

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Online Data Supplement

Bacterial Challenge in Smoke Exposed Mice Exacerbates Inflammation and Skews the Inflammatory Profile

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Methods

Cigarette smoke exposure

Mice were exposed for 50 minutes to the smoke generated from twelve 2R4F reference cigarettes with the filters removed, twice daily, 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.

Immediately, or 24 hours, following cigarette smoke exposure, 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). Levels of COHb were reported in reference (E1). 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. Levels of cotinine were measured to be 18.5±3.3 ng/ml in control BALB/c mice, 16.4±1.4 ng/ml in control C57BL/6 mice, 503.7±55.6 ng/ml in cigarette smoke exposed BALB/c mice, and 376.9±60.1 ng/ml in cigarette smoke exposed C57BL/6 mice.

Preparation of, and innoculation of mice with, NTHI

The strain of bacteria used was nontypeable *Haemophilus influenzae* strain 11P6 (E2, E3). This is a clinical strain of NTHI isolated from sputum of a patient with COPD experiencing an acute exacerbation. Demonstration of specific immune response and an inflammatory response to this

strain establish it as causative of exacerbation (E3, E4) 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 hemin and 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+Hemin+NAD broth, and the culture was incubated with rotary shaking at 37°C until an OD_{600} value of 0.7-0.8 units was obtained. Bacteria quantification was verified by plating serial dilutions of broth cultures onto chocolate agar plates. For intranasal delivery, the broth was washed three times with phosphate buffered saline (PBS). Isoflurane-anesthetized mice were inoculated intranasally with 10^6 cfu of NTHI in a total volume of 35 μL of PBS. Animals were inoculated at least 4 hours after the last smoke-exposure.

Bronch-Alveolar Lavage

At the time of killing, lungs were removed and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). Lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of BAL fluid was consistently recovered and total cell counts were determined using a haemocytometer. For differential cell counting, BAL fluid was centrifuged, supernatants collected and stored at -20, cell pellets resuspended in PBS, and cytospins prepared from the resuspended cells by cyto-centrifugation at 300 rpm for 2 min (Shandon Inc., Pittsburgh, PA). Cytopins were stained with HEMA-3 (Biochemical Sciences Inc., Swedesboro, New Jersey, USA) and differential counts were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells (lymphocytes, or macrophages/monocytes).

Histological Assessment

For histological assessment the left lung was inflated with 10% formalin at a constant pressure of 20 cm H_2O, 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 hematoxylin and eosin or picosirius red. Picro sirius red picutres were taken under polarized light. Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). Image analysis for measurment of Picro Sirius Red and epithelial thickness was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada)

Morphometric Measurement of Alveolar Macrophages

At the time of killing BAL was isolated. BAL fluid was centrifuged, cell pellets resuspended in PBS, and cytospins with a density of $1x10^5$ cells per slide were prepared from the resuspended cells by cyto-centrifugation at 300 rpm for 2 min (Shandon Inc., Pittsburgh, PA). Cytopin slides were stained with HEMA-3 (Biochemical Sciences Inc., Swedesboro, New Jersey, USA). Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). Pictures were taken at 400x magnification and image analysis was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada). Cellular area was determined by quantifying the total number of pixels than an object

occupies, and the pixel count was calibrated to real world dimensions by use of a graticule. Cellular shape was derived by use of the formula: shape factor = (4*Pi*Area)/(perimeter^2). The shape factor gives an indication of the objects shape, where circles have the greatest area to perimeter ratio and the value will approach 1 for a perfect circle. Neutrophils, based on standard haemocytological criteria, and groups of more than 1 cell were excluded from the analysis.

Measurent of Hydroxyproline

Hydroxyproline concentration in BAL fluid was measured by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). 100ul of BAL fluid was mixed with the internal standard solution (13C, 15N-labelled hydroxyproline) and concentrated hydrochloric acid and heated at 100 °C overnight. After evaporation of the acidic solution the residue was dissolved in the LC mobile phase and 10ul injected into the LC-MS/MS system. The peak area ratios of hydroxyproline over the internal standard were measured and quantification made from a standard curve in the range 1.0 - 40.0mmol/L.

