CIGARETTE SMOKE IMPAIRS INNATE HOST DEFENSE AGAINST PNEUMOCCAL PNEUMONIA

by

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To My Family

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ABSTRACT

Exposure to cigarette smoke (CS) is associated with increased risk of many infectious diseases including those caused by *Streptococcus pneumoniae*. Such pneumococcal infections are the leading cause of community-acquired pneumonia and deaths from invasive bacterial infections. Since CS exposure also impairs the function of the alveolar macrophage (AM), a cell central to innate host defense in the lung, we used *in vivo* and *in vitro* approaches to determine the effects of CS exposure in a murine model of pneumococcal pneumonia, and to elucidate how CS-mediated impairment of AM function might contribute to this relationship. Mice exposed to CS over 5 weeks developed more serious infections, with 4-fold and 35-fold higher pulmonary bacterial burdens at 24 hr and 48 hr post-infection, respectively. In separate experiments, we compared the effects of low-tar versus regular cigarettes on cellular recruitment and AM function, finding no evidence to support the perception that low-tar cigarettes might be

less harmful. *In vitro*, AMs from CS-exposed animals displayed impaired cytokine production following pneumococcal challenge and reduced phagocytosis of bacteria but not IgG-opsonized microspheres, indicating intact FcγR-mediated phagocytosis. To remove possible effects from other cells, naive AMs were treated *in vitro* with cigarette smoke conditioned media (CSCM), yielding similar impairments in phagocytosis that were specific to complement-opsonized pneumococcus, but no impairment in FcγR-mediated microsphere phagocytosis. However in another experiment, CSCM-pretreated rat AMs did display impaired phagocytosis of IgG-opsonized *E.coli*, suggesting CS may interfere with TLR-mediated bacterial recognition. CSCM pretreatment impaired cytokine synthesis and reactive oxygen intermediate generation following challenge with LTA, a bacterial ligand for TLR2. However no differences were seen in TLR2 transcription suggesting that CSCM may impair TLR-mediated AM activation through disruption of downstream reactive oxygen intermediate-mediated signal transduction. Such a mechanism would indicate common themes underlying some of the diverse effects of CS exposure on immune function. Overall, the novel finding of impaired pulmonary innate host defense following CS exposure, and observation of AM bacterial recognition and phagocytosis impairments, may suggest new directions for understanding the effects of CS exposure on human health.

CHAPTER 1

Introduction

The Pneumococcus

The Gram-positive bacterium *Streptococcus pneumoniae* was first identified in 1881 by Louis Pasteur and George Sternberg, and within 5 years had been termed the pneumococcus, because of its frequent presence in respiratory infections (1). The organism is widespread, generally causing asymptomatic nasopharyngeal colonization that can last for several months before resolving without dissemination to the lower respiratory system or other body compartments (2). A first instance of colonization confers a measure of acquired resistance to subsequent encounter with the same strain, but not to a serologically distinct strain. Thus, carriage rates tend to decline from childhood to adulthood, following the accumulation of previous exposures. The prevalence of pneumococcal carriage varies widely between geographical areas, with rates for children higher than 80% observed in parts of Africa (3). Carriage is also higher in crowded environments such as military barracks and nursing homes.

The spread of pneumococcal colonization beyond the oropharynx can lead to other infections including pneumonia, otitis media, meningitis and bacteremia, placing pneumococcus as the leading cause of death from invasive bacterial infections (4).

Pneumococcal pneumonia is the most common form of community-acquired pneumonia, both in the US (5) and worldwide (6). Young children and the elderly are particularly vulnerable as are individuals with immunodeficiencies from splenectomy (7), or diseases such as HIV (8) and genetic complement deficiencies (9), and those with certain chronic diseases including chronic obstructive pulmonary disease (COPD) (10). Most deaths from pneumococcal disease are in the developing world, with a 2006 estimate of approximately 800,000 deaths annually among children under 5 years of age in these countries (11).

Recent years have seen the introduction of effective pneumococcal vaccines, however geographic coverage is incomplete. More than 90 serotypes of pneumococcus have been identified, and there are indications that strains used to generate the vaccine may be replaced by non-vaccine strains over time (11). The growth of susceptible populations and the emergence of multi-drug resistant strains (12) point to the continuing relevance of this pathogen to public health. The need for an improved understanding of the mechanisms of pathogenesis and determinants of susceptibility to pneumococcal infection call for additional study.

Protecting the Airways

The respiratory system is under constant exposure to the external environment and the many potential pathogens found there. To reduce penetration of foreign material and microorganisms into the deep airspaces of the lung, animals use a series of filtration, exclusion, and removal systems. After gross filtration by the nasal vibrissae (whiskers), architectural features including nasal turbinates direct the flow of air entering the

nasopharyngeal cavity, leading to impaction and deposition on mucosal surfaces (13) containing ciliated, pseudostratified columnar epithelial cells and mucus-producing goblet cells. Particles trapped within the mucus layer are subject to multiple antimicrobial factors including beta-defensins, cathelicidin, and lysozyme, and are propelled by ciliated epithelium to the oropharynx, where they are swallowed or expelled from the body (14). Particles not removed in the nasal cavity may pass through the larynx and into the trachea, which undergoes repeated bifurcation into bronchi and successively-smaller bronchioles. Particles with sufficient inertia deposit through impaction, while slowing airflow provides additional deposition through sedimentation. The respiratory epithelium in these areas constitutes a "mucocilliary escalator" which acts to move contaminant-containing mucus upward and out of the respiratory tract. The bronchiolar epithelium also includes Clara cells, which produce secretory products including surfactant protein-(SP-)A, and SP-D, as well as other proteins with immunomodulatory and antiinflammatory functions (15). The transition from air-conducting to gas-exchange regions of the lungs occurs in the terminal bronchioles, where epithelial morphology changes from columnar to simple cuboidal epithelium without goblet cells. Beyond the terminal bronchioles lie respiratory bronchioles, leading through alveolar ducts to alveolar sacs and individual alveoli. The alveoli themselves are lined with simple squamous cells (type I), marked by a thin morphology that facilitates gas exchange. Also present are the cuboidal type II pneumoctyes, which proliferate to replace type I cells, as well as producing surfactant proteins.

Despite the efficiency of the conducting airways in excluding foreign material, some particles, particularly those smaller than 1 μm (16), may penetrate into the alveolar

compartment. Protecting the alveolus from infection necessitates immune surveillance and coordinated activity of a variety of cells including alveolar macrophages (AMs), as well as epithelial cells, endothelial cells, neutrophils and other leukocytes. In the healthy individual, AMs comprise the vast majority of leukocytes present within the alveolar space, and persist for a comparatively long span of approximately 3 months (17). Figure 1.1 depicts an AM within the alveolar milieu. Alveolar macrophages play a variety of roles including removal of particulates, phagocytosis and killing of pathogens, and secretion of antimicrobial peptides. They also serve to regulate other cells through the release of soluble mediators including proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and members of the interleukin (IL-) family (18-20). Importantly, through the phagocytic removal of noxious stimuli and secretion of anti-inflammatory mediators including IL-10 and the lipid prostaglandin E 2 (PGE_2), AMs also participate in the resolution of inflammation, as an excessive immune response could lead to insufficient gas-exchange within the lung, resulting in death of the host (18).

The AM and Pneumococcal Infection

Although the specific roles played by AMs during innate host defense against this organism remain incompletely understood, their participation has been shown by depletion of these cells prior to infection. In a high-dose infection, increased mortality was seen among AM-depleted animals (19). However, increased inflammation and neutrophil recruitment, combined with the lack of difference in measured bacterial burden, led the authors to conclude that AMs function primarily to remove apoptotic cells and resolve inflammation, rather than to clear bacteria directly. In contrast, others have

shown increased outgrowth of bacteria after AM depletion in a low-dose, resolving model of pulmonary pneumococcal infection (20), despite increased recruitment of neutrophils. Importantly, in this model animals with normal AM numbers were able to clear the bacteria without detectable recruitment of neutrophils, while a higher inoculum resulted in inflammation and neutrophil recruitment. Together, these data suggest that the role played by the AM in host defense against *S. pneumoniae* may vary with the severity of the infection.

AM Receptors for Unopsonized Bacteria

The capacity of AMs to identify targets innately (*i.e.*, without prior exposure), is conferred by a broad array of receptors that recognize highly-conserved pathogen-associated molecular patterns (PAMPs). These pattern-recognition receptors (PRRs) may be cytoplasmic, such as the nucleotide-binding oligomerization domain proteins (NODs) (21), or membrane-bound, as in the case of toll-like receptors (TLRs), and allow the cell to sense and respond to specific stimuli (22, 23). For example, TLR2 on the AM surface binds lipoteichoic acid (LTA) from the cell wall of gram-positive bacteria such as *S. pneumoniae*. Association with its ligand activates signaling motifs in the cytoplasmic region of the receptor, allowing the recruitment of the adaptor proteins MyD88 (24), IL-1 receptor associated kinase (IRAK) 1, IRAK2, and IRAK4. Assembly of this complex allows the initiation of multiple kinase-dependent signal cascades, resulting in the nuclear translocation of transcription factors including nuclear-factor-kappa B (NF-κB) and activator protein-1 (AP-1) (25). Many genes are upregulated as a result of the binding of these transcription factors to nuclear DNA, including TNF-α, IL-1β and IL-6, which are

important in host-defense against this organism as discussed below. AMs express multiple TLRs, and can differentially regulate the degree of expression in response to inflammatory stimuli such as TNF-α, IL-1β, and bacterial lipopolysaccharide (LPS) (26). In humans, genetic defects in NF-κB function and IRAK4 are associated with susceptibility to pneumococcal infections (9, 30), demonstrating the importance of these PRR-dependent pathways.

In addition to ligand-specific PRRs such as the TLRs, AMs also express scavenger receptors (SRs), such as SR-A, which bind to a wide range of polyanionic ligands including both PAMPs and non-biological particles (27). Binding of these receptors facilitates phagocytosis of bacteria (28). Despite the lack of any known intracellular signaling motifs within its short cytoplasmic domain (29), binding of SR-A to a specific monoclonal antibody has been shown to modulate cytokine production in murine AMs (30). Comparing peritoneal macrophages from wild-type and SR-A -/- mice, another study found no difference in cytoplasmic protein tyrosine kinase activities following treatment with the SR-A ligand fucoidin (31). In contrast, the authors observed that this fucoidin-elicited activity was nearly absent in mice genetically lacking CD14, an accessory protein involved in TLR signaling. This result implied that some cytoplasmic signaling activity previously attributed to SR-A was mediated through TLRs. Both LTA and polyinosinic:polycytidylic acid (Poly I:C) have been used to demonstrate SR-A mediated signal transduction in a macrophage-derived cell line (32), however these results must be interpreted with caution due to the presence of TLRs which also recognize these ligands. Recently, it has been shown that SR-A physically associates with Mer receptor tyrosine kinase (MertK), a cell-surface receptor with known

cytoplasmic signaling activity (29). This led the authors to suggest that SR-A may serve as an accessory protein by bringing MertK monomers into close proximity, thus facilitating their autophosphorylation.

In a mouse model of pneumococcal pneumonia, SR-A -/- mice were found to have higher pulmonary bacterial burdens and reduced survival compared with wild-type animals (33). Instillation of heat-killed, fluorescently-labeled *S. pneumoniae* in these animals revealed impaired AM phagocytosis of the bacteria. The macrophage receptor with collagenous structure (MARCO) is closely related to SR-A, and also expressed on AMs. Mice lacking this receptor displayed increased pulmonary bacterial numbers as well as reduced survival following pulmonary infection with *S. pneumoniae* (34). SR-mediated clearance of normally pyogenic bacterial products such as LPS has been observed without the induction of proinflammatory responses (35). Because of this, some have proposed that SRs play an important role in limiting excessive inflammation by preventing the interaction of these moieties with proinflammatory PRRs (36). Interestingly, it has been recently reported that bone marrow-derived dendritic cells from mice which are heterozygous SR-A +/- and TLR2 +/- display impaired phagocytosis of unopsonized gram-positive *Staphylococcus aureus* (37). The authors recapitulate this effect with SR-A +/-, MyD88 +/- cells, demonstrating for the first time that TLR-mediated signaling can regulate SR-A dependent bacterial phagocytosis.

AM Receptors for Opsonized Bacteria

Within the alveolar environment, extracellular factors such as complement, immunoglobulin (Ig), and SP-D, can opsonize particles by recognizing and binding

PAMPs, and each of these has been found to participate in host defense against *S. pneumoniae*, as described below. The complement system is composed of a large number of proteins which interact with one another sequentially and in parallel to facilitate the removal and killing of exogenous organisms [reviewed in (38)]. The opsonic function of complement can be activated by three distinct mechanisms, termed the classical, alternative, and mannose-binding lectin pathways. Because the alternative pathway involves direct deposition of complement on the pathogen surface, while the classical pathway is associated with antibody deposition, it has been assumed that the alternative pathway played a more prominent role in innate immunity to *S. pneumoniae*. Likewise, several in vitro studies demonstrated activation of the alternative pathway in the presence of pneumococcal components (39, 40). More recent data generated with mice genetically deficient in specific complement components has demonstrated the importance of the classical pathway in both intravenous and intranasal infection models (41). Further, the first component in the classical pathway (C1q) has been shown to bind pneumococcal cell wall in the absence of Ig (42).

Regardless of the initiating pathway of complement fixation, all culminate with the cleavage of complement component 3 (C3), and deposition of the resulting fragments C3b and, more importantly for phagocytosis, iC3b on the surface of the pathogen. In addition to opsonization, the C3b fragment possesses enzymatic activity that can lead to the generation of the membrane attack complex, a complement-derived protein complex capable of killing cells through disruption of cell membranes. However, *S. pneumoniae* is resistant to the cytolytic activity of this complex due to the distance between the outer capsule and cell wall. The binding of C3 fragments and surfactant proteins to a variety of

dedicated leukocyte surface receptors greatly facilitates phagocytic uptake of opsonized particles and transduces opsonin- and receptor-specific signals to the cytoplasm, conferring additional sensory functionality (43, 44). Consistent with the central role of complement in host defense against *S. pneumoniae*, genetic defects in complement function have been associated with increased susceptibility to pneumococcal infection in humans, and are most severe when disrupting the classical pathway of complement fixation (9, 45,46). The importance of C3 in pulmonary pneumococcal infection was demonstrated by using C3-/- mice (47). These mice were found to have increased pulmonary bacterial loads, increased dissemination out of the alveolar space, and reduced survival compared with wild-type animals. Because these outcomes were not associated with a change in pulmonary leukocyte recruitment, the authors concluded that C3 likely exerts its effects through facilitation of opsonophagocytosis or direct microbial killing in this model.

While complement components are serum proteins produced in abundance by the liver, they can be rapidly carried into the lung during inflammation, with significant increases of C3 in the airways observed as early as 1 hr following infection in a murine model of *S. pneumoniae* pneumonia (47). Additionally, functional complement proteins have been observed in lavage fluid recovered from the lungs of healthy human subjects (48), and C3 has been found in lavage fluid from healthy mice, although at much lower levels than in serum (49). Isolated lung sections (50), AMs (51), and type II alveolar epithelial-derived cells (52, 53), have been shown to produce functional complement in vitro, leading some to speculate that locally-produced complement proteins may contribute to host defense during early stages of respiratory infection (48, 50).

Monocytes express three primary forms of complement receptor (CR), which function as heterodimers of distinct α chains with a common β chain, designated CD18. The relative expression of these receptors changes with maturation of the cell, and is tissue-specific in resident macrophages (54, 55). In circulating monocytes, CR1 levels are the highest, followed by CR3 and CR4. In AMs, however, the order is reversed, and CR4 is expressed at the highest levels (56). Macrophages maintain a substantial portion of their CR3 and CR4 within cytoplasmic pools, and activation of the cell with a stimulus such as phorbol ester rapidly induces receptor translocation to the plasma membrane and enhancement of iC3b binding (54). In a mouse model of systemic serotype 3 pneumococcal infection, CR3 -/- and CR4 -/- animals had reduced survival compared with wild-type controls (57).

Immunoglobulins bind *S. pneumoniae*, and the development of type-specific antibody underlies acquired immunity from both infection and vaccination (58). The presence of anticapsular antibodies in the blood reduces the risk of bacteremia during pneumococcal pneumonia (59), although recovery from pneumococcal infection can occur in the absence of such antibodies (60). Alveolar macrophagess express receptors for the Fc portion of Ig, permitting opsonophagocytosis of Ig-opsonized targets. Further, the rate of such phagocytosis can be modulated independently of receptor-ligand binding, through the activity of immune modulators such as leukotrienes (61).

Working together, these systems enable context-dependent responses to specific stimuli. For example, SP-A can attenuate TLR-mediated NF-κB activation and TNF-α production (62, 63), but also enhance Ig- and complement-mediated phagocytosis (64).

Alveolar epithelial cells also maintain AMs in a quiescent state through integrin-mediated interactions that are terminated by the presence of PAMPs (65; 66). As a result of this high degree of regulation, AMs typically carry out their functions without inducing an overt inflammatory reaction (67), however when the clearance capacity of the resident AMs is exceeded, they respond with the generation of proinflammatory signals with both autocrine and paracrine effects resulting in the recruitment of other cells such neutrophils and interstitial or circulating monocytes to the site of insult(68, 69).

Roles for Specific Cytokines

In animal models, the use of monoclonal antibodies (mAb) and genetically-engineered mice have provided evidence for the roles of several cytokines in host-defense against pneumococcal infections. Treatment with TNF-α blocking mAb prior to infection lead to higher pulmonary bacterial burdens after 40 hr, and significantly earlier death in a murine model of pneumococcal pneumonia (70). In IL-1 receptor knockout mice, the pulmonary bacterial burden was 2 orders of magnitude higher than wild-type counterparts 24 and 48 hr after infection, although survival did not differ (71). Administration of anti-TNF-α mAb prior to infection profoundly reduced survival of these mice compared with wild-type, with simultaneous blockade of both IL-1β and TNF-α resulting in the death of all the animals in the group. Another study found both increased bacterial loads and reduced survival of IL-1β knockout mice, which was significantly improved with the administration of recombinant IL-1β (72). Interleukin-6 has also been shown to play a positive role in innate host defense against pneumococcal pneumonia, as IL-6 -/- displayed higher pulmonary bacterial loads 40 hr post-infection,

and reduced survival times (73). Because these mice had higher pulmonary levels of TNF-α and IL-1β than the corresponding wild-type animals, the authors concluded that IL-6 serves primarily an antiinflammatory function. If this interpretation is correct, it would stand in contrast to the antiinflammatory cytokine IL-10, which was found to impair innate host defense against pneumococcal pneumonia (74). Here, the addition of recombinant IL-10 to instillation with *S. pneumoniae* resulted in higher pulmonary bacterial burdens and reduced survival time. Similarly, treatment with anti-IL-10 prior to infection reduced the number of pulmonary bacteria, and increased survival time.

The cytokines IL-12, IL-18, and interferon-gamma (INF-γ) have typically been associated with development of a T-helper (Th) 1 mediated response, promoting the activity of phagocytes, and early host defense functions (75). The role of these cytokines in pneumococcal infection is less clear, with conflicting results in different models. In a model using INF-γ -/- mice developed on a C57BL/6 background, the absence of INF-γ was associated with significantly increased mortality following pulmonary pneumococcal infection (76). Conversely, another study compared BALB/C-derived INF-γ -/- mice with wild-type counterparts in a murine pneumococcal pneumonia model, finding no increased mortality, and fewer bacteria in the lungs of knockout animals (77). Likewise, INF-γ receptor-deficient mice showed reduced pulmonary load and no difference in mortality compared with wild-type counterparts.

One study on the role of IL-12 found no reduction in pulmonary pneumococcal clearance in IL-12 -/- mice, nor significant effects of prior treatment with IL-12 neutralizing mAb (78). In contrast, others have observed reduced pulmonary clearance of

S. pneumoniae and decreased survival time in mice lacking p40, a monomer of IL-12 (79). This defect was restored with the addition of recombinant IL-12. The authors of another report found that pulmonary instillation of recombinant IL-12 given 24 hr prior to infection reduced bacterial load and improved survival in wild-type mice, although no such effects were seen when INF-γ knockout mice were similarly treated

A positive role for IL-18 was suggested by the finding that IL-18 -/- mice had significantly higher pulmonary bacterial burdens and dissemination into the blood at 24 and 48 hr following infection (78). Similarly, wild-type mice treated with anti-IL-18 mAb displayed significantly higher bacterial burdens in the lungs. However, a report comparing the effects of IL-18 on nasal colonization, pneumonia, and bacteremia caused by *S. pneumoniae* found lower pulmonary bacterial burdens in IL-18 knockout mice (80). The authors note that the effects of IL-18 vary with both the location of infection and the pneumococcal strain.

Neutrophils

Neutrophils share a common myeloid progenitor cell with macrophages, and are rapidly recruited into the alveolar space following bacterial infection or other inflammatory stimuli [reviewed in (81)]. Neutrophils and AMs share many common features such as the expression of PRRs (*e.g.* TLRs, NODs) and perform common functions such as bacterial phagocytosis and killing, and elaboration of cytokines. There are important dissimilarities as well, such as the production of neutrophil extracellular traps (NETs) containing antimicrobial proteins attached to a lattice of nucleic acid filaments (82). These structures are thought to function both in physically preventing the

spread of pathogens, as well as bringing them into close proximity to the associated antimicrobial factors. Alveolar deposition of NETs has been observed in pneumococcal pneumonia (83), however it is believed that *S. pneumonia* is resistant to them in part due to positively-charged moieties on the capsular surface (84). Neutrophils may have detrimental effects under conditions of excessive inflammation, but a positive role in pneumococcal pneumonia has been demonstrated be depleting these cells with a neutralizing antibody prior to infection (85). In this model, the depletion of neutrophils led to significantly higher pulmonary bacterial burdens in a high-dose infection, but not in lower-dose infections.

Reactive Oxygen and Nitrogen Species

Both neutrophils and AMs can generate reactive oxygen and nitrogen species through the actions of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and nitric oxide synthase (NOS), respectively. Generation of reactive oxygen species contributes to bacterial killing, and defects in components of NADPH-oxidase lead to chronic granulomatous disease, with increased susceptibility to infection (86). Emerging evidence suggests that reactive oxygen species may function not only in direct microbial killing, but also in signal transduction (87, 88). Components of the NADPH-oxidase system assemble on the maturing phagosome, and there are indications that the complex can cooperate with TLRs to guide phagosome maturation (89). Reactive nitrogen species kill *S. pneumoniae* in vitro, and mice deficient in NOS had lower bacterial loads in a systemic pneumoccal infectioin model, but higher pulmonary burden in a pneumonia model (90).

Cigarette Smoke and Infectious Disease

CS exposure has been associated with higher rates of pneumococcal carriage (91), and multiple epidemiological studies have shown increased incidence of pneumococcal infections in CS exposed populations. In a retrospective case-control study of male patients with culture-confirmed pneumococcal infections (10), Lipsky *et al.* determined that the relative risk of current smokers was 4.00 compared with non-smokers, while former smokers had a relative risk of 2.14, which did not reach significance. It should be noted that this group was composed of adult males, and most studies find that males are more frequently affected by pneumococcal infections (60). Additionally, as the study examined men who were referred to a clinic from services for the homeless, it represents a subgroup distinct from the general population.

Pastor *et al.* (92) used an active surveillance program to study the population of Dallas County, Texas in 1995, and identify cases of invasive pneumococcal disease (defined as a positive culture isolated from a normally sterile body fluid such as blood). In adults aged between 25 and 64 years, current smoking was associated with significant odds ratios of 2.6, with a 95% confidence interval (CI) of 1.9 to 3.5, and in those 65 years or older 2.2 (95% CI, 1.4 - 3.4). This study did not collect data on past or passive CS exposure, which may have lowered the magnitude of the observed effect, due to the presence of exposed individuals in the "nonsmoker" group.

In a large population-based case control study (93), Nuorti *et al.* examined active surveillance data from Baltimore, Atlanta and Toronto, Canada for cases of invasive pneumococcal disease, defined as in the previous report. The odds ratio among current

smokers was determined to be 4.1 (95% CI, 2.4 - 7.3), and among passively-exposed nonsmokers it was 2.5 (95% CI, 1.2 - 5.1). In smokers, 51% of cases were attributable to CS exposure. This should be considered the key study due to the large population size, extensive controls for possible confounding factors, and the collection of information on smoking behaviors, history and passive smoke exposures. These additional data allowed the authors to demonstrate dose response relationships between pneumococcal disease risk and the number of cigarettes smoked per day, as well as total pack-years. Strikingly, while an inverse relationship was found between incidence and time since cessation in former smokers, levels did not achieve parity with never-smokers until 13 years following cessation.

The above studies are distinct from others which have found an increased incidence of community-acquired pneumonia in smokers (94, 95) in that all cases were confirmed to be of pneumococcal origin. Pneumococcal infections are not unique in being influenced by CS exposure. Such associations have been demonstrated in human populations for many infectious diseases (96), including tuberculosis (97), influenza (98), legionnaires disease (99), upper respiratory infections (100), *Helicobacter pylori* infection (101), meningococcal disease (102), and sexually-transmitted diseases (103). While epidemiological associations of this type can provide many useful insights, they cannot fully control for the inherent diversity of physiological and pathophysiological processes simultaneously occurring in human populations.

The pathology of COPD is an example of such diversity. COPD is chronic respiratory disease, marked by airway obstruction, leading to shortness of breath,

punctuated by acute exacerbations of symptoms that can be induced by infections or other inflammatory stimuli (104). Such acute exacerbations of COPD (AECOPD) can lead to shortness of breath, hospitalization or death. Most of the estimated 12 million cases of COPD in the US (105) are attributable to smoking, and in 2004, the disease is believed to have caused over 100,000 deaths among the US population. It has been projected to become the world's fifth-leading cause of chronic morbidity and mortality by 2020 (106). For unknown reasons, only 15-20% of smokers will develop clinically-significant symptoms of COPD, demonstrating the contribution of individual susceptibility to disease progression (107). Much research has focused on identifying genetic and environmental determinants of susceptibility to the development and progression of COPD. Unfortunately, the factors underlying susceptibility to COPD remain very poorly understood.

Acute exacerbations are accompanied by pulmonary and systemic inflammation, and undoubtedly there are mechanistic relationships between immune function and COPD. For example, on study of patients with AECOPD found bacterial or viral infections in 78% of cases and airway neutrophilia in all cases (108). The magnitude of neutrophilia was correlated with exacerbation severity, independent of positive or negative finding of infection. Elevated serum levels of TNF-α and IL-6 have been found in COPD patients compared with healthy controls even in the absence of acute exacerbation (109). The mechanisms responsible for AECOPD are not fully understood, but it has recently been proposed that they could involve both ineffectual immune responses leading to lung infections and overly forceful reactions leading to excessive inflammation (110).

Pneumococcal carriage (111) is associated with development of acute exacerbations, and COPD patients are at increased risk for pneumococcal pneumonia(10). Similarly, antibiotics (112) and pneumococcal vaccination (113) have proven therapeutic benefits in AECOPD. A better understanding of the effects of CS exposure on host defense against pneumococcal infection may ultimately be useful in the study of COPD.

Animal Models

Animal models have been used to study relationships between cigarette smoke and a human health effects including COPD (114), cancer (114), fertility (115), nicotine addiction (116), and sudden infant death syndrome (117). While such models unavoidably differ from human subjects, they offer the benefits of greater control of experimental conditions and reduced genetic variability. Modeling human smoking represents a particular challenge, due to the volitional nature of dosing in human smokers. Because few non-primate models involve direct, active smoking by the subject, most models use some form of apparatus to mechanically generate smoke. This smoke must then pass into a chamber or nose-only exposure system, and thus such models represent a compromise between smoking and exposure to environmental tobacco smoke (ETS). Additionally, practical considerations typically preclude duplicating human dosing patterns, which often involve smoking throughout the day, every day, sometimes for many years. Decisions regarding duration, magnitude, and timing of exposures are dictated by the goals of the individual experiment, and thus wide variability is found from model to model. This presents a challenge when comparing data from different systems.

In vivo studies of experimentally-induced pulmonary infection have found increased burdens of the viral pathogens respiratory syncytial virus (118), influenza A (119), the fungal pathogen *Pneumocystis murina*, (120), and the bacterium *Pseudomonas aeruginosa* (121) in smoke-exposed animals. Surprisingly, this approach has not been applied to elucidate the effects of CS exposure on host defense against pneumococcal pneumonia. One report exists in which rats were intratracheally infected with *S. pneumoniae* following 8 weeks of CS exposure as a control group in a larger study of ethanol-mediated host defense impairments (122). No impairment was found in the CS group, however the animals were euthanized one hour following infection. More importantly, the model involved pre-recruitment of neutrophils by administration of Gram-negative lipopolysaccharide into the lungs several hours prior to infection, making this model more closely resemble polymicrobial rather than pneumococcal pneumonia.

Many more reports describe effects of CS exposure on specific functional parameters such as elastin fragment-mediated AM chemotactic activity (123), increased pulmonary TNF-α and IL-6 protein levels (124), and elevated markers of lipid peroxidation (125) within the lungs of CS-exposed mice. Cigarette smoke produces extensive effects on AM functions with inferable relevance to pneumococcal infections, including alteration of surface receptor expression (126), pathogen recognition and subsequent cytokine production (127-129), phagocytosis (130; 131), and microbial killing (132).

Project Focus

The goal of this project was to test the hypothesis that CS exposure impairs innate host defense against pneumococcal pneumonia, at least in part through a mechanism involving disruption of alveolar macrophage AM phagocytosis and cytokine production. AMs are known to accumulate in the lungs of smokers (133), creating an apparent paradox in light of impaired host defense in these individuals. With their comparatively long life spans, AMs are likely to experience particularly heavy cumulative exposures relative to other cells. However, few studies have directly addressed the effects of CS on AM function in the context of pneumococcal pneumonia, despite the importance of this pathogen, and the heightened infection risk in smokers. This fact formed part of the rationale for the project, as such an animal model would be useful in the exploration of pathophysiological mechanisms, and perhaps ultimately, translation to therapeutic applications.

The decision to focus on AMs as a mechanism when examining CS-mediated susceptibility to infection with *S. pneumoniae* reflects the early and central role of these cells in innate host defense, as well as the lack of published reports addressing the question. However, CS is a complex mixture containing literally thousands of individual compounds (134), with many effects not directly mediated through the AM. Therefore, the effects described here will constitute only one aspect of CS-mediated host defense impairment. Hopefully these observations will contribute to the growing interest in the effects of environmental exposures on immune function, and suggest new questions to ask as we seek to better understand these effects.

