Mechanisms of Moraxella catarrhalis Induced Immune Signaling in the Pulmonary Epithelium

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Chronic Obstructive Pulmonary Disease (COPD) is the fourth leading cause of death in the United States and is becoming increasingly more common. It has a global prevalence of 10% of the general population. COPD is a condition of irreversible airflow limitation due to airway obstruction and lung destruction, making breathing difficult.

Exacerbations of COPD are characterized by an increase in symptoms, a worsening of lung function, and an increase of inflammation. The most common causes of acute exacerbations are infection and air pollution. Moraxella catarrhalis is one of the leading causes of acute exacerbations, responsible for approximately 10%. There are currently no treatments that can stop the progression of COPD or suppress the inflammation. A better understanding of the mechanism behind the inflammation is needed to identify new therapeutic targets.

Little is known about how M. catarrhalis interacts with the host epithelium to lead to exacerbations. The airway epithelium recognizes pathogens via receptors such as the toll-like receptors (TLR). Many pathogens signal through TLRs to activate NF-κB which translocates to the nucleus and activates transcription of inflammatory mediators.
It has been shown that *Haemophilus influenzae*, another cause of acute exacerbations, can induce the production of inflammatory mediators from the bronchial epithelium through the p38 MAPK (mitogen activated protein kinase) and ERK1/2 (extracellular signal-regulated kinase) pathways. PI3K (phosphoinositide-3 kinase) has also been implicated in airway inflammation, though the literature is conflicting about whether it functions as pro- or anti-inflammatory.

We have found that *M. catarrhalis* can induce the production of the pro-inflammatory chemokines IL-8 and MCP-1. TLR2 is at least partly responsible for the chemokine expression. Three kinases, PI3K, p38 MAPK, and ERK are all activated in response to *M. catarrhalis*. PI3K is activated downstream of TLR2 and shows differential regulation of MCP-1 and IL-8. PI3K has a suppressive effect on p38 MAPK activation but has no effect on ERK. P38 MAPK positively regulates the expression of MCP-1 in response to *M. catarrhalis*, but has no effect on IL-8. ERK is a positive regulator of *M. catarrhalis*-induced MCP-1 and IL-8 production. We are currently working to elucidate these pathways.

Our work aims to understand the lung epithelium’s response to infection by *M. catarrhalis* and the signaling pathways involved in that response in hopes of being able to better treat or prevent exacerbations and improve the quality of life for people with COPD.
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INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is the fourth leading cause of death in the United States and is becoming increasingly more common. COPD affects millions of people and results in billions of dollars spent on health care (Heron and Smith, 2007). It is a global problem; with an estimated prevalence of 10% in the general population worldwide (Menezes et al., 2005; Xu et al., 2005).

COPD is a progressive inflammatory disease characterized by irreversible airflow limitation. It is a condition encompassing either emphysema, a progressive loss of lung function due to the destruction of the lung parenchyma and enlargement of airspaces, or chronic bronchitis, obstruction of the small airways and mucus hypersecretion, or both (Barnes et al., 2003).

COPD is a disease of chronic inflammation in the small airways and lung. It is very complex with involvement of many different inflammatory cell types and inflammatory mediators (Barnes et al., 2003). There is infiltration of leukocytes including macrophages and neutrophils into the airways that contribute to the tissue damage. Leukocyte chemoattractants such as MCP-1 (monocyte chemoattractant protein-1/CCL2) and IL-8 (interleukin-8/CXCL8) are increased in the sputum and bronchoalveolar lavage fluid of COPD patients (Di Stefano et al., 1998).

Acute exacerbations of COPD are characterized by an increase of symptoms and a worsening of lung function (Barnes et al., 2003). Exacerbations may be prolonged and
can have a deleterious effect on quality of life for the patient (Groenewegen et al., 2003; Seemungal et al., 1998), and may accelerate progression of COPD. Severe exacerbations can result in death, with inpatient mortality rates ranging between 4-30%; the highest mortality is seen in patients with respiratory failure (Donaldson and Wedzicha, 2006).

The most common causes of an exacerbation are infection of the airway and air pollution. At least 50% of patients have high concentrations of pathogenic bacteria in their lower airways during exacerbation (Sethi et al., 2002). The predominant bacteria recovered during acute exacerbations from lower airways are *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (Verduin et al., 2002)

*M. catarrhalis* is an aerobic Gram-negative diplococcus. Very little is known about the pathogenesis of *M. catarrhalis* because it was once thought to be a nonpathogenic, commensal organism that colonized the nasopharynx in healthy adults. It has since been shown that *M. catarrhalis* is an important cause of disease. Aside from causing approximately 10% of exacerbations in COPD, *M. catarrhalis* is also one of the main causes of otitis media (Verduin et al., 2002). Most research to date on *M. catarrhalis* has focused on genetic studies, its outer membrane proteins, and vaccine candidates. It is not yet fully understood how *M. catarrhalis* induces the host immune response.

Immune signaling in the lower airway epithelium is very important in the host’s defense to infection. Airway epithelial cells recognize pathogens with the aid of pattern recognition receptors such as the Toll-like receptors (TLR) (Zhang and Ghosh, 2001). When TLR is activated by a pathogen, it sets off a signal transduction cascade leading to activation of the transcription factor, NF-κB. NF-κB activates the expression of pro-inflammatory mediators including cytokines and chemokines (Liang et al., 2004). Two
such chemokines important in COPD pathogenesis are MCP-1 and IL-8. MCP-1 is a chemokine that recruits and activates monocytes/macrophages. IL-8 is a chemoattractant for neutrophils.

Other signaling pathways are also activated in response to pathogen recognition. These include the PI3K/Akt pathway that has been shown to have both pro- and anti-inflammatory effects (Ruse and Knaus, 2006), the Raf/MEK/ERK pathway, and the p38 MAPK pathway. These pathways are used by the cell to control the inflammatory response to a pathogen. Understanding which pathways are activated in response to *M. catarrhalis* and the inflammatory mediators released will help elucidate how *M. catarrhalis* causes COPD exacerbations. This knowledge could lead to advances in the treatment of diseases such as COPD, improved quality of life for patients, and perhaps development of a therapy to prevent exacerbations.

The research presented here shows that *M. catarrhalis* can induce the production of the pro-inflammatory chemokines, MCP-1 and IL-8. TLR2 is at least partly responsible for the chemokine expression. Three kinases, PI3K, p38 MAPK, and ERK are all activated in response to *M. catarrhalis*. PI3K is activated downstream of TLR2 and shows differential regulation of MCP-1 and IL-8. PI3K has a suppressive effect on p38 MAPK activation but has no effect on ERK. P38 MAPK positively regulates the expression of MCP-1 in response to *M. catarrhalis*, but has no effect on IL-8. ERK is a positive regulator of *M. catarrhalis*-induced MCP-1 and IL-8 production.
LITERATURE REVIEW

Chronic Obstructive Pulmonary Disease

Prevalence

Chronic obstructive pulmonary disease (COPD) is an important global health problem; a major cause of morbidity and mortality worldwide (Barnes, 2003; Rabe et al., 2007). The global prevalence is estimated to be 10% in the general population (Menezes et al., 2005; Xu et al., 2005). COPD is currently ranked as the fifth most common cause of death worldwide and is predicted by the World Health Organization to become the fourth most common by the year 2030, behind ischemic heart disease, cerebrovascular disease, and HIV/AIDS (Barnes, 2007; Lopez et al., 2006). COPD is estimated to have been responsible for the deaths of 2.74 million people worldwide in 2000 (Hurd, 2005). COPD is also extremely prevalent in the United States, affecting approximately 16.4 million people in 1994. COPD is estimated to be the fourth leading cause of death in the United States (Sethi et al., 2007). It is becoming increasingly common; in the past 20 years the COPD death rate for women has nearly tripled, from 20.1 to 56.7/100,000 (Hurd, 2005). COPD is also a large financial burden on society. In the United States an estimated $14.7 billion is spent on direct medical costs, and another $15.7 billion on
indirect costs (e.g. loss of productivity) each year (Hurd, 2005). This amount is likely to increase as the population ages. In developed countries exacerbations of COPD account for a huge burden on the health care system (Rabe et al., 2007).

**Definition**

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease as a disease “…characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases” (Barnes, 2007). This airflow limitation is caused by a combination of narrowing of small airways and parenchymal destruction (Rabe et al., 2007). COPD is an umbrella encompassing three conditions: (i) chronic obstructive bronchiolitis with fibrosis and obstruction of small airways, (ii) emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways, and (iii) chronic bronchitis characterized by a productive cough (mucus hypersecretion) of more than three months duration for two or more successive years (American Thoracic Society, 1995; Barnes et al., 2003). Many patients have all three conditions. The progressive loss of lung function is caused by emphysema due to destruction of the lung parenchyma and by narrowing of the small airways resulting from chronic inflammation, fibrosis, and a loss of elastic recoil (Barnes, 2007). Characteristic symptoms of COPD include chronic and progressive dyspnea, cough, and sputum production (Rabe et al., 2007). While considered to be a preventable disease,
there is no cure for COPD, and no current treatments or therapies can stop or reverse the inevitable progression of disease (Barnes, 2003; Rabe et al., 2007).

Disease progression

COPD is divided into four stages based on severity. Stage 1, mild COPD, is characterized by mild airflow limitation and may be accompanied by chronic cough and sputum production. Stage 2, moderate COPD, is characterized by increasing limitation of airflow and shortness of breath with exertion. Chronic cough and sputum production are usually present at this stage. Stage 3, severe COPD, is characterized by further worsening of airflow limitation; presenting as shortness of breath, fatigue, and reduced exercise capacity. Repeated exacerbations are common at this stage and negatively affect the patient’s quality of life. The final stage, Stage 4, is very severe COPD. It is characterized by severe airflow limitation and chronic respiratory failure that may affect the heart. Exacerbations occurring at this stage can be life threatening (Rabe et al., 2007).

Risk Factors

Smoking tobacco is a common precedent of COPD, with 80% of cases related to smoking (Murphy, 2000). The prevalence of COPD is much higher in smokers and ex-smokers than in those who have never smoked (Menezes et al., 2005). Cigarette smoking is the most widely encountered risk factor for COPD and, because of this, smoking cessation programs are considered to be the most important and effective element of prevention. COPD is also more prevalent in men than women (though this becoming more equal), and in people older than 40 (Rabe et al., 2007). Other risk factors include
air pollution, poor diet, and occupational exposure (Barnes et al., 2003; Salvi and Barnes, 2009).

**Pathology**

COPD is a disease of chronic inflammation in the small airways and lung. It is very complex with involvement of many different inflammatory cell types and inflammatory mediators (Barnes et al., 2003).

