Mechanisms of induction of CCL20/MIP3- in lung epithelial cells by Moraxella catarrhalis

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Mechanisms of Induction of CCL20/MIP3-alpha in Lung Epithelial Cells by *Moraxella catarrhalis*

Submitted by: Pablo Serrano

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

**Examination Committee**

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Date of Defense: June 18, 2008
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Dedication

Le dedico este proyecto a mi madre, Carmen Aybar, por su dedicacion y esfuerzo durante su vida.
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# Table of contents

Dedication ii  
Acknowledgements iii  
Table of Contents iv  
Introduction 1  
Background 3  
Material and Methods 31  
Results 42  
Discussion 65  
Conclusion 72  
References 74  
Abstract 93  
Appendices 95
Introduction

The pathophysiology of chronic obstructive pulmonary disease (COPD) is not well understood. Many inflammatory cells are present in the lungs and broncho alveolar lavage (BAL) fluid of patients with COPD including macrophages, neutrophils, natural killer cells and dendritic cells. Until recently, however, no correlation was found between cell counts in BAL fluid and the symptoms and extent of the disease [COPD]. Studies have now shown that a large number of dendritic cells collected from BAL fluid of patients with COPD not only correlate with the severity of disease but they are also present from the beginning of the disease process. The number of dendritic cells in the BAL fluid also correlates with the histopathology obtained from study patients, as well as the cigarette-smoke-mice model of COPD.[1-3]. Dendritic cells are the most important link between innate and adaptive immunity since they are the first cells to encounter, take up and process microbes in order then to present them to T lymphocytes in peripheral lymph nodes and thereby to initiate the adaptive arm of immunity. In the event of inflammation, dendritic cells are chemoattracted to the lung by a specific group of cytokines – chemokines. The most important chemokine responsible for this attraction is CCL20/MIP3-α through its specific receptor CCR6. The level of this cytokine has also been found to be increased in patients and mice with COPD. The source of this highly expressed cytokine has not yet been analyzed. Some propose lung macrophages as the primary source of CCL20/MIP3-α; more likely, however, alveolar and airway epithelial cells are responsible. [4].
The main stimulus for the secretion of these high amounts of CCL20/MIP3-α is also currently being studied. Chronic airway colonization with bacteria is suggested as one of the most important causes of chronic airway stimulation with subsequent secretion of inflammatory cytokines, which eventually lead to COPD. *Moraxella catarrhalis* is one of the most important bacteria to colonize the airway and to produce exacerbations in patients with COPD. [5].

This project proposes that *Moraxella catarrhalis* stimulates lung epithelial cells to express CCL20/MIP3-α. The proposed receptors used by this bacterium to stimulate lung epithelial cells as well as the resulting intracellular signaling pathways are described.
Background

Chronic obstructive pulmonary disease

COPD remains one of the leading causes of death and disability in the United States and the world. It was responsible for approximately 3 million deaths worldwide in 2002, ranking 5th as a cause of death. COPD prevalence is expected to increase as the deaths from tobacco use continue to rise. In the United States, it is the fourth leading cause of death – with 119,000 deaths, 726,000 hospitalizations and 1.5 million hospital emergency department visits annually. In 2004, the cost to the nation for COPD was approximately $37.2 billion, including expenditures of $20.9 billion in direct health care costs, $7.4 billion in indirect morbidity costs and $8.9 billion in indirect mortality costs [6-8]

The etiology of COPD is long-term exposure to toxic gases and particles, where cigarette smoke contributes to approximately 90% of the cases of COPD. The cessation of smoking slows down the progression of the disease only if performed early in the course of the disease. Smoking cessation once the disease is already in the chronic inflammatory phase, however, is ineffective in curbing disease progression due to irreversible changes to the lung anatomy and physiology. [9, 10] Chronic inflammation is the basic pathophysiological principle that affects patients with COPD; it leads to eventual scarring of the lining of the bronchial apparatus and excessive production of mucus from the airway epithelium, which creates an ideal breeding place for bacterial infections within the airways. [11, 12] COPD is characterized by two clinically
distinctive syndromes, with symptoms that may overlap in any given patient: chronic bronchitis and emphysema. Chronic bronchitis refers to a disease process associated with purulent hyper-secretion and small airway obstruction. Histologically it is characterized by infiltration of inflammatory cells, from both the innate and adaptive components of immunity, into the central airway epithelium, which are those airways larger than 4 mm in central diameter. Emphysema refers to the destruction of the bronchiolar and lung tissue beyond the terminal bronchioles without signs of fibrosis, resulting in a decrease of the elastic recoil force and air trapping.[13] The airflow limitation found in COPD is due to chronic obstructive bronchiolitis and the loss of this elastic recoil found in emphysema, which predisposes the alveoli to collapse during exhalation following a reduction in the amount of interstitial and alveolar attachments.[14] Patients with COPD also have structural alterations of the small airways (airway remodeling) as well as systemic inflammation.

COPD consists of four separate anatomic lesions (emphysema, small airway remodeling, pulmonary hypertension, and chronic bronchitis, which includes chronic mucus hypersecretion).[15] Airway remodeling manifests in airway wall fibrosis, squamous metaplasia, hyperplasia and hypertrophy, mucous metaplasia of the epithelium, and smooth muscle hypertrophy. This process occurs mainly in small airways (less than 2 mm in diameter).[14]. The most important component of epithelial remodeling is mucous metaplasia, where mucous is over-excreted in response to inflammatory signals. This process may be due to cigarette smoke exposure, viral and bacterial infections (acute or chronic) or altered mucin gene transcription due to inflammation.[14, 16].
COPD exacerbations are important components of this disease morbidity and mortality.[17] The inflammation seen in these exacerbations are important at both the local and systemic levels, with an increased expression of cytokines in the blood, correlating with the severity of pulmonary symptoms. The rate of concomitant bacterial infection in the lungs of patients with acute exacerbation varies but ranges between 23-57%. It remains, however, unclear when to give antibiotics to treat COPD exacerbations.

A study performed in 1987 [18] suggests that antibiotic therapy is beneficial in all instances; improvements were shown in the time of recovery for normal pulmonary function tests as well as in symptoms for those who took antibiotic therapy as opposed to placebo. The antibiotic therapy should include those that are effective against the three most common bacterial isolates in COPD exacerbations, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Attention to less common forms of bacterial infections (*Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila*) should be considered if improvement is not seen soon after starting treatment. [19-21].

There are a number of proteins and cells that are found to increase in the blood and BAL fluid of patients with COPD. Some of these proteins serve as a marker for the severity of the disease and others represent a prognosis factor of morbidity and mortality. There is no single, best biomarker to identify patients with COPD nor to determine the pathophysiology of the problem, but rather it is the combination of different proteins that underlie the basis for diagnosis and prognosis in different patient subsets. This study refers to CCL20/MIP3-α as one of the most important proteins in the pathogenesis of the disease, although other cytokines and acute phase proteins have also been studied and
therefore deserve explanation. For example, C-reactive protein is an acute phase protein secreted by the liver in response to the primary cytokine burst that follows infection, but it is also seen at elevated levels in chronic disease processes like COPD. It induces pro-inflammatory cytokines and chemokines, and it upregulates C3, the main adhesion molecule of the complement system and the membrane attack complex, C5-C9. [22]. TNF-α is one of the major cytokines induced in COPD; it stimulates inflammatory cells at the site of injury to secrete more cytokines and chemokines that will then continue with the inflammatory process, eventually leading to tissue destruction, such as that seen in emphysema.[23] IL-8 is released by airway and lung epithelial cells, alveolar macrophages and neutrophils. It attracts mainly neutrophils to the site of pathology, and it is associated with the high levels of neutrophils seen in the lung tissue and BAL fluid of patients with acute exacerbations of COPD. [24].

**Innate and adaptive immune response in the lung**

There are two types of immune responses in the human body – the innate and the adaptive immune responses. The innate response to pathogens in the lungs begins with the mucociliary clearance mechanism, which is the epithelial barrier and the inflammatory response associated with macrophages, polymorphonuclear cells (PMN), eosinophils, natural killer cells, monocytes, mast cells and epithelial cells. [12, 25]. These cells are attracted to the site of injury following the activation of pattern recognition receptors in airway and lung epithelial cells and airway macrophages. These
receptors are germline encoded and recognize conserved and invariant features of microorganisms.[26]. Proteins such as defensins, collectin, lisozymes and other antibiotic polypeptides are also very important for the control of pathogen invasion in the lung. They exhibit broad spectrum antimicrobial activity against bacteria, fungi and some viruses. Some of the antimicrobial functions of defensins are attributable to a positively charged cystein rich region of their structure, which damages bacterial cell walls. This destructive characteristic is also shared by some chemokines, including CCL20/MIP3-α.[27, 28]. Finally, cytokines and chemokines orchestrate the host defense by stimulating phagocytes, inducing the production of acute phase proteins by the liver, inducing the hypothalamus to increase body temperature, stimulating the subsequent cells from the adaptive immune response, activating the local endothelium to induce vasodilation and to increase permeability of the blood vessels, thereby allowing proteins and cells to reach the site of inflammation, and ultimately by initiating a repair process in the injured tissue, primarily through the synthesis of collagen by fibroblasts and the stimulation of endothelial cells to initiate angiogenesis. [29]. Some of the most important cytokines in this innate immunity process are TNF-α and IL-1.

The innate immune response lacks memory, is not specific and has limited diversity as opposed to the adaptive response, which is highly specific and has excellent memory.[29]. This latter part of the immune response is directed by T and B lymphocytes, which are primed by antigen presenting cells (APC). The link between the innate and adaptive immunity is to be found on APC, which are capable of internalizing antigens, processing them and presenting them to the cells of the adaptive immunity system, in a similar fashion to T lymphocytes that possess antigen receptors. The genes
encoding these receptors are assembled from gene segments in the germline, a process in which somatic recombination enables the generation of a wide variety of receptors with random, but narrow specificities.[26]. The two types of antigen receptors in the adaptive immunity arm are the T-cell receptors and the B cell receptors. There are two types of αβ T cells: the Th helper cells, which are identified by expressing CD4 co-receptors that recognize antigenic peptides bound to major histocompatibility complex (MHC) class II, and cytotoxic T cells, which express CD8$^+$ molecules that recognize MHC class I. B cells, on the other hand, recognize a great number of antigens by binding to a specific epitope.[26]. Dendritic cells (DCs) are an excellent example of APC; they circulate in the blood and then localize to mucosal surfaces. In the lungs, they are arranged in a complex and extensive network at the base of the epithelium and lamina propria (approximately 400-800 DCs/mm$^2$)[30], where they engulf antigens, become activated, mature and migrate to peripheral lymph nodes where they encounter T lymphocytes. [13, 31]. The T cells, activated by an antigen, migrate from the parafolliular areas of the lymph node to the edge of the follicle where they meet B cells that have been recognized by the same antigen. This encounter between CD4 Th1 cells and B cells initiates B cell activation and proliferation, leading them to the center of the follicle, called the germinal center, where they begin to produce antibodies. A positive selection of those B cells that produce a high affinity antibody capable of binding to the antigen presented by DCs occurs, and such cells become memory B cells or plasma cells. The antibodies IgM and IgG can neutralize microbial toxins and initiate a high quality opsonization and phagocytic killing process. Depending on the stimulus that B cells receive from either Th1 or Th2 cells, the antigen production would lean towards IgE (by IL-4) or IgA (by
TGF-β and IL-5)[32]. The cell-mediated response of adaptive immunity is induced when alveolar macrophages, which have taken up microbes as part of the innate immunity, are activated by IFN-γ, which is secreted by CD4 Th1 and CD8+ lymphocytes that have recognized the antigen presented by macrophages in their cell surface. This activation of macrophages accelerates their intracellular killing capacity. A further component of the cell-mediated immune response includes a subpopulation of CD8+ cytotoxic T cells, which recognize cells that are infected by intracellular organisms such as viruses, and which then express them in their membrane. CD8+ cells then start a cytotoxic process that ends with the apoptosis of the target cell.[13, 33].