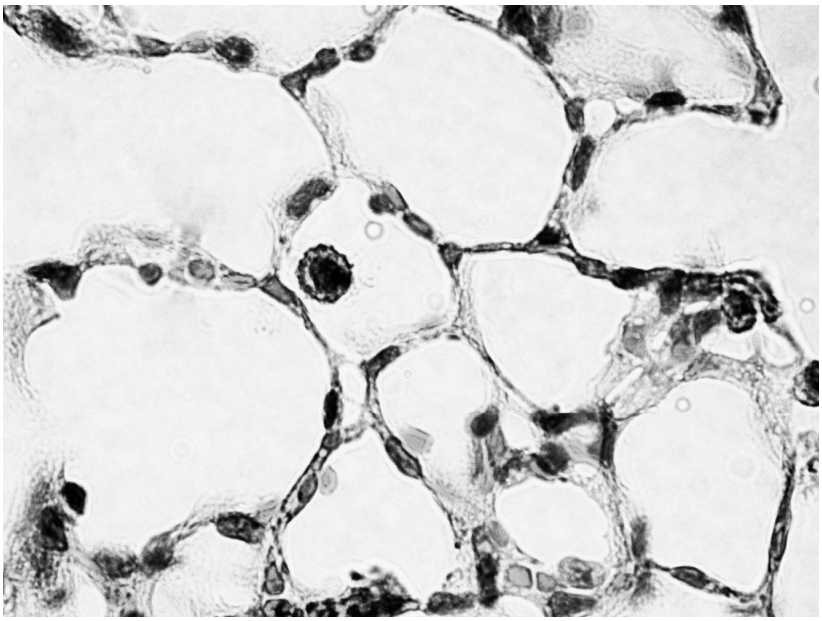

Fig 1.1 Alveolar macrophage in situ
Murine lung section stained with hematoxilin and eosin (400 x magnification)

References

1. Watson, D.A., D.M. Musher, J.W. Jacobson, and J. Verhoef. 1993. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. *Clin.Infect.Dis.* 17:913-924.

2. McCullers, J.A., and E.I. Tuomanen. 2001. Molecular pathogenesis of pneumococcal pneumonia. *Front.Biosci.* 6:D877-89.

3. Cardozo, D.M., C.M. Nascimento-Carvalho, F.R. Souza, and N.M. Silva. 2006. Nasopharyngeal colonization and penicillin resistance among pneumococcal strains: a worldwide 2004 update. *Braz.J.Infect.Dis.* 10:293-304.

4. Kadioglu, A., and P.W. Andrew. 2004. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 25:143-149.

5. Mandell, L.A., R.G. Wunderink, A. Anzueto, J.G. Bartlett, G.D. Campbell, N.C. Dean, S.F. Dowell, T.M. File Jr, D.M. Musher, M.S. Niederman, A. Torres, C.G. Whitney, Infectious Diseases Society of America, and American Thoracic Society. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin.Infect.Dis.* 44 Suppl 2:S27-72.

6. Anonymous . 1999. Pneumococcal vaccines. WHO position paper. *Wkly.Epidemiol.Rec.* 74:177-183.

7. Cadili, A., and C. de Gara. 2008. Complications of splenectomy. *Am.J.Med.* 121:371-375.

8. Klugman, K.P., S.A. Madhi, and C. Feldman. 2007. HIV and pneumococcal disease. *Curr.Opin.Infect.Dis.* 20:11-15.

9. Picard, C., A. Puel, J. Bustamante, C.L. Ku, and J.L. Casanova. 2003. Primary immunodeficiencies associated with pneumococcal disease. *Curr.Opin.Allergy Clin.Immunol.* 3:451-459.

10. Lipsky, B.A., E.J. Boyko, T.S. Inui, and T.D. Koepsell. 1986. Risk factors for acquiring pneumococcal infections. *Arch.Intern.Med.* 146:2179-2185.

11. Scott, J.A. 2007. The preventable burden of pneumococcal disease in the developing world. *Vaccine.* 25:2398-2405.

12. Van Bambeke, F., R.R. Reinert, P.C. Appelbaum, P.M. Tulkens, and W.E. Peetermans. 2007. Multidrug-resistant Streptococcus pneumoniae infections: current and future therapeutic options. *Drugs.* 67:2355-2382.

13. Schwab, J.A., and M. Zenkel. 1998. Filtration of particulates in the human nose. *Laryngoscope.* 108:120-124.

14. Cantin, A.M. 2001. Biology of respiratory epithelial cells: role in defense against infections. *Pediatr.Pulmonol.* Suppl 23:167-169.

15. Singh, G., and S.L. Katyal. 2000. Clara cell proteins. *Ann.N.Y.Acad.Sci.* 923:43-58.

16. National Center for Environmental Assessment. 1999. Exposure factors handbook CDROM. 99/001

17. Thomas, E.D., R.E. Ramberg, G.E. Sale, R.S. Sparkes, and D.W. Golde. 1976. Direct evidence for a bone marrow origin of the alveolar macrophage in man. *Science.* 192:1016-1018.

18. Zhang, X., and D.M. Mosser. 2008. Macrophage activation by endogenous danger signals. *J.Pathol.* 214:161-178.

19. Knapp, S., J.C. Leemans, S. Florquin, J. Branger, N.A. Maris, J. Pater, N. van Rooijen, and T. van der Poll. 2003. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am.J.Respir.Crit.Care Med.* 167:171-179.

20. Dockrell, D.H., H.M. Marriott, L.R. Prince, V.C. Ridger, P.G. Ince, P.G. Hellewell, and M.K. Whyte. 2003. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J.Immunol.* 171:5380-5388.

21. Opitz, B., A. Puschel, B. Schmeck, A.C. Hocke, S. Rosseau, S. Hammerschmidt, R.R. Schumann, N. Suttorp, and S. Hippenstiel. 2004. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J.Biol.Chem.* 279:36426-36432.

22. Seo, H.S., S.M. Michalek, and M.H. Nahm. 2008. Lipoteichoic acid is important in innate immune responses to gram-positive bacteria. *Infect.Immun.* 76:206-213.

23. Knapp, S., S. von Aulock, M. Leendertse, I. Haslinger, C. Draing, D.T. Golenbock, and T. van der Poll. 2008. Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor. *J.Immunol.* 180:3478-3484.

24. Brikos, C., and L.A. O'Neill. 2008. Signalling of toll-like receptors. *Handb.Exp.Pharmacol.* (183):21-50.

25. Takeda, K., and S. Akira. 2004. TLR signaling pathways. *Semin.Immunol.* 16:3-9.

26. Oshikawa, K., and Y. Sugiyama. 2003. Gene expression of Toll-like receptors and associated molecules induced by inflammatory stimuli in the primary alveolar macrophage. *Biochem.Biophys.Res.Commun.* 305:649-655.

27. Platt, N., and S. Gordon. 2001. Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. *J.Clin.Invest.* 108:649-654.

28. Dunne, D.W., D. Resnick, J. Greenberg, M. Krieger, and K.A. Joiner. 1994. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc.Natl.Acad.Sci.U.S.A.* 91:1863-1867.

29. Todt, J.C., B. Hu, and J.L. Curtis. 2008. The scavenger receptor SR-A I/II (CD204) signals via the receptor tyrosine kinase Mertk during apoptotic cell uptake by murine macrophages. *J.Leukoc.Biol.* 84:510-518.

30. Jozefowski, S., and L. Kobzik. 2004. Scavenger receptor A mediates H2O2 production and suppression of IL-12 release in murine macrophages. *J.Leukoc.Biol.* 76:1066-1074.

31. Kim, W.S., C.M. Ordija, and M.W. Freeman. 2003. Activation of signaling pathways by putative scavenger receptor class A (SR-A) ligands requires CD14 but not SR-A. *Biochem.Biophys.Res.Commun.* 310:542-549.

32. Coller, S.P., and D.M. Paulnock. 2001. Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. *J.Leukoc.Biol.* 70:142-148.

33. Arredouani, M.S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik. 2006. The Macrophage Scavenger Receptor SR-AI/II and Lung Defense against Pneumococci and Particles. *Am.J.Respir.Cell Mol.Biol.*

34. Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik. 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J.Exp.Med.* 200:267-272.

35. Hampton, R.Y., D.T. Golenbock, M. Penman, M. Krieger, and C.R. Raetz. 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature.* 352:342-344.

36. Haworth, R., N. Platt, S. Keshav, D. Hughes, E. Darley, H. Suzuki, Y. Kurihara, T. Kodama, and S. Gordon. 1997. The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *J.Exp.Med.* 186:1431-1439.

37. Amiel, E., A. Alonso, S. Uematsu, S. Akira, M.E. Poynter, and B. Berwin. 2008. Toll-like receptor regulation of scavenger receptor-A-mediated phagocytosis. *J.Leukoc.Biol.*

38. Janeway, C. 2005. Immunobiology : the immune system in health and disease, 6th ed. New York, Garland Science,

39. Hummell, D.S., A.J. Swift, A. Tomasz, and J.A. Winkelstein. 1985. Activation of the alternative complement pathway by pneumococcal lipoteichoic acid. *Infect.Immun.* 47:384-387.

40. Winkelstein, J.A., and A. Tomasz. 1977. Activation of the alternative pathway by pneumococcal cell walls. *J.Immunol.* 118:451-454.

41. Brown, J.S., T. Hussell, S.M. Gilliland, D.W. Holden, J.C. Paton, M.R. Ehrenstein, M.J. Walport, and M. Botto. 2002. The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proc.Natl.Acad.Sci.U.S.A.* 99:16969-16974.

42. Prellner, K. 1981. C1q binding and complement activation by capsular and cell wall components of S. pneumoniae type XIX. *Acta Pathol.Microbiol.Scand.[C].* 89:359-364.

43. Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, F. Genovese, F. Tomasello, T.H. Flo, T. Espevik, and G. Teti. 2000. Beta 2 integrins are involved in cytokine responses to whole Gram-positive bacteria. *J.Immunol.* 164:5871-5876.

44. Rezzonico, R., R. Chicheportiche, V. Imbert, and J.M. Dayer. 2000. Engagement of CD11b and CD11c beta2 integrin by antibodies or soluble CD23 induces IL-1beta production on primary human monocytes through mitogen-activated protein kinase-dependent pathways. *Blood.* 95:3868-3877.

45. Vassallo, G., R.W. Newton, S.E. Chieng, M.R. Haeney, A. Shabani, and P.D. Arkwright. 2007. Clinical variability and characteristic autoantibody profile in primary C1q complement deficiency. *Rheumatology (Oxford).* 46:1612-1614.

46. Roy, S., K. Knox, S. Segal, D. Griffiths, C.E. Moore, K.I. Welsh, A. Smarason, N.P. Day, W.L. McPheat, D.W. Crook, A.V. Hill, and Oxford Pneumoccocal Surveillance Group. 2002. MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet.* 359:1569-1573.

47. Kerr, A.R., G.K. Paterson, A. Riboldi-Tunnicliffe, and T.J. Mitchell. 2005. Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. *Infect.Immun.* 73:4245-4252.

48. Watford, W.T., A.J. Ghio, and J.R. Wright. 2000. Complement-mediated host defense in the lung. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 279:L790-8.

49. Younger, J.G., S. Shankar-Sinha, M. Mickiewicz, A.S. Brinkman, G.A. Valencia, J.V. Sarma, E.M. Younkin, T.J. Standiford, F.S. Zetoune, and P.A. Ward. 2003. Murine complement interactions with Pseudomonas aeruginosa and their consequences during pneumonia. *Am.J.Respir.Cell Mol.Biol.* 29:432-438.

50. Strunk, R.C., D.M. Eidlen, and R.J. Mason. 1988. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J.Clin.Invest.* 81:1419-1426.

51. Hetland, G., E. Johnson, and U. Aasebo. 1986. Human alveolar macrophages synthesize the functional alternative pathway of complement and active C5 and C9 in vitro. *Scand.J.Immunol.* 24:603-608.

52. Zhao, Y.X., A. Andoh, M. Shimada, H. Takaya, K. Hata, Y. Fujiyama, and T. Bamda. 2000. Secretion of complement components of the alternative pathway (C3 and factor B) by the human alveolar type II epithelial cell line A549. *Int.J.Mol.Med.* 5:415-419.

53. Rothman, B.L., A.W. Despins, and D.L. Kreutzer. 1990. Cytokine regulation of C3 and C5 production by the human type II pneumocyte cell line, A549. *J.Immunol.* 145:592-598.

54. Brown, E.J. 1991. Complement receptors and phagocytosis. *Curr.Opin.Immunol.* 3:76-82.

55. Gil, M., F.X. McCormack, and A.M. Levine. 2009. Surfactant protein-A modulates cell surface expression of CR3 on alveolar macrophages and enhances CR3-mediated phagocytosis. *J.Biol.Chem.*

56. Arnaout, M.A. 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood.* 75:1037-1050.

57. Ren, B., M.A. McCrory, C. Pass, D.C. Bullard, C.M. Ballantyne, Y. Xu, D.E. Briles, and A.J. Szalai. 2004. The virulence function of Streptococcus pneumoniae surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *J.Immunol.* 173:7506-7512.

58. Casal, J., and D. Tarrago. 2003. Immunity to Streptococcus pneumoniae: Factors affecting production and efficacy. *Curr.Opin.Infect.Dis.* 16:219-224.

59. AlonsoDeVelasco, E., A.F. Verheul, J. Verhoef, and H. Snippe. 1995. Streptococcus pneumoniae: virulence factors, pathogenesis, and vaccines. *Microbiol.Rev.* 59:591-603.

60. Catterall, J.R. 1999. Streptococcus pneumoniae. *Thorax.* 54:929-937.

61. Mancuso, P., and M. Peters-Golden. 2000. Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C-dependent. *Am.J.Respir.Cell Mol.Biol.* 23:727-733.

62. Yamada, C., H. Sano, T. Shimizu, H. Mitsuzawa, C. Nishitani, T. Himi, and Y. Kuroki. 2006. Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response. Importance of supratrimeric oligomerization. *J.Biol.Chem.* 281:21771-21780.

63. Murakami, S., D. Iwaki, H. Mitsuzawa, H. Sano, H. Takahashi, D.R. Voelker, T. Akino, and Y. Kuroki. 2002. Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-alpha secretion in U937 cells and alveolar macrophages by direct interaction with toll-like receptor 2. *J.Biol.Chem.* 277:6830-6837.

64. Kuroki, Y., M. Takahashi, and C. Nishitani. 2007. Pulmonary collectins in innate immunity of the lung. *Cell.Microbiol.* 9:1871-1879.

65. Morris, D.G., X. Huang, N. Kaminski, Y. Wang, S.D. Shapiro, G. Dolganov, A. Glick, and D. Sheppard. 2003. Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. *Nature.* 422:169-173.

66. Munger, J.S., X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, J.F. Pittet, N. Kaminski, C. Garat, M.A. Matthay, D.B. Rifkin, and D. Sheppard. 1999. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell.* 96:319-328.

67. Ali, F., M.E. Lee, F. Iannelli, G. Pozzi, T.J. Mitchell, R.C. Read, and D.H. Dockrell. 2003. Streptococcus pneumoniae-associated human macrophage apoptosis after bacterial internalization via complement and Fcgamma receptors correlates with intracellular bacterial load. *J.Infect.Dis.* 188:1119-1131.

68. Thomassen, M.J., L.T. Divis, and C.J. Fisher. 1996. Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10. *Clin.Immunol.Immunopathol.* 80:321-324.

69. Mizgerd, J.P. 2002. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin.Immunol.* 14:123-132.

70. van der Poll, T., C.V. Keogh, W.A. Buurman, and S.F. Lowry. 1997. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. *Am.J.Respir.Crit.Care Med.* 155:603-608.

71. Rijneveld, A.W., S. Florquin, J. Branger, P. Speelman, S.J. Van Deventer, and T. van der Poll. 2001. TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. *J.Immunol.* 167:5240-5246.

72. Kafka, D., E. Ling, G. Feldman, D. Benharroch, E. Voronov, N. Givon-Lavi, Y. Iwakura, R. Dagan, R.N. Apte, and Y. Mizrachi-Nebenzahl. 2008. Contribution of IL-1 to resistance to Streptococcus pneumoniae infection. *Int.Immunol.* 20:1139-1146.

73. van der Poll, T., C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, and S.F. Lowry. 1997. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J.Infect.Dis.* 176:439-444.

74. van der Poll, T., A. Marchant, C.V. Keogh, M. Goldman, and S.F. Lowry. 1996. Interleukin-10 impairs host defense in murine pneumococcal pneumonia. *J.Infect.Dis.* 174:994-1000.

75. Spellberg, B., and J.E. Edwards Jr. 2001. Type 1/Type 2 immunity in infectious diseases. *Clin.Infect.Dis.* 32:76-102.

76. Rubins, J.B., and C. Pomeroy. 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect.Immun.* 65:2975-2977.

77. Rijneveld, A.W., F.N. Lauw, M.J. Schultz, S. Florquin, A.A. Te Velde, P. Speelman, S.J. Van Deventer, and T. Van Der Poll. 2002. The role of interferon-gamma in murine pneumococcal pneumonia. *J.Infect.Dis.* 185:91-97.

78. Lauw, F.N., J. Branger, S. Florquin, P. Speelman, S.J. van Deventer, S. Akira, and T. van der Poll. 2002. IL-18 improves the early antimicrobial host response to pneumococcal pneumonia. *J.Immunol.* 168:372-378.

79. Yamamoto, N., K. Kawakami, Y. Kinjo, K. Miyagi, T. Kinjo, K. Uezu, C. Nakasone, M. Nakamatsu, and A. Saito. 2004. Essential role for the p40 subunit of interleukin-12 in neutrophil-mediated early host defense against pulmonary infection with Streptococcus pneumoniae: involvement of interferon-gamma. *Microbes Infect.* 6:1241-1249.

80. Paterson, G.K., C.E. Blue, and T.J. Mitchell. 2005. Role of interleukin-18 in experimental infections with Streptococcus pneumoniae. *J.Med.Microbiol.* 54:323-326.

81. Craig, A., J. Mai, S. Cai, and S. Jeyaseelan. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect.Immun.* 77:568-575.

82. Wartha, F., K. Beiter, S. Normark, and B. Henriques-Normark. 2007. Neutrophil extracellular traps: casting the NET over pathogenesis. *Curr.Opin.Microbiol.* 10:52-56.

83. Beiter, K., F. Wartha, B. Albiger, S. Normark, A. Zychlinsky, and B. Henriques-Normark. 2006. An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. *Curr.Biol.* 16:401-407.

84. Wartha, F., K. Beiter, B. Albiger, J. Fernebro, A. Zychlinsky, S. Normark, and B. Henriques-Normark. 2007. Capsule and D-alanylated lipoteichoic acids protect

Streptococcus pneumoniae against neutrophil extracellular traps. *Cell.Microbiol.* 9:1162-1171.

85. Garvy, B.A., and A.G. Harmsen. 1996. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation.* 20:499-512.

86. Shatwell, K.P., and A.W. Segal. 1996. NADPH oxidase. *Int.J.Biochem.Cell Biol.* 28:1191-1195.

87. Gwinn, M.R., and V. Vallyathan. 2006. Respiratory burst: role in signal transduction in alveolar macrophages. *J.Toxicol.Environ.Health B Crit.Rev.* 9:27-39.

88. Forman, H.J., and M. Torres. 2001. Signaling by the respiratory burst in macrophages. *IUBMB Life.* 51:365-371.

89. Blander, J.M., and R. Medzhitov. 2004. Regulation of phagosome maturation by signals from toll-like receptors. *Science.* 304:1014-1018.

90. Kerr, A.R., X.Q. Wei, P.W. Andrew, and T.J. Mitchell. 2004. Nitric oxide exerts distinct effects in local and systemic infections with Streptococcus pneumoniae. *Microb.Pathog.* 36:303-310.

91. Greenberg, D., N. Givon-Lavi, A. Broides, I. Blancovich, N. Peled, and R. Dagan. 2006. The contribution of smoking and exposure to tobacco smoke to Streptococcus pneumoniae and Haemophilus influenzae carriage in children and their mothers. *Clin.Infect.Dis.* 42:897-903.

92. Pastor, P., F. Medley, and T.V. Murphy. 1998. Invasive pneumococcal disease in Dallas County, Texas: results from population-based surveillance in 1995. *Clin.Infect.Dis.* 26:590-595.

93. Nuorti, J.P., J.C. Butler, M.M. Farley, L.H. Harrison, A. McGeer, M.S. Kolczak, and R.F. Breiman. 2000. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N.Engl.J.Med.* 342:681-689.

94. Almirall, J., I. Bolibar, M. Serra-Prat, J. Roig, I. Hospital, E. Carandell, M. Agusti, P. Ayuso, A. Estela, A. Torres, and Community-Acquired Pneumonia in Catalan Countries (PACAP) Study Group. 2008. New evidence of risk factors for community-acquired pneumonia: a population-based study. *Eur.Respir.J.* 31:1274-1284.

95. Almirall, J., C.A. Gonzalez, X. Balanzo, and I. Bolibar. 1999. Proportion of community-acquired pneumonia cases attributable to tobacco smoking. *Chest.* 116:375-379.

96. Arcavi, L., and N.L. Benowitz. 2004. Cigarette smoking and infection. *Arch.Intern.Med.* 164:2206-2216.

97. Slama, K., C.Y. Chiang, D.A. Enarson, K. Hassmiller, A. Fanning, P. Gupta, and C. Ray. 2007. Tobacco and tuberculosis: a qualitative systematic review and meta-analysis. *Int.J.Tuberc.Lung Dis.* 11:1049-1061.

98. Kark, J.D., M. Lebiush, and L. Rannon. 1982. Cigarette smoking as a risk factor for epidemic a(h1n1) influenza in young men. *N.Engl.J.Med.* 307:1042-1046.

99. Straus, W.L., J.F. Plouffe, T.M. File Jr, H.B. Lipman, B.H. Hackman, S.J. Salstrom, R.F. Benson, and R.F. Breiman. 1996. Risk factors for domestic acquisition of legionnaires disease. Ohio legionnaires Disease Group. *Arch.Intern.Med.* 156:1685-1692.

100. Blake, G.H., T.D. Abell, and W.G. Stanley. 1988. Cigarette smoking and upper respiratory infection among recruits in basic combat training. *Ann.Intern.Med.* 109:198-202.

101. Bateson, M.C. 1993. Cigarette smoking and Helicobacter pylori infection. *Postgrad.Med.J.* 69:41-44.

102. Fischer, M., K. Hedberg, P. Cardosi, B.D. Plikaytis, F.C. Hoesly, K.R. Steingart, T.A. Bell, D.W. Fleming, J.D. Wenger, and B.A. Perkins. 1997. Tobacco smoke as a risk factor for meningococcal disease. *Pediatr.Infect.Dis.J.* 16:979-983.

103. Wolf, R., and D. Freedman. 2000. Cigarette smoking, sexually transmitted diseases, and HIV/AIDS. *Int.J.Dermatol.* 39:1-9.

104. Wilson, R. 2000. Evidence of bacterial infection in acute exacerbations of chronic bronchitis. *Semin.Respir.Infect.* 15:208-215.

105. National Heart, Lung, and Blood Institute. 19uu. Morbidity and mortality : chartbook on cardiovascular, lung, and blood diseases.

106. Rabe, K.F., S. Hurd, A. Anzueto, P.J. Barnes, S.A. Buist, P. Calverley, Y. Fukuchi, C. Jenkins, R. Rodriguez-Roisin, C. van Weel, J. Zielinski, and Global Initiative for Chronic Obstructive Lung Disease. 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am.J.Respir.Crit.Care Med.* 176:532-555.

107. Decramer, M., L. Nici, S. Nardini, J. Reardon, C.L. Rochester, C.M. Sanguinetti, and T. Troosters. 2008. Targeting the COPD exacerbation. *Respir.Med.* 102 Suppl 1:S3-15.

108. Papi, A., C.M. Bellettato, F. Braccioni, M. Romagnoli, P. Casolari, G. Caramori, L.M. Fabbri, and S.L. Johnston. 2006. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am.J.Respir.Crit.Care Med.* 173:1114-1121.

109. Karadag, F., A.B. Karul, O. Cildag, M. Yilmaz, and H. Ozcan. 2008. Biomarkers of systemic inflammation in stable and exacerbation phases of COPD. *Lung.* 186:403-409.

110. Curtis, J.L., C.M. Freeman, and J.C. Hogg. 2007. The immunopathogenesis of chronic obstructive pulmonary disease: insights from recent research. *Proc.Am.Thorac.Soc.* 4:512-521.

111. Bogaert, D., P. van der Valk, R. Ramdin, M. Sluijter, E. Monninkhof, R. Hendrix, R. de Groot, and P.W. Hermans. 2004. Host-pathogen interaction during pneumococcal infection in patients with chronic obstructive pulmonary disease. *Infect.Immun.* 72:818-823.

112. Martinez, F.J., M.K. Han, K. Flaherty, and J. Curtis. 2006. Role of infection and antimicrobial therapy in acute exacerbations of chronic obstructive pulmonary disease. *Expert Rev.Anti Infect.Ther.* 4:101-124.

113. Nichol, K.L., L. Baken, J. Wuorenma, and A. Nelson. 1999. The health and economic benefits associated with pneumococcal vaccination of elderly persons with chronic lung disease. *Arch.Intern.Med.* 159:2437-2442.

114. Churg, A., M. Cosio, and J.L. Wright. 2008. Mechanisms of cigarette smoke-induced COPD: insights from animal models. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 294:L612-31.

115. Huang, J., M. Okuka, M. McLean, D.L. Keefe, and L. Liu. 2008. Effects of cigarette smoke on fertilization and embryo development in vivo. *Fertil.Steril.*

116. Slotkin, T.A. 2002. Nicotine and the adolescent brain: insights from an animal model. *Neurotoxicol.Teratol.* 24:369-384.

117. Sayers, N.M., and D.B. Drucker. 1999. Animal models used to test the interactions between infectious agents and products of cigarette smoked implicated in sudden infant death syndrome. *FEMS Immunol.Med.Microbiol.* 25:115-123.

118. Phaybouth, V., S.Z. Wang, J.A. Hutt, J.D. McDonald, K.S. Harrod, and E.G. Barrett. 2006. Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 290:L222-31.

119. Gualano, R.C., M.J. Hansen, R. Vlahos, J.E. Jones, R.A. Park-Jones, G. Deliyannis, S.J. Turner, K.A. Duca, and G.P. Anderson. 2008. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir.Res.* 9:53.

120. Christensen, P.J., A.M. Preston, T. Ling, M. Du, W.B. Fields, J.L. Curtis, and J.M. Beck. 2008. Pneumocystis murina infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement, and pulmonary inflammation in mice. *Infect.Immun.* 76:3481-3490.

121. Drannik, A.G., M.A. Pouladi, C.S. Robbins, S.I. Goncharova, S. Kianpour, and M.R. Stampfli. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am.J.Respir.Crit.Care Med.* 170:1164-1171.

122. Vander Top, E.A., G.A. Perry, M.U. Snitily, and M.J. Gentry-Nielsen. 2006. Smoke exposure and ethanol ingestion modulate intrapulmonary polymorphonuclear leukocyte killing, but not recruitment or phagocytosis. *Alcohol.Clin.Exp.Res.* 30:1599-1607.

123. Houghton, A.M., P.A. Quintero, D.L. Perkins, D.K. Kobayashi, D.G. Kelley, L.A. Marconcini, R.P. Mecham, R.M. Senior, and S.D. Shapiro. 2006. Elastin fragments drive disease progression in a murine model of emphysema. *J.Clin.Invest.* 116:753-759.

124. Valenca, S.S., and L.C. Porto. 2008. Immunohistochemical study of lung remodeling in mice exposed to cigarette smoke*. *J.Bras.Pneumol.* 34:787-795.

125. Valenca, S.S., F. Silva Bezerra, A.A. Lopes, B. Romana-Souza, M.C. Marinho Cavalcante, A.B. Lima, V.L. Goncalves Koatz, and L.C. Porto. 2008. Oxidative stress in mouse plasma and lungs induced by cigarette smoke and lipopolysaccharide. *Environ.Res.* 108:199-204.

126. Droemann, D., T. Goldmann, T. Tiedje, P. Zabel, K. Dalhoff, and B. Schaaf. 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir.Res.* 6:68.

127. Chen, H., M.J. Cowan, J.D. Hasday, S.N. Vogel, and A.E. Medvedev. 2007. Tobacco smoking inhibits expression of proinflammatory cytokines and activation of IL-1R-associated kinase, p38, and NF-kappaB in alveolar macrophages stimulated with TLR2 and TLR4 agonists. *J.Immunol.* 179:6097-6106.

128. Hodge, S., G. Hodge, J. Ahern, H. Jersmann, M. Holmes, and P.N. Reynolds. 2007. Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am.J.Respir.Cell Mol.Biol.* 37:748-755.

129. Gaschler, G.J., C.C. Zavitz, C.M. Bauer, M. Skrtic, M. Lindahl, C.S. Robbins, B. Chen, and M.R. Stampfli. 2008. Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages. *Am.J.Respir.Cell Mol.Biol.* 38:218-226.

130. Ortega, E., F. Hueso, M.E. Collazos, M.I. Pedrera, C. Barriga, and A.B. Rodriguez. 1992. Phagocytosis of latex beads by alveolar macrophages from mice exposed to cigarette smoke. *Comp.Immunol.Microbiol.Infect.Dis.* 15:137-142.

131. Ortega, E., C. Barriga, and A.B. Rodriguez. 1994. Decline in the phagocytic function of alveolar macrophages from mice exposed to cigarette smoke. *Comp.Immunol.Microbiol.Infect.Dis.* 17:77-84.

132. King, T.E.,Jr, D. Savici, and P.A. Campbell. 1988. Phagocytosis and killing of Listeria monocytogenes by alveolar macrophages: smokers versus nonsmokers. *J.Infect.Dis.* 158:1309-1316.

133. Barnes, P.J. 2004. Alveolar macrophages as orchestrators of COPD. *COPD.* 1:59-70.

134. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. 2004. Tobacco smoke and involuntary smoking. *IARC Monogr.Eval.Carcinog.Risks Hum.* 83:1-1438.

CHAPTER 2

Low and regular tar cigarette smoke impairs alveolar macrophage TNF-α and PGE_2 synthesis and phagocytosis of opsonized bacteria

Introduction

Cigarette smoking is a well-known cause of lung cancer and chronic obstructive pulmonary disease (1). It is also a significant cause of pulmonary infections due to its ability to suppress the immune response (2, 3). Cigarette smoke (CS) exposure suppresses the host defense mechanisms of the upper and lower respiratory tract and is associated with an increased rate of colonization with *Streptococcus pneumoniae* (4). Pneumonia caused by *S. pneumoniae*, or pneumococcal pneumonia, is the most common cause of community-acquired pneumonia and is the leading cause of death from invasive bacterial infections (5, 6). In a study by Nuorti et al., exposure to CS was the single most important controllable risk factor for invasive pneumococcal disease in the immunocompetent non-elderly host (7). While the association between cigarette smoke exposure and an increased incidence of bacterial pneumonia is well established, much remains unknown regarding the mechanisms by which cigarette smoking impairs pulmonary host defense.