Inflammatory Mediator Measurements by Bead array analysis

Samples from the BAL or AM cell supernatants were prepared and bead array analysis measured by Rules Based Medicine with the Rodent multi-Analyte Profile (Austin, Texas, USA). The least detectable dose (LDD) was determined as the mean + 3 standard deviations of 20 blank readings. Results below the LDD were analyzed as not detected (ND). Data from the RBM RodentMap analyses were visualized in a heat map using Spotfire* DecisionSite* 9.1 (Spotfire, Inc, Somerville,

MA, USA). Individual scales were applied to account for the large numerical differences between the mediators. Data are expressed as mean, or mean ± SEM as indicated in the Table legends. Statistical analysis was performed with SPSS statistical software version 16.2 (Chicago, IL, USA) with the general linear model with LSD post hoc test for multiple group comparison. Individual p values and fold change are shown in the Tables.

FIGURE E1

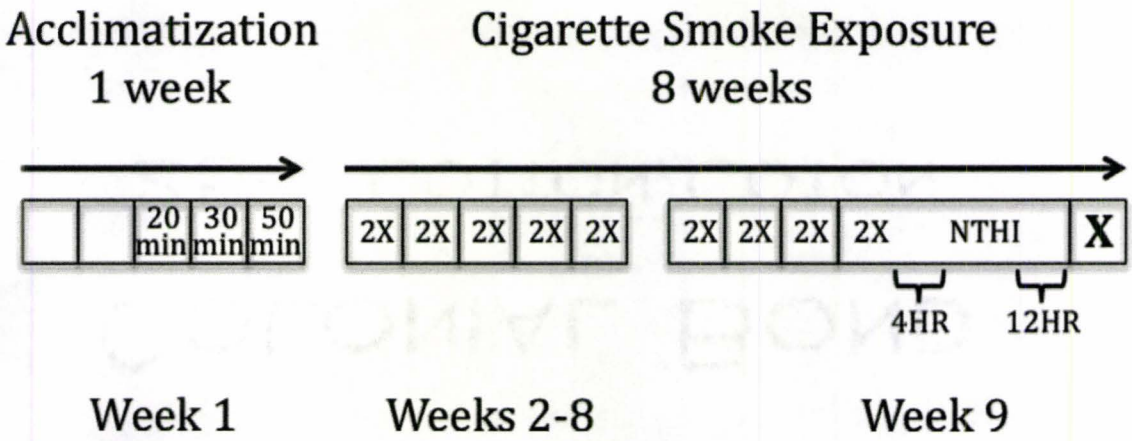

Figure E1: *General experimental time line.* In an initial 3-day acclimatization period (week 1), mice were placed in restrainers only for 20 minutes on day 1, 30 minutes on day 2, and 50 minutes on day 3. During the cigarette smoke exposure period (weeks 2-9) mice were exposed for 50 minutes, twice daily, 5 days per week, for a total of 8 weeks. Control animals were exposed to room air only. During the 8th week of cigarette smoke exposure (week 9) mice were allowed to rest for approximately 4 hours following the second cigarette smoke exposure and subsequently challenged intranasaly with PBS or 10^6 CFU NTHI. 12 hours post challenge mice were killed. Deviations of this general time line are specified in individual result sections and Figure Legends.

FIGURE E2

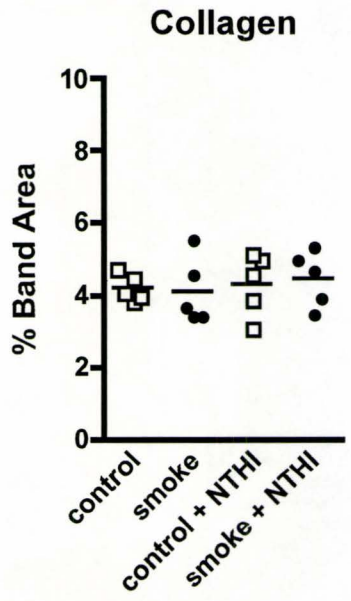

Figure E2: *Airway remodeling in cigarette smoke exposed NTHI challenged mice.* A) Representative light photomicrographs or paraffin-embedded cross-sections of lung tissue 12 hours post PBS or NTHI challenge. Panes show Picro Siruis red (PSR)- stained sections visualized under polarized light indicating subepithelial collagen deposition. B) Morphometric analysis of lung histology. Data represent the percentage of the area of interest that is stained with PSR. All images were taken at 200x magnification. Data are expressed as mean ± SD, n=5/group. Statistical analysis was completed by 2-way ANOVA.