**Moraxella catarrhalis**

*Moraxella catarrhalis* is an aerobic Gram negative diplococcus responsible for approximately 20% of all cases of childhood bacterial otitis media and up to 20% of adult lung infections with COPD.[34-37]. This bacterium colonizes approximately 32% of all patients with COPD in lower respiratory airway and it is the third leading cause of exacerbations in this population. Pneumonia caused by this pathogen tends to be mild in healthy adults but can prove devastating in patients with COPD.[38]. There are several important cell wall structures in *Moraxella catarrhalis*: lipoooligosaccharides (LOS), peptidoglycan, outer membrane proteins, pericellular structures and the capsule. The LOS seems to be the most important virulence factor.[38]. The cell wall of a bacterium is the structure that defines the exterior of the
cytoplasm. In Gram negative organisms it is composed of three structures: the cytoplasmic membrane, a thin inner wall composed of peptidoglycan, which is generally 2-3 nanometers thick and contains just a few layers of peptidoglycan (as opposed to Gram positive bacteria where this layer is very thick), and an outer membrane, which is approximately 7 nm thick and is composed of a lipid bilayer of phospholipids, lipoproteins, other proteins, and lipopolysaccharides, with this latter component located in the outermost portion of the outer membrane. Gram positive bacteria have only two layers, the cytoplasmic membrane and a thick layer of peptidoglycan as the outermost component. [39].

*Moraxella catarrhalis* has become an important pathogen in a society for which there exists insufficient research focusing on its genome and vaccine therapy. The lack of a satisfactory animal model makes this research more difficult since it is therefore not possible to determine which protein or antigen is best suited for vaccine therapy. [40, 41]. *Moraxella catarrhalis* has the ability to colonize the airway of patients with COPD, immunocompromised children and the elderly. The significance of this colonization is not well understood, although several studies have suggested the possibility that continuous airway colonization with *Moraxella catarrhalis*, among other bacteria, is the main reason for disease progression in patients with COPD. This airway colonization is associated with higher levels of inflammatory cells and cytokines, as measured in the sputum and the BAL fluid, with a higher number of exacerbations, and a steady decline in lung function. It is thought that bacterial colonization occurs independent of continuing tobacco exposure as a contributing factor for chronic airway inflammation in patients with COPD. It is important to notice that bacterial colonization is also prevalent
among healthy smokers; whether or not this reflects a possible etiologic factor for chronic inflammatory disease of the lung remains to be proven. [5]. Only 33% of patients with COPD are colonized with bacteria at any given point in time. It is thought that this is a cyclic pattern in which all patients with COPD will eventually have bacterial colonization and airway inflammation but only during a specific length of time. The period of time in which the patient with COPD will be colonized by bacteria seems to change depending on the individual immune function of the patient. [42].

**Moraxella catarrhalis, CCL20/MIP3-α and COPD**

The chronic presence of inflammatory cells in the airway and lung parenchyma leads to tissue deterioration from destructive enzymes excreted by neutrophils and macrophages such as elastases, serine and cystein proteases, and metalloproteinases, among others. Chemokines play an important role in this process. Antigens like lipopolysaccharide (LPS) are able to stimulate the secretion of chemokines, leading to the attraction of several different inflammatory cells to the lung. [43-45].

Since COPD is a complex inflammatory disease, there is not a unique cell responsible for the inflammatory process, but rather a number of inflammatory cells that act in an inter-regulated manner to create the environment necessary for disease progression. Many inflammatory cells are overexpressed in the airway and lung parenchyma of patients with COPD, including macrophages, neutrophils, T lymphocytes, B lymphocytes and dendritic cells. Epithelial cells are a very important component of innate immunity since they are
the first ones to encounter the antigens that will eventually lead to the development of adaptive immunity. They secrete a number of inflammatory mediators and proteases that attract other inflammatory cells. They are activated by cigarette smoke, bacterial infection and colonization and produce TNF-α, IL-1β, GM-CSF, and IL8 among other cytokines. They also produce defensins and other cationic peptides that have antimicrobial properties important in innate immunity. These cells are also important in adaptive immunity since they transport immunoglobulin A (IgA) and secrete chemokines that specifically attract DCs, the key cell linking both arms of immunity. They secrete CCL20/MIP3-α in response to cytokines such as TNF-α and in response to particulate matter found in cigarette smoke. [44, 46]. It is expected that these cells would respond to bacterial stimulation (Moraxella catarrhalis infection) and to have similar patterns of chemokine secretion. [47]. There is also an LPS-induced animal model of COPD, although this animal model is more accurate for an acute process rather than a chronic one. Multiple bouts of LPS stimulation are required to obtain a COPD-like histopathology. [15].

Some of the cytokines that are found more frequently in the sputum of patients with COPD are CCL20/MIP3-α. It is the most important chemokine to attract DCs through interaction with its specific receptor CCR6. It is most likely secreted by airway epithelial cells, Langerhans cells and also DCs themselves once they become activated. DCs and CCL20/MIP3a are found at higher levels in patients with COPD, as compared to smokers without COPD and non-smokers. The number of DCs in the airway of patients with COPD increases as the severity of the disease progresses, suggesting an important pathophysiological role of this cell type in COPD. They interact with the major
pathophysiological components of COPD, including chronic inflammation, proteolytic activity and oxidative stress. DCs loose their tolerance as a result of activation by cigarette smoke, producing a chronic inflammatory state in the lung. They also secrete metalloproteinases, for example MMP-12, in conjunction with macrophages, thereby contributing to the tissue destruction found in emphysema. Animal models, using scid-mice that lack functional B and T lymphocytes but have normal components of innate immunity, have shown that chronic exposure to cigarette smoke induces emphysema to the same extent as control mice with normal adaptive immunity. The BAL fluid of these mice was rich in macrophages, neutrophils as well as DCs and cytokines such as CCL20/MIP3-α, suggesting the importance of DCs in the primary development of COPD.

CCR6 knockout (KO) mice exposed to cigarette smoke show a decrease inflammatory profile in the lungs with lower numbers of evidenced DCs, activated CD8$^+$ lymphocytes and neutrophils. With chronic cigarette smoke exposure these mice have partial protection against pulmonary emphysema probably due to a decreased activation of macrophages and DCs with the subsequent decrease in secretion of MMP-12, a decreased number of neutrophils that produce proteinases, and a decreased number of CD8$^+$ lymphocytes T, which induce apoptosis of alveolar epithelial cells. The number of CD8$^+$ T cells in the lungs of patients with COPD does not correlate with the severity of symptoms, therefore not providing evidence for a causal relationship. However, it is thought that these cells do play a role in the pathogenesis of COPD since they have the capacity to cause cytolysis through perforins and granzymes as well as apoptosis via a Fas-Fas ligand interaction or caspase activation.
CCL20/MIP3-α and dendritic cells

CCL20/MIP3a belongs to the family of chemotactic cytokines, which are a group of small (6-14 kDa, 70-90 amino acids), basic, and heparin-binding chemotactic cytokines that contain 4 conserved cysteine residues linked by disulfide bonds. [49]. Chemokines are classified according to the position and the structure of the first two cysteines at the N-terminal region and whether or not they are separated by other amino acids. There are currently four classes of chemokines: CXC or chemokines-α, located in chromosome 4q12-21; CC or chemokines-β, located in chromosome 17q11-22; CX3C or fraktalkine, which is membrane-bound and chemotactic to NK cells and CD8^+ T lymphocytes and C chemokines, located in chromosome 1q33, of which the only one identified so far is lymphotactin, which attracts CD8^+ T lymphocytes. [50, 51]. Even though CCL20/MIP3-α is a CC chemokine, it is not clustered at chromosome 17q11-22, but rather it is located at chromosome 2q33-37. Apparently, this gene was generated before the amplification of the CC chemokines on chromosome 17. [52]

The promoter site for CCL20/MIP3-α contains a NF-κB binding site, which is responsible for the TNF-α dependent induction of cytokines, with additional transcription factors involved: AP-1, AP-2, C-EBP, Sp1 and ESE-1. [50, 53]. CCL20/MIP3-α has only one specific receptor, CCR6, a seven-transmembrane domain G protein coupled receptor, making CCL20/MIP3-α unique among chemokines, as they usually have multiple receptors and each of these receptors is activated by many different
CCL20/MIP3-α is mainly expressed in inflamed epithelial surfaces, such as in the small bowel and the lung. CCL20/CCR6 interaction functions as one of the most potent mechanisms for regulation and recruitment of immature DCs. [2]. The signaling pathways that induce the release of CCL20/MIP3-α in response to cytokines, particulate matter, and bacteria are incompletely described. It is known that CCL20/MIP3-α can be released from the airway epithelium in response to a series of stimuli, including TNF-α, IL-1β, the Th2 cytokines IL-4 and IL-13 through activation of the p38 MAPK pathway. [30].

DCs of the airway epithelium and lung parenchyma are very dynamic populations, which are highly reactive to locally-produced cytokines and at the same time are very distinct from one another. Lung parenchyma dendritic cells, as contrasted with those of the airway epithelium, are a much larger population, which is in direct contact with alveolar and parenchymal tissue macrophages, although these macrophages are not thought to play a major role in the antigen traffic to lymph nodes. [31]

The intraepithelial DC population exhibits the dendritiform morphology of mature interdigitating DCs, and expresses MHC II. Once an inhaled antigen comes into the alveolar space, intraepithelial or alveolar space, semi-mature dendritic cells engulf the antigen and start a maturation process that is dependent on both the stimulation from various cytokines produced locally in the alveolar epithelium as well as by a back-communication between the DCs and memory T cells through CD40. This interaction between memory T cells and the DCs seems to be the rate limiting step for cell maturation in its stimulated expression of CD86, which is located on the plasma membrane of maturing DCs. The alveolar epithelial cells produce cytokines such as...
CCL20/MIP3-α, RANTES and eotaxin that, in the unique case of CCL20/MIP3-α, attract more immature DCs and memory T cells, thereby amplifying the maturation process and enhancing the inflammatory response. These maturing DCs become attracted by different chemokines and migrate to the draining lymphatics, where Th cells bearing IL2-R progress to the lung parenchyma and start the secretion of cytokines, directed by their immunologic memory. The continuous communication between DCs and memory T cells enhances the maturation process. Data available suggest that the interaction between DCs and Th1 cells in lymph nodes could occur as early as 30 minutes to 2 hours after stimulation of the lung epithelium. This interaction may be a result of the interaction between the constitutive DC population of the lung parenchyma with the incoming antigen, and also of the rapid incoming traffic of immature DCs to the site of inflammation, which are both caused by the locally produced chemokines, where CCL20/MIP3-α plays a vital role. [56-60].

**Pathogen recognition receptors as a link between innate and adaptive immunity**

Lung epithelial cells may use a wide variety of receptors in order to initiate the inflammatory response. The receptors that epithelial cells use to recognize bacterial antigens have recently been discovered and analyzed. Toll-like receptors (TLRs) are pathogen-recognition receptors (PRR) that interact with pathogen-associated molecular patterns (PAMP) and that activate a series of intracellular signaling cascades that culminate in the activation of transcription factors, resulting in the expression of
cytokines and chemokines. It has been found that lung epithelial cells express many TLRs, such as TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9, which are highly expressed and functionally active.[61]. They are distinguished from other PRR by their ability to recognize and discriminate between structures that are unique to microorganisms. They are interesting receptors because they can bind to many different organisms and molecules if they have a common structure or pattern (PAMP). The detection of these PAMP by the PRR signals the presence of microorganisms in the host through a process whereby the innate immune system unleashes a series of events, culminating in the destruction of the invading pathogen. [26, 62].

