PROTEASE DYSREGULATION: ROLE IN NEUTROPHILIC INFLAMMATION IN CYSTIC FIBROSIS

by

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A DISSERTATION

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Cystic fibrosis (CF) is a lethal disorder characterized by abnormal epithelial ion transport; this disorder is characterized by an ongoing airway remodeling and neutrophilic inflammation. Much of the airway remodeling is due to activation of a group of enzymes known as proteases; human neutrophil elastase (HNE) is a prominent protease found in the CF airway. While specific cytokines/chemokines are well-known for their role in the inflammation seen in CF, it is unknown how the protease-rich environment in CF lung disease influences airway inflammation. Recently, our group has characterized a novel collagen-derived fragment, proline-glycine-proline (PGP), which causes neutrophil influx through mechanisms similar to interleukin-8 (IL-8) in an experimental model of airway inflammation. However, the role of this peptide in clinical disease and the mechanism of PGP generation are unknown. In the current work, we first examine the protease signature seen in airway secretions from CF individuals, determining notable increases in members of the matrix metalloprotease (MMP) family. One member of this family, MMP-9, has increased activity in CF. The importance of increased MMP-9 activity in airway inflammation is reinforced in a murine model of F. tularensis infection in which MMP-9 deficiency leads to decreased airway neutrophilia; PGP, which was elevated in MMP-9$^+/+$ mice, were significantly diminished in MMP-9 knockout mice. Administration of MMP-9 or MMP-8, in concert with prolyl endopeptidase (PE), is capable
of generating PGP in mice. In addition, sputum from CF individuals is also capable of generating PGP from intact collagen ex vivo. The use of specific inhibitors can significantly attenuate PGP generation both in vivo and ex vivo. Finally, we demonstrate that PGP is elevated in CF patients compared to normal controls. In addition to these findings, we also show that MMP-9 also is capable of cleaving IL-8 in vitro, leading to the generation of a new, more potent IL-8 species. These studies underscore the importance of MMP-9 in airway inflammation seen in CF lung disease; indeed, as many of these proteases are found in other conditions with ongoing inflammation and extracellular remodeling, it is possible that these findings may be more generalized to chronic neutrophilic inflammatory conditions.
DEDICATION

This dissertation is dedicated to my parents, Satish and Mithlesh Gaggar, who have always loved and supported me throughout my life and in all of my endeavors; my brother, Anuj Gaggar, who is my best friend and an inspiration in all aspects of my life; and especially my wife, Shilpa K. Gaggar and son, Akash Shah Gaggar, who are and always will be my heart and soul.
ACKNOWLEDGMENTS

I sincerely thank all of the friends, family, and colleagues whose help has made this work possible and enjoyable. This dissertation and training has truly been a testament to all of the wonderful people in my life. First, I’d like to thank Bhagvan (God), with whom all things are possible. My wife, Shilpa, has been a source of strength and support throughout this process- without her patience and understanding, this work would not be possible. In addition, Ed Blalock stands as a testament to what a mentor truly is: his guidance in all things have led to the development of mutual respect and a wonderful friendship. J.P. Clancy has also been crucial to my development as both a scientist and physician: I thank him for his wisdom and friendship. Others whose advice and collaboration have been essential to the completion of the work contained herein include my committee members Patricia Jackson, Philip J. O’Reilly, Lisa M. Schweibert, and F. Shawn Galin. In addition, I would like to thank members of both the Gregory Fleming James CF Center (Eric J. Sorscher, Yao Li, Michele Kong, Steve Rowe, Jennifer Guimbellot, Louisa Pyle), the Blalock lab (Nathaniel Weathington, Brett Noerager, Xin Xu, Matt Hardison), the Department of Physiology/Biophysics Graduate program (Dale Be- nos, Peter Smith, Lisa Schweibert, F. Shawn Galin, and all of the graduate students), and Department of Medicine, Pulmonary/Critical Care at UAB for their continued support. Finally, this work would not be possible without the inspiration from my son, Akash Gaggar.
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<td>Aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ABSL-3</td>
<td>vertebrate biosafety level 3</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
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<tr>
<td>Ala</td>
<td>alanine</td>
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<tr>
<td>ALI</td>
<td>acute lung injury</td>
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<tr>
<td>APMA</td>
<td>aminophenylmercuric acetate</td>
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<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
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<tr>
<td>ARF</td>
<td>acute respiratory failure</td>
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<tr>
<td>Arg</td>
<td>arginine</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAL/BALF</td>
<td>broncho alveolar lavage fluid</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSL-3</td>
<td>biosafety level 3</td>
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<tr>
<td>C57/Bl6</td>
<td>C57/black 6</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
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</table>
Ca+ calcium
CC CC chemokine
CDR complementarity-determining region
CF cystic fibrosis
cftr cystic fibrosis gene
CFTR cystic fibrosis transmembrane conductance regulator
CFU colony forming units
Cl- chloride
COOH carboxyl
COPD chronic obstructive pulmonary disease
CXC CXC chemokine
CXCL CXC chemokine ligand
CXCR CXC chemokine receptor
Deg degree
DMSO dimethyl sulfoxide
ECL enhanced chemiluminescence
ECM extracellular matrix
EDTA ethylenediamine tetraacetic acid
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<tr>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ELR</td>
<td>glutamate-leucine-arginine</td>
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<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>F508</td>
<td>phenylalanine 508</td>
</tr>
<tr>
<td>F. tularensis</td>
<td>Francisella tularensis</td>
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<td>FEV1</td>
<td>forced expiratory volume in 1 second</td>
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<td>FVB/NJ</td>
<td>friend virus B/NIH Jackson</td>
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<td>FVC</td>
<td>forced vital capacity</td>
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<tr>
<td>Gly</td>
<td>glycine</td>
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<td>GRO</td>
<td>growth-related oncogene</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
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<tr>
<td>H&amp;E</td>
<td>hematolysin and eosin</td>
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<td>HNE</td>
<td>human neutrophil elastase</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>In</td>
<td>intranasal</td>
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<td>Ip</td>
<td>intraperitoneal</td>
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<tr>
<td>It</td>
<td>intratracheal</td>
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<tr>
<td>LC</td>
<td>liquid chromatography</td>
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<td>Leu</td>
<td>leucine</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LVS</td>
<td>live viral strain</td>
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<td>MAB</td>
<td>monoclonal antibody</td>
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<tr>
<td>MCP</td>
<td>macrophage chemoattractant protein</td>
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<tr>
<td>MH</td>
<td>Mueller-Hinton</td>
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<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
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<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
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<tr>
<td>MRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MTD</td>
<td>mean time to death</td>
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<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteases</td>
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<tr>
<td>N-α-PGP</td>
<td>n-acetyl-PGP</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>Na+</td>
<td>Sodium</td>
</tr>
<tr>
<td>N/A</td>
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<tr>
<td>N-terminal</td>
<td>amino terminal</td>
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<td>amino</td>
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<tr>
<td>P(x)</td>
<td>amino acid residues (for enzyme specificity)</td>
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<tr>
<td>PA</td>
<td>Pseudomonas aeruginosa</td>
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<td>PAGE</td>
<td>polyacrylamide gel</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PE</td>
<td>prolyl endopeptidase</td>
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<td>PGP</td>
<td>proline-glycine-proline</td>
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<tr>
<td>PI</td>
<td>post-infection</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte, neutrophil</td>
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<tr>
<td>Pro-</td>
<td>proenzyme or proline</td>
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<tr>
<td>Pro-Gly-Pro</td>
<td>PGP</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
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<tr>
<td>SA</td>
<td>Staphylococcal aureus</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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LIST OF ABBREVIATIONS (Continued)

sLPI  secretory leukoproteinase inhibitor

TGF-β  transforming growth factor beta

TIMP  tissue inhibitor of metalloprotease

TNFα  tumor necrosis factor alpha

UAB  University of Alabama-Birmingham

Val  valine

Zn  zinc
INTRODUCTION

Background: Cystic Fibrosis.

Cystic fibrosis (CF) is a common lethal genetic disease in persons of northern European descent, with a carrier frequency in this population of 1:25 and an incidence of ~1:2,500 births. Over 30,000 CF-affected individuals have been identified in the U.S., and the worldwide prevalence is estimated to be ~70,000. The disease is characterized by abnormal transport of sodium (Na+) and chloride (Cl-) across the epithelia of many tissues, including the lungs, pancreas, GI tract, liver, sweat glands, and male reproductive ducts [1]. The pulmonary manifestations of CF are responsible for substantial morbidity and most CF-related mortality. Present therapies directed towards treatment of the pulmonary symptoms of CF (including airway clearance medications and techniques, bronchodilators, anti-inflammatory agents, antibiotics, and multidisciplinary care at CF care centers) have helped increase the life expectancy of CF patients approximately three-fold since the early 1960s. Unfortunately, respiratory failure remains the cause of death in >90% of CF patients [2], with a median survival of 36.5 years.

CF is caused by defective function of the cftr gene product named the Cystic Fibrosis Transmembrane conductance Regulator, or CFTR [3]. CFTR is a member of the traffic-ATPase family, functions as a cAMP-regulated Cl- channel, and also regulates other ion conductance pathways including epithelial Na+ channels, Cl- channels, and possibly ATP transport [4]. The CFTR protein is normally expressed in those tissues with clinical CF
symptomatology, including the airway submucosal glands and surface airway epithelial cells. Defects in CFTR function are felt to negatively influence the airway surface fluid composition and/or mucus found in the CF airway [5]. This, in turn, is felt to lead to poor mucociliary clearance, chronic bacterial infection and inflammation within the airways, and the progressive airway destruction characteristic of CF with epithelial necrosis, progressive acquired small and large airway malacia, and ultimately pan-bronchiectasis [6].

**Cytokines and Chemokines in Cystic Fibrosis.**

The airways of CF patients, while normal at birth, quickly become inflamed and chronically colonized with characteristic bacterial pathogens. Inherent, exaggerated inflammation or early bacterial colonization may initiate this process, and it is well established that massive neutrophil infiltration defines the cellular pathology seen in the CF airway [7]. In addition to this cellular influx, increased production of inflammatory mediators, including TNF-α, and IL-1β [8], combined with reduced levels of anti-inflammatory cytokines such as IL-10 [9], produce a milieu that intensifies and perpetuates the airway injury characteristic of CF.

A group of molecules which have been of interest in chronic inflammatory diseases are chemokines. Chemokines are small heparin-binding proteins that direct the movement of circulating leukocytes to sites of inflammation or injury. There are 2 major classes of chemokines: CC chemokines and CXC chemokines [10]. A subgroup of CXC chemokines are neutrophil-specific chemoattractants. These molecules have a glutamate-leucine-arginine (ELR) motif in their N-terminal region and, as such, are termed ELR+
CXC chemokines [11]. An example of a classic ELR+ chemokine is interleukin-8 (IL-8 or CXCL8), a prominent chemokine found in a variety of neutrophilic lung diseases such as chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and CF [12]. The source of these chemokines is believed to be from the airway epithelium itself, along with inflammatory cells including macrophages and neutrophils. IL-8 is such a prominent mediator in CF that it seems to correlate with clinical lung function in CF individuals and often serves as a biochemical endpoint in CF-related intervention trials [13]. However, the use of IL-8 as a biologic marker of inflammation is often criticized for its lack of specificity for both chronic and acute inflammatory conditions.

Human neutrophils demonstrate 2 major CXC receptors (CXCR) on their cell surface, CXCR1 and CXCR2 (mice only have CXCR2). Both of these receptors can induce neutrophil chemotaxis upon binding with an agonist (i.e. IL-8) though actin polymerization and depolymerization via Rho/Rac GTP-ases [14]. In addition, CXCR1 binding can induce the release of reactive oxygen species from neutrophils, leading to local oxidative damage [15]. Therefore, the binding of chemokines to their receptors on neutrophils have important functional consequences to host defense and airway damage.

**Proteases in Cystic Fibrosis.**

Another group of molecules which have attracted increased interest in airway damage and remodeling in chronic lung disease are proteases. Proteases are enzymes which have the capacity to degrade proteins to either smaller-sized proteins or to peptides. The major classes of proteases are aspartic, cysteine, serine, threonine, and metalloproteases. These categories are based on the characteristics of the active site of these proteases [16]. Each
of these classes is composed of subclasses of proteases and substrate specificity may differ even amongst individual members of subclasses of proteases. In addition, proteases often have specific antiproteases which are involved in the regulation of the enzyme’s proteolytic activity.

Recent models of airway inflammation indicate that protease:antiprotease imbalance and protease dysregulation are prime contributors to pulmonary diseases, with detrimental effects on resolution of inflammation and remodeling of the pulmonary architecture [17]. Alpha1-antitrypsin deficiency is a classic example of this process [18], with unchecked and unrelenting pulmonary protease activity producing emphysema and premature pulmonary failure. CF is another lung disease in which unchecked protease activity has been hypothesized to damage the airway architecture and contribute to progressive bronchiectasis [19]. Human Neutrophil Elastase (HNE) is a well described serine protease that has been shown to be a biomarker of pulmonary inflammation in both alpha-1 antitrypsin deficiency and CF. Dysregulation of HNE propagates inflammation and is believed to disrupt the pulmonary architecture through damage of structural proteins in both diseases [18, 20].

Several other proteases have been postulated to contribute to CF lung disease and identification of those responsible enzymes remains an area of intense investigation. One group of proteases receiving attention in other chronic inflammatory lung diseases is the matrix metalloproteases (MMPs), a subclass of the metalloprotease family. MMPs perform numerous biologic functions, including degradation of matrix components and remodeling of tissues, release of cytokines, growth factors and chemokines, and modulation of cell mobility and migration. There are > 20 MMPs that have been identified and,
based on structure and substrate specificity, the members can be subdivided into collagenases, gelatinases, stromelysins, matrilysin, macrophage elastase, and membrane-type MMPs (MT-MMPs, which are expressed on the cell surface). Together they have the combined ability to degrade essentially all connective tissue components. While MMPs are involved in many normal homeostatic mechanisms, they are commonly elevated in their expression and activities in conditions in which inflammation and tissue destruction are operative [21].

MMPs are regulated at the level of transcription (through a variety of specific cytokines including IL-1 and TNF-a), translation, and post-translational modification (cleavage of prodomain from enzyme to induce activation). Perhaps the best recognized mechanism of MMP regulation is through the binding of small, specific protein inhibitors (tissue inhibitor of metalloproteases (TIMPs)) to the active site of the MMP [22]. Data suggest that dysregulated cellular production, secretion and activation of MMPs, and/or dysfunction of their inhibitors are involved in pathologic conditions within the lung parenchyma. Through degradation of extracellular matrix components, MMPs can function in both the destruction of alveolar epithelium, as well as its reorganization during the repair process. Animal studies, in addition to human studies in adults, support a role for MMPs and an imbalance between MMPs and their inhibitors in the pathogenesis of several well-recognized pulmonary disorders including COPD and asthma [23].

MMPs, however, have not been systematically studied in CF lung disease. Some initial studies have demonstrated elevated levels and activity of MMPs (MMP-8 and MMP-9) from lower airway secretions from CF patients [24, 25] and others have described elevated MMP-7 on the airway epithelial surface of patients with CF [26]. Despite these
novel findings in CF, these studies do not adequately address two major concerns derived from determining these elevated MMP levels: (1) What cells are responsible for release of these proteases and what causes the activation of the quiescent zymogen to active enzyme and (2) What are the functional consequences of elevated MMP activity in the airways of CF patients? Addressing these questions will require information regarding the release and activation of MMPs from various airway cells and consideration of whether MMPs can modulate inflammation and the innate immune response.

**MMPs and Airway Cells: Regulation and Release.**

MMPs are prominent proteases which are found in a variety of airway cell types including neutrophils, macrophages (both alveolar and interstitial), fibroblasts, airway epithelial cells, and vascular endothelial cells [23]. While some MMPs are particularly localized to specific cell types (ie MMP-7 is found predominately in airway epithelia and fibroblasts) [26], other MMPs are found in a variety of cell types. As these MMPs act extracellularly, it is also of importance to determine mechanisms by which they are released. Unfortunately, to date, very few mechanisms regulating MMP release from cells have been well-characterized.

One mechanism of MMP release which is well-characterized is the release of MMP-9 from neutrophils. While MMP-9 is found in a variety of airway cells, its highest concentrations are found in neutrophils [27]; of note, MMP-8 is also found in neutrophils. As such, MMP-8 and MMP-9 are often termed “neutrophil-derived MMPs”. MMP-9 is stored in tertiary granules in neutrophils and it has been found that if a CXCR2 agonist binds to the receptor, it will induce degranulation of neutrophil tertiary granules via a
protein kinase C (PKC) pathway [28]. This pathway of MMP-9 release is specific for binding to CXCR2 but not CXCR1. Neutrophils, therefore, serve as rich reservoirs for a variety of proteases (ie serine proteases and MMPs) and the mechanism for MMP-9 release seems mediated through chemokine binding.