Macrophages are very important in the pathology of COPD and could account for nearly all of its known features (Shapiro, 1999; Shapiro and Senior, 1999; Tetley, 2002). COPD patients have an increased number of macrophages in sputum, bronchoalveolar lavage (BAL) fluid, lung parenchyma, and airways (Ito et al., 2007; Retamales et al., 2001). There is also a correlation between the severity of COPD and the number of macrophages in the airways (Di Stefano et al., 1998). The increased severity of disease could be due to enzymes secreted by the macrophages such as metalloproteinases -2, -9, -12, and cathepsins K, L, S (Punturieri et al., 2000; Russell et al., 2002; Tetley, 2002). Alveolar macrophages from patients with COPD have higher activation levels and secrete more inflammatory mediators than those from smokers without COPD (Aldonyte et al., 2003; Finlay et al., 1997; Russell et al., 2002). The increase in macrophage numbers seen in COPD could be due to increased recruitment of circulating monocytes (Capelli et al., 1999; Traves et al., 2002) perhaps in response to higher levels of the chemokine MCP-1 (Monocyte Chemoattractant Protein-1/CCL2). CCL2/MCP-1 levels are increased in sputum, BAL, and lungs of COPD patients (Capelli et al., 1999; de Boer et al., 2000; Traves et al., 2002). The increased numbers of macrophages could also be due to
Activated neutrophils are found in increased numbers in the sputum and BAL fluid of COPD patients (Keatings et al., 1996; Lacoste et al., 1993), but in low numbers in the lung parenchyma (Finkelstein et al., 1995), perhaps because of rapid migration through the airways. Sputum neutrophil numbers correlate with disease severity and the rate of lung function decline (Di Stefano et al., 1998; Stanescu et al., 1996). Neutrophils secrete elastase, cathepsin G, proteinase-3, and metalloproteinases 8 and 9 (Barnes, 2004; Cowburn et al., 2008; Stockley, 2002) and may therefore contribute to tissue destruction (Wang et al., 2003). However, there is a negative association between neutrophil counts and the amount of alveolar destruction in COPD (Finkelstein et al., 1995) and neutrophils are not a major part of parenchymal inflammation in COPD (Barnes et al., 2003). Neutrophil migration is controlled by the chemokine interleukin-8 (IL-8/CXCL8) which is also elevated in sputum and BAL fluid of COPD patients (Hollander et al., 2007; Yamamoto et al., 1997). CXCL8/IL-8 levels are further increased during exacerbations, though the cellular source is uncertain (Nocker et al., 1996; Soler et al., 1999).

Other inflammatory cell types involved in COPD include T lymphocytes and dendritic cells. T cells numbers are increased in the lung parenchyma and airways of patients with COPD, with a greater increase in CD8+ than CD4+ cells. The role of the increased T cell numbers on COPD severity is not yet known (Retamales et al., 2001). Dendritic cells are present in high numbers in the airways, and due to their ability to activate so many other inflammatory cell types, are likely to have an important role in COPD. Dendritic cells have been found in increased numbers in the airways of smokers,
but their contribution to COPD needs further study (Barnes et al., 2003).

Treatment

As of yet, no treatment exists that can stop the progression of COPD or completely suppress the inflammation (Barnes, 2008a). Current treatments only ameliorate the symptoms of disease. The best current drug therapy is long-lasting bronchodilators. Common treatments for COPD include corticosteroids, which are widely prescribed for this purpose. However, only about 10% of COPD patients show symptomatic improvement with oral corticosteroids, and this effect could be due to concomitant asthma (Barnes et al., 2003). Corticosteroids are not effective at suppressing this inflammation because macrophages from COPD patients are nonresponsive to them, in contrast to macrophages from healthy individuals (Culpitt et al., 1999; Keatings et al., 1997). However, systemic corticosteroids have shown a beneficial effect on acute exacerbations (Niewoehner, 2006).

There are many novel drugs being considered for the treatment of COPD. Longer lasting inhaled β₂-agonists and muscarinic antagonists, both of which cause airway dilation via smooth muscle relaxation (Barnes, 2008a, b). Anti-IL-8 therapy has had little benefit in clinical trials. TNF-α inhibitors are currently undergoing trials (Chung, 2006). Other promising drugs in early stages include CXCR2 (IL-8 receptor) antagonists, phosphodiesterase 4 inhibitor, and pathway inhibitors of p38 MAPK, NF-κB, and PI3K. Another approach is the reversal of corticosteroid resistance by increasing histone deacetylase activity (Barnes, 2003, 2008a, b, c).
Exacerbation

Acute exacerbations of COPD are characterized by an increase of symptoms and a worsening of lung function (Barnes et al., 2003). Burge and Wedzicha proposed the definition: “an exacerbation of COPD is a sustained worsening of the patient’s condition, from the stable state and beyond normal day-to-day variations that is acute in onset and may warrant additional treatment in a patient with underlying COPD” (Burge and Wedzicha, 2003). They are a common cause of hospital admission. Acute exacerbations may be prolonged and can have a deleterious effect on quality of life for the patient (Groenewegen et al., 2003; Seemungal et al., 1998) and may accelerate progression of COPD. Increased frequency of exacerbations is found in more severe COPD, with an average of 2.68 exacerbations per year in moderate COPD increasing to 3.43 per year in severe COPD (Donaldson and Wedzicha, 2006). Exacerbations are characterized by increased breathlessness and are often accompanied by wheezing and chest tightness, increase in cough and sputum production, increased purulence of sputum, or fever (Rabe et al., 2007). Purulent exacerbations are usually related to bacterial infection and are associated with increased neutrophil inflammation. Levels of CXCL8/IL-8 in the sputum have been found to be unchanged in mild exacerbations (Gompertz et al., 2001), but are greatly elevated in severe exacerbations requiring hospital admission (Hill et al., 1999). Bhowmik et al. found levels of CXCL8/IL-8 during exacerbation to positively correlate with all sputum cell counts (Bhowmik et al., 2000). Severe exacerbations can result in death, with inpatient mortality rates ranging between 4-30%; the highest mortality is seen
in patients with respiratory failure (Donaldson and Wedzicha, 2006).

Treatments of COPD exacerbations include bronchodilators, glucocorticosteriods, and antibiotics. Short-acting inhaled $\beta_2$-agonists are the preferred treatment, with addition of an anticholinergic if needed. Methylxanthines, bronchodilators with an unknown mechanism of action, are often used, but their effectiveness is unclear. Intravenous glucocorticosteriods are a recommended addition to other therapies in hospital management of exacerbations (Niewoehner, 2006; Rabe et al., 2007). Antibiotics are recommended only for exacerbations with purulent sputum (Celli and Barnes, 2007).

Causes of Exacerbation

The most common causes of an exacerbation are infection of the airway and air pollution, but for nearly a third of severe exacerbations, a cause could not be determined. Infectious agents in exacerbation can be either viral or bacterial; in one study of patients admitted to a hospital with severe exacerbation, 78% showed evidence of either viral or bacterial infection (Celli and Barnes, 2007). The contribution of bacterial infection to COPD is unclear and is a source of controversy (Murphy, 2000). However, at least 50% of patients have been shown to have high concentrations of pathogenic bacteria in their lower airways during exacerbation (Sethi et al., 2002). The predominant bacteria recovered during acute exacerbations from lower airways are *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (Verduin et al., 2002). Viruses also play a role in exacerbations, with rhinovirus being implicated the most often (Currie
and Wedzicha, 2006; Greenberg et al., 2000). Respiratory syncytial virus and influenza have also been associated with exacerbations (Wedzicha, 2004). Many patients with acute exacerbation have concomitant viral and bacterial infection. About 25% of patients admitted to a hospital for an exacerbation had both viral and bacterial infection, and their exacerbations were more severe (Celli and Barnes, 2007).

**Moraxella catarrhalis**

**History**

*Moraxella catarrhalis* was first described in 1896, when it was given the name *Micrococcus catarrhalis* (Karalus and Campagnari, 2000). It is morphologically similar to the *neisseriae*, and was moved to this genus as *Neisseria catarrhalis*. *N. catarrhalis* belonged to the group of nongonococcal, nonmeningococcal neisseriae, and was considered to be a part of the normal upper respiratory tract flora (Karalus and Campagnari, 2000; Verduin et al., 2002). In 1963, it was discovered that the bacterium originally called *Micrococcus catarrhalis* was actually two distinct species, *N. catarrhalis* and *N. cinerea*. There was found to be a wide phylogenetic separation between the two species so *N. catarrhalis* was moved to a new genus and renamed *Branhamella catarrhalis* (Verduin et al., 2002). In 1963, *B. catarrhalis* was moved yet again, to the genus *Moraxella*. *Moraxella catarrhalis* was not a perfect fit in its new genus as it is coccoid, and the moraxellaceae are rod shaped (Catlin, 1990). This caused considerable controversy with many scientists preferring the genus *Branhamella* for this bacterium.
DNA sequencing and comparison of 16S rDNA has now confirmed that \textit{M. catarrhalis} is closely related to the other moraxellaceae despite the differences in cell morphology (Enright and McKenzie, 1997).

\textbf{Characteristics}

\textit{M. catarrhalis} is an aerobic Gram-negative diplococcus with flattened abutting sides (Verduin et al., 2002). \textit{M. catarrhalis} possesses LOS (lipoooligosaccharide) on its surface. This differs from the LPS (lipopolysaccharide) of many pathogenic Gram-negative bacteria in that LOS has, at most, only a single repeating O antigen (Fomsgaard et al., 1991; Holme et al., 1999; Karalus and Campagnari, 2000). The O antigen of \textit{M. catarrhalis} is more antigenically conserved among strains than the LOS of other Gram-negative bacteria. The LOS falls into three types, A (61%), B (29%), and C (5%); accounting for 95% of strains (Edebrink et al., 1995; Vaneechoutte et al., 1990a). The peptidoglycan of \textit{M. catarrhalis} is very immunogenic to macrophages. Though \textit{M. catarrhalis} is a Gram-negative bacterium, it has been shown to have the a similar amount of peptidoglycan as two Gram-positive organisms, \textit{Staphylococcus aureus} and \textit{Bacillus subtilis} under identical culture conditions (Keller et al., 1992). Gram-positive bacteria normally have between 20-40 layers of peptidoglycan, so this finding suggests \textit{M. catarrhalis} may have a thick, multilayered peptidoglycan architecture. Between strains of \textit{M. catarrhalis} there is little variation in the outer membrane proteins. Eight major outer membrane proteins have been identified and designated as OMPs A through H (Murphy et al., 2005a), and more recently a few other outer membrane proteins have
been described. These are currently the target of much study as vaccine candidates (McMichael, 2000a, b).

*M. catarrhalis* has a high incidence (90%) of β-lactamase, allowing it to resist β-lactam antibiotics including penicillin, amoxicillin, ampicillin, and piperacillin (Felmingham and Gruneberg, 2000; Verduin et al., 2002). The majority of isolates still show susceptibility to amoxicillin-clavulanic acid (a β-lactamase inhibitor), cefixime, chloramphenicol, ciprofloxacin, and ofloxacin (Felmingham and Gruneberg, 2000). Of clinical concern, is the observation that the β-lactamase of *M. catarrhalis* not only protects it from penicillins, but also protects other bacteria such as *S. pneumoniae* or *H. influenzae* during concomitant infection. This effect is referred to as the indirect pathogenicity of *M. catarrhalis* (Patel et al., 1995; Van Hare et al., 1987; Verduin et al., 2002). Though the mechanism by which *M. catarrhalis* can confer β-lactam resistance is unknown, evidence suggests the β-lactamase enzyme may be released into the extracellular environment (Budhani and Struthers, 1998).