TLRs are a family of trans-membrane receptors that are characterized by having multiple copies of leucine-rich repeats (LRR), which are involved in ligand recognition in the extra-cellular domain and an intra-cytoplasmic Toll/IL-1R (TIR) motif (belonging to the IL-1R family), which then transmits the signal to other intracellular adaptor proteins.[63]. The members of the TIR superfamily are defined by the presence of an intracellular TIR domain and they are divided in two main groups: the immunoglobulin domain group and the leucine rich repeat domain group. This first group includes the IL-1R, IL-18R and “single immunoglobulin interleukin 1 receptor related molecule” (SIGIRR).[64] IL-1R is very important in inflammation physiology as it stimulates the expression of MIP-2, KC and C-reactive protein. IL-18R promotes the differentiation of Th1 cells and the activation of natural killer cells. SIGGIR does not activate NF-κB or interferon regulatory factor 3 (IRF3), but it negatively regulates the TIR signaling. The subgroup of leucine rich domain consists of at least 13 TLRs. [65].
According to previous research, TLRs diverged from a common mammalian ancestral gene. TLR1 and 6 diverged 95 million years ago. TLR4 emerged as a distinct entity 180 million years ago. TLR3, 5, 7 and 8 diverged 150 million years ago. During the early vertebrate evolution there existed very few TLRs. These TLRs originated from a primary prevertebral TLR-like protein, with pro-inflammatory components, approximately 500 million years ago. [66]. The canonical TLR pathway has a much earlier origin than previously assumed as it is present in some of the simplest of true animals including corals and sea anemones, members of the basal phylum Cnidaria.[67].

Toll-like receptors were first described as an important component of the *Drosophila melanogaster* embryogenesis, where they determine the differentiation of dorsal structures from ventral structures, leading to the formation of important elements in the adult fly. These ground-breaking findings lead to the awarding of the 1995 Nobel Prize in Physiology and Medicine to Cristiane Nusslein-Volhard and his research group. [68] Later research, conducted by Jules Hoffman, lead to the recognition of Toll-like Receptors as an important protein in the immune system of the adult fly for fighting fungal infections. [69] In 1997, Charles Janeway and Ruslan Medzhitov identified the first mammalian TLR now called TLR4. [70]. These findings were further investigated by Bruce Beutler [71] at the Scripts Institute in 1998. Beutler used positional cloning to prove that particular mice – those that could not respond to LPS – illustrated mutations that abolished the function of TLR4, thereby demonstrating the role of TLR4 as the LPS receptor in mammals. Beutler’s earlier research in the early 1980’s had also purified, identified and recognized TNF-α as an inflammatory protein. Further research eventually led to the theory that the toxic effects of LPS in the body were caused primarily by the
massive release of TNF-α into the circulation by inflammatory cells like macrophages. [72].

The recognition of LPS is principally mediated by either a membrane-bound or soluble form of CD14 through a serum factor, called LPS binding protein (LBP). [73]. CD14 only has an extra-cellular domain; therefore it is dependent on TLR4 in order to transduce the signal to the intracellular domain. MD-2 acts as an adaptor molecule to specifically activate TLR4. It is after LPS has associated with the LPS binding protein and then formed a ternary complex with CD14 that MD-2 recognizes LPS and subsequently activates TLR4. MD2, but not CD14, is essential for LPS recognition and activation of TLR4, suggesting therefore that there is a CD14 alternate MD2-TLR4 pathway. [74]. TLR2 forms homodimers and physically associates with CD14. It triggers a response that ends in the production of reactive oxygen species and the transcription of several inflammatory cytokines in response to LPS, LAM from mycobacteria, BLP from *Mycobacterium tuberculosis*, cell wall components of gram positive bacteria (peptidoglycan, lipoproteins/lipopeptides), mannans, glycosyl-phosphatidylinositol from *Trypanosoma cruzi* and zymosan, a component of yeast cell walls. [75-79]. It is thought that the intracellular signaling pathways between TLR2 and TLR4 are very similar with the exception of the molecule MD2, which is needed for TLR4, but not TLR2.[80]

Other types of PRR that are present in lung epithelial cells and that may also be important for recognition of Gram negative organisms, such as *Moraxella catarrhalis*, are the nucleotide binding oligomerization domain (NOD) proteins, or NOD like receptors (NLR). [81, 82]. NLR are localized in the cytosol but they are able to recognize intracellular and also extracellular organisms through the intracellular delivery of
mucopeptides by a type III or type IV secretion apparatus. These apparatuses are transport channels that deliver virulence factors into cytosol, although the exact underlying mechanisms remain elusive. [82]. They also contain a region of leucine rich repeats located at the C-terminal region. There are 23 known mammalian NLR identified so far and they are classified according to their effector domain, caspase-recruitment domain (CARD) for NOD, the pyrin domain (PYD) for NALP, and the baculovirus-inhibitor of apoptosis repeats (BIR) for NAIP. NOD1 and NOD2 sense molecules found in bacterial PGN; NOD1 detects PGN containing meso-diaminopimellic acid present mainly in Gram negative bacteria and NOD2 detects the muramyldipeptide MurNac-L-Ala-D-esoGln (MDP-LD) present in Gram negative and Gram positive bacteria. Once NLR are activated, they continue their intracellular signaling through activation and oligomerization of receptor-interacting protein 2 (RIP2), which is independent of TLR signaling and results in the activation of the Iκb kinase complex. They also activate the caspase-1 complex, named inflammasome, in charge of cleaving the inactive precursors of cytokines to covert them in active proteins. [83].

There is also another type of receptor that is activated by double-stranded RNA from viruses – the cytoplasmic retinoic acid inducible (RIG) like receptors (RLR). This receptor type completes the trilogy of PRR. [84].
**Downstream signaling of toll-like receptors**

Once TLRs are activated, they form homodimers or heterodimers, resulting in a conformational change in the cytoplasmic TIR domain and the recruitment of adaptor molecule myeloid differentiation response element 88 (MyD88). It has long been thought that the Toll protein shared the same intracellular signaling pathway as the IL-1R, since both receptors are almost identical in the extracytoplasmic region; therefore it is not considered surprising that TLRs would continue their signal to activate MyD88. [85]. This MyD88 molecule associates with the TLR via a homophilic interaction using the TIR domain. The death domain of MyD88 recruits downstream serine threonine kinases interleukin-1 receptor-associated kinase-4 (IRAK4) to the receptor complex. IRAK4 is autophosphorylated, and then acts to phosphorylate IRAK1. IRAK1 is dissociated from the receptor complex to recruit TNF receptor associated factor 6 (TRAF6). [86]. The dissociated IRAK-TRAF6 complex activates downstream kinases like the mitogen activated protein kinase/ERK kinase kinase 1 (MEKK1) or the transforming growth factor beta activated kinase (TAK1). This latter molecule is then capable of activating the inhibitory-binding protein kB kinase (IKK) complex. [74, 87].

MyD88 is composed of a C-terminal TIR domain, separated from an N-terminal death domain (DD) by a short chain of amino acids without a transmembrane region, suggesting thereby its adapter function in the cytoplasm. This DD has been associated since its discovery with the cytoplasmic tail of TNF receptors, which is required in order to induce apoptosis. The DD of the MyD88 interacts with other adaptor molecules through their respective DD in order to activate MAPK and other transcription factors,
such as NF-kB. [74]. This adaptor molecule was originally identified as one of the myeloid differentiation primary response genes rapidly induced by IL-6 in M1 myeloleukemic cells. Then, MyD88 was shown to be associated with the IL-1R in activating IRAK1, resulting thereby in the activation of transcription factors NF-kB and AP-1. [80]. Two years following its discovery, in 1997, MyD88 was associated with TLR signaling when the researchers Kawai, et al. found that MyD88-deficient mice were unresponsive to endotoxin. [88].

The IRAK family consists of two active kinases – IRAK4 and IRAK – and two kinases that are inactive – IRAK2 and IRAKM. [89]. IRAK, IRAK2 and IRAK4 are ubiquitously expressed in humans, whereas IRAKM is only present in macrophages when the Toll signaling is activated.

TRAF6 belongs to a family of adaptor proteins that share a common structural domain at their C-terminal region. This region allows this family of factors to interact with cell surface receptors or other signaling molecules. TRAF6 was initially discovered as a signal transducer adaptor molecule for IL-1. [90]. This molecule activates not only NF-κB but also other kinases, like JNK and p38. TRAF6 activates IKK through interaction with a separate heterodimeric protein complex, the ubiquitin conjugating enzyme Ubc13 and the Ubc-like protein Uev1A. The polyubiquitinated TRAF6, associated with TAB-2, acts as the ubiquitin E3 ligase to activate TAK1, a member of the MAP kinase kinase kinase family that is associated with TAB-1. The activation of TAK1 leads to the phosphorylation of IKK complex and NF-kB activation but also to the phosphorylation of the p38, JNK and ERK MAPK pathways, which lead to the activation of AP-1. [91, 92].
TLRs can also initiate mitogen activated protein kinase (MAPK) signaling pathways, thereby activating multiple transcription factors, such as AP-1 and Elk-1, involving p38, ERK1/2 and c-Jun NH2 terminal kinase (JNK). [93]. Additionally, there is a pathway that is MyD88-independent and that is used by both TLR3 and TLR4. This pathway leads to the activation of the transcription factor IRF3, which is a member of those IRF transcription factors that play an important role in the immune system. Such transcription factors determine cell growth control and immunomodulation by stimulating the expression of type I IFN, DC maturation, cross-presentation of exogenous antigens for the proliferation of CD8+ T cells, NK cell activation and apoptosis. [94]. IRF3 activates the promoter of interferon β and the chemokine ‘regulated on activation normal T cell expressed and secreted’ (RANTES) when a viral infection is taking place. IRF3 is phosphorylated by serine-threonine kinases TANK-binding kinase 1 (TBK1) and IκB kinase related kinase-ε (IKKe) when TLR3 or TLR4 are activated by double-stranded RNA from viruses or LPS. These kinases are recruited to the N-terminal region of TRIF by NF-κB activating kinase associated protein-1 (NAP1) and lead to the dimerization of IRF3 and its interaction with binding proteins, such as p300. This complex translocates to the nucleus where it associates with IRF promoter sites in the DNA. [65]. The TLR3 pathway also leads to the activation of NF-κB by using the adaptor molecule TRIF and the receptor interacting protein 1 (RIP1), both of which associate with TRAF6-TAK1 and TAB2. RIP1 is a kinase that contains a death domain that associates with TRIF via the RIP homotypic interaction motif (RHIM) domain in the C-terminal region. The TRAF6-TAK1 complex dissociates into the cytosol where TAK1 is phosphorylated and activated, leading then to
the activation of IKK and NF-κB. TRIF is the molecule in which the signal to NF-κB or to IRF3 diverges. (See appendix 1 and 2) [84].