Cigarette smoke exposure suppresses the antibacterial activities of the alveolar macrophage (AM) through mechanisms that are poorly understood (8-10) The limited

numbers of studies that have assessed the impact of cigarette smoke exposure on relevant AM functional endpoints have not provided consistent results. For example, a number of investigators have demonstrated that cigarette smoke exposure inhibits the elaboration of cytokines (TNF-α, IL-1β, IL-6, and IL-12) and lipid mediators (PGE_2) (11, 9, 12, 13). In contrast, others have shown that cells obtained from smokers or cells exposed to cigarette smoke conditioned media (CSCM) in vitro produce increased levels of proinflammatory cytokines and PGE_2 (14-16).

Tobacco smoke is a complex mixture of over 4800 chemicals and little is known regarding the immunosuppressive effects of these compounds. The tar fraction of cigarette smoke is a non-aqueous component of cigarette smoke condensate that has received considerable attention in regard to its role in lung cancer (17). Light, ultra-light, or low tar cigarettes, which account for 82% of cigarette sales in the U.S., have been erroneously perceived by smokers as a less hazardous product that could be used as a step toward cessation (18, 19). Surprisingly, there are very few studies that have compared the effects of low tar (LT) and regular tar (RT) cigarette smoke on health outcomes in human subjects and in animal models of cigarette smoke-induced lung disease (19-23). There are no studies, to our knowledge, that have explored the effects of LT and RT cigarette smoke on AM innate immune responses against *S. pneumoniae*. In the present study, we tested the hypothesis that the tar content of cigarettes might play an important role in modulating AM inflammatory mediator synthesis and phagocytosis of *S. pneumoniae* in vitro.

Methods

Animals

Specific pathogen-free male C57BL/6 mice, 8-12 weeks of age, were obtained from Jackson Laboratories, (Barharbor, ME) and female Wistar rats, weighing 125-150g, were acquired from Charles River Laboratories, (Portage, MI). All experiments were conducted in compliance with the Animal Care and Use Committee of the University of Michigan.

Cigarette smoke exposure

Mice were exposed to main-stream cigarette smoke from regular tar (RT) (2R4F, 9.7 mg of tar per cigarette) or low tar (LT) (1R5F, 1.7 mg of tar per cigarette) (University of Kentucky, Lexington, KY) cigarettes generated by a smoking machine, mixed with room air as previously described (24), and adapted for mice (25) 4 h per day, 5 days per wk for 2 weeks in a whole-body exposure chamber. During exposure, the mice were in standard caging units with wire tops and water *ad libidum*, however food and bedding were removed to reduce non-respiratory routes of exposure. Control animals were in identical caging units, but exposed to room air rather than CS. Total suspended dry particulate mass during the 4 h exposures was 1.8 mg/m^3 for RT and 1.7 for mg/m^3 LT. For the short-term (1 wk), high-dose, tobacco smoke exposure experiments, cigarette smoke was generated from RT cigarettes using a TE-2 (Teague Enterprises, Davis, CA) smoke generator at a concentration of 50 mg/m^3, with controls housed as above.

AM harvest and culture

AMs were obtained by bronchoalveolar lavage (BAL) as previously described from rats or mice exposed to tobacco smoke for 2 weeks or room air (26). After lavage fluid centrifugation at 4°C at 400 *g* for 5 min, the cell pellet was resuspended in Hanks' balanced salt solution (HBSS, GIBCO, Grand Island, NY), and the cells were enumerated using a hemocytometer. The cells were centrifuged a second time and resuspended in RPMI 1640 (GIBCO) to a concentration of 5×10^5 cells/ml. Murine or rat AMs were adhered to cell culture wells (2×10^5/well) in RPMI 1640 for 1 h. After 1 h, the cell culture medium was replaced with complete medium (RPMI containing 10% fetal bovine serum and 1% penicillin/streptomycin, GIBCO), and the macrophages were cultured for 16 h.

Lung leukocyte isolation and assessment

Following exposure to tobacco smoke or room air, lungs were excised from each mouse, washed with PBS, minced with scissors, and digested enzymatically for 30 min in 15 ml digestion buffer (RPMI 1640, 5% FCS, 1 mg/ml collagenase (Boehringer Mannheim, Chicago, IL) and 30 µg/ml DNase (Sigma-Aldrich, St. Louis, MO), as previously described (27). A purified population of leukocytes was obtained after subsequent sample processing involving tissue fragmentation, erythrocyte lysis, filtration, and density gradient centrifugation to remove cell debris and epithelial cells. The total number of viable lung leukocytes was determined by trypan blue exclusion using a Neubauer hemocytometer. Differential counts were determined after cells had been cytospun onto glass slides using a cytocentrifuge (Cytofuge 2, StatSpin, Inc, Norwood, MA) and stained using a modified Wright-Giemsa stain (DiffQuick; American Scientific

Products, McGraw Park, IL). A total of 200–400 cells were counted from randomly chosen fields using light microscopy (x1000). The total number of cells of a particular leukocyte subset was determined by multiplying the percentage of each population in a particular sample by the total number of lung leukocytes collected from each mouse.

Preparation of CSCM

CSCM was prepared by drawing (bubbling) the smoke from five 2R4F cigarettes, secured to the inlet port of a glass impinger (Ace Glass, Vineland, NJ), through 50 ml of RPMI 1640 contained in a reservoir. Aliquots of the CSCM were stored at –70°C.

In vitro CSCM exposure

Rat AMs (2×10^5 per well) were cultured over night with 1% CSCM in complete medium. Using the XTT assay (Roche Diagnostics, Indianapolis, IN), we observed that culturing AMs with 1% CSCM, as compared with media alone, did not significantly influence cell viability (data not shown).

Stimulation of AMs with lipoteichoic acid or heat killed Streptococcus pneumoniae for cytokine and PGE_2 synthesis

Isolated leukocytes recovered from collagenase digest of the lungs of mice exposed to either room air or tobacco smoke generated from RT, or LT cigarettes and 1×10^6 cells were adhered to cell culture dishes for 1 h. Following adherence, the non-adherent cells were washed away and the cells were cultured with complete media alone or with heat-killed *S. pneumoniae* (4×10^8/well) [serotype 3, (ATCC 6303, American

Type Culture Collection, Manassas, VA)], for 16 h at 37°C with 5% CO_2 in room air. Rat AMs were stimulated to produce TNF-α using 10 μg/ml lipoteichoic acid derived from *S. aureus* (Sigma, St. Louis, MO) in the presence or absence of 1% CSCM and N-acetyl cysteine (NAC) diluted in cell culture media (Calbiochem, San Diego, CA). Aliquots of cell culture supernatants were collected from each well and stored at –70°C.

Cytokine and PGE_2 determinations

Cytokines (TNF-α and IL-6) and PGE_2 were determined in cell culture supernatants using commercially-available enzyme-linked immunoassay kits (Assay Designs, Ann Arbor, MI).

Phagocytosis Assays

Phagocytosis of fluorescently-labeled *S. pneumoniae* was performed using the methods as previously described (Mancuso et al., 2006). In brief, 2 x 10^5 AMs were incubated with 2 x 10^6 CFUs of fluorescently labeled *S.pneumoniae* with 2.5% autologous serum in RPMI (total volume 1 ml) for 30 min. The percentage of AMs containing fluorescent *S. pneumoniae* was determined by counting 200 macrophages in random fields using fluorescent microscopy (1000x) by an observer who was blinded to the identity of the samples. The data was normalized to the values obtained for the percentage of AMs with bacteria of the control animals.

Phagocytosis of FITC-labeled *Escherichia coli (E.coli)* was performed with rat AMs cultured with and without 1% CSCM and NAC (0.5 mM) overnight. On the following day, the AMs were assessed for their ability to phagocytose IgG-opsonized

FITC-labeled *E.coli* using the Bioparticle phagocytosis kit (Invitrogen, Molecular Probes, Eugene, OR) as previously described. The data was normalized to the values obtained for AMs cultured in medium without CSCM.

Statistical analysis

Where appropriate, mean values were compared using a paired t-test or Kruskal-Wallis test on ranks from nonparametric data. In all cases, a P value of <0.05 was considered significant.

Results

Effect of cigarette smoke exposure on lung cell populations obtained from BAL fluid and collagenase digest of the lungs of mice

As shown in Figure 2.1, the majority (>95%) of cells obtained from the BAL fluid of mice exposed to either room air or tobacco smoke from either RT or LT cigarettes were identified as AMs and the total number and each subtype did not differ among treatment groups. While there was a trend for lower numbers of AMs in mice exposed to RT tobacco smoke, this difference can be explained by the variability in the total number of cell harvested from each mouse. Using the collagenase digest the of lung procedure, we assessed the effects of tobacco smoke on the total lung leukocyte population. We did not observe a significant difference between any of the treatment groups in either the total number or subtype of lung leukocytes.

Both RT and LT cigarette smoke exposure impair lung leukocyte TNF-α and PGE_2 synthesis

As the synthesis of cytokines TNF-α and IL-6 synthesis is required for an effective innate immune response against *S. pneumoniae*, and emerging evidence supports a role for PGE_2 in regulating pulmonary innate immunity (28-30), we assessed the ability of lung leukocytes recovered from mice exposed to either room air or tobacco smoke generated from RT or LT cigarettes to produce TNF-α, IL-6 and PGE_2 in the absence and presence of heat-killed bacteria. As shown in Figure 2.2, TNF-α production was absent in lung leukocytes that were cultured with medium alone. However, when these cells were cultured with heat-killed *S. pneumoniae,* we observed a robust production of this cytokine in AMs obtained from mice exposed to room air. This response was reduced by approximately 30% in AMs from either RT or LT tobacco smoke exposure. IL-6 production was reduced in lung leukocytes obtained from mice exposed to RT tobacco smoke with and without heat-killed *S. pneumoniae*.

Interestingly, IL-6 production, with and without heat killed *S. pneumoniae*, was not affected by LT tobacco smoke exposure. We next evaluated the production of PGE_2 by lung leukocytes. This eicosanoid has been shown to suppress the ability of macrophages to produced TNF-α (31). We observed that the production of PGE_2 by lung leukocytes recovered from mice exposed to tobacco smoke from either RT or LT cigarettes was significantly reduced following culture with heat killed-*S. pneumoniae*. Finally, we observed no differences in cell viability among our treatment groups suggesting that this does not explain the effects of tobacco smoke exposure on lung inflammatory mediator production (data not shown).

Cigarette smoke exposure impairs murine AM phagocytosis of S. pneumoniae but not IgG-coated microspheres

The AM plays a critical role in the defense of the lung, clearing microbial pathogens through the process of phagocytosis and killing. To determine if cigarette smoke affects the capacity of AMs to phagocytose *S. pneumoniae*, we compared the phagocytic ability of AMs recovered from mice exposed to the smoke generated from either RT or LT cigarettes in vitro. As mentioned in the methods section of this report, mice were exposed to equal amounts of smoke generated from these cigarettes as indicated by the amount of particulate collected during the exposure periods. As shown in Figure 2.3, both RT and LT cigarette smoke exposure for 2 wks, as compared with exposure to room air (control), reduced the ability of AMs to phagocytose *S. pneumoniae*. The ability of AMs to phagocytose bacteria was reduced by approximately 50% in the RT and 40% in the LT treatment groups.

Next, we examined whether a higher level of cigarette smoke (50 mg/m^3 for 4 h) exposure administered during a shorter period of time (1 wk) could reduce phagocytosis of *S. pneumoniae.* As shown in Figure 2.4, as compared with the control, 30% fewer AMs from mice exposed to a high-dose of RT smoke were able to phagocytose serum opsonized *S. pneumoniae*. To determine whether the effect of RT smoke was specific for phagocytosis of serum opsonized bacteria or affected the AM's ability to phagocytose other targets, we assessed AM Fcγ receptor-mediated phagocytosis by determining the ability of AMs to phagocytose inert targets (polystyrene microspheres) coated with the immunoglobulin G (IgG). In contrast to serum opsonized *S. pneumoniae*, there was no difference between our treatment groups in AM phagocytosis of IgG-opsonized microspheres.

CSCM impairs AM phagocytosis of IgG-opsonized FITC-labeled E.coli

As an alternative approach to assess the ability of tobacco smoke exposure to impair AM Fcγ receptor-mediated phagocytosis, we next assessed AM phagocytosis of FITC-labeled *E.coli* opsonized with IgG following treatment with CSCM (Figure 2.5). We used rat AMs for these studies since much greater quantities of these cells can be obtained per animal and much is known about the regulation of Fcγ receptor-mediated phagocytosis in these cells (28, 32-34). We also employed the use of CSCM, which is a common in vitro model for cigarette smoke exposure. This in vitro treatment with CSCM also permitted us to culture cells in the presence of NAC to determine if these agents could block the immunosuppressant effects of tobacco smoke exposure. A previous report by Green indicated that the addition of glutathione or cysteine to AMs inhibits the reduction in phagocytosis caused by CSCM (35). As shown in Figure 2.5, culturing AMs overnight with 1% CSCM impaired phagocytosis of FITC-labeled *E.coli* by approximately 45%. Culturing the AMs with CSCM and NAC, at doses previously shown to maintain normal levels of reduced glutathione levels (NAC) did not restore phagocytosis. The inhibitory effect of CSCM was not due to differences between our treatment groups in cell viability as determined by the XTT assay (data not shown). Together with Figure 2.4, the finding that CSCM impairs the ability of AMs to phagocytose opsonized bacteria rather than an inert target opsonized with IgG suggests that CSCM inhibits the ability of AMs to respond to bacteria in vitro.

CSCM induced impairment of AM TNF-α synthesis is not reversed with NAC in vitro.

We have already observed a reduction in TNF-α production by lung leukocytes obtained from mice exposed to RT tobacco smoke following stimulation with heat-killed *S. pneumoniae*. In the next set of experiments, we asked if NAC could restore TNF-α synthesis in AMs cultured with CSCM. We also used the TLR2 ligand, lipoteichoic acid, a component of the cell wall of gram-positive bacteria, to stimulate these cells to produce TNF-α since TLR2 expression is reduced in the AMs of smokers (36). As shown in Figure 2.6, culturing AMs with CSCM inhibited TNF-α synthesis by approximately 50%. Culturing these cells with CSCM and NAC did not restore TNF-α synthesis. This result suggests that CSCM inhibits cytokine synthesis via a TLR2 dependent mechanism.

Discussion

The AM plays a critical role in orchestrating the innate immune response against bacteria that reach the alveolar surface. The population of these cells increases up to 5-fold in human cigarette smokers (37). Despite this fact, smokers exhibit an increased risk of pneumococcal pneumonia (38). In the present study, we did not observe any changes in the total number of leukocytes obtained from BAL fluid or collagenase digest of the lungs after two weeks of either tobacco smoke or room air exposure. Although others have observed increases in macrophages, neutrophils, and lymphocytes using a longer duration or higher level of smoke exposure (39, 40), our findings suggest that the alterations in AM phagocytosis of bacteria and cytokine and PGE_2 production are not due to changes in the population of cells recruited to the lungs in response to tobacco smoke exposure.

The production of inflammatory cytokines such as TNF-α and IL-6 during pneumococcal pneumonia is known to play a critical role in host defense (29, 30, 41). While a number of studies have demonstrated that tobacco smoke decreases alveolar macrophage cytokine production, very few studies have compared the effects of tar levels in cigarettes on this response. The reduction in TNF-α and IL-6 following RT tobacco smoke and CSCM exposure in lung leukocytes and AMs, respectively, is in agreement with previous reports (12, 13). However, we also found that LT smoke exposure reduced TNF-α but not IL-6 synthesis in lung leukocytes. This finding is novel and suggests that LT tobacco smoke lacks a component that is present in the tar fraction cigarette smoke that inhibits an essential component of IL-6 activation or synthesis. However, this result does not suggest that LT, as compared with RT, cigarette smoke exposure is less immunosuppressive since we observed no differences in other endpoints.

PGE_2 is a potent immunomodulatory lipid derived from the cyclooxygenase pathway of arachidonic acid metabolism known to inhibit TNF-α synthesis in macrophages (31). Prostaglandin production is significantly induced at sites of inflammation by the rapid up-regulation of enzymes such as cyclooxygenase-2 and microsomal PGE_2 synthase-1 (42). Exposure of human fibroblasts to CSCMs has also been shown to enhance the expression of cyclooxygenase-2, microsomal PGE_2 synthase-1, and subsequent PGE_2 synthesis.(43). In addition, Mao and colleagues have demonstrated that AMs recovered from active smokers produce greater amounts of PGE_2 following stimulation with a calcium ionophore (15). Our observation that PGE_2 synthesis was reduced in lung leukocytes recovered from mice exposed both LT and RT tobacco smoke indicates that the suppression of TNF-α synthesis was not due to

enhanced prostanoid synthesis. This seems to contradict previous reports by Martey and Mao. However, in our experiments, heat-killed *S. pneumoniae*, known to activate macrophages via the toll-like receptors 2 and 4, was employed to stimulate cytokine and PGE_2 in lung leukocytes (44, 45) . We speculate that the observed reduction in TNF-α and PGE_2 synthesis following tobacco smoke exposure may have been due to a reduction in the expression of toll-like receptors 2 whose expression in AMs is reduced in cigarette smokers (36). Our results showing that CSCM inhibits TNF-α synthesis in AMs stimulated with the TLR2 agonist, LTA, supports this speculation.

Our results showing similar suppression of AM phagocytosis of *S. pneumoniae* following exposure to either RT or LT smoke confirms a previous report by Gonzalez-Rothi and Harris who observed that phagocytosis of *S. cerevisase* was impaired in AMs obtained from rats exposed to the smoke generated from low yield cigarettes (1.7 mg of tar) (20). In this study, the vents in the cigarettes were sealed to ensure that all of the smoke generated from these cigarettes reached the exposure chamber and to simulate the compensatory behavior of humans who smoke low tar cigarettes and block the vents with their fingers or lips to increase the yield of smoke from these exposures (46). In our experiments, mice were exposed to nearly identical levels of particulates generated from each type of cigarette (RT 1.8 and LT 1.7 mg/m^3). These results indicate that tobacco smoke exposure, from either RT or LT cigarettes, would equally impair AM function in human smokers.

Normal murine serum contains complement and many other potential opsonins. Upon activation of the alternative complement cascade, complement fragments C3b and

iC3b bind to bacterial surfaces. Phagocytosis of serum opsonized *S. pneumoniae* is mediated by complement receptors known to be expressed by murine AMs such as CR1, CR3, and CR4 (47). We observed an impairment in AM phagocytosis of *S. pneumoniae* following two weeks of tobacco smoke exposure, and exposure to high levels of smoke after one week, suggesting a rapid impairment of complement receptor mediated phagocytosis. Keast and Taylor have also reported a similar reduction in complement mediated phagocytosis of *S. aureus* using PMNs from mice exposed to high tar tobacco smoke (48). In contrast, a study by Klut and co-workers demonstrated that CR3 (CD11b/CD18) expression increased in intravascular pulmonary neutrophils (PMN) following tobacco smoke exposure in rabbits (49).

Cigarette smoke has been shown to induce oxidative stress and produce free radicals (50, 51). We attempted to block the CSCM induced oxidative stress using a dose of NAC that has been shown to maintain intracellular levels of reduced glutathione (16, 50). Nether of these treatments could block the suppressive effects of CSCM on TNF-α synthesis or phagocytosis. These data imply that the oxidative stress induced by CSCM could not be blocked by NAC or that CSCM inhibits AM cytokine synthesis and phagocytosis through mechanisms that are independent of oxidative stress.

While we have demonstrated that smoke exposure reduced phagocytosis of serum opsonized *S. pneumoniae*, we also found no deficiency in the ability of AMs from tobacco smoke exposed mice to phagocytosis IgG-coated microspheres. This result does not imply that tobacco smoke exposure selectively impairs phagocytosis of serum opsonized *S. pneumoniae* since we observed an impairment in AM phagocytosis of

FITC-labeled IgG opsonized *E.coli* following overnight exposure to CSCM in vitro. However, it may indicate that moieties, such as lipopolysaccharides, lipoteichoic acid, and muramyl peptides, that are present on the surface of *E. coli* and *S. pneumoniae* (respectively) and absent in IgG-coated microspheres, may play an important role in enhancing the ability of AMs to phagocytose *S. pneumoniae*. In fact, phagocytosis of *S.pneumoniae* is delayed in PMNs obtained from TLR2 deficient mice indicating an important role of TLR2 in the activation of the phagocytic response (52). Based on our results, future investigation, regarding the mechanism by which tobacco smoke exposure impairs AM phagocytosis of *S. pneumoniae*, is warranted.

In summary, we have observed similar impairments in AM cytokine and PGE_2 synthesis and phagocytosis of bacteria following exposure to either RT and LT tobacco smoke in vivo suggesting no health benefit from smoking low tar or "light" cigarettes. Finally, the observed defects in AM function in our studies may provide a partial explanation for the increased incidence of pneumococcal pneumonia in smokers.

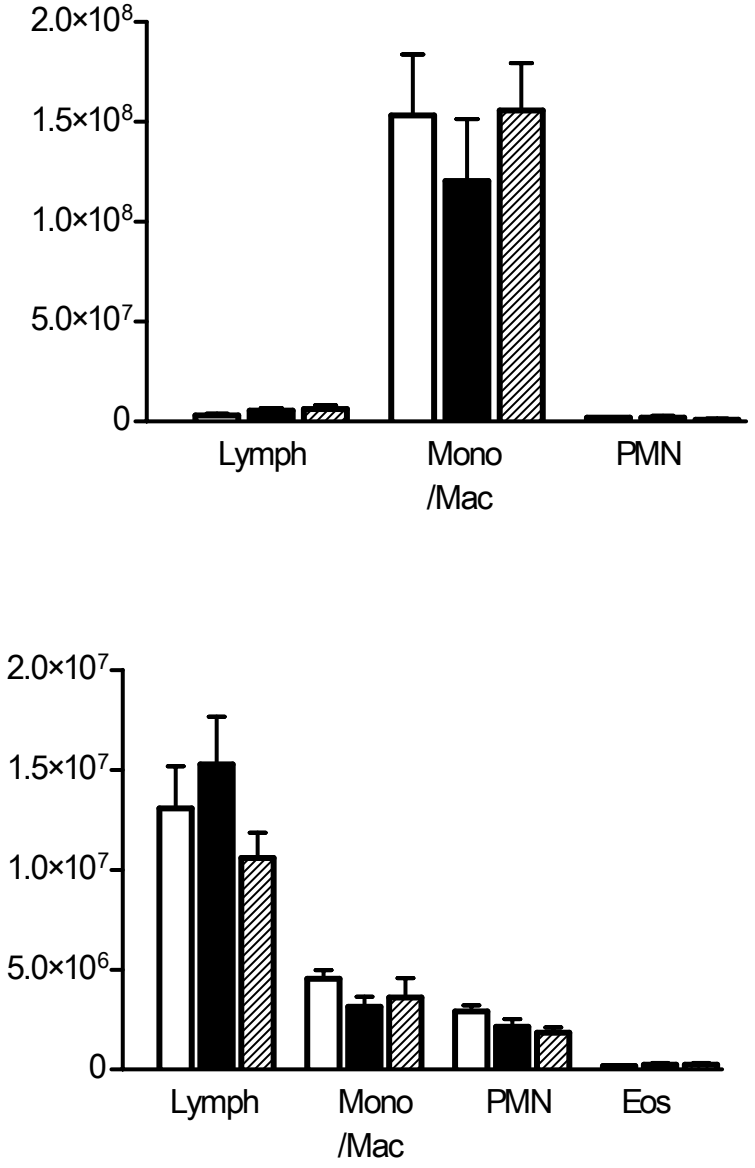

Figure 2.1. Composition of pulmonary leukocyte populations following 2 weeks of CS exposure. Lung leukocyte subtype counts obtained from bronchoalveolar lavage fluid (A) or collagenase digest of the lungs (B) in mice following 2 weeks of exposure to room air (control), smoke generated from regular tar (RT), or low tar (LT) cigarettes.

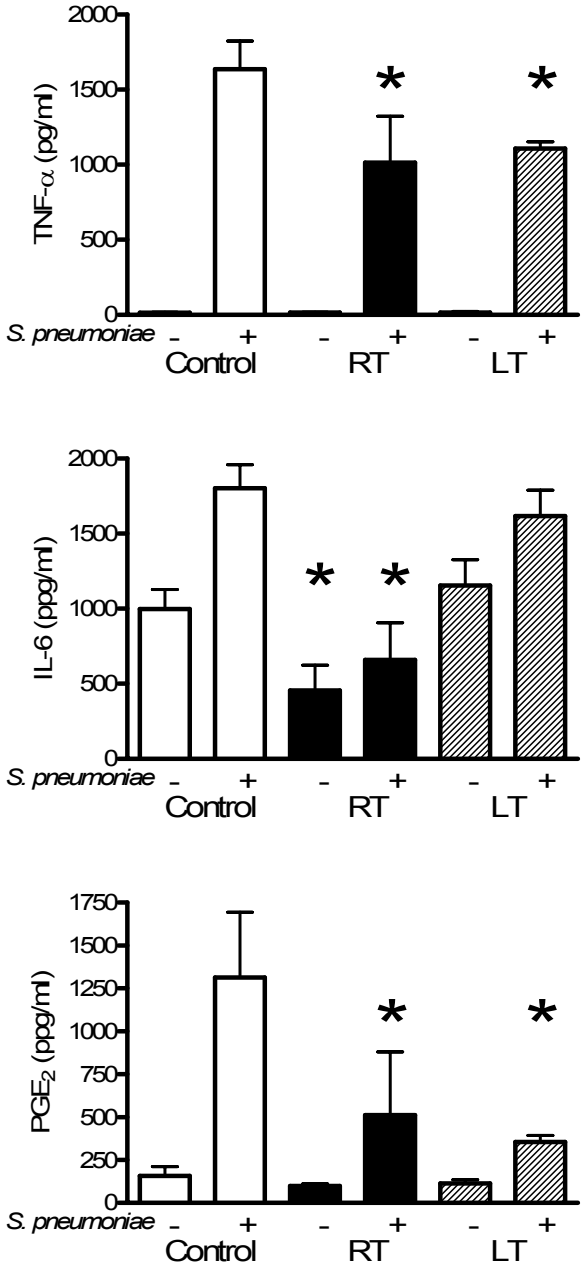

Figure 2.2. Cigarette smoke impairs lung leukocyte cytokine and PGE_2 production. Cytokine and PGE_2 production was assessed in lung leukocytes obtained from mice following 2 weeks of exposure to room air (control), smoke generated from regular tar (RT), or low tar (LT) cigarettes. Lung leukocytes were obtained from collagenase digest of the lungs, purified by adherence, and cultured overnight with or without heat-killed *S. pneumoniae*. Cell culture supernatants were assayed for cytokines TNF-α, IL-6, or PGE_2 using commercially available EIA kits. Each value represents the mean ± SEM of 5 separate experiments. *, $P<0.05$ vs. corresponding control.

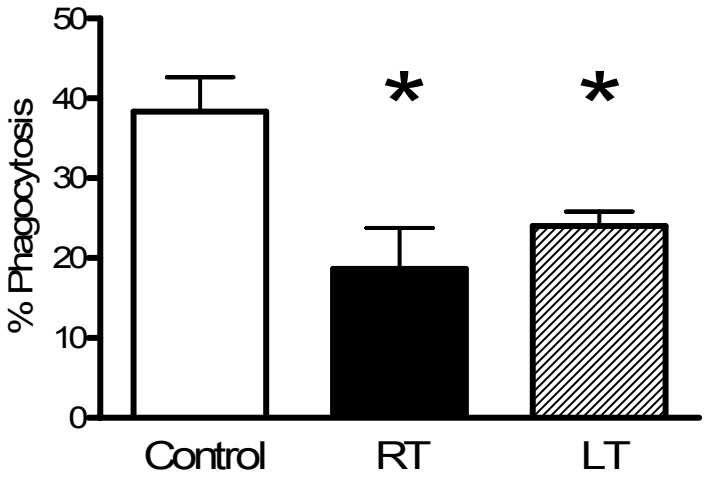

Figure 2.3. Cigarette smoke impairs AM phagocytosis of serum-opsonized *S. pneumoniae*. Phagocytosis of serum opsonized *S. pneumoniae* in alveolar macrophages (AM) obtained from mice following 2 weeks of exposure to room air (control), smoke generated from regular tar (RT), or low tar (LT) cigarettes. Each value represents the mean ± SEM of three separate experiments. *, $P<0.05$ vs. control. AM phagocytosis of fluorescent *S. pneumoniae* is expressed as the percentage of cells containing bacteria.

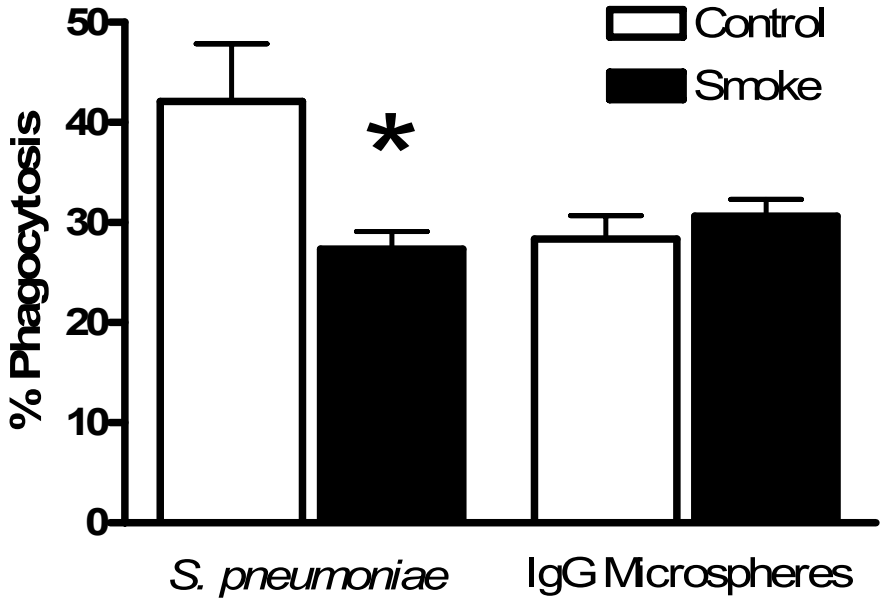

Figure 2.4. Cigarette smoke impairs AM phagocytosis of serum-opsonized *S. pneumoniae*, but not IgG-opsonized microspheres. Phagocytosis of serum opsonized *S. pneumoniae* or IgG-opsonized microspheres in alveolar macrophages (AM) obtained from mice following 1 week of exposure to room air (control) or smoke generated from regular tar cigarettes. Each value represents the mean ± SEM of three separate experiments. *, $P<0.05$ vs. control. Phagocytosis is expressed as the percentage of cells containing at least one particle.

