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Molecular Mechanism of Cytokine Production by Human Lung Fibroblast in Response to Pseudomonas Aeruginosa Elastase

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MOLECULAR MECHANISM OF CYTOKINE PRODUCTION BY HUMAN LUNG FIBROBLAST IN RESPONSE TO *PSEUDOMONAS AERUGINOSA* ELASTASE

by

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MOLECULAR MECHANISMS OF CYTOKINE PRODUCTION BY HUMAN LUNG FIBROBLASTS IN RESPONSE TO *PSEUDOMONAS AERUGINOSA* ELASTASE

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Pathogenicity of *Pseudomonas aeruginosa* infections can be heavily influenced by the host inflammatory responses. *P. aeruginosa* secretes several extracellular products such as lipopolysaccharide, exotoxin A, and elastase. This bacterium is widely studied in acute and chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and Cystic Fibrosis (CF). In order to understand part of the underlying mechanism, we focused on the role of *Pseudomonas aeruginosa* elastase (PE) in provoking the host inflammatory response in an *in vitro* model. We hypothesized that PE stimulates cytokine and chemokine production by activating mitogen activated protein kinase (MAPK) cascade through nuclear factor kappa B (NFkB). We used biochemical, as well as

immunological techniques, to confirm or reject our hypothesis. We found that PE provokes the fibroblast through the epidermal growth factor receptor (EGFR) which leads to the activation of the extracellular signal-regulated kinase (ERK) arm of the MAPK and translocation of NFkB to the nucleus. Our data indicate that PE stimulates gene and protein expression of the proinflammatory cytokine interleukin 8 (IL-8) through the MAPK pathway. Furthermore, the use of specific inhibitors of EGFR, MEK, and NFkB, AG 1478, U0126, and BAY 11-7085 respectively, confirmed the hypothesis. The study confirms the importance of PE in *P. aeruginosa* induced infection and inflammation in the human lungs.

Chapter One
Introduction

Overview of the Project

Microbial infections are prominent throughout the world and demand continuous investigation into the pathology of bacterial, viral, fungal and parasitic infections. Pathogens can have a significant impact on the lifespan of humans by invading and potentially destroying healthy organ systems (Li et al., 2012). Therefore, the number of infections must be limited and therapeutic modality must be improved to decrease infection related mortality and morbidity worldwide. A particular case of interest in regards to pulmonary infections is Cystic Fibrosis (CF), where detrimental effects occur from not only a genetic defect, but with recurrent microbial infections (Ratjen et al., 2009). Unfortunately, the most common cause of death in CF population is respiratory failure as a result of recurrent pulmonary infection and inflammation (Scheid et al., 2001; Hogardt & Hessemann, 2013). Similarly, acute nosocomial, as well as community pneumonias, among immunocompromised population are challenging health issues (Camps Serra et al., 2008).

Cystic Fibrosis is the most common fatal autosomal, recessive disease in the United States (Eidelman et al., 2001; Ren, L., 2008). Mutation in cystic fibrosis transmembrane conductance regulator gene (CFTR) and defective CFTR ion channel are the culprit gene and protein in CF disorder. The mutation causes an abnormal

physiological environment including thick mucus and low level of extracellular glutathione (Griese et al., 2004). These change the molecular pathology of the cells which leads to a greater sensitivity to reactive oxygen species (ROS) and increased proinflammatory chemokines and cytokines including interleukin (IL) 8 because of activation of signal transduction pathways such as mitogen activated protein kinase (MAPK) (Pongnimitprasert et al., 2008). At the organismal level, these changes compromise the ability of an individual to clear pulmonary infections among other health issues (Roussel et al., 2011; Scheid et al., 2001). Establishment of a proinflammatory environment as a possible sequelae of CFTR defect or response to microbial infections, is suggested to play a significant role in further injury and remodeling of the lungs (Dalton et al., 2012). The combination of compromised nutrient absorption and pulmonary functions due to recurrent infections and inflammation increases the morbidity and mortality in the CF population (Eidelman et al., 2001; Azghani et al., 2002).

The lung environment requires persistent flora flow just like any other system of the human body. Normal fluid movement, cytokines and chemokines secretion and ciliary motion are necessary components to maintain homeostasis in the lungs (Livraghi & Randell, 2007). Mutation in CFTR, changes in mucus composition and the defect in epithelial ciliary function creates a niche for opportunistic microbes such as *Staphylococcus aureus, Hemophilus influenza,* and *Pseudomonas aeruginosa* in the lungs (Govan & Nelson, 1992).

The purpose of this study was to investigate the role of elastase from *Pseudomonas aeruginosa*. In the pathogenesis of Cystic Fibrosis, we studied the role of bacterial elastase in inflammatory response of the structural cells of the lungs.

Pseudomonas aeruginosa

Pseudomonas aeruginosa (PA), a Gram-negative bacillus, is an opportunistic pathogen that causes necrotizing inflammation and systemic infections in immunocompromised individuals with high mortality (Galle et al., 2008). The organism causes acute nosocomial pneumonia as well as chronic pulmonary infection in CF. The primary target of PA is immunocompromised patients, including individuals affected by chronic obstructive pulmonary disease (COPD) and CF (Azghani et al., 2002; Qin et al., 2012). Several antibiotics including macrolides and beta lactams are available to treat pseudomonal infections effectively, but multidrug resistant PA in the CF community as well as in a hospital setting is on the rise (Smith et al., 2001; Alvarez et al., 2010).

Most of the chronic PA infections are biofilm associated which leads to interrogation of the affected tissue from the numerous counts of PA. Biofilms are structured communities of bacterial cells enclosed in an extracellular polymeric matrix consisting of secreted proteins, exopolysaccarides and nucleic acids, whichcan adhere both to abiotic and biotic surfaces, serving as a permanent source of infections (Lima et al., 2011). These modes of growth by pathogenic bacteria allow microbes to proliferate even in the most hostile environments, including anaerobic environments and in CF patients' lungs where the supply of oxygen is lower (King et al., 2010). The biofilm community causes chronic endobronchiolitis, is resistant to the innate immune defense, and resists antibiotic therapy as well (Hoiby et al., 2001). *P. aeruginosa* is considered as a well-rounded organism that thrives in a hostile environment, uncommon to some typical bacteria that provoke human immune responses (Costerton et al., 1999).

In addition to exploiting factors that limit the efficacy of the host immune responses, PA has developed several mechanisms to escape the host innate and adaptive immune responses. The formation of impenetrable biofilms, the intrinsic ability to develop resistance to antibiotics, and the release of a large number of virulence factors (e.g. lipopolysaccharide (LPS), exotoxins, and proteases) are included in PA arsenals (Delden & Iglewski, 1998; Clark et al., 2011). An understanding of cellular signaling events of the host in response to bacterial metabolites may have a profound effect in discovering novel therapeutic modality.

The infection process for PA is similar to many other host-pathogen interaction. The intrinsic bacterium first colonizes disrupted epithelium of the host organ system, such as the lung tissue of a CF individual. After bacterial colonization and failure of the innate immune system to overcome the pathogen, an acute infection arises (Delden & Iglewski, 1998). In the acute phase, PA first colonizes the tissue at the beginning of the invasion, where biofilms could be seen as well. The secretion of extracellular virulence factors by the bacterium in the infection scenario causes extensive tissue damage, bloodstream invasion, and bacterial dissemination to distant tissues (Delden & Iglewski, 1998; Pressler et al., 2011). *P. aeruginosa* initiates the onset of the not only acute infection, but the recurrent chronic infection seen in CF patients because current therapy is unable to eradicate PA infection (Qin et al., 2012).