Mechanisms of MMP Activation.

In addition to release of MMPs into the extracellular environment, these proteases must have their pro-domain cleaved to generate enzymatic activity. Their latency is maintained by a cysteine sulfhydryl group in the prodomain binding to Zn. The “cysteine switch” is the mechanism by which this bond is broken and MMPs are activated [21]. While a variety of in vitro mechanisms have been described for MMP activation (detergents, mercury-based compounds), in vivo activation centers predominately on protease-based activation. While specific serine proteases (ie trypsin, chymotrypsin) have been well-characterized as MMP activators in both in vitro and murine models, other MMPs may also serve as potent activators [22]. For example, MMP-9 has been shown to be activated by MMP-3 (stromelysin-1) and MMP-2 (gelatinase A) [27]. Interestingly, a novel pathway of activation of MMP-2 has been described by MT-MMPs, pointing to a unique avenue of cell-surface bound MMPs as gelatinase-activating agents; there are varied reports of this similar activation with the other gelatinase, MMP-9 [29]. Regardless, MMPs are unique proteases which can be activated by a variety of proteases and have even been shown to autocatalyze zymogen once activated. Despite these findings, pinpointing a defined proteolytic mechanism for MMP activation in clinical human samples has proven to be difficult.
Protease/Protease Potentiation and Regulation of Inflammatory Molecules.

An area of increased interest is the role proteases may play in the modulation of the innate immune response in patients with chronic neutrophilic lung diseases such as CF. As mentioned earlier, IL-8 is a prominent chemoattractant found in a variety of neutrophilic lung diseases including cystic fibrosis. IL-8 is released from epithelial cells predominately as a 77 aa form. It undergoes proteolytic or oxidant-related processing which helps to create a smaller, more potent chemokine at 72 aa, although the exact mechanism of this processing remains unknown [30]. HNE, a serine protease, has also been found to upregulate transcription of MMP-2, an MMP found to be elevated in neutrophilic inflammatory conditions such as acute lung injury and corneal keratitis [31]. In addition, HNE also upregulates transcription of cathepsin-B, a serine protease thought to be involved in trauma-related inflammation [31]. Interestingly, MMP-2 has also been shown to be activated by HNE from a zymogen to an active protease extracellularly [32].

Extracellular Matrix Fragmentation: A Novel Avenue of Innate Immunity.

Recently, our group has characterized a novel neutrophil chemoattractant, proline-glycine-proline (PGP), which acts via a CXCR-mediated mechanism [33]. This peptide was generated in a murine model of inflammation utilizing nebulized lipopolysaccharide (LPS). This unique chemoattractant is derived from collagen fragmentation and demonstrates structural homology with known ELR+, CXC chemokines such as IL-8. In both in vivo and in vitro experiments, PGP was found to induce neutrophil chemotaxis and to induce superoxide production. While the unique biologic properties of this neutrophil
chemokine ligand are understood, the proteolytic pathway by which PGP is liberated from collagen remains unclear.

Brief Overview of Thesis.

The first manuscript presented in this thesis more completely examines the specific MMP profile seen from sputum samples of patients from CF patients. This paper begins with a description of the proteolytic dysregulation in cystic fibrosis and the implications related to airway remodeling. Our paper extends these observations by undergoing an extensive examination of a variety of MMP isotypes, utilizing a variety of techniques including immunoblotting, gelatin zymography, and ELISA. We demonstrate a specific MMP profile (MMP-8, MMP-9, MMP-11, and MMP-12) in CF sputum which differed from non-CF patients, either with or without respiratory failure. MMP-9 was the most prominent isoform present and demonstrated significant dysregulation relative to its natural inhibitor, TIMP-1. Our data demonstrated a pattern of elevated HNE activity which corresponded with this elevated MMP-9 activity in vivo. We then extend these observations to show that HNE has the capability of activating pro-MMP-9 to an active isoform and degrading TIMP-1 in vitro. These two independent mechanisms demonstrate a system which is likely operative for in vivo activation of MMP-9 in CF.

The potential importance of the role of MMP-9 in the generation of PGP is examined in the second manuscript which is co-authored by this candidate. This paper utilizes a novel model of neutrophilic inflammation and collagen breakdown by Francisella tularensis infection. These features of inflammation mimic changes seen in the lungs of cystic fibrosis individuals and, as such, offer novel insights to the inflammatory pathways
seen in chronic neutrophilic lung diseases. *F. tularensis* is a gram negative intracellular organism which, when inhaled, causes notable inflammation and collagen turnover, leading to high rates of death. It is so potent, in fact, that it has been considered as an agent in biological warfare. Our experiments show that inhalation of *F. tularensis* in wild-type FVB/NJ mice demonstrate increased collagen turnover, neutrophil influx, and death vs. the MMP-9 knockout mice. However, both groups of mice demonstrated similar levels of traditional murine ELR+ CXC chemokines (KC and MIP-2). There is, however, a difference in the PGP levels, with MMP-9 knockout mice with significantly less PGP compared to wild-type, pointing to a possible role of MMP-9 in PGP generation.

The final manuscript presented in this thesis extends the observations of dysregulated proteolytic activity in CF and the data implicating MMP-9 in PGP generation in the *F. tularensis* inflammation model by examining (1) Is PGP found in the sputum of CF patients? and (2) Do the dysregulated proteases found in CF, such as MMP-9, contribute to the generation of PGP? This paper begins by a basic description of chemokines and characteristics of PGP as a novel neutrophil chemoattractant. PGP was then measured in CF sputum samples and compared to normal controls, demonstrating a significantly increased amount in CF vs normals. The CF samples were then assayed for their ability to generate PGP *de novo* from intact type I and II collagens *in vitro*. CF samples demonstrated notable PGP generation compared to very little generation seen with normal control sputum, indicating that the appropriate proteolytic machinery was present in CF sputum for PGP liberation from collagen. Extensive *in vitro* and *in vivo* experiments showed that PGP is generated in a stepwise process with the initial cleavage of collagen mediated by either MMP-8 or MMP-9 and a second cleavage event mediated by prolyl endopepti-
dase (PE), a serine protease which had previously not been described as a modulator of the innate immune response. This paper demonstrates the potential role of PGP as a biomarker for CF and the specific proteolytic mechanisms by which PGP is liberated from collagen during the inflammatory response and, subsequently, augments this response.

Together, this dissertation examines the unique avenue of proteases regulating the inflammatory response, utilizing a classic neutrophilic lung disorder as the model disease for these studies. The dissertation examines modulation of a chemokine response through biochemical and pharmacologic activities of proteases, murine models of inflammation, novel proteolytic activation cascades, and inhibition studies of proteases. Indeed, these studies may serve as the underpinnings from which an understanding of protease biology may lead to a more robust knowledge of how proteases modulate innate immune response in chronic neutrophilic lung diseases such as CF. This work also identifies novel potential therapeutic targets for the modulation of the inflammatory response seen in this chronic destructive lung disease.
MATRIX METALLOPROTEASE-9 DYSREGULATION IN LOWER AIRWAY SECRETIONS OF CYSTIC FIBROSIS PATIENTS

by

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Format adapted for dissertation
Abstract.

Matrix metalloproteases (MMPs) are proteolytic enzymes that regulate extracellular matrix turnover and aid in restoring tissue architecture following injury. There is an emerging role for extracellular matrix destruction in the pathogenesis of chronic neutrophilic lung diseases. In this study, we examined the expression and activity profiles of MMPs in lower airway secretions from cystic fibrosis (CF) patients, patients with acute respiratory failure (ARF), and normal controls. A discrete repertoire of MMP isoforms was found in the CF samples, with robust MMP-9 expression compared to normal controls and ARF. CF samples possessed increased levels of active MMP-9, as well as decreased amounts of Tissue Inhibitor of Metalloprotease-1, (TIMP-1), a natural inhibitor of MMP-9. The CF inpatient samples demonstrated fully active MMP-9 activity compared to CF outpatients, ARF, and normal controls. CF samples also demonstrated increased Human Neutrophil Elastase (HNE) levels compared to ARF and normal controls. To examine potential mechanisms for the protease dysregulation seen in the CF clinical samples, in vitro studies demonstrated that HNE could activate pro-MMP-9 and also degrade TIMP-1; this HNE-based activation, however, was not seen with MMP-8. A strong correlation was seen between HNE and MMP-9 activity in CF inpatient samples. Finally, the dysregulated MMP-9 activity seen in CF inpatient sputum samples could be significantly reduced by the use of MMP-9 inhibitors. Collectively, these findings further emphasize the proposed protease/antiprotease imbalance in chronic neutrophilic lung disease, providing a potential mechanism contributing to this proteolytic dysregulation.
Introduction.

Several chronic inflammatory conditions are characterized by an imbalance of proteases with their naturally occurring antiproteases. This protease/antiprotease hypothesis is believed to be an important component of pulmonary pathology seen in chronic lung diseases such as \( \alpha \)-1-antitrypsin deficiency [1], bronchopulmonary dysplasia [2, 3, 4], asthma [5, 6, 7], and emphysema [8, 9]. Although different families of proteases have been, in part, implicated in the end-organ damage described in these conditions, recent attention has turned to matrix metalloproteases (MMPs) as mediators of this process. This family of proteases plays important roles in paracrine and distal hormone signaling, cell matrix turnover, and restoration of normal tissue architecture and function after injury [10]. MMPs are released as tightly regulated zymogens, with secondary regulation provided by direct interactions with proteins such as Tissue Inhibitors of MMPs (TIMPs) and \( \alpha \)-macroglobulin. MMP-8 and -9 are stored and released from neutrophil granules [11], and MMP-7 is expressed and released from the airway epithelium where it serves an important role in epithelial repair [12]. Left unchecked, MMPs have the capacity to degrade essentially all components of the extracellular matrix, potentially contributing to the tissue destruction and dysfunction seen in a variety of chronic lung diseases [13].

Structural airway changes are a prominent feature of cystic fibrosis (CF), a chronic genetic disorder in which abnormal ion transport predisposes patients to infection and neutrophilic inflammation [14]. The widening of airways and loss of normal mucociliary clearance mechanisms leads to the development of bronchiectasis [15]. These structural changes are a direct cause of the pulmonary morbidity and mortality seen in the
vast majority of CF patients and are felt to be a consequence of unrelenting airway inflammation in the disease [16]. Previous studies have demonstrated increased sputum neutrophil accumulation in CF patients versus normal and disease controls, with inverse relationships between neutrophil counts and lung function [17, 18, 19]. Neutrophil influx occurs early in CF lung disease, with a significant burden seen in many CF infants prior to established infection with CF pathogens [15, 16, 17]. Neutrophils are the predominant source of human neutrophil elastase (HNE), a well-described serine protease that contributes to CF lung disease. HNE expression and activity is elevated in CF sputum with reduced levels of compensatory antiproteases such as secretory leukoproteinase inhibitor (sLPI) and α-1 antitrypsin [16, 19]. This excessive and untempered protease activity can disrupt bacterial clearance, degrade elastin, and contributes to a perpetuation of airway inflammation and destruction [1, 20].

A limited number of studies have examined MMPs in CF lung disease. An earlier study suggested an increase in MMP-9 activity in CF sputum compared to asthmatics and normal controls [21]. Dunsmore et al reported that MMP-7 was elevated in CF airway epithelium compared to normal controls and that this was localized predominately to alveolar type II cells [22]. Ratjen and colleagues examined BAL fluid from CF patients and found increased MMP-8, MMP-9, and MMP-9/TIMP-1 ratios in children with CF compared to healthy controls [23]. A recent report by Sagel and colleagues demonstrated excessive MMP-9 expression in the lower airway secretions of CF patients, and a negative correlation between MMP-9 levels and lung function (FEV1) [24].

In this report, we examined the protease/antiprotease balance with regards to MMP activity in CF lung disease compared with normal subjects and subjects with acute
respiratory failure (ARF). We hypothesized that CF patients had a unique sputum MMP profile compared to ARF and normal control individuals, and that CF inpatients samples demonstrated increased proteolytic activity compared with CF outpatients samples. A unique MMP profile, including neutrophil-derived MMPs -8 and -9, were identified in the majority of CF samples, with particularly elevated MMP-9 activity demonstrated. HNE activity was also high in CF samples relative to control, with a notable correlation demonstrated between HNE and MMP-9 activity in CF inpatient samples. Investigating potential relationships between these proteases, our studies demonstrate HNE is sufficient to activate MMP-9 and degrade TIMP-1 at physiologic concentrations seen in CF secretions. Finally, MMP-9 activity in CF samples could be reduced by specific and nonspecific small molecule inhibitors. Together, the results provide support for an important regulatory relationship between HNE and MMP-9, and provide mechanistic evidence for the dysregulated MMP-9 activity observed in CF.
Methods.

*Patient samples*

UAB Institutional Review Board approval was obtained prior to all studies involving human subjects and samples. Informed consent was obtained from all subjects or caregivers of subjects with CF and/or ARF. Samples from normal controls were remnant secretions obtained from well pediatric subjects undergoing elective surgical procedures (no diagnosis of known lung disease) via suctioning of endotracheal tubes (as part of routine airway maintenance). No identifiers were collected with these controls.

*ARF Subjects*

Lower airway secretions were collected, via endotracheal suctioning, from intubated pediatric patients with a known diagnosis of ARF requiring intubation and ventilatory support. Samples were collected every 12 hours for the period of intubation. The samples included in this report were pooled from the first five days of intubation. The details regarding this population are summarized in Table I.

*CF Subjects*

All subjects carried the diagnosis of CF based on routine diagnostic criteria, including two sweat Cl- values > 60 mM, and a minimum of two clinical features consistent with the diagnosis. Genotype and sputum bacterial culture information was available in the majority of subjects, and is included in the demographic summary provided in Table I. Sputum was obtained by spontaneous expectoration (i.e.: no sputum induction). When possible, CF inpatients provided three separate sputum samples at three distinct times during the course of hospitalization for treatment of routine pulmonary exacerbations.
tions (Days 0-1, 5-7, and 10-14). CF outpatients provided a single sputum sample during routine outpatient clinic visits.

**Sputum and Endotracheal Aspirate Processing**

Once sputum was collected, the sputum was diluted 1:2 with 0.9% normal saline and centrifuged at 1000 RPM for 10 minutes with separation of pellet from supernatant. The supernatant was collected, protein concentration was measured, and then separate aliquots were saved for measurements (-80° C).

**Protein detection**

All samples were electrophoresed through SDS-polyacrylamide gels (both reducing and nonreducing conditions) and electroblotted onto Immobilon-P PVDF membranes. Membranes were blocked in Tris buffer (pH=7.4) containing 5% powdered milk for one hour. Once washed, they were incubated with primary antibody (anti-MMP or anti-TIMP) overnight at 4°C. After incubation, samples were washed with borate saline (100 mM boric acid, 25 mM Na borate, 75 mM NaCl) and incubated with secondary antibody for one hour. Species-specific IgG horseradish peroxidase conjugates were used as secondary antibody at dilutions of 1:5000. Immunoblots were then developed using ECL chemiluminescent kits (GE Healthcare, Piscataway, NJ). Unless otherwise stated, 16 µg of sample was loaded for each immunoblot lane.
**MMP activity and TIMP quantification assays**

Briefly, MMP-8 and MMP-9 specific ELISA-based activity assays were used to quantify specific MMP activity (R and D systems, Minneapolis, MN). Samples were diluted to fit manufacturer’s sensitivity for individual kits (0-25 ng/ml for MMP-8 and 0-16 ng/ml for MMP-9 kits). Both samples and recombinant enzyme standards were prepared and incubated for two hours at room temperature in 96-well plates coated with monoclonal antibodies for the MMP(s) (MAB 908 for MMP-8 and MAB 911 for MMP-9) of interest. After incubation, samples and standards were activated with 1 mM amino-phenylmercuric acetate (APMA), a chemical activator of MMPs, and further incubated for two hours at 37°C. After incubation, a fluorogenic substrate (Fluor-Pro-Leu-Gly-Leu-Ala-Arg-NH2) was placed in each well and the plate was incubated at 37°C for 18 hours. The plate was then read on a spectrophotometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA; excitation and emission wavelength of 320 and 405, respectively) and data was quantified using standard curves provided with the kits. Standardized kits for measuring the activity and concentration of other MMPs identified during this study were not available.