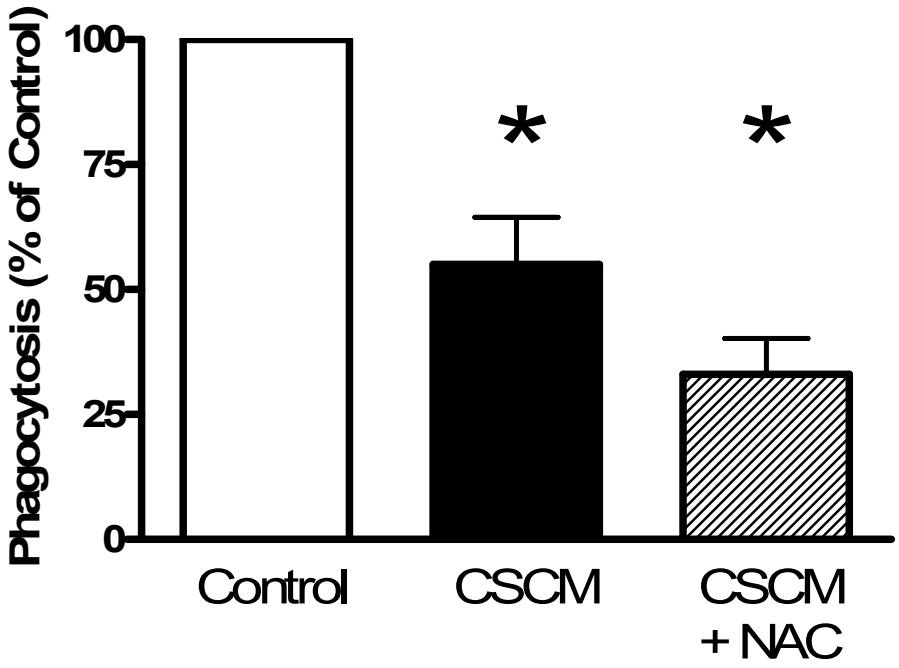

Figure 2.5. Impaired phagocytosis in CSCM-cultured AMs is not restored by NAC treatment. Rat AMs were cultured overnight in media alone (control), or in 1% CSCM with or without NAC (0.5mM). On the following day, phagocytosis of IgG-opsonized FITC labeled *E.coli* was assessed using a fluorimeter. Each value represents the mean ± SEM of three separate experiments. *, $P<0.05$ vs control. Phagocytosis was calculated as described and expressed as a percentage of the control value to which no CSCM was added.

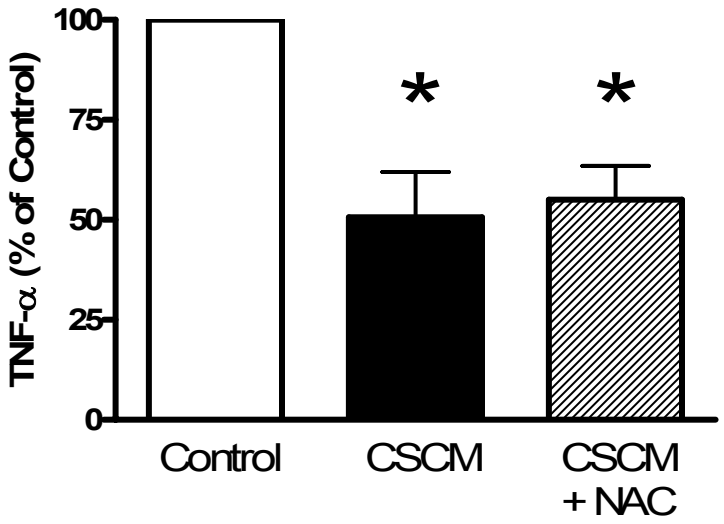

Figure 2.6. Impaired TNF-α production in CSCM-cultured AMs is not restored by NAC treatment. AMs were cultured overnight with medium alone (control) or 1% CSCM and NAC (0.5mM). Cell culture supernatants were assayed for TNF-α using a commercially available EIA kit. The data represent the mean ± SEM of 3 separate experiments. *, $P<0.05$ vs control.

References

1. Brody, J.S., and A. Spira. 2006. State of the art. Chronic obstructive pulmonary disease, inflammation, and lung cancer. *Proc.Am.Thorac.Soc.* 3:535-537.

2. Daniele, R.P., J.H. Dauber, M.D. Altose, D.T. Rowlands Jr, and D.J. Gorenberg. 1977. Lymphocyte studies in asymptomatic cigarette smokers. A comparison between lung and peripheral blood. *Am.Rev.Respir.Dis.* 116:997-1005.

3. Ruiz, M., S. Ewig, A. Torres, F. Arancibia, F. Marco, J. Mensa, M. Sanchez, and J.A. Martinez. 1999. Severe community-acquired pneumonia. Risk factors and follow-up epidemiology. *Am.J.Respir.Crit.Care Med.* 160:923-929.

4. Greenberg, D., N. Givon-Lavi, A. Broides, I. Blancovich, N. Peled, and R. Dagan. 2006. The contribution of smoking and exposure to tobacco smoke to Streptococcus pneumoniae and Haemophilus influenzae carriage in children and their mothers. *Clin.Infect.Dis.* 42:897-903.

5. Kadioglu, A., and P.W. Andrew. 2004. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 25:143-149.

6. Niederman, M.S., L.A. Mandell, A. Anzueto, J.B. Bass, W.A. Broughton, G.D. Campbell, N. Dean, T. File, M.J. Fine, P.A. Gross, F. Martinez, T.J. Marrie, J.F. Plouffe, J. Ramirez, G.A. Sarosi, A. Torres, R. Wilson, V.L. Yu, and American Thoracic Society. 2001. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am.J.Respir.Crit.Care Med.* 163:1730-1754.

7. Nuorti, J.P., J.C. Butler, M.M. Farley, L.H. Harrison, A. McGeer, M.S. Kolczak, and R.F. Breiman. 2000. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N.Engl.J.Med.* 342:681-689.

8. Drannik, A.G., M.A. Pouladi, C.S. Robbins, S.I. Goncharova, S. Kianpour, and M.R. Stampfli. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am.J.Respir.Crit.Care Med.* 170:1164-1171.

9. Green, G.M., and D. Carolin. 1967. The depressant effect of cigarette smoke on the in vitro antibacterial activity of alveolar macrophages. *N.Engl.J.Med.* 276:421-427.

10. Thomas, W.R., P.G. Holt, and D. Keast. 1978. Cigarette smoke and phagocyte function: effect of chronic exposure in vivo and acute exposure in vitro. *Infect.Immun.* 20:468-475.

11. Balter, M.S., G.B. Toews, and M. Peters-Golden. 1989. Multiple defects in arachidonate metabolism in alveolar macrophages from young asymptomatic smokers. *J.Lab.Clin.Med.* 114:662-673.

12. Ouyang, Y., N. Virasch, P. Hao, M.T. Aubrey, N. Mukerjee, B.E. Bierer, and B.M. Freed. 2000. Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts. *J.Allergy Clin.Immunol.* 106:280-287.

13. Soliman, D.M., and H.L. Twigg 3rd. 1992. Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *Am.J.Physiol.* 263:L471-8.

14. Karimi, K., H. Sarir, E. Mortaz, J.J. Smit, H. Hosseini, S.J. De Kimpe, F.P. Nijkamp, and G. Folkerts. 2006. Toll-like receptor-4 mediates cigarette smoke-induced cytokine production by human macrophages. *Respir.Res.* 7:66.

15. Mao, J.T., M.D. Roth, K.J. Serio, F. Baratelli, L. Zhu, E.C. Holmes, R.M. Strieter, and S.M. Dubinett. 2003. Celecoxib modulates the capacity for prostaglandin E2 and interleukin-10 production in alveolar macrophages from active smokers. *Clin.Cancer Res.* 9:5835-5841.

16. Yang, S.R., A.S. Chida, M.R. Bauter, N. Shafiq, K. Seweryniak, S.B. Maggirwar, I. Kilty, and I. Rahman. 2006. Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 291:L46-57.

17. Kaufman, D.W., J.R. Palmer, L. Rosenberg, P. Stolley, E. Warshauer, and S. Shapiro. 1989. Tar content of cigarettes in relation to lung cancer. *Am.J.Epidemiol.* 129:703-711.

18. Commission., F.T. 2000. Federal Trade Commission report to Congress for 1998. Pursuant to the Federal Cigarette Labeling and Advertising Act. . Washington, DC, Federal Trade Commission,

19. Kozlowski, L.T., M.E. Goldberg, B.A. Yost, E.L. White, C.T. Sweeney, and J.L. Pillitteri. 1998. Smokers' misperceptions of light and ultra-light cigarettes may keep them smoking. *Am.J.Prev.Med.* 15:9-16.

20. Gonzalez-Rothi, R.J., and J.O. Harris. 1986. Effects of low-yield-cigarette smoke inhalation on rat lung macrophages. *J.Toxicol.Environ.Health.* 17:221-228.

21. Holt, P.G., J.E. Chalmer, L.M. Roberts, J.M. Papadimitriou, W.R. Thomas, and D. Keast. 1976. Low-tar and high-tar cigarettes. *Arch.Environ.Health.* 31:258-265.

22. Valenca, S.S., P. Castro, W.A. Pimenta, M. Lanzetti, S.V. Silva, C. Barja-Fidalgo, V.L. Koatz, and L.C. Porto. 2006. Light cigarette smoke-induced emphysema and NFkappaB activation in mouse lung. *Int.J.Exp.Pathol.* 87:373-381.

23. Withey, C.H., A.O. Papacosta, A.V. Swan, B.A. Fitzsimons, G.A. Ellard, P.G. Burney, J.R. Colley, and W.W. Holland. 1992. Respiratory effects of lowering tar and nicotine levels of cigarettes smoked by young male middle tar smokers. II. Results of a randomised controlled trial. *J.Epidemiol.Community Health.* 46:281-285.

24. Simani, A.S., S. Inoue, and J.C. Hogg. 1974. Penetration of the respiratory epithelium of guinea pigs following exposure to cigarette smoke. *Lab.Invest.* 31:75-81.

25. Hautamaki, R.D., D.K. Kobayashi, R.M. Senior, and S.D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* 277:2002-2004.

26. Peters-Golden, M., R.W. McNish, R. Hyzy, C. Shelly, and G.B. Toews. 1990. Alterations in the pattern of arachidonate metabolism accompany rat macrophage differentiation in the lung. *J.Immunol.* 144:263-270.

27. Traynor, T.R., W.A. Kuziel, G.B. Toews, and G.B. Huffnagle. 2000. CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection. *J.Immunol.* 164:2021-2027.

28. Aronoff, D.M., C. Canetti, and M. Peters-Golden. 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J.Immunol.* 173:559-565.

29. van der Poll, T., C.V. Keogh, W.A. Buurman, and S.F. Lowry. 1997. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. *Am.J.Respir.Crit.Care Med.* 155:603-608.

30. van der Poll, T., C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, and S.F. Lowry. 1997. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J.Infect.Dis.* 176:439-444.

31. Kunkel, S.L., M. Spengler, M.A. May, R. Spengler, J. Larrick, and D. Remick. 1988. Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. *J.Biol.Chem.* 263:5380-5384.

32. Aronoff, D.M., C. Canetti, C.H. Serezani, M. Luo, and M. Peters-Golden. 2005. Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1. *J.Immunol.* 174:595-599.

33. Canetti, C., B. Hu, J.L. Curtis, and M. Peters-Golden. 2003. Syk activation is a leukotriene B4-regulated event involved in macrophage phagocytosis of IgG-coated targets but not apoptotic cells. *Blood.* 102:1877-1883.

34. Mancuso, P., and M. Peters-Golden. 2000. Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C-dependent. *Am.J.Respir.Cell Mol.Biol.* 23:727-733.

35. Green, G.M. 1968. Cigarette smoke: protection of alveolar macrophages by glutathione and cysteine. *Science.* 162:810-811.

36. Droemann, D., T. Goldmann, T. Tiedje, P. Zabel, K. Dalhoff, and B. Schaaf. 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir.Res.* 6:68.

37. Shapiro, S.D. 1999. The macrophage in chronic obstructive pulmonary disease. *Am.J.Respir.Crit.Care Med.* 160:S29-32.

38. Lipsky, B.A., E.J. Boyko, T.S. Inui, and T.D. Koepsell. 1986. Risk factors for acquiring pneumococcal infections. *Arch.Intern.Med.* 146:2179-2185.

39. Bracke, K.R., A.I. D'hulst, T. Maes, K.B. Moerloose, I.K. Demedts, S. Lebecque, G.F. Joos, and G.G. Brusselle. 2006. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *J.Immunol.* 177:4350-4359.

40. D'hulst, A.I., K.Y. Vermaelen, G.G. Brusselle, G.F. Joos, and R.A. Pauwels. 2005. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur.Respir.J.* 26:204-213.

41. Bergeron, Y., N. Ouellet, A.M. Deslauriers, M. Simard, M. Olivier, and M.G. Bergeron. 1998. Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect.Immun.* 66:912-922.

42. Desouza, I.A., C.F. Franco-Penteado, E.A. Camargo, C.S. Lima, S.A. Teixeira, M.N. Muscara, G. De Nucci, and E. Antunes. 2005. Inflammatory mechanisms underlying the rat pulmonary neutrophil influx induced by airway exposure to staphylococcal enterotoxin type A. *Br.J.Pharmacol.* 146:781-791.

43. Martey, C.A., S.J. Pollock, C.K. Turner, K.M. O'Reilly, C.J. Baglole, R.P. Phipps, and P.J. Sime. 2004. Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E2 synthase in human lung fibroblasts: implications for lung inflammation and cancer. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 287:L981-91.

44. Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H.W. Pfister, and C.J. Kirschning. 2003. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J.Immunol.* 170:438-444.

45. Pathak, S.K., A. Bhattacharyya, S. Pathak, C. Basak, D. Mandal, M. Kundu, and J. Basu. 2004. Toll-like receptor 2 and mitogen- and stress-activated kinase 1

are effectors of Mycobacterium avium-induced cyclooxygenase-2 expression in macrophages. *J.Biol.Chem.* 279:55127-55136.

46. Scherer, G. 1999. Smoking behaviour and compensation: a review of the literature. *Psychopharmacology (Berl).* 145:1-20.

47. Greenberg, S., and S. Grinstein. 2002. Phagocytosis and innate immunity. *Curr.Opin.Immunol.* 14:136-145.

48. Keast, D., and K. Taylor. 1983. The effects of chronic tobacco smoke exposure from high-tar cigarettes on the phagocytic and killing capacity of polymorphonuclear cells of mice. *Environ.Res.* 31:66-75.

49. Klut, M.E., C.M. Doerschuk, S.F. Van Eeden, A.R. Burns, and J.C. Hogg. 1993. Activation of neutrophils within pulmonary microvessels of rabbits exposed to cigarette smoke. *Am.J.Respir.Cell Mol.Biol.* 9:82-89.

50. Aoshiba, K., J. Tamaoki, and A. Nagai. 2001. Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 281:L1392-401.

51. Machiya, J., Y. Shibata, K. Yamauchi, N. Hirama, T. Wada, S. Inoue, S. Abe, N. Takabatake, M. Sata, and I. Kubota. 2007. Enhanced expression of MafB inhibits macrophage apoptosis induced by cigarette smoke exposure. *Am.J.Respir.Cell Mol.Biol.* 36:418-426.

52. Letiembre, M., H. Echchannaoui, F. Ferracin, S. Rivest, and R. Landmann. 2005. Toll-like receptor-2 deficiency is associated with enhanced brain TNF gene expression during pneumococcal meningitis. *J.Neuroimmunol.* 168:21-33.

CHAPTER 3

Cigarette smoke impairs complement-mediated alveolar macrophage phagocytosis and pulmonary innate host defense against pneumococcal pneumonia

Introduction

Pneumococcal pneumonia, caused by the gram-positive pathogen *Streptococcus pneumoniae*, is the most common form of community-acquired pneumonia in the US (1) and worldwide (2). This organism can disseminate from the respiratory system, and is the leading cause of death from invasive bacterial infections (3), with antibiotic resistant strains becoming increasingly common (1). Cigarette smoke (CS) exposure increases the risk of serious pneumococcal infections in humans (4, 5), although the mechanisms underlying this effect are not known. Consistent with increased risk of many infectious diseases among smokers (6), animal models have been used to demonstrate impairments in host defense against viral (7, 8), fungal (9), and bacterial infections (10) in smoke-exposed animals. To our knowledge, no reports exist which demonstrate the effects of CS exposure on host defense in an animal model of pneumococcal pneumonia, despite the clinical significance of this pathogen.

The alveolar macrophage (AM) is a specifically differentiated resident phagocyte in the pulmonary alveoli which acts to maintain an environment free of pathogens and debris (11). In the non-infected individual, AMs constitute the majority of immune cells

within the alveolar space and act as a first line of innate host defense in the lung, using an array of receptors to recognize pathogen-associated molecular patterns (PAMPs) and to facilitate phagocytic uptake (12). Normally, AM function is tightly regulated to prevent inappropriate inflammation that could result in lung damage (13), however under conditions which overwhelm their clearance capacity, AMs play additional roles in the generation and subsequent resolution of inflammation and leukocyte recruitment (14, 15). Murine models of pulmonary pneumococcal infection have shown increased mortality (16), and bacterial burden (17) following AM depletion, indicating their importance in innate host defense against such infections. Phagocytosis of pneumococcus is enhanced following opsonization with factors such as complement fragments, making complement a necessary factor for effective host defense against pneumococcal pneumonia (18, 19).

While no reports to date describe the effects of CS exposure on AM phagocytosis of pneumococcus, many studies exist showing impairments in phagocytosis of other targets (20-24). CS has been shown to cleave complement directly, making it unavailable to participate in phagocytosis, suggesting the possibility that CS exposure may alter rates of AM phagocytosis through this mechanism.

Because the effects of CS exposure on pulmonary innate host defense against pneumococcal pneumonia are incompletely understood and have not been described in an animal model, we sought to determine whether CS exposed mice would show altered responses to subsequent pulmonary infection. We also used in vitro systems to determine effects on AM phagocytosis and complement opsonization.

Methods

Animals

Female BALB/C mice aged 8-12 weeks were purchased from Charles River Laboratories (Portage, MI). In vitro determination AM phagocytosis after in vivo CS exposures used male C57BL/6 mice, obtained at 8-12 weeks of age (Jackson Laboratories, Barharbor, ME). All experiments were conducted in accordance with the Animal Care and Use Committee of the University of Michigan.

CS exposure model

Smoke from standardized 2R4F research cigarettes (University of Kentucky, Lexington, KY) was generated by a TE-2 cigarette smoking machine (Teague Enterprises, Davis, CA), at a mean concentration of 19.6 mg/m^3. Animals were exposed 4 hr/day, 5 day/week for 5 weeks in a whole-body exposure chamber, with water available *ad libidum*. Control animals were housed in an identical chamber, but exposed to room air with no smoke. For in vitro determination phagocytosis after in vivo CS exposure, animals were exposed for one week at 50 mg/m^3, using a custom-built cigarette smoking machine, as previously described (25). Exposure levels were determined gravimetrically.

Infection model

S. pneumoniae, serotype 3, (ATCC 6303, American Type Culture Collection, Manassas, VA) was grown to mid-log phase in Todd Hewett broth supplemented with 0.5% yeast extract (Difco, Detroit, MI) at 37°C, with 5% CO_2. For in vitro studies, bacteria were washed in sterile water and heat-killed at >90°C for 30 min. Infection was

performed via intratracheal instillation as previously described (26), at a dose of $1x10^4$ colony-forming units (CFU). Animals were monitored for health at least once every 6 hours for 48 hours following infection. Core temperatures were determined immediately after euthanasia, by insertion of a probe into a surgical incision in the peritoneum.

In vitro CS exposures: CS-conditioned media (CSCM)

Smoke from five cigarettes was drawn through 50 ml RPMI 1640 (Invitrogen, Carlsbad, CA) using a glass impinger (Ace Glass, Vineland, NJ), after which the fluid was aliquoted and stored at -70ºC. Pilot dose-response studies were conducted to determine non-cytotoxic levels using the XTT assay (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's directions. Direct cytotoxicity of CSCM was not observed in this model at levels up to 2.0% CSCM, although it was seen at 5.0% and above (data not shown). All experiments were conducted with 1% or less CSCM, added to the cell culture media after adherence and incubated 18-24 hours.

Bronchoalveolar lavage (BAL) and AM culture

Mice were euthanized by CO_2 inhalation or intraperitoneal pentobarbital injection, and lungs removed surgically after exsanguination. The trachea was cannulated onto a hypodermic needle/syringe combination. In 0.5 ml increments, 10 ml of ice-cold HEPES-buffered saline solution containing dextrose, sodium EDTA, and penicillin/streptomycin (Invitrogen, Carlsbad, CA) was instilled into the lung and removed to a separate syringe. In experiments measuring cellular recruitment following pneumococcal challenge, 2 ml total lavage volume was used. After recovery by BAL,

cells were centrifuged at approximately 400 x g, resuspended in RPMI1640 (Invitrogen), enumerated with a hemocytometer, and plated at the desired concentration. After 1h at 37ºC, 5% CO_2, the media was replaced with warm RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and returned to the incubator. For cell recruitment studies, differential stains were also performed on the BAL cells using a modified Wright-Giemsa stain (American Scientific Products, McGraw Park, IL).

Determination of pulmonary bacterial load and cytokines

Homogenization of lung or spleen tissue for determination of cytokine levels was performed as previously described (27) except lungs were not perfused prior to homogenization. Lungs and spleen were homogenized, serially diluted, and plated on blood agar plates for determining bacterial burdens. Samples were removed for CFU determination prior to addition of detergent. After centrifugation, supernatants were stored at -80°C until analysis. Determination of cytokine levels in whole lung homogenate was performed at the University of Michigan Immunology Core, using commercially available reagents (R&D Systems, Minneapolis, MN).

Phagocytosis

Preparation and phagocytosis determinations of IgG-opsonized polystyrene microspheres were performed as previously described (28). Microscopic determination of pneumococcal phagocytosis was performed as previously described (29), at a ratio of 25 bacteria per AM, with externalize fluorescence quenched with trypan blue. Plate-reader based phagocytosis determinations were made with an adaptation of a previously

described method (30). Briefly, AMs were cultured overnight at $1x10^5$ AM/well, in 384 well microplate (Corning, Lowell, MA) with or without 1% CSCM in the media. The number of replicate wells was determined by AM harvest, and ranged from 5 to 10 wells per condition per experiment. The next day, the media was removed and replaced with RPMI 1640 without serum or antibiotic. FITC-labeled pneumococcus was either unopsonized, or opsonized with normal or heat-treated rat, or complement-component 3-deficient human (Sigma) serum for 30 minutes at 37°C, and washed by repeated centrifugation before being added to the wells at ratio of 200 bacteria per AM (or 300:1 for unopsonized bacteria). After 3 hours incubation, plates were placed on ice to halt phagocytosis, and 500 μg/ml trypan blue (Sigma) was added to quench extracellular fluorescence before reading the plate. In these experiments, since control and CSCM groups were from the same original pool and thus present in the same numbers, the PI corresponds to the non-quenchable (i.e. intracellular) fluorescence intensity, relative to untreated control cells.

Statistical Analysis

Data was analyzed using Prism 4 (GraphPad Software, La Jolla, CA) using a paired or unpaired t test where appropriate, or one-way analysis of variance with post-hoc tests as noted. In all cases, a *P* value of less than 0.05 was considered significant.

Results

Cigarette Smoke Exposure Leads to Impaired Pneumococcal Clearance and More Severe Illness

To determine the effect of CS exposure on bacterial clearance, animals were exposed for 5 weeks to CS or room air (control) as described in the methods section, prior to infection with *S. pneumoniae*. Figure 3.1 shows that levels of viable bacteria recovered from the lungs of smoke-exposed animals were approximately 4-fold and 35-fold higher than control animals at 24 and 48 hr post-infection, respectively. Neither death nor pneumococcal dissemination, as measured by splenic bacterial burden, was seen at these timepoints (data not shown). Because hypothermia has been associated with increased severity of pneumococcal infection (31), we measured core temperature at the time of euthanasia (Figure 3.1, panel B). Smoke-exposed mice had significantly reduced core temperatures at 24 hr post infection, consistent with the higher bacterial burden at this timepoint, while at 48 hr, the trend toward lower temperatures in smoke-exposed animals did not reach significance. Elevated levels of cytokines, including IL-1β and TNF-α, have been observed in pneumococcal pneumonia (32, 33). These cytokines are produced locally (34, 35), therefore we measured the levels of these mediators in whole lung homogenates from infected animals. Smoke-exposed animals had a trend toward higher levels of TNF-α at 24 hr post-infection, and a significant increase above control at 48 hr, with IL-1β levels significantly higher at both timepoints (Figure 3.1, panel C & D), further confirming a more severe infection in these animals. (Levels of IL-6, MIP-2, and PGE_2 were also measured, but did not show significant differences).

Impaired Pneumococcal Clearance in Smoke-exposed Animals is Not Due to Reduced Alveolar Leukocyte Numbers or Viability

To determine whether the impairments in bacterial clearance were due to a CS-mediated deficit in resident alveolar leukocyte numbers or viability, BAL cells from

animals exposed to CS or room air for 5 weeks were examined. Exposure did not significantly alter the numbers of lymphocytes or neutrophils recovered from BAL fluid (Figure 3.2). While AMs, which constituted > 95% of the recovered leukocytes in both groups of animals, were 57.4% higher in the CS-exposed group, this difference did not reach statistical significance. Additionally, the viability of these cells as determined by XTT assay did not differ (data not shown).

Because leukocyte recruitment to the lungs following pulmonary pneumococcal infection is typically rapid and robust (34), we sought to compare the capacity of CS-exposed and control animals to recruit leukocytes to the alveolar compartment in response to pneumococcal challenge. In the infection model described above, the higher bacterial burden among the smoke-exposed animals created an unequal challenge at the measured timepoints. Therefore, to create conditions of similar challenge, animals were exposed to CS or room air for 5 weeks and subsequently instilled with heat-killed, rather than viable, pneumococcus at a dose equivalent to 10^6 CFU/animal. Figure 3.2 shows BAL leukocytes at 24 and 48 hr post-challenge, and indicates that smoke-exposed mice do not have reduced recruitment of immune cells to the lungs after pneumococcal challenge. Recruitment of neutrophils was higher in CS-exposed animals at 24 and 48 hours after infection. In contrast to uninfected animals, monocyte/macrophage numbers were not different between CS and control groups following pneumococcal challenge, likely reflecting an influx of recruited monocytes, which are known to migrate to the lungs following pneumococcal infection (36).

Impaired Phagocytosis in Pulmonary Leukocytes from Smoke-exposed Mice

To better understand whether CS exposure alters the phagocytic capacity of AMs, we isolated cells from CS-exposed and control animals, and assessed phagocytosis in vitro. Figure 3.3 shows that BAL-derived AMs from one-week CS-exposed animals had approximately half the phagocytic activity of AMs from control animals, measured as percentage of cells participating in phagocytosis (*i.e.* containing at least one bacterium).

Cigarette smoke mediated functional impairments can be reproduced in vitro using primary AM cultures and CSCM pretreatment

To confirm a direct effect of CS on AMs in the absence of other cell types, we harvested AMs from the BAL fluid of naïve animals, and exposed them to CSCM in vitro before performing functional assays. Figure 3.4 shows that CSCM caused a dose-dependent decrease in the percentage of cells participating in phagocytosis of serum-opsonized, fluorescently-labeled pneumococcus that reached statistical significance at a level of 1.0% CSCM. These experiments measured the proportion of cells containing at least one internalized bacterium on slides scored by an observer blinded to the treatment group. This approach was used because the morphological characteristics of the pneumococcus make accurate manual counting of internalized bacteria problematic. However, because even a single bacterium can be seen with this method, it provides an extremely sensitive assay.

In vitro Pretreatment of AMs with CSCM Impairs Complement-mediated Phagocytosis

To determine the nature of the phagocytic impairment caused by CS, we assessed phagocytosis of disparate targets by CSCM-pretreated AMs in vitro, expressed as the phagocytic index (PI), reflecting the amount of target taken up by AMs. For bacterial

phagocytosis, we used a fluorescence-based assay to determine the effect of CSCM pretreatment on the PI of AMs with heat-killed, fluorescently-labeled pneumococcus as the target. Figure 3.5 shows that when pneumococcus was opsonized with normal rat serum, a modest but significant phagocytic reduction is observed in CSCM-pretreated AMs in this assay. However, when opsonization was performed with heat-inactivated rat serum or C3-depleted human serum, no such impairment was detectable. Similarly, in experiments using unopsonized pneumococcus as the phagocytic target, no impairment was seen with CSCM pretreatment. This assay yielded more modest CSCM-mediated phagocytic impairments than found with direct microscopic enumeration, however the results are not directly comparable. In addition to measuring different endpoints (proportion of positive cells versus PI), there were significant methodological differences in the two assays, including much higher numbers of bacteria used in the plate-based assay to achieve sufficient fluorescent intensity for the spectrophotometric hardware.

Using direct microscopic observation, no difference was seen in the PI of IgG-opsonized polystyrene microspheres by AMs cultured with 1.0% CSCM versus control AMs (Figure 3.5). The lack of observable effect persisted when the data was analyzed as the percentage of cells participating in phagocytosis (data not shown).

Discussion

Because of the association between CS exposure and increased risk of serious pneumococcal infection, we sought to determine whether a murine model could be used to recreate a CS-mediated impairment in anti-pneumococcal innate pulmonary host defense in the laboratory. We found that mice exposed to CS for 5 weeks prior to

infection had a substantially higher lung burden of this pathogen, accompanied by physiological signs of more severe infection including hypothermia and elevated pulmonary cytokines. This finding is in broad agreement with studies examining the effect of CS exposure on murine models of non-pneumococcal lung infections (7, 9-10).

The observed defect in pneumococcal clearance in CS-exposed animals was not due to decreased numbers or viability of resident leukocytes prior to infection. On the contrary, there were more AMs present in the lungs of exposed mice. While this trend did not reach statistical significance using our sample size, elevated AM numbers have also been reported in human smokers (37) and longer-duration mouse models (38) of CS exposure, so it is likely that the increases observed in our model represent an early stage in the accumulation of AMs. Additionally, under conditions of similar challenge using heat-killed pneumococcus, CS-exposed animals displayed no impairment in leukocyte recruitment post-infection. Rather, these animals had higher levels of neutrophil recruitment, consistent with other published murine models of pneumococcal pneumonia in which AM function was artificially impaired (39) or the number of AMs was reduced experimentally (16). A paradox is posed by the CS-associated increase in AMs, which have been reported to be as much as six-fold higher in smokers (40), and increased susceptibility to pulmonary infections.

Because of the variety of components contributing to host defense in vivo, we examined AMs isolated from CS-exposed animals, and found reduced in vitro phagocytosis of serum-opsonized, fluorescently-labeled pneumococcus, as measured by the proportion of cells participating in phagocytosis. Notably, the effect appeared after

only one week of CS exposure. These results demonstrate impaired phagocytosis in AMs isolated from CS-exposed animals, however they do not formally rule out the participation of other cell types present in the lungs during the period CS of exposure. As an example, it is known that alveolar epithelial cells can maintain AMs in a hyporesponsive state through an integrin-mediated mechanism (41). In another study, pretreatment of macrophages with extracellular matrix proteins modified in vitro by CS impaired phagocytosis of apoptotic neutrophils (21).