As stated earlier and depicted in Figure 1, *P. aeruginosa* strains produce several extracellular toxins and proteases that contribute towards its virulence (Wretlind & Paviovskis, 1983). Lipopolysaccharide is one of the strongest virulence factors, which induces the expression of certain chemoattractants (including IL-8), by immune cells,

epithelial, and possibly some interstitial cells, therefore leading to neutrophils recruitment to the site of infection (Figure 1; Kon et al., 1999).

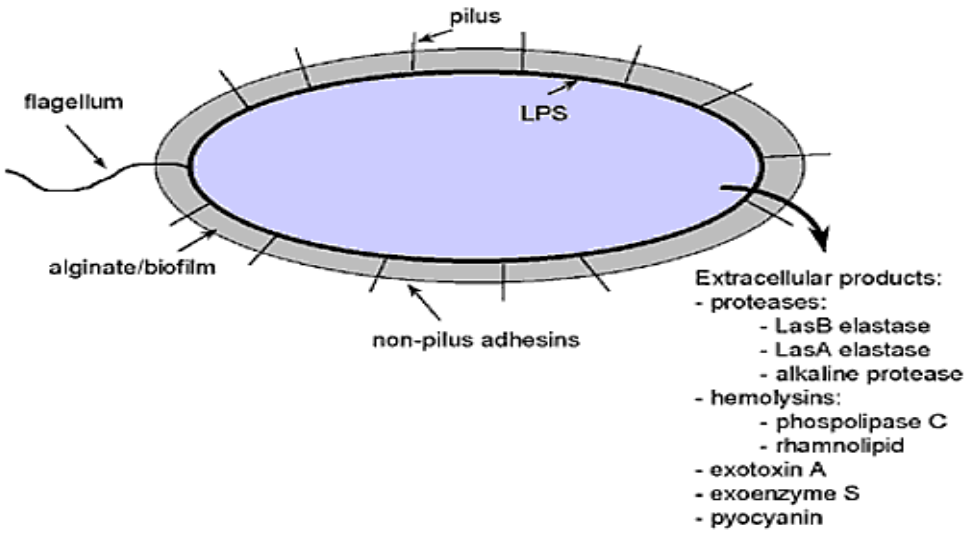

Figure 1. Virulence factors of *Pseudomonas aeruginosa.* This bacterium has both cell-associated (flagellum, pilus, nonpilus adhesins, alginate, lipopolysaccharide, and extracellular virulence factors (including proteases, hemolysins, exotoxin A, exoenzyme S, and pyocyanin) (Adopted from Deldon, 1998).

It has been recognized that PA provokes the strongest inflammatory response in the respiratory track which sometimes leads to septic shock or necrotizing (Kon et al., 1999). We sought to investigate the potential role of *Psuedomonas* elastase and the interstitial cells of the lungs in induction of the profound inflammatory response in PA pulmonary infection and inflammation.

Characteristics of *Pseudomonas aeruginosa* elastase

P. aeruginosa produces three proteases, protease I (Las A), elastase (Las B), and alkaline protease (protease III) (Wretlind & Paviovskis, 1983). The metalloproteinase elastase (PE), produced as a cell wall-associated, inactive proenzyme, is one of the

strongest virulence factors produced by PA (Wretlind & Paviovskis, 1983; Kon et al., 1999). Being a metal chelator-sensitive neutral proteinase that contains zinc, PE is active against casein, elastin, hemoglobin, fibrinogen, and other proteins in neutral pH environments such as the lungs and *in vitro* (Wretlind & Paviovskis, 1983; Clark et al., 2011).

Pseudomonas elastase is capable of degrading various plasma proteins involved in host innate immune system (Kon et al., 1999). Inactivation of complement factors including C1, C3, C5, C8, and C9 by PE has been reported as well (Wretlind & Paviovskis, 1983). This particular host defense tags invading pathogens for destruction, and when PE inhibits the process, the host loses its ability to develop specific adaptive immune responses.

PE causes leukocyte accumulation and increased vascular permeability which leads to severe and damaging inflammation at the site of infection (Cohen & Prince, 2013). Therefore, identification of host cell receptors and PE-induced downstream signaling pathways in the lungs parenchymal and stromal cells are essential steps in understanding the pathogenesis of PA-induced lung injury and therapy.

Human Lung Fibroblast

The human pulmonary system is a multi-facetted collaboration of many cells including epithelial cells and fibroblasts. The lung fibroblasts play a major role in tissue repair by proliferating and secreting extracellular matrix components in the lungs' parenchyma (Noordhoek et al., 2003). The remodeling of damaged tissue is primarily dependent on the ability of the lung fibroblast to degrade the damaged extracellular matrix components and replace them with the new and functional matrices (Kaufman et

al., 2001). Extracellular matrix elements have been an extreme concern in embryogenesis, wound healing, and fibro-proliferative responses to tissue injury due to infection and inflammation (Zhao, 1999). Alteration in fibroblast repair processes can lead to extensive scarring, loss of utilities, and compromised pulmonary functions, which lead to further investigation relative to their immunomodulary action (Kaufman et al., 2001).

In response to bacterial infections, fibroblasts produce proinflammatory cytokines and chemokines including IL-8 (Bedke et al., 2009). Similar to macrophages, the fibroblasts are activated through a specific receptor, which activates intracellular signaling cascades and cytokines' gene expression and protein synthesis and secretion (Glaros et al., 2009). The transcription factor nuclear factor kappa B (NFkB) has been found to transcribe particular proinflammatory cytokine genes in response to pathogens, but little is known about its effects in lung fibroblasts' cytokine production (Kaufman et al., 2001; Barnes, 2009). Therefore, we explored the effect of PE on NFkB activation in human lung fibroblasts *in vitro*, along the hypothesized signaling route described below.

Several extracellular receptors provoke host intracellular signaling to regulate particular gene transcriptions. The gene of interest could be for cytokine expression in response to microbes or allergens or it may be essential for carrying on a homestatic cellular function. Majority of human cells, including fibroblasts, express epidermal growth factor receptor (EGFR) which is stimulated by growth factors including its specific ligand the epidermal growth factor (EGF) (Mitsudomi & Yatabe, 2010). Once activated, the EGFR signals the intracellular kinases such as the mitogen activated protein kinase (MAPK) intermediates involved in cellular growth and differentiation

(Robinson and Cobb, 1997; Yarden, 2001). *Pseudomonas* elastase activates cytokines gene expression in epithelial cells as reported by Azghani et al (2002); however, the mechanism of action of PE-induced IL-8 gene expression is not known at this time. The recent discoveries have led us to examine PE/EGFR activation of MAPK signaling cascade in human lung fibroblasts.