TLR2 is a unique molecule because it has the capacity to bind a wide variety of exogenous ligands, bacterial, viral, mycobacterial and fungal products. TLR2 is expressed in many different cell lines, including immune effector cells, epithelial surfaces, and hemopoietic cells. [95-97]. TLR2 is also unusual because it partners with at least two other types of TLRs to form heterodimers as the functional elements for intracellular signaling. [98]. TLR2-TLR1 ligands include triacyl lipopeptides from bacterial outer membranes and TLR2-TLR6 ligands include diacyl-lipopeptides found in Mycoplasma spp. and zymosan, a cell wall component of fungi. [80, 99]. It has been proposed that Moraxella catarrhalis signals through TLR2 in airway epithelial cells.[81] It could be possible that the LOS of this bacterium interacts preferentially with TLR2, although other cell wall proteins and lipopeptides can not be excluded. It is possible, due to the wide array of pathogen patterns that TLR2 recognizes, that it has different adaptor molecules that would interact with each of the pathogen patterns. For example, CD36 senses microbial diacylglycerides, but not other TLR2/6 ligands. For the most part, TLR2 mediates its signal through adaptor molecule MyD88 and TIRAP (TIR adaptor protein). [87, 100].

TLR4 has two different downstream pathways, MyD88-dependent and MyD88-independent, the latter constituting 75% of the signaling pathways in monocytes. Additionally, MyD88-independent pathways signals through the adapter TRIF, which is the same pathway as TLR3, resulting in the production of IFN-β, IFN-γ inducible protein 10 (one of the CXC chemokines), glucocorticoid-attenuated response gene 16
(GARG16), immune-responsive gene 1 (IRG-1), and other chemokines regulated by IFN-γ synthesis, through a slower and delayed stimulation of NF-κB. [101]. Another important process that results from the activation of the MyD88-independent pathway is the expression of MHC-II and accessory molecules (B7 antigens), on APCs and T-cells, thus priming the adaptive immune response. [98]. The most important TLR4 agonist is LPS; therefore, TLR4 has been called the LPS receptor, even though there are other TLRs that recognize certain types of LPS as well, such as TLR2-recognizing cylindrically-shaped LPS or other atypical LPS from *Legionella*, *Leptospira* and *Porphyromonas*. LPS is a major component of the outer membrane of Gram-negative bacteria; it is not synthesized by Gram positive bacteria. LPS is the most pyrogenic of all antigens found in the membrane of bacteria, including lipopeptides, lipoteichoic acid, and lipoproteins, among others. The lipid A component of LPS is the only toxic substance of this molecule, and its toxicity depends on the quantity and density of secondary acyl chains. LPS is composed of primary and secondary acyl chains, which are linked to each other by a disaccharide phosphate backbone, a ketodeoxyoctulosonic acid and a polysaccharide. [102]. It plays an important role in Gram negative sepsis and it is also one of the major stimuli for reactive airway diseases including asthma and COPD. The transduction of the signal from LPS-MD2-TLR4 to NF-κB involves MyD88, which is highly associated with IRAK and the TIRAP/MyD88 adapter molecule (Mal). [103]. TLR4 has also been linked to many inflammatory diseases, since it is stimulated by a wide variety of ligands, including free fatty acids, heat shock proteins and others. The link between obesity, inflammation and insulin resistance has now been attributed to TLR4, when activated by excessive amounts of free fatty acids. [104].
The negative regulation of the Toll pathway is exemplified by SIGIRR, also known as TIR8. It was first discovered in 1999 by Thomassen et al from the Immunex Corporation. [64]. It was found that this molecule does not have the same function as the other Toll domain containing proteins because SIGIRR inhibits TIR-mediated signaling as opposed to activating it, as found with the other such proteins. This protein forms a complex with the IL-1 receptor, IRAK and TRAF6 after IL-1 stimulation. [65]. IRAKM can also be considered a negative regulator of the Toll signaling, [105] where it prevents the dissociation of the IRAK-IRAK4 complex from MyD88, thereby inhibiting the interaction between TRAF6 and IRAK. [86].

**NF-κB activation**

NF-κB is a ubiquitously expressed molecule that plays a central role in regulating the expression of many genes related to inflammation, such as cytokines, chemokines, adhesion molecules, acute phase proteins and antimicrobial peptides. [106]. It was discovered in 1986, by 1975 Nobel Physiology and Medicine prize winner David Baltimore, via its interaction with the Ig kappa light chain in mature B cells. [107]. The existence of five NF-κB proteins has been found: Rel (c-Rel), RelA (p65), RelB, NFκB1 (p50 and its precursor p105) and NFκB2 (p52 and its precursor p100). They can exist as homo or heterodimers.

The activation of NF-kB begins with the assembly of a high molecular weight protein complex, known as signalosome. This complex is made up of IKK-α and IKK-β,
together with a scaffolding protein named IKK-γ (also known as NEMO). The subsequent phosphorylation of a set of inhibitory binding proteins (IkB) by IKK-α and IKK-β results in the recognition of IkB by the β-transducin repeat, containing protein SKp1/Cullin/F-box ubiquitin ligase complex for further ubiquitination and degradation by the 26S proteasome, then releasing NF-kB, thereby unmasking its nuclear localization signal, which then allows this molecule to translocate into the nucleus in order to initiate gene expression by binding to the promoters of target genes. [62, 65].

Pharmacotherapy in COPD

COPD is a very disabling disease and none of the existing medications have been shown to modify the long-term decline in lung function characteristic of this disease. The goal of pharmacotherapy at this moment is to provide relief of symptoms and to prevent complications secondary to the progression of the disease. [11]. Treatments include bronchodilators, antibiotics, oxygen therapy, and systemic glucocorticoids. [6, 108, 109]. Pneumonia and influenza vaccines are routinely given to patients with COPD in order to prevent de-compensations related to bacterial infections. A vaccine against Moraxella catarrhalis has not yet been developed, although several groups are working together on this due to the alarming consequences of this infection in patients with COPD. It has been shown that major protein targets in *Moraxella catarrhalis* are UspA1, Hag, and UspA2; however, it has also been found that patients with COPD usually become re-
infected by different strains of *Moraxella catarrhalis*, suggesting that the vaccine should contain a mixture of antigens expressed by this un-encapsulated bacterium.[110].

A concept that could be explored further is the development of adjuvants for vaccines. An enhancement of the immune response in a vaccine against *Moraxella catarrhalis* could be achieved using mixtures of bacterial products that stimulate the specific receptors used by these bacteria in the human innate immune system. Some TLR ligands are efficacious as vaccine adjuvants, such as lipid A analogues (TLR4 agonists), which therefore indicates the merits of identifying synthetic TLR agonists.[111, 112].

The understanding of the molecular mechanism of infection, intracellular signaling pathways and ulterior production of cytokines is of great importance in order to understand the pathogenesis of this microbe and its relationship with human disease. It is evident that medications for patients with COPD are lacking and the possibility of finding receptor-specific or protein-specific inhibitors to treat patients with COPD is compelling. Longer lasting treatments afforded by manipulating the innate immune system would provide great advancement. Immunotherapy would result in decreased symptoms, a reduced need for medications, and thereby a better quality of life. It may also prevent the transition from acute to chronic inflammatory disease of the airways.

Another possible therapeutic approach is to target innate immune pathways in the acute setting of infection, blunting the pro-inflammatory cascade by using receptor antagonists based on inhibitory antibodies or antagonistic ligands. [87].

Inflammatory mediators such as TNF-α activate NF-κB and AP-1 transcription factors and enhance the expression of both pro-inflammatory and protective antioxidant genes. [113]. These pro-inflammatory genes, CCL20/MIP3-α for example, propagate the
inflammatory response in a sequence of events that eventually link the innate immune response with the adaptive immune response in an attempt to eliminate the invasive pathogen.

Eritoran (E5564), a synthetic lipid A analogue that has been designed to antagonize the effects of LPS has been found to interact with the MD-2 molecule, inhibiting the dimerization of TLR4. [114]. It is being investigated clinically for the treatment of severe sepsis, septic shock and other endotoxin-mediated diseases such as chronic airway hyper-reactivity in COPD. Eritoran is now passing a phase III double-blind, placebo-controlled clinical trial in patients undergoing cardiac surgery with cardiopulmonary bypass. [115, 116].

Another molecule that has been discovered, TAK-242, has been shown to inhibit LPS-induced intracellular signaling without inhibiting LPS binding to cells. Additionally, TAK-242 is known to strongly suppress TLR-4 mediated cytokine and nitric oxide production from monocytes and macrophages. This molecule is being investigated as part of an animal mouse endotoxin shock model. [117].

It can be seen that COPD is not a curable disease. The symptoms of COPD can be treated and its progression halted but the main pathophysiological state of chronic inflammation will always prevail. By far the most effective way to treat patients with COPD is primary prevention. Since about 90% of the cases of COPD are known to be associated with tobacco exposure, it is intuitive that strategies developed to prevent the use of tobacco would have a major influence on the incidence of COPD. Smoking cessation is the most significant intervention in patients with COPD since it results in a slower lung function decline and improvement in survival rates. [118]. Major public
health organizations in the United States and the world are working to reduce the prevalence of tobacco smoking. Tobacco-control laws consistent with the World Health Organization (WHO) Framework Convention on Tobacco Control would contribute invaluably in the prevention of COPD. In conjunction with national and transnational law, surveys could be conducted in order to detect the most vulnerable groups in their populations, which would thereby allow for the construction of focused research strategies. [119, 120]. The involvement of health care professionals such as medical doctors, nurses, dentists, bio-technicians and pharmacists in tobacco use prevention and cessation is of extreme importance in order to reduce the number of smoking-related diseases. [121]. Some successful examples of health professionals’ involvement in tobacco cessation are the WHO’s no tobacco day (WNTD) 2005 and the Doctors’ Manifesto for Tobacco control 2002.
Materials and methods

Reagents and Antibodies

E. coli (0111:B4) ultra pure LPS (LPS) was purchased from InvivoGen (San Diego, CA). Pam³CSK⁴ synthetic bacterial lipoprotein TLR2-TLR1 ligand was purchased from Invivogen (San Diego, CA). MyD88 rabbit polyclonal antibody against amino acid lysine 119 and TRAF6 (D10): sc-8409 mouse monoclonal antibody against amino acids 1-274 at the N-terminus were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). β-actin antibody was purchased from Sigma (Saint Louis, MO). Mouse anti–goat IgG was purchased from BioRad (Hercules, CA). Goat anti- mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Inhibitors PDTC and MG-132 were purchased from Sigma-Aldrich. TLR2 and TLR4 functional antibodies were purchased from eBioscience (San Diego, CA).

Cell Culture

The human lung epithelial A549 cells (ATCC CCL-185) were maintained in Ham’s F12 (Cellgrow, Mediatech Inc, Hemdon, VA), supplemented with 10% FBS and 1.2% penicillin-streptomycin (Invitrogen, Carlsbad, CA). HEK293 cells, stably transfected with cDNA3, TLR2 and TLR4-MD2-CD14 (ATCC), were maintained in DMEM (Invitrogen) with 10% FBS, 5% penicillin-streptomycin, and 50 μg/mL G418 (Gibco, Carlsbad, CA) for HEK-cDNA3 and HEK-TLR2 cells and with Hygromycin B 200
μg/mL and Blasticidin 10 μg/mL (Invivogen) for HEL-TLR4-MD2-CD14 cells. All cell lines were maintained at 37°C in a humidified incubator with 5% saturated CO₂. Cells were passed every 2 or 3 days by treating them with 0.25% trypsin (Gibco).

To assess the multiplicity of infection (MOI) of Moraxella catarrhalis, a complete cell count of A549 cells in a 24 well plate was performed. Cell counts were 1x10⁵ cells per well and the experiments were performed in 500 µL per well, and therefore the infection dose of 10⁶ cfu·mL⁻¹ of Moraxella catarrhalis corresponded with a MOI of 1.