For the studies of TIMP-1, samples were diluted to fit manufacturer’s sensitivity for ELISA (0-10 ng/ml). Both samples and recombinant TIMP-1 standards were prepared and incubated for two hours at room temperature in 96-well plates coated with TIMP-1 monoclonal antibodies. Bound TIMP-1 was then conjugated with a horseradish peroxidase-based secondary antibody for one hour. A colorimetric substrate (hydrogen peroxide and chromagen) was placed in each well and color change was assessed after 30 minutes. Color changes were quantified on a colorimeter via standard curves.
**Human Neutrophil Elastase (HNE) assays**

Briefly, HNE-specific ELISA activity kits were used to quantify HNE concentrations in clinical samples (Calbiochem, San Diego CA). Samples were diluted to fit manufacturer’s kit specificity (0-20 ng/ml). Both samples and recombinant HNE standards were prepared and incubated for two hours at room temperature in 96-well plates coated with a monoclonal antibody for HNE. A fluorogenic substrate (MeOSuc-Ala-Ala-Pro-Val-AMC) was then added and the plate was incubated for 2 hours. Finally, the plate was read on a spectrophotometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA; excitation and emission wavelength of 360 and 460, respectively) and data was quantified using standard curves provided with the kits.

**MMP-8 and MMP-9 Activation**

For in vitro studies involving activation of pro-MMPs with APMA, 1mM APMA (R and D Systems, Minneapolis) was incubated with recombinant pro-MMPs (R and D Systems, Minneapolis) in a 37°C incubator for 2 hours. For pro-MMP activation with HNE, recombinant pro-MMPs were incubated with specified concentrations of recombinant HNE (Calbiochem, San Diego) at 37°C for 2 hours.

**Statistical analysis**

Descriptive statistics including mean and standard error of the mean (SEM) were determined for all continuous data. An exponential correlation was generated between HNE and MMP-9 activity. Paired and unpaired t-tests were used for comparisons of MMP, HNE, and TIMP activities utilizing Sigmastat statistical software (Jandel, CA).
p-value ≤ 0.05 was used to determine statistical significance. As this was an exploratory study to examine MMP expression and activity in the three study groups, no formal power calculations were performed in advance of sample collection.

**Material**

All primary monoclonal antibodies and recombinant MMPs were purchased through either R and D Systems (Minneapolis, MN) or Chemicon (Temacula, CA). HNE, HNE-specific inhibitor (N-(2-(4-(2,2-Dimethylpropionyloxy) phenyl-sulfonylamino)benzoyl)aminoacetic acid N-(o-(p-Pivaloyloxybenzene) sulfonylaminobenzoyl) glycine), MMP-9 inhibitor (C$_{27}$H$_{33}$N$_3$O$_5$S (anthranilic MMP inhibitor, Catalog) were purchased via Calbiochem (San Diego, CA).
Results.

Demographic information summarizing the study groups is shown in Table I. CF samples were derived from patients who were hospitalized for uncomplicated pulmonary exacerbations (n = 33) and patients seen in outpatient clinics as part of routine (‘well’) follow-up visits (n = 13). ARF samples (n = 26) included pooled samples during days 01-05 of intubation, and control samples (n = 26) were obtained from subjects undergoing routine outpatient surgical procedures (no known lung disease). As expected, the CF study groups were significantly older than the pediatric ARF subjects (p<0.05). Both inpatient and outpatient CF groups had a female predominance. The inpatient CF group was enriched for severe mutations (particularly delta F508 homozygous mutation) and had significantly reduced lung function (FEV1, FVC) at the beginning of hospitalizations relative to the CF outpatient controls (p<0.01). All CF inpatient and outpatient subjects were pancreatic insufficient as judged by their treating physician, and pulmonary function of the CF inpatients was reduced relative to the outpatients.

**MMP-9 activity is increased in the airway secretions of CF inpatients.**

Using specific antibodies to detect various MMP isoforms (MMP-1,-2,-3,-7,-8,-9,-10,-11,-12,-13,-19,-20,-26,-27,-28), screening Western blots from CF and ARF samples were performed. Positive controls with recombinant enzymes were utilized for all screened MMPs. CF patient samples demonstrated detectable MMP-8, MMP-9, MMP-11, MMP-12, and TIMP-1 expression; ARF samples demonstrated a similar pattern with detectable MMP-2, -8, -9, -11 and TIMP-1. Normal controls had only sporadic staining for MMP-8, MMP-9, and TIMP-1. Together, the results point towards a limited
<table>
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<th>CF Inpatient</th>
<th>CF Outpatient</th>
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<td>33</td>
<td>11</td>
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<td>22.1yr (1.3)</td>
<td>25.8 yr (2.21)</td>
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<td></td>
<td>(1mo – 19yr)</td>
<td>(9yr – 34yr)</td>
<td>(15yr – 40yr)</td>
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<td>PA+ and SA- (4)</td>
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Table I. Demographic information of study groups

P values in chart (CF inpatients) refer to changes from admission values
• N/A: Not applicable
• †P<0.01: ARF compared with CF inpatient and outpatients.
• ‡P<0.01: CF inpatient compared with CF outpatients.
• PA’ (sputum culture positive for Pseudomonas aeruginosa), ‘SA’ (sputum culture positive for Staphylococcus aureus), ‘PA’ and SA’ (sputum culture positive for both Pseudomonas aeruginosa and Staphylococcus aureus), ‘PA’ and SA’ (sputum culture negative for both Pseudomonas aeruginosa and Staphylococcus aureus).
• Normal controls patients included patients undergoing elective procedures without known lung disease.
repertoire of detectable MMP isoforms in the lower airways of the two disease states with MMP-8 (Figure 1A) and MMP-9 (Figure 1C) demonstrating increased staining relative to other isoforms in both CF and ARF. To quantify MMP activity, we performed MMP-9 and MMP-8 activity assays. MMP-9 activity was in general higher in CF inpatient samples compared with ARF (Figure 1D), and all disease groups demonstrated high activity (three to seven-fold increases) relative to the normal controls. Parallel studies were performed to compare MMP-8 activity in CF and control samples (Figure 1B). MMP-8 activity was elevated in the ARF samples compared with normal controls and both populations had elevated MMP-8 activity compared to CF patients. Of note, MMP-9 activity was elevated relative to MMP-8 activity within each disease group.

**MMP-9 activity does not correlate with clinical disease measures**

In our study, no clear change in sputum MMP-9 activity could be detected within the inpatient CF group across the period of hospitalization (mean MMP-9 activity = 1213.5 ng/mg (+/- 369) on Day 0-1 samples, mean MMP-9 activity = 1376.8 ng/mg (+/- 314.1) on days 10-14), despite clear improvements in lung function (FEV1 or FVC) (Table 1). There was also no correlation with MMP-9 activity and lung function in CF outpatient samples (data not shown). In addition, no clear association between sputum microbiology, gender, age, or disease-treatment and MMP-9 activity was identified in either of the CF groups (data not shown). A positive correlation between MMP-9 and the pulmonary inflammatory biomarker human neutrophil elastase (HNE) was seen (and is discussed in further detail below).
Figure 1: MMP-8 and MMP-9 detection and activity in CF lower airway secretions.

1A. Western blot (7.5% SDS nonreduced gel) demonstrates faint MMP-8 staining (60 KDa, black arrow) in CF and ARF samples. Each lane is sputum from a separate subject (+ = recombinant MMP-8 positive control, C = normal control).

1B. MMP-8 activity levels were quantified as described in Methods. CF inpatients (n=33), CF outpatients (n=11), ARF (n=26), and normal controls (n=10) were compared. Individual points are from separate subjects (* p<0.02, † p<0.005 compared to normal controls). Dashed line is the mean value of normal controls. MMP-8 activity was significantly reduced in CF inpatients and outpatient samples.

1C. Western blot (7.5% non-reduced gel) demonstrates staining of 78 KDa band (white arrow) corresponding with active MMP-9 isoforms in CF and ARF patients. Each lane represents a separate patient. (+ = recombinant 92 KDa MMP-9 positive control, C = normal control). 92 KDa band represents MMP-9 proband (gray arrow). 130 KDa molecular weight band likely represent lipocalin complexes with MMP-9 (black arrow).

1D. MMP-9 levels were quantified as described in the Methods section. CF inpatients (n=33), CF outpatients (n=11), ARF (n=26), and normal controls (n=10) were compared. Individual points are from separate subjects (* p<0.001 compared with normal controls). Dashed line is the mean value of normal controls. CF inpatients demonstrate approximately 7-fold increase in MMP-9 activity compared to normal controls.
**MMP-9 in CF inpatient sputum is constitutively active**

Since MMP-9 activity was increased in CF sputum, we examined whether MMP-9 regulation was maintained in CF lower airway secretions. MMP-9 activity is normally limited in part by secretion of its zymogen form, followed by conversion from pro-enzyme to active MMP-9 in vivo. Utilizing 1mM aminophenylmercuric acetate (APMA), a typical activator of pro-MMP-9 for in vitro studies, the data showed that MMP-9 was fully and constitutively active in CF inpatient samples relative to clinically stable CF outpatients and controls (Figure 2).

Specifically, MMP-9 function was enhanced by 57 % (+/- 29.1) and 52% (+/-8.2) following chemical stimulation with APMA in normal controls and ARF patients, respectively. In contrast, less APMA-induced activation of MMP-9 was seen in CF outpatient samples (39%, +/-18.0), and this activation was absent in samples from CF inpatients (2.6% over basal activity +/- 5.9, p<0.01 compared to normals).  

**HNE activates MMP-9 but not MMP-8 in vitro**

In order to examine if relationships between HNE and MMP-8 or MMP-9 were present in CF sputum, we compared HNE activity across the study populations, and confirmed that HNE levels were elevated in CF inpatient and outpatient sputum samples compared to normal controls (Figure 3A). We next examined whether HNE had stimulatory effects on pro-MMP-9 and pro-MMP-8 zymogen proteins. In functional studies, purified pro-MMP-8 was readily activated by the nonspecific chemical stimulus APMA, but failed to demonstrate activation following incubation with increasing concentrations of
Figure 2: MMP-9 is constitutively active in CF inpatient samples. MMP-9 activity levels were measured at baseline and following stimulation with 1 mM APMA to activate latent MMP-9 proenzyme. CF inpatient samples demonstrate little activation following APMA treatment compared to other conditions (*p<0.01 compared to inducible activation in normal controls).
HNE (Figure 3B). In contrast, pro-MMP-9 was activated by all concentrations of HNE, with maximal activation seen following incubation with 2.5 ng/ml of HNE (Figure 3C). In additional control experiments, activation of pro-MMP-9 by HNE was reduced by 47% (+/-3.2) following co-treatment with an HNE-specific inhibitor (p<0.01), while APMA activation of pro-MMP-9 was insensitive to similar treatment (Supplemental figure 1). The concentrations of HNE capable of activating Pro-MMP-9 were within the range detected in CF inpatient sputum samples (Figure 3A). HNE incubation had little effect on pro-MMP-8, as all protein remained in its inactive zymogen form (Figure 3B). In contrast, increasing concentrations of HNE selectively depleted the pro-MMP-9 band (92 KDa) relative to active MMP-9 enzyme (78KDa) (Figure 3D).

**MMP-9 and HNE activity correlates in vivo**

Our in vitro (Figure 3) results demonstrated that HNE alone was sufficient to activate pro-MMP-9 in vitro at physiologic concentrations of HNE. Figure 4 demonstrates a close exponential correlation between HNE and MMP-9 in CF inpatient samples (n=16), with a correlation coefficient (R^2) of 0.74. In contrast, neither MMP-9 nor HNE activity correlated with measures of lung function (FEV1, FVC) in both CF inpatient and outpatient samples (R^2 for MMP-9/FEV1=0.01, MMP-9/FVC=0.01 for CF inpatient samples; R^2 for HNE/FEV1=0.1, HNE/FVC=0.1 for CF outpatient samples; data not shown).

**TIMP-1 concentration is reduced in CF lung disease**

MMP-9 is also known to be negatively regulated by TIMP-1, a naturally occurring MMP inhibitor. Figure 5A compares TIMP-1 immunoblots of CF and control sputum,
Figure 3: HNE cleaves and activates pro-MMP-9 but does not activate pro-MMP-8.

3A. Mean HNE levels in sputum samples (±SE) were measured as described in Methods. CF inpatients (n=9) demonstrate approximately 40-fold and CF outpatients (n=10) demonstrate 6-fold increased activity compared to normal controls (n=10) (* p<0.01).

3B. MMP-8 activity (±SE) measurements (10 ng/ml) demonstrate MMP-8 activation following treatment with APMA (1 mM) compared to MMP-8 alone, but increasing concentration of HNE fail to stimulate MMP-8 activity.

3C. MMP-9 activity (±SE) measurements (10 ng/ml) demonstrate MMP-9 activation following treatment with APMA (1 mM) or increasing concentration of HNE compared to MMP-9 alone (* p<0.001). HNE alone (far right, 100 ng/ml) shows no measurable MMP-9 activity.

3D. Recombinant human pro-MMP-8 (R and D Systems at 10 ng/lane, top gel) was also incubated with increasing concentrations of recombinant HNE (2.5, 5, 10) for 2 hours. These samples were separated on a 7.5% reducing gel and subsequently stained with anti-MMP-8 monoclonal antibody (MAB 908, R and D Systems). The 75 KDa proband (black arrow) is not depleted by increasing concentrations of HNE. Recombinant human pro-MMP-9 (R and D Systems at 10 ng/lane, bottom gel) was incubated with increasing concentrations of recombinant HNE (2.5, 5, 10, 100 ng/lane) for 2 hours. These samples were separated on a 7.5% reducing gel and subsequently stained with anti-MMP-9 monoclonal antibody (MAB 911, R and D Systems). The 92 KDa proband (black arrow) is depleted relative to the 78 kDa active enzyme (white arrow) over the HNE concentrations of 2.5-10 ng/lane.
Supplemental Figure 1: MMP-9 activation by HNE is mitigated by HNE inhibitor
MMP-9 activity (±SE) with HNE (10 ng/ml, left panel: white bar) is mitigated by 2 hour co-incubation with an HNE specific inhibitor (1 ug/ml, left panel: gray bar) by approximately 50% († p<0.01). MMP-9 activity is not sensitive to HNE inhibition at baseline (middle) or after activation APMA (1 mM) (right panel) (* p=ns). The dashed line represents baseline MMP-9 activity (prior to HNE or APMA treatment).
Figure 4: MMP-9 activity correlates with HNE activity in vivo.
Recently collected CF inpatient samples (n=16) were examined for endogenous (non-APMA stimulated) MMP-9 activity and HNE activity; these values were normalized for total protein in each sample. A Spearman correlation demonstrated a correlation coefficient of 0.738
confirming variable TIMP-1 detection in all conditions. TIMP-1 concentrations in the CF specimens were depleted relative to control specimens as quantified by ELISA (Figure 5B). When a ratio of total active MMP-9 to TIMP-1 concentration (an established measure of MMP-9 regulation [7, 24]) was calculated for samples in each group, ratios from CF inpatients and outpatients were significantly increased compared to ARF (Figure 5C). Together, these findings suggest that loss of MMP-9 inhibition by TIMP-1 may contribute to the increased MMP-9 activity seen in CF sputum samples.

**TIMP-1 is degraded by HNE in vitro**

TIMP-1 has been previously shown to serve as a substrate for HNE activity [25, 26]. To confirm this as another possible mechanism for MMP-9 dysregulation in CF, recombinant TIMP-1 was incubated with HNE for increasing time periods (Figure 6). HNE (27 kDa) and TIMP-1 (28 kDa), are shown in Figure 6 (lanes H and T, respectively). No change in HNE bands were seen through the course of the experiments (24 hr co-incubation). In contrast, TIMP-1 bands were rapidly degraded by co-incubation with HNE, directly demonstrating that active HNE is sufficient to degrade TIMP-1.

**MMP-9 in CF sputum is sensitive to MMP inhibitory molecules.**

The results shown in Figures 1-4 indicate that MMP-9 is expressed at high levels and is dysregulated in CF sputum, with constitutive activity seen relative to both ARF and normal control samples. To examine whether excessive MMP-9 activity in CF sputum could be reduced or normalized, samples were treated with various established
Figure 5: TIMP-1 levels are reduced and MMP-9/TIMP-1 ratios are increased in CF lower airway secretions.

5A. Western blot (12% SDS nonreduced gel) demonstrates TIMP-1 staining (28 KDa, black arrow) in ARF samples (n=6), CF inpatient (n=3), and CF outpatient (n=3) samples. Each lane is sputum from a separate subject. (“+” = recombinant TIMP-1 control).

5B. Mean TIMP-1 levels (±SE) are shown for CF inpatients (n=22), CF outpatients (n=11), ARF (n=26) and normal controls (n=3) (* p<0.001 for CF inpatient and CF outpatients compared with ARF).