Process of Inflammation

As part of the innate immune system, inflammation consists of an elaborate collaboration of cellular and chemical responses. The leukocytes, lymphocytes, macrophages, dendritic cells, mast cells, as well as structural cells within tissue(s) play a role in an effective immune response. Pathogens, including bacteria, viruses, fungi and parasites, are able to set off the host immune signals and once initiated, inflammation begins with local erythema, edema and pain. Inflammation is a phenomenal process in the aid to clear an infection; however, hyperactivation of the system could cause tissue destruction. For example, high neutrophil accumulation can cause host tissue destruction observed in acute lung injury (Smith et al., 1997; Guo & Ward, 2002). Cytokine and chemokine secretion by lung epithelia and fibroblast has also been reported with potential benefit and harm, including a compromise in the metabolism of extracellular matrix components (Wang et al., 2007).

The lungs of CF patients are prone to microbial infection, PA in particular, because of the mutation in CFTR gene and dysfunctional CFTR ion channels. Consequently, the CF lungs integrity is lessoned with recurrent infections and persistent inflammation. The inflammation in the host can be determined by chemical markers, such as cytokines and an increase in vascular permeability is instigated by infiltration of

proinflammatory cytokines and onset of inflammation and edema (Barnes, 2009). *Pseudomonas aeruginosa* elastase has been found to be present in the lungs of infected cystic fibrosis (CF) individuals and more than likely affects their inflammatory responses (Azghani et al., 2002; Delden & Iglewski, 1998). Since PA infections of CF patients comes with high morbidity and mortality, understanding the mechanisms of PE induced inflammatory responses and the pathogenesis of the lung injury, due to PE, will aid in determining therapies for PA infection within immunocompromised individuals and the CF population.

Mediators of Inflammatory Response

Cytokines are mediators that are characterized as low molecular weight proteins that are expressed upon cell activation by stimuli. Derived from the Greek root "to set in motion", cytokines use specific cell receptors and intercellular signaling mediators of the effector cells in the endocrine, autocrine, and paracrine range (Sherry and Cerami, 1988). More than 80 peptide members exist within the cytokine family that are released from microbe-stimulated leukocytes or structural cell (Cannon, 2000). Along with their Greek terminology, cytokines can be considered for their unique name because nearly all eukaryotic cells have the potential of producing these proteins as well as responding to them (Dinarello, 2000). In addition to their role in infection and inflammation, cytokines are involved in a variety of cellular functions including proliferation and differentiation (Liao et al., 2011). Cytokines have been classified based of their receptors, therefore leading to the assorted nomenclature such as hematopoietic growth factors, chemokines, and interleukins (Oppenheim, 2001; Liles & Voorhis, 1995). Interleukins (ILs) are produced by host immunological aids, such as macrophages, monocytes, and some tissue

cells, and bind to specific receptors and are involved in inter-cellular communication in the immune system in response to extracellular stimuli (Akdis et al., 2011; Rainsford et al., 1996). Chemokines, on the other hand, act as stimulators or inhibitors of inflammation by recruiting or inhibiting of specific populations of leukocytes to the site (Gouwy et al., 2005; Smith et al., 1997). Several of the proinflammatory cytokines, and/or chemokines, accumulate in the airways of patients with acute and chronic pulmonary diseases and when infection occurs (Teijaro et al., 2011; Hartl et al., 2007; Guo and Ward, 2002). Microbial toxins and metabolites commence gene expression by the host in an effort to initiate an array of inflammatory responses through signaling molecules (Gouwy et al., 2005). Cytokines and chemokines both initiate the recruitment and retention of bone marrow-derived immune effector cells such, as T-cells and B-cells. A sustained accumulation of neutrophils in the airways of individuals with chronic lung diseases is an inflammatory response observed when microbe-induced IL-8 secretions occur (Hartl et al., 2007; Kaufman et al., 2001; Roussel et al., 2011). It has been noted that high accumulation of IL-8 is associated with the chronic inflammation seen in CF patients (Smith et al., 1997). Since sub-epithelial fibroblasts are continuously exposed to microbial products released in the lumen of the airways, understanding the consequence of the fibroblast activation and their effector molecules will provide an effective treatment modality in pulmonary infection and inflammation (Scheid et al., 2001).

Inflammation can be a response to specific proinflammatory cytokines and/or chemokines stimuli that trigger an initial molecular signaling event. The gene for IL-8, a highly recognized cytokine in immune response, is transcribed by several transcription factors including a commonly observed factor known as nuclear factor kappa B (NF_kB)

(Roussel et al., 2011; Akdis et al., 2011). Recent studies indicate that the cells of non-blood origin, including fibroblasts, can also secrete this prominent cytokines (Dinarello, 2000). In CF studies, IL-8 plays a crucial role in the inflammation of the lungs as well as tissue destruction because of the continual recruitment and activation of immune effector cells and neutrophils in particular (Cohen & Prince, 2012). Since the fibroblasts are the chief producer of extracellular matrix components necessary for cell migration and tissue remodeling, they may lag in making the necessary materials to rebuild the impaired tissue while assisting to resolve an infection (Wang et al., 2007). Though the cells of blood origin are primarily recognized as the IL-8 source, the mechanism, magnitude, and consequence of the contribution of structural cells, such as fibroblasts, to the inflammatory process is not yet clear (Akdis et al., 2011). Studies have shown that the transcription of IL-8 depends on specific stimuli, host cells' extracellular receptor, cell type, and possibly all three variables combined (Groessl et al., 2012). For instance, lipopolysaccharide utilizes the toll-like receptor 4 (TLR4) on the human lung fibroblast to provoke an inflammatory response to Gram-negative bacteria (Zhang et al., 2011). Also, the CD40 transmembrane domain of fibroblasts is known as a crucial element in the process of fibroblast activation and cytokine and chemokine production (Kaufman et al., 2001). Investigation into novel fibroblast receptors and their downstream signaling related to immune response are cellular events that need further investigation.

In addition to IL-8, other proinflammatory cytokines and chemokines are responsible for tissue destruction at the site of infection. Interleukin 1 Beta (IL-1ß) is a common proinflammatory cytokine produced by several immune cells including resident macrophages (Akdis et al., 2011). Interleukin 1ß plays an important role in the

coordination of local and systemic inflammation by inducing the expression of other proinflammatory cytokines such as IL-6 (Akdis et al., 2011). Tumor necrosis factor alpha (TNFα) is another proinflammatory cytokine with a conserved genetic sequences in several cell types (Akdis et al., 2011). This cytokine, combined with other immune mediators, produces a local state of inflammation to aid the eradication of an invading pathogen(s) (Parham & Janeway, 2009). Tumor necrosis factor alpha (TNFα) is primarily released by macrophages, as a result of toll-like receptors stimulation by bacterial products including LPS and DNA and later needs to be explored in other reactions (Parham & Janeway, 2009). Regulation of immune effector molecules and cells during inflammatory response *in vitro* have been seen to be interrupted in the presence of *P. aeruginosa* (Azghani et al., 2002). This study will seek to identify the mechanism of a bacterial stimuli to potentially provoke an inflammatory response in a lung structural cell through the observance of cytokine secretion.

Epidermal Growth Factor Receptor (EGFR) and Inflammatory Response

Eukaryotic cell response to extracellular stimuli by means of cellular receptors and consequent intracellular signaling pathways propagate gene activation (Robinson & Cobb, 1997). These genetic responses may include cell growth and differentiation, cellular repair, induction of inflammation, and destruction of the pathogen (Mutalik & Venkatesh, 2006).