Pathogenicity

Very little is known about the virulence of *M. catarrhalis*. There are considered to be five requirements for virulence of mucosal pathogens: (i) binding and colonization of mucosal surfaces, (ii) entry into host tissues, (iii) multiplication *in vivo*, (iv) interference with host defense mechanisms, and (v) production of damage to the host (Smith, 1995). Of these, the most is known about *M. catarrhalis’* adherence mechanisms, and even this is not fully understood (Karalus and Campagnari, 2000; Verduin et al., 2002). The outer membrane protein UspA1 is involved in *M. catarrhalis’*
ability to form biofilms, and could be an important factor in virulence (Lafontaine et al., 2000; Pearson et al., 2006). Much more research on *M. catarrhalis* is needed. However, because it is a strict human pathogen, there has been little progress in finding a good animal model in which to study *M. catarrhalis*. In most cases the bacteria are cleared in 24-48 hours and the animal stays healthy (Karalus and Campagnari, 2000; Verduin et al., 2002).

In the past few decades *M. catarrhalis* has been increasingly recognized as a pathogen. It was once thought to be a nonpathogenic, commensal organism which colonized the nasopharynx in healthy adults (Catlin, 1990; Johnson et al., 1981). It is now known to be an important cause of upper respiratory tract infections in otherwise healthy children and elders (Verduin et al., 2002). In addition, *M. catarrhalis* can cause lower respiratory tract infections, particularly in people with underlying disease such as COPD. There has been much controversy over the past century as to whether *M. catarrhalis* is commensal or pathogenic (Karalus and Campagnari, 2000). Because of this controversy, most clinical laboratories did not test for *M. catarrhalis*, especially if a known pathogen such as *S. pneumoniae* or *H. influenzae* was present (Karalus and Campagnari, 2000). As a result, there is relatively little known about *M. catarrhalis*’ virulence factors and pathogenic characteristics.

In many recent studies, it has been shown that *M. catarrhalis* is an important cause of disease, particularly in children and adults with underlying respiratory disease or infection. In immunocompromised patients, *M. catarrhalis* can cause many severe infections including pneumonia, endocarditis, septicemia, and meningitis (Catlin, 1990; Verduin et al., 2002). It has also been implicated in hospital outbreaks of respiratory
Epidemiology

*M. catarrhalis* is very common in the population, with upper respiratory tract carriage rates highest in the fall and winter (Van Hare et al., 1987; Verduin et al., 2002). Fewer than 4% of healthy adults are thought to carry *M. catarrhalis* (Karalus and Campagnari, 2000; Vaneechoutte et al., 1990b; Verduin et al., 2002). However, children have up to a 75% carriage rate (Murphy et al., 2005b; Vaneechoutte et al., 1990b; Verduin et al., 2002). Using a monthly sampling technique, it was revealed that 77.5% of children were colonized by *M. catarrhalis* at least once during the first two years of life (Faden et al., 1994). There is also a correlation between frequency of colonization and occurrence of upper respiratory tract infections (Van Hare et al., 1987; Varon et al., 2000; Verduin et al., 2002). *M. catarrhalis* is considered to be an important pathogen in respiratory tract infections, both in children and in adults with underlying disease, such as COPD. In children *M. catarrhalis* is a common cause of otitis media (middle ear infection), sinusitis, tracheitis, bronchitis, and pneumonia (Faden et al., 1994; Verduin et al., 2002). Otitis media is a very frequent infection in young children and is considered the most serious and frequent infection caused by *M. catarrhalis* in children (Berman, 1995; Faden et al., 1994; Klein, 1999). *M catarrhalis* causes an estimated 3-4 million cases of otitis media in the United States (Klein, 1994; Murphy, 1996). Otitis media also accounts for much antibiotic use and has a high financial toll (Klein, 1994; Murphy, 1996; Stool and Field, 1989; Van Hare et al., 1987). In adults, *M. catarrhalis* is associated with a variety of diseases. It is the most frequent bacterial cause of laryngitis
(about 55%) (Schalen et al., 1980). While *M. catarrhalis* is not a common cause of lower respiratory tract infection in adults, it can cause infection in three separate situations: (i) in COPD patients, (ii) in pneumonia in the elderly, and (iii) in nosocomial infections (Murphy, 1998).

**M. catarrhalis in COPD**

*M. catarrhalis* is the third most common isolate from acute exacerbations in COPD, behind *H. influenzae* and *S. pneumoniae* (Verduin et al., 2002). The frequency of *M. catarrhalis* isolation from sputum is rising (Boyle et al., 1991; Verduin et al., 2002). One study found *M. catarrhalis* to be the most isolated pathogen in COPD (Sportel et al., 1995). There appears to be a subset of patients (around 20%) who are chronically colonized with *M. catarrhalis*. Often (40-50% of the time) *S. pneumoniae* or *H. influenzae* can be isolated from sputum in addition to *M. catarrhalis* (Barreiro et al., 1992; Pollard et al., 1986; Verduin et al., 2002).

There are three indications that *M. catarrhalis* is a causative agent of COPD exacerbations. First, a subset of exacerbated patients has sputum Gram stains consisting predominantly of Gram-negative diplococci. When cultured, these yield nearly pure *M. catarrhalis* (Bakri et al., 2002). Second, specimens obtained from transtracheal aspirates and protected brush during COPD exacerbations yield pure cultures of *M. catarrhalis* (Aitken and Thornley, 1983; Fagon et al., 1990; Monso et al., 1995). Third, exacerbated patients with sputum cultures positive for *M. catarrhalis* show improvement when treated with *M. catarrhalis*-targeted antibiotics (McLeod et al., 1986; Nicotra et al., 1986).
**M. catarrhalis Inflammation**

*M. catarrhalis* is cleared from the lungs much more slowly than other bacteria (Verduin et al., 2002). *In vivo*, *M. catarrhalis* has shown the ability to induce expression of inflammatory mediators. *M. catarrhalis* induces expression of IL-6, CXCL8/IL-8, CCL2/MCP-1, GM-CSF, and PGE₂ from A549 lung epithelial cells (Fink et al., 2006; Rosseau et al., 2005; Slevogt et al., 2007). Polymyxin B treatment did not alter the levels of cytokine released, suggesting that this process may not involve the bacterium’s LOS (Fink et al., 2006). Macrophages exposed to *M. catarrhalis*, or to culture supernatants, released IL-1β, IL-6, CXCL8/IL-8, and PGE₂ (Fink et al., 2006). A549 cells infected with *M. catarrhalis* secreted the chemokine CCL2/MCP-1, and upregulated the adhesion molecules ICAM-1 and VCAM-1. The same study also showed the ability of *M. catarrhalis*-infected epithelial cells to increase the inflammatory capacity of monocytes, inducing a strong respiratory burst and transepithelial migration (Rousseau et al., 2005).

**Immunity and Inflammation**

**Innate Immunity**

The airway epithelium is an important barrier to infection. In addition to providing a mechanical barrier, it signals the presence of a pathogen to other immune cells (Rastogi et al., 2001; Tosi, 2005). The lower airway is normally sterile, yet nasal concha
(turbinates) and septum only catch particles greater than 10 μm in size, allowing most microbes to pass through (Rastogi et al., 2001).

The tracheal airway is lined with epithelium consisting of columnar ciliated cells, secretory goblet cells, and cells with microvilli that provide mechanisms for mucociliary clearance (Hippenstiel et al., 2006). The bronchiole epithelium is made up of cuboidal cells and secretory Clara cells. The alveolar epithelium consists of Type I cells (95%) that are fused to endothelial cells via the basement membrane and which are responsible for gas exchange. Type II alveolar epithelial cells are secretory and are responsible for the alveolar fluid content (Hippenstiel et al., 2006).

More than just passive mechanical barrier, airway epithelial cells are a major source of antimicrobial peptides important in local host defense (Tosi, 2005). Epithelial cells that make up the mucus membranes express human β-defensins. These are small cationic peptides with the ability to disrupt the membrane of a broad range of pathogens (Tosi, 2005). Epithelial cells also express the antimicrobial proteins lysozyme, which attacks the peptidoglycan cell walls of bacteria, and cathelicidin, with broad antimicrobial activity (Tosi, 2005). Lactoferrin, lactoperoxidase, and surfactant are also broad range antimicrobial proteins expressed by the airway epithelium (Rastogi et al., 2001).

The airways between the larynx and the bronchioles are covered by a 5-100μm thick fluid secreted by the epithelium. A layer of mucus floats on top of this fluid. Many bacteria become bound to the mucins (Rastogi et al., 2001). The coordinated beating of the cilia on the columnar ciliated cells provides clearance of inhaled particles trapped in the fluid (Diamond et al., 2000). In healthy individuals most of the bacteria become trapped in the mucus and are expelled from the body via what is termed the ‘mucociliary
escator’ (Rastogi et al., 2001). Unfortunately this mucociliary clearance often breaks down in people with underlying disease, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis, allowing bacteria to persist. Certain pathogens are able to resist being expelled by the mucociliary escalator, even in healthy individuals. Epithelial cells must recognize pathogens and mount a response against them to protect the body.

**Recognizing Pathogens**

Potential pathogens are recognized with the aid of Pattern Recognition Receptors (PRR) that recognize and bind to the Pathogen Associated Molecular Patterns (PAMP) found on microbes (Akira et al., 2006; Kawai and Akira, 2005; Zhang and Ghosh, 2001). One such family of PRRs is the Toll-like receptors (TLR). The two Nod receptors, Nod1 and Nod2, are also important for recognition of bacteria (Hippenstiel et al., 2006). When activated, such as by a bacterial pathogen, TLRs and Nods start an intracellular signaling cascade leading to activation of NF-κB (Figure 1).

**NF-κB**

NF-κB is an important mediator of immune signaling. It can also activate expression of anti-apoptotic genes and has been implicated in some cancers. Therefore NF-κB needs to be tightly regulated (Vanden Berghe et al., 2006). It is present in the cytoplasm of cells, bound to an inhibitor, IκB. When this inhibitor is phosphorylated by IKK, it is targeted to the proteasome where it is degraded. This frees NF-κB to translocate to the nucleus (Hayden et al., 2006). In the nucleus, NF-κB activates the expression of proinflammatory genes by binding to response elements and recruiting
transcription factors. This results in expression of cytokines including IL-1, IL-2, IL-6, IL-12, TNF-α, IFN-γ and chemokines such as CXCL8/IL-8 and CCL2/MCP-1 (Liang et al., 2004).

**Signal Transduction**

Cells have a complex system for responding to external stimuli. The signal must go from outside of the cell to the nucleus, where the cell can alter expression of genes to appropriately respond to the stimulus. Receptors often span the plasma membrane of the cell with a domain to recognize the stimulus on the outer surface of the membrane and a domain to activate a signal transduction cascade on the inner surface of the membrane. For the receptors concerned in this study, the intracellular domain of the receptors has tyrosine kinase activity. Thus, when the receptor binds to its ligand the intracellular portion becomes phosphorylated and activated. The phosphorylated residues now act as a docking site for proteins with SH-2 domains. These bind, become phosphorylated and active, and in turn, phosphorylate the next protein down the line. In this way a signal from the surface is transduced and amplified—the ‘cascade’ (Lee and McCubrey, 2002); (Figure 1).
Figure 1. Overview of recognition and response to pathogen. See text for details.
Nucleotide-binding Oligomerization Domain Proteins

The Nod (nucleotide-binding oligomerization domain) proteins have three domains. Ligand recognition is mediated by a carboxy-terminal leucine-rich repeat domain similar to that in the TLRs. The central domain exhibits ATPase activity and facilitates oligomerization with other Nod proteins. Protein-protein interactions are mediated by the amino-terminal caspase-recruitment domain (CARD) (Hippenstiel et al., 2006). The Nod proteins reside in the cytosol of the cell, where they recognize bacterial cell wall components. Nod1 is activated by peptides containing γ-D-glutamyl-meso-diaminopimelic acid derived from peptidoglycan of Gram-negative bacteria. Nod2 recognizes the muramyl-dipeptide MurNAc-L-Ala-D-isoGln that is found in the peptidoglycan of almost all bacteria (Hippenstiel et al., 2006). Nod1 is ubiquitously expressed and Nod2 is primarily expressed in antigen presenting cells and epithelial cells. Activation of the Nod proteins leads to recruitment of the kinase RICK/RIP2 through association of the CARD domains. This leads to interaction with the regulatory subunit of IKK, IKKγ/NEMO, and NF-κB activation and proinflammatory gene transcription (Zhao et al., 2008). *M. catarrhalis* has been shown to activate proinflammatory gene expression through the action of Nod1. Knock down of Nod1 decreased the secretion of IL-8 by 30-35% from *M. catarrhalis* stimulated A549 cells (Slevogt et al., 2007).