5C. MMP-9 activity was measured in each sample and these values were divided by TIMP-1 concentration for the given samples. The mean values for this ratio are shown for CF inpatients (n=22), CF outpatients (n=11), ARF (n=26), and normal controls (n=3). CF inpatient values are increased approximately 30-fold for CF inpatients († p<0.01 compared to ARF) and 5-fold for CF outpatients (* p<0.05 compared to ARF). Statistical comparisons were limited to CF vs. ARF due to low numbers of normal samples for these analyses.
Figure 6: TIMP-1 is degraded by HNE.
Coomassie blue stain of recombinant human TIMP-1 (100 μg/ml, 28 kDa gray arrow) incubated with recombinant HNE (50 μg/ml, 27 kDa white arrow) for increasing time points (hours as noted). Lane H = HNE alone (4 hours, 37 degrees C), Lane T = TIMP-1 alone (4 hours, 37 degrees C). Black arrow shows the 31 KDa molecular weight marker.
MMP-9 inhibitory molecules [27]. MMP-9 activity in CF sputum was blocked following treatment with EDTA (1 mM), doxycycline (an orally available antimicrobial, 100 µg/ml), and MMP-9 Inhibitor ((C$_{27}$H$_{33}$N$_3$O$_5$S) [Calbiochem, Catalog #444278], a commercially available specific inhibitor of MMP-9, 50 ng/ml)) (Figure 7). The results confirm that the MMP-9 inhibitory effects of these small molecules are retained in CF sputum samples, providing a potential avenue to restore regulation to this protease in vivo.
Figure 7. Dysregulated MMP-9 Activity in CF Sputum is Normalized with MMP-9 Inhibitors
CF sputum samples (n=5) with high MMP-9 activity (±SE) were examined in each of the conditions noted. Samples were incubated with vehicle (DMSO, 1:1000), doxycycline (100 µg/ml), EDTA (5 mM), or MMP-9 specific inhibitor (Calbiochem, Catalog #444278 at 50 ng/ml) for 2 hours followed by measurements of MMP-9 activity (* p<0.01 compared with basal activity).
Discussion

In this report, we examined the expression of MMP isoforms in CF lower airway secretions compared to pediatric patients with ARF and normal controls. A distinct pattern of MMP isoform expression was observed in both CF and ARF patient populations, with neutrophil-derived MMPs (MMP-8,-9) demonstrating prominent expression (Figure 1A and 1C). MMP-9 was the predominant MMP isoform identified in CF samples, with three-fold increased activity in CF outpatient and seven-fold increased activity in CF inpatients compared to normal controls (Figure 1B). Interestingly, MMP-9 was constitutively active in CF inpatients samples, demonstrating little inducible zymogen in these samples relative to the other disease conditions (Figure 2), and pointing toward an important difference in the proteolytic profile seen in CF inpatients.

HNE, a well-established pro-inflammatory serine protease, demonstrated 40-fold increased activity in CF inpatient samples compared to normal controls. In order to examine possible contributions of high HNE activity to the increased MMP-9 activity seen in vivo, we tested whether regulatory relationships between HNE and pro-MMP-9 could be demonstrated in vitro. Our experiments showed that HNE, at physiologic concentrations seen in CF sputum, was sufficient to cleave and activate pro-MMP-9 (Figure 3C and 3D) and degrade TIMP-1 (Figure 6). CF inpatient sputum demonstrated a strong correlation between HNE and MMP-9 activity (Figure 4). Small molecule inhibitors of MMP-9 were active in CF sputum, reducing protease activity by approximately 80% (Figure 7). Together, our results identify markedly elevated MMP-9 activity in CF sputum, and describe two potential mechanisms that contribute to this dysregulation which are mediated by HNE.
**HNE activates MMP-9**

In vivo mixing experiments with purified MMP-9 zymogen and HNE demonstrated that HNE treatment was sufficient to activate MMP-9, converting pro-enzyme to active protease (Figure 3). This effect was specific, as parallel experiments with pro-MMP-8 showed no activating effects via HNE. HNE targets a variety of substrates, and has also been shown to have activating effects on MMP-2 and cathepsin B [28, 29]. Previous work by Ferry et al. have suggested that HNE, a serine protease well known to contribute to CF lung inflammation and damage [20, 30, 31], can enhance MMP-9 activity both in vitro and in a murine model [32]. In that study, activation of MMP-9 was performed in a murine model of acute lung injury (following LPS administration), demonstrating an increased 75 KDa gelatinolytic band. Additionally, the 92 KDa pro-enzyme was prominent when an HNE inhibitor was co-administered to the mice. This study also demonstrated conversion of recombinant pro-MMP-9 to enzyme with HNE incubation. Our studies extend these observations by examining the effects of various physiologic concentrations of HNE found in CF sputum on the conversion of pro-MMP-9 to active protease. Additionally, we utilized an HNE inhibitor to block activation of pro-MMP-9 by HNE, further demonstrating specificity of HNE effects for MMP-9 (Supplemental Figure 1). We then examined HNE and MMP-9 activity in CF inpatient samples, reporting a strong positive correlation between the two proteases in vivo (Figure 4). Together, the results provide strong support for the hypothesis that increased HNE activity in CF sputum can disrupt MMP-9 regulation via pro-enzyme activation.
**HNE degrades TIMP-1**

TIMP-1 is a 184 residue protein containing two N-glycosylated sites [33] and five disulfide linkages [34]. Previous work by Nagase suggests that TIMP-1 is degraded by HNE. In these studies, the degraded TIMP-1 was unable to inhibit active MMP-3 in a dose-dependent manner [25, 26]. Additionally, Shapiro et al have shown in chronic smoke-exposed mice that HNE can cleave and inactivate TIMP-1 and that MMP-12 can cleave and inactivate α-1 antitrypsin, the natural inhibitor of HNE in vivo [35].

Our findings demonstrate a similar pattern of TIMP degradation by HNE. Based on known HNE substrate specificity, at least six potential cleavage sites can be mapped in TIMP-1. Although the crystal structure of the MMP-9:TIMP-1 complex has not been fully characterized, it has been postulated that the interaction is similar to that of MMP-3:TIMP-1 [36].

**Clinical implications of dysregulated MMP-9 activity**

Previous work has led to the protease/antiprotease model of disease, in which antiprotease inactivation by proteases generates a pro-proteolytic environment seen in chronic inflammatory lung diseases [1, 35]. One important observation from our study was the increased protease activity (both total and constitutive) measured in CF inpatients compared with CF outpatients (Figures 1-3), with little change in MMP-9 activity seen over the course of standard inpatient pulmonary care. These results suggest that during periods of acute pulmonary decline, MMP-9 dysregulation is accentuated, possibly due to increased HNE activity. We speculate that this excessive proteolytic activity during inpatient exacerbations may contribute to an augmentation of structural lung dam-
age and airway remodeling in CF patients. Additionally, MMP-9 activity failed to decline in association with treatments shown to reduce other inflammatory markers in the CF airway [18]. One could speculate, therefore, that constitutive MMP-9 activity may serve a pro-inflammatory role in CF. MMP-9 can clip the amino terminus from IL-8, creating a peptide product with chemotactic potency that is enhanced approximately ten-fold above the parent molecule [37]. More recently, our group has characterized a novel collagen-derived peptide in COPD and other diseases of the airways, proline-glycine-proline (PGP), that has prominent neutrophil chemotaxis activity [38]. Based on the substrate specificity of MMP-9, it is plausible that high levels of active enzyme may play a proinflammatory role in CF and other neutrophilic lung diseases through cleavage of structural proteins, leading to a variety of neutrophil chemotactic molecules. A model of this hypothesis is summarized in Figure 8. Clearly, more work is needed to clarify the importance of MMP-9 activity in CF airway disease, as high levels of unregulated enzyme activity has the potential to increase inflammatory signaling, neutrophil load, and to accelerate bronchiectasis.

While this study examined important proteases which are present in CF lung disease, we acknowledge limitations of our study. First, there were differences in the study populations. The CF samples were from significantly older subjects compared with the ARF population; additionally, the CF inpatients had an increased proportion of delta F508 homozygous individuals compared to CF outpatients (although no evidence of milder disease (e.g. pancreatic sufficiency) was observed in either group). Second, the samples collected in CF patients were spontaneously expectorated compared to samples from ARF and control patients, which were obtained through endotracheal suctioning. In
Figure 8. Model of MMP-9, TIMP-1 and HNE cross-regulation.
Both pro-MMP-9 and HNE are released from PMNs. Pro-MMP-9 is converted to active MMP-9 through direct activation via HNE. Additionally, HNE also inactivates TIMP-1, a natural inhibitor of MMP-9. This process of proteolytic cross-talk can lead to increased activation of MMP-9. In addition to augmenting proteolytic damage to the lung parenchyma, dysregulated MMP-9 activity can enhance IL-8 activity and release/modification of other chemotactic molecules/peptides to increase neutrophil influx into the airways.
addition, the ARF samples were pooled over a 5 day period for a given patient as opposed to CF and control samples, which were obtained at single time points. While extensive qualitative bacteriology was available for the CF samples, quantitative cultures were not performed. Finally, while our sputum processing ensured that TIMPs are maintained in the supernatant, it is possible that other nonspecific MMP inhibitors may have been depleted during processing. Each of these potential confounders could influence the protease measurements of the airway, and should be considered in future studies of MMP biology and pathophysiology in CF airway disease.

In summary, our studies demonstrate a unique profile of MMPs expressed in CF sputum. MMP-9 was found to be the predominant active MMP isoform and demonstrated elevated activity compared to its antiprotease, TIMP-1. HNE may disrupt normal regulation of MMP-9 through direct activation of pro-MMP-9 zymogen and inactivation of TIMP-1 in CF airway disease. Restoration of the protease/antiprotease balance in chronic CF airway inflammation may represent a viable strategy to reduce the excessive inflammatory phenotype, and potentially impact tissue remodeling and destruction produced by unopposed MMP-9 proteolytic activity.
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References


13. Shapiro SD. Diverse roles of macrophage matrix metalloproteinases in tissue


MATRIX METALLOPROTEINASE 9 ACTIVITY ENHANCES HOST SUSCEPTIBILITY TO PULMONARY INFECTION WITH TYPE A AND B STRAINS OF FRANCISELLA TULARENSIS

by

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Abstract.

A striking feature of pulmonary infection with the Gram-negative intracellular bacterium *Francisella tularensis*, a category A biological threat agent, is an intense accumulation of inflammatory cells, particularly neutrophils and macrophages, at sites of bacterial replication. Given the essential role played by host matrix metalloproteinases (MMPs) in modulating leukocyte recruitment and the potentially indiscriminate destructive capacity of these cells, we investigated whether MMP-9, an important member of this protease family released by neutrophils and activated macrophages, plays a role in the pathogenesis of respiratory tularemia. We found that *F. tularensis* induced expression of MMP-9 in FVB/NJ mice and that the action of this protease is associated with higher bacterial burdens in pulmonary and extrapulmonary tissues, development of more extensive histopathology predominated by neutrophils, and increased morbidity and mortality compared with mice lacking MMP-9 (MMP-9<sup>−/−</sup>). Moreover, MMP-9<sup>−/−</sup> mice were able to resolve infection with either the virulence-attenuated type B (live vaccine strain) or the highly virulent type A (SchuS4) strain of *F. tularensis*. Disease resolution was accompanied by diminished leukocyte recruitment and reductions in both bacterial burden and proinflammatory cytokine production. Notably, neutrophilic infiltrates were significantly reduced in MMP-9<sup>−/−</sup> mice, owing perhaps to limited release of Pro-Gly-Pro (PGP), a potent neutrophil chemotactic tripeptide released from extracellular matrix through the action of MMP-9. Collectively, these results suggest that MMP-9 activity plays a central role in modulating the clinical course and severity of respiratory tularemia and identifies MMPs as novel targets for therapeutic intervention as a means of modulating neutrophil recruitment.
Introduction.

*Francisella tularensis* exists in two clinically relevant forms, the European biovar B (*holarctica*) that produces acute though mild self-limiting infections and the more virulent U.S. biovar A (*tularensis*) which often is associated with respiratory tularemia and a more severe clinical course [1]. The first reference to pleuropulmonary tularemia is a 1924 report by Verbrycke [2], wherein a number of nodules were described in the lungs of a dying patient. Subsequent studies have revealed that cellular infiltrates within these nodules are composed primarily of neutrophils and macrophages with scattered lymphocytes, red cells, desquamated epithelial cells, and plasma cells [3, 4]. Aerosol challenge with a virulence-attenuated type B (live vaccine strain (LVS) [3]) and the highly virulent type A (SchuS4) strain of *F. tularensis* elicit a comparable clinical picture in the lungs of infected mice [5, 6, 7]. Pulmonary infection with either of these strains causes inflammation of peribronchiolar tissues and is characterized by the exudation of dense infiltrates of neutrophils and macrophages into the bronchiolar lumen. Organisms can be found inside macrophages as early as 20 min postinfection (PI) and focal bronchiolitis is apparent by 24 h at which point considerable replication of bacteria has ensued [8, 9]. It is likely that the explosive replicative capacity of *F. tularensis* contributes to the greater morbidity and mortality associated with pulmonary infection.

It is generally recognized that pro- and anti-inflammatory cytokines play a critical role in modulating the activation state and effector functions of innate inflammatory cells which migrate into sites of bacterial infection. In addition to cytokines, matrix metalloproteinases (MMPs) and a number of potent chemokines along with complement and extracellular matrix (ECM) components are generated within the inflammatory milieu. It is
the coordinate action of these molecules which orchestrate leukocyte extravasation into the inflammatory focus [10, 11, 12, 13, 14]. MMPs comprise a large family of Zn$^{2+}$- and Ca$^{2+}$-dependent endopeptidases whose capacity to degrade or process ECM is associated with tissue remodeling, chronic inflammation, tumor cell metastasis, and more recently, the progression of various infectious diseases [10, 15, 16]. MMP-9, an important member of this protease family, is a 92-kDa type B gelatinase that is secreted as an inactive proenzyme by both neutrophils and macrophages [10, 17]. This pro-MMP-9, subsequently activated by proteolytic cleavage, degrades both ECM (type I, IV, V, VII, XI collagens, fibrin, and laminin) and nonmatrix substrates [18, 19]. MMP-9 acts to establish a concentration gradient of CXCL8 (formerly human IL-8 or murine KC) which directs the diapedesis of neutrophils across vascular endothelium [10, 13], activates proinflammatory cytokines such as TNF-a and IL-6 [10], and is associated with tissue destruction [16].

Chemokines such as KC and MIP-2 initiate the influx of neutrophils into sites of infection. In turn, these cells release collagenase and MMPs (e.g., MMP-9) to generate chemotactic fragments from ECM proteins that amplify the recruitment of inflammatory cells [10, 17]. In 1995, Pfister et al. [20] demonstrated that one such fragment, Pro-Gly-Pro (PGP), is chemotactic for neutrophils and likely results from the hydrolysis of collagen. More recently, it was shown that intratracheal instillation of PGP stimulates neutrophil infiltration into the lungs of C57BL/6 mice [21]. Currently, it is thought that upon its generation, PGP contributes to the maintenance and extension of the neutrophil influx during periods of declining chemokine levels (e.g., during late-stage disease).
There is growing appreciation of the role played by MMPs in infectious disease, particularly their importance in orchestrating the recruitment of innate inflammatory cells and regulating their effector functions subsequent to cellular activation [22]. Therefore, we evaluated the role of MMP-9 in the pathogenesis of respiratory tularemia relying upon the well-characterized mouse model of infection [6, 23, 24, 25, 26]. In the present study, we propose that MMP-9-mediated regulation of the matrix environment, which coordinates the influx of neutrophils, plays a more destructive than protective role in host defense against pulmonary infection with *F. tularensis*. This notion is supported by the higher bacterial burden, increased histopathology, and greater susceptibility to infection seen in wild-type mice compared with those deficient for MMP-9.
Materials and Methods

Mice

Wild-type and congenic MMP-9−/− FVB/NJ mice (The Jackson Laboratory) were housed in the Animal Resources Facility (Albany Medical College, Albany, NY). Food and water were provided ad libitum. All animal procedures conformed to the Institutional Animal Care and Use Committee guidelines. All experiments were conducted using equal numbers of male and female mice of 4–8 wk of age per group.

Bacteria

*F. tularensis* LVS (ATCC 29684; American Type Culture Collection) was provided by Dr. K. Elkins (U.S. Food and Drug Administration, Bethesda, MD). *F. tularensis* SchuS4, originally isolated from a human case of tularemia [27], was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD). All experiments using SchuS4 were conducted within a Centers for Disease Control-certified ABSL-3/BSL-3 facility at Albany Medical College. The bacteria were cultured on modified Mueller-Hinton (MH) agar plates or in modified MH broth (Difco Laboratories) supplemented with ferric pyrophosphate and IsoVitalex (BD Biosciences) and maintained as previously described [28, 29].