The tyrosine-kinase epidermal growth factor receptor (EGFR), a member of the ErbB family receptors, is a multi-function receptor that regulates key cellular processes such as proliferation, survival, and differentiation during development and potentially

inflammation (Pastore et al., 2007; Schneider & Wolf, 2009). Ligation of the EGFR by extracellular stimuli initiates activation of downstream signaling pathways including MAPK. Epidermal growth factor receptor can be activated by several extracellular mediators including metalloproteinases from neutrophils, but whether or not bacterial proteases, including elastase from *P. aeruginosa*, utilize this receptor to induce the host inflammatory response is not yet clear (Pastore et al., 2007). This study investigated the role of EGFR in PE-induced inflammatory response by the lung fibroblast.

Mitogen Activated Protein Kinase Pathway

The mitogen activated protein kinase pathway is a conserved cellular signaling cascade in eukaryotes (Plotnikov et al., 2011). The pathway serves as a regulatory element that mediates the transduction of signals from the cell surface to the nucleus (Mutalik & Venkatesh, 2006; Robinson & Cobb, 1997). The MAPK cascade regulates numerous cellular processes including cell differentiation, movement, division, and apoptosis (Mutalik & Venkatesh, 2006). The MAPKs are grouped into subfamilies on the basis of sequence similarity, mechanisms of upstream regulation, and sensitivity to activation by different mitogen activated kinase kinases (MEKs) (Robinson & Cobb, 1997; Mutalik & Venkatesh, 2006). Multiple arms of the MAPK pathways, ERK, JNK/SAPK, and p38, can be individually stimulated by the upstream signaling events with different outcomes. For example, the p38 MAP kinase and the JNK/SAPKs are recognized as stress sensors and sometimes can promote apoptosis (Goldsmith et al., 2004). The extracellular signal-related kinase (ERK) arm of MAPK is involved in cell growth, proliferation and differentiation (Napoli et al., 2012). Two proteins with molecular weight of 42 KDs and 44 KDs known as ERK ½ are activated by MAPK

kinase known as MEK, which in turn is activated by a MEK kinase (MEKK) (Robinson & Cobb, 1997). Phosphorylation of particular amino acids of ERK1/2 activates or inhibits particular downstream mediators of the pathway to transcribe appropriate gene expressions (Figure 2) (Mutalik & Venkatesh, 2006). Given the right stimuli, the EGFR could activate upstream intermediate molecules of ERK, which in turn can cause activation and translocation of NFkB into the nucleus for further gene expression (Oekinghaus et al., 2011). PE might elicit this response; however, the mechanism of PE-induced EGFR activation and its outcome is not clear at this time (Azghani et al., 2002; Azghani et al., 2014).

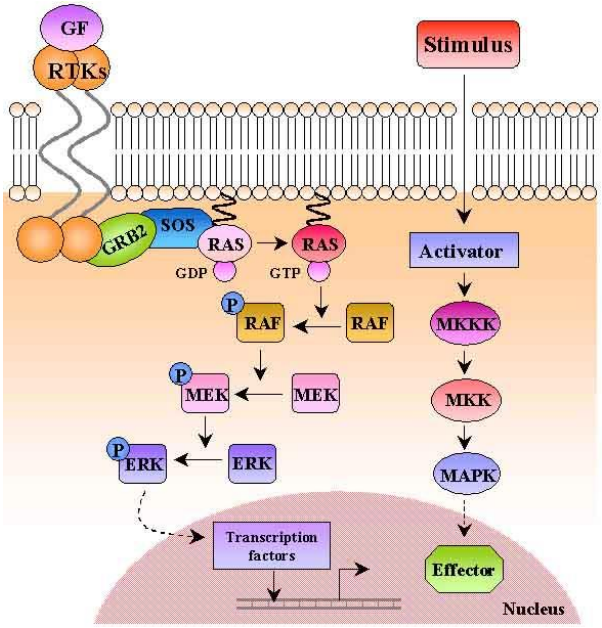

Figure 2. Schematic presentation of the RAF/ERK/MAPK signaling pathway. (Kabarrah & Chin, 2006)

Nuclear Factor Kappa B

Numerous transcription factors (TFs) are triggered to initiate specific gene(s) expression following ERK activation. The nuclear factor kappa B (NFkB) is a known TF involved in inflammatory responses observed in *in vitro* and in acute lung injury models

(Chen et al., 2011; Azghani et al., 2002). Activation and translocation of NFkB to nucleus occur in response to pathogens, including *P. aeruginosa,* and/or their virulence factors including LPS (Roussel et al., 2011). Translocation of NFkB to the nucleus occurs after phosphorylation of its bound inhibitor I-kappa B (IkB) and release of NFkB (Guma et al., 2011). Once in the nucleus, NFkB transcribes specific genes including proinflammatory and inflammatory chemokines and cytokines (Goh et al., 2012).

In addition to EGFR, microbial virulence factors ligate TLRs and activate specific signaling cascades and promote gene expression. For instance, *P. aeruginosa* LPS activates several cytokines' gene expression and protein synthesis via TLR4/MD2/CD14/MyD88 complex which may cause septic shock via NFkB induced transcription of cytokines including tumor necrosis factor (TNF-α) and IL-6 (Paraham & Janeway, 2009). Accordingly, we sought to observe NFkB's involvement in the fibroblasts' inflammatory response to *P. aeruginosa*.

Objectives

The human population will continue to witness episodes of common, emerging, and reemerging infectious diseases. With continual microbial infections occurring as secondary complications in immunocompromised individuals, persistent research in pathogen/host interaction is a necessity. An opportunistic-bacterial infection of soft tissues and the human pulmonary system will be studied. In particular, we specifically focused on the mechanism of the human lung fibroblast's inflammatory response to *Pseudomonas aeruginosa* elastase along a targeted, known signaling pathway. It is hypothesized that PE provokes an inflammatory response along the MAPK signaling route in the human lung fibroblast, measured by the secretions of IL-8 (Figure 3). At this

time, PE's involvement in the highly utilized MAPK signaling event in other types of cells or tissues is unclear. This study will give us more insight of *Pseudomonas aeruginosa's* mechanistic involvement in a normal host's physiological environment and raise awareness to undetermined events in an immunocompromised pulmonary system.