**Stimulation, RNA extraction and cDNA reverse transcription**

A549 cells were plated at a density of 1x10⁵ per well of a 24 well plate in 1 mL of complete media overnight. After changing to antibiotic, free media cells were stimulated with Moraxella catarrhalis at different concentrations. The concentration of bacteria was measured by counting the amount of colony forming units that can grow at a specific wavelength measurement and adjusting that to the number of cells in the wells. Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Cells were rinsed with 1x PBS to wash excess medium and lysed by adding 0.25 mL of the TRIzol reagent per well. RNA was isolated by chloroform extraction and precipitated using isopropanol and finally washed in ethanol. The RNA was resuspended in 25 µL of RNase-free water (Invitrogen, Carlsbad, CA). Any residual DNA contaminant in the resulting RNA was removed by DNase I treatment using the DNA-free kit (Ambion, Austin, TX). Three µL of 10x DNase buffer and 1 µL of rDNase1 were added to 25 µL of the RNA and incubated for 30 min at 37°C with occasional shaking. After rDNase1 treatment, 3 µL of DNase inactivation reagent was added and the mix was incubated for
another 4 min at room temperature (RT) with frequent vortexing. Finally, it was centrifuged at 13000 rpm for 1.5 min at RT and the supernatant was collected. Total RNA was quantified and its quality was assessed by NanoDrop (ND-100 spectrophotometer) (Thermo Fisher Scientific, Wilmington, DE). Only RNA with an A260/280 ratio ≥ 1.8 was used for reverse transcription.

cDNA was synthesized using 1 µg RNA for cDNA reverse-transcription by using random hexamer primer (RHP) and the Omniscript RT kit (Qiagen, Harts worst, CA). After heating the samples for 5 min at 65°C, cDNA master mix was added until the total equaled 20µL. The master mix consisted of 2 µL of 10 x buffer, 2 µL of dNTPs, 1 µL reverse transcriptase enzyme, 0.25 µL RNASin, and 0.25 µL RHP and the necessary amount of nuclease free water (Ambion, Austin, TX). The mix was then incubated for 1 h at 37°C. Following incubation, the mix was heated at 95°C for 5 min, rapidly cooled on ice for 2 min and centrifuged at 1300 rpm for 1 min at RT. The cDNA was finally diluted with 80 µL of nuclease free water and stored at -20°C for further use.

**Real Time Semi Quantitative Reverse Transcription-PCR (RTSQ-PCR)**

Semi quantitative real-time PCR was carried out in triplicates with 2.5µL cDNA and using SYBR Green 1, (Molecular Probes, Eugene, Oregon) master mix containing hot start Taq (Denville Scientific Inc., Metuchen, NJ) enzyme diluents and dNTP’s (Idaho Technologies, Salt Lake City, Utah) and gene-specific primers (1 µM) in a final volume of 25 µL, and they were analyzed in a 96-well optical reaction plate (Applied Biosystems) in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sequence were as follows: GAPDH forward 5’-GGGAA-
GGTGAAGGTCGGAGT-3’, reverse 5’-TCCACTTTACCAGAGTTAAAGCAG-3’, and CCL20/MIP3α forward 5’-GAGTTTGCTCCTGGCTGCTTTG-3’, reverse: 5’AA GTTGCTTGCTGCTTCTGATTTCG-3’ purchased from Integrated DNA Technology (IDT, Coralville, IA). The amplification was programmed such that the mix was heated to 95°C for 8 min (activating Taq enzyme) for 1 cycle followed by 40 cycles of heating at 95°C for 30 sec, 52°C for 30 sec (annealing), 72°C for 30 sec (extension) and 85°C for 1 min (acquiring fluorescence). The product was finally melted from 60°C to 95°C (dissociation curve) for 1 cycle to obtain the specifically amplified target. The cycle threshold (Ct) value was calculated from amplification plots, and gene expression was normalized using the Ct of the housekeeping gene GAPDH. Relative quantity (fold induction) of the target gene mRNA was then calculated using the comparative Ct method \(2^{-\Delta\Delta Ct}}\).

**Enzyme-Linked Immuno Sorbent Assay (ELISA)**

A549 cells were plated at a density of 2 x 10^5 cells per well per ml in 24 well tissue culture plates and stimulated with 100 MOI of *Moraxella catarrhalis* for 0 (not stimulated control group), 6 and 18 hours. Cell supernatants were collected at respective time points and frozen at -80°C. The day the ELISA (R&D Systems, Minneapolis, MN) was performed, the supernatants were centrifuged at 13000 rpm for 1.5min at 4°C and the supernatant was collected for further analysis for secreted CC20/MIP3α, according to the manufacturer’s instructions. Ninety-six well polystyrene microplates, coated with a monoclonal antibody specific for human CC20/MIP3α, were provided with the kit. Fifty µL of Assay Diluent was mixed with 50 µl of standard, control or sample (supernatant)
per well by gentle tapping and incubated for 2 h at RT. Following incubation, the wells were aspirated, washed and 100 µL of human CC20/MIP3α conjugate (provided with the kit) was added to each well and incubated for another 2 h at RT. Then the wells were again aspirated, washed and 100 µL of CC20/MIP3α substrate solution was added and incubated for 30 min at RT, protected from light. Finally, 100 µL of stop solution (provided with the kit) was added into each well and the optical density of the samples was determined within 30 min using the VersaMax microplate reader (Molecular Devices), set to 450nm. Assay diluents, controls, standards, conjugates, substrates and stop solution were provided with the kit. Sample cytokine concentrations were calculated from the standard curve ($R^2 \geq 0.99$), using the SoftMax Pro 4.8 software. Each sample was run in triplicates and 96 well polystyrene microplates, coated with a monoclonal antibody specific for human CC20/MIP3α, were provided with the kit.

**Fluorescence Microscopy**

A549 cells were co-transfected with the siGLO (stained with Texas red) and the respective siRNA. Following transfection (24 hours later), the cells were visualized under the Olympus IX70 fluorescence microscope (Olympus America, Inc., Melville, NY). After focusing the cells, the picture was acquired at the same spot twice: firstly under Texas red filter and secondly under white light. The two images were then superimposed with the background artificially illuminated with green fluorescence (all the cells) and Texas red (transfected cells got illuminated) and the picture was taken again. Thus the green cells with red spots indicated the transfected cells. The procedure
was repeated for different regions and the transfection efficiency was calculated on the average.

**Western Blotting**

A549 cells were either transfected or left untransfected after having been stimulated with 100 MOI of *Moraxella catarrhalis* for three hours. Following stimulation, culture media was discarded, the cells washed with 1X PBS (Invitrogen, Carlsbad, CA) and then treated with 100 μL of lysis buffer (150 mM NaCl, 5mM EDTA, pH 8, 1% Triton-X100, 10 mM Tris-HCl, pH 7.4, 1 mM sodium orthovanadate and one tablet of protease inhibitor cocktail in 50ml of lysis buffer) for 15 min on ice and then centrifuged at 13000 rpm, 4°C for 15 min. Supernatants were collected and the protein concentrations were determined by Bradford assay using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL) and the Bio-Rad spectrophotometer. 5X SDS buffer (Santa Cruz Biotechnology, CA) was added to the lysate, containing equal amounts of protein and boiled at 95°C for 6 min, placed on ice for 2 min and then centrifuged for 1 min at 13000 rpm. Equal amounts of protein (4μg) were loaded in each lane and separated by SDS-polyacrylamide gel (10%) electrophoresis. Fractionated proteins were blotted onto a nitrocellulose membrane. Blots were probed with specific primary antibodies overnight followed by horseradish peroxidase–labeled respective secondary antibody and visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and the Fluorchem 890 (Alpha Innotech, San Leandro, CA) detection system. To re-confirm an equal loading, the probed membrane was stripped (2% SDS, 62.5 mM Tris-HCl, 100mM 2-ME and pH
adjusted to 6.5) and re-probed with anti-human β-actin monoclonal antibody followed by horseradish peroxidase–labeled goat anti–human IgG (BioRad, Hercules, CA), and visualized as above.

**RNA interference (RNAi)**

The siRNAs specific to MyD88, TRAF-6, TLR2 and TLR4 were purchased from Dharnacon (Lafayette, CO). Each siRNA is essentially a pooled siRNA mixture called siGENOME SMARTpool, which contains four siRNA pre-designed using a SMARTselection™ algorithm to silence the specific target gene. As for negative controls, we utilized non-targeting siRNA, which contains at least 4 mismatches to any human, mouse or rat gene and RISC-free, which is also a non-targeting siRNA with impaired ability for RISC interaction. They were transfected individually or together with DharmaFECT 2 transfection reagent (Dharmacon, Lafayette, CO). Transfection efficiency of the siRNA’s in A549 cells was determined by co-transfecting siGLO control with either of the siRNA (MyD88 or TRAF-6) and then visualizing the effect using fluorescence microscopy. To determine the efficiency of gene silencing, the whole cell lysate of the above co-transfected cells was used for western blotting as described above and probed using specific antibodies. The control groups were co-transfected with siRNA RISC (RNA Induced Silencing Complex) free, non targeting control and mock-non transfected group. β-actin was used as loading control and the protein expressions were also normalized to it to determine the silencing efficiency.
Plasmids

The cDNAs encoding the dominant negative mutants of MyD88 (pcDNA3-MyD88 DN), TRAF-6 (pcDNA3-TRAF-6 DN), IKK-α (pcDNA3- IKK-α DN) and IKK-β (pcDNA3-IKK-α DN) were cloned into the mammalian expression vector pCDNA3. The reporter construct NF-κB-Luc was generated by inserting three copies of the NF-κB binding site from the E-selectin promoter into pGL2 vector upstream of the Firefly luciferase gene (pGL2-κB-Luc). Located downstream from PTAL (TATA-like promoter region from the herpes simplex virus thymidine kinase promoter) is the firefly luciferase (luc) reporter gene. The luciferase assay internal control pRL-null-luc was ordered from Promega (Madison WI).

Transient Transfection

Transient transfection of plasmids was performed using Fugene 6 (Roche Applied Science, Indianapolis, IN). For transfection in 24-well plates, 1x10⁵ RAW 264.7 cells per well were seeded the day before the transfection and maintained in Ham’s F12 containing 10% FBS. On the day of the transfection, for each well, 3 μL of Fugene 6 was used for each 1 μg of plasmid, including the pGL2-NF-κB-luc, along with pRL-null-luc and siRNA specific for MyD88 and TRAF-6. The mixture was then incubated for 20 min at RT. The medium in the 24 well plates was changed and 500 μL of fresh Ham’s F12, with 10% FBS and 20 μL of the transfection mixture, was added into each well. The cells were used for experiments 24 h post-transfection.
For transient transfection of only siRNA into A549 cells, Dharmafect 2 transfection reagent was used. Cells were seeded at a density of $1 \times 10^5$ cells per well in 24 well plates the day before the transfection. On the day of the transfection, 10 µL of 2 mM siRNA was mixed with 22.5 µL of 1x siRNA buffer and 25 µL of DMEM and incubated for 5 min at RT. In another tube 2 µL of Dharmafect 2 was diluted in 48µL of Ham’s F12 and incubated for 5 min at RT. The contents of the two tubes were then mixed gently by slow pipetting and incubated for an additional 30 min at RT. Finally 400 µL of DMEM with 10% FBS was added to it and the whole transfection medium (0.5 mL) was then gently poured over the cells. The cells were utilized for experimentation 48 or 72 h post transfection.