Infection

All infection experiments used groups of 6–10 mice that were monitored for survival or euthanized at designated time points PI. An aliquot of *F. tularensis* was thawed, diluted in sterile PBS and mice were inoculated intranasally (i.n.) with 10^1 and 10^2 CFU of *F. tularensis* SchuS4 or 10^3 and 10^4 CFU of *F. tularensis* LVS in a volume of 20 μl of
PBS (10 µl/nare); actual dosages were confirmed by plating. Before i.n. inoculation, animals were deeply anesthetized via i.p. injection of a mixture of ketamine (20 mg/ml) and xylazine (1 mg/ml). Sham-inoculated controls received an equal volume of uninoculated MH broth diluted in PBS. For survival experiments, mice were examined twice daily for morbidity and mortality for a period of 14 days and the median survival was calculated for each group. Euthanized mice were necropsied at various times PI and lung, liver, and spleen were excised aseptically.

**Collection of bronchoalveolar lavage (BAL) fluid (BALF)**

Following infection as described above, BAL was performed on euthanized mice using a 1-cc syringe fitted with a flexible capillary tube attached to a 23 G needle. The capillary tubing was inserted into the anterior portion of the trachea of the mouse. The lungs were slowly perfused with 1 ml of PBS (37°C) taking care to avoid hyperinflation of the lungs. Once instilled, the volume of PBS is recovered using the same syringe and the process repeated once with a new syringe and an additional 1 ml volume of PBS. Aliquots of BALF recovered from individual animals were pooled and stored short-term (<24 h) at 4°C or long-term (>24 h) at –80°C. The volume of lavage fluid recovered is recorded for each mouse and the number of cells contained in the BALF, as enumerated by hemacytometry, was normalized on the basis of the volume recovered. Total protein concentrations in BALF samples were measured using a commercially available assay (Bio-Rad).
**Electron spray ionization-liquid chromatography-mass spectrometry/mass spectrometry (ESI-LC-MS/MS)**

BALF samples were run on an MDS Sciex API-4000 Q-Trap (Applied Biosystems) equipped with a Shimadzu HPLC (Shimadzu). This instrumentation has a limit of detection in the range of 10–20 pg/ml. The HPLC is performed using a Develosil RP-Aqueous C30 (2.0 x 150 mm, 5 µm) column (Phenomenex) with a 20–100% gradient of water containing 0.1% formic acid:acetonitrile. Gradients are run at 0.2 ml/min for 6 min followed by a 4-min equilibration. Mass transitions for acetylated PGP were observed at 312-112 and 312-140. Mass transitions for nonacetylated PGP were observed at 270-70 and 270-116. Peak areas were integrated and quantified based upon standard curves determined using known amounts of both forms of PGP under these conditions.

**MMP-9 zymography**

Gelatinase activity was assayed by the method of Hibbs et al. [30]. Briefly, 50 µg of total protein from lung homogenates was incubated with 1 ml of sterile H2O and 100 µl of gelatin-agarose beads (Sigma-Aldrich) overnight at 4°C to enrich for MMPs. Beads then were centrifuged at 10,000 x g for 1 min, resuspended in nonreducing Laemmli sample buffer (lacking 2-ME) and incubated for 30 min at room temperature (samples are not boiled before loading). After centrifugation at 14,000 x g for 2 min, equal volumes of the eluate were resolved on a 7.5% nonreducing SDS-PAGE containing 4 mg/ml gelatin (Sigma-Aldrich). Following electrophoresis, gels were washed three times with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100, 5 mM CaCl2, and 1 µM ZnCl2 and subsequently incubated for 24 h at 37°C in the same buffer containing 1% Triton X-100. This denaturation/renaturation step promotes MMP activity without proteolytic cleavage of
pro-MMP-9. Gelatin activity was visualized by staining gels with 0.5% Coomassie blue and destaining with methanol/acetic acid; band intensities were quantified using a Fluorochem 8000 Imaging system (Alpha Innotech). Zymography results were confirmed using a mouse pro-MMP-9 ELISA (R&D Systems).

**Bacterial burden**

Portions of lung (20 mg), liver (30 mg), and spleen (5 mg) were homogenized after the addition of 0.5 ml of PBS containing protease inhibitor mixture (Roche Diagnostics) using a mechanical homogenizer (Mini Bead Beater; Biospec Products) and sterile inert Zirconia beads. Homogenates were spun for 10 s in a microcentrifuge at 1000 rpm and 10-fold serial dilutions of clarified supernatant were made in sterile PBS. Ten 1-µl aliquots of each dilution were spotted onto duplicate MH chocolate agar plates as described elsewhere [28, 29]. Quantification was done by counting the colonies on the plates after 48–72 h incubation at 37°C and results were expressed as log_{10} CFU/ml. The remaining lung homogenate was spun in a microcentrifuge at 14,000 rpm for 20 min and the clarified supernatant was used immediately to assay for the presence of cytokines and to measure MMP-9 activity.

**Histopathology**

Lungs, liver, and spleen from *F. tularensis* LVS-infected and sham-inoculated mice sacrificed at day 9 were excised and fixed in 10% neutral-buffered formalin. Lungs were inflated by instillation of PBS into the trachea before fixation. Tissues were processed using standard histological methods to obtain 5-µm-thick paraffin sections and were stained with H&E. For some experiments, the extent of collagen deposition and structural
integrity of fibrillar collagen was assessed in lung sections following Masson’s Trichrome staining using the Accustain Trichrome stain kit (Sigma-Aldrich). Lungs also were assessed microscopically using a histopathologic scoring system and a numerical score for each animal was assigned by adding the subscores from nine parameters as described elsewhere [29]. Each of these parameters was graded from 0 to 3 and then combined for a maximum cumulative score of 27. Lung scores were based on the extent and degree of peribronchiolar/bronchiolar inflammation, bronchial lumen exudation, extent of perivascular infiltration, the frequency and extent of necrotizing/organizing bronchopneumonic patches, the degree of alveolar inflammation, and the type and numbers of inflammatory cells (i.e., neutrophils and/or macrophages) involved in peribronchovascular areas and in the lung parenchyma. Liver sections were evaluated with respect to the quantity and quality (discrete/nondiscrete) of granuloma formation, their distribution and cellular composition. Spleen sections were similarly evaluated for granulomatous lesions within the red and white pulp.

**Differential cell counting**

The percentage of neutrophils infiltrating the lungs of infected mice was determined by modified Wright’s staining of leukocytes isolated from enzymatically digested tissue. Briefly, lungs were perfused with sterile PBS, collected aseptically, finely cut with a scalpel, and incubated for 60 min at 37°C with digestion buffer (PBS containing 0.1% BSA, 0.01 M MgCl₂, and 7 mM NaN₃) to which 250 µg/ml DNase I and 3.3 mg/ml collagenase A was added. Cell suspensions were passed through a 70-µm nylon cell strainer (BD Biosciences) and cytospun onto polylysine-coated slides. Slides were prepared using
a standard, modified Wright’s staining protocol and leukocytes were enumerated by light microscopy.

**Cytokine measurements**

Lung homogenates were assayed for the presence of pro- and anti-inflammatory cytokines using Cytometric Bead Array flex sets (BD Pharmingen) which allow for simultaneous measurement of MCP-1, KC, TNF-α, IL-6, IFN-γ, IL12-p70, and IL-10 levels. Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Immunocytometry Systems (BDIS)). Data were acquired and analyzed using BD FACSAarray software and FCAP Array software, version 1.0 (BDIS), respectively. The limits of detection for MCP-1, KC, TNF-α, IL-6, IFN-γ, IL-12p70, and IL-10 are 29, 16.2, 17.1, 6.5, 5.2, 9.2, and 16.4 pg/ml, respectively. Murine MIP-2 was measured using a commercially available kit (BioSource International) following the manufacturer’s instructions.

**Statistical analysis**

A log-rank test was used to determine the level of significance for the Kaplan-Meier survival analyses. All other results were expressed as mean ± SEM and comparisons between the groups were made using one-way ANOVA followed by Bonferroni’s correction, nonparametric Mann-Whitney $U$ test, or Student’s $t$ test. Differences between control and experimental groups were considered significant at a $p < 0.05$ level.
Results.

*F. tularensis* LVS induces expression of MMP-9

To begin evaluating the role of MMP-9 in tularemia pathogenesis we determined whether *F. tularensis* has the capacity to promote its release during infection. Lungs were excised from mice at various times following i.n. infection and tissue homogenates were assayed by gelatin zymography which specifically reveals MMP-9 and MMP-2 expression. It was observed that MMP-9 levels were substantially elevated above sham-inoculated controls by day 3 and remained so for the duration of the experiment (Fig. 1, A and B). In contrast, MMP-2 levels were unaltered from baseline. As expected, no MMP-9 expression was associated with lung homogenates recovered from mice with a targeted mutation of the *mmp9* gene (Fig. 1A). A more quantitative way of measuring MMP-9 levels than gel zymography is by ELISA, therefore, this latter methodology was used to corroborate the findings presented in Fig. 1, A and B. As measured by ELISA, the pattern of pro-MMP-9 expression in lung homogenates was similar to that observed in zymograms, beginning at day 3 PI and continuing through day 9 (Fig. 1C).
Figure 1. *F. tularensis* induces in vivo production of MMP-9. FVB/NJ mice were infected with $10^3$ CFU of *F. tularensis* LVS, sacrificed at various time points and lung homogenates were assayed by gelatin zymography for the presence of MMP-9 and -2 (A). MMP-9/ mice served as a negative control. MMP-9 band intensities were normalized to MMP-2 levels and shown as a fold increase above baseline levels (B). Pro-MMP-9 levels were measured by commercial ELISA (C). Results are derived from lung homogenates pooled from four to six individual animals and are representative of three independent experiments (12–18 mice total).
F. tularensis-induced MMP-9 activity associated with greater morbidity and mortality

MMP-9 activity may engender extensive cellular infiltration of the lungs by neutrophils and macrophages, the latter of which supports bacterial replication and thus would increase susceptibility to tularemia. Further, the combination of degradative molecules released by activated neutrophils and MMP-induced tissue destruction contributes to pathophysiological changes in the lung. To test these notions, MMP-9+/+ and congenic MMP-9−/− mice were infected i.n. with 10³ and 10⁴ CFU of F. tularensis LVS and their relative susceptibility was evaluated on the basis of the cumulative proportion of mice surviving and the median time to death (MTD) of the experimental group. MMP-9+/+ mice began to die in response to a 10⁴ CFU dose 9 days PI. The MTD for this group was 10 days and 83% of the mice were dead by day 14 (Fig. 2A). In contrast, no mortality was recorded in the MMP-9−/− group until day 13 and by day 14 only 17% of the mice had succumbed to infection with a MTD of >14 days, a significant difference compared with the wild-type controls (p < 0.01). Following infection with 10³ CFU of F. tularensis LVS, 33% of the MMP-9+/+ mice died while all of those deficient for MMP-9 survived (p < 0.05) (Fig. 2A).

Given the highly virulent nature of type A strains of F. tularensis and their potential for use as a biological threat agent, it was of interest to determine whether MMP-9 deficiency could alter the course of disease caused by infection with SchuS4. When mice were inoculated with 10¹ CFU of F. tularensis SchuS4, 100% of the MMP-9+/+ mice succumbed to infection by the eighth day with a MTD of 6 days. In contrast, only 50% of the MMP-9−/− mice died by day 14 with a MTD of 11 days (Fig. 2B). However, no difference was observed between the two groups when challenged with 10² CFU suggesting that
Figure 2. MMP-9 deficiency enhances host resistance to *F. tularensis* infection.
MMP-9+/+ and MMP-9−/− mice were inoculated i.n. with 10^3 and 10^4 CFU of *F. tularensis* LVS (A) or 10^1 and 10^2 CFU of *F. tularensis* SchuS4 (B) and monitored for morbidity and mortality. Results are expressed as Kaplan-Meier curves and *p* values determined using log-rank test. The results shown are representative of three independent experiments (*n* = 6 mice per group or 18 mice total).
greater numbers of *F. tularensis* can overcome the resistance phenotype conferred by MMP-9 deficiency. All mice surviving challenge with either the LVS or SchuS4 strain were sacrificed at day 21 and infection was confirmed by ELISA-based serological measurement of anti-*F. tularensis*-specific IgG Ab levels (our unpublished data). These results demonstrate that MMP-9<sup>−/−</sup> mice exhibit a diminished morbidity and mortality associated with pulmonary infection of *F. tularensis*. Importantly, MMP-9 deficiency does not alter the mRNA levels of other MMPs or tissue inhibitors of metalloproteases [31], nor are compensatory changes in the enzymatic activity of other MMPs observed in MMP-9<sup>−/−</sup> animals [32]. Thus, the more resistant phenotype in this model of *F. tularensis* infection can be attributed to the lack of MMP-9 activity. To our knowledge, this is the first report of a host gene whose absence enhances resistance to challenge with a type A strain of *F. tularensis*.

**F. tularensis LVS-infected MMP-9<sup>−/−</sup> mice have a significantly lower bacterial burden in pulmonary and extrapulmonary tissues**

Based upon the greater resistance observed in MMP-9<sup>−/−</sup> animals, we postulated this disease phenotype might be associated with a lower bacterial burden in infected tissues. In fact, the number of bacteria found in tissues from MMP-9<sup>−/−</sup> mice were 1–1.5 log<sub>10</sub> CFU lower than that found in MMP-9<sup>+/+</sup> mice (Fig. 3). In lung and liver, a significant difference in bacterial burden was observed between groups as early as 7 days PI, while splenic burdens did not differ significantly until the ninth day. In other experiments, it was determined that, unlike in MMP-9<sup>+/+</sup> mice, *F. tularensis* LVS and SchuS4 were cleared entirely from the tissues of surviving MMP-9<sup>−/−</sup> animals by day 21 PI (unpublished data).
Figure 3: MMP-9+/mice harbor significantly fewer bacteria in affected tissues. MMP 9+/ and MMP-9−/− mice were inoculated i.n. with 10^3 CFU of F. tularensis LVS. At the times indicated, mice were sacrificed and homogenates of the lungs, liver, and spleen were plated for determination of bacterial burden. Results shown are the mean SEM and are representative of two independent experiments (n =6 mice per time point or 12 mice total). p <0.001 using the one-way ANOVA.
MMP-9<sup>−/−</sup> mice develop less severe pathology in lung, liver, and spleen in response to *F. tularensis* LVS

MMP-9<sup>−/−</sup> mice infected i.n. with 10<sup>3</sup> CFU of *F. tularensis* LVS underwent histological evaluation to determine whether increased survival and a lower bacterial burden was associated with diminished tissue pathology (Table I and Fig. 4). Beginning at day 3 and continuing throughout the course of infection, MMP-9<sup>−/−</sup> mice exhibited less severe histopathological changes in the lungs compared with their MMP-9<sup>+/+</sup> counterparts (*p* < 0.001) (Table I). The difference in mean histopathological scores was greatest at day 9 PI (MMP-9<sup>+/+</sup> mice, 23.6 ± 0.2 vs MMP-9<sup>−/−</sup>, 9.6 ± 1.3) as corroborated by the extent of inflammation in the lungs of infected wild-type vs MMP-9<sup>−/−</sup> mice (Fig. 4). Wild-type lungs showed severe pathology characterized by infiltration of macrophages and lymphocytes around the majority of bronchi/bronchioles and accompanying blood vessels. Hypertrophy of bronchiolar walls, thickening of the alveolar septa and exudation of macrophages and neutrophils within the alveolar lumen were a consistent finding. Additionally, many discrete to diffuse disseminated necrotic pneumonic patches predominated by neutrophils (see inset) were seen within the lung parenchyma. The alveoli around such necrotic patches contained fibrinous exudate admixed with neutrophilic debris and macrophages. In marked contrast, the lungs of MMP-9<sup>−/−</sup> mice showed a lesser degree of pathological changes in the airways and parenchyma (Fig. 4). The inflammatory reaction showed mild to moderate interrupted cuffs of mononuclear cells (lymphocytes, macrophages, and fibroblasts) and only slight thickening of alveolar walls. Few chronic pneumatic patches comprised almost exclusively of macrophages and fibroblasts were seen mostly adjacent to inflamed airways and blood vessels. One of the more intriguing histopathological fea
Table I. Histological scores reflecting the degree of F. tularensis-induced lung pathology

<table>
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<th>Days PI</th>
<th>MMP-9(^{+/+})</th>
<th>MMP-9(^{-/-})</th>
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<tr>
<td>1</td>
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<td>4.1 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>16.1 ± 0.6(^{a})</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>17.0 ± 0.4(^{a})</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>7</td>
<td>18.8 ± 0.8(^{a})</td>
<td>10.8 ± 0.9</td>
</tr>
<tr>
<td>9</td>
<td>23.6 ± 0.2(^{a})</td>
<td>9.6 ± 1.3</td>
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\(^a\), *p < 0.001.*
Figure 4. MMP-9−/− mice exhibit less severe histopathological changes than their wild-type counterparts.
Lungs, liver, and spleen of MMP-9−/− and MMP-9+/+ mice were evaluated at day 9 post-i.n. inoculation with 10^3 CFU of *F. tularensis* LVS. Sham-inoculated FVB/NJ mice served as a control. *Inset* in MMP-9+/+ lung panel shows a necrotic lesion containing neutrophilic debris. *Inset* in MMP-9−/− lung panel has an accumulation of macrophages with evidence of only minimal neutrophilic infiltration. Arrows in liver panels indicate nascent granulomas and those in the spleen indicate an area of intense lymphoproliferation that is absent in the spleens of infected MMP-9−/− mice. Magnification is as follows: lung (40 X), liver and spleen (100 X), and insets (200 X).
tures that distinguished the MMP-9\(^{-/-}\) mouse from its wild-type counterpart is the decreased number of neutrophils in the lungs of the former.