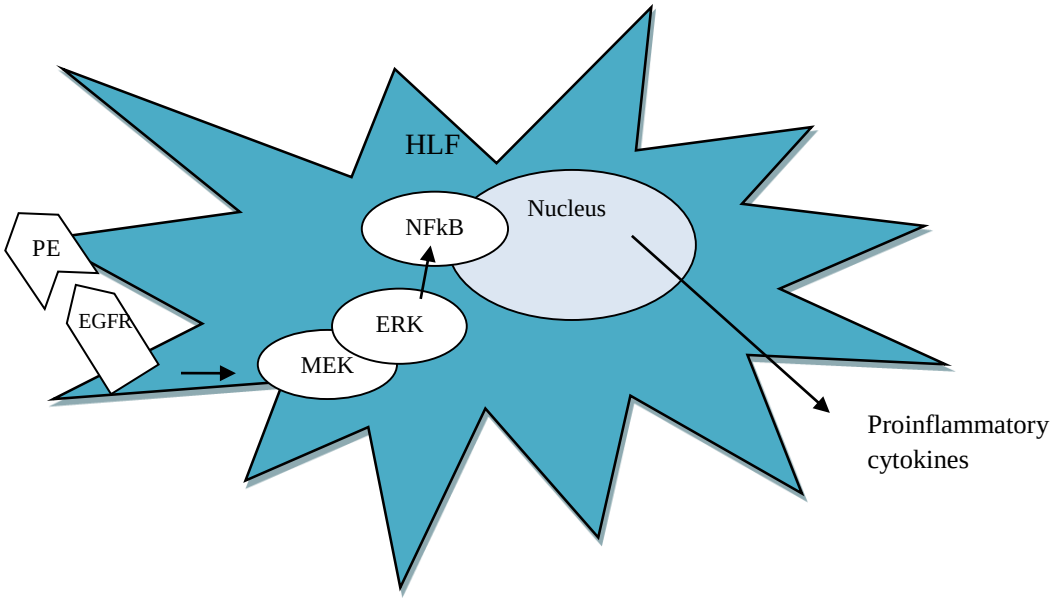

Figure 3. Hypothesized signal transduction pathway in the human lung fibroblasts (HLF) for production of proinflammatory cytokines.

Chapter Two
Material and Methods

Cell Culture

An established line of human lung cells, IMR-90, isolated from normal fetal lung tissue were used at the fifth through the sixteenth passages. The cell line was obtained from the American Type Culture Collection (ATCC Manassas, VA). The fibroblasts were grown in Minimum Essential Medium (MEM Sigma-Aldrich St. Louis, MO) supplemented with 1% nonessential amino acids, 10% fetal bovine serum (FBS Life Technologies Grand Island, NY), 1% L-glutamine, 1% penicillin and streptomycin antibiotics, and 2.5 μg/mL Plasmocin (Invivogen, San Diego, CA). The cells were maintained in T25 cm^2 and T75 cm^2 cell culture flasks in the complete MEM medium and kept in a humidified incubator at 37 °C in 5% CO_2. Media were removed and replaced every two days. The lung fibroblasts were transferred to new flasks and/or plates when the cells formed confluent monolayers on the bottom of the flask.

Mediators and Treatment Modality

The fibroblasts were serum-starved for 18 hours prior to treatments. Cells were treated with PA products, lipopolysaccharide (LPS) or elastase (PE) from *P. aeruginosa*, for selected time periods and post-treatment sampling times. Purified PE was obtained from Elastin Products Company (EPC, Owensville, MO). Lipopolysaccharide was

purchased from Sigma-Aldrich Company and was used as a known positive control for cell activation (Zhang et al., 2011; Azghani et al., 2002). TRIzol (Invitrogen, Grand Island, NY) was used for nuclear extraction, ribonucleic acid (RNA), along with high grade chloroform.

Three specific MAPK inhibitors were used to investigate the role of selected cell signaling pathways. The MEK inhibitor U0126 was used to prevent MEK phosphorylation. The inhibitor AG 1785 was used in order to block the EGFR, while BAY 11708 was incorporated to prevent NFkB translocation to the nucleus. All inhibitors were purchased from Calbiochem, Darmstadt, Germany. Pre-confluent cell monolayers, grown in 24, 48, and 6-well cell culture plates were treated as described (Table 1) and supernatants were collected and centrifuged to remove cell fragments and were stored at -80˚C for further analysis.

Table 1. Treatments and corresponding concentrations used. A (+) indicates the positive controls within the design. Serum free Minimal Essential Media (MEM) alone used as negative control. All treatments were diluted FBS-free MEM.

Treatment	Concentration
MEM-	**Non-treated media**
PE	**1.2 U/mL**
LPS+	**5 µg/mL**
FBS+	**20%**
EGF+	**1 µg/mL**
U0126	**10 µM**
AG 1478	**10 µM**
BAY 11-7085	**10 µM**

Total RNA Extraction

Fibroblasts grown in cell culture dishes, as described previously, were lysed with TRIzol solution (Life Technologies Grand Island, NY) for total RNA extraction according to an in-house laboratory protocol. After the completion of treatments, the supernatant was removed from each cell culture dish and frozen until later protein analysis. Next, 1 mL of TRIzol solution was added to each dish and incubated at room temperature for 10 minutes. The solution of TRIzol and lysed cells' content were transferred to a 2.0 mL centrifuge tube where we added 0.3 mL of chloroform to each 1mL solution. Each vial was then agitated for 20 seconds and incubated at room temperature for 10 minutes. Afterwards, each vial was centrifuged at 1400 rpm at 4°C for 15 minutes. The RNA separated into the top, aqueous phase (clear supernatant) where the DNA and proteins settled at the interphase. The clear top layer was carefully removed without breaking into the interphase and put the sample into a clean, autoclaved centrifuge vial. 500 μL of isopropyl alcohol (500 μL per 1 mL of TRIzol used) was added and each vial was agitated gently before the solution was stored in -20°C for 10 minutes to aid in detecting the RNA isolate. Next, we centrifuged the alcohol mixture at 1400 rpm for 15 minutes at 4°C and the RNA pellet formed at the tip of the vial was mixed with 1mL ethanol (1:3 parts DEPC autoclaved water to 75% ethanol). Centrifugation was completed two more times to keep the RNA pellet whole and to separate the pellet from the alcohol to allow faster drying. Lastly, the centrifuge vial cap was gently left open for all alcohol to completely evaporate and leave behind the RNA. The RNA pellet was

resuspended with 20 μL of preheated (60°C) RNA Secure and the sample was heated at 60°C for 10 minutes as suggested by the manufacturer.

The resuspended RNA was measured right away using a nanospectrophotometer (Nanodrop 1000, Thermo Scientific Wilmington, DE)at the optical density (OD) of 261/280 nm. 2 μL of RNA sample to read the total RNA with 2 μL autoclaved DEPC water flushes between each reading. For the OD ratio of 261/280, a reading of 1.8-2.2 was determined as a good RNA sample and a reading lower than the suggested range was considered as having protein or DNA contamination.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

A high-capacity reverse transcription kit was used to convert extracted RNA molecules to single-stranded DNA molecules (ssDNA or cDNA). This kit was purchased from Applied Biosystems (Life Technologies Grand Island, NY) and protocol was followed from manufacturer's instructions.

For each run, 1μg of each treated sample was used to perform the reverse transcription and then added to the RNA Secure and DEPC-treated water solution resulting in a final volume of 5μL. Each reaction was kept at 37°C for 2 hours and afterwards immediately set at 85°C to deactivate the transcriptase. All completed reactions were stored at -20°C for future qRT-PCR assay.

A primer set of the target cytokine was used in order to detect the particular gene expression. The forward and reverse primers for IL-8 were from Bioneer, Alameda, CA. The primers were rehydrated to establish a 100 pmoles/μL concentration and stored at -20°C until the assays were run.