Table E1: *Bead array analysis of BAL protein concentrations from cigarette smoke exposed mice challenged with NTHI.*

		control	smoke	ratio	p	control + NTHI	smoke + NTHi	ratio	p
MCP-5 (Monocyte Chemoattractant Protein-5)	pg/mL	ND	ND	NA	NA	10.2±5.9	84.5±18.7	8.3	<.01
MCP-1 (Monocyte Chemoattractant Protein-1)	pg/mL	4.8±0.3	56.0±9.0	11.7	0.22	58.7±14.5	446.3±51.1	7.6	<.01
MCP-3 (Monocyte Chemoattractant Protein-3)	pg/mL	10.0±0.5	49.8±6.6	5	0.44	62.9±8.3	385.7±68.7	6.1	<.01
IP-10 (Inducible Protein-10)	pg/mL	16.1±1.1	109.1±88.0	6.8	0.72	311.0±140.8	1330±319	4.3	<.01
MIP-1gamma (Macrophage Inflammatory Protein-1gamma)	ng/mL	0.1±0.0	3.5±0.9	34	0.07	5.6±0.6	22.7±2.0	4	<.01
Osteopontin	ng/mL	17.2±2.5	161.7±75.2	9.4	0.06	50.7±20.3	165.3±51.5	3.3	0.12
IgA (Immunoglobulin A)	ug/mL	0.1±0.0	0.9±0.5	6	0.22	0.8±0.3	2.5±0.6	3.1	0.02
MPO (Myeloperoxidase)	ng/mL	1.9±0.3	512±100.5	273	0.27	1068±202	3283±565	3.1	<.01
RANTES (Regulation Upon Activation, Normal T-Cell Expressed and Secreted)	pg/mL	ND	5.3±5.3	NA	0.68	20.2±12.4	59.8±10.8	3	0.01
Calbindin	pg/mL	11.7±7.4	28.9±14.8	2.5	0.31	9.0±4.5	26.4±14.2	2.9	0.30
CD40	pg/mL	5.5±0.4	18.5±2.9	3.3	0.19	23.6±2.3	68.5±12.2	2.9	<.01
MDC (Macrophage-Derived Chemokine)	pg/mL	14.2±2.0	132.7±24.7	9.3	0.11	239±59.5	698.7±68.4	2.9	<.01
VCAM-1 (Vascular Cell Adhesion Molecule-1)	ng/mL	1.6±0.3	7.7±0.6	4.8	<.01	7.3±1.2	19.7±1.0	2.7	<.01
CRP (C Reactive Protein)	ng/mL	2.1±2.1	11.6±2.0	5.6	0.15	14.3±2.3	37.3±7.6	2.6	0.01
Fibrinogen	ug/mL	0.4±0.4	1.6±0.2	4.4	0.37	2.5±0.7	6.5±1.6	2.6	0.01
TIMP-1 (Tissue Inhibitor of Metalloproteinase Type-1)	ng/mL	0.0±0.0	0.7±0.5	14.7	0.61	2.1±1.0	5.2±1.2	2.5	0.03
Apo A1 (Apolipoprotein A1)	ug/mL	0.1±0.1	0.4±0.1	4	0.42	0.9±0.3	2.2±0.4	2.4	0.01
LIF (Leukemia Inhibitory Factor)	pg/mL	44.4±1.3	66.2±4.4	1.5	0.62	93.5±15.5	211.3±56.6	2.3	<.01
OSM (Oncostatin M)	pg/mL	36.6±1.3	48.3±6.0	1.3	0.39	55.5±7.8	126.7±15.0	2.3	0.02
SCF (Stem Cell Factor)	pg/mL	30.7±2.4	41.6±0.3	1.4	0.29	42.3±5.1	92.5±12.3	2.2	<.01