The co-transfection of plasmid ($pGL2-\kappa B$-luc) and siRNA (MyD88 or TRAF-6) was performed using Fugene 6 (Roche Applied Science, Indianapolis, IN). $1 \times 10^5$ A549 cells per well were seeded in 24 well plates the day before the transfection and maintained in Ham’s F12 containing 10% FBS. On the day of the transfection, the siRNA was diluted to 2 µM using 1X siRNA buffer (Dharmacon, Lafayette, CO). For each well, 2 µM siRNA was added to 0.3 µg of $pGL2-\kappa B$-luc and 0.05 µg of $pRL-null$-luc and Ham’s F12 to make a total volume of 50 µL, and the tube was placed at room temperature for 5 min. In another tube, 1 µL Dharmafect Duo was added into 49 µL of Ham’s F12 and incubated for 5 min. After 5 min the contents of above two tubes were mixed gently by carefully pipetting and incubated for another 30 min at room temperature. The mixture was finally diluted with 400 µL of DMEM with 10% FBS and added to each well. 24 h after transfection, the cells were ready for experiments.
**Dual Luciferase Activity Assay**

Luciferase reporter assay was performed using the Dual Luciferase Assay kit from Promega (Madison, WI). Cells were transfected with luciferase reporter plasmids as described above, internal control *pRL-null-luc* and different siRNAs. Forty-eight hours after transfection, cells were stimulated with *Moraxella catarrhalis* for three hours. The cells were washed once with PBS and lysed with 100 μL passive lysis buffer per well (included in the kit). The cells were then placed on a rocker for 20 min at RT. A mix of 50 μL of the lysate and 50 μL of LARII substrate was then utilized to determine the firefly luciferase activity using the Monolight 3010 luminometer (BD Sciences, San Jose, CA). Subsequently, 50 μL Stop and Glo substrate was added into the reaction to determine the internal control renilla luciferase activity. The relative luciferase activity was expressed as a ratio of the firefly luciferase readings to the renilla luciferase readings.

**Flow Cytometry Analysis for the TLR4 and TLR2 Receptor**

Flow cytometry was performed on A549 cells transfected with siRNA specific for TLR2 and TLR4 receptors and on HEK293 cells transfected with TLR2 and TLR4-MD2-CD14, in order to prove the presence of these receptors on the cell membrane. A cell density of 1x10^6 cells per sample was used. The cells were washed once in cold PBS (Invitrogen) and once with FACS buffer. Each sample was resuspended in 2 mL FACS buffer and placed in FACS tubes. The tubes were centrifuged for 6 min at 50g at 4ºC. The buffer was decanted, and 3μg FitC conjugated mouse anti-human TLR4 (Imgenex, San Diego, CA) or mouse anti-human TLR2 (Imgenex) monoclonal antibody was added to the
appropriate samples. The samples were then gently vortexed and placed on ice. After one hour, the cells were washed twice with buffer and resuspended in 1 mL buffer. Each sample was then measured in an EPICS Elite flow cytometer. A sample of A549 cells, HEK293-TLR2, HEK293-TLR4-MD2-CD14 was not incubated with the antibody for the specific protein, but rather with a control mouse IgG 2A isotype antibody (Imgenex).

**Statistical analysis**

Data are shown as mean and the standard error of the mean of at least three independent experiments as indicated. Stimulatory effects of Moraxella catarrhalis and inhibitory effects of siRNA, DN mutant forms, functional antibodies and chemical inhibitors were evaluated using paired t-test.
Results

*Moraxella catarrhalis* induces CCL20/MIP3-α transcription in lung epithelial cells

To study the host-pathogen interaction between *Moraxella catarrhalis* and lung epithelial cells in vitro, A549 cell line was used as the cell culture model. This is a hypotriploid human cell line from the lung carcinoma of a 58 year old male with a modal chromosome number of 12. This cell line resembles the alveolar epithelial cells type II (ATII) of the human lung. They cover only 7% of the alveolar surface but they form 67% of the number of epithelial cells within the alveoli. They are important in the initiation of the innate immune response because of the secretion of surfactant protein-A (SP-A) and SP-D, which bind to pathogens. [122]. They also secrete a number of cytokines that attract inflammatory cells to the lung area in order to initiate the innate component of immunity. [47, 123]. The incubation of A549 cells with *Moraxella catarrhalis* (MOI 0, 0.1, 0.01, 0.001, 1, 10, 50, 100, 150, 200, 250, and 300) produced a dose-dependent increase in CCL20/MIP3-α mRNA transcription. (Fig 1A). A time-dependent increase in CCL20/MIP3-α transcription can also be seen when A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* at 0, 0.5, 1, 3, 6 and 9 hours. (Fig. 1B). The complete time-dose dependent increase in the transcription of CCL20/MIP3-α is shown in Fig 1C. Even by increasing the MOI of bacteria at 6 and 9 hours, the expression of CCL20/MIP3-α can not be observed, indicating the early activation of CCL20/MIP3-α gene after infection with *Moraxella catarrhalis*. TNF-α was used as a positive control for the
expression of CCL20/MIP3-α by A549 cells since CCL20/MIP3-α is known to be activated after stimulation with TNF-α. (Fig 1D). [30, 53]. The expression of CCL20/MIP3-α was also evaluated at the protein level using enzyme linked immunosorbent assay (ELISA) of the supernatant after incubation of A549 cells with Moraxella catarrhalis at three different time points (3, 6 and 18 hours) with 0 and 100 MOI. (Fig. 1E).

**Figure 1A**

A549 cells were incubated with different MOI of Moraxella catarrhalis wild type strain O35E for three hours. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
Figure 1B

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* wild type strain O35E for the indicated time periods. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.

![Graph showing fold induction of CCL20](image)

Figure 1C

A549 cells were incubated with different multiplicity of infection (MOI) of *Moraxella catarrhalis* wild type strain O35E for the indicated time periods. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
Figure 1D

TNF-α 6ng/mL was incubated for 3 hours with A549 cells and used as a positive control. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
**Figure 1E**

A549 cells were incubated with different MOI of *Moraxella catarrhalis* wild type strain O35E for the indicated time periods. CCL20/MIP3-α protein levels were determined by ELISA. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.

*Moraxella catarrhalis* signals mainly through TLR2 not TLR4 in A549 cells

There are multiple pattern recognition receptors that are involved in recognizing Gram negative bacteria, among which are TLR4, TLR2, and other intracellular receptors. [81]. Previous reports suggest the possibility that *Moraxella catarrhalis* signals preferentially through TLR2 in lung epithelial cells, most likely due to the scarcity of TLR4 receptors in its cell membrane. In other epithelial surfaces where TLR4 is predominant, *Moraxella catarrhalis* signals through TLR4. The role of TLR2 and TLR4 in the CCL20/MIP3-α
signaling of *Moraxella catarrhalis* in A549 cells was evaluated using siRNA experiments to decrease the expression of TLR2 and TLR4 protein. Figure 2A shows the transfection efficiency of siRNA by fluorescence microscopy. The transfection of siRNA TLR2 and TLR4 was performed concomitant with the transfection of siRNA GLO, a commercially available product that serves as a control for nuclear transfection in RNA interference. It can be seen that siRNA glow is located inside the nucleus at 48 hours of transfection, indicating that siRNA specific for TLR2 and TLR4 are transfected as well. Figure 2B shows that the expression of TLR2 and TLR4 protein are decreased by siRNA specific for TLR2 and siRNA specific for TLR4 respectively and not siRNA control detected by flow cytometry with specific FITC associated antibodies against TLR2 and TLR4 receptors. The siRNA specific for TLR2 significantly blocked the expression of CCL20/MIP3-α by RT-PCR and the decrease was even more significant when combined with siRNA specific for TLR4. The incubation of siRNA specific for TLR4 alone was not sufficient to decrease the expression of CCL20/MIP3-α in A549 cells. (Fig 2C). A similar experiment was performed using functional antibodies against TLR2 and TLR4. The incubation of TLR2 antibody for one hour prior to stimulation resulted in a significant decrease of the expression of CCL20/MIP3-α, which was even more pronounced when the incubation was performed along with TLR4 functional antibody, although TLR4 functional antibody alone failed to show a decrease in the expression of CCL20/MIP3-α once stimulated with 100 MOI of *Moraxella catarrhalis* for three hours. (Fig. 2D). PAM³CSK⁴, a specific agonist of TLR2, was used as a positive control for the expression of CCL20/MIP3-α by A549 cells and LPS, the specific agonist of TLR4 was used as a control as well. A549 cells respond poorly to LPS, most likely as a result of the
relatively low expression of TLR4 in their cell membrane. (Fig 2E). *Moraxella catarrhalis* is also capable of stimulating TLR4 receptors when they are available. [124]. HEK293 cells stably transfected with TLR4-MD2-CD14 respond to the stimulation of 100 MOI of *Moraxella catarrhalis* by expressing CCL20/MIP3-α. These cells also respond to commercially available LPS and not to TLR2 agonist PAM$^3$CSK$^4$. HEK293 cells transfected with TLR2 also respond to *Moraxella catarrhalis* and specific TLR2 agonist PAM$^3$CSK$^4$ and not to LPS. Flow cytometry analysis of these cells show that they only express one of the two receptors, either TLR2 or TLR4. The difference between HEK293 TLR2 and HEK293 TLR4-MD2-CD14 cell response to *Moraxella catarrhalis* was not statistically significant. As a control, HEK293 cells transfected with cDNA3 (the plasmid vector) were used. HEK293-cDNA3 cells do not respond to any of the previous stimulus used. (Fig. 2F).

**Figure 2A**

A549 cells were co-transfected with the siGLO (stained with Texas red) and siRNA specific for TLR2 and TLR4. Twenty-four hours following transfection, the cells were visualized under fluorescence microscopy and two pictures were taken: one under white light (i), and another with Texas red filter (ii). Another picture was taken with the background artificially illuminated with green fluorescence to denote all the cells (iii) and a further picture superimposing B with C (iv), indicating that the green cells with red spots are the transfected cells.
Figure 2B

Gene silencing efficiency was evaluated using FACS. Cells were incubated with FITC conjugated TLR2, TLR4 or isotype antibody. A549 cells were transfected with siRNA control or siRNA specific for TLR2 (i) and TLR4 (ii) during 72 hours. Figures are representative of three different experiments.
Figure 2C

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. TLR2 and TLR4 knockdown was performed using specific siRNAs. They were incubated with A549 cells for 72 hours prior to stimulation. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
**Figure 2D**

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. TLR2 and TLR4 knockdown was performed using specific functional antibodies against those receptors. They were incubated with A549 cells for 1 hour prior to stimulation. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.

![RT-PCR of the expression of CCL20](image)

**Figure 2E**

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis*, 1 μg/mL of LPS or PAM₃CSK₄ for 3 hours. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared to non-stimulated cells.
HEK293 cells stably transfected with cDNA, TLR2 or TLR4-MD2-CD14 were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. CCL20/MIP3-α mRNA levels were detected using RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
MyD88 is necessary for the expression of CCL20/MIP3-α

The expression of CCL20 is decreased, but not completely abolished after inhibition of adaptor molecule MyD88, which could be due to synchronous activation of a secondary TIR adaptor molecule TRAM and TRIF, important for TLR3 and TLR4 signaling pathway only. The use of another TIR adaptor molecule, TIRAP or MAL, is not a reasonable explanation because the pathway is MyD88 dependent. [80, 125]. MyD88 molecule is important for all TLRs, including TLR2 and TLR4 pathways. The knockdown of this protein was performed using siRNA specific for MyD88. Western Blot of this protein shows the knockdown efficiency of the siRNA, which is almost 100%. (Fig. 3A). The incubation of Moraxella catarrhalis for three hours with A549 cells, which were previously incubated with mock, siRNA control or siRNA MyD88, shows the decrease in the expression of CCL20/MIP3-α when siRNA MyD88 and not siRNA control or mock are present. The measurement of the expression of CCL20/MIP3-α mRNA was performed using RT-PCR. (Fig. 3B). The transfection of dominant negative mutant forms of MyD88 into A549 cells was shown to cause a decrease in the expression of CCL20/MIP3-α mRNA by RT-PCR. (Fig. 3C). This dominant negative mutant form of MyD88 was created by truncating the N-terminal region, making it thereby inefficient to transmit any kind of signal downstream. The importance of MyD88 for NF-κB activity was further evaluated by dual luciferase assay. Cells transfected with siRNA for MyD88 and not for siRNA control showed decreased
activity of NF-κB, suggesting the importance of transcription molecule NF-κB for CCL20/MIP3-α mRNA expression. (Fig. 3D).