Toll-like Receptors

TLRs are transmembrane proteins with N-terminal leucine-rich repeats, which recognize PAMPs, and a C-terminal TIR (Toll-IL-1 receptor) domain, which is required for signaling (Kaisho and Akira, 2006; Kawai and Akira, 2005; O'Neill and Greene,
There are 10 TLR family members in humans that recognize patterns such as bacterial cell wall components, flagellin, and viral nucleic acids. TLR 2 recognizes Gram-positive bacteria by binding to cell wall components such as peptidoglycan and lipoproteins (but recognizes components also found on some Gram-negative bacteria) (Takeuchi et al., 1999). Gram-negative bacteria are usually detected through TLR 4, via indirect binding of lipopolysaccharide (LPS) (Akira and Takeda, 2004; Munford and Varley, 2006; Zhang and Ghosh, 2001). CD14 and LPS binding protein (LBP) bind to LPS (Fenton and Golenbock, 1998). TLR4 can form either homo- or hetero-dimers and physically interacts with CD14 (Aliprantis et al., 1999; Yang et al., 1999). This triggers a signal leading to production of inflammatory mediators.

Classical TLR 2 and 4 signaling pathways are similar, with the main difference being the need for accessory protein MD2 in TLR 4 but not TLR 2 signaling (Munford and Varley, 2006; Takeuchi and Akira, 2001). MD2 and CD14 are both required for TLR4 functionality, but are not expressed by many airway epithelial cells, including A549, used in this study (Chen, M. Personal Communication, 2009). However, a soluble version of these molecules is expressed by alveolar macrophages, allowing for epithelial TLR4 function in situ (Fenton and Golenbock, 1998). After agonist binding there is a conformational change in the TIR domain and TIRAP (TIR-domain containing adapter protein) binds the intracellular TIR domain of the TLR, resulting in recruitment of MyD88 (myeloid differentiation primary-response protein 88) (Kaisho and Akira, 2006). The death domain (DD) of MyD88 binds IRAK-4 (IL-1 associated kinase-4), a serine/threonine kinase. IRAK-4 forms a complex with IRAK-1 and recruits TRAF6 (TNF receptor-associated factor 6) (Hayden et al., 2006; O'Neill and Greene, 1998).
TRAF6 activates TAK1 (TGFβ-activated kinase 1) which activates NIK (NF-κB-inducing kinase). NIK phosphorylates the IKK complex (IκB kinase). IKK is responsible for targeting IκB for proteasomal degradation (Hayden et al., 2006; Liang et al., 2004). This releases NF-κB to travel to the nucleus and activate gene expression by binding to its response element in the DNA and activates the transcription of proinflammatory genes.

Phosphoinositide-3 Kinase

PI3K (phosphoinositide-3 kinase) is a lipid kinase that phosphorylates phosphoinositides (PtdIns) to generate second messengers involved in many cellular functions including survival, proliferation, and metabolism (Deane and Fruman, 2004). PI3Ks are divided into three classes (I, II, and III) based on structure and specificity. Class I is activated by cell surface receptors and represents a major signaling pathway (Hawkins et al., 2006). Class I is further broken down into class IA and IB (Wymann and Marone, 2005). PI3K has a catalytic subunit and a regulatory subunit. The class IA regulatory subunits are coded by three genes (α, β, γ) which give rise to five splice variations; p85α, p55α, p50α, p85β, and p55γ. They have two src homology domains with a coiled-coil region in between allowing for tight binding to three possible catalytic subunits; p110α (PI3Kα), p110β (PI3Kβ), and p110δ (PI3Kδ) (Wymann and Marone, 2005). PI3Kα and PI3Kβ are ubiquitously expressed in the body and the lack of both together is embryonic lethal. PI3Kδ is mainly expressed in cells of the immune system (Patton et al., 2007). There is only a single class IB member, PI3Kγ, with a p101 regulatory subunit or a p87 adapter protein. PI3Kγ is highly expressed throughout the
hematopoietic system, and seems to be important for inflammation and allergy (Wymann et al., 2003). The function of class II PI3Ks is still largely unknown. There is evidence that they may be involved in cell-surface receptor activation and endocytosis (Falasca and Maffucci, 2007; Hawkins et al., 2006). Class III PI3Ks belong to a major regulatory pathway involved in endosome and lysosome activity (Hawkins et al., 2006).

Inactive PI3K is located in the cytosol of the cell. The SH-2 domains of the p85 regulatory subunit bind to an activated receptor tyrosine kinase. Interestingly, this signaling complex often includes the active, GTP-bound Ras directly associating with a Ras-binding domain motif in the p110 catalytic subunit (Hawkins et al., 2006). Ras is often activated in parallel with class I PI3K activation. Ras can play an important activating role for all the class I isoforms except for PI3Kβ (Hawkins et al., 2006). There is evidence that PI3Kβ is regulated differently from the other isoforms. It can be activated by G proteins and appears to directly activate Rab5, suggesting a role in endocytosis (Shin et al., 2005). Class IB is activated by G-protein-coupled receptors and has also been shown to interact with GTP-Ras (Suire et al., 2002). Once activated, PI3K generates phosphorylated lipids in the membrane (Hawkins et al., 2006; Lee and McCubrey, 2002). Class I PI3K phosphorylates PtdIns(4,5)P₂ to create PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ recruits and anchors proteins, including Akt (also called Protein Kinase B), to the plasma membrane through interaction with its pleckstrin homology domain (Wymann et al., 2003). This interaction is required to make the Thr308 activation loop available to the PDK kinases (Hawkins et al., 2006). Once Akt is anchored in the membrane it is phosphorylated on Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and then on Ser473 by PDK2 (Alessi et al., 1997; Franke et al., 1997; Lee and
Activated Akt is a serine/threonine kinase and can phosphorylate many proteins giving rise to a variety of different effects (Du and Tsichlis, 2005; Li, 2003); (Figure 1).

A major role of class I PI3Ks is in the control of cell motility. The production of PIP3/PIP2 regulates the activity of multiple guanine exchange factors and GTPase-activating proteins. These in turn regulate the Arf and Rac GTPases which are involved in cytoskeletal and membrane delivery events in the formation of lamellipodia (Barber and Welch, 2006; Hawkins et al., 2006). The main isoform involved in this process is PI3Kγ (Smith et al., 2007). Leukocytes from PI3Kγ deficient mice show severe defects in chemotaxis of neutrophils, monocytes, and T cells (Medina-Tato et al., 2007; Oak et al., 2007). PI3Kδ is the main isoform controlling chemotaxis in B cells (Oak et al., 2007; Okkenhaug et al., 2006). PI3Kδ is also a key regulator of T cell development and differentiation (Okkenhaug et al., 2006; Patton et al., 2007).

PI3Ks also play a major role in cell growth, proliferation, and survival. Akt is involved in multiple cell regulation pathways; including activation of mTORC1 (an integrator of cell growth decisions), regulation of FOXO transcription factors, activation of GSK3, and activation of p27 KIP (Hawkins et al., 2006). All of these are key regulators of cell cycle progression and survival. This pivotal role in controlling cell cycle also means that PI3K-Akt pathway mutations are found in many cancers.

**Extracellular Signal-Regulated Kinase**

The extracellular signal-regulated kinases (ERKs) were the first of the mitogen-activated protein kinase (MAPK) family to be discovered (Lee and McCubrey, 2002).
The dual-specificity, serine/threonine and tyrosine kinase, MEK (MAP/ERK kinase) family acts directly upstream to activate the MAPKs like ERK. Raf activates MEK and is in turn activated by Ras, (Lee and McCubrey, 2002). Active GTP-bound Ras recruits Raf to the membrane. There, Raf is phosphorylated on tyrosine and/or serine/threonine residues and dimerizes. The activated Raf dimer phosphorylates MEK1 and MEK2 on serines S218 and S222 (Lee and McCubrey, 2002). The inhibitor U0126 (used in this study) blocks this activation through an unknown mechanism (Lee and McCubrey, 2002). Directly downstream of MEK is ERK1 (p44) and ERK2 (p42). MEK activates ERK1/2 by phosphorylating the T183 and Y185 residues (Lee and McCubrey, 2002). Once active, ERK2 dimerizes and translocates to the nucleus, where it can activate a variety of targets (Lee and McCubrey, 2002); (Figure 1).

P38 Mitogen-Activated Protein Kinase

P38 is another member of the MAPK family. P38 MAPK has four different isoforms, α, β, γ, and δ. All the isoforms are activated by either MEK3 or MEK6 (Adcock et al., 2006). MEK6 can activate all the isoforms, but MEK3 seems to be selective for p38 α and β (Lee and McCubrey, 2002). The p38 α and β isoforms activate downstream kinases such as MAPKAP2/3 (MAPK activated protein) and MSK1/2 (mitogen- and stress-activated kinase). The γ and δ isoforms generally target transcription factors rather than MAPKAP. Like the other MAPKs, p38 activation can lead to expression of proinflammatory mediators (Lee and McCubrey, 2002); (Figure 1).
Crosstalk between signal transduction pathways

Signaling pathways in the cell are often interconnected with one another. A signal may initially activate one pathway, a member of which may either activate or inhibit a member of a different pathway. Another aspect to consider is that many proteins may form large groups around receptors or other key pathway molecules. These signal transduction complexes allow for interaction of multiple pathway members at any one time. Increasingly, cell signal transduction is being thought of as a signaling web rather than a linear signaling pathway. This allows for great specificity and fine tuning of the response to the stimulus. In the same way, the signaling pathways mentioned above have shown themselves to be interconnected, or to have crosstalk between them.

Recently PI3K has been implicated in PRR signaling, though the exact pathway is unclear. PI3K can directly interact with some TLRs or TLR adapter proteins to regulate signaling downstream to NF-κB or MAPK (Liew et al., 2005). The PI3K-Akt pathway was shown to be activated in response to TLR2 or TLR4 agonists (Martin et al., 2003). The cytosolic domain of TLR2 contains multiple p85 docking sites for the binding of PI3K. These sites are also present in TLR1 and TLR6, but are not found in TLR3, TLR4, or TLR5. A putative p85 docking site has been identified in the MyD88 adaptor molecule, possibly allowing involvement of many of the TLRs (Arbibe et al., 2000).