The livers of MMP-9\(^{+/+}\) mice showed rarefied hepatocytes around the central veins, prominent Kupffer cells and stray lymphocytes in the sinusoids (Fig. 4). Many discrete microgranulomas (more than five per field) were found disseminated in different hepatic lobules adjacent to the vessels in the portal areas and a few within the lobules. These granulomas consisted of macrophages (of mostly >50 cells), neutrophils, few lymphocytes, red cells, and occasional apoptotic hepatocytes. Hepatocytes surrounding the granulomas were swollen and had eosinophilic cytoplasm. In contrast, liver changes in MMP-9\(^{-/-}\) mice were considerably less severe as evidenced by the presence of minute and fewer numbers of microgranulomas (two to three per field) having loosely arranged macrophages (mostly 10–15 cells) and lymphocytes.

The spleen of MMP-9\(^{+/+}\) mice revealed hypertrophy of the majority of the white pulp with pale areas and an increased number of marginal zone cells (Fig. 4). The red pulp was moderately engorged and had mild to moderate neutrophil, macrophage, and lymphocytic infiltration. MMP-9\(^{-/-}\) mice showed considerably fewer hypertrophied splenic follicles with pale areas, while the red pulp contained reduced numbers of macrophages and no infiltrating neutrophils.
The lungs of F. tularensis-infected MMP-9<sup>−/−</sup> mice harbor quantitatively fewer neutrophils than are found in MMP-9<sup>+/+</sup> mice

In addition to differences in the overall extent of cellular infiltration, histological evaluation suggested that MMP-9 deficiency results in neutrophils representing a lower percentage of the total leukocyte population in the lungs. To quantify the percentage of different inflammatory cell types in the lung, we determined cell numbers after modified Wright’s staining of leukocytes recovered from enzymatically digested tissues. As seen in Fig. 5, as early as 6 h PI neutrophils represented a significantly lower percentage of leukocytes in MMP-9<sup>−/−</sup> mice than in their wild-type counterparts. This difference also was observed at 1, 7, and 9 days PI and just missed statistical significance at days 3 and 5. In contrast, the percentage of macrophages comprising the inflammatory infiltrate was unaltered by MMP-9 deficiency and lymphocytes represented a larger percentage of the total cell population compared with MMP-9<sup>+/+</sup> mice (our unpublished data).

MMP-9<sup>−/−</sup> mice exhibit an altered profile of cytokine production following i.n. challenge with F. tularensis LVS

The elaboration of pro- and anti-inflammatory mediators is a reflection of the host immune response to pathogens. To explore whether the production of immunomodulators is altered by MMP-9 deficiency, we determined the levels of MCP-1, KC, TNF-α, IL-6, IFN-γ, IL-12p70, and IL-10 at various time points post-i.n. inoculation with 10<sup>3</sup> CFU of F. tularensis LVS, a dosage found to be nonlethal during the first week of infection. Given the initial "centralized" nature of the immune response to pulmonary infection, we chose to measure tissue-resident levels of these cytokines/chemokines. As seen in Fig. 6, MCP-1, KC, TNF-α, and IL-6 were similarly elevated in both MMP-9<sup>+/+</sup> and MMP-9<sup>−/−</sup>
Figure 5. MMP-9 deficiency impairs neutrophil recruitment into the lungs of mice infected with *F. tularensis*.
Differential cell counting was performed on leukocytes liberated from the enzymatically digested lungs of MMP-9 *+/+* and MMP-9 *−/−* mice inoculated i.n. with $10^3$ CFU of *F. tularensis* LVS. Results represent the mean percentage ± SEM of neutrophils determined for each group and are cumulative results of two independent experiments ($n$ =3 mice per group or 6 mice total), $p < 0.01$ using the one-way ANOVA.
Figure 6. MMP-9+/+ mice produce higher levels of proinflammatory cytokines than those deficient for MMP-9.
MMP-9 +/+ and MMP-9 −/− mice were inoculated i.n. with 103 CFU of *F. tularensis* LVS. At the times indicated, mice were sacrificed and homogenates of the lungs were used to measure cytokine levels with sham inoculated mice serving as controls. Results shown are the mean SEM and are representative of two independent experiments (*n* = 6 mice per time point or 12 mice total), *p* < 0.05, *p* < 0.01 using the one-way ANOVA.
mice until day 9 at which point their levels returned to baseline in MMP-9\(-/-\) mice, but remained elevated in their wild-type counterparts. Production of another potent neutrophil chemoattractant, MIP-2, mirrored that of KC insofar as no difference was observed between mice until day 9 PI at which point levels of this chemokine were higher in wild-type animals (unpublished data). Also in MMP-9\(^{+/+}\) mice, IFN-\(\gamma\) levels rose faster, achieved higher levels and remained elevated as compared with that found in MMP-9\(-/-\) mice where IFN- release returned to preinfection levels by day nine PI (Fig. 5). F. tularensis LVS stimulated IL-12p70 release in both groups of mice, however, only at day 3 were the levels higher in the MMP-9\(^{+/+}\) than in MMP-9\(-/-\) mice. It is worth noting that the level of IL-10 in both mouse genotypes remained below detectable limits under these experimental conditions.

**MMP-9 deficiency impairs degradation of collagen whose deposition is increased during F. tularensis infection**

Respiratory infection of mice by *F. tularensis* LVS results in considerable deposition of collagen beneath the basement membrane and throughout the extracellular space surrounding the bronchioles and venules of the lung (Fig. 7A). By day 9 PI, both deposited collagen and dense cellular infiltrates remain prominent features of inflammation in the lungs of wild-type mice. In contrast, a lesser degree of collagen deposition and many fewer inflammatory cells are observed in MMP-9\(-/-\) mice and this inflammatory response to *F. tularensis* appears self-limiting as it is almost entirely resolved by the ninth day of infection. Another striking and consistent difference is that the structural integrity of collagen fibrils in MMP-9\(^{+/+}\) mice indicates extensive proteolytic cleavage into ECM frag-
ments while dense collagen bundles are essentially intact within the lungs of mice lacking MMP-9 (Fig. 7B).

**Infection of mice with *F. tularensis* LVS induces the MMP-9-dependent generation of PGP**

Based upon the findings presented in Fig. 7B, one would predict that PGP levels in the lungs of wild-type mice would be higher than in their MMP-9−/− counterparts. To determine whether this is the case, BALF was recovered from mice infected i.n. with 10^3 CFU of the LVS at different times PI and were assayed by ESI-LC-MS/MS for the presence of PGP. Consistent with the lack of MMP-9 activity and the intact state of collagen fibrils, there was significantly less PGP in the lungs of MMP-9−/− mice at days 1, 3, 5, 7, and 9 PI compared with their wild-type counterparts (p < 0.0001, 0.0236, 0.0037, 0.0083, and 0.0034, respectively) (Fig. 8).
Figure 7. MMP-9<sup>−/−</sup> mice exhibit less intense and self-limiting inflammatory cell recruitment and minimal collagen degradation in response to <i>F. tularensis</i> infection. Lung sections of MMP-9<sup>+/+</sup> and MMP-9<sup>−/−</sup> mice inoculated i.n. with 10<sup>3</sup> CFU of <i>F. tularensis</i> LVS were stained with Masson’s Trichrome. Sections were evaluated on the basis of leukocyte recruitment and collagen deposition at various times PI (A, magnification, 200 X), and the integrity of collagen fibrils on the fifth day (B, magnification 1000 X, oil immersion).
Figure 8. Significantly less PGP is generated in the *F. tularensis*-infected lungs of MMP-9⁻/⁻ mice. BALF was recovered from mice infected i.n. with $10^3$ CFU of the LVS at different times PI and was assayed by ESI-LC-MS/MS for the presence of PGP. Results shown are the mean ± SEM ($n=6$ mice per time point), $p=0.001$ using the one-way ANOVA.
Discussion.

Within the lung, epithelia and alveolar macrophages are resident cellular responders to inhaled bacteria. Activation of these cell types, along with endothelial cells lining the pulmonary vasculature, results in the up-regulation of IL-1, CXCL8, TNF-α, MCP-1, and members of the selectin, integrin, and Ig superfamily of adhesion molecules. *F. tularensis* LVS potently induces the secretion of CXCL8 by and the expression of adhesion molecules on HUVECs [33]. Collectively, these adhesion molecules and chemokines play a critical role in directing the emigration of neutrophils and peripheral blood macrophages into infected tissues. Recruitment of host cells into an inflammatory focus is an important first step in tularemia pathogenesis because *F. tularensis* replicates within macrophages and dendritic cells [34, 35]. Leukocyte recruitment is facilitated by the action of a large family of metalloproteinases whose members are responsible for remodeling the ECM, conversion of inactive cytokines/chemokines to their active form, and establishment of chemotactic gradients that direct the movement of leukocytes across epithelial and endothelial barriers [19]. One such member of this family is MMP-9, a type B gelatinase released from granular stores within activated neutrophils and also secreted by activated macrophages [17].

Herein, we report that *F. tularensis* LVS triggers an influx of innate inflammatory cells, principally neutrophils and macrophages, in wild-type mice that far exceed the number found in MMP-9−/− mice. The greater density of cells found in the lungs of mice expressing MMP-9 is associated with a higher bacterial burden and prolonged release of a number of proinflammatory cytokines and chemokines, an observation consistent with the fact that activated neutrophils and macrophages are the primary source of these potent
immunomodulators. With respect to the production of neutrophil chemoattractants, one of the more unexpected results was the similarity in KC and MIP-2 levels seen in wild-type and MMP-9−/− mice for much of the course of disease. A newly described mechanism for neutrophil recruitment involves the generation of PGP via MMP-9-mediated collagen degradation [21]. Sharing sequence homology with CXCL8/KC [21], PGP binds to the same CXCR1/2 receptors on neutrophils as does CXCL8/KC thus directing their movement into inflammatory sites [36, 37]. Histological evaluation of collagen integrity in the lungs of F. tularensis-infected MMP-9−/− mice suggests limited breakdown of collagen compared with that observed in the lungs of wild-type mice. Entirely consistent with this difference in ECM degradation are the significantly lower levels of PGP found in mice lacking MMP-9 (Fig. 8) and the lower number of neutrophils infiltrating the lung parenchyma of these animals (Fig. 3). The notion that generation of PGP plays an important auxiliary role in neutrophil recruitment during the acute phase of respiratory tularemia is currently under investigation.

In other studies, MMP-9−/− mice exhibit altered patterns of neutrophil migration in a model of allergic lung inflammation [38], less severe Ab-induced arthritis [39], and are protected against lethal endotoxic shock [31]. The ability of MMP-9 deficiency to protect mice against challenge with LPS is particularly intriguing and consistent with our findings. Dubois et al. [31] found that LPS stimulates the release of MMP-9 both in vitro and in vivo. Further, the release of preformed stores of MMP-9 by degranulating neutrophils precedes secretion of proinflammatory cytokines such as TNF-α suggesting a direct effect. Although F. tularensis possess LPS this molecule is quite impotent as a proinflammatory agonist [40, 41, 42], and recently was demonstrated to be incapable of activating
neutrophils due to its inability to engage the cell surface [43]. The capacity of other *F. tularensis*-associated molecular patterns to trigger the release of granular stores or to stimulate de novo production of MMP-9 is being evaluated. Other bacterial components known to induce MMP-9 activity, which *F. tularensis* also possess [44], include lipoproteins such as those found in *Borrelia burgdorferi*, a Gram-negative bacterium which happens to lack LPS [45].

The release of inflammatory mediators (e.g., cytokines, lytic enzymes, and reactive oxygen and nitrogen species, etc.) from neutrophils and the subsequent degradation of ECM are believed to be key factors contributing to multiple organ failure [46]. Elegant studies conducted by Bosio et al. [47], further support the notion that neutrophils play a key pathogenic role in mediating host susceptibility to *F. tularensis* LVS insofar as B cell-deficient mice succumb to infection more readily than their wild-type counterparts. Enhanced susceptibility could not be mitigated by transfer of specific Abs and was not related to differences in macrophage phagocytic/antimicrobial function or cytokine production. Rather, transfer of primed B cells re-established a resistant phenotype and was associated with diminished bacterial burden and decreased neutrophilia, findings consistent with our studies using MMP-9<sup>−/−</sup> mice. It was postulated that excessive neutrophilia during *F. tularensis* infection of B cell-deficient mice is detrimental and results in marked tissue pathology [47], a notion supported by similar findings in a B cell-deficient murine model of *Leishmania donovani* [48].

Notwithstanding the current study and these other reports, it also is recognized that neutrophils are critical for host defense against lethal challenge with *F. tularensis*
[23, 49, 50, 51]. Considering this dichotomy in the role of neutrophils in tularemia pathogenesis we propose that the host’s immune response to \( F. \textit{tularensis} \) must strike a fine balance between one which is too muted and thus ineffectual in killing and clearing organisms and one which is overexuberant to the point of causing organ failure and death. This notion of achieving a "just right" response likely holds true for other cellular and soluble mediators of the innate and adaptive immune response to infection as well.

In summary, our study suggests a mechanism whereby MMP-9 production, whose activity may enhance infiltration of \( F. \textit{tularensis} \)-infected lungs by neutrophils and macrophages, enhances the morbidity and mortality associated with respiratory tularemia. The former cell type likely contributes to the greater destruction of the lung parenchyma in MMP-9\(^{+/+}\) mice while the latter exacerbates this pathology by supporting greater bacterial replication than is found in mice lacking MMP-9. These results argue strongly that MMP-9 plays an important role in modulating the intensity of the inflammatory response to both type A and B strains of \( F. \textit{tularensis} \). Selective blockade of this ECM-degrading protease through pharmacological or molecular genetic strategies may provide a novel adjunct to antibiotic-based therapeutic intervention in respiratory tularemia.
Acknowledgements.

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A NOVEL PROTEOLYTIC CASCADE GENERATES AN EXTRACELLULAR MATRIX-DERIVED NEUTROPHIL CHEMOATTRACTANT IN CYSTIC FIBROSIS

by

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Abstract.

Chronic neutrophilic inflammation is a manifestation of a variety of lung diseases, such as cystic fibrosis (CF), and is responsible for increased morbidity/mortality worldwide. Recently, we have described a novel collagen-derived peptide, proline-glycine-proline (PGP), which acts as a neutrophil chemoattractant through CXC receptors (CXCR)-1 and -2 (Weathington et al. 2006. *Nature Medicine* 12:317-323). Here, we explore PGP levels in CF and the mechanisms involved in PGP generation. Sputum from CF subjects has elevated levels of PGP and these secretions can generate PGP from collagen. We further demonstrate that PGP production is a multi-step process. Two matrix metalloproteases (MMPs), MMP-8 and MMP-9, are involved in the initial cleavage of collagen to fragments. The liberation of PGP from these fragments is then catalyzed by prolyl endopeptidase (PE), a unique serine protease elevated in CF secretions. The generation of PGP correlates closely with airway neutrophil counts after administration of proteases in vivo. Finally, we show that the generation of PGP can be blocked by the use of PE, MMP-8, or MMP-9 specific inhibitors. The determination of this mechanism highlights unique protease/protease interactions regulating innate immunity and allows for the identification of logical targets for disease-modifying therapeutics for inflammatory lung diseases such as CF.
Introduction.

Neutrophils are important mediators in a variety of chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) [1] and cystic fibrosis (CF) [2]. Neutrophil influx into these chronically inflamed airways propagates damage via multiple mechanisms including oxidant injury and the release of proteolytic enzymes [3].