To establish a standard curve and positive controls for the PCR, we carried out a serial dilution of the plasmid carrying the IL-8 gene and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (primers: forward 5'-TCGGAGTCAACGGATTTGGTCGTA, REVERSE 5'AGCCTTCCTCCATGGTGGTGGTGAAGA) was used as a regulatory control and establishing a baseline for the test RNA molecules. Quantitative PCR of human IL-8 was performed in triplicate. A 15 μL final volume of PCR mix containing 200 nM of each forward 5' TGCGCCAACACAGAAATTATTGTA and reverse 5' ATTCTCAGCCCTCTTCAAAAACTT primer and 50 ng of cDNA in a master mix (10 μL) containing 7.5 μL of 2 x iTaqSYBR Green Supermix with ROX (Bio-Rad Hercules, CA) and 2.5 μL of the primer set at a final concentration of 0.05 μM for each amplicon were added to the wells of a MicroAmp Fast 96-well reaction plate (Applied Biosystems – Life Technologies). The plates were carefully sealed with optical adhesive cover 4360954 (Applied Biosystems – Life Technologies Grand Island, NY) and placed in a StepOnePlus real-time PCR system with data collection software v2.1 (Applied Biosystems – Life Technologies Grand Island, NY). The expression of IL-8 in treatment samples was compared to that of untreated control cells. Cycler setup was established according to the iTaqSYBR Green Supermix with ROX manufacturer's protocol.

Single-Analyte Enzyme-linked immunosorbent assay (ELISA)

The replaced media, or supernatants, from cell culture wells were used to quantify secreted IL-8 protein after treatments. A 96-well plate was pre-coated with IL-8 capture antibody (primary antibody) overnight. A wash buffer, consisting of 1x PBS and Dulbecco buffered salts, was used as a wash solution between each step to rinse excess

reagents from the treatment plate according to the manufacturer's protocol (Biolegend San Diego, CA). A protein blocking agent (assay diluent) was added to each well of the 96-well plate, and allowed to sit at room temperature in wells for one hour while rocking at 200 rpm. The assay diluent was removed from the plate and another rinse with wash buffer was applied. A standard curve was established by a 1:2 serial dilution of 1000 pg/mL of IL-8 protein as well. The sample supernatants were removed from frozen storage and thawed in ice and spun in a microcentrifuge for 10 minutes at 1000 rpm and 22°C. Each sample was added to the well according to our design of the run and was incubated for two hours while shaking at 200 rpm. The plate was washed with wash buffer and the detection antibody (secondary antibody) was added to each well for one hour while shaking at 200 rpm. Detection antibody was washed from the plate and Avidin-HRP enzyme was added to sit in the wells for thirty minutes while rocked in the dark. The Avidin-HRP leftover solution was washed with wash buffer and TMB Substrate Solution was added for 15 minutes in the dark without shaking. A blue color in the wells would appear with the addition of the substrate which turned stable yellow with the addition of 2N H_2SO_4 solution. The plate was read with the Beckman Coulter AD 340 microplate reader at the OD=450 nm absorbance and subtracted from the data at OD=570 nm in order to eliminate the plastic plate absorption, if any. The data were normalized log arithmetically by the AD 340 software and optical densities were translated into concentrations of IL-8 in pg/mL.

Statistical Analysis

A one-way ANOVA and Dunnett's post-test analysis were performed to test the differences among the treatment groups in comparison with the control. Results are

presented as means of at least three independent experiments. Standard errors were calculated to observe the means of each treatment represented in an experiment and were represented by error bars. P-values less than 0.05 were considered statistically significant for the means of each treatment.

Chapter Three
Results

Effect of *P. aeruginosa* elastase on Cytokine Production by Human Lung Fibroblasts: Dose and Time Course Study

Different concentrations of enzymatically active and inactive PE were utilized in order to determine the optimal PE concentration and exposure time in cytokine production by IMR-90 monolayer in culture. Three different concentrations of PE were used as suggested by previous studies in our laboratory which included 0.6 U/mL, 1.2 U/mL, and 2.0 U/mL. Based on the preliminary data, the 1.2 U/mL concentration was found to be the optimal dose in this project.

As for the exposure time, based on previous signal transduction study reports from our laboratory, we carried out PE exposure experiments at 10 and 20 minutes and measured IL-8 protein expression at 6 and 24 hours post incubation (Azghani et al., 2002). At the 10 minute treatment time, we found a significant increase in IL-8production while doubling PE treatment time did not change the IL-8 production significantly (Figure 4).

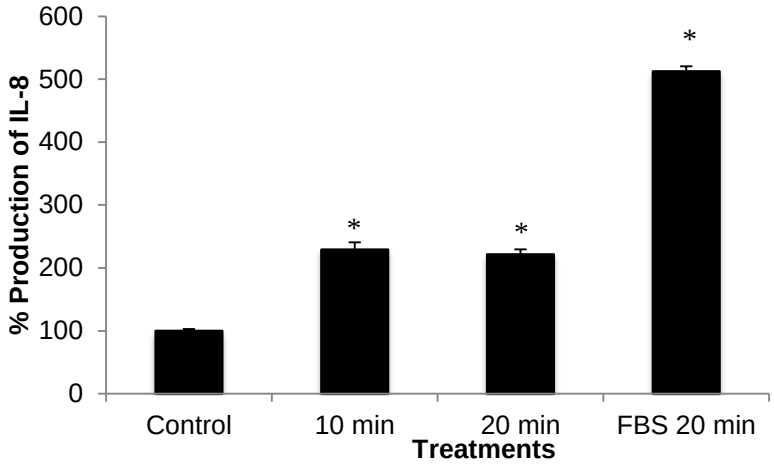

Figure 4. IL-8 production at 24 hours post treatment with PE (1.2 U/mL). IMR-90 grown to confluence in culture plates produced more IL-8 when they were exposed to PE for 10 minutes. Doubling exposure time did not affect the cell response. Error bars represent the standard errors of the means. Asterisks indicate significant difference between treated and control cells at $p<0.05$, n=3 independent experiments.

To determine whether enzymatic activity of PE is necessary PE-induced IL-8 expression, we compared IL-8 protein expression by the monolayers exposed to active enzyme to that of 1.2 U/mL of PE after heat-inactivation in a boiling water bath for 20 minutes. As shown in Figure 5, results indicated that only the active form of elastase provoked the lung fibroblasts to secrete IL-8 significantly. Based on these findings, we used enzymatically active PE in preceding experiments.

Optimal post-treatment incubation times for cytokine production were evaluated at 6 hours, 16 hours, and 24 hours after 10 minutes PE exposure and rinse. Cells in culture plate were tested with different cell holding capacity and found that both a brief 6 hours as well as a 24 hours post-treatment periods were sufficient to statistically differentiate the PE effect on IL-8 production by cultured IMR-90 monolayers. The 24 hours post-treatment time; however, was sufficient when cells were confluent in a 48-well plate, while the 6 hours post-exposure time was suitable when more cells were

grown in a 6-well plate. Thus in proceeding experiments, IL-8 protein secretions were measured from treated confluent monolayers of IMR-90 cells grown in 48-well plates.