When we removed other components from the model system by pre-treating naive AMs with CSCM, the phagocytic impairment remained, and was found to be dose-dependent. In contrast, no difference was seen in phagocytosis of IgG-coated polystyrene microspheres with CSCM pretreatment, suggesting that the impairment was not a generalized effect as might be seen with disruption of cytoskeletal function or membrane trafficking. The plate-based assay confirmed a significant reduction in the phagocytosis of serum-opsonized pneumococcus by AMs pretreated with CSCM measured as PI. Significantly, when these experiments were conducted using unopsonized pneumococcus, no differences were observed between CSCM-pretreated and control AMs.

Although AM phagocytosis of unopsonized pneumococcus can occur through receptors such as scavenger receptor A (SR-A) (42) and macrophage receptor with collagenous structure (MARCO) (43), it is inefficient, and multiple lines of evidence support the importance of C3 in anti-pneumococcal innate host defense. For example, genetic defects in C3 are associated with susceptibility to pneumococcal infections in humans (18) and mice (44), and mutant strains of pneumococcus lacking the anti-

complement factors pneumococcal surface protein A and pneumolysin display reduced virulence in wild-type, but not C3-knockout mice (45). Neither of these reports specifically addresses the role of AMs in this effect, however increased bacterial burdens were seen in C3-deficient mice within 1 hr of infection, arguing against a major role for recruited cells such as neutrophils.

Because CSCM impaired phagocytosis of serum opsonized, but not unopsonized pneumococcus, we undertook experiments to clarify the role of complement opsonization in this impairment. To this end, we compared the effect of CSCM pretreatment on AM phagocytosis of pneumococcus opsonized with normal rat serum, heat-inactivated rat serum, and human serum depleted of C3 by immunoprecipitation. We found that in the absence of C3, CSCM pretreatment did not impair in vitro phagocytosis of pneumococcus, identifying this complement component as the relevant serum factor. It was of interest that in experiments run side-by-side on the same microplate, opsonization with normal rat serum produced roughly twice the level of phagocytosis seen with either form of complement-depleted serum (data not shown), affirming the importance of this opsonin in our system.

Our data establish a role for complement in CSCM-mediated impairment of AM phagocytosis of pneumococcus, which is a novel finding. When interpreting the relevance of these in vitro data to the living animal, it bears consideration that opsonization of the phagocytic targets was carried out in isolation from either cells or CS constituents, and thus could not be affected by CSCM treatment. However, in vivo, opsonization occurs in the same pulmonary milieu, and CS is known to directly cleave

C3 (46-48), suggesting that complement-mediated effects may be more pronounced at the organismal level.

The data presented here demonstrate clearly that CS exposure impairs pulmonary innate host defense against pneumococcal pneumonia in living animals, and that this effect is not due to reduced leukocyte numbers or viability. Using in vitro approaches, we showed impairment of complement-mediated AM phagocytosis of this organism. Given the important role played by AMs in maintaining a pathogen-free alveolar environment, the functional defects we describe here may well contribute to increased pneumococcal susceptibility in CS-exposed humans.

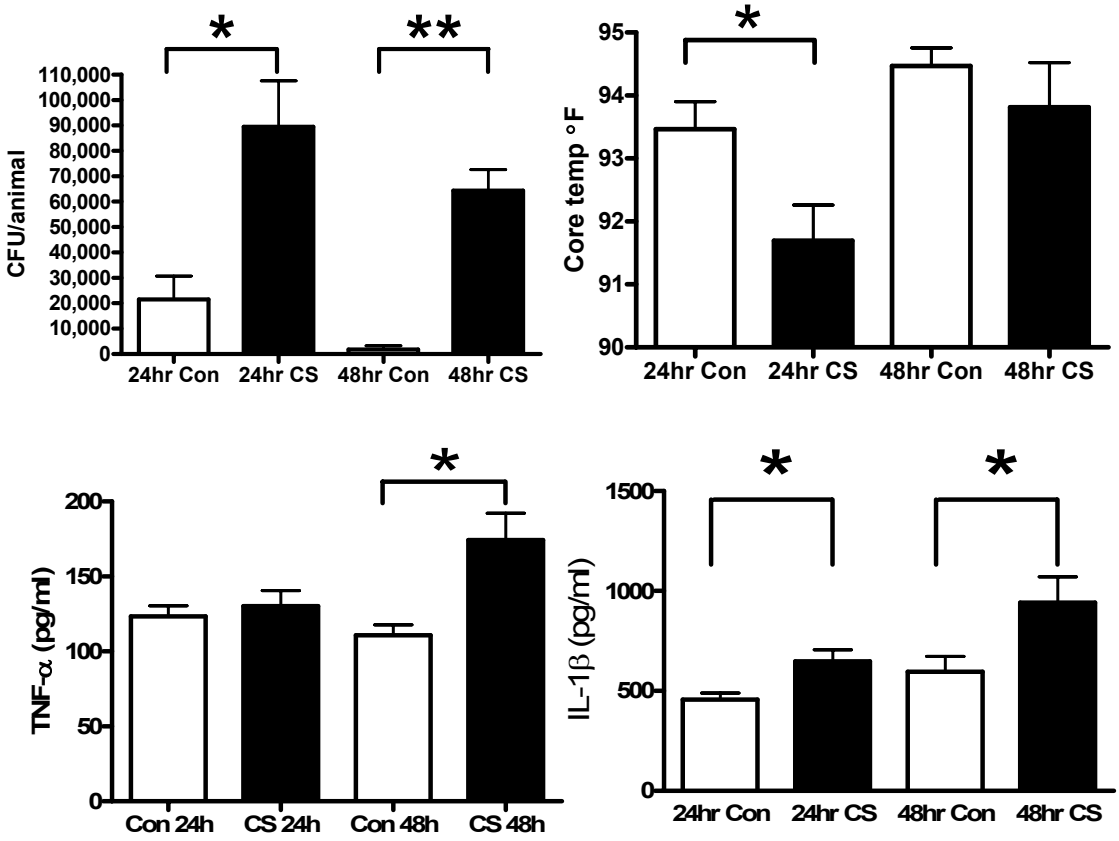

Figure 3.1. Cigarette smoke increases pulmonary bacterial burden and worsens clinical signs of pneumococcal pneumonia. Female BALB/C mice were exposed to CS (solid bars) or room air (open bars) for 5 weeks followed by intratracheal instillation with 10^4 CFU *S. pneumoniae*. (A) Lung homogenate was assessed for bacterial CFU at 24 and 48 hours post-infection. (B) Core temperatures were taken at the time of euthanasia. (C,D) Levels of TNF-α and IL-1β are expressed as the sum of whole lung homogenate and BAL fluid levels. Bars represent the mean ± standard error of the mean; n = 7–8 mice per group; **P* < 0.05 by unpaired t test; ***P* < 0.05 by Mann-Whitney test for non-parametric data.

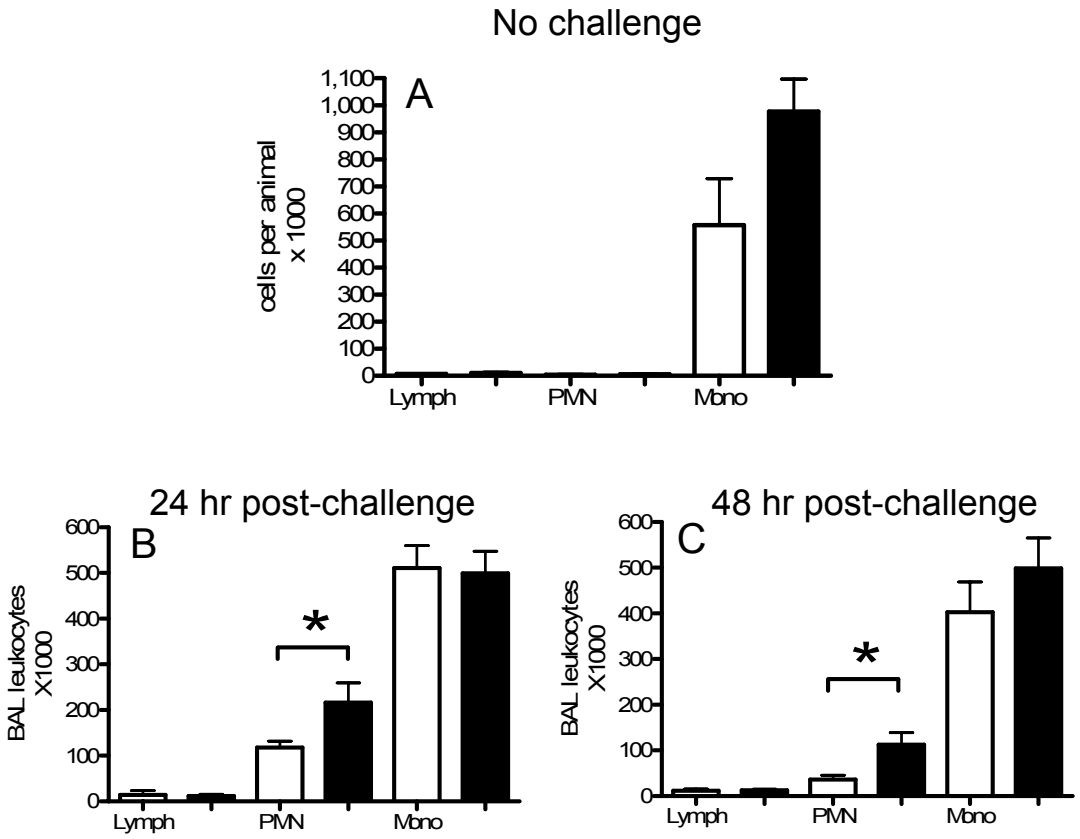

Figure 3.2. Composition of alveolar leukocyte population before and after pneumococcal infection. Female BALB/C mice were exposed to CS (solid bars) or room air (open bars) for 5 weeks with no subsequent challenge or followed by intratracheal instillation with heat-killed *S. pneumoniae* (10^6 CFU-equivalent dose). BAL was performed following the final CS exposure (A) or at 24 hr (B) and 48 hr (C) post-challenge. Total leukocyte numbers were determined on a hemocytometer, and differential staining was performed to distinguish lymphocytes (Lymph), neutrophils (PMN) and monocyte/macrophages (Mono). Bars represent the mean ± standard error of the mean; n = 4–6 mice per group in panel A, and 7-8 mice per group in panels B and C; *$P < 0.05$ by unpaired t test.

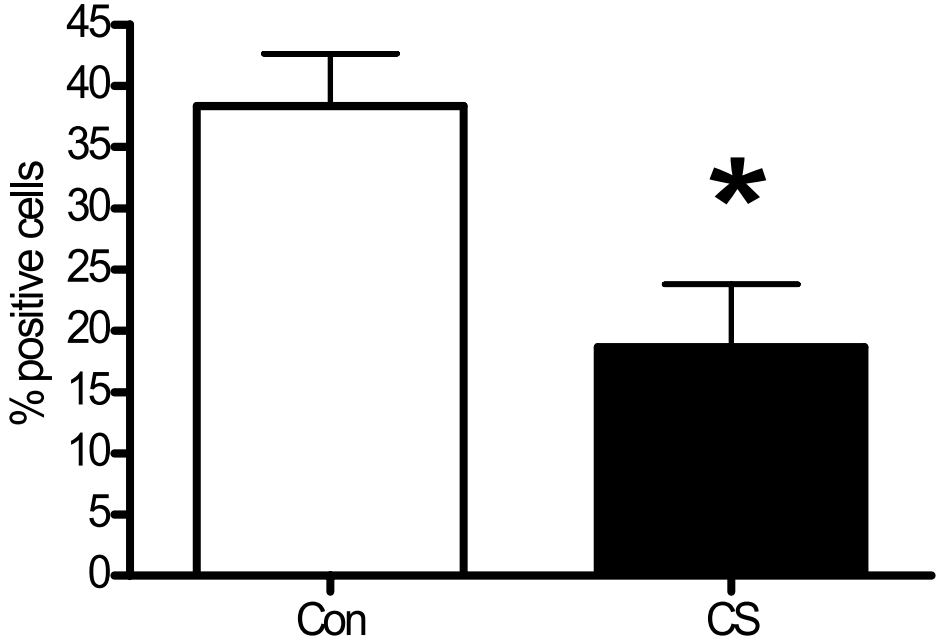

Figure 3.3. Cigarette smoke impairs AM phagocytosis of serum-opsonized *S. pneumoniae*. Male C57BL/6 mice were exposed to CS or room air (Con) for one week prior to cell harvest by BAL and determination of phagocytosis. Bars represent the mean ± standard error of the mean; n = 3-5 mice per group; *$P < 0.05$ vs control by unpaired t test.

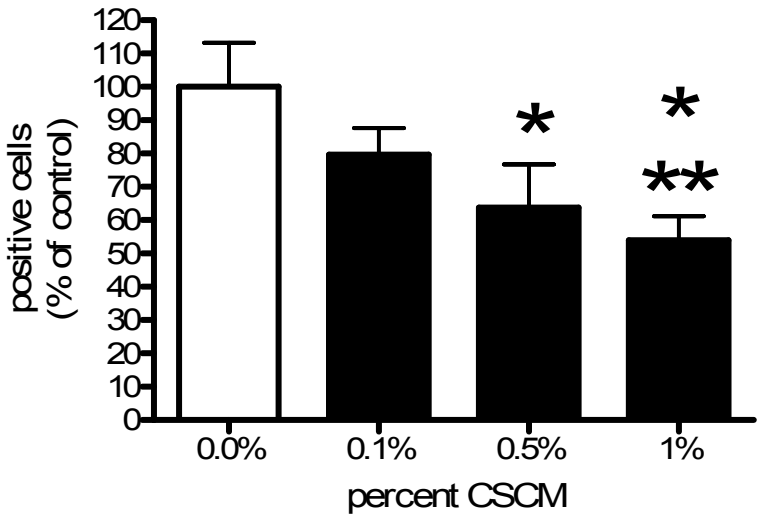

Figure 3.4 Cigarette smoke-conditioned media impairs AM phagocytosis of serum-opsonized *S. pneumoniae*. Murine AMs were cultured for 24 hr with varying concentrations of CSCM (solid bars) or without CSCM (open bars). Proportion of cells participating in phagocytosis of serum-opsonized, FITC-labeled pneumococcus was determined microscopically. Bars represent the mean ± standard error of the mean of four replicate experiments; **P* < 0.05 compared with control by unpaired t test; ***P* < 0.05 compared with 0.1% CSCM by one-way ANOVA with Dunnett's post-hoc test.

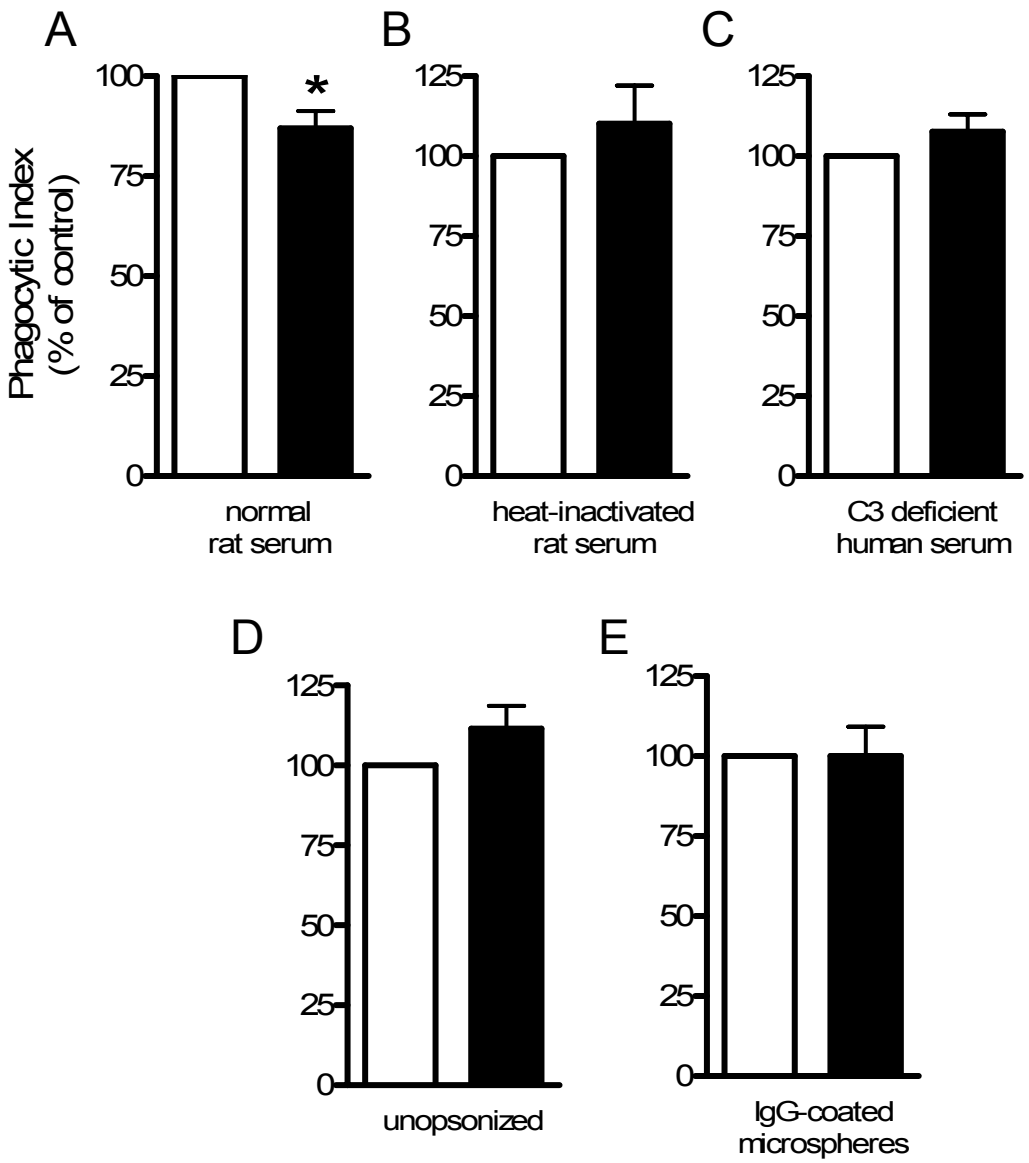

Figure 3.5. Cigarette smoke-conditioned media impairs AM phagocytosis of iC3b-opsonized *S. pneumoniae*. AMs cultured in 1% CSCM (solid bars) show reduced levels of phagocytosis compared with controls (open bars) when normal rat serum-opsonized pneumococcus is used (A), but not when the bacteria is opsonized with heat-inactivated rat serum (B) or C3-deficient human serum (C), nor in the absence of opsonization (D). Uptake of IgG-coated microspheres was not different between groups (E). Data are mean ± SEM of 3-5 experiments, each normalized to same-plate controls. *$P < 0.05$ compared with control AMs by paired t test.

References

1. Mandell, L.A., R.G. Wunderink, A. Anzueto, J.G. Bartlett, G.D. Campbell, N.C. Dean, S.F. Dowell, T.M. File Jr, D.M. Musher, M.S. Niederman, A. Torres, C.G. Whitney, Infectious Diseases Society of America, and American Thoracic Society. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin.Infect.Dis.* 44 Suppl 2:S27-72.

2. Anonymous . 1999. Pneumococcal vaccines. WHO position paper. *Wkly.Epidemiol.Rec.* 74:177-183.

3. Kadioglu, A., and P.W. Andrew. 2004. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 25:143-149.

4. Nuorti, J.P., J.C. Butler, M.M. Farley, L.H. Harrison, A. McGeer, M.S. Kolczak, and R.F. Breiman. 2000. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N.Engl.J.Med.* 342:681-689.

5. Almirall, J., I. Bolibar, M. Serra-Prat, J. Roig, I. Hospital, E. Carandell, M. Agusti, P. Ayuso, A. Estela, A. Torres, and Community-Acquired Pneumonia in Catalan Countries (PACAP) Study Group. 2008. New evidence of risk factors for community-acquired pneumonia: a population-based study. *Eur.Respir.J.* 31:1274-1284.

6. Arcavi, L., and N.L. Benowitz. 2004. Cigarette smoking and infection. *Arch.Intern.Med.* 164:2206-2216.

7. Phaybouth, V., S.Z. Wang, J.A. Hutt, J.D. McDonald, K.S. Harrod, and E.G. Barrett. 2006. Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 290:L222-31.

8. Gualano, R.C., M.J. Hansen, R. Vlahos, J.E. Jones, R.A. Park-Jones, G. Deliyannis, S.J. Turner, K.A. Duca, and G.P. Anderson. 2008. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir.Res.* 9:53.

9. Christensen, P.J., A.M. Preston, T. Ling, M. Du, W.B. Fields, J.L. Curtis, and J.M. Beck. 2008. Pneumocystis murina infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement, and pulmonary inflammation in mice. *Infect.Immun.* 76:3481-3490.

10. Drannik, A.G., M.A. Pouladi, C.S. Robbins, S.I. Goncharova, S. Kianpour, and M.R. Stampfli. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am.J.Respir.Crit.Care Med.* 170:1164-1171.

11. Marriott, H.M., and D.H. Dockrell. 2007. The role of the macrophage in lung disease mediated by bacteria. *Exp.Lung Res.* 33:493-505.

12. Stuart, L.M., and R.A. Ezekowitz. 2005. Phagocytosis: elegant complexity. *Immunity.* 22:539-550.

13. Ali, F., M.E. Lee, F. Iannelli, G. Pozzi, T.J. Mitchell, R.C. Read, and D.H. Dockrell. 2003. Streptococcus pneumoniae-associated human macrophage apoptosis after bacterial internalization via complement and Fcgamma receptors correlates with intracellular bacterial load. *J.Infect.Dis.* 188:1119-1131.

14. Thomassen, M.J., L.T. Divis, and C.J. Fisher. 1996. Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10. *Clin.Immunol.Immunopathol.* 80:321-324.

15. Mizgerd, J.P. 2002. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin.Immunol.* 14:123-132.

16. Knapp, S., J.C. Leemans, S. Florquin, J. Branger, N.A. Maris, J. Pater, N. van Rooijen, and T. van der Poll. 2003. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am.J.Respir.Crit.Care Med.* 167:171-179.

17. Dockrell, D.H., H.M. Marriott, L.R. Prince, V.C. Ridger, P.G. Ince, P.G. Hellewell, and M.K. Whyte. 2003. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J.Immunol.* 171:5380-5388.

18. Picard, C., A. Puel, J. Bustamante, C.L. Ku, and J.L. Casanova. 2003. Primary immunodeficiencies associated with pneumococcal disease. *Curr.Opin.Allergy Clin.Immunol.* 3:451-459.

19. Kerr, A.R., G.K. Paterson, A. Riboldi-Tunnicliffe, and T.J. Mitchell. 2005. Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. *Infect.Immun.* 73:4245-4252.

20. Hodge, S., G. Hodge, J. Ahern, H. Jersmann, M. Holmes, and P.N. Reynolds. 2007. Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am.J.Respir.Cell Mol.Biol.* 37:748-755.

21. Kirkham, P.A., G. Spooner, I. Rahman, and A.G. Rossi. 2004. Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products. *Biochem.Biophys.Res.Commun.* 318:32-37.

22. Hodge, S., G. Hodge, R. Scicchitano, P.N. Reynolds, and M. Holmes. 2003. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in

their ability to phagocytose apoptotic airway epithelial cells. *Immunol.Cell Biol.* 81:289-296.

23. Ortega, E., F. Hueso, M.E. Collazos, M.I. Pedrera, C. Barriga, and A.B. Rodriguez. 1992. Phagocytosis of latex beads by alveolar macrophages from mice exposed to cigarette smoke. *Comp.Immunol.Microbiol.Infect.Dis.* 15:137-142.

24. Ortega, E., C. Barriga, and A.B. Rodriguez. 1994. Decline in the phagocytic function of alveolar macrophages from mice exposed to cigarette smoke. *Comp.Immunol.Microbiol.Infect.Dis.* 17:77-84.

25. Wright, J.L., and A. Churg. 1990. Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig. *Am.Rev.Respir.Dis.* 142:1422-1428.

26. Hsu, A., D.M. Aronoff, J. Phipps, D. Goel, and P. Mancuso. 2007. Leptin improves pulmonary bacterial clearance and survival in ob/ob mice during pneumococcal pneumonia. *Clin.Exp.Immunol.* 150:332-339.

27. Greenberger, M.J., R.M. Strieter, S.L. Kunkel, J.M. Danforth, R.E. Goodman, and T.J. Standiford. 1995. Neutralization of IL-10 increases survival in a murine model of Klebsiella pneumonia. *J.Immunol.* 155:722-729.

28. Mancuso, P., and M. Peters-Golden. 2000. Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C-dependent. *Am.J.Respir.Cell Mol.Biol.* 23:727-733.

29. Mancuso, P., G.B. Huffnagle, M.A. Olszewski, J. Phipps, and M. Peters-Golden. 2006. Leptin corrects host defense defects after acute starvation in murine pneumococcal pneumonia. *Am.J.Respir.Crit.Care Med.* 173:212-218.

30. Aronoff, D.M., C. Canetti, and M. Peters-Golden. 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J.Immunol.* 173:559-565.

31. Martens, P., S.W. Worm, B. Lundgren, H.B. Konradsen, and T. Benfield. 2004. Serotype-specific mortality from invasive Streptococcus pneumoniae disease revisited. *BMC Infect.Dis.* 4:21.

32. Rijneveld, A.W., S. Florquin, J. Branger, P. Speelman, S.J. Van Deventer, and T. van der Poll. 2001. TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. *J.Immunol.* 167:5240-5246.

33. Tokairin, Y., Y. Shibata, M. Sata, S. Abe, N. Takabatake, A. Igarashi, T. Ishikawa, S. Inoue, and I. Kubota. 2008. Enhanced immediate inflammatory response to Streptococcus pneumoniae in the lungs of mice with pulmonary emphysema. *Respirology.* 13:324-332.

34. Bergeron, Y., N. Ouellet, A.M. Deslauriers, M. Simard, M. Olivier, and M.G. Bergeron. 1998. Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect.Immun.* 66:912-922.

35. Dehoux, M.S., A. Boutten, J. Ostinelli, N. Seta, M.C. Dombret, B. Crestani, M. Deschenes, J.L. Trouillet, and M. Aubier. 1994. Compartmentalized cytokine production within the human lung in unilateral pneumonia. *Am.J.Respir.Crit.Care Med.* 150:710-716.

36. Taut, K., C. Winter, D.E. Briles, J.C. Paton, J.W. Christman, R. Maus, R. Baumann, T. Welte, and U.A. Maus. 2008. Macrophage Turnover Kinetics in the Lungs of Mice Infected with Streptococcus pneumoniae. *Am.J.Respir.Cell Mol.Biol.* 38:105-113.

37. Koyama, S., S.I. Rennard, D. Daughton, S. Shoji, and R.A. Robbins. 1991. Bronchoalveolar lavage fluid obtained from smokers exhibits increased monocyte chemokinetic activity. *J.Appl.Physiol.* 70:1208-1214.

38. Hautamaki, R.D., D.K. Kobayashi, R.M. Senior, and S.D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* 277:2002-2004.

39. Dockrell, D.H., M. Lee, D.H. Lynch, and R.C. Read. 2001. Immune-mediated phagocytosis and killing of Streptococcus pneumoniae are associated with direct and bystander macrophage apoptosis. *J.Infect.Dis.* 184:713-722.

40. Fisher, G.L., K.L. McNeill, G.L. Finch, F.D. Wilson, and D.W. Golde. 1982. Functional evaluation of lung macrophages from cigarette smokers and nonsmokers. *J.Reticuloendothel.Soc.* 32:311-321.

41. Morris, D.G., X. Huang, N. Kaminski, Y. Wang, S.D. Shapiro, G. Dolganov, A. Glick, and D. Sheppard. 2003. Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. *Nature.* 422:169-173.

42. Arredouani, M.S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik. 2006. The Macrophage Scavenger Receptor SR-AI/II and Lung Defense against Pneumococci and Particles. *Am.J.Respir.Cell Mol.Biol.*

43. Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik. 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J.Exp.Med.* 200:267-272.

44. Kerr, A.R., G.K. Paterson, A. Riboldi-Tunnicliffe, and T.J. Mitchell. 2005. Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. *Infect.Immun.* 73:4245-4252.

45. Yuste, J., M. Botto, J.C. Paton, D.W. Holden, and J.S. Brown. 2005. Additive inhibition of complement deposition by pneumolysin and PspA facilitates Streptococcus pneumoniae septicemia. *J.Immunol.* 175:1813-1819.

46. Robbins, R.A., K.J. Nelson, G.L. Gossman, S. Koyama, and S.I. Rennard. 1991. Complement activation by cigarette smoke. *Am.J.Physiol.* 260:L254-9.

47. Kew, R.R., B. Ghebrehiwet, and A. Janoff. 1985. Cigarette smoke can activate the alternative pathway of complement in vitro by modifying the third component of complement. *J.Clin.Invest.* 75:1000-1007.

48. Perricone, R., C. de Carolis, G. de Sanctis, and L. Fontana. 1983. Complement activation by cigarette smoke condensate and tobacco infusion. *Arch.Environ.Health.* 38:176-179.

CHAPTER 4

Cigarette smoke impairs alveolar macrophage cytokine production through a TLR2-mediated mechanism

Introduction

Epidemiological reports have linked cigarette smoke (CS) exposure with a wide variety of infectious diseases (1). Laboratory studies found impaired host defense against viral (2, 3), fungal (4), and bacterial (5) pathogens in smoke-exposed animals. Surprisingly, there is a paucity of experimental data describing the effect of CS exposure on innate host defense against the gram-positive bacterial pathogen *Streptococcus pneumoniae*. In human populations, exposure to cigarette smoke (CS) is independently associated with increased risk of serious pneumococcal infections (6-8), although the specific nature of this association has yet to be elucidated. Pneumococcal pneumonia is the most common cause of community-acquired pneumonia the US (9) and globally (10), and is the leading cause of death from invasive bacterial infections (11). The growth of particularly susceptible populations such as the elderly and immunocompromised, as well as the increase in antibiotic-resistant strains (9), make pneumococcal disease a continuing cause for concern.

Within the lung, the alveolar macrophage (AM) plays an important role as a sentinel cell of innate immunity requiring the capacity to identify and respond to targets

without prior exposure. For this purpose, AMs express a large number of receptors that recognize molecular motifs including pathogen-associated molecular patterns (PAMPs). Examples relevant to pneumococcus include toll like receptor (TLR) 2, TLR4 (12), scavenger receptor (SR)-A (13), macrophage receptor with collagenous structure (MARCO) (14), as well as a cytoplasmic receptor Nucleotide-binding oligomerization domain-2 (NOD2) (15). Engagement of these receptors with ligands initiates signal transduction cascades leading to AM activation. In some cases, such as TLR2 and TLR4 this signaling is dependent on reactive oxygen intermediates (ROI) (16, 17).

An unequivocal consensus is lacking regarding the effects of CS exposure on AM cytokine production, due at least in part to wide variation in models and methodology. Some studies have found that CS activates AMs to produce proinflammatory cytokines (18). More commonly, CS is associated with impairments in cytokine production, particularly in response to microbial constituents (19-21).