The major chemoattractants for neutrophils in these conditions are Glu-Leu-Arg positive (ELR+) CXC chemokines including IL-8, GRO-α, GRO-β, and GRO-γ (human) and KC and MIP-2 (mouse) [4]. Collagen-derived fragments have also been reported to induce neutrophil chemotaxis [5]. Our group has recently described the role of a specific collagen-derived peptide, proline-glycine-proline (PGP), in neutrophilic lung inflammation [6]. This peptide can be acetylated to create a 4 to 10-fold more potent chemotactic peptide (N-α-PGP), although the mechanism of this acetylation is unknown. N-α-PGP and PGP (N-α-PGP/PGP), via structural homology to most ELR+ CXC chemokines, act as neutrophil chemoattractants through CXCR1 and CXCR2 on neutrophils. This novel CXC ligand demonstrates the ability to not only induce neutrophil chemotaxis but also to induce superoxide release from neutrophils via CXCR1 binding. The kinetics of aerosolized lipopolysaccharide (LPS) administration to mice demonstrates that initial neutrophil influx is dependent on traditional ELR+ chemokines (KC and MIP-2) but is maintained and augmented by PGP until neutrophils are cleared from the airways, concomitant with declining PGP levels in the airway.
Despite the presence of PGP in models of LPS-induced airway inflammation, it is unknown whether PGP may serve as a biomarker in CF. In addition, while the biological properties of these peptides are becoming increasingly understood, the system by which PGP is generated is not known. One possible mechanism by which PGP is produced is through the cleavage of collagen (where there are many ‘PGP’ repeats in the sequence) through the action of proteolytic enzymes.

Recent models of airway inflammation indicate that protease/antiprotease imbalance is a prime feature in several pulmonary diseases including CF [7] and COPD [8]. One class of proteases felt to play an important role in airway remodeling in lung disease is matrix metalloproteases (MMPs), a family of zinc containing endopeptidases with the capacity to degrade multiple components of the extracellular matrix [9]. Recently, our laboratory has shown the presence of discrete MMPs in the sputum of patients with CF [10]. Due to their capacity for matrix degradation and the presence of these proteases in chronic neutrophilic lung diseases, we hypothesized that MMPs may play a role in PGP liberation from collagen.

Therefore, the aim of this study was to determine whether N-α-PGP/PGP may serve as biomarkers in CF and to determine the proteolytic mechanisms involved in PGP generation from collagen. In this report, we describe the presence of PGP-containing peptides in significantly increased quantities in the sputum of CF individuals compared to healthy subjects and demonstrate the capacity of CF sputum to generate PGP from intact collagen in vitro. We further describe the proteolytic system involved in PGP generation as a step-wise process utilizing the coordinated efforts of MMPs (MMP-8 and MMP-9) and prolyl endopeptidase (PE), a serine protease herein described for the first time with a
possible central role in airway inflammation. Finally, we demonstrate that inhibition of these specific proteases blocks *ex vivo* generation of PGP by CF sputum, pointing to a possible role of these inhibitors as therapeutics in chronic neutrophilic lung diseases such as CF.
Methods.

Materials

Recombinant MMP-9, MMP-8, and MMP-12 were purchased from R and D Systems (Minneapolis, MN). Recombinant HNE, HNE specific inhibitor, MMP-9 specific inhibitor, MMP-8 specific inhibitor, MMP-2 specific inhibitor, and PE inhibitor were purchased from Calbiochem (San Diego, CA). PE was purchased from US Biologicals. PE substrate was purchased from Chem-Impex (Woodale, IL). Type I and II collagen were purchased from Sigma (St Louis, MO).

Enzyme Inhibitors:

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<tr>
<td>MMP-9</td>
<td>C$<em>{27}$H$</em>{33}$N$_3$O$_5$S (Calbiochem)</td>
<td>$IC_{50} = 5$ nM</td>
<td>[27]</td>
</tr>
<tr>
<td>MMP-2</td>
<td>cis-9-Octadecenoyl-N-hydroxylamide (Calbiochem)</td>
<td>$K_i = 1.7$ $\mu$M</td>
<td>[28]</td>
</tr>
<tr>
<td>HNE</td>
<td>N-(2-(4-(2,2Dimethylpropionyloxy)phenylsulfonylamino)benzoyl) aminoacetic acid N-(o-(p-Pivaloxybenzene) sulfonylaminobenzoyl) glycine (Calbiochem)</td>
<td>$IC_{50} = 50$ nM</td>
<td>[29]</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>a-6-Deoxy-5-hydroxytetracycline (Calbiochem)</td>
<td>Nonspecific MMP inhib.</td>
<td>[30]</td>
</tr>
</tbody>
</table>

ESI-LC/MS/MS for PGP detection:

PGP and N-$\alpha$-PGP were measured in *in vitro* and in sputum samples using a MDS Sciex (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu HPLC. HPLC was done using a 2.1 x 150 mm Develosil C30 column (with A: 0.1% formic acid and B: acetonitrile +0.1% formic acid). 0 min-0.6 min 20% B, 0.6-5 min the gradient is up to 100% B. Background removed by flushing with 100% isopropanol +0.1% formic. 
acid. Positive electrospray mass transitions are at 270-70 and 270-116 for PGP and 312-140 and 312-112 of N-α-PGP.

**In vivo murine administration:**

Mice underwent intratracheal protease administration as described in figure legend 3. The concentrations of proteases administered were: MMP-8, MMP-9, MMP-12: 55.6 ug/kg, HNE: 200 ug/kg, PE: 18.4 mg/kg. The relative enzyme activities are:

- **PE enzyme activity:** 1 unit=1mM pNA/min @ 30 deg C, pH 7; specific activity of 22.6 units/mg of PE
- **MMP-9 enzyme activity:** 10 uM ES001 (substrate) and 20 ng MMP-9 = 1300 pmoles/min/ug at 37 deg C
- **MMP-8 enzyme activity:** 10 uM ES001 (substrate) and 50 ng MMP-8 = 250 pmoles/min/ug at 37 deg C
- **MMP-12 enzyme activity:** 10 uM ES001 (substrate) and 20 ng MMP-12 = 500 pmol/min/ug at 37 deg C
- **HNE enzyme activity:** 22 units/mg protein. (1 unit=hydrolysis of 1.0 umol pNA/min at 25 deg C, pH 8.0)

**Bronchoalveolar lavage:**

After mice were euthanized with phenobarbital, mice underwent bilateral thoracotomy and were lavaged with four 1-ml aliquots of cold phosphate-buffered saline.

**Sputum processing:**

Sputum was collected and diluted 1:2 with 0.9% normal saline, centrifuged at 1000 RPM for 15 minutes, and supernatant collected. Protein concentration was measured, and then separate aliquots were saved for measurements (MMP, elastase, PE) and mass spectrometry (N-α-PGP/PGP).

**Prolyl endopeptidase activity assay:**

20 µl of sputum was incubated with a specific substrate (2mM Z-Gly-Pro-pNa) at 37°C and 5% CO₂ and cleavage of para-nitroaniline (pNa) from the substrate by PE de-
detected using a spectrophotometer at 410nm and compared to a generated standard curve for PE activity.

**Ex vivo collagen assay:**

100 µl of saline-diluted sputum was incubated with extensively dialyzed, intact type I or II collagen (50 µl, 1 mg/ml) for 24 hours at 37°C and 5% CO₂. The samples were filtered through a 10 kDa filter, washed with 20 µl of 1N HCl and analyzed using ESI- LC-MS/MS for levels of PGP and N-α PGP. Amounts of PGP and N-α PGP generated by each sputum sample were determined by subtracting basal levels already present in each sample.

Thereafter, CF sputum samples were individually evaluated and the most active samples were pooled. For the inhibitor experiments, these pooled sputums were treated with noted inhibitor and allowed to incubate for 6 hours. At 6 hours, dialyzed collagen was added to the sample and sample was further incubated for 18 hours. Inhibitor concentrations utilized: MMP-8, -9, -2 @ 50 uM, PE @ 100 uM, and doxycycline at 1mM.

**Statistical testing:**

We used Instat software (Graphpad). Two-tailed Student t-test were used for simple significant testing and ANOVA for comparing 3 or more groups. Means presented ±SEM; statistical significance is considered for p<0.05. Pearson correlation was utilized to compare PE activity and PGP generation.
Results.

*N-α-PGP and PGP are elevated in sputum from CF individuals*

In order to examine N-α-PGP/PGP in sputum from CF and normal controls, we modified our published mass spectrometry technique of electronspray ionization liquid chromatography-mass spectrometry/mass spectrometry (ESI LC-MS/MS) for simultaneous detection of these peptides in clinical samples (Supplemental Figure 1). Figure 1a shows that 8/10 (80%) CF samples had N-α-PGP above our threshold for positivity versus 1/10 (10%) of normal controls with mean values of each group 3.78 ng/ml (+/- 1.84) and 0.13 ng/ml (+/- 0.12), respectively (p<0.01). The mean values for PGP in the CF samples were 204.76 ng/ml (+/- 83.9) versus 16.2 ng/ml (+/- 19.8) in normal controls (p=0.05, data not shown). These results demonstrate elevated N-α-PGP/PGP in CF patients, and led to inquiry regarding the capability of CF sputum to generate these inflammatory peptides from collagen ex vivo.

*CF sputum is capable of generating PGP from collagen*

To examine whether CF sputum had the necessary components to generate PGP from collagen, both CF and normal control sputum samples were incubated with either Type I or Type II collagen and examined via mass spectrometry for PGP or N-α-PGP. The amount of generated N-a-PGP/PGP ex vivo was determined by subtracting basal N-α-PGP/PGP of each clinical sample from N-α-PGP/PGP values detected with sample co-incubation on collagen. Figure 1b shows that 8/10 sputum samples from CF individuals generated PGP from type I collagen (average: 148ng/ml, range: 130-530% of basal PGP) compared to 1/10 normal patient samples (average: 1.3 ng/ml, (range: 0-117% of basal
Supplemental Figure 1: PGP and N-α-PGP can be detected via mass spectrometry. ESI-LC-MS/MS simultaneously detects N-α-PGP (312-140 and 312-112) and PGP (270-116 and 270-70) in a single sample.
Figure 1: PGP detection and generation are increased in CF samples.

1a: N-α-PGP is increased in CF samples compared to normal control samples.
CF (n=10) and normal control (n=10) sputum samples were analyzed utilizing electrospray ionization liquid chromatography-mass spectrometry/mass spectrometry (ESI LC-MS/MS) for N-α-PGP detection. CF samples demonstrated 8 of 10 (80%) positive for N-α-PGP versus normal controls having 1 of 10 (10%) positive for N-α-PGP. Threshold for positivity (0.825 ng/ml) was determined as two standard deviations above mean (95% confidence interval) for control sputum values.

1b: PGP production is significantly increased in CF samples compared to normal control samples on type I collagen.
CF sputum (n=10) and normal control sputum (n=10) were each incubated on extensively dialyzed type I collagen for 24 hours at 37°C. PGP values of the samples on PBS were subtracted from PGP values of samples incubated on type I collagen to determine PGP production. PGP generated from CF samples were significantly increased compared to normal control samples on type I collagen (* p<0.05).

1c: PGP production is significantly increased in CF samples compared to normal control samples on type II collagen.
CF sputum (n=10) and normal control sputum (n=10) were each incubated on extensively dialyzed type II collagen for 24 hours at 37°C. PGP values of the samples on PBS were subtracted from PGP values of samples incubated on type II collagen to determine PGP production. PGP generated from CF samples were significantly increased compared to normal control samples on type II collagen (* p<0.05).
Similarly, figure 1c demonstrates 8/10 CF sputum samples generated PGP from type II collagen (average: 240 ng/ml, range 140-675% of basal PGP) compared to PGP generation from 2/10 normal controls (average 9.8 ng/ml, range 0-155% of basal PGP), p<0.05). Slightly lower but statistically significant % increases in N-α-PGP levels were seen in CF sputum incubated with both type I and type II collagen (data not shown). These data demonstrate that CF sputum contains the proteolytic enzymes necessary to generate PGP from collagen and led to further examination of the proteases required for PGP production.

**Prolyl endopeptidase is elevated in CF sputum and correlates with PGP levels**

To our knowledge, the only enzyme directly capable of cleaving PGP from the often repeated ‘PPGP’ motif in collagen is prolyl endopeptidase (PE), a serine protease which provides specific cleavage to the right of a proline [11]. PE is an enzyme implicated in neuropeptide processing and specific neurologic conditions [12]. While its location has been reported to be cytosolic [11], many reports describe extracellular activity of the enzyme [12]. This enzyme has been identified in lung parenchyma [13], alveolar macrophages [14], and bronchoalveolar lavage (BAL) fluid [15].

To determine if PE may be playing a role in PGP generation in the CF lower airway, we attempted to assay its presence in CF sputum. Utilizing a very specific substrate for PE (Z-Gly-Pro-pNa) [16], we found a five-fold increase in PE activity in CF patients (n=10) compared to normal control (n=10) samples (Figure 2a). When we correlated PGP production and PE activity in the CF samples capable of generating PGP (n=8), the correlation coefficient was 0.72 (Figure 2b). This exponential correlation fits with the
Figure 2: Prolyl endopeptidase activity is elevated in CF samples and correlates with PGP levels

2a: Prolyl endopeptidase activity is elevated in CF samples compared to normal controls.
CF (n=10) and normal control (n=10) sputum samples were examined for prolyl endopeptidase (PE) activity utilizing a colorimetric assay. CF samples demonstrated a five-fold increase in PE activity compared to normal controls (* p<0.01).

2b: Prolyl endopeptidase activity correlates with PGP.
Concentration of PGP was correlated with PE activity in CF samples (n=8). The samples demonstrated a correlation coefficient (R²) of 0.718 via exponential curve.
expected biology of an enzymatic process. Together, these results implicate increased PE activity with PGP generation in vivo. However, PE is only capable of cleaving substrates of 30-100 amino acids or less [17]; therefore, it alone could not directly take collagen to a tripeptide. We hypothesized that PGP generation from intact collagen was a step-wise process involving initial proteolytic cleavage of collagen with subsequent activity by PE.

**PGP production correlates with PMN influx and is a stepwise processes involving MMPs and PE**

Recently, our laboratory has reported increased protease activity in CF sputum; human neutrophil elastase (HNE) was elevated along with the MMP isoforms collagenase-2 (MMP-8), gelatinase B (MMP-9), stromelysin-3 (MMP-11), and macrophage metalloelastase (MMP-12) [10]. Consequently, we examined whether airway exposure to specific MMPs and PE would generate PGP in vivo. Figure 3a shows that following intratracheal delivery to murine lungs, MMPs alone, HNE alone, or PE alone does not generate PGP. However, when either MMP-8 or MMP-9 is combined with PE, PGP is generated at levels significantly higher than PBS control or either enzyme alone. Of note MMP-12 and HNE, two prominent proteases found in a variety of chronic neutrophilic lung diseases including CF [10, 18] did not generate significant amounts of PGP in the presence of PE. Figure 3b demonstrates an extremely strong correlation ($R^2 = 0.996$) between PMN influx and PGP generation following exposure to enzyme combinations involving PE in our murine model. These results verify the stepwise generation of PGP with specific MMPs in combination with PE, and point toward dysregulated proteolytic activity in driving neutrophil influx into the lungs.
**Figure 3**: PGP is generated by proteases in vivo and correlates with neutrophil influx

**3a: In vivo PGP generation.**

In vivo PGP production was examined using matrix metalloproteases (MMPs) or human neutrophil elastase (HNE), with or without prolyl endopeptidase (PE). Various proteases (50 ul) were administered intratracheally into murine (4-6 week old Balb-C mice) airways and bronchoalveolar lavage (BAL) fluid was collected 24 hours later. PGP levels were determined using ESI LC-MS/MS.

PGP production was significantly increased in MMP-9 with PE (* p<0.05 versus PBS control, † p<0.05 versus to MMP-9 alone) and MMP-8 with PE (* p<0.05 versus PBS control, † p<0.05 versus MMP-8 alone) compared to other proteases with or without PE. PBS control and PE alone had similar PGP production.

**3b: PGP production correlates with neutrophil influx.**

PGP production levels (black boxes) were compared to neutrophil counts (gray boxes) in mice treated with a combination of the indicated protease and PE from Fig 3a. There is a notable correlation between PGP production and neutrophil counts for each condition (R2=0.996, inset).
Inhibition of proteases blocks PGP production

In order to further examine the physiologic importance of these proteases towards PGP generation, we utilized the CF sputum ex vivo assay for PGP production. CF sputum was incubated with enzyme inhibitors and then incubated with collagen. After 24 hours, the supernatant from this incubation was collected and the quantity of PGP was determined by mass spectrometry. The results were reported as percent inhibition compared to PGP generation by CF sputum alone (Figure 4). PE inhibition completely blocked generation of PGP, pointing to its central importance in PGP generation. PGP production is inhibited almost 80% by both MMP-8 and MMP-9 inhibitors individually, and over 90% when these inhibitors are combined. That MMP-8 and -9 inhibitors alone block PGP production suggests that the two proteases act in concert to generate an optimal substrate for PE. MMP-2 and HNE inhibitors did not have significant effects on PGP generation compared to MMP-8, -9 and PE inhibitors. Doxycycline, a nonspecific small molecule MMP inhibitor [19] causes approximately 75% inhibition of PGP production and may serve as an important well-tolerated therapeutic agent to modify this pathway of inflammation in CF patients.
**Figure 4:** Prolyl endopeptidase, MMP-8 and -9 inhibitors can block the production of PGP. Inhibitors were incubated for 2 hours with pooled CF sputum and these sputums were placed on type I collagen for 24 hours as previously described. PGP concentrations from these groups were compared to pooled CF sputum on type I collagen not treated with inhibitor. PE inhibitor demonstrated complete blockade of PGP production, MMP-8 and -9 specific inhibitors individually demonstrated 80-90% inhibition with their combination resulting in complete blockade of PGP generation. Doxycycline, a nonspecific MMP inhibitor, demonstrated comparable PGP inhibition as MMP-8 alone. Neither MMP-2 nor HNE specific inhibitors resulted in significant changes in PGP production.
Discussion.