**Figure 3A**

Knockdown efficiency of the siRNA MyD88 demonstrated using Western Blot. MyD88 specific siRNA, siRNA control and mock were incubated with A549 cells for 48 hours. Figure is representative of three different experiments.

![Mock si control siRNA MyD88](image)

**Figure 3B**

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. MyD88 knockdown was performed using specific siRNA, which was incubated with A549 cells for 48 hours prior to stimulation. CCL20/MIP3-α mRNA level was determined by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
Figure 3C

MyD88 functional activity was abolished using dominant negative forms of MyD88, transiently transfected into A549 cells. Cells were incubated with the dominant negative form for 24 hours and then incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. CCL20/MIP3-α mRNA level was determined by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
Figure 3D

A549 cells were cotransfected with NF-κB luciferase plasmid, renilla plasmid and siRNA MyD88 or siRNA control. After incubation for 24 hours, cells were stimulated with 100 MOI of Moraxella catarrhalis for 6 hours. NF-κB activity was detected by dual luciferase assay. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
**TRAF 6 is necessary for the expression of CCL20/MIP3-α**

The decrease of the expression of CCL20/MIP3-α by knocking down TRAF6 is more pronounced than the decrease seen by knocking down MyD88 and this difference is statistically significant in each of the three experiments. (Fig 4B-4D). The reason for this finding is not completely understood, since the only possible alternative pathway is the MyD88 independent pathway that is also TRAF6 dependent. However, this pathway is slow reacting and the activation of NF-κB would not be seen at the three hours that the experiment was performed. There is another TIR adaptor molecule – TIRAP – but this molecule is MyD88 dependent as well. (Appendix). The Western Blot of the knockdown efficiency of TRAF6 after incubating A549 cells with siRNA specific for TRAF6 is shown in Fig. 4A. The expression of CCL20/MIP3-α by A549 cells is completely abolished after knocking down TRAF6 with siRNA. (Fig 4B). This effect is also seen
when TRAF6 is overexpressed as a dominant negative mutant form. This result is also dependent on the transfection efficiency of this plasmid. (Fig. 4C). The activation of NF-κB is decreased when siRNA specific for TRAF6 is added to A549 cells incubated with 100 MOI of *Moraxella catarrhalis* for three hours. This effect was not seen when mock or siRNA control was added. (Fig 4D).

**Figure 4A**

Knockdown efficiency of the siRNA MyD88 demonstrated by Western Blot. TRAF6 specific siRNA, mock and siRNA control were incubated with A549 cells for 48 hours. Figure is representative of three different experiments.

![Mock siRNA control siRNA TRAF 6](image)

**Figure 4B**

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. TRAF6 knockdown was performed using specific siRNA, which was incubated with A549 cells for 48 hours prior to stimulation. CCL20/MIP3-α mRNA level was determined by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
Figure 4C

TRAF6 functional activity was abolished using dominant negative mutant forms of TRAF6, transiently transfected into A549 cells. Cells were incubated with the dominant negative mutant form for 24 hours and then incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. CCL20/MIP3-α mRNA level was determined by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.
Figure 4D

A549 cells were cotransfected with NF-κB luciferase plasmid, renila plasmid and siRNA specific for TRAF6 or siRNA control. After incubation for 24 hours, cells were stimulated with 100 MOI of *Moraxella catarrhalis* for 6 hours. NF-κB activity was detected by dual luciferase assay. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate. * = p<0.05 compared with control.

**Activation of NF-kB by dual luciferase assay**

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**NF-κB is necessary for the expression of CCL20/MIP3-α**

The ubiquitously expressed molecule NF-κB, understood to play a central role in regulating the expression of many genes related to inflammation, was shown to be involved in the expression of CCL20/MIP3-α in A549 cells following stimulation with
When A549 cells are incubated with NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC) and MG132 and then stimulated with Moraxella catarrhalis, the mRNA and protein expression of CCL20/MIP3-α is completely blunted. (Fig. 5A and 5B). PDTC is an antioxidant that modulates the activity of redox-sensitive transcription factors, like NF-κB. PDTC inhibits the activation of IκB through oxidative stress, impeding the release of NF-κB to the nucleus. [126]. MG132 is a proteasome inhibitor not specific for NF-κB, which prevents the degradation of IκB, with the 26 proteasome making impossible the release of NF-κB. When dominant negative mutant forms of IKK-α and IKK-β are expressed in A549 cells, the protein IκB can not be phosphorylated in its NH2-regulatory domain, keeping it attached to NF-κB, thereby preventing its release to the nucleus and thus making it inactive. In this experiment, cells incubated with dominant negative mutant forms of IKK-α and IKK-β for 48 hours showed a marked decrease in the expression of CCL20/MIP3-α after stimulation with 100 MOI of Moraxella catarrhalis for three hours. (Fig. 5C).

**Figure 5A**

A549 cells were incubated with 100 MOI of Moraxella catarrhalis for 3 hours. NFκB inhibitors PDTC 50 nM and MG132 1 µM were incubated with A549 cells for 1 hour prior to stimulation. CCL20/MIP3-α mRNA levels were detected by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
Figure 5B

A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* wild type strain O35E for the indicated time periods with NF-κB inhibitors PDTC 50 nM and MG132 1 µM. CCL20/MIP3-α protein levels were determined using ELISA. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
A549 cells were incubated with 100 MOI of *Moraxella catarrhalis* for 3 hours. Dominant negative IKKα and IKKβ were incubated with A549 cells for 48 hours prior to stimulation. CCL20/MIP3-α mRNA levels were detected by RT-PCR. Data presented are the mean and the standard error of the mean of three independent experiments done in triplicate.
RT-PCR of the expression of CCL20 regulated by the IKK complex

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Discussion

Recent studies suggest the role of CCL20/MIP3-α in the pathogenesis of COPD, not only for the initiation of the inflammatory process but also for the maintenance of the chronic inflammation seen in the lungs of patients with COPD. [1, 2]. *Moraxella catarrhalis* is an important pathogen associated with exacerbations in patients with COPD, including chronic bronchitis. [127]. The association between *Moraxella catarrhalis* and CCL20/MIP3-α has not been clarified as of yet. The current study evidences the link and a proposed intracellular signaling mechanism between *Moraxella catarrhalis* stimulation of lung epithelial cells and the expression and secretion of CCL20/MIP3-α. This places *Moraxella catarrhalis* as an indirect cause of the chronic inflammation, remodeling and tissue destruction seen in the progression of COPD. The direct cause may reside in the increased number of DCs present in the airways of patients with COPD, which express CCR6 and are attracted by the high levels of CCL20/MIP3-α in the lungs as expressed by airway epithelial cells. This interaction between CCL20/MIP3-α and CCR6 may provide a possible mechanism for the accumulation of DCs in the lungs of patients with COPD. [16, 128].

In the current study, the incubation of *Moraxella catarrhalis* with lung epithelial cells led to the expression CCL20/MIP3-α mRNA and protein in a time- and dose-dependent manner. These results suggest that CCL20/MIP3-α is a gene that is stimulated very early in the course of infection, thereby implicating a very rapid intracellular pathway involved in this process. The expression of CCL20/MIP3-α mRNA appeared at one hour, peaked
at three hours and decreased thereafter. The longer the cells were incubated with *Moraxella catarrhalis*, the less expression of CCL20/MIP3-α was seen. Those cells that were kept longer (six and nine hours after stimulation) were still viable and showed active signs of mitosis, which implies that the transcription of CCL20/MIP3-α gene is halted at an early stage, preventing the cell from continuing to transcribe this gene. The levels of mRNA in the cell are regulated by the rate at which the mRNA decays, a process called mRNA turnover. [129]. The specific mechanisms that help regulate this mRNA turnover are not completely understood, but it is clear that the presence of regulatory proteins – those that adapt to specific mRNA regions, such as the adenylate and uridylate rich elements (ARE) in the mRNA 3’ untranslated region (UTR) – help to regulate gene expression by increasing mRNA stability or by accelerating its decay. There are other cis-acting elements that modulate transcript stability and that are found in the 5’ untranslated region (5’UTR) and coding region of mRNA. [130]. Other processes that may be implicated in mRNA decay are the following: the deadenylation by decapping [131, 132], the regulation of translation, where inhibition of the initiation process destabilizes mRNA, but the inhibition of translation elongation, for example, with cycloheximide, promotes mRNA stabilization [129], and the non-sense mediated decay, where mRNA with premature stop codons are eliminated before translation [133]. Other theories suggest that the type of stimulus to the receptor determines the type of gene that is going to be transcribed: early (3 hours) versus late gene (6-24 hours). There are two different types of NF-κB activation patterns: the monophasic and the oscillatory patterns. [134]. In the monophasic mode, the NF-κB activation results from a brief stimulation of the cell membrane receptor; NF-κB enters the nucleus and induces IκB,
whose translation results in a redistribution of NF-κB back into the cytoplasm. On the other hand, NF-κB activation by the oscillatory manner results from tonic stimulation of a cell membrane receptor, producing continuous IKK activation, IκB degradation and multiple rounds of NF-κB translocation and recapture. This mode of activation induces the so called “late genes” that may peak 6 hours after stimulation. In the experiments performed in this study, the bacterium was incubated the whole time with the cells, suggesting a tonic activation of the receptors. The dynamics might be different between TLRs and TNF-α receptors, where the initial NF-κB activation modes were originally described and a process of tolerance might take place. Even though the mRNA peaked at three hours and then decayed, the protein level of CCL20/MIP3-α appeared at three hours, peaked at eighteen hours after stimulation and then remained at the same levels thereafter. As opposed to mRNA levels, protein levels once secreted can not be controlled by the cell. Since CCL20/MIP3-α is an early gene, the mRNA expression shots down shortly after stimulation (a period that may take three to six hours), but the already transcribed protein remains in the supernatant where it was then measured. LPS is a glycolipid present in the membrane of Gram negative bacteria. Lipid A is the most important component of LPS and is responsible for the inflammatory response when it interacts with pattern recognition receptors. [135]. Certain epithelial surfaces have been reported to respond to stimulation by LPS, including some reports that A549 cells respond at some level to extremely high doses of LPS. [136, 137]. However, a large number of studies, including the current, LPS is not able to stimulate A549 cells to express CCL20, nor other inflammatory cytokines like TNF-α and IL-8 (data not shown). This lack of effect is primarily due to the scarcity of TLR4 receptors, and not due to a
lack of CD14, as A549 cells express CD14 in the cell membrane. CD14 is the specific receptor of LPS that lacks a transmembrane domain, and therefore it adapts to TLR4 in order to continue with the intracellular signaling. [138, 139]. Flow cytometry analysis performed on A549 cells show that a relatively low number of cells express TLR4, but since even this small amount of TLR4 receptors could initiate a signaling cascade that would eventually lead to expression of inflammatory cytokines, this study took into consideration the possibility that a synergistic effect may exist between TLR2 and TLR4, producing the observed inflammatory response. At least in A549 cells, Moraxella catarrhalis interacts with TLR2 receptors to express CCL20/MIP3-α, although TLR4 may also be important to a lesser degree in this process. When TLR2 was blocked, either by the use of siRNA or with functional antibodies, there was decreased expression of CCL20/MIP3-α, although it was not completely abrogated. However, when both TLR2 and TLR4 were blocked at the same time and A549 cells were stimulated with Moraxella catarrhalis, the expression of CCL20/MIP3-α was completely blunted, reflecting a possible involvement, although small of TLR4 receptor in this signaling pathway. Moraxella catarrhalis is capable of interacting and signaling through at least these two receptors, TLR2 and TLR4, if they are available in large amounts in the cell surface. This capability was demonstrated when HEK293 cells transfected with TLR2 and TLR4-MD2-CD14 were shown to express CCL20/MIP3-α when exposed to Moraxella catarrhalis. A yet greater response was evidenced in HEK 293 cells transfected with TLR2, although the difference was not statistically significant from those HEK293 cells transfected with TLR4-MD2-CD14, besides these two cell lines should not be compared since it is not known the extent of receptor expression.
What component of the bacterium is actually stimulating the TLR2 is not known. It is clear that TLR2 can respond to bacterial peptidoglycan [140], lipotheicoic acid [141] and certain bacterial lipopeptides [142], all commonly found in the cell wall of Gram negative bacteria. The possibility of recognition of the *Moraxella catarrhalis* LOS molecule by TLR2 is a valid explanation. Multiple recent articles have described that certain non-enterobacterial organisms signal through a TLR4 independent mechanism involving TLR2. Among these bacteria are: *Porphyromonas gingivalis*, *Leptospira interrogans*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Chlamidia trachomatis* and *Bacteroides fragilis*. [143-146]. The toxic effects of an LPS that signals through TLR2 is much less potent than those that signal through TLR4, as measured by TNF-α expression. This lowered toxicity could prove advantageous to the pathogen as an extreme inflammatory response is not produced, thereby improving its chances of survival in a living host. One of the proposed reasons for this decreased activation of the immune system as a result of TLR2 is the lack of induction of IL-12 and IFN-γ in this pathway. Most of the signaling from Gram positive bacteria originates from stimulation of TLR2/TLR6 by the lipoteichoic acid (LTA) and the peptidoglycan (PGN). [147]. Previous research has shown that TLR4 signals through lipid A molecules that have a biphosphorylated-hexa-acyl 12-16 carbons architecture while TLR2 appears to recognize a larger number of lipid A moieties, such as the biphospho-pentaacyl format and even biphospho-hexaacyl formats with longer carbon chains (13 to 28). [143]. Further analysis of the chemical structure of the lipid A from *Moraxella catarrhalis* and the TLR2 and TLR4 response need to be performed in order to determine whether this bacterium LOS preferentially uses TLR2, or whether it is rather that, in the absence of TLR4 receptors, it
signals primarily with the lipopeptides, lipoproteins and lipotheicoic acid found in the cell membrane.