PI3K can be either a positive or negative regulator of TLR signaling, possibly depending on cell type and stimulus (Ruse and Knaus, 2006). As a positive regulator, PI3K activation was found to promote activation of NF-κB p65 (Arbibe et al., 2000). Much evidence also exists for PI3K as a negative regulator of the NF-κB pathway. Inhibition of PI3K-Akt pathway results in increased NF-κB p65 activation in LPS-
stimulated monocytes (Guha and Mackman, 2002). A downstream kinase of PI3K, Akt, has been implicated in the NF-κB pathway, possibly through increasing transactivation of NF-κB (Kane et al., 1999; Thomas et al., 2002).

The PI3K pathway has been tied to the MAPK pathways. Inhibition of PI3K was shown to block ERK1/2 phosphorylation, but not activation of p38 MAPK (Martin et al., 2003). Additionally, use of PI3K inhibitors prevent ERK1/2 activation, and PI3K activity is required for Mycobacterium tuberculosis-induced MAPK activation in human macrophages (Mendez-Samperio et al., 2008). The PI3K/Akt pathway was also shown to inhibit the Raf/MEK/ERK pathway, but the effect was dependent upon differentiation state of the cell. Akt was observed to form a complex with Raf (Rommel et al., 1999). Zimmermann showed that Akt interacts with and phosphorylates Raf on Ser259, leading to inhibition of the Raf/MEK/ERK pathway (Zimmermann and Moelling, 1999). PI3K is often a negative regulator of p38 MAPK. Akt activation can decrease p38 MAPK activity (Li, 2003). Haemophilus influenzae-induced activation of the PI3K-Akt pathway leads to inhibition of p38 MAPK (Li, 2003).

Activation of p38 MAPK has been shown to inhibit ERK activation. Phosphorylated p38 bound to ERK and kept it from being activated by MEK (Zhang et al., 2001). NF-κB activity can be regulated by phosphorylation of p38 MAPK (Vanden Berghe et al., 2006). P38 MAPK and ERK positively regulate NF-κB activity without affecting NF-κB activation and DNA binding. NF-κB still must be activated in the cytosol by an IκBa-specific kinase. An additional step may occur, whereby the p38 MAPK and ERK pathways contribute to transcriptional activation by modulating the transactivation of the p65 subunit of NF-κB (Vanden Berghe et al., 2006). P38 MAPK
was not found to phosphorylate IκB, the NF-κB DNA-binding subunit p50, or the transactivating half of p65. This suggests a kinase downstream in the p38 MAPK or ERK pathways is responsible for the phosphorylation (Vanden Berghe et al., 2006). The MAPK pathway upstream of ERK can lead to activation of NF-κB by another mechanism; Raf-1 can act as an IκB kinase to induce NF-κB activation (Li and Sedivy, 1993).

**Inflammatory Mediators**

Activation of transcription leads to production of proinflammatory mediators, including chemokines, which are the focus of this study. Chemokines are low molecular weight proteins involved in leukocyte migration (Tosi, 2005). The superfamily is split into four major groups based on structure. The groups are CC, CXC, C, and CXXXXC named for the number and spacing of N-terminal cysteine residues (Curnock et al., 2002). They signal through seven-transmembrane domain G-protein coupled receptors to induce cell migration (Tosi, 2005). IL-8 (Interleukin 8, CXCL8) and MCP-1 (Monocyte chemoattractant protein 1, CCL2) are two chemokines important in COPD and other chronic inflammatory conditions. Neutrophils, macrophages, and CD8 T cells are found increased in number in the airway of COPD patients, though the role of macrophages and neutrophils in disease is still not completely understood (Medina-Tato et al., 2006; Stockley, 2002; Tetley, 2002).
The Purpose of this Study

The purpose of this study is to understand how infection with *M. catarrhalis* leads to acute exacerbations in COPD. The ability of *M. catarrhalis* to cause inflammation is examined, specifically the expression of the chemokines MCP-1, and IL-8; both of which are important in COPD inflammation. This study helps to elucidate the mechanism by which an airway epithelial cell recognizes and responds to infection with *M. catarrhalis* by examining the roles of the signaling molecules described above. Once the mechanism is known, targets for anti-inflammation therapy can be identified and new, more effective, treatments for acute exacerbations may be developed.
MATERIALS AND METHODS

Antibodies and Reagents

Phospho-PI3K p85 (Tyr458)/p55 (Tyr199) antibody, total PI3K p85 antibody, phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (197G2) antibody, phospho-Akt (Ser473) antibody, Akt (pan) (11E7) antibody, phospho-p38 MAPK (Thr180/Tyr182) (28B10) antibody, p38 MAP Kinase antibody, PI3K p110α (C73F8) antibody, and PI3K p110β (C33D4) antibody were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibody was purchased from Sigma (Saint Louis, MO). ERK 2 (p42 MAPK) (C-14) antibody, PI3K p110δ (A-8) antibody, PI3K p85α (B-9) antibody, and PI3K p85β (T15) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PI3K p110γ antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Goat anti-mouse IgG-HRP, goat anti-mouse IgG1-HRP, and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Chemical inhibitors LY294002, SB203580, and U0126 were purchased from Calbiochem (San Diego, CA). Wortmannin was purchased from Cell Signaling Technology (Beverly, MA). Functional grade mouse-anti-human Toll-like receptor 2 IgG2a, κ, functional grade mouse-anti-human Toll-like receptor 4 IgG2a, κ, and
functional grade mouse IgG2a, κ isotype control were purchased from eBioscience (San Diego, CA).

**Cell Strains and Culture Conditions**

The human lung epithelial cell line A549 (ATCC CCL-185) was kindly provided by Dr. Eric Lafontaine (University of Georgia, Athens, GA) and maintained in Ham’s F-12 cell culture medium purchased from Cellgrow, Mediatech, Inc. (Herndon, VA) and supplemented with 10% heat-inactivated FBS, 100U/mL penicillin and 100μg/mL streptomycin (Gibco, Grand Island, NY), 2mM L-glutamine (Gibco), and 0.15% sodium bicarbonate (Cellgrow). *Moraxella catarrhalis* wild type strain O35E (biofilm-negative (Pearson et al., 2006)) was kindly provided by Dr. Eric Lafontaine (University of Georgia, Athens, GA) and cultured using Todd Hewitt agar (Bacto Todd Hewitt broth with Bacto Agar both from BD, Sparks, MD) at 37°C and 7.5% CO₂ overnight. For infection experiments, *M. catarrhalis* was suspended in PBS supplemented with 1.5% Difco gelatin (BD) and adjusted to an OD₆₀₀ of 0.750 (corresponding to approximately 10⁹ cfu/mL). The indicated multiplicity of infection (MOI) of *M. catarrhalis* was added to A549 monolayers immediately after adding fresh, antibiotic free culture medium. Plates were centrifuged at 170 x g for 1 or 5 minutes to ensure bacterial contact with the monolayer.

**Real-time RT PCR**
A549 cells were plated at a concentration of 5x10^5 cells per well of a 6-well plate and grown overnight. *M. catarrhalis* was added to wells at indicated MOI. After the indicated length of time the supernatant was removed and TRIzol Reagent from Invitrogen (Carlsbad, CA) was used as directed by manufacturer’s instructions to extract RNA. Contaminating DNA was removed using Ambion’s (Austin, TX) DNA-free kit according to manufacturer’s instructions. Next 1μg RNA was reverse transcribed using Omniscript Reverse Transcriptase from Qiagen as directed by package insert with 40 U/μL RNasin Plus RNase inhibitor purchased from Promega (Madison, WI) and 10 μM pd(N)_6 random hexamer primer from Amersham Biosciences (Piscataway, NJ). cDNA was analyzed using real-time RT-PCR using SYBR Green I from Molecular Probes (Eugene, OR), dNTP from Amersham Biosciences (Piscataway, NJ), Hot-Start Taq DNA Polymerase from Denville Scientific, Inc. (Metuchen, NJ), and gene-specific primers from IDT (Coralville, IA). Primer sequences: **GAPDH**: forward 5’-GGG AAG GTG AAG GTC GGA GT-3’ reverse 5’-TCC ACT TTA CCA GAG TTA AAA GCA G-3’; **CXCL8/IL-8**: forward 5’-TGA CTT CCA AGC TGG CCG TG-3’ reverse 5’-CGC AGT GTG GTC CAC TCT CA-3’; **MCP-1**: forward 5’-CGC CTC CAG CAT GAA AAG TCT-3’ reverse 5’-ATG AAG GTG GCT GCT ATG AGC-3’. Fold change in expression relative to the housekeeping gene, GAPDH, was calculated using the comparative C_\text{T} method, (e.g. 2^{-\Delta\Delta C_\text{T}}) (Livak and Schmittgen, 2001).

**Cytometric Bead Array**
A549 cells were plated at a concentration of $5 \times 10^5$ cells per well in a 6-well plate and incubated overnight. *M. catarrhalis* was added to cells at the indicated MOI for the indicated length of time. Supernatants were removed and chemokine concentration was analyzed with the Cytometric Bead Array Human Chemokine I kit purchased from BD Biosciences (San Diego, CA) and used according to the manufacturer’s instructions. Briefly, five populations of capture beads specific to CXCL8/IL-8, MCP-1, RANTES, IP-10, or MIG are mixed together and incubated with supernatants and standards along with PE detection reagent. After three hours beads are washed and analyzed by flow cytometry using a FACSCalibur instrument manufactured by BD Biosciences. Each specific bead population has an unique fluorescence intensity allowing the different chemokines to be distinguished from one another. Data was analyzed using BD CBA software from BD Biosciences.

**ELISA**

A549 cells were plated at a concentration of $5 \times 10^5$ cells per well in a 6-well plate and incubated overnight. Cells were stimulated with the indicated MOI of *M. catarrhalis*. After the indicated time supernatants were removed and an ELISA was performed using a DuoSet ELISA development kit for human CXCL8/IL-8 or human MCP-1 from R&D Systems, Inc. (Minneapolis, MN) as directed by manufacturer.

**Western Blotting**
A549 cells were plated at a concentration of 5 x 10^5 cells per well in a 6-well plate and allowed to grow overnight. *M. catarrhalis* was added at the indicated MOI. For experiments using chemical inhibitors the monolayers were pretreated with the indicated concentration of inhibitor for one hour prior to infection. For experiments using functional antibodies the monolayers were pretreated with 10μg/mL of indicated antibody for one hour prior to addition of bacteria. After indicated length of stimulation with *M. catarrhalis* cells were washed with cold PBS and treated with lysis buffer for 15 minutes on ice. Cell debris was pelleted by centrifugation and SDS was added to the protein-containing supernatant. SDS-protein solutions were loaded on 10% polyacrylamide gels at the appropriate volume for an equal amount of protein in each lane and separated using electrophoresis. Protein was then transferred to a nitrocellulose membrane. Blots were probed with specific primary antibodies overnight at 4°C followed by horseradish peroxidase-labeled secondary antibody for one hour at room temperature and exposed using the SuperSignal West Femto kit or the SuperSignal West Pico kit from Pierce (Rockford, IL). After phospho-protein detection blots were stripped and re-probed for total protein or β-actin.

**RNA Interference**

A549 cells were plated at a concentration of 7.5 x 10^4 cells per well in a 24-well plate. After overnight incubation cells were transfected using the transfection reagent DharmaFECT 1 with 100 nM ON-TARGETplus SMARTpool siRNA purchased from Dharmaco, Inc. (Lafayette, CO) targeting the PI3K isoforms p110α (*PIK3CA*), p110β
(PIK3CB), p110δ (PIK3CD), p85α (PIK3R1), p85β (PIK3R2) and the control siCONTROL Non-Targeting siRNA Pool (Dharmacon) (Table 1). After 72 hours cells were infected with indicated MOI of *M. catarrhalis*. After the indicated time period supernatants were removed and analyzed using ELISA.