Pneumococcal pneumonia provokes robust pro-inflammatory cytokine production in the lungs, and suppression of this response has been shown to impair host defense in animal models (22-25), while experimental inducement of proinflammatory cytokines prior to infection can improve pneumococcal clearance and host survival (26). Furthermore, less virulent pneumococcal strains which rarely disseminate from the lungs have been shown to elicit a greater proinflammatory cytokine response than strains which more frequently spread into systemic infections (27), suggesting that even partially impaired cytokine production may be important in determining the course of infection.

Because AMs are a major source of cytokines in the lung (28, 29), and because cytokine production is an indicator of macrophage activation following bacterial challenge, we sought to determine the effects of CS exposure on the ability of AMs to produce specific mediators, as a relevant indicator of innate immune function. Further, we examined AM TLR expression as well as ROI production, a response mediated downstream of the TLRs, in order to provide mechanistic insights into bacterially-induced AM activation following CS exposure.

Methods

Animals

Female BALB/C mice aged 8-12 weeks were purchased from Charles River Laboratories (Portage, MI). In vitro determination of leukocyte cytokine release after in vivo CS exposures used male C57BL/6 mice, obtained at 8-12 weeks of age (Jackson Laboratories, Barharbor, ME). All experiments were conducted in accordance with the Animal Care and Use Committee of the University of Michigan.

CS exposure model

Smoke from standardized 2R4F research cigarettes (University of Kentucky Tobacco Research Institute, Lexington, KY) was generated by a TE-2 cigarette smoking machine (Teague Enterprises, Davis, CA), at a mean concentration of 19.6 mg/m^3. Animals were exposed 4 hr/day, 5 day/week for 5 weeks in a whole-body exposure chamber, with water available *ad libidum*. Control animals were housed in an identical chamber, but exposed to room air with no smoke. For cytokine release from isolated

pulmonary leukocytes, exposures were two weeks at 1.8 mg/m^3, and used a custom-built cigarette smoking machine, previously described (30). Exposure levels were determined gravimetrically.

Bacteria

S. pneumoniae, serotype 3, 6303, (American Type Culture Collection, Manassas, VA) was grown to mid-log phase in Todd Hewett broth supplemented with 0.5% yeast extract (Difco, Detroit, MI) at 37°C, with 5% CO_2, washed in sterile water and heat-killed at >90°C for 30 min. In vivo challenge was performed via intratracheal instillation as previously described (31), at a dose equivalent to $1X10^6$ CFU.

In vitro CS exposures: CS-conditioned media (CSCM)

Smoke from five cigarettes was drawn through 50 ml RPMI 1640 (Invitrogen, Carlsbad, CA) using a glass impinger (Ace Glass, Vineland, NJ), after which the fluid was aliquoted and stored at -70ºC. Pilot dose-response studies were conducted to determine non-cytotoxic levels using the XTT assay (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's directions. Direct cytotoxicity of CSCM was not observed in this model at levels up to 2.0% CSCM, although it was seen at 5.0% and above (data not shown). All experiments were conducted with 1% or less CSCM, added to the cell culture media after adherence and incubated 18-24 hours.

Bronchoalveolar lavage (BAL) and AM culture

Mice were euthanized by CO_2 inhalation or intraperitoneal pentobarbital injection, and lungs removed surgically after exsanguination. The trachea was cannulated onto a hypodermic needle/syringe combination. In 0.5 ml increments, 10 ml of ice-cold HEPES-buffered saline solution containing dextrose, sodium EDTA, and penicillin/streptomycin (Invitrogen, Carlsbad, CA) was instilled into the lung and removed to a separate syringe. After recovery by BAL, cells were centrifuged at approximately 400 x g, resuspended in RPMI1640 (Invitrogen), enumerated with a hemocytometer, and plated at the desired concentration. After 1h at 37ºC, 5% CO_2, the media was replaced with warm RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and returned to the incubator. For digest-derived AMs, enzymatic digestion of lung tissue was performed as previously described (32), followed by filtration, gradient centrifugation, and adherence purification, and were then cultured as with BAL-derived AMs. For in vitro CS exposure, AMs were first cultured 24 hr with or without CSCM, and subsequently challenged with 50 μg/ml purified LTA (Sigma Aldrich, St. Louis, MO).

Determination of pulmonary cytokines

Homogenization of lung was performed as previously described (33) except lungs were not perfused prior to homogenization. After centrifugation, supernatants were stored at -80°C until analysis. Determination of TNF-α, IL-6 and PGE_2 release from digest-derived leukocytes and PGE_2 levels in lung homogenate were performed in our lab using commercial ELISA kits (Assay Designs, Ann Arbor, MI). Determination of cytokine release from BAL-derived AMs and in whole lung homogenates was performed

at the University of Michigan Immunology Core, using commercially available reagents (R&D Systems, Minneapolis, MN).

RT-PCR

After 24 hour incubation with or without 1% CSCM, primary cultures of AMs challenged with 50 μg/ml LTA for 4 h followed by processing with RNeasy kit (Qiagen, Valencia, CA), and RT-PCR was performed as previously described (34).

ROS generation

After 24 hr incubation with or without 1% CSCM, primary cultures of AMs were loaded with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate H_2DCFDA (Invitrogen), according to the manufacturer's guidelines, and subsequently challenged with 50 μg/ml LTA for 1 h.

Statistical Analysis

Data was analyzed using Prism 4 (GraphPad Software, La Jolla, CA) using a t test where appropriate, or one-way analysis of variancewith post-hoc tests as noted. In all cases, a *P* value of less than 0.05 was considered significant.

Results

Reduced Pulmonary Cytokine Levels in CS-exposed Mice

To determine the effect of CS exposure on pulmonary cytokine levels in vivo, we challenged mice with heat-killed pneumococcus after 5 week CS or room air (control)

exposure, and measured pulmonary cytokines in BAL fluid and whole lung homogenate. Levels shown in Figure 4.1 are the sum of BAL and homogenate values, and show a trend toward lower levels of TNF-α, IL-1β and IL-10 in CS-exposed animals, which reached significance at 48 hr. The reduction was specific to these cytokines, as levels of IL-6, MIP-2, MCP-1, TGF-β, and PGE_2 showed no significant differences between CS and control groups at either 24 or 48 hours post challenge (data not shown). Differences between groups were not due to reduced numbers or viability of pulmonary leukocytes (data not shown).

Impaired cytokine production by Lung-digest Derived AMs from Smoke-exposed Mice

To better understand whether CS-exposure alters the production of cytokines by AMs, we harvested these cells from CS-exposed and control animals, challenged them with heat-killed pneumococcus in vitro, and assessed levels of select mediators in the culture medium. In collagenase digest-derived primary leukocyte cultures which had been gradient and adherence purified for AMs, cells from CS-exposed animals produced reduced levels of the important pro-inflammatory cytokines TNF-α and IL-6, as well as the anti-inflammatory PGE_2 in response to challenge with heat-killed pneumococcus (Figure 4.2). The cells were plated in equal numbers, and viability as measured by the XTT assay was not different between groups (data not shown).

CS-mediated cytokine impairments can be reproduced in vitro using primary AM cultures and CSCM pretreatment.

To confirm a direct effect of CS on AMs in the absence of other cell types, we harvested AMs from the BAL fluid of naive animals, and exposed them to CSCM in

vitro. After subsequent challenge with the TLR2 ligand LTA, supernatants were collected for determination of TNF-α, a prototypical indicator of macrophage activation. AMs cultured for 24 hours in media containing CSCM displayed impaired TNF-α production in response to LTA stimulation (Figure 4.3). This effect was dose-dependent, and became significant at a concentration of 0.5% CSCM in the culture media. Here we used the TLR2 ligand LTA as the challenge, which is a more specific activating stimulus than whole bacteria. This approach served to isolate the observed response from effects of other TLRs, or other receptors responsive to pneumococcal activation.

AM transcription of TLR2 and TLR4 is not reduced by CSCM pretreatment

Since TLR2 is a receptor for LTA, and both TLR2 and TLR4 are known to play important roles in innate recognition of pneumococcus, we cultured AMs with or without CSCM to determine whether transcription of these receptors was affected. We also compared TLR2 expression in CSCM-pretreated and control AMs after challenge with LTA. Figure 4.4 shows that CSCM-pretreated and control AMs displayed similar levels of TLR2 mRNA, either in the presence or absence of LTA. As seen in Figure 4.4, CSCM pretreatment did not reduce the level of TLR4 mRNA relative to control. Interestingly, there was a non-significant trend toward higher levels of TLR4 transcript in CSCM-pretreated AMs.

Pretreatment of AMs with CSCM In vitro Impairs AM Production of Intracellular Reactive Oxygen Intermediates.

To assess the effect of CS exposure on the AM respiratory burst, we loaded CSCM-pretreated or control AMs with the fluorescent indicator H_2DCFDA, followed by

challenge with either gram-positive LTA or media alone. Figure 4.5 shows that while levels of ROI generation in untreated AMs were very similar between control and CSCM pretreated cells, LTA challenge of control cells resulted in a significant induction of intracellular ROS generation (about 2-fold greater than saline challenge). In contrast, challenge of CSCM-pretreated cells induced a non-significant 40% increase over unchallenged cells.

Discussion

Following pneumococcal challenge, lower levels of IL-1β, TNF-α and IL-10 were found in the lungs of CS-exposed animals than in lungs from control animals, but IL-6 and MIP-2 (the murine equivalent of IL-8, normally associated with neutrophil recruitment), as well as the immunomodulatory prostaglandin PGE_2, which are all known to be produced by AMs in response to pneumococcal challenge (35, 31, 36, 23, 37) were not. The pattern cannot be described simply in terms of exacerbated or diminished inflammatory status in the lung, as IL-1β and TNF-α are proinflammatory, whereas IL-10 acts to moderate inflammation.

These data are useful in that they demonstrate a cytokine-suppressive effect of CS-exposure at the organismal level, however they do not specifically demonstrate a role for AMs, due to the presence of other cell types capable of producing these mediators. Further, these data derive from whole lung homogenate, which is relevant because of the compartmentalization of specific cytokine responses within the lung. For example, in a study of human patients with unilateral bacterial pneumonia, AMs from the involved lung spontaneously produced greater IL-1β, IL-6 and TNF-α than AMs from the non-infected

lung, consistent with higher levels of all three cytokines found in the involved lung. However, only IL-6 was higher in the serum of these patients than healthy controls (35). Thus, differences in cytokine levels within specific pneumococcus-containing microenvironments are likely greater than can be observed from integrated whole-lung levels, particularly as the heat-killed bacteria used in these experiments cannot proliferate and colonize additional areas.

AMs were more specifically implicated by obtaining these cells from CS-exposed animals and subsequently challenging them in vitro with heat-killed pneumococcus. In these experiments, we found that each of the mediators we measured (IL-6, TNF-α, and PGE_2) was produced at lower levels in cells from CS-exposed animals compared with controls, an effect that was not explainable by reduced cell viability or alterations in the composition of the pulmonary leukocyte population. Because we measured lower levels of IL-6 and PGE_2 from isolated cells in this model, but not in whole lung homogenate, it may be that reduced AM production of these mediators in the whole-lung model was masked by production from other cell types. In subsequent experiments we replaced the in vivo CS exposure with CSCM pretreatment of AMs harvested from naive animals, and found that this widely-used in vitro CS surrogate also impaired production of TNF-α. Here, involvement of a TLR2-mediated mechanism is implied, because the effect was seen with purified LTA as the challenge.

Previous reports have described in vitro impairments in bacterially-induced AM pro-inflammatory cytokine production following exposure to CS components (38, 39). Although these studies focus on gram-negative bacteria or LPS, a recent report indicates

AMs from human smokers display impaired synthesis of IL-1β, IL-6 and TNF-α following challenge with a TLR2-specific synthetic ligand (20). Interestingly, this study found impaired cytokine responses to both TLR2 and TLR4 ligands, but increased sensitivity to TLR3-mediated activation, pointing to altered intracellular signaling pathways rather than defective biosynthetic capacity in smoker's AMs. In contrast, a study using a murine model of CS exposure found that AMs from exposed mice had diminished cytokine responses to both TLR3 and TLR4 ligands in vitro (21). Neither of these studies found altered expression of TLRs on CS-exposed AMs, although others (40) have shown reduced expression of TLR2, but not TLR4, with AMs from human smokers.

Because TLR2 and TLR4 are both known to act as sensors for pneumococcal PAMPs, we sought to determine whether the observed in vitro impairments in cytokine production were due to reduced expression of these receptors in our model. Using RT-PCR, we found no significant differences in expression of either TLR between CSCM and control AMs that would explain the earlier observation. Exposure to bacterial PAMPs can modulate expression of TLRs by murine macrophages, as seen by increased expression of TLR2 following TLR4-mediated activation with LPS (41). This effect may be relevant in human CS exposure, as AMs from nonsmokers but not smokers displayed increased expression of TLR2 mRNA and protein following in vitro LPS challenge (40). LTA has also been shown to induce expression of TLR2 and TLR4 (42, 43) in human monocytes. For this reason, we examined the expression of these receptors after challenge with LTA, and found no significant effect of CSCM pretreatment. Consistent with previous reports, we did find a trend toward increased TLR2 expression after LTA exposure, but CSCM did not appear to modify this effect. Intriguingly, CSCM-pretreated

AMs displayed a non-significant trend toward higher levels of TLR4, an effect which has not been previously reported. While this is not an unreasonable finding, given that exposure to related toxicants including ozone (44) and residual oil fly ash (45) have been shown to upregulate TLR4 in AMs, such an effect would not be expected to result in impaired AM cytokine production.

Since CSCM-mediated impairment of cytokine production in our model was not explained by altered TLR expression, we next examined an intracellular response downstream of TLR activation. We found that CSCM pretreatment substantially impaired production of ROI by AMs in our model. Macrophages are known to produce ROI under stimulation with microbial products (46), damaged host proteins (47), and airborne particulate matter (48). In the past, ROI were often assumed to function primarily in direct microbial killing, but recent years have seen an emerging recognition of their role in signal transduction, particularly in macrophages (reviewed in 49, 50). ROI have been shown to participate in TLR2-stimulated generation of proinflammatory cytokines by monocytes (51), with activation of MAP kinases being an important ROS-dependent signaling step (52, 53). Thus, CS-mediated defects in ROI generation by AMs may well contribute to the reduction of proinflammatory cytokines we observed in vivo and in vitro with pneumococcal and LTA challenge by disrupting signal transduction downstream of TLRs.

The role of ROI in TLR-mediated signaling has been compared to the narrow point in an hourglass (54), with many TLRs and adaptor proteins signaling through a very limited number of ROI-sensitive initial kinases, leading to activation of a broader range

of distal kinases and transcription factors. The utility of innate immunity's overlapping PAMP-sensing mechanisms is illustrated by the fact that while TLR2 is undoubtedly the primary leukocyte sensor for LTA, mice lacking this receptor can still mount an effective response to pneumococcus through TLR4-mediated recognition of pneumolysin (55). In contrast, experimental blockade of ROI has been shown to impair TLR-mediated activation of NF-κB and production of downstream cytokines (46, 47, 56, 57) while exogenous reactive oxygen species in the form of H_2O_2 can activate MAP kinases and NF-κB (reviewed in (58)). In another study using in vitro pneumococcal infection of vital human lung tissue (29), it was found that AM production of pro-inflammatory cytokines IL-6, TNF-α and IL-8 were significantly reduced by pharmacological inhibition of MAP kinase, but only modestly by anti-TLR2 antibodies.

The data presented here demonstrate that CS exposure impairs AM production of immune mediators in response to heat-killed *S. pneumoniae*. Because CSCM-pretreated AMs were also hyporesponsive to purified LTA, it is likely that disruption of TLR2-mediated pathways is involved in the observed impairments, although reduced expression of TLR2 was not apparent. Defective production of ROI was observed in CSCM-pretreated AMs, which may be a mechanism underlying the reduced cytokine response in these cells. Given the important role played by cytokines in the course of pneumococcal infection, and the role of AMs in producing these mediators, the functional defects we describe here may well contribute to increased pneumococcal susceptibility in CS-exposed humans.

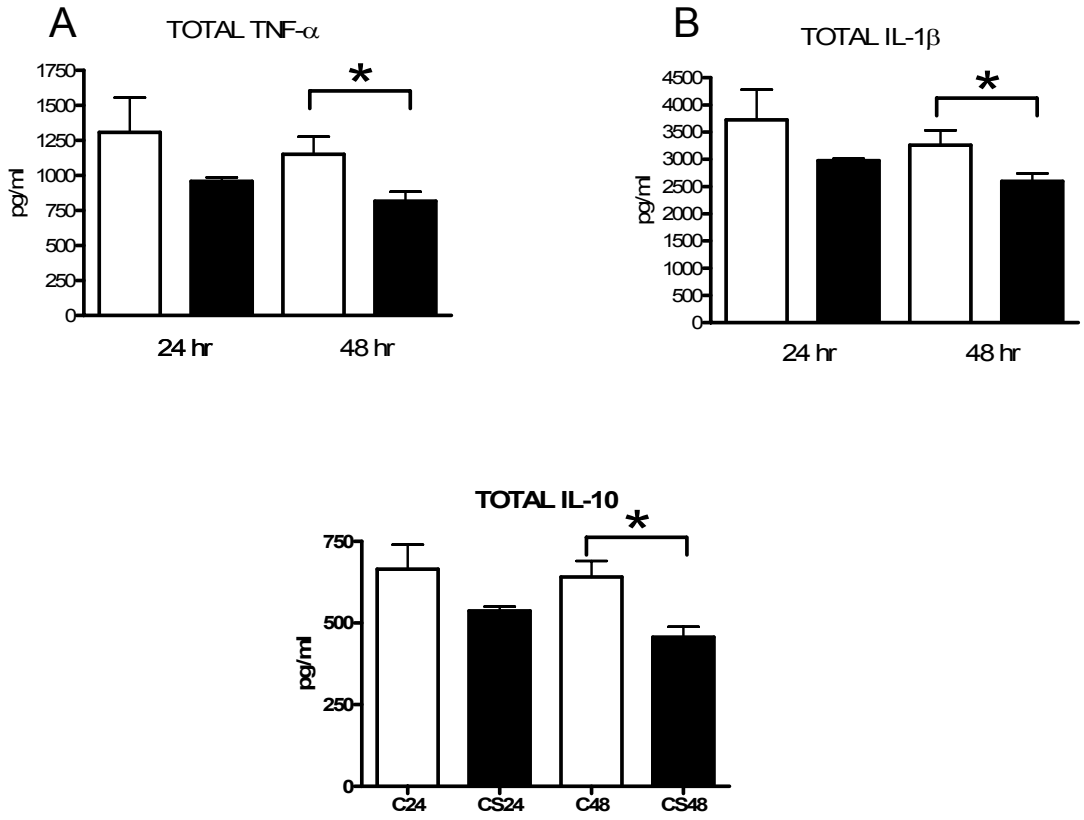

Figure 4.1. Cigarette smoke reduces pulmonary cytokine levels following pneumococcal challenge. Female BALB/C mice were exposed to CS (solid bars) or room air (open bars) for 5 weeks followed by intratracheal instillation with heat-killed *S. pneumoniae* (10^6 CFU-equivalent dose). Levels of TNF-α and IL-1β are expressed as the sum of whole lung homogenate and BAL fluid levels. Bars represent the mean ± standard error of the mean; n = 7–8 mice per group; **P* < 0.05 by unpaired t test.

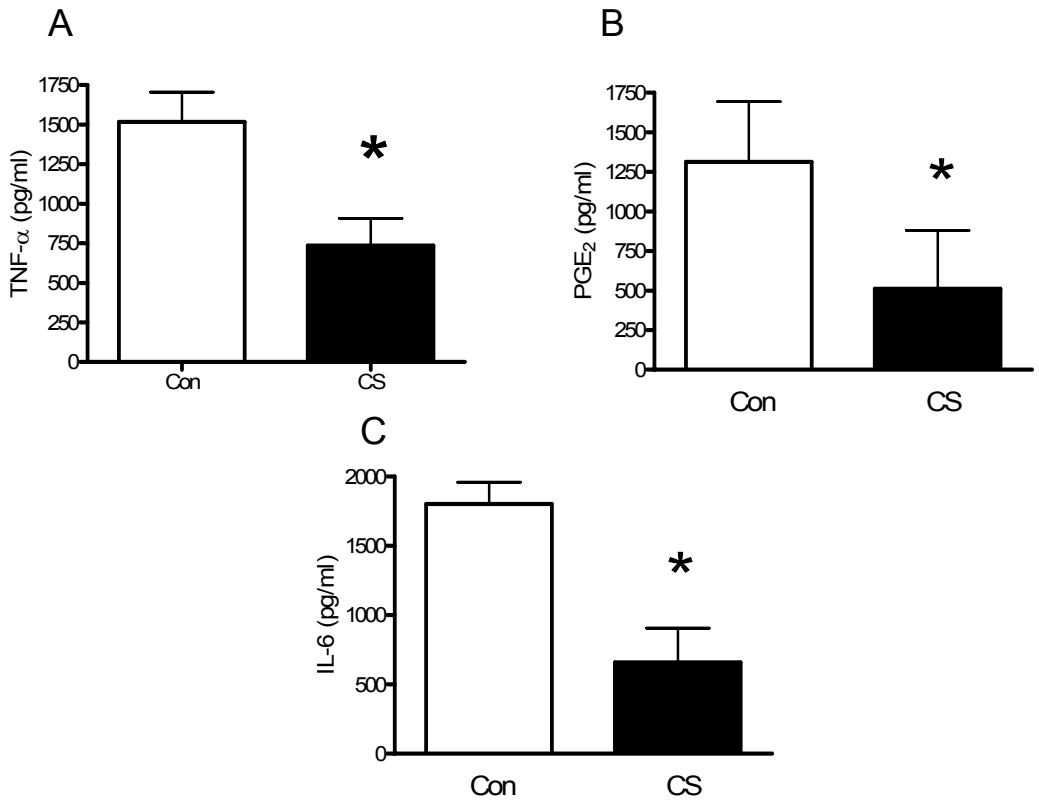

Figure 4.2. Cigarette smoke impairs cytokine and PGE2 production in digest-derived AMs. After 2 weeks vivo CS exposure, cells were harvested and purified as described in methods, followed by 24 hour challenge with heat-killed pneumococcus, prior to collection of supernatants for determination of TNF-α (A), PGE_2 (B) and IL-6 (C) Bars represent the mean ± standard error of the mean; n = 4-5 mice per group; *$P < 0.05$ by unpaired t test.

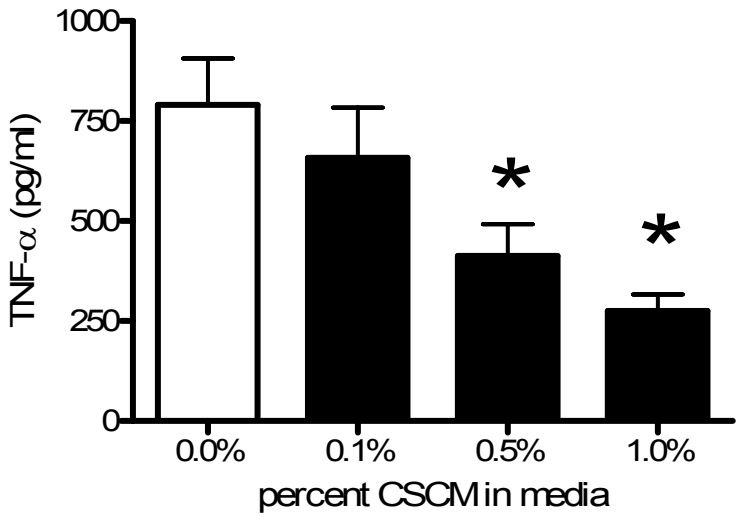

Figure 4.3 Cigarette smoke-conditioned media impairs AM production of TNF-α. Murine AMs were cultured for 24 hr with varying concentrations of CSCM (solid bars) or without CSCM (open bar). TNF-α levels were measured by ELISA after overnight challenge with purified LTA. Bars represent the mean ± standard error of the mean of four replicate experiments; *****$P < 0.05$ compared with control by one-way ANOVA with Dunnett's post-hoc test.

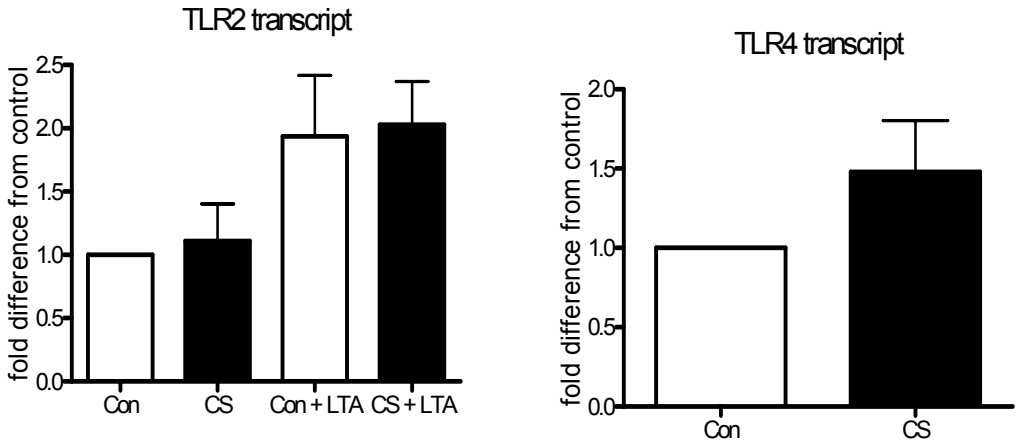

Figure 4.4 Cigarette smoke-conditioned media does not reduce expression of TLR2 or TLR4. Murine AMs were cultured for 24 hr with varying concentrations of CSCM (solid bars) or without CSCM (open bar), and with or without 50 μg/ml LTA. Bars represent the mean ± standard error of the mean of four replicate experiments.

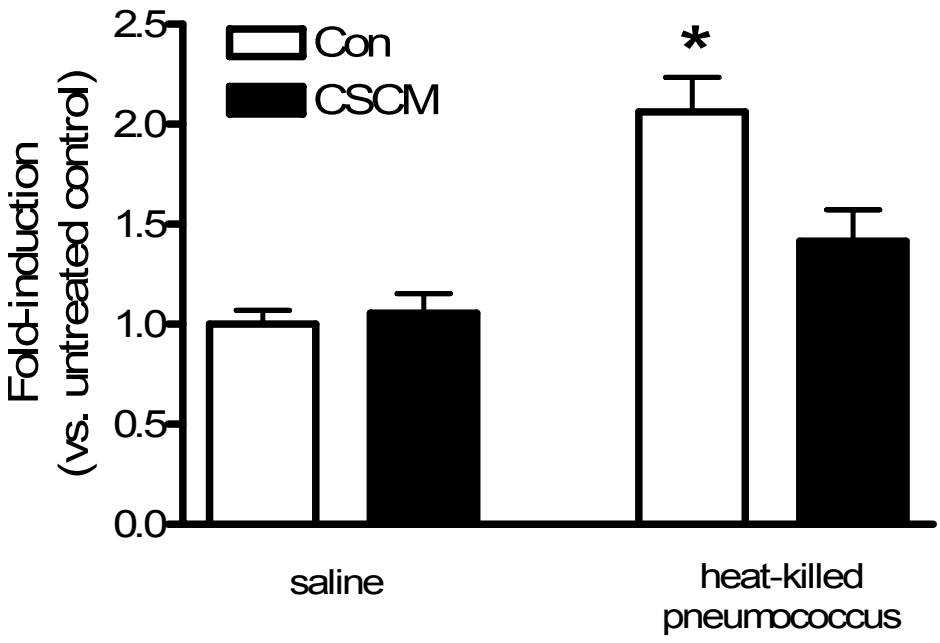

Figure 4.5. Cigarette smoke-conditioned media impairs LTA-induced AM production of intracellular ROI**.** Using the fluorescent indicator H_2DCFDA, AMs cultured in 1% CSCM produced lower levels of ROI relative to control cells after 2 hr challenge with 50 μg/ml LTA. Values are mean ± SEM of 4 identical experiments (final reading minus initial reading), normalized to saline challenge and analyzed by one-way ANOVA with Dunnett's post-hoc test. $*P < 0.05$ compared with saline-challenged control AMs by t test.

References

1. Arcavi, L., and N.L. Benowitz. 2004. Cigarette smoking and infection. *Arch.Intern.Med.* 164:2206-2216.

2. Phaybouth, V., S.Z. Wang, J.A. Hutt, J.D. McDonald, K.S. Harrod, and E.G. Barrett. 2006. Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 290:L222-31.

3. Gualano, R.C., M.J. Hansen, R. Vlahos, J.E. Jones, R.A. Park-Jones, G. Deliyannis, S.J. Turner, K.A. Duca, and G.P. Anderson. 2008. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir.Res.* 9:53.

4. Christensen, P.J., A.M. Preston, T. Ling, M. Du, W.B. Fields, J.L. Curtis, and J.M. Beck. 2008. Pneumocystis murina infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement, and pulmonary inflammation in mice. *Infect.Immun.* 76:3481-3490.

5. Drannik, A.G., M.A. Pouladi, C.S. Robbins, S.I. Goncharova, S. Kianpour, and M.R. Stampfli. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am.J.Respir.Crit.Care Med.* 170:1164-1171.

6. Nuorti, J.P., J.C. Butler, M.M. Farley, L.H. Harrison, A. McGeer, M.S. Kolczak, and R.F. Breiman. 2000. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N.Engl.J.Med.* 342:681-689.

7. Almirall, J., I. Bolibar, M. Serra-Prat, J. Roig, I. Hospital, E. Carandell, M. Agusti, P. Ayuso, A. Estela, A. Torres, and Community-Acquired Pneumonia in Catalan Countries (PACAP) Study Group. 2008. New evidence of risk factors for community-acquired pneumonia: a population-based study. *Eur.Respir.J.* 31:1274-1284.

8. Pastor, P., F. Medley, and T.V. Murphy. 1998. Invasive pneumococcal disease in Dallas County, Texas: results from population-based surveillance in 1995. *Clin.Infect.Dis.* 26:590-595.

9. Mandell, L.A., R.G. Wunderink, A. Anzueto, J.G. Bartlett, G.D. Campbell, N.C. Dean, S.F. Dowell, T.M. File Jr, D.M. Musher, M.S. Niederman, A. Torres, C.G. Whitney, Infectious Diseases Society of America, and American Thoracic Society. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin.Infect.Dis.* 44 Suppl 2:S27-72.

10. Anonymous . 1999. Pneumococcal vaccines. WHO position paper. *Wkly.Epidemiol.Rec.* 74:177-183.

11. Kadioglu, A., and P.W. Andrew. 2004. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 25:143-149.

12. Schnare, M., M. Rollinghoff, and S. Qureshi. 2006. Toll-like receptors: sentinels of host defence against bacterial infection. *Int.Arch.Allergy Immunol.* 139:75-85.