Lymphotactin	pg/mL	28.3±1.2	30.7±2.1	1.1	0.86	38.7±7.9	81.0±17.3	2.1	0.01
GCP-2 (Granulocyte Chemotactic Protein-2)	ng/mL	0.2±0.1	0.7±0.0	3.1	0.84	2.9±2.0	6.0±2.4	2	<.01
IL-11 (Interleukin-11)	pg/mL	ND	13.2±6.6	NA	0.08	21.9±2.3	44.3±5.6	2	0.21
IL-1alpha (Interleukin-1alpha)	pg/mL	20.3±1.6	58.5±22.1	2.9	0.67	131.4±52.0	257.3±110.0	2	0.01
SAP (Serum Amyloid P)	ng/mL	42.7±4.2	112.5±19.2	2.6	0.08	129.7±22.2	265.7±39.2	2	0.19
Eotaxin	pg/mL	4.4±0.8	9.6±2.9	2.2	0.50	18.8±7.1	35.7±7.0	1.9	0.05
Cystatin-C	ng/mL	32.5±2.4	71.8±10.1	2.2	0.02	75.8±8.2	133.0±12.9	1.8	<.01
IL-17 (Interleukin-17)	pg/mL	ND	ND	NA	NA	22.1±22.1	34.4±19.0	1.6	0.08
IL-1beta (Interleukin-1beta)	ng/mL	0.5±0.1	1.2±0.3	2.6	0.11	1.4±0.3	2.3±0.4	1.6	1.00
MIP-3beta (Macrophage Inflammatory Protein-3beta)	ng/mL	ND	0.1±0.0	NA	0.21	0.2±0.0	0.3±0.0	1.5	0.01
FGF-basic (Fibroblast Growth Factor-basic)	ng/mL	0.3±0.0	0.6±0.0	1.6	0.01	0.7±0.1	1.0±0.1	1.4	<.01
Leptin	pg/mL	13.4±6.7	28.1±0.8	2.1	0.02	32.8±2.2	46.9±2.5	1.4	0.03
Clusterin	ug/mL	0.1±0.0	0.6±0.1	4.4	0.21	1.3±0.4	1.7±0.3	1.3	0.31
M-CSF (Macrophage-Colony Stimulating Factor)	ng/mL	0.1±0.0	0.3±0.2	6.2	0.09	0.5±0.1	0.7±0.1	1.3	0.27
VEGF (Vascular Endothelial Cell Growth Factor)	pg/mL	237±24.3	687±181.9	2.9	0.12	1203±283.3	1527±124.1	1.3	0.24
Haptoglobin	ug/mL	1.1±0.3	1.7±0.3	1.6	0.19	2.0±0.2	2.4±0.3	1.2	0.35
IL-18 (Interleukin-18)	ng/mL	0.1±0.1	0.8±0.1	6.6	<.01	0.9±0.1	1.0±0.0	1.2	0.05
Tissue Factor	ng/mL	0.6±0.1	1.0±0.1	1.6	0.02	1.1±0.1	1.3±0.1	1.2	0.20
TPO (Thrombopoietin)	ng/mL	1.7±0.4	5.0±0.3	3	<.01	6.3±0.6	7.6±0.1	1.2	0.12
Factor VII	ng/mL	0.3±0.0	0.5±0.0	1.8	<.01	0.6±0.0	0.6±0.0	1.1	0.41
FGF-9 (Fibroblast Growth Factor-9)	ng/mL	0.8±0.0	0.6±0.1	0.8	0.08	0.6±0.0	0.7±0.1	1.1	0.81
GST-Mu	ng/mL	9.3±1.1	9.3±1.8	1	1.00	6.5±2.5	7.2±1.9	1.1	0.31
IL-5 (Interleukin-5)	ng/mL	0.1±0.0	0.1±0.0	1.4	0.06	0.2±0.0	0.2±0.0	1.1	0.63
NGAL (Lipocalin-2)	ng/mL	9.0±0.2	8.9±0.4	1	0.95	8.3±0.4	9.2±1.3	1.1	0.60