Toll-like receptors recruit five different Toll-IL1 receptor (TIR) domain adaptors in order to continue with their signals: MyD88; TIR domain adaptor protein (TIRAP), also known as MyD88 adaptor-like (MAL), which mediates the activation of the MyD88 dependent pathway associated with TLR2 and TLR4; TIR domain containing adaptor inducing interferon β (TRIF), or TIR containing adaptor molecule -1 (TICAM1); TRIF-related adapter molecule (TRAM), or TICAM2; and SAM- and ARM containing protein. [80, 148-151]. It is very likely that the signal transduction from *Moraxella catarrhalis* -TLR2 to CCL20/MIP3-α expression goes through MyD88. There is only one pathway independent of MyD88, which is the TLR4-TRAM-TRIF pathway. Since TLR4 contribution to the signaling of *Moraxella catarrhalis* in A549 cells is insignificant, it was expected that the expression of CCL20/MIP3-α would prove to proceed uniquely through MyD88 dependent pathways. The findings suggest, however, that this MyD88 independent pathway (dependent on TRAF6) might be important for CCL20/MIP3-α expression after *Moraxella catarrhalis* stimulation of A549 cells. Yet, in order to prove this hypothesis, further experiments need to be performed. This hypothesis is not consistent with the findings that CCL20/MIP3-α is an early gene since the MyD88 independent pathway usually stimulates genes through a delayed NF-κB activation pattern. When TRAF6 was blocked, the expression of CCL20/MIP3-α was decreased to almost negligible level, likely due to the fact that this molecule is involved in all pathways associated with TLRs, even those that are MyD88 independent. Other kinases that may be involved in the activation of A549 cells by this bacterium include PI3 kinase,
MAPKs extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The decrease in the expression of CCL20/MIP3-α when blocking either MyD88 or TRAF6 was not complete, leaving open this possibility. In addition, it is known that *Moraxella catarrhalis* is capable of stimulating these kinases in lung epithelial cells; whether these are important for CCL20/MIP3-α expression is not known. [47, 152]. Many studies published in the literature suggest the importance of NF-κB for CCL20/MIP3-α signaling. [153, 154]. They also suggest that NF-κB is not the only transcription factor that may be involved in this pathway, proposing AP-1 as an alternate possibility, depending on the type of stimulus. [155]. It is interesting that CCL20/MIP3-α is involved in many different inflammatory pathways in human pathology, involving colon cancer, pancreatic cancer, chronic gastritis, asthma, and inflammatory bowel disease, among others. [156-160]. The involvement of transcription factor NF-κB was initially evident when the dual luciferase NF-κB assay was performed. In this experiment, the activity of NF-κB was determined by the measurement of the luminescence of the luciferase with the transfection efficiency controlled with the renilla activity. When knocking down MyD88 and TRAF6, the activity of NF-κB is decreased at 6 hours – more so with TRAF6 than with MyD88 – and yet they are both decreased. Experiments using commercially available inhibitors of NF-κB and laboratory created dominant negative mutant forms of IKK-α and IKK-β further demonstrated the role of this transcription factor in *Moraxella catarrhalis* activation of lung epithelial cells, leaving very little importance to the possibility of involvement by other transcription factors, such as AP-1, at least as concerns the early response phase.
Conclusion

The current study provides novel information on the molecular pathways that are used by *Moraxella catarrhalis* in lung epithelial cells in order to activate them and thereby to secrete CCL20/MIP3-α. These results support the body of literature that suggests an important role of this cytokine in the pathogenesis of COPD. This study proposes that chronic colonization with *Moraxella catarrhalis* stimulates cells to produce large amounts of CCL20/MIP3-α, that then result in an unbalanced inflammatory process contributing to the chronic inflammation found in patients with COPD as well as chronic bronchitis. No existing research has suggested the possibility that *Moraxella catarrhalis* colonization is a major cause of chronic inflammation in COPD, despite the fact that many research articles have suggested the possibility that CCL20/MIP3-α is a major culprit in this process. The association between *Moraxella catarrhalis* and CCL20/MIP3-α provides a new insight into the pathogenesis of COPD. Studies with animal models and experiments with humans are required in order to further evaluate this relationship. A major drawback of the current study is the lack of an animal model for the infection with *Moraxella catarrhalis*; although it has been shown before that there is a significant correlation between in-vitro experiments with lung epithelial cells and in-vivo findings.

This study shows that *Moraxella catarrhalis* stimulates lung epithelial cells primarily through TLR2, whereas TLR4 might also be important. Following this stimulation, the signal initiated by TLR2 continues through a MyD88-TRAF6 dependent pathway, finally activating transcription factor NF-κB. The activation of other intracellular receptors,
such as NOD1, can not be excluded with this research. The evidence of the involvement of these particular pathways opens a window for future pharmacologic research towards novel therapies for COPD. The uncontrolled activation of inflammatory adaptor molecules inside the cells are the most likely culprits of the chronic inflammation found in patients with COPD. Further research, directed towards targeted therapy to block specific intracellular signaling pathways (e.g.: CCL20/CCR6 axis), may therefore prove useful in the treatment of chronic lung disease.
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Abstract

The chemokine CCL20 is crucial for the development of the inflammatory response in the disease COPD. The alveolar epithelium is an important source of CCL20 after stimulation from particulate matter and bacteria. This process contributes to lymphocyte and dendritic cell activation, which is the link between innate and adaptive immunity, characteristic of chronic inflammation and chronic lung disease. *Moraxella catarrhalis* causes approximately 20% of COPD exacerbations; however, the cellular sources of the inflammatory mediators and the pathways by which it induces production of these mediators are as of yet unknown. This project examines the cytokine-inducing capacity of a wild-type *Moraxella catarrhalis* strain using the A549 lung epithelial cell line. *Moraxella catarrhalis* induces CCL20 expression at both mRNA and protein levels in A549 cells in a dose and time dependent manner as detected using RT-PCR and ELISA. TLR2 is the most important receptor for *Moraxella catarrhalis* in A549 cells, and the TLR4 receptor plays a minor role in this recognition. This was demonstrated by TLR2- and TLR4-specific RNA interference and functional antibodies. The finding that A549 cells express few TLR4 receptors in their cell membrane and that HEK293 cells transfected with TLR2 or TLR4-MD2-CD14 respond equally to stimulation with *Moraxella catarrhalis* suggests that *Moraxella catarrhalis* can signal equally through TLR2 or TLR4, when available. To elucidate the intracellular signaling pathways that are activated after infection with *Moraxella catarrhalis*, dominant negative and siRNA specific forms of MyD88 and TRAF6 were used, showing a significant decrease in the expression of CCL20 by RT-PCR. Dual luciferase assays show increase activity of NF-
κB luciferase when A549 cells were incubated with Moraxella catarrhalis, suggesting an important role of NF-κB in CCL20 expression. Moreover, the incubation of siRNA specific for MyD88 and TRAF6 in A549 cells decreased the activity of NF-κB luciferase, confirming the importance of these two adaptor molecules in NF-κB activation. We further investigated the role of NFκB using dominant negative mutant forms of IKK-α and IKK-β, which showed a dramatic decrease in the expression of CCL20. In addition, the use of NFκB inhibitors MG132 and PDTC showed a substantial decrease in CCL20 mRNA and protein level expression by RT-PCR and ELISA. Conclusions: Moraxella catarrhalis induces CCL20 expression primarily through TLR2 receptors, with a small role for TLR4 receptors. This is a MyD88-, TRAF6- and NF-κB-dependent pathway. We demonstrated the close relationship between innate and adaptive immunity and its importance for the development of chronic inflammation in the lung. Further research, directed towards targeted therapy to block specific intracellular signaling pathways (e.g.: CCL20/CCR6 axis), may therefore prove useful in the treatment of chronic lung disease.
Appendices

Apexix 1. TLR signal transduction pathways. TLR2 and TLR4 signal through MyD88-dependent pathway associated with TIRAP to activate TRAF6. The polyubiquitination of this molecule leads to TAK1 activation with subsequent phosphorylation of IKK-α and IKK-β. Once IκB is phosphorylated and degraded, NF-κB is free to proceed to the nucleus and activate the transcription of inflammatory cytokines.

\[ \text{TLR2} \quad \text{TLR4} \]

\[ \text{MyD88} \quad \text{TIRAP} \]

\[ \text{IRAK} \quad \text{TRAF6} \]

\[ \text{IKK} \alpha/\beta \]

\[ \text{MAPK} \]

\[ \text{IκB} \]

\[ \text{NF-κB} \]

\[ \text{CCL20} \]
Appendix 2. TLR4 and TLR3 signal through a MyD88 independent pathway. TLR4, as shown before, also signals through a MyD88 dependent pathway. They can induce IFN-β through the TRIF-IRF3 pathway that involves the IRF3 activating kinase TBK1 (TANK binding kinase1). The receptor interacting protein 1 (RIP1) is a crucial signaling molecule for TRIF mediated NF-κB activation.