13. Arredouani, M.S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik. 2006. The Macrophage Scavenger Receptor SR-AI/II and Lung Defense against Pneumococci and Particles. *Am.J.Respir.Cell Mol.Biol.*

14. Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik. 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J.Exp.Med.* 200:267-272.

15. Opitz, B., A. Puschel, B. Schmeck, A.C. Hocke, S. Rosseau, S. Hammerschmidt, R.R. Schumann, N. Suttorp, and S. Hippenstiel. 2004. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J.Biol.Chem.* 279:36426-36432.

16. Amory-Rivier, C.F., J. Mohler, J.P. Bedos, E. Azoulay-Dupuis, D. Henin, M. Muffat-Joly, C. Carbon, and P. Moine. 2000. Nuclear factor-kappaB activation in mouse lung lavage cells in response to Streptococcus pneumoniae pulmonary infection. *Crit.Care Med.* 28:3249-3256.

17. Ogura, Y., N. Inohara, A. Benito, F.F. Chen, S. Yamaoka, and G. Nunez. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J.Biol.Chem.* 276:4812-4818.

18. Valenca, S.S., P. Castro, W.A. Pimenta, M. Lanzetti, S.V. Silva, C. Barja-Fidalgo, V.L. Koatz, and L.C. Porto. 2006. Light cigarette smoke-induced emphysema and NFkappaB activation in mouse lung. *Int.J.Exp.Pathol.* 87:373-381.

19. Kent, L., L. Smyth, C. Clayton, L. Scott, T. Cook, R. Stephens, S. Fox, P. Hext, S. Farrow, and D. Singh. 2008. Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. *Cytokine.* 42:205-216.

20. Chen, H., M.J. Cowan, J.D. Hasday, S.N. Vogel, and A.E. Medvedev. 2007. Tobacco smoking inhibits expression of proinflammatory cytokines and activation of IL-1R-associated kinase, p38, and NF-kappaB in alveolar macrophages stimulated with TLR2 and TLR4 agonists. *J.Immunol.* 179:6097-6106.

21. Gaschler, G.J., C.C. Zavitz, C.M. Bauer, M. Skrtic, M. Lindahl, C.S. Robbins, B. Chen, and M.R. Stampfli. 2008. Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages. *Am.J.Respir.Cell Mol.Biol.* 38:218-226.

22. van der Poll, T., C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, and S.F. Lowry. 1997. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J.Infect.Dis.* 176:439-444.

23. Jones, M.R., B.T. Simms, M.M. Lupa, M.S. Kogan, and J.P. Mizgerd. 2005. Lung NF-kappaB activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia. *J.Immunol.* 175:7530-7535.

24. van der Poll, T., C.V. Keogh, W.A. Buurman, and S.F. Lowry. 1997. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. *Am.J.Respir.Crit.Care Med.* 155:603-608.

25. Rijneveld, A.W., S. Florquin, J. Branger, P. Speelman, S.J. Van Deventer, and T. van der Poll. 2001. TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. *J.Immunol.* 167:5240-5246.

26. Reppe, K., T. Tschernig, A. Luhrmann, V. van Laak, K. Grote, M.V. Zemlin, B. Gutbier, H.C. Muller, M. Kursar, H. Schutte, S. Rosseau, R. Pabst, N.W. Suttorp, and M. Witzenrath. 2008. Immunostimulation with Macrophage-Activating Lipopeptide-2 Increased Survival in Murine Pneumonia. *Am.J.Respir.Cell Mol.Biol.*

27. Burgess, T.S., A.F. Hirschfeld, G.J. Tyrrell, J.A. Bettinger, and S.E. Turvey. 2008. Commonly invasive serotypes of Streptococcus pneumoniae trigger a reduced innate immune response compared with serotypes rarely responsible for invasive infection. *FEMS Immunol.Med.Microbiol.* 53:136-139.

28. Thomassen, M.J., L.T. Divis, and C.J. Fisher. 1996. Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10. *Clin.Immunol.Immunopathol.* 80:321-324.

29. Xu, F., D. Droemann, J. Rupp, H. Shen, X. Wu, T. Goldmann, S. Hippenstiel, P. Zabel, and K. Dalhoff. 2008. Modulation of the inflammatory response to Streptococcus pneumoniae in a model of acute lung tissue infection. *Am.J.Respir.Cell Mol.Biol.* 39:522-529.

30. Wright, J.L., and A. Churg. 1990. Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig. *Am.Rev.Respir.Dis.* 142:1422-1428.

31. Hsu, A., D.M. Aronoff, J. Phipps, D. Goel, and P. Mancuso. 2007. Leptin improves pulmonary bacterial clearance and survival in ob/ob mice during pneumococcal pneumonia. *Clin.Exp.Immunol.* 150:332-339.

32. Traynor, T.R., W.A. Kuziel, G.B. Toews, and G.B. Huffnagle. 2000. CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection. *J.Immunol.* 164:2021-2027.

33. Greenberger, M.J., R.M. Strieter, S.L. Kunkel, J.M. Danforth, R.E. Goodman, and T.J. Standiford. 1995. Neutralization of IL-10 increases survival in a murine model of Klebsiella pneumonia. *J.Immunol.* 155:722-729.

34. Ballinger, M.N., L.L. Hubbard, T.R. McMillan, G.B. Toews, M. Peters-Golden, R. Paine 3rd, and B.B. Moore. 2008. Paradoxical role of alveolar macrophage-derived granulocyte-macrophage colony-stimulating factor in pulmonary host defense post-bone marrow transplantation. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 295:L114-22.

35. Dehoux, M.S., A. Boutten, J. Ostinelli, N. Seta, M.C. Dombret, B. Crestani, M. Deschenes, J.L. Trouillet, and M. Aubier. 1994. Compartmentalized cytokine production within the human lung in unilateral pneumonia. *Am.J.Respir.Crit.Care Med.* 150:710-716.

36. Mohler, J., E. Azoulay-Dupuis, C. Amory-Rivier, J.X. Mazoit, J.P. Bedos, V. Rieux, and P. Moine. 2003. Streptococcus pneumoniae strain-dependent lung inflammatory responses in a murine model of pneumococcal pneumonia. *Intensive Care Med.* 29:808-816.

37. Sorrell, T.C., C.P. Rochester, F.N. Breen, and M. Muller. 1989. Eicosanoids produced during interactions between Pseudomonas aeruginosa and alveolar macrophages are species-dependent. *Immunol.Cell Biol.* 67 (Pt 3):169-176.

38. Dubar, V., P. Gosset, C. Aerts, C. Voisin, B. Wallaert, and A.B. Tonnel. 1993. In vitro acute effects of tobacco smoke on tumor necrosis factor alpha and interleukin-6 production by alveolar macrophages. *Exp.Lung Res.* 19:345-359.

39. Higashimoto, Y., Y. Shimada, Y. Fukuchi, K. Ishida, C. Shu, S. Teramoto, E. Sudo, T. Matsuse, and H. Orimo. 1992. Inhibition of mouse alveolar macrophage production of tumor necrosis factor alpha by acute in vivo and in vitro exposure to tobacco smoke. *Respiration.* 59:77-80.

40. Droemann, D., T. Goldmann, T. Tiedje, P. Zabel, K. Dalhoff, and B. Schaaf. 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir.Res.* 6:68.

41. Matsuguchi, T., T. Musikacharoen, T. Ogawa, and Y. Yoshikai. 2000. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *J.Immunol.* 165:5767-5772.

42. Moller, A.S., R. Ovstebo, K.B. Haug, G.B. Joo, A.B. Westvik, and P. Kierulf. 2005. Chemokine production and pattern recognition receptor (PRR) expression in whole blood stimulated with pathogen-associated molecular patterns (PAMPs). *Cytokine.* 32:304-315.

43. Ryu, Y.J., E.J. Kim, S.H. Lee, S.Y. Kim, G.Y. Suh, M.P. Chung, H. Kim, O.J. Kwon, and W.J. Koh. 2007. Impaired expression of Toll-like receptor 2 in nontuberculous mycobacterial lung disease. *Eur.Respir.J.* 30:736-742.

44. Williams, A.S., S.Y. Leung, P. Nath, N.M. Khorasani, P. Bhavsar, R. Issa, J.A. Mitchell, I.M. Adcock, and K.F. Chung. 2007. Role of TLR2, TLR4, and MyD88 in murine ozone-induced airway hyperresponsiveness and neutrophilia. *J.Appl.Physiol.* 103:1189-1195.

45. Cho, H.Y., A.E. Jedlicka, R. Clarke, and S.R. Kleeberger. 2005. Role of Toll-like receptor-4 in genetic susceptibility to lung injury induced by residual oil fly ash. *Physiol.Genomics.* 22:108-117.

46. Sadikot, R.T., H. Zeng, F.E. Yull, B. Li, D.S. Cheng, D.S. Kernodle, E.D. Jansen, C.H. Contag, B.H. Segal, S.M. Holland, T.S. Blackwell, and J.W. Christman. 2004. p47phox deficiency impairs NF-kappa B activation and host defense in Pseudomonas pneumonia. *J.Immunol.* 172:1801-1808.

47. Eberlein, M., K.A. Scheibner, K.E. Black, S.L. Collins, Y. Chan-Li, J.D. Powell, and M.R. Horton. 2008. Anti-oxidant inhibition of hyaluronan fragment-induced inflammatory gene expression. *J.Inflamm.(Lond).* 5:20.

48. Zhang, Y., J.J. Schauer, M.M. Shafer, M.P. Hannigan, and S.J. Dutton. 2008. Source apportionment of in vitro reactive oxygen species bioassay activity from atmospheric particulate matter. *Environ.Sci.Technol.* 42:7502-7509.

49. Gwinn, M.R., and V. Vallyathan. 2006. Respiratory burst: role in signal transduction in alveolar macrophages. *J.Toxicol.Environ.Health B Crit.Rev.* 9:27-39.

50. Forman, H.J., and M. Torres. 2001. Signaling by the respiratory burst in macrophages. *IUBMB Life.* 51:365-371.

51. Lee, I.T., S.W. Wang, C.W. Lee, C.C. Chang, C.C. Lin, S.F. Luo, and C.M. Yang. 2008. Lipoteichoic acid induces HO-1 expression via the TLR2/MyD88/c-Src/NADPH oxidase pathway and Nrf2 in human tracheal smooth muscle cells. *J.Immunol.* 181:5098-5110.

52. Song, C.H., J.S. Lee, S.H. Lee, K. Lim, H.J. Kim, J.K. Park, T.H. Paik, and E.K. Jo. 2003. Role of mitogen-activated protein kinase pathways in the production of tumor necrosis factor-alpha, interleukin-10, and monocyte chemotactic protein-1 by Mycobacterium tuberculosis H37Rv-infected human monocytes. *J.Clin.Immunol.* 23:194-201.

53. Yang, S.R., A.S. Chida, M.R. Bauter, N. Shafiq, K. Seweryniak, S.B. Maggirwar, I. Kilty, and I. Rahman. 2006. Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. *Am.J.Physiol.Lung Cell.Mol.Physiol.* 291:L46-57.

54. Kolls, J.K. 2006. Oxidative stress in sepsis: a redox redux. *J.Clin.Invest.* 116:860-863.

55. Dessing, M.C., S. Florquin, J.C. Paton, and T. van der Poll. 2008. Toll-like receptor 2 contributes to antibacterial defence against pneumolysin-deficient pneumococci. *Cell.Microbiol.* 10:237-246.

56. Liu, L.L., L.K. Gong, H. Wang, Y. Xiao, X.F. Wu, Y.H. Zhang, X. Xue, X.M. Qi, and J. Ren. 2008. Baicalin inhibits macrophage activation by lipopolysaccharide and protects mice from endotoxin shock. *Biochem.Pharmacol.* 75:914-922.

57. Kim, J.H., H.J. Na, C.K. Kim, J.Y. Kim, K.S. Ha, H. Lee, H.T. Chung, H.J. Kwon, Y.G. Kwon, and Y.M. Kim. 2008. The non-provitamin A carotenoid, lutein, inhibits NF-kappaB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-kappaB-inducing kinase pathways: role of H(2)O(2) in NF-kappaB activation. *Free Radic.Biol.Med.* 45:885-896.

58. Gloire, G., S. Legrand-Poels, and J. Piette. 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem.Pharmacol.* 72:1493-1505.

CHAPTER 5

Conclusion

Project Rationale and Purpose

Over one billion people are active smokers, consuming more than 5 trillion cigarettes annually (1). There is a broad recognition that smoking is harmful, and recent decades have seen increased understanding of the dangers posed by ETS exposure. While rates of smoking are declining in many industrialized nations, global rates are growing due to increases in the developing world. The profound burden of disease caused by exposure to CS has lead the World Health Organization to describe exposure to CS as the leading cause of preventable death in the world (1). While much attention has been focused on diseases such as cancer and cardiovascular disease, both smoking and ETS have also been associated with increased incidence of infectious diseases (2, 3). It has been estimated that the increased burden of infectious disease due to CS exposure may rival cancer and heart disease as causes of CS-related morbidity and mortality (4).

S. pneumoniae is the leading cause of community-acquired pneumonia and death from invasive bacterial infections in the world, with developing countries bearing the largest burden. Both active smoking and ETS have been associated with increased susceptibility to pneumococcal infection. In the lung, the AM plays a central role in innate host defense, and previous research has indicted that CS can alter the normal function of these cells. Therefore, we sought to examine the effects of CS on innate host defense against pneumococcal pneumonia using a combination of in vivo and in vitro approaches. The goal of this project was to test the hypothesis that CS exposure impairs innate host defense against pneumococcal pneumonia, at least in part through a mechanism involving disruption of alveolar macrophage AM phagocytosis and cytokine production.

Key Findings

Increased Susceptibility to Pneumococcal Pneumonia

In these studies, mice exposed to cigarette smoke for 5 weeks prior to pulmonary infection with *S. pneumoniae* were found to have significantly higher pulmonary bacterial burdens 24 and 48 hr after infection (4-fold and 35-fold, respectively). This was accompanied by clinical signs of more severe illness, such as hypothermia and elevated levels of the inflammatory cytokines TNF-α, IL-1β, and IL-6. The increased pneumococcal burden was specific to the lung, as no systemic dissemination of bacteria was seen at these timepoints. This would seem to suggest that like humans, mice exposed to CS are more susceptible to pneumococcal infection. While a positive relationship between CS and impaired host defense against *S. pneumoniae* might be

expected given previous epidemiological associations, it is striking that an effect of this magnitude can be measured after only 5 weeks of exposure.

In the development of the exposure model, it was found that 5 weeks of CS exposure did not reduce the number or viability of BAL leukocytes relative to unexposed mice. Rather, greater numbers of AMs were present in CS-exposed mice, as has been reported in human smokers (5). Likewise, higher bacterial burdens in CS-exposed mice cannot be explained by reduced leukocyte number or viability following infection. This was shown by experiments in which mice were challenged with heat-killed *S. pneumoniae* following 5 weeks of CS exposure. Under these conditions, no impairment in leukocyte viability or recruitment was seen. On the contrary, these experiments revealed similar numbers of monocytes/macrophages and greater numbers of neutrophils in BAL fluid from CS-exposed animals following challenge.

Neutrophils are rapidly recruited to the lung following bacterial pneumonia (6), and have been shown to be important in host defense against pneumococcal pneumonia specifically (7). In COPD patients, neutrophils are commonly found in sputum during acute exacerbations, possibly due to bacterial infection(8). Some murine models of CS exposure are characterized by neutrophilia in the absence of infection (9), however in our model no such phenomenon was seen in the absence of bacterial challenge. Determination of leukocyte recruitment was performed because lowered neutrophil or monocyte numbers might have been a plausible mechanism to explain higher bacterial burden. The observation of significantly increased, rather than decreased, neutrophil recruitment following challenge in CS-exposed mice has possible implications. In

models of pneumococcal pneumonia with prior AM depletion, neutrophils are recruited to the alveoli in higher numbers. Consequently, this finding tends to support for the hypothesis that AM function is impaired by CS-exposure. It has been suggested that neutrophils may have detrimental effects by causing excessive inflammation and tissue damage. Such an effect is not ruled out in our model, however the lack of bacterial dissemination out of the lung suggests that this is not a major factor over the observed time span.

Impairment of Complement-mediated Phagocytosis

Since AMs are the primary resident phagocyte in the alveolar compartment, experiments were undertaken to determine the effects of CS on AM phagocytic functions. When AMs were recovered from mice exposed to CS, a significantly smaller proportion of cells participated in phagocytosis of serum-opsonized *S. pneumoniae*. Compared with the infection experiment, this result demonstrates that a phagocytic impairment is measurable at an earlier time (2 weeks versus 5 weeks), and lower dose of CS (approximately 2 mg/m^3 versus approximately 20 mg/m^3).

This phagocytic impairment was not dependent on other cell types, since very similar results were seen in AMs from unexposed mice following culture in CSCM at noncytotoxic concentrations. This is relevant because CS exposure may lead to increased epithelial cell apoptosis within the lung (10)(11). Phagocytosis of apoptotic cells by AMs results in increased PGE_2 production and suppression of phagocytosis and bacterial killing. When apoptotic cells are instilled in a model of pneumococcal pneumonia, bacterial clearance is impaired (12). However in that model, neutrophil recruitment is

also impaired, whereas we observed enhanced neutrophil recruitment following pneumococcal challenge in CS-exposed mice.

When AMs were recovered from mice exposed to CS for 1 wk, a significantly lower proportion of cells participated in phagocytosis of serum-opsonized *S. pneumoniae*, but no difference was found in the phagocytosis of IgG-opsonized microspheres. This result further argues against a role for apoptotic cells or PGE_2 in the observed impairment, as PGE_2 has been shown to impair AM phagocytosis of both IgG-opsonized erythrocytes and IgG-opsonized *E. coli* (13). The contrast between phagocytosis of serum-opsonized and IgG-opsonized targets raised the possibility that the defect was opsonin-specific, and not due to a more general defect such as cytoskeletal or membrane effects. Comparing AM phagocytosis of *S. pneumoniae* opsonized with rat serum, heat-inactivated rat serum, and C3-deficient serum demonstrated CSCM-mediated impairment only in the presence of functional C3.

These findings are consistent with the known role for complement in host defense against pneumococcal infection (14). In a previous report, impairment of complement-mediated phagocytosis of a gram-positive pathogen was observed in neutrophils from CS-exposed mice (15). Additionally, a model of pneumococcal pneumonia in cirrhotic rats demonstrated reduced levels of C3 in BAL fluid accompanied by increased bacterial burdens and mortality(16). This study also found impaired bacterial killing prior to the recruitment of neutrophils, suggesting the involvement of AMs, and reduced complement deposition on recovered bacteria from cirrhotic rats. In a mouse model of pneumococcal

pneumonia, C3 -/- mice developed higher pulmonary bacterial burdens, elevated IL-6, and had reduced survival relative to wild-type counterparts.

The results of the in vitro phagocytosis assays used in this project could easily under-represent the degree of phagocytic impairment present in vivo, because opsonization was carried out in the absence of CSCM. Cigarette smoke can activate complement directly in a mechanism that is not dependent on the presence of host cells or pathogens(17). Further, CS has been shown to chemically modify C3 specifically (18). Reduced serum levels of C3 and C4 have been found in COPD patients, however the effect of recurrent infection cannot be controlled for in this context (19). The pneumococcus posses anticomplement activity through secreted and surface-bound proteins such as pneumococcal surface protein (Psp) A, PspC, pneumolysin which are important virulence factors (20-22). Activation of C3 at a distance from the organism by secreted factors such as pneumolysin reduces deposition on the pathogen surface (23). Thus, CS itself recapitulates part of the pneumococcal anticomplement mechanisms, and in the pulmonary environment bacterial opsonization may well be further compromised.

An intriguing contrast emerged when assessing Fcγ-mediated phagocytosis. In a model using IgG-opsonized polystyrene microspheres, neither AMs from CS-exposed animals nor CSCM-pretreated AMs displayed impaired phagocytosis of this target. However in an assay using rat AMs, CSCM caused significant impairment of IgG-opsonized *E. coli*. While speculative, the discordance between results with *E. coli* and microspheres may relate to factors present on the bacterial surfaces, but which are absent in the microspheres.

Impairment of AM Cytokine Production

Lung digest-derived AMs from CS-exposed animals displayed impaired production of the cytokines TNF-α and IL-6 as well as the immunomodulatory lipid PGE_2, in response to heat-killed pneumococcus, although their viability was no different than controls. It is notable that these three mediators constitute the entire panel for which we tested. It is unclear how these in vitro impairments relate to findings in the whole animal, as only partial concordance was seen between the two data sets. Additionally, the cytokine reductions seen in whole animals was moderate compared with decreases seen in isolated AMs. We speculated that mediator production by other cells could compensate for impaired synthesis by AMs in vivo. Another possibility is that more substantial differences may exist within local microenvironments than are apparent from whole-lung measurements, due to compartmentalization of cytokine responses. Future studies could clarify these issues by using immunohistochemical or PCR-based approaches to localize cytokine production by region or cell type. Such an approach would be particularly informative if combined with simultaneous localization of the pneumococcus itself.

It is relevant that proinflammatory cytokines such as TNF-α, IL-1β and IL-6 have been shown to be important in host defense against pneumococcal infection (24-26). Many important pulmonary innate immune functions, including neutrophil recruitment are mediated through the transcription factor NF-κB (27). Activation of this factor in the lung during pneumococcal pneumonia requires TNF-α and IL-1β receptor signaling (28). Within the cytoplasm, second-messenger cascades initiated by these two receptors merge

and synergize with TLR-mediated signaling pathways (29). Further, CS has been found to inhibit the activation of downstream second messengers including kinases such as p38 and NF-κB, leading to suppression of cytokine production (30).

Impairment of TLR2 signaling

Several recent reports indicate that CS exposure impairs TLR-dependent signaling (31)(32)(30). The primary surface PRR for gram-positive LTA is TLR2, and expression of this receptor is reduced on AMs from smokers (33).

In this project, assays using LTA to challenge CSCM-pretreated AMs indicated a dose-dependent impairment of TLR2-mediated TNF-α production. As noted above, AMs from CS-exposed mice also produced reduced TNF-α following challenge with heat-killed *S. pneumoniae*, which can activate TLR4 as well as TLR2 signaling (34-36). However, using RT-PCR, no decrease of either TLR2 or TLR4 transcription was found in CSCM-pretreated AMs, implying disruption of downstream signaling pathways.

Impairment of ROI Generation

Alveolar macrophage production of ROI following pneumococcal challenge was also impaired in CSCM-cultured AMs. This may play a role in impaired signaling, as H_20_2 scavengers have been found to reduce TLR4-depndent activation of NF-κB and subsequent transcription of TNF-α, IL-1β and cyclooxygenase-2 (COX-2) in RAW 264.7 cells and murine peritoneal macrophages (37). Impaired COX-2 production is also consistent with the reduced levels of PGE_2 seen in vitro with AMs from CS-exposed

animals. Similar effects may occur in TLR2 signaling, since LTA-induced gene transcription has also been shown to be dependent on ROI some cells (38).

Additional Findings:

Scavenger Receptor-A Does Not Mediate Impairment

During the course of this project, data were generated which did not ultimately become part of a larger chapter. Some of these results warrant discussion as additional findings. The involvement of SR-A was considered as a possible mechanism underlying CS-mediated phagocytic suppression, because SR-A binds to LTA. This possibility was supported by a report of increased susceptibility to pneumococcal infection in SR-A -/- mice compared with wild-type controls (39). The study found impaired AM phagocytosis in vivo, increased pulmonary bacterial burden and decreased survival in these mice. Such a mechanism might also have explained the impaired production of ROI in CSCM-cultured AMs observed in our study. This is because ligation of SR-A with a specific mAb results in H_20_2 production in murine AMs (40).

However, flow cytometry revealed no significant difference in surface expression of SR-A on CSCM-cultured versus control AMs (Figure 5.1). Likewise, RT-PCR did not demonstrate a difference in SR-A mRNA between CSCM-cultured and control AMs (Figure 5.2). Additionally, blocking studies were performed with fucoidin, a competitive ligand for SR-A, to assess rates of non-opsonic phagocytosis in CSCM-cultured and control AMs (Figure 5.3). Blockade of SR-A did not change the relationship between

CSCM-cultured and control cells. Significantly, CSCM did not impair phagocytosis of unopsonized *S. pneumoniae* in these experiments. Therefore, this line of study was not pursued further.

Lack of "Safe Smoking"

Smoking is a learned behavior, and as such is mediated by perceptions. Extensive effort has been expended to inform the public on the dangers associated with smoking, with some measure of success evidenced by the widespread recognition of cigarettes as harmful (41). However, smoking is also highly addictive, leading to interest in approaches that might permit the smoker to reduce the harmful effects of exposure without quitting the habit. Some individuals choose products marketed as "light" cigarettes, based on a perception that they are less injurious than conventional cigarettes (42). Vitamins, antioxidants, or other supplements have also been explored as a way to mitigate the effects of CS exposure (43-45), in some cases with negative consequences (46-47). While not integral to the project hypothesis, a means of limiting CS-induced immunosuppression would be useful in reducing burdens of morbidity and mortality, and as a tool to understand the underlying pathology.

For this reason, experiments were undertaken to compare the effects of regular and low-tar cigarettes on pulmonary leukocyte populations and AM phagocytosis and production of TNF-α, IL-6 and PGE_2. Additionally, in vitro treatment with the antioxidant N-acetyl-cysteine, was assessed as a possible modifier of CSCM-mediated suppression of phagocytosis and TNF-α production. No significant difference was seen in the composition of resident leukocyte populations in the alveolar space or lung

parenchyma in any of the treatment groups. One limitation of this model is that in subsequent studies, clear trends toward pulmonary leukocyte accumulation did not become apparent until 5 weeks of exposure. It is possible that differences between regular and light cigarettes could appear if longer exposures were used.

In contrast, AM phagocytosis of serum-opsonized *S. pneumoniae* was significantly impaired in CS-exposed mice, and the level of impairment was similar in regular and low-tar groups. Likewise, regular and low-tar smoke impaired AM production of TNF-α and PGE_2 to a similar degree. The only difference between regular and low-tar groups was in AM production of IL-6. Production of this cytokine was impaired in the regular CS group, but only trended lower in mice exposed to low-tar smoke. The biological significance of this finding is not clear, because in the absence of bacteremia, IL-6 levels during bacterial pneumonia are less compartmentalized than other cytokines such as TNF-α and IL-1β (48). The term "tar" can be misleading, since in the context CS, it refers to the total particulate fraction, and not a specifically hydrophobic portion. The vapor and particulate phases of CS contain different constituents (49), and have been shown to induce different genes in a human epithelial-derived cell line (50). The differential effects of regular and low-tar smoke on IL-6 production could be investigated by using glass-fiber or charcoal filters to produce vapor phase or particulate phase CS exposures, respectively. Such an approach might also be used to generate CSCM for in vitro studies. In vitro antioxidant treatment did not prevent impairment of phagocytosis or TNF-α in CSCM-cultured AMs. Together, these data offer little support for efforts to make CS exposure less harmful.

Model Strengths and Limitations

Understanding Mechanisms

To best interpret the data presented here, it is worth considering some of the strengths and weakness of the approaches used. This does not detract from the value of the experimental findings, but rather provides additional value by placing them in the appropriate biological context. Largely these considerations fall into categories: factors that affect our ability to discern mechanisms, and others that affect our ability to extrapolate to human exposures and disease. Overall, this study was strengthened by using a combination of in vivo and in vitro models, so that in vivo experiments could demonstrate a biologically relevant effect, while in vitro assays could dissect mechanisms more specifically.

While the purpose of the project was related to understanding overall innate host defense in CS-exposed animals, the specific focus was on mechanisms involving AMs. Therefore, situations in which AM effects could not be separated from effects mediated through other cell types represent a limitation. Largely, this was the case only in vivo, as in other assays AMs were studied in isolation. The ability to determine individual AM effects, therefore, represents a strength of the project.

In particular, in vivo determinations of pulmonary bacterial load, cell recruitment and cytokine production were performed at 24 and 48 h after infection. This is a common practice, but represents a time by which other cells, primarily neutrophils, have

already been recruited to the alveolar space. Additional experiments were planned to determine bacterial load, cytokine levels, and leukocyte recruitment at an early time (6 hr) at which point the effects of recruited cells would be much lower. These experiments were not carried out only because of the substantial time and effort required to mount multiple, successive 5 week exposures. Such studies would be extremely useful, however, and would be a logical next step in this line of inquiry. Significantly, survival in mouse models of pneumococcal pneumonia is associated with early bacterial clearance (51; 52).

In vitro, it is not possible to fully reproduce the living environment. Relevant factors such as alveolar epithelial cells, dendritic cells, lymphocytes, surfactant proteins, as well as acute phase reactants produced by the liver during infection were not present in our models. Future studies could use approaches such as epithelial co-culture models or the addition of surfactant proteins to determine the importance of these factors. Also, in order to produce functional assays, bacterial challenges were in some cases not entirely naturalistic. For example, in vitro pneumococcal challenges used large doses of heat-killed bacteria. Alternative methods could use live bacteria for in vitro pneumococcal challenge. In the case of phagocytosis, fluorescently-transfected *S. pneumoniae* could be a useful tool. Also, phagocytosis assays were carried out with levels of bacteria several orders of magnitude higher than those used in vivo, and during the course of assay development, it was found that CS-induced phagocytic impairments could be obscured with very large bacterial doses.

More simply, in vivo determination of phagocytosis could be performed with the same bacteria used in our study. Aside from including all the factors normally present in vivo, this approach has another advantage. When phagocytosis assays are performed with AMs adhered to plastic or glass surfaces, phagocytosis is substantially slowed. Our results indicate that TLR2-mediated signaling is impaired in CS-exposed animals, and in experiments with neutrophils, it was found that genetic deficiency of TLR2 delays pneumococcal phagocytosis (53). Therefore, it is possible that the longer phagocytosis assays are under-representing the phagocytic defect present in vivo.

In vivo CS exposures

A perfect model of human smoke exposure is a theoretical impossibility, due to the great variety of exposure patterns, unrelated co-exposures, and genetic diversity within the human species. In these studies, mice were exposed to machine-generated CS in whole body exposure chambers, an approach which has been widely used in research. While a detailed history of the development of this model did not ultimately become a part of this dissertation, some salient points warrant consideration before final evaluation of the data. First among them is the use of mice as a model for humans. Mice have many similarities with humans, and have been used extensively in immunological and toxicological research. However, important differences exist between humans and rodent models, such as the respiratory deposition of smoke particles. Particles the size of ETS (approximately 0.2 μm mass median aerodynamic diameter) deposit more deeply in the pulmonary space in humans than rodents, and are retained for a longer period (54).

Since exposures occurred in a chamber rather than by direct smoking behavior of the animal, this model bears many similarities with ETS. Environmental tobacco smoke is composed of both sidestream smoke from the combustion zone, and exhaled mainstream smoke. Likewise the smoking machine used in most of these experiments was configured to collect smoke from both streams and deliver the combined mixture into the chamber. This becomes particularly relevant in light of internal tobacco industry research showing that sidestream smoke is two to six times more toxic than mainstream smoke, depending on the biological endpoint (55).