CD40 Ligand	pg/mL	193.7±53.9	333±75.6	1.7	0.21	358.7±65.3	359.0±87.6	1	0.74
IL-10 (Interleukin-10)	pg/mL	604.7±26.3	520.3±4.3	0.9	0.06	527±40.5	501±22.1	1	1.00
Insulin	uIU/mL	1.2±0.0	1.3±0.0	1.1	<.01	1.4±0.0	1.4±0.0	1	1.00
vWF (von Willebrand Factor)	ng/mL	ND	1.5±0.2	NA	0.83	2.8±0.7	2.8±0.4	1	0.51
MIP-1alpha (Macrophage Inflammatory Protein-1alpha)	ng/mL	ND	0.3±0.2	NA	0.44	0.8±0.4	0.7±0.1	0.9	0.83
Myoglobin	ng/mL	1.0±0.5	2.0±0.8	2	0.21	1.1±0.4	1.0±0.3	0.9	0.87
IL-4 (Interleukin-4)	pg/mL	28.2±1.0	16.3±8.5	0.6	0.21	7.2±7.2	5.1±5.1	0.7	0.53
KC/GROalpha (Melanoma Growth Stimulatory Activity Protein)	ng/mL	ND	0.1±0.1	NA	<.01	0.3±0.1	0.2±0.1	0.7	0.82
MIP-1beta (Macrophage Inflammatory Protein-1beta)	pg/mL	67.3±3.3	489.3±395.4	7.3	0.44	1180±615.3	829±93.8	0.7	0.52
MMP-9 (Matrix Metalloproteinase-9)	ng/mL	0.6±0.1	82.0±59.6	138.5	0.58	371.3±173.4	278.3±83.4	0.7	0.45
MIP-2 (Macrophage Inflammatory Protein-2)	pg/mL	9.7±3.0	62.9±55.0	6.5	0.46	174.4±76.9	107.1±21.7	0.6	0.36
IL-6 (Interleukin-6)	pg/mL	ND	79.3±79.3	NA	0.62	202.7±112.5	93.8±32.7	0.5	0.31
Growth Hormone	ng/mL	ND	1.3±0.2	NA	<.01	1.2±0.1	0.4±0.4	0.3	0.32
GST-alpha (Glutathione S-Transferase alpha)	pg/mL	35.3±35.3	148.3±44.9	4.2	0.09	92.0±50.7	30.6±30.6	0.3	0.03
TNF-alpha (Tumor Necrosis Factor-alpha)	ng/mL	0.1±0.0	0.5±0.5	8.7	0.50	1.7±0.8	0.5±0.1	0.3	0.09
GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	pg/mL	0.6±0.6	12.6±10.6	21.2	0.32	30.3±12.0	6.9±1.4	0.2	0.07
IL-2 (Interleukin-2)	pg/mL	8.9±4.5	10.6±5.3	1.2	0.97	ND	5.0±5.0	NA	0.01
IFN-gamma (Interferon-gamma)	pg/mL	9.1±4.6	ND	NA	0.98	ND	14.0±8.2	NA	0.31
IL-7 (Interleukin-7)	pg/mL	ND	ND	NA	NA	ND	22.8±22.8	NA	<.01
Endothelin-1	pg/mL	ND	4.5±4.5	NA	NA	ND	ND	NA	NA
Beta-2 Microglobulin	ug/mL	ND	ND	NA	NA	ND	ND	NA	NA
EGF (Epidermal Growth Factor)	pg/mL	ND	ND	NA	NA	ND	ND	NA	NA
IL-12p70 (Interleukin-12p70)	ng/mL	ND	ND	NA	NA	ND	ND	NA	NA
IL-3 (Interleukin-3)	pg/mL	ND	ND	NA	NA	ND	ND	NA	NA
SGOT (Serum Glutamic-Oxaloacetic Transaminase)	ug/mL	ND	ND	NA	NA	ND	ND	NA	NA