Table 1. SiRNA Target Sequences

<table>
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<tr>
<th>Gene Targeted</th>
<th>Target Sequence</th>
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<tr>
<td>PIK3CA</td>
<td>5’-GCGAAAAUCUCACACUAUU-3’ 5’-GUGGUAAAGUUCCCAGAUA-3’ 5’-GCUUAGAGUUGGAGUUUGA-3’ 5’-GACCCUAGCCUUAGAUAA-3’</td>
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<tr>
<td>PIK3CB</td>
<td>5’-GAUUCAGUUGAGAUGAUAU-3’ 5’-GUGCUGUGGUAUUCAGAUA-3’ 5’-GAUUAGUGUGAGCAAGUCA-3’ 5’-CAAAGAGGUCUGCCAUAAA-3’</td>
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<td>PIK3CD</td>
<td>5’-ACGAUGAGCUGUUCACAGUAAU-3’ 5’-CCGAAGACACAGGCAGUA-3’ 5’-GCGUGGGCAGACAUUCUUUA-3’ 5’-CGAGUGGAGUUUAACGAAG-3’</td>
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<tr>
<td>PIK3R1</td>
<td>5’-AGUAAGCAUUGUGUCAUA-3’ 5’-GGAACGGUGAGGUAUGAAUA-3’ 5’-GACGAGAGAACCACUUCUUG-3’ 5’-UAAUGUGAGCUGUAGGAAA-3’</td>
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<tr>
<td>PIK3R2</td>
<td>5’-GCGCCAGCUUAGGUCUA-3’ 5’-GGAACGCACUUUGGUAUCGUAG-3’ 5’-GGACACAGAGCCGAGUAU-3’ 5’-GGAAGGGGCGAAGAAUA-3’</td>
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<tr>
<td>Control</td>
<td>5’-AUGAAGCGUAGAUAUUGCUCAUU-3’</td>
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</table>
Statistical Analysis

Data was statistically analyzed using a Student $t$-test and statistical significance was accepted at $P<0.05$ (indicated by asterisks). Data is shown as mean of at least three independent experiments with error bars representing plus and minus one standard deviation.
RESULTS

*Moraxella catarrhalis* induces CCL2/MCP-1 and CXCL8/IL-8 chemokine expression in human lung epithelial cells

Chemokines are an integral component of the immune response to a pathogen. They are not only a product of the immune response, but can also activate other immune cells and recruit leukocytes to the site of infection, resulting in inflammation. In COPD, macrophages and neutrophils make dominate the cellular infiltration into the airways during acute exacerbations. CCL2/MCP-1 is a chemokine that attracts and activates monocytes/macrophages, while CXCL8/IL-8 is a chemoattractant for neutrophils. Both chemokines are found at elevated levels in sputum and bronchoalveolar lavage fluid of COPD patients (Capelli et al., 1999; Yamamoto et al., 1997). *Moraxella catarrhalis* can cause acute exacerbations of COPD, which are a heightened inflammatory state. To determine whether the interaction of *M. catarrhalis* with the lower airway epithelium could be responsible for this inflammation, via inducing release of MCP-1 or IL-8, the human lung epithelial cell line, A549, was used as an *in vitro* cell model.

For these experiments, monolayers of A549 cells were stimulated over a range of MOI (multiplicities of infection; bacteria per A549 cell). Bacteria remained in the culture with the A549 cells for the length of the experiment and significant growth was expected.
Therefore the MOI noted is an initial MOI. During its exponential phase, *M. catarrhalis* can multiply from an OD$_{600}$ 0.05 to OD$_{600}$ 1.0 in about 5 h (Heiniger et al., 2005). Real-time RT-PCR was used to determine the increase of MCP-1 (Figure 2 A) and IL-8 (Figure 2 B) mRNA relative to the control mRNA for the housekeeping enzyme GAPDH. Both chemokine mRNA levels increased greatly between 6 and 30 h after infection at 100 MOI *M. catarrhalis*. This increase is significant at 30 h for both MCP-1 (p=.0001) and IL-8 (p=.0006) compared to unstimulated cells. When infected with fewer bacterial cells, at 0.1 MOI, mRNA levels show a smaller, but still significant increase (p=.02, MCP-1; p=.01, IL-8).

We next measured the MCP-1 (Figure 2 C) and IL-8 (Figure 2 D) protein secreted after infection with a range of doses of *M. catarrhalis* using a cytometric bead array. *M. catarrhalis* at 100 MOI induced the greatest secretion of both MCP-1 (Figure 2 C); (p=.02, 18h; p=.004, 30h; p=.03, 48h) and IL-8 (Figure 2 D); (p=.01, 18h; p=.003, 30h; p=.02, 48h) and a lower secretion level with 0.1 MOI (MCP-1: p=.04, 18h; p=.04, 48h; IL-8: p=.01, 18h; p=.03, 48h), suggesting a dose-dependent response. Chemokine secretion was seen as early as 18 h after infection, increasing over time. The cytometric bead array also measured levels of the chemokines RANTES (*Regulated upon activation normal T cell expressed and secreted*, also called CCL5, a chemoattractant for T cells, eosinophils, and basophils), IP-10 (*IFN gamma induced protein 10*, also called CXCL10, a chemoattractant for monocytes and T cells), and MIG (*Monokine induced by gamma IFN*, also called CXCL9, attracts T cells); all of which were below detection limits.

Thus *M. catarrhalis* can induce cultured lung epithelial cells *in vitro* to release chemoattractants for both macrophages and neutrophils.
Figure 2. MCP-1 and IL-8 induced in response to *M. catarrhalis*. A and B: After infection of A549 monolayers with 0, 0.1, or 100 MOI *M. catarrhalis*, RNA was extracted at 0, 6, or 30 h. Real time RT-PCR was used to measure the fold induction of MCP-1 (A) and IL-8 (B) relative to GAPDH. C and D: A549 monolayers were infected with 0, 0.1, 100 MOI *M. catarrhalis* for 0, 18, 30, or 48 h. The supernatant was removed and used in a cytometric bead array to measure secretion of MCP-1 (C), IL-8 (D), RANTES, IP-10, and MIG. RANTES, IP-10, and MIG all had levels below detection limits (data not shown). Figures show the means of five independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined compared to unstimulated cells at same time, as p≤0.05.
TLR2, but not TLR4, signaling is important for *Moraxella catarrhalis*-induced MCP-1 and IL-8 expression

Epithelial cells recognize bacteria such as *M. catarrhalis* with the aid of pattern recognition receptors (PRR). The Toll-like receptor family is one of the most important and most studied of the PRR. Of the Toll-like receptors, TLR2, which recognizes lipopeptides and peptidoglycan, and TLR4, which recognizes lipopolysaccharide (Takeda and Akira, 2004), are the most likely to be involved in recognition of *M. catarrhalis*.

To determine if either TLR2 or TLR4 signaling is involved in A549 cell expression of MCP-1 and IL-8 in response to *M. catarrhalis*, functional antibodies were used to block the receptors. After 18 h of stimulation with *M. catarrhalis* at 100 MOI, there was a decrease in both MCP-1 (Figure 3 A) and IL-8 (Figure 3 B) secretion compared to the isotype control when TLR2 was blocked (p=.04, MCP-1; p=.001, IL-8). No change was seen when TLR4 was blocked (p=.43, MCP-1; p=.14, IL-8). This suggests that the TLR2 pathway is involved in the expression of MCP-1 and IL-8 induced by *M. catarrhalis*. However, secretion was not completely abrogated by the functional antibody. This could simply mean the antibody did not completely block TLR2 function, or there could be additional receptors involved, or both. This differential effect of blocking antibodies suggests that the A549 cells are responding to *M. catarrhalis* lipoproteins, though in the absence of CD14 or MD2 it is difficult to interpret the role of TLR4.
Figure 3. TLR2, but not TLR4, signaling is important for *M. catarrhalis* induced MCP-1 and IL-8 expression. A549 monolayers were pretreated for 1 h with 10 μg/mL blocking antibodies against TLR2, TLR4, or a mouse IgG2a, κ isotype control. After infection with 0 or 100 MOI *M. catarrhalis* for 18 h, supernatants were removed and an ELISA was used to measure MCP-1 (A) and IL-8 (B) secretion. Due to the high variability of IL-8 levels between experiments, the data were normalized to the 100 MOI isotype control value. Figures show the means of five independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined compared to isotype control at 100 MOI, as p≤0.05.

PI3K is involved in *Moraxella catarrhalis*-induced MCP-1 and IL-8 expression

PI3K, a lipid kinase that phosphorylates PIP2, has been implicated in inflammation. The product, PIP3, is a second messenger that recruits proteins such as Akt to the membrane where they can be activated and begin signaling cascades (Wymann and Marone, 2005).

To test the involvement of the PI3K signaling pathway, A549 cells were infected with *M. catarrhalis* at 100 MOI. Protein levels were then measured using western blotting (Figure 4 A, 4 B). β-Actin is shown as a loading control. *M. catarrhalis* induces activation of PI3K very early, with phosphorylation of both p85 (Fig 4 A, top band) and
p55 (Fig 4 A, second band from top) occurring after 5 min of stimulation and lasting until 30 min. There is a low level of basal activation, probably due to the many other roles PI3K has in the cell (Hawkins et al., 2006). Phosphorylation of Akt occurs within 5 min of exposure to *M. catarrhalis*, and activation continues for at least 120 min (Figure 4 B).

Figure 4. Involvement of the PI-3 Kinase signaling pathway. A549 monolayers were infected with *M. catarrhalis* at 100 MOI for 0, 5, 10, 15, 30, 60, 90, 120, and 180 min. Then cells were lysed and protein was extracted. The protein was used in a western blot probed with antibodies to phospho-PI3K, PI3K, and β-Actin (A) or to phospho-Akt and Akt (B).
To further test the role of PI3K in A549 response to *M. catarrhalis*, PI3K was inhibited using the small chemical inhibitor LY294002 (Figure 5 A), a selective inhibitor of PI3K (Vlahos et al., 1994). Cells were pretreated with LY294002 for 1 h and then stimulated with *M. catarrhalis* at 100 MOI for 18 h. Supernatants were removed, and secretion of MCP-1 and IL-8 was measured using ELISA (Figure 5 B, 5 C). As expected, in the absence of *M. catarrhalis* there was very little chemokine protein secreted. Also as expected, *M. catarrhalis* increased the levels of both MCP-1 (Figure 5 B) and IL-8 (Figure 5 C) (second bar). PI3K inhibition decreased levels of MCP-1 (Figure 5 B) in a dose-dependent manner (10μM p=.01, 20μM p=.01), suggesting a positive role for PI3K in this pathway. In contrast, when PI3K was inhibited, IL-8 levels increased in a dose-dependent manner (Figure 5 C); (0.1μM p=.02, 1μM p=.02, 5μM p=.001, 10μM p=.003, 20μM p=.0001), suggesting a negative regulatory role for PI3K. In both cases, the regulatory role may be direct or indirect.
Figure 5. Involvement of the PI-3 Kinase signaling pathway. A: A549 monolayers were pretreated for 1 h with a PI3K inhibitor, LY294002, at 0, 0.1, 1, 5, 10, or 20 μM and infected with *M. catarrhalis* at 100 MOI for 18 h then cells were lysed and protein was extracted. The protein was used in a western blot probed with antibodies to phospho-PI3K, and PI3K (A). B and C: Cells were incubated with LY294002 (LY), at concentrations of 0, 0.1, 1, 10, or 20 μM for 1 h prior to infection with *M. catarrhalis* at 100 MOI. After 18 h the supernatant was removed and used in an ELISA. Amounts of MCP-1 (B) and IL-8 (C) protein secreted are shown. Figures show the means of five independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined compared to 0 μM LY/100 MOI *M. cat*, as p≤0.05.
Involvement of PI3K isoforms in MCP-1 and IL-8 expression after *M. catarrhalis* stimulation

Figure 5 suggested PI3K has a positive regulatory role on MCP-1 expression, but a negative regulatory role on IL-8 expression. We postulated that this difference might be at the level of PI3K isoforms. PI3K is divided into three classes. Class IA includes the catalytic subunit isoforms p110α, p110β, and p110δ, and the regulatory subunit isoforms p85α and p85β. Class IB includes a single isoform, p110γ (Wymann and Marone, 2005). Western blotting showed that all of the Class IA isoforms are expressed in A549 cells, and not the Class IB isoform, p110γ (Figure 6). This is consistent with observations that p110γ is mainly found in cells of hematopoietic lineage (Wymann et al., 2003).