Another important consideration is the level of exposure. The experiments described here reflect the development and refinement of these methods over time. Early experiments included low-level exposures of approximately 2 mg/m^3, as well as high-dose exposures of 50 mg/m^3. Later, a level of approximately 20 mg/m^3 was used. This level was chosen based on observation of the animals' behavior and biological responses as well as the behavior of the exposure system itself, which provided the most stable performance under these conditions. It should be noted that these levels are higher than what is typically measured in ETS. While levels in the milligram range have been reported in some indoor environments, more commonly levels in the tens or hundreds of micrograms are seen (56). In this aspect, the models used here more closely resemble the higher exposures of active smokers. However, in some environments ETS levels can be much higher than what is found in homes and workplaces. For example, a level of 4 mg/m^3 was measured in a moving automobile (57). This is unsurprising considering a recent report which carefully measured the combined particulate emissions of several commercial cigarettes and found approximately 18 mg per cigarette (58).

While exposure concentrations are largely under control of the researcher, practical considerations often dictate other aspects of the model such as timing and duration of exposures. In these models, mice were exposed 4 hr/day, with no exposures over the weekends. This is in marked contrast to smokers, who typically have their first cigarette soon after waking and continue to smoke periodically throughout the day and on weekends. However some ETS exposures, particularly in workplace environments, may mimic such a pattern more closely.

Many smokers continue the habit for years, whereas the longest set of exposures in these experiments was 5 weeks. This duration was chosen because elevated numbers of AMs were apparent by this time. Alveolar macrophages are known to accumulate in the lungs of human smokers (5), which is paradoxical in light of smokers' increased susceptibility to respiratory infections. To account for the possibility that AM accumulation somehow contributed to immune impairment, it was decided to recapitulate this phenomenon in the model. However, other experiments used exposures as short as one week. It is noteworthy than significant effects were seen even after the shortest period of exposure.

Cigarette smoke-conditioned media

In some experiments, CS-conditioned media (CSCM) was used as a model for CS. While more dissimilar to native CS, it is a widely used surrogate. Further, since a substantial portion of CS passes through the media, this model is likely to bias against producing all the effects of CS. Additionally, to provide uniformity of conditions, CSCM in these experiments was prepared in a large batch and stored as frozen aliquots, further reducing

volatile smoke components. Finally, our findings support the validity of the model in this context, in that AMs isolated from CS-exposed animals displayed impairments similar to those pre-treated with CSCM.

Pneumonia model

All experiments in this project were carried out with a single strain of *S. pneumoniae* (serotype 3). Hence, different results could be possible with other pneumococcal variants. While most of these studies were carried out on BALB/C mice, there was general concordance with effects seen in the limited number of C57BL/6 experiments. Another possible limitation of our model was its dissimilarity with early stages of human infection. In order to demonstrate inter-group differences with a reasonable number of animals, models such as intranasal instillation or the intratracheal instillation used here introduce large inocula into the respiratory system, creating an infection that is both massive and instantaneous. Typically, nasopharyngeal colonization precedes the development of lower respiratory infection, and exposure of the host to the pathogen during this period likely alters the subsequent immune response as infection develops in the lung. Further, the response of AMs to pneumococcal stimulation varies with the magnitude of the provocation, such that large infections induce inflammation and recruitment of additional immune cells, whereas much smaller infections can be cleared without these responses.

If one assumes that the fulminant models of pneumococcal pneumonia typically used in the laboratory represent a particularly harsh onset of infection relative to the typical natural history of the disease, then lower-dose models could potentially be more informative of human subjects. In such models, macrophages resolve infection without obvious inflammation, emphasizing the importance of phagocytic impairments in the initial stages of a pulmonary infection (59). This raises the possibility that our model under-represents the impairment of AM function, due to the rapid recruitment of distal cells, which were more numerous in CS-exposed animals following pneumococcal challenge. In one study, AMs from COPD patients and asymptomatic smokers displayed phagocytic impairment compared with nonsmokers, while blood monocytes between the groups did not differ (60).

It is worth noting however, that pneumococcal pneumonia is a particularly serious infection, with mortality rates as high as 35% (61). By comparison, the model used here produced approximately 50% mortality over a two-week period during initial experiments. Therefore it is likely to bear significant similarities with human pneumonia. As our ability to measure subtle physiological endpoints improves, it may be feasible to demonstrate more pronounced and specific impairments of AM function in vivo, through the use of increasingly naturalistic models of pneumococcal infection. In one example of such an endpoint, colonic temperature was compared with independent clinical assessment in mice following bacterial infection (62). In all cases, hypothermia agreed with, but preceded a finding of moribund status.

The Question is "Why"?

While this project confirmed impairments in anti-pneumococcal innate host defense, and AM function, many questions remain. The mechanisms by which CS exerts suppression of complement-mediated phagocytosis and TLR2-dependent signaling are unclear. An important observation may be that neither of these effects was seen under conditions that failed to cause the other. This suggests that there may be common mechanisms underlying both effects.

There are several lines of reasoning that support this possibility. For example, complement receptors, including CR3 and CR4 which are expressed on AMs, have been shown to interact with gram-positive ligands (63) and activate NF-kB (64). It has also been suggested found that CR3 is necessary for full induction of inflammatory genes including TNF-α and COX-2 with low doses of LPS (65). This project did not measure expression of AM receptors for complement. However, because at least two major CRs (CR3 and CR4) are maintained in cytoplasmic pools, and have the capacity to undergo activation or deactivation independently of expression, such experiments alone may not provide an answer (66-68). Future study could shed additional light on these effects by measuring expression and activation of these receptors and pharmacological inhibition of TLR- and cytokine-mediated signaling pathways.

Final Thoughts

In sum, the results of this project can reasonably be interpreted as confirming the hypothesis that CS exposure impairs pulmonary innate host defense in a murine model of pneumococcal pneumonia, and that AMs are likely to be involved in this impairment. The experiments described here represent the first demonstration of this relationship in an animal model, and thus constitute a novel finding. Further, the heavy toll of disease caused by this pathogen and the large number of people exposed to CS lend practical significance to these observations. Because of the model's similarities with ETS, the appearance of immunosuppression after such a limited number of exposures has meaningful implications for those who come into contact with smokers as well.

This model may be useful in the future as a means to explore the immunological effects of smoking and ETS exposure. Specific lines of inquiry are suggested by the observation of impairments in complement-mediated phagocytosis and TLR-mediated signaling. Ideally, such investigations could help to inform decisions regarding tobacco policy, and potentially aid in the development of therapies.

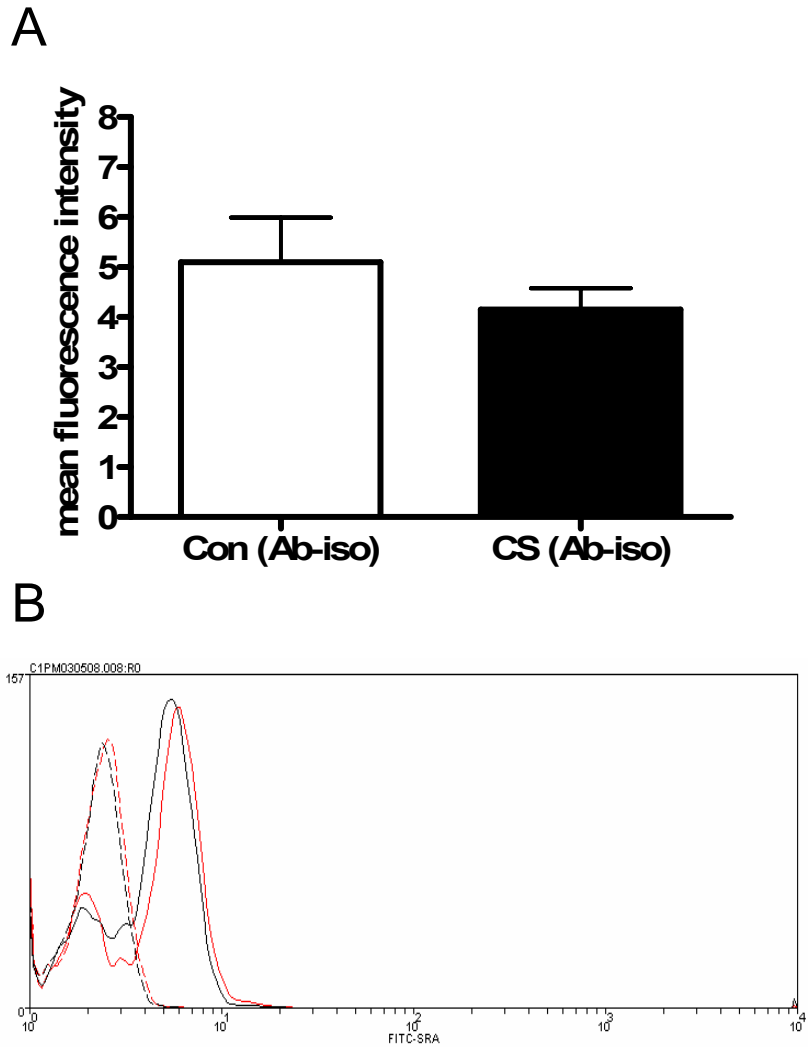

Fig 5.1. Expression of SR-A on CSCM-cultured and control AMs.
Alveolar macrophages were isolated from BAL fluid of naïve female BALB/C mice as described in Chapter 2, followed by culture in media alone or 1% CSCM in ultralow-adherence plates. Cells were labeled with FITC-conjugated rat anti-mouse SR-A (clone 2F8) or isotype control (AbD Serotec, Raleigh, NC) according to manufacturer's recommendations, and fixed on ice prior to analysis on a BD Biosciences FACSAria by a trained operator. A. Expression of SR-A with subtraction of isotype control, n = 7 experiments. B. A representative histogram showing SR-A (solid) and isotype-control (dashed) signal for CSCM (light) and control (dark) AMs.

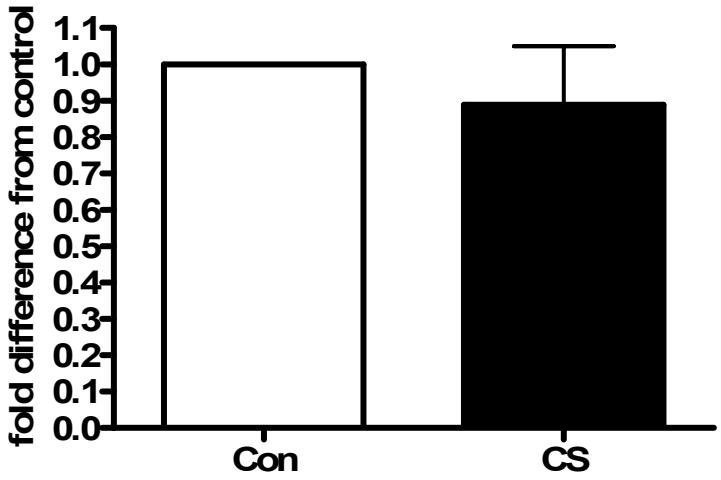

Fig 5.2. Transcription of SR-A in CSCM-cultured and control AMs.
Alveolar macrophages were isolated from BAL fluid of naïve female BALB/C mice and cultured in media alone or 1% CSCM. Treatment, mRNA isolation and RT-PCR analysis were carried out simultaneously with TLR transcription studies described in Chapter 4. Bars represent the mean ± standard error of the mean of four replicate experiments.

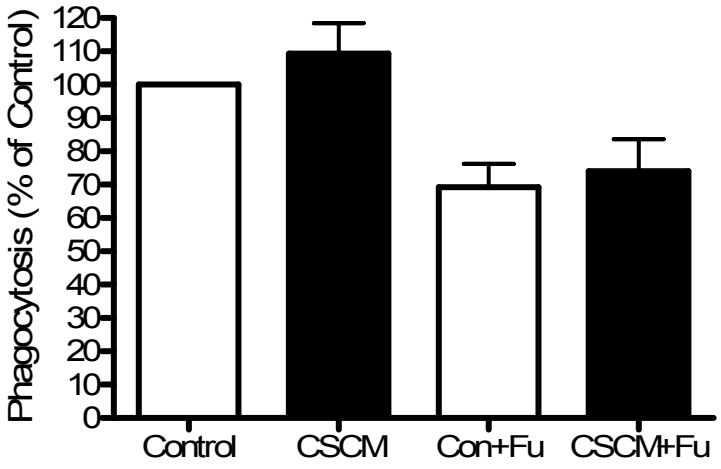

Fig 5.3. Blockade of SR-A does not modify the relative rates of non-opsonic phagocytosis in CSCM-cultured and control AMs. Alveolar macrophages were isolated from BAL fluid of naïve female BALB/C mice and cultured in media alone or 1% CSCM. Phagocytosis of unopsonized FITC-labeled *S. pneumoniae* was determined as described in Chapter 3. In Con + Fu and CSCM + Fu, groups cells were treated with 40 μg/ml fucoidin (Sigma) for 30 minutes prior to phagocytosis.

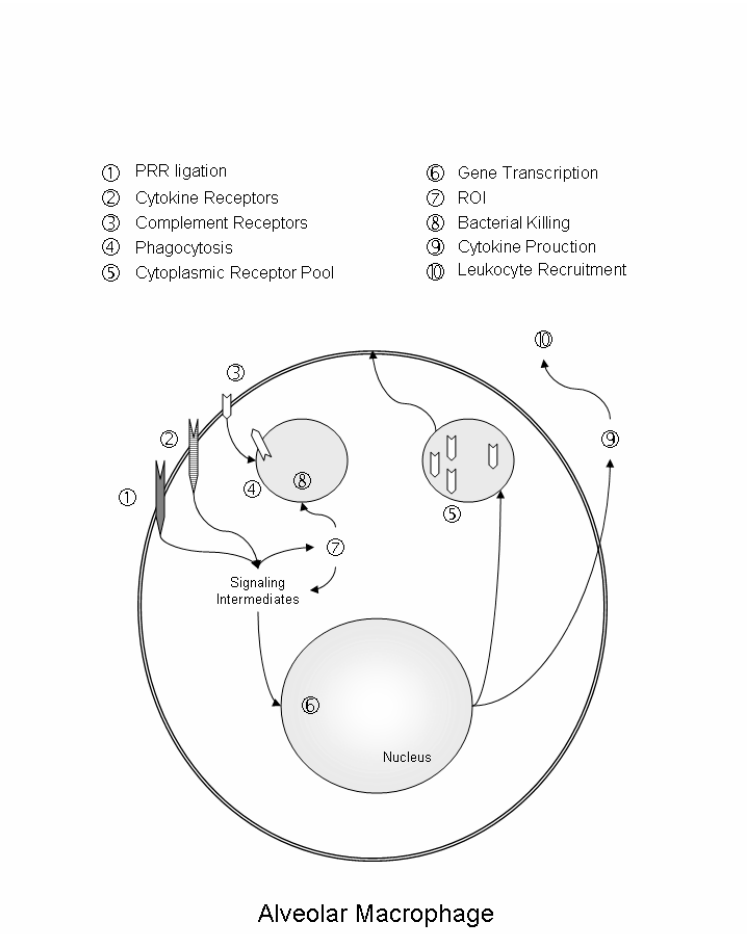

Fig 5.4. Selected mechanisms in CS-mediated impairments of innate host defense.

References

1. World Health Organization, and Research for International Tobacco Control. 2008. WHO report on the global tobacco epidemic, 2008 : the MPOWER package. Geneva, World Health Organization,

2. Wu-Williams, A.H., and J.M. Samet. 1990. Environmental tobacco smoke: exposure-response relationships in epidemiologic studies. *Risk Anal.* 10:39-48.

3. Li, J.S., J.K. Peat, W. Xuan, and G. Berry. 1999. Meta-analysis on the association between environmental tobacco smoke (ETS) exposure and the prevalence of lower respiratory tract infection in early childhood. *Pediatr.Pulmonol.* 27:5-13.

4. Arcavi, L., and N.L. Benowitz. 2004. Cigarette smoking and infection. *Arch.Intern.Med.* 164:2206-2216.

5. Barnes, P.J. 2004. Alveolar macrophages as orchestrators of COPD. *COPD.* 1:59-70.

6. Craig, A., J. Mai, S. Cai, and S. Jeyaseelan. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect.Immun.* 77:568-575.

7. Garvy, B.A., and A.G. Harmsen. 1996. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation.* 20:499-512.

8. Papi, A., C.M. Bellettato, F. Braccioni, M. Romagnoli, P. Casolari, G. Caramori, L.M. Fabbri, and S.L. Johnston. 2006. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am.J.Respir.Crit.Care Med.* 173:1114-1121.

9. Morris, A., G. Kinnear, W.Y. Wan, D. Wyss, P. Bahra, and C.S. Stevenson. 2008. Comparison of cigarette smoke-induced acute inflammation in multiple strains of mice and the effect of a matrix metalloproteinase inhibitor on these responses. *J.Pharmacol.Exp.Ther.* 327:851-862.

10. Wu, C.H., H.H. Lin, F.P. Yan, C.H. Wu, and C.J. Wang. 2006. Immunohistochemical detection of apoptotic proteins, p53/Bax and JNK/FasL cascade, in the lung of rats exposed to cigarette smoke. *Arch.Toxicol.* 80:328-336.

11. Jiao, Z.X., Q.L. Ao, and M. Xiong. 2006. Cigarette smoke extract inhibits the proliferation of alveolar epithelial cells and induces apoptosis. *Sheng Li Xue Bao.* 58:244-254.

12. Medeiros, A.I., C.H. Serezani, S.P. Lee, and M. Peters-Golden. 2009. Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. *J.Exp.Med.* 206:61-68.

13. Aronoff, D.M., C. Canetti, and M. Peters-Golden. 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J.Immunol.* 173:559-565.

14. Picard, C., A. Puel, J. Bustamante, C.L. Ku, and J.L. Casanova. 2003. Primary immunodeficiencies associated with pneumococcal disease. *Curr.Opin.Allergy Clin.Immunol.* 3:451-459.

15. Keast, D., and K. Taylor. 1983. The effects of chronic tobacco smoke exposure from high-tar cigarettes on the phagocytic and killing capacity of polymorphonuclear cells of mice. *Environ.Res.* 31:66-75.

16. Propst-Graham, K.L., L.C. Preheim, E.A. Vander Top, M.U. Snitily, and M.J. Gentry-Nielsen. 2007. Cirrhosis-induced defects in innate pulmonary defenses against Streptococcus pneumoniae. *BMC Microbiol.* 7:94.

17. Robbins, R.A., K.J. Nelson, G.L. Gossman, S. Koyama, and S.I. Rennard. 1991. Complement activation by cigarette smoke. *Am.J.Physiol.* 260:L254-9.

18. Kew, R.R., B. Ghebrehiwet, and A. Janoff. 1985. Cigarette smoke can activate the alternative pathway of complement in vitro by modifying the third component of complement. *J.Clin.Invest.* 75:1000-1007.

19. Chauhan, S., M.K. Gupta, A. Goyal, and D.J. Dasgupta. 1990. Alterations in immunoglobulin & complement levels in chronic obstructive pulmonary disease. *Indian J.Med.Res.* 92:241-245.

20. Quin, L.R., Q.C. Moore 3rd, and L.S. McDaniel. 2007. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. *Infect.Immun.* 75:2067-2070.

21. Yuste, J., M. Botto, J.C. Paton, D.W. Holden, and J.S. Brown. 2005. Additive inhibition of complement deposition by pneumolysin and PspA facilitates Streptococcus pneumoniae septicemia. *J.Immunol.* 175:1813-1819.

22. Ren, B., M.A. McCrory, C. Pass, D.C. Bullard, C.M. Ballantyne, Y. Xu, D.E. Briles, and A.J. Szalai. 2004. The virulence function of Streptococcus pneumoniae surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *J.Immunol.* 173:7506-7512.

23. Rubins, J.B., and E.N. Janoff. 1998. Pneumolysin: a multifunctional pneumococcal virulence factor. *J.Lab.Clin.Med.* 131:21-27.

24. Kafka, D., E. Ling, G. Feldman, D. Benharroch, E. Voronov, N. Givon-Lavi, Y. Iwakura, R. Dagan, R.N. Apte, and Y. Mizrachi-Nebenzahl. 2008. Contribution of IL-1 to resistance to Streptococcus pneumoniae infection. *Int.Immunol.* 20:1139-1146.

25. Rijneveld, A.W., S. Florquin, J. Branger, P. Speelman, S.J. Van Deventer, and T. van der Poll. 2001. TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. *J.Immunol.* 167:5240-5246.

26. van der Poll, T., C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, and S.F. Lowry. 1997. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J.Infect.Dis.* 176:439-444.

27. Quinton, L.J., M.R. Jones, B.T. Simms, M.S. Kogan, B.E. Robson, S.J. Skerrett, and J.P. Mizgerd. 2007. Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J.Immunol.* 178:1896-1903.

28. Mizgerd, J.P. 2002. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin.Immunol.* 14:123-132.

29. O'Neill, L.A. 2002. Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr.Top.Microbiol.Immunol.* 270:47-61.

30. Chen, H., M.J. Cowan, J.D. Hasday, S.N. Vogel, and A.E. Medvedev. 2007. Tobacco smoking inhibits expression of proinflammatory cytokines and activation of IL-1R-associated kinase, p38, and NF-kappaB in alveolar macrophages stimulated with TLR2 and TLR4 agonists. *J.Immunol.* 179:6097-6106.

31. McMaster, S.K., M.J. Paul-Clark, M. Walters, M. Fleet, J. Anandarajah, S. Sriskandan, and J.A. Mitchell. 2008. Cigarette smoke inhibits macrophage sensing of Gram-negative bacteria and lipopolysaccharide: relative roles of nicotine and oxidant stress. *Br.J.Pharmacol.* 153:536-543.

32. Gaschler, G.J., C.C. Zavitz, C.M. Bauer, M. Skrtic, M. Lindahl, C.S. Robbins, B. Chen, and M.R. Stampfli. 2008. Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages. *Am.J.Respir.Cell Mol.Biol.* 38:218-226.

33. Droemann, D., T. Goldmann, T. Tiedje, P. Zabel, K. Dalhoff, and B. Schaaf. 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir.Res.* 6:68.

34. Knapp, S., S. von Aulock, M. Leendertse, I. Haslinger, C. Draing, D.T. Golenbock, and T. van der Poll. 2008. Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor. *J.Immunol.* 180:3478-3484.

35. Srivastava, A., P. Henneke, A. Visintin, S.C. Morse, V. Martin, C. Watkins, J.C. Paton, M.R. Wessels, D.T. Golenbock, and R. Malley. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect.Immun.* 73:6479-6487.

36. Malley, R., P. Henneke, S.C. Morse, M.J. Cieslewicz, M. Lipsitch, C.M. Thompson, E. Kurt-Jones, J.C. Paton, M.R. Wessels, and D.T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc.Natl.Acad.Sci.U.S.A.* 100:1966-1971.

37. Kim, J.H., H.J. Na, C.K. Kim, J.Y. Kim, K.S. Ha, H. Lee, H.T. Chung, H.J. Kwon, Y.G. Kwon, and Y.M. Kim. 2008. The non-provitamin A carotenoid, lutein, inhibits NF-kappaB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-kappaB-inducing kinase pathways: role of H(2)O(2) in NF-kappaB activation. *Free Radic.Biol.Med.* 45:885-896.

38. Lee, I.T., S.W. Wang, C.W. Lee, C.C. Chang, C.C. Lin, S.F. Luo, and C.M. Yang. 2008. Lipoteichoic acid induces HO-1 expression via the TLR2/MyD88/c-Src/NADPH oxidase pathway and Nrf2 in human tracheal smooth muscle cells. *J.Immunol.* 181:5098-5110.

39. Arredouani, M.S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik. 2006. The Macrophage Scavenger Receptor SR-AI/II and Lung Defense against Pneumococci and Particles. *Am.J.Respir.Cell Mol.Biol.*

40. Jozefowski, S., and L. Kobzik. 2004. Scavenger receptor A mediates H2O2 production and suppression of IL-12 release in murine macrophages. *J.Leukoc.Biol.* 76:1066-1074.

41. Bala, M., L. Strzeszynski, and K. Cahill. 2008. Mass media interventions for smoking cessation in adults. *Cochrane Database Syst.Rev.* (1):CD004704.

42. Smith, S.Y., B. Curbow, and F.A. Stillman. 2007. Harm perception of nicotine products in college freshmen. *Nicotine Tob.Res.* 9:977-982.

43. Olatunji-Bello, I.I., S.O. Olayemi, A.O. Daramola, and A.O. Ogungbemi. 2008. Ascorbic acid and the effect of cigarette smoke on tissues--a preliminary report. *West Afr.J.Med.* 27:78-81.

44. Hennekens, C.H., J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, J.M. Gaziano, P.M. Ridker, W. Willett, and R. Peto. 1996. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N.Engl.J.Med.* 334:1145-1149.

45. Lee, I.M., N.R. Cook, J.E. Manson, J.E. Buring, and C.H. Hennekens. 1999. Beta-carotene supplementation and incidence of cancer and cardiovascular disease: the Women's Health Study. *J.Natl.Cancer Inst.* 91:2102-2106.

46. Omenn, G.S., G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens Jr, B. Valanis, J.H. Williams Jr, S. Barnhart, M.G. Cherniack, C.A. Brodkin, and S. Hammar. 1996. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *J.Natl.Cancer Inst.* 88:1550-1559.

47. Albanes, D., O.P. Heinonen, P.R. Taylor, J. Virtamo, B.K. Edwards, M. Rautalahti, A.M. Hartman, J. Palmgren, L.S. Freedman, J. Haapakoski, M.J. Barrett, P. Pietinen, N. Malila, E. Tala, K. Liippo, E.R. Salomaa, J.A. Tangrea, L. Teppo, F.B. Askin, E. Taskinen, Y. Erozan, P. Greenwald, and J.K. Huttunen. 1996. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. *J.Natl.Cancer Inst.* 88:1560-1570.

48. Dehoux, M.S., A. Boutten, J. Ostinelli, N. Seta, M.C. Dombret, B. Crestani, M. Deschenes, J.L. Trouillet, and M. Aubier. 1994. Compartmentalized cytokine production within the human lung in unilateral pneumonia. *Am.J.Respir.Crit.Care Med.* 150:710-716.

49. Bi, X., G. Sheng, Y. Feng, J. Fu, and J. Xie. 2005. Gas- and particulate-phase specific tracer and toxic organic compounds in environmental tobacco smoke. *Chemosphere.* 61:1512-1522.

50. Vulimiri, S.V., M. Misra, J.T. Hamm, M. Mitchell, and A. Berger. 2009. Effects of Mainstream Cigarette Smoke on the Global Metabolome of Human Lung Epithelial Cells. *Chem.Res.Toxicol.*

51. Dallaire, F., N. Ouellet, Y. Bergeron, V. Turmel, M.C. Gauthier, M. Simard, and M.G. Bergeron. 2001. Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia. *J.Infect.Dis.* 184:292-300.

52. Dallaire, F., N. Ouellet, M. Simard, Y. Bergeron, and M.G. Bergeron. 2001. Efficacy of recombinant human granulocyte colony-stimulating factor in a murine model of pneumococcal pneumonia: effects of lung inflammation and timing of treatment. *J.Infect.Dis.* 183:70-77.

53. Letiembre, M., H. Echchannaoui, P. Bachmann, F. Ferracin, C. Nieto, M. Espinosa, and R. Landmann. 2005. Toll-like receptor 2 deficiency delays pneumococcal phagocytosis and impairs oxidative killing by granulocytes. *Infect.Immun.* 73:8397-8401.

54. Oberdorster, G., and F. Pott. 1987. Extrapolation from rat studies with environmental tobacco smoke (ETS) to humans: comparison of particle mass deposition and of clearance behavior of ETS compounds. *Toxicol.Lett.* 35:107-112.

55. Schick, S., and S. Glantz. 2005. Philip Morris toxicological experiments with fresh sidestream smoke: more toxic than mainstream smoke. *Tob.Control.* 14:396-404.

56. Jenkins, R.A., M.R. Guerin, B.A. Tomkins, and Center for Indoor Air Research. 2000. The chemistry of environmental tobacco smoke : composition and measurement, 2nd ed. Boca Raton, Lewis Publishers,

57. Ott, W., N. Klepeis, and P. Switzer. 2008. Air change rates of motor vehicles and in-vehicle pollutant concentrations from secondhand smoke. *J.Expo.Sci.Environ.Epidemiol.* 18:312-325.

58. Charles, S.M., C. Jia, S.A. Batterman, and C. Godwin. 2008. VOC and particulate emissions from commercial cigarettes: analysis of 2,5-DMF as an ETS tracer. *Environ.Sci.Technol.* 42:1324-1331.

59. Opitz, B., A. Puschel, B. Schmeck, A.C. Hocke, S. Rosseau, S. Hammerschmidt, R.R. Schumann, N. Suttorp, and S. Hippenstiel. 2004. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J.Biol.Chem.* 279:36426-36432.

60. Hodge, S., G. Hodge, J. Ahern, H. Jersmann, M. Holmes, and P.N. Reynolds. 2007. Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am.J.Respir.Cell Mol.Biol.* 37:748-755.

61. Marrie, T.J. 1999. Pneumococcal pneumonia: epidemiology and clinical features. *Semin.Respir.Infect.* 14:227-236.

62. Soothill, J.S., D.B. Morton, and A. Ahmad. 1992. The HID50 (hypothermia-inducing dose 50): an alternative to the LD50 for measurement of bacterial virulence. *Int.J.Exp.Pathol.* 73:95-98.

63. Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, F. Genovese, F. Tomasello, T.H. Flo, T. Espevik, and G. Teti. 2000. Beta 2 integrins are involved in cytokine responses to whole Gram-positive bacteria. *J.Immunol.* 164:5871-5876.

64. Thieblemont, N., N. Haeffner-Cavaillon, A. Haeffner, B. Cholley, L. Weiss, and M.D. Kazatchkine. 1995. Triggering of complement receptors CR1 (CD35) and CR3 (CD11b/CD18) induces nuclear translocation of NF-kappa B (p50/p65) in human monocytes and enhances viral replication in HIV-infected monocytic cells. *J.Immunol.* 155:4861-4867.

65. Perera, P.Y., T.N. Mayadas, O. Takeuchi, S. Akira, M. Zaks-Zilberman, S.M. Goyert, and S.N. Vogel. 2001. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. *J.Immunol.* 166:574-581.

66. Darcissac, E.C., G.M. Bahr, M.A. Parant, L.A. Chedid, and G.J. Riveau. 1996. Selective induction of CD11a,b,c/CD18 and CD54 expression at the cell surface of human leukocytes by muramyl peptides. *Cell.Immunol.* 169:294-301.

67. Detmers, P.A., S.D. Wright, E. Olsen, B. Kimball, and Z.A. Cohn. 1987. Aggregation of complement receptors on human neutrophils in the absence of ligand. *J.Cell Biol.* 105:1137-1145.

68. Wright, S.D., and B.C. Meyer. 1986. Phorbol esters cause sequential activation and deactivation of complement receptors on polymorphonuclear leukocytes. *J.Immunol.* 136:1759-1764.