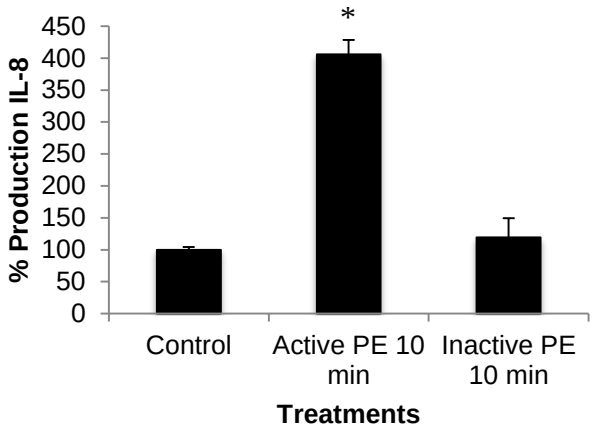

Figure 5. Comparison of the effects of enzymatically active of PE to that of the heat-inactivated protease on IL-8 production. Cells were treated with PE (active and inactive) for 10 minutes, washed and left alone in MEM for 24 hours. Data indicate % increase in IL-8 concentration in the supernatants. Error bars represent standard error of the means (n=3 independent experiments). Asterisks indicate significant difference from the control at $p<0.05$.

P. aeruginosa elastase Triggers IL-8 Gene Expression in the Human Lung Fibroblast through EGFR/ERK 1/2 stimulation

In an effort to investigate the mechanism of action of PE-induced IL-8 secretion, we hypothesized that PE activates IL-8 gene expression through EGFR/MAPK signaling pathways in the lung fibroblast. Specific blockers were used in different stages of these pathways to address the mechanism of action of PE-induced IL-8 gene expression. The cells were exposed to PE in the absence and presence of specific EGFR and MAPK signaling inhibitors outlined below. At the conclusion of each experiment, the cell lysates were used to quantitate IL-8 gene expression using quantitative real time polymerase chain reaction assays (QRT-PCR).

To determine if IL-8 gene expression was dependent upon ERK1/2 activation, the monolayers were pretreates with a MEK inhibitor (U0126, 10 μM, 15 min) prior to PE treatment. As shown in Figure 6, pretreatment with specific MEK inhibitor completely abrogated PE-induced IL-8 mRNA expression. Blocking EGFR activation by its specific inhibitor AG 1478 (300 nM, 60 min) confirmed the involvement of EGFR in PE-induced IL-8 mRNA expression (Figure 6). The relative expression of IL-8 mRNA was estimated by quantitative real time polymerase chain reaction assays of the RNA samples isolated from cells treated with PE or positive control EGF in the presence or absence of AG1478 pretreatment. The data shown in Figure 6 revealed that both PE and EGF caused significant increase in IL-8 gene expression and the induction of IL-8 mRNA was repressed by the signaling inhibitor in both instances. We should point out that the IL-8 mRNA expression for each treatment was presented as a ratio of the untreated control (relative quantity control). The variance in RNA quantity was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

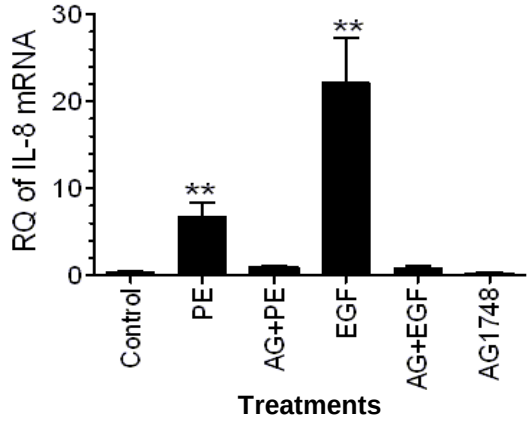

Figure 6. PE-enhanced IL-8 mRNA expression is abrogated by inhibition of EGFR activation. Confluent monolayers of IMR-90 cells grown in six-well plates were serum starved overnight. The monolayers were then treated with PE (1.2 U/mL) or EGF (1µg/mL) in the presence or absence of AG 1748 (10 µM). Monolayers treated with the medium or AG 1478 alone were used as controls. At the end of 2 hours treatment, total RNA was isolated and analyzed for IL-8 gene expression by QRT-PCR analysis. The IL-8 mRNA expression for each treatment was presented as a ratio of the untreated control (relative quantity control). The variance in RNA quantity was normalized by GAPDH gene expression. Asterisks indicate significant difference from the control at $p<0.05$.

Effect of EGFR/ERK/NFkB Activation on IL-8 Protein Secretion by the Human Lung Fibroblast from PE exposure

We next sought to determine the influence of EGFR/ ERK/NFkB signaling pathways on IL-8 protein production and secretion by cultured lung fibroblasts. The supernatants from MEM (negative control), FBS and LPS (positive controls) and PE (1.2 U/mL, 10 min)-treated cells were analyzed for IL-8 protein content. PE enhanced IL-8 protein secretion in an amount comparable to that of *P. aeruginosa* LPS, a well-known activator of IL-8 expression (Figure 7, LPS and PE). The concentration of IL-8 secreted in the medium was significantly ($p<0.05$) higher with PE or LPS treatment compared to the control. The MEK inhibitor U0126 blocked PE-induced IL-8 production (Figure 7, U0126 + PE). The influence of PE on IL-8 production by fibroblasts in the presence of

specific inhibitors of EGFR (10 µM, 15 min), and NFkB (BAY 11-7085, 10 µM, 15 min) are shown in Fig. 7 as well. All above-mentioned inhibitors suppressed IL-8 production significantly ($p<0.05$), suggesting a link between PE-induced activation of EGFR with MAPK and NFkB signaling pathways leading to *de novo* synthesis and secretion of IL-8.

The data depicted in Figure 8 confirm the notion that PE activates EGFR signaling evens in a manner similar to that of its specific ligand EGF. Just like PE, EGF utilizes EGFR/ERK/NFkB signaling mediators to increase IL-8 protein secretion in human lung fibroblasts. Specific inhibitors of the aforementioned pathways reduced IL-8 production to the lever of MEM-treated control cells (Figure 8, U0126+EGF, AG1478+EGF, and BAT 11-7085+EGF).

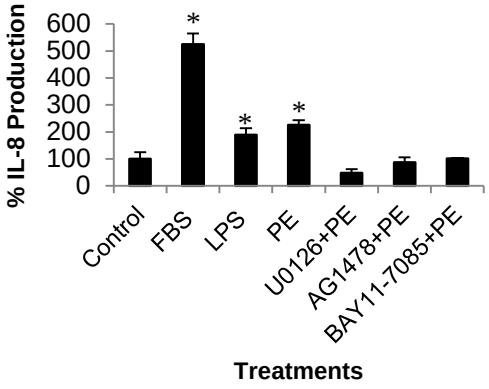

Figure 7. PE-induced IL-8protein secretions in the presence and absence of specific signal transduction inhibitors; U0126 inhibits MEK, AG1478 inhibits EGFR ligation, and BAY11-7085 inhibits NFkB translocation. Error bars represent the standard error of the means (n=3 independent experiments, $p<0.05$). Asterisks indicate groups statistically different from the control group.

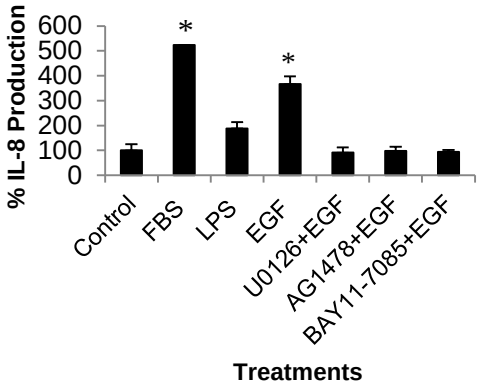

Figure 8. EGF-induced IL-8protein secretions in the presence and absence of specific signal transduction inhibitors (a known induction used as our positive control); U0126 inhibits MEK, AG1478 inhibits EGFR ligation, and BAY11-7085 inhibits NFkB translocation. Error bars represent the standard error of the means (n=3 independent experiments, $p<0.05$). Asterisks indicate groups statistically different from the control group.