![Western blot images showing PI3K isoforms](image)

**Figure 6. PI3K isoforms expressed in A549 cells.** A549 monolayers were stimulated with 100 MOI *M. catarrhalis* for 24 hours or not stimulated (NS). The cells were then lysed and protein was extracted. Equal concentrations of protein were used in a western blot probed with antibodies to PI3K p110α, p110β, p110γ, p110δ, p85α, or p85β. β-actin was used as a loading control.
Each of the isoforms expressed in A549 cells was individually “knocked down” using siRNA. Western blotting confirmed the knock down efficiency with the percent protein remaining (normalized to the siControl non targeting siRNA) indicated below each lane (Figure 7). After stimulation with *M. catarrhalis* the supernatants were removed and ELISA was used to measure levels of secreted MCP-1 and IL-8 (Figure 8). Knockdown of PI3K isoform p110β was associated with a large decrease in MCP-1 protein secretion. Knockdown of PI3K isoforms p110α and p110δ also had similar but lesser effects on MCP-1 secretion (Figure 8, left column). P110δ was the only PI3K isoform knocked down to have any effect on IL-8, indicating p110δ suppresses its expression (Figure 8, right column). Knockdown of PI3K has opposing effects on MCP-1 and IL-8, consistent with the effects of chemical inhibition if PI3K observed in Figure 5. This suggests PI3K has a differential regulatory role, either directly or indirectly, on MCP-1 and IL-8 expression in A549 cells in response to *M. catarrhalis*. 
Figure 7. siRNA knock down of the PI3K isoforms. A549 monolayers were transfected for 48, 72, or 96 h with siRNA targeting each PI3K isoform (siRNA), a nontargeting control (siCon), or mock transfected (Mock). The cells were then lysed and the protein was used in a western blot probed with antibodies to PI3K p110α, p110β, p110δ, p85α, or p85β. β-actin was used as a loading control. Band density was measured and normalized to β-actin. SiRNA knockdown is shown as a percentage of siControl for each transfection time.
Fig 8. Involvement of the PI-3 Kinase isoforms. A549 monolayers were transfected for 72 h with siRNA targeting PI3K p110α, p110β, p110δ, p85α, p85β, or a nontargeting control (siCon), then infected with *M. catarrhalis* at 100 MOI for 6, 12, 18, or 24 hours. The supernatants were used in ELISAs to measure MCP-1 (left) and IL-8 (right) protein secreted. Figures show the means of four independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined as $p \leq 0.05$ compared to siCon for each condition.
PI3K activation is dependent on TLR2, but not TLR4, functionality

Figure 2 showed that A549 cells respond to *M. catarrhalis*, via TLR2, to produce MCP-1 and IL-8. Figures 4 and 5 show that PI3K signaling is involved in the production of MCP-1 and IL-8. Some studies have suggested PI3K may function downstream of TLRs (Arbibe et al., 2000; Kane et al., 1999; Martin et al., 2003). To see if activation of PI3K by *M. catarrhalis* requires TLR, functional antibodies were used to block the receptors (Figure 9). A549 cells were pretreated for 1 h with 10 μg/ml of anti-TLR2 (Figure 9 A) or anti-TLR4 (Figure 9 B) antibody. *M. catarrhalis* was used at 100 MOI to stimulate the cells for 5, 15, 30, 60, 90, or 120 min. Protein was used in a western blot probed for phospho-PI3K, PI3K, and β-actin as a loading control. TLR2 inhibition prevented the normal phosphorylation of PI3K in the presence of *M. catarrhalis* (Figure 9 A). Phosphorylation of PI3K was evident after 90 min, but was greatly delayed relative to uninhibited cells. As seen before (Figure 3), TLR4 inhibition had no effect on PI3K activation (Figure 9 B), though it is not clear whether sufficient CD14 or MD2 are present for TLR4 to be functional. Phosphorylation of PI3K occurred between 5 and 15 min after exposure to *M. catarrhalis*, consistent with the results seen in the absence of blocking antibody (Figure 4 A).
Figure 9. PI3K involvement downstream of TLR. A549 monolayers were pretreated for 1 h with functional antibodies at 10 μg/mL targeting TLR2 (A) or TLR4 (B) and then stimulated with *M. catarrhalis* at 100 MOI for 5, 15, 30, 60, 90, or 120 min. Cells were lysed and the protein used in a western blot probed for phospho-PI3K, PI3K, and β-actin as a loading control.

P38 MAPK signaling is involved in *Moraxella catarrhalis*-induced MCP-1 and IL-8 expression

The p38 MAPK pathway has been implicated in inflammatory signaling in response to some bacteria. N’Guessan showed *M. catarrhalis* could activate p38 in BEAS 2B bronchial epithelial cells but that its inhibition had no effect on PGE₂ release (N’Guessan et al., 2007). To determine if p38 is involved in the response to *M. catarrhalis*, A549 cells were infected with bacteria at 100 MOI for 1, 5, 15, 30, 60, 120,
or 180 min. P38 MAPK was activated in response to *M. catarrhalis*, peaking between 5 and 15 min (Figure 10 A). To see if p38 activation is involved in the production of MCP-1 and IL-8 in response to *M. catarrhalis*, an inhibitor of p38 MAPK, SB203580, was used. SB203580 is a pyridinyl imidazole, selective inhibitor of α and β p38; inhibiting catalytic activity by competitive binding in the ATP pocket (Adcock et al., 2006). A549 cells were pretreated for 1 h with 0.1, 1, 5, 10, or 20 μM SB203580, and then stimulated with *M. catarrhalis* at 100 MOI for 18 h. An ELISA was used to measure MCP-1 (Figure 10 B) or IL-8 (Figure 10 C) protein secreted. As expected, *M. catarrhalis* infection caused increased secretion of both MCP-1 and IL-8. Inhibition of p38 significantly decreased the level of secretion of MCP-1 (Figure 10 B) (5μM p=.05, 10μM p=.002, 20μM p=.0005), but had no statistically significant effect on IL-8 protein levels (Figure 10 C).
Figure 10. Involvement of the p38 MAPK signaling pathway. A: A549 monolayers were infected with *M. catarrhalis* at 100 MOI for 0, 1, 5, 15, 30, 60, 120, and 180 min, then cells were lysed and protein was extracted. The protein was used in a western blot probed with antibodies to phospho-p38 and p38 MAPK (A). B and C: A549 cells were pretreated for 1 h with a p38 inhibitor, SB203580 (SB), at 0, 0.1, 1, 5, 10, or 20 μM and then infected with *M. catarrhalis* at 100 MOI for 18 h, after which the supernatant was removed and used in an ELISA. Amounts of MCP-1 (B) and IL-8 (C) protein secreted are shown. Figures show the means of five independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined compared to 0 μM SB/100 MOI *M. cat*, as p≤0.05.

PI3K involvement in the p38 MAPK pathway

There is some evidence that the negative effects PI3K can have on the inflammatory response may be due to it negatively regulating other pro-inflammatory transcriptional regulators such as p38 MAPK and ERK (Li, 2003; Zimmermann and
Moelling, 1999).

To test whether PI3K may be acting upstream of p38 MAPK, PI3K was inhibited and the effect on p38 activation was examined. A549 cells were pretreated for 1 h with the PI3K inhibitor, LY294002 at 20\(\mu\)M and then stimulated with \textit{M. catarrhalis} at 100 MOI for 5, 15, 30, 60, 90, or 120 min. Western blotting shows an increase of p38 phosphorylation upon inhibition of PI3K (Figure 11). This suggests that PI3K may have a suppressive role on p38 activation. There is no further increase in phosphorylation of p38 seen with addition of \textit{M. catarrhalis} as one would expect, perhaps because the peak level of activation of p38 had already been reached in response to the PI3K inhibitor.

![Figure 11. Dependence of p38 MAPK activation on PI3K.](image)

**Figure 11. Dependence of p38 MAPK activation on PI3K.** A549 cells were pretreated for 1 h with the PI3K inhibitor LY294002 at a concentration of 20\(\mu\)M, and then stimulated with \textit{M. catarrhalis} at 100 MOI for 5, 15, 30, 60, 90, or 120 min. Cells were lysed and protein was used in a western blot probed for phospho-p38, total p38 MAPK, and \(\beta\)-actin as a loading control. The figure is representative of three independent experiments.
ERK1/2 (p44/42 MAPK) signaling is involved in Moraxella catarrhalis-induced MCP-1 and IL-8 expression

The ERK signaling pathway is involved in expression of inflammatory mediators in response to some bacteria. In bronchial epithelial cells, M. catarrhalis induced ERK-dependent activation of COX-2 and PGE2 release (N'Guessan et al., 2007). To determine if ERK is involved in the response to M. catarrhalis, A549 cells were infected with 100 MOI of the bacteria for 1, 5, 15, 30, 60, 120, or 180 min. Activation of ERK is seen in response to M. catarrhalis, peaking at 5 min (Figure 12 A). To see if this activation of ERK may be involved in the induction of MCP-1 and IL-8 expression by M. catarrhalis, the selective inhibitor of MEK1/2 (the kinase that activates ERK), U0126, was used. A549 cells were pretreated for 1 h with 0.1, 1, 5, 10, or 20 μM of U0126 and then stimulated with M. catarrhalis at 100 MOI for 18 h. An ELISA was used to measure MCP-1 (Figure 12 B) or IL-8 (Figure 12 C) protein secreted. M. catarrhalis infection increased secretion of both MCP-1 and IL-8. Inhibition of ERK significantly decreased the level of secretion of both chemokines.
Figure 12. Involvement of the ERK signaling pathway. A: A549 monolayers were infected with *M. catarrhalis* at 100 MOI for 0, 1, 5, 15, 30, 60, 120, and 180 min then cells were lysed and the protein was used in a western blot probed with antibodies to phospho-ERK1/2, ERK, and β-actin as a loading control (A). B and C: A549 cells were pretreated for 1 h with an ERK inhibitor, U0126, at 0, 0.1, 1, 5, 10, or 20 μM, and then infected with *M. catarrhalis* at 100 MOI for 18 h after which the supernatant was analyzed using an ELISA. Amounts of MCP-1 (B) and IL-8 (C) protein secreted are shown. Figures show the means of five independent experiments, and the error bars represent one standard deviation. Statistical significance is represented by an asterisk and was determined compared to 0 μM SB/100 MOI *M. cat*, as p≤0.05.
PI3K involvement in the ERK1/2 (p44/42 MAPK) pathway

To test whether PI3K may be acting upstream of ERK, PI3K was inhibited and the effect on ERK activation was examined. A549 cells were pretreated for 1 h with the PI3K inhibitor, LY294002 at 20μM and then stimulated with 100 MOI *M. catarrhalis* for 5, 15, 30, 60, 90, or 120 min. Western blotting shows no effect on ERK activation (Figure 13). There was no increase in phosphorylation of ERK in the presence of LY294002; in contrast to that seen for p38 (Figure 11). After stimulation with *M. catarrhalis* activation of ERK increased in a manner consistent with activation seen in the absence of LY294002 (Figure 12 A). This suggests that *M. catarrhalis* induced activation of ERK is independent of PI3K, in other words, PI3K is not upstream of ERK.