TABLE E1: BALB/c mice were smoke exposed for 8 weeks and subsequently challenged intranasaly with 10^6 cfu of NTHI. 12 hours post challenge mice were killed and BAL was taken. Data are expressed as mean ± SD; n=3 pooled samples of 3/group. Statistical analysis was

completed by 2-way ANOVA. Ratio: smoke value divided by control value, ND: not detected, NA: not applicable when one or both of control or smoke values were not detected.

Table E2: *Bead array analysis of AM cell supernatant protein concentrations from cigarette smoke exposed mice stimulated with NTHI.*

		control	smoke	ratio	control + NTHI	smoke + NTHI	ratio
MDC (Macrophage-Derived Chemokine)	pg/mL	ND	69.4	NA	17.3	358	20.7
MMP-9 (Matrix Metalloproteinase-9)	ng/mL	ND	0.792	NA	0.325	1.76	5.42
IL-1alpha (Interleukin-1alpha)	pg/mL	15.8	147	9.3	77.8	418	5.37
MCP-3 (Monocyte Chemoattractant Protein-3)	pg/mL	ND	17	NA	34.7	165	4.76
MCP-1 (Monocyte Chemoattractant Protein-1)	pg/mL	4.82	25.2	5.23	61.1	250	4.09
MIP-1gamma (Macrophage Inflammatory Protein-1gamma)	ng/mL	0.109	0.932	8.55	0.934	3.2	3.43
GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	pg/mL	ND	ND	NA	5.15	14.7	2.85
IL-18 (Interleukin-18)	ng/mL	ND	0.231	NA	0.373	0.763	2.05
Factor VII	ng/mL	ND	ND	NA	0.279	0.562	2.01
IL-5 (Interleukin-5)	ng/mL	ND	ND	NA	0.0972	0.19	1.95
TPO (Thrombopoietin)	ng/mL	ND	ND	NA	1.83	3.09	1.69
FGF-basic (Fibroblast Growth Factor-basic)	ng/mL	0.165	0.228	1.38	0.404	0.673	1.67
Fibrinogen	ug/mL	ND	1.71	NA	1.19	1.91	1.61
IL-1beta (Interleukin-1beta)	ng/mL	ND	ND	NA	0.492	0.775	1.58
IL-11 (Interleukin-11)	pg/mL	ND	ND	NA	56.2	84.5	1.5
M-CSF (Macrophage-Colony Stimulating Factor)	ng/mL	ND	0.0059	NA	0.0105	0.0156	1.49
VEGF (Vascular Endothelial Cell Growth Factor)	pg/mL	20.9	41.2	1.97	123	177	1.44
IP-10 (Inducible Protein-10)	pg/mL	ND	14.8	NA	53.3	73.8	1.38
OSM (Oncostatin M)	ng/mL	0.0288	0.0478	1.66	0.157	0.217	1.38
SCF (Stem Cell Factor)	pg/mL	ND	26.4	NA	113	154	1.36
TIMP-1 (Tissue Inhibitor of Metalloproteinase Type-1)	ng/mL	ND	0.0646	NA	0.355	0.466	1.31
GCP-2 (Granulocyte Chemotactic Protein-2)	ng/mL	ND	0.0069	NA	0.237	0.31	1.31
Tissue Factor	ng/mL	0.402	0.43	1.07	0.894	1.16	1.3
LIF (Leukemia Inhibitory Factor)	pg/mL	14.6	19.3	1.32	64.5	82.2	1.27
IL-7 (Interleukin-7)	ng/mL	ND	ND	NA	0.0736	0.0922	1.25
Insulin	uIU/mL	0.981	0.932	0.95	0.942	1.16	1.23
Lymphotactin	pg/mL	ND	ND	NA	35	41.9	1.2
NGAL (Lipocalin-2)	ng/mL	4.74	3.61	0.76	4.03	4.82	1.2
Cystatin-C	ng/mL	5.59	14.1	2.52	15.5	17	1.1
IFN-gamma (Interferon-gamma)	pg/mL	ND	ND	NA	17.7	19.4	1.1
GST-Mu	ng/mL	6.45	5.94	0.92	4.51	4.91	1.09