Chapter 4
Discussion

Determining the effector molecules in host-pathogen interaction in acute and chronic infections could be the key to understanding underlying mechanisms of disease. The opportunistic human pathogen, *P. aeruginosa* (PA), causes nosocomial pneumonia and recurrent pulmonary infection in individuals with hereditary CF disease (Murray et al., 2007). Build-up of a dehydrated mucus in an environment and accumulation of activated as well as dead immune cells, provides a suitable microbial growth medium (Bharnsholt et al., 2009). *P. aeruginosa's* ability to thrive in such an environment for the rest of the host's life cycle will depend on several virulence factors including elastase, the protease of interest in this project.

Virulence factors are bacterial weapons to resist host innate and adaptive immune responses. *P. aeruginosa* elastase is a well-known virulence factor that is implicated in pathogenesis *Pseudomonas*-induced injury in individual affected by CF (Delden & Iglewski, 1998; Pressler et al., 2011). Pulmonary epithelia is the first line of defense in the fight against bacterial infection and invasion. In the case that epithelial are impaired, bacteria will disseminate to the lungs parenchyma as well as stroma, and the blood stream (Smith et al., 1996; Parsons et al., 1987; Azghani et al., 1990; Rejman et al., 2007).

Likewise, the lung fibroblasts are important in the system's homeostasis, support of epithelia, and parenchymal tissue reconstruction. Furthermore, fibroblasts' role in local

immune defense is now appreciated through production of the proinflammatory chemokine IL-8 (Noordhoek et al., 2003; Bedke et al., 2009). An initial, external stimulus is needed to prompt cytokine production by the fibroblast in their efforts to recruit the effector cells of the immune system. Lipopolysaccharide of *P. aeruginosa* is a well-known stimulus of such a response (Kon et al., 1999). Previously published reports and the data reported here indicate that PE is a potent immune response activator as well. The mechanism of action of PE-induced immune response in structural cells of the lungs are not yet clear.

Eukaryotic cells respond to extracellular stimuli by means of cellular receptors and activation of downstream signal transduction pathway(s), which results in specific gene expression (Robinson & Cobb, 1997). The MAPK pathway is a conserved signaling cascade that mediates the transduction of signals from the cell surface to the nucleus (Plotnikov et al., 2011; Mutalik & Venkatesh, 2006; Robinson & Cobb, 1997). The MAPK is composed of multiple arms, ERK1/2/JNK, and p38, which are involved in cell proliferation and differentiation, and the transcription factor NFkB is observed as the transcription factor for many of these cellular mechanisms (Napoli et al., 2012; Chen et al., 2011; Nasu et al., 2007; Azghani et al., 2002).

Activation and translocation of NFkB to the nucleus occur in response to pathogens, including *P. aeruginosa*, and its LPS (Roussel et al., 2011; Akdis et al., 2011). Elastase from PA also induces NFkB activation and IL-8 gene expression and propose that the pathway is activated by upstream signaling events involving EGFR and MAKP (ERK1/2). Several extracellular stimuli activate the MAPK cascade and can thereby elicit a wide range of responses dependent upon the properties of the target cells (Cobb, 1999;

Kumasaka et al., 1996; Rosenfeld et al., 2001). In inflammation, activation of the ERK/MAPK in host cells stimulates transcription of several cytokine genes through activation of nuclear transcription factors such as NFkB (Blackwell et al., 2001; Li et al., 1998; Pendurthi et al., 2000). In the cultured human lung fibroblasts utilized in this study, PE likewise enhances IL-8 gene expression by activation of NFkB. However, the involvement of other transcription factors in PE-induced IL-8 gene expression remains probable and requires further exploration.

Fibroblasts comprise over 40% of the parenchymal cells in human lungs and their role in maintenance of the structural integrity and function of the lungs has been confirmed by several investigators (Behzad et al, 1996; Dulon et al., 2005). Induction of chemokine secretion by lung fibroblasts may contribute to the migration of leukocytes into the parenchyma of the injured lung (Dulon et al., 2005). Under normal conditions, the intact respiratory epithelial lining sequesters lung fibroblasts from direct exposure to bacterial cells or their metabolic products (Gauldi et al., 1992). In respiratory infections, a breach in the epithelial lining occurs under the influence of host factors and bacterial products, allowing bacteria and their toxins access to submucosal and interstitial lung fibroblasts (Azghani et al., 1990; Pearson, et al., 2000; Poynter et al., 2003). PE is one such bacterial product that can disrupt the epithelial lining and may contribute to pathogenesis of *Pseudomonas* infection (Azghani, 1996; Azghani et al., 2000; Donald et al., 1982).

High concentrations of IL-8 in the lungs have been linked to the pathogenesis of CF as well as acute pulmonary diseases including the acute or adult respiratory distress syndrome (ARDS) and sepsis (Armstrong et al., 1997; Meyer-Hoffert et al., 2004). In

animal models of *P. aeruginosa* lung infections, a marked polymorphonuclear neutrophil (PMN) infiltration that appears to be due to IL-8 production from lung fibroblasts, epithelial cells, as well as macrophages (Blackwell & Christman, 1996; DiCamillo et al., 2002; Tang et al., 1993). Several bacterial metabolites including LPS, elastase, autoinducer N-3- Oxododecanoyl homoserine lactone, pyocyanin, as well as flagella and pili stimulate respiratory epithelial cells to produce IL-8 (Azghani et al., 2000; Noah et al., 1997; Smith et al., 2001). However, the effects of PE on expression of IL-8 by lung fibroblasts is poorly understood, a gap in current knowledge that is addressed by the observations reported herein.

P. aeruginosa elastase enhances IL-8 production in human lung fibroblasts in culture, is in part, via activation of the ERK1/2 arm of MAPK pathway and activation of NFkB. These data confirm the role of PE in pathogenesis of pulmonary inflammation and reconcile with recent *in vivo* observations. In a DPB model of lung infection, pulmonary inflammation induced by a *P. aeruginosa* mutant strain with reduced active elastase was compared with that of a wild type strain. Although both strains survived equally well, a relatively more intense infiltration of mononuclear inflammatory cells occurred in the bronchi of the wild type *P. aeruginosa* treated animals on day 90 post-incubation (Witko-Sarsat et al., 1999). Similarly, in a rat air pouch model of acute infection, enzymatically active PE significantly increased the host inflammatory response as evidenced by a higher exudate volume and an increase in the number of neutrophils and the IL-8 concentration (Kida et al., 2007). The mechanisms of PE-induced inflammatory responses in these models; however, are not yet clear.

In conclusion, our data suggest that PE may in part modulate lung inflammation by enhancing IL-8 production by lung fibroblasts. This physiologic alteration may occur via PE-induced activation of ERK1/2 and nuclear translocation of NFkB that eventually binds the enhancer region and activates IL-8 gene expression and protein synthesis. The observed data represent a newly recognized pathway by which lung fibroblasts can influence local expression of IL-8 and inflammatory cell traffic within the injured lung.

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