![Figure 13. Dependence of ERK activation on PI3K. A549 cells were pretreated for 1 h with the PI3K inhibitor LY294002 at a concentration of 20μM, and then stimulated with *M. catarrhalis* at 100 MOI for 5, 15, 30, 60, 90, or 120 min. Cells were lysed and protein was used in a western blot probed for phospho-ERK, total ERK, and β-actin as a loading control. The figure is representative of three independent experiments.](image-url)
DISCUSSION

COPD is the fourth leading cause of death in the United States and is becoming increasingly more common. It affects millions of people and results in billions of dollars spent on health care (Heron and Smith, 2007). Acute exacerbations of COPD negatively affect the quality of life for patients and can lead to death in up to 8-11% of cases (Groenewegen et al., 2003). Current treatments do not prevent exacerbations nor do they stop the disease progression (Barnes, 2008a). The purpose of this study is to elucidate the mechanism by which exacerbations are induced in the hopes that a therapeutic target may be identified.

Exacerbations of COPD yield a state of increased inflammation. Yamamoto et al. showed higher levels of MCP-1 and IL-8 and increased infiltration of macrophages and neutrophils in airway tissues of COPD patients (Yamamoto et al., 1997). *M. catarrhalis* is one of the major causes of acute exacerbations in COPD. We show that *M. catarrhalis* infection of A549 alveolar epithelial cells induces expression of MCP-1 and IL-8 (Figure 14). This suggests that, in situ an infection by *M. catarrhalis* would lead to inflammation and infiltration of macrophages and neutrophils like that seen in COPD. Our data is consistent with previous studies that have shown *M. catarrhalis* induces MCP-1 in AEpC (alveolar epithelial cells) (Rosseau et al., 2005) and IL-8 from A549 cells (Fink et al., 2006).
Figure 14. Summary of A549 response to *M. catarrhalis*. See text for details.

*M. catarrhalis* is a Gram-negative bacterium and so might be expected to signal through TLR4. However, it does not have the typical LPS (the TLR4 agonist) on its surface and instead has LOS that is lacking the repeating O side chains (Fomsgaard et al., 1991). For this reason it might activate TLR2 which recognizes cell wall components such as peptidoglycan and lipoprotein. *H. influenzae* has a similar LOS to *M. catarrhalis* and activates TLR2, and not TLR4 (Li, 2003). When TLR2 activation was inhibited by blocking antibody, the *M. catarrhalis*-induced chemokine expression was diminished.
Inhibition of TLR4 had no effect on MCP-1 or IL-8 expression. This indicates *M. catarrhalis* signals through TLR2 to produce chemokines in A549 cells (Figure 14), though in the absence of CD14 and MD2 it is difficult to interpret the role of TLR4. However, TLR2 is probably not the only receptor involved; when inhibited, chemokine expression dropped, but was not abrogated. The intracellular receptor, Nod1 also has been shown to recognize *M. catarrhalis*. Knock down of Nod1 decreased the secretion of IL-8 by 30-35% from *M. catarrhalis* stimulated A549 cells (Slevogt et al., 2007).

Aside from the classical, well-studied TLR signaling pathway, several other pathways have been implicated in inflammatory signaling, particularly the kinases PI3K, ERK, and p38 MAPK. We decided to examine the PI3K-Akt pathway, the Raf/MEK/ERK pathway, and the p38 MAPK pathways for involvement in *M. catarrhalis* induced inflammation.

The Raf/MEK/ERK signaling pathway positively regulates NF-κB activity (Vanden Berghe et al., 2006). One mechanism for this is the activation of Raf-1 as an IκB kinase (Li and Sedivy, 1993). We found that phosphorylation of ERK occurs in response to *M. catarrhalis* stimulation. Inhibition of ERK diminished the expression of both MCP-1 and IL-8. This shows that *M. catarrhalis* signals through ERK (directly or indirectly) to produce MCP-1 and IL-8 (Figure 14).

P38 MAPK signaling can activate of NF-κB, leading to expression of proinflammatory mediators (Vanden Berghe et al., 2006). We show that *M. catarrhalis* induces activation of p38. The expression of MCP-1, but not IL-8, in response to *M. catarrhalis* is dependent upon p38 phosphorylation (Figure 14).
PI3K/Akt was activated in response to *M. catarrhalis*. When LY294002 inhibition was used, MCP-1 expression decreased, but IL-8 expression increased suggesting PI3K differentially regulates these chemokines (Figure 14).

We hypothesized that the apparent differential regulation of MCP-1 and IL-8 might be at the isoform level. It is useful to know which isoforms are responsible for the effects of PI3K. This would allow a specific isoform to be targeted by a drug and not the entire class of PI3K to limit toxicity. Class I PI3K has four catalytic subunit isoforms, p110α, p110β, p110γ, and p110δ, and two regulatory subunit isoforms, p85α, p85β (Wymann and Marone, 2005). We found p110γ to be the only isoform not expressed in A549 cells. Using siRNA knock down we found that only p110δ has any effect on IL-8, suppressing its expression (Figure 14). P110β was the most responsible for MCP-1 expression, but the other catalytic subunit isoforms had some effect (Figure 14). It should be noted that in some cases inhibition of one isoform is compensated for by the other isoforms and this may mask the effect of individual isoforms.

PI3K can be activated by the TLRs to regulate NF-κB activation (Liew et al., 2005). We found that PI3K activation induced by *M. catarrhalis* occurs downstream of TLR2 (Figure 14). TLR4 is not involved in *M. catarrhalis*-induced PI3K signaling. When TLR2 activation was blocked, PI3K phosphorylation did not occur in response to *M. catarrhalis* stimulation until after a long, 90-120 minute delay. This eventual activation could be due to secondary effects such as autocrine cytokine signaling through a receptor other than TLR2.
We have shown that PI3K can be both a positive and negative regulator of inflammation by its differential regulation of MCP-1 and IL-8. PI3K/Akt signaling is involved in many different cellular functions. It can enhance inflammation or suppress inflammation. The difference seems to depend on cell type and stimulus. Several groups have shown PI3K to have a positive role in inflammation. Duan et al show that PI3K is involved in airway hyperresponsiveness and chemokine production and when PI3K is inhibited the inflammation is suppressed (Duan et al., 2005). PI3K can interact with TLR and lead to activation of NF-κB (Ruse and Knaus, 2006).

PI3K also can have a negative role in inflammation. PI3K was shown to suppress the NO and TNF-α production in macrophages stimulated with LPS. With PI3K inhibition, the macrophages increased NO and TNF-α production (Park et al., 1997). Ward found that PI3K/Akt has a negative regulatory role on inflammatory gene expression in macrophages (Ward and Finan, 2003). PI3K is also involved in TH1/TH2 balance by suppressing IL-12 in dendritic cells (Fukao et al., 2002). Guha showed inhibition of the PI3K/Akt pathway enhances LPS induced gene expression through increased activation of Egr-1, AP-1, and NF-κB (Guha and Mackman, 2002).

This negative role of PI3K/Akt activation may be due to its effects on p38 MAPK. *Haemophilus influenzae* signals through TLR2 to activate NF-κB and proinflammatory mediators. It activates PI3K and this leads to inhibition of p38 MAPK (Li, 2003). Several studies have suggested that PI3K can suppress the MAPK pathways including p38 and ERK (Blum et al., 2001; Ito et al., 2007; Li, 2003; N'Guessan et al., 2007). Like *M. catarrhalis*, *Salmonella* induces activation of PI3K in epithelial cells.
Inhibition of PI3K increases levels of IL-8 (Huang et al., 2005).

We found that activation of p38 MAPK in response to *M. catarrhalis* was enhanced after pretreatment with LY294002, indicating that PI3K has an inhibitory effect on p38 MAPK (Figure 14). However, we found PI3K inhibition had no effect on ERK activation. This supports the findings by others that the PI3K/Akt pathway can modulate the activation of MAPK (Mendez-Samperio et al., 2008).

Slevogt et al. show *M. catarrhalis* can be internalized by epithelial cells using macropinocytosis (Slevogt et al., 2007). Macropinocytosis is a PI3K-dependent process. PI3K controls the contractile mechanism that allows the ruffles to become intracellular organelles (Araki et al., 2003). The PI3K inhibitor, wortmannin, prevents the formation of macropinosome-like structures (Araki et al., 1996). Pretreatment of Beas 2B bronchial epithelial cells with wortmannin significantly inhibits the invasion of the cells by *M. catarrhalis* (Slevogt et al., 2007). This role of PI3K in internalizing *M. catarrhalis* could account for many of the effects inhibiting PI3K has on inflammation. The role of PI3K in inflammation needs further study as much of the literature seems to be conflicting.

So far no therapy has been found that can stop the progression of COPD or suppress the inflammation (Barnes, 2008a). Understanding the mechanism by which *M. catarrhalis* causes inflammation is important. If the mechanism is known then drug targets can be identified to prevent inflammation such as occurs during exacerbations. This study has helped elucidate the signaling pathways involved in *M. catarrhalis*-induced inflammation in the airway epithelium. It is important to consider, however, that there may be a difference in the epithelial cells of COPD patients that would cause them
to react differently to *M. catarrhalis* than healthy epithelial cells. More work is needed to fully understand the path the inflammatory signal is taking from the bacteria to the nucleus and how this results in both pro- and anti-inflammatory effects.
CONCLUSIONS

1. *Moraxella catarrhalis* induces CCL2/MCP-1 and CXCL8/IL-8 expression in human lung epithelial cells.

2. TLR2, but not TLR4, signaling is important for *M. catarrhalis*-induced MCP-1 and IL-8 expression.

3. *M. catarrhalis* induces activation of the PI3K/Akt pathway. PI3K inhibition diminishes MCP-1 expression, but enhances IL-8 expression.

4. The PI3K isoforms p110α, p110β, and p110δ are involved in the positive regulation of MCP-1 expression. The PI3K isoform p110δ is involved in the negative regulation of IL-8 expression.

5. PI3K activation is dependent on TLR2, but not TLR4.

6. *M. catarrhalis* induces activation of the p38 MAPK pathway. P38 inhibition diminishes MCP-1 expression, but has no effect on IL-8 expression. PI3K suppresses p38 MAPK activation.

7. *M. catarrhalis* induces activation of the ERK pathway. ERK inhibition diminishes MCP-1 and IL-8 expression. PI3K has no effect on ERK activation.
REFERENCES


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