MIP-1alpha (Macrophage Inflammatory Protein-1alpha)	ng/mL	0.059	0.96	16.3	98.8	107	1.08
FGF-9 (Fibroblast Growth Factor-9)	ng/mL	ND	ND	NA	2.18	2.23	1.02
IgA (Immunoglobulin A)	ug/mL	0.353	0.43	1.22	0.434	0.442	1.02
Haptoglobin	ug/mL	0.288	0.246	0.85	0.294	0.298	1.01
MIP-1beta (Macrophage Inflammatory Protein-1beta)	pg/mL	45.3	117	2.58	20900	20700	0.99
Clusterin	ng/mL	0.459	0.618	1.35	0.808	0.781	0.97
Osteopontin	ng/mL	11.9	37.5	3.15	32.2	29.4	0.91
MCP-5 (Monocyte Chemoattractant Protein-5)	pg/mL	ND	ND	NA	26	22.6	0.87
IL-4 (Interleukin-4)	pg/mL	17.1	ND	NA	30.6	26.3	0.86
Calbindin	ng/mL	0.0454	0.0446	0.98	0.043	0.0352	0.82
IL-10 (Interleukin-10)	pg/mL	42.2	80.7	1.91	645	498	0.77
IL-3 (Interleukin-3)	pg/mL	ND	ND	NA	8.78	6.59	0.75
IL-12p70 (Interleukin-12p70)	ng/mL	ND	ND	NA	0.241	0.171	0.71
TNF-alpha (Tumor Necrosis Factor-alpha)	ng/mL	0.044	0.0953	2.17	19.5	12.6	0.65
RANTES (Regulation Upon Activation, Normal T-Cell Expressed and Secreted)	pg/mL	ND	ND	NA	736	459	0.62
KC/GROalpha (Melanoma Growth Stimulatory Activity Protein)	ng/mL	ND	0.0527	NA	25.9	15.4	0.59
MIP-2 (Macrophage Inflammatory Protein-2)	pg/mL	122	468	3.84	58600	33000	0.56
IL-6 (Interleukin-6)	pg/mL	ND	ND	NA	11100	4050	0.36
MIP-3beta (Macrophage Inflammatory Protein-3beta)	ng/mL	ND	ND	NA	ND	0.138	NA
MPO (Myeloperoxidase)	ng/mL	ND	4.09	NA	ND	5.03	NA
VCAM-1 (Vascular Cell Adhesion Molecule-1)	ng/mL	ND	ND	NA	ND	0.281	NA
CD40	pg/mL	ND	ND	NA	2.42	ND	NA
Apo A1 (Apolipoprotein A1)	ug/mL	ND	ND	NA	ND	ND	NA
Beta-2 Microglobulin	ug/mL	ND	ND	NA	ND	ND	NA
CD40 Ligand	pg/mL	ND	ND	NA	ND	ND	NA
CRP (C Reactive Protein)	ug/mL	ND	ND	NA	ND	ND	NA
EGF (Epidermal Growth Factor)	pg/mL	ND	ND	NA	ND	ND	NA
Endothelin-1	pg/mL	ND	ND	NA	ND	ND	NA
Eotaxin	pg/mL	ND	ND	NA	ND	ND	NA
Growth Hormone	ng/mL	ND	ND	NA	ND	ND	NA
GST-alpha (Glutathione S-Transferase alpha)	ng/mL	ND	ND	NA	ND	ND	NA
IL-17 (Interleukin-17)	ng/mL	ND	ND	NA	ND	ND	NA
IL-2 (Interleukin-2)	pg/mL	ND	ND	NA	ND	ND	NA
Leptin	ng/mL	ND	ND	NA	ND	ND	NA
Myoglobin	ng/mL	ND	ND	NA	ND	ND	NA
SAP (Serum Amyloid P)	ug/mL	ND	ND	NA	ND	ND	NA
SGOT (Serum Glutamic-Oxaloacetic Transaminase)	ug/mL	ND	ND	NA	ND	ND	NA
vWF (von Willebrand Factor)	ng/mL	ND	ND	NA	ND	ND	NA

TABLE E2: BALB/c mice were cigarette smoke exposed for 8 weeks, AMs isolated and stimulated with media alone or media plus NTHI. 12 hours post-stimulation, supernatants were collected for analysis. Data represent pooled samples of 3. Ratio: smoke value divided by control value, ND: not detected, NA: not applicable when one or both of control or smoke values were not detected.

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