Proteolytic pathway and biomarker: Unique aspects of PGP

Our experimental data points to the generation of PGP as a multi-step process involving both members of the matrix metalloprotease family (MMP-8, MMP-9) and serine protease family (PE). Both MMP-8 and MMP-9 are released from neutrophils in the airway and cleave collagen fragments as modeled in Figure 5. These collagen fragments are derived from well-documented cleavage sites for these proteases [20, 21] and conform well to the size specification for a PE substrate. We hypothesize that macrophages are activated and release PE into the airway causing the further degradation of the collagen fragment to PGP, which then acts on CXC receptors to drive neutrophil influx into the airway. This system, therefore, may involve the coordination of both neutrophils and macrophages for the release and regulation of the PGP generating enzymes. While some studies have examined the monocyte influx through elastin fragmentation [22], to our knowledge, this is the first manuscript to directly implicate a specific protease pathway in the generation of specific neutrophil chemotactic collagen peptides and to describe a specific collagen peptide as a biomarker in clinical disease.

PE demonstrates a unique role in this system for the generation of a collagen-derived CXC ligand. This enzyme is necessary for the generation of PGP from intact collagen. CF clinical samples demonstrate a strong correlation between PE activity and PGP production, recapitulating with fidelity our in vivo murine and ex vivo data. This is the first report of this enzyme as a possible modulator of the inflammatory response in any organ system. It is conceivable that the biologic compartment of other chronic neutron
Figure 5: PGP generation is a multi-step process.
The generation of PGP is a multi-step process initially involving release of MMP-8 or MMP-9 from activated neutrophils. These proteases proteolytically cleave collagen to fragments 30-100 amino acids in length. These fragments are then further cleaved to PGP by PE, possibly released by alveolar macrophages [14]. The PGP generated then acts as a neutrophil chemoattractant and the system perpetuates itself.
philic inflammatory conditions (ie arthritis, atherosclerotic disease) may have elevated levels of this enzyme and MMP-8 or MMP-9, leading to the generation of PGP. The ability of proteases generating bioactive products from collagen underlies important pathways regulating innate immunity in chronic inflammatory conditions.

**PGP as a novel component of innate immunity: Therapeutic implications**

Our results comprise part of a growing body of data implicating MMPs in regulation of inflammation [23, 24]. Unlike MMPs, PE is an enzyme which has not been implicated in inflammation biology or lung pathology. However, our current data suggests that PE has a central role in PGP generation and pulmonary neutrophilic inflammation. Interestingly, the mechanism of PGP generation showcases possible interactions between neutrophils (source of MMPs) and macrophages (source of PE) in a unique avenue of lung innate immunity. Further investigation of this enzyme and its role in pulmonary immunology and host defense appears warranted.

The increased levels of N-α-PGP/PGP seen in CF sputum suggest a role for these peptides in CF-related inflammation. The enzymes involved in the generation of PGP may serve as future therapeutic targets in the treatment of the unrelenting inflammation seen in conditions such as CF and COPD. Regulation of collagen turnover and reduction of inflammatory peptides may help identify disease-specific endpoints and even alter the natural history of these conditions. While most MMP or PE inhibitors are not currently available for testing in human trials, well-tolerated antimicrobials such as doxycycline may be useful in clinical trials as inhibitors of MMP and N-α-PGP/PGP generation.
Acknowledgements.

We would like to thank Dr. Nathaniel Weathington and Dr. F. Shawn Galin for their insight into aspects of protease biology and Dr. EJ Sorscher for his thoughtful comments.

AG is funded through the UAB CIFA Award and the Cystic Fibrosis Foundation (GAGGA07A0). JPC is funded through The Thrasher Award. JEB is funded through the Cystic Fibrosis Foundation (R464-CR02) and NIH (HL077783 and HL090999). Funds for the purchase of mass spectrometers and the operation of the Mass Spectrometry Shared Facility came from the following NIH grants to the University of Alabama at Birmingham: S10 RR19231, P30 CA13148, P50 AT00477, U54 CA100949, P30 AR050948, and P30 DK74038.
References


GENERAL DISCUSSION

Summary.

These manuscripts have discussed the biology of MMPs, demonstrated a specific pattern of MMPs in CF with a novel mechanism of MMP-9 activation, and shown a specific proteolytic system for PGP generation operative in CF. This work builds to demonstrate the importance of proteases as potential modulators of inflammation. The first paper thoroughly examines the MMP profile seen in patients with CF, showing a difference between patients with acute respiratory failure (secondary to either acute lung injury or pneumonia) and normal controls. In addition, this paper demonstrates what we feel is an important activation mechanism for MMP-9 in vivo. The second paper looks at the importance of dysregulated MMP-9 activity in an impressive model of acute airway inflammation utilizing F. tularemia, an inflammatory system which manifests both the matrix turnover and neutrophilic inflammation seen in CF. The notable change in murine collagen turnover, airway inflammation, and mortality in MMP-9 knockout vs wildtype animals underscores a crucial role of this enzyme in this model of inflammation, implicating MMP-9 as a new therapeutic target for this condition. More importantly, the finding of decreased PGP in MMP-9 knockout animals demonstrated both the importance of PGP in this model of inflammation and pointed to MMP-9 as a protease involved in PGP generation. The third manuscript extended these observations by determining the specific
protease mechanism for PGP generation utilizing MMP-8 and/or MMP-9 in conjunction with PE. In addition, PGP seems to serve as a biomarker in CF and the enzymes which generate PGP are elevated in CF sputum.

The Determination of Specific Cleavage Sites of MMP-9 and TIMP-1 by HNE.

We extended our project goals to examine the specific cleavage sites of the MMP-9 and TIMP-1 by HNE to help further characterize the specific relationships between these proteins. Our work has demonstrated specific cleavage sites on these molecules where HNE can exert its effects, which conform to substrate specifications for HNE and size of cleaved products detected. Since the time of submission of the first article for publication, we have conducted experiments to determine these specific cleavage sites and will present the unpublished findings from these studies.

HNE is a member of the serine protease family with well characterized catalytic and inhibitory regions. HNE normally cleaves peptide bonds in which the P1 residue is a small alkyl group. Substrate specificity is also affected by residues P4-P2’ around the scissile bond, and the specificity of HNE for these sites has previously been demonstrated. The initial experiments involved incubating recombinant TIMP-1 (10 ug/ml) with recombinant HNE (100 ng/ml) over a variety of time points (2, 4, 8, 24 hours) in vitro. These results were shown by coomassie stain of protein products, as demonstrated
in the first manuscript. A band for HNE was identified and demonstrated no change over the incubation. The TIMP-1 band showed cleavage and prominent protein product (approx 16 kDa) was cut from gel and underwent N-terminal sequencing, showing a consistent cleavage site at Val 69 (Figure 1a).

To determine the cleavage site of pro-MMP-9 for HNE, a peptide of 15 amino acids was synthesized (Gly 84 to Arg 98) to represent a region where cleavage would result in the liberation of proenzyme, leading to MMP-9 activation with appropriate size, as determined by MMP-9/HNE incubation experiments from the first article. 1 mg of this peptide was incubated with 100 ng/ml of HNE over various time points (0, 15 min, 30 min, 1 hour, 2 hour) and these samples were examined via mass spectrometry. A distinct cleavage site for HNE cleavage was determined as Ala 89 (Figure 1b).

These results extend our observations of HNE activation of MMP-9 and degradation of TIMP-1 by determining the specific sites where HNE causes molecular cleavage. These findings have implications in the development of future therapeutics and may lead to the development of “designer molecules” with site specific mutations to prevent either MMP-9 activation or TIMP-1 degradation in the HNE-rich environment, possibly leading to a balance of proteases/antiproteases in a variety of chronic neutrophilic diseases.
MMP-9 Potentiates IL-8 Activity.

As mentioned in the first article of this work, MMP-9 was a prominent protease found in the lower airway secretions of CF individuals. As a result of the second and third article of this thesis, we show a central role MMP-9 plays in modifying inflammation by the release of PGP from collagen. With this important role in the generation of a novel CXC ligand determined, we asked if MMP-9 could play a role in modification of a traditional neutrophil chemokine, IL-8, and will present the unpublished findings from these studies.

IL-8 is released predominately as a 77 aa isoform from epithelial cells and neutrophils and, subsequently, undergoes processing to the predominant and more potent 72 aa isoform. The mechanism by which this processing occurs is not fully understood but extracellular proteases are felt to play a role [30].

The first question in these studies was if full-length (77aa) IL-8 incubation with MMP-9 demonstrated a change in the ability to cause neutrophil chemotaxis relative to either molecule alone. 77 aa IL-8 (300 ng/ml) was incubated with activated MMP-9 (10 ng/ml) at 37 deg C for 24 hours and this sample was compared to 77 aa IL-8 alone (300 ng/ml), 72 aa IL-8 (300 ng/ml), MMP-9 alone (10 ng/ml), or media control (negative control) for neutrophil chemotaxis utilizing a chemotaxis assay (as described in methods section of the second manuscript). Figure 2 showed that the chemotactic activity is enhanced in the IL-8+MMP-9 incubated sample versus the 77 aa IL-8 but less than the 72 aa IL-8 species. MMP-9 alone demonstrated no endogenous chemotactic activity.
The incubated IL-8/MMP-9 sample was then examined via mass spectrometry and compared to spectra for both known IL-8 species. Figure 3 summarizes mass spectrometry results from in-vitro co-incubation of 77aa IL-8 with MMP-9 compared to 77 aa IL-8 (blue) and 72 aa IL-8 (green) alone. Interestingly, the addition of MMP-9 produces a new shortened IL-8 derivative (red). This proposed N-terminal cleavage site (Figure 4) for IL-8 conforms for the substrate specificity for MMP-9 [34]. Another paper has also demonstrated MMP-9 mediated cleavage of IL-8 but shows a 70 aa product [38]; the size of this product, however, is lower than is expected in biologic systems and does not conform to a favorable MMP-9 cleavage site. Although these experiments are of interest, further work needs to be done to verify these findings.

This unique 74 aa IL-8 species offers insight into the role MMP-9 may play in the post-translational processing of this prominent chemokine in disease. The generation of this novel IL-8 species also underlies a pattern of increased IL-8 potency with increased number of amino acids cleaved from the N-terminus; an improved understanding of why this occurs may offer unique insights into relative potencies of these species. More importantly, these findings point to another important role proteases play in modulation of the innate immune response- often, proteases cleave cytokines/chemokines, leading to both increased or decreased activity. This area of study, termed “degradomics”, is an area of increased interest in inflammation biology [34].
Figure 1a: MMP-3 interaction with TIMP-1 (RCSB 1009). While the crystal structure for MMP-9/TIMP-1 interaction is not completely known, it is thought to be similar to MMP-3/TIMP-1 interaction. The pink colored molecule is MMP-3 and the light blue colored molecule is TIMP-1. The red amino acid is threonine 98, an important amino acid in TIMP-1 recognition of MMPs. HNE cleavage site for TIMP-1 is Val-69 (yellow, with white circle) as determined via N-terminal sequencing of TIMP cleavage products.

Figure 1b: MMP-9 Crystal Structure (RCSB structure IRJ). The red motif represents the MMP-9 prodomain while the yellow region represents the remainder of MMP-9 molecule. The light blue region represents the catalytic site. Previously mapped cleavage sites for trypsin (violet) and APMA (dark blue) are shown in the prodomain. The light green (with white circle) shows the determined HNE cleavage sites (Ala-89), determined via mass spectrometry.
MMP-9 Dysregulation and CF Lung Disease: Implications to Innate Immunity and Therapeutics.

The findings outlined above and in the three papers in this thesis describe a very central role played by proteases in CF lung disease. Proteases and antiproteases, specifically MMP-9, demonstrates significant dysregulation in CF lung disease and this dyregulation seems mediated by HNE, another prominent protease found in CF. This system typifies an important corollary to the protease/antiprotease hypothesis: with multiple proteases/antiproteases in a given biologic compartment (ie alveolus, vasculature, airways), there are bound to be protease/protease or protease/antiprotease interactions which alter the primary, secondary, and tertiary structure of these enzymes. Our findings highlight the importance of these interactions through both in vitro and in vivo studies.

Beyond generalized airway remodeling, our work describes two distinct but related mechanisms by which MMP-9 activity leads to modulation of the innate immune response. MMP-9, in conjunction with PE, leads to the generation of PGP; the importance of MMP-9 in the generation of this ligand has been reinforced by the second paper in this thesis. MMP-9 can also directly cleave 3 aa from IL-8, leading to a more potent IL-8 isoform. While distinct, these mechanisms lead to binding to CXCR on neutrophils and causing increased neutrophil chemotaxis. While we have shown the presence of the PGP peptide in clinical samples from CF patients, an examination of CF sputum for various IL-8 species would seem prudent for the future.
Ultimately, the data generated from this work points to specific therapeutic targets for the treatment of CF lung disease and inflammation. One possible target which has received attention has been anti-HNE therapy. Unfortunately, while a logical choice, HNE-specific therapeutics has been met with mixed success [35]. Another possible therapeutic might be to develop an HNE-resistant TIMP-1 molecule to be given to CF patients to help restore protease/antiprotease balance in vivo. Anti-IL-8 therapy has also been considered in a variety of lung diseases but has been met with poor success [36]; a possible reason for this is that the PGP pathway may not have been adequately blocked in these trials.

At this point, we can only speculate about PE as a possible therapeutic target. As our paper is the first paper to describe a central role of PE in inflammation, there have been no clinical trials examining PE inhibition looking at inflammation endpoints. However, the degree by which PE inhibition reduces PGP generation in sputum ex vivo reinforces its central role in PGP production and certainly warrants further consideration as a therapeutic target.

As such, perhaps the most interesting target described thus far is MMP-9. This protease may be involved in two unique and important systems of innate immunity and is well-known to be responsible for some of airway structural changes seen in chronic lung diseases. While there are no current MMP-9 specific inhibitors being tested in Phase 3 trials for lung disease, there are other options for possible MMP-9 inhibition. Doxycycline, a well-characterized antibiotic, can act as a small-molecule inhibitor for MMPs
[37]. Perhaps this well-tolerated antimicrobial may be used as a possible adjunct to CF related therapy in the near future.
Figure 2: IL-8 co-incubated with MMP-9 demonstrate increased chemotaxis. Neutrophil chemotaxis of IL-8 species were assessed utilizing a transwell chemotaxis assay. 77 amino acid IL-8+ MMP-9 (green box) demonstrated increased chemotaxis compared to media control (red box, far left) and 77 amino acid isoform (yellow box). Note that MMP-9 has no inherent chemotactic properties (black box, far right).
Figure 3: MMP-9/IL-8 co-incubation leads to a novel IL-8 species.
Co-incubation of 77 amino acid IL-8 (blue spectra, with molecular weight) with activated MMP-9 (10 ng/ml) for 24 hours yields a new IL-8 isoform (red spectra, with molecular weight), possibly 74 aa in mass. This spectra is compared to spectra of a known biologically active IL-8 isoform, 72 amino acid (green spectra, with molecular weight).
Interleukin-8

NH2-avl/pr/sake1rcqcktyskpfhpkfikelsrviesgphcanteiivklstdgrelcldpkenwrvqrvvekflkrae-COOH

Figure 4: Amino acid sequence of IL-8 and proposed MMP-9 cleavage site. The amino acid sequence of full-length IL-8 (77aa) and the proposed cleavage site for MMP-9, leading to a 74 aa product (red). The cleavage site for 72 aa IL-8 is shown in green.
General References


NOTICE OF APPROVAL

DATE: December 19, 2006

TO: J. Edwin Blalock, Ph.D.
MCLM-896  0005
FAX: 934-1446

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: A New Pathway for Neutrophil-Induced Airway Inflammation
Sponsor: NIH
Animal Project Number: 061107715

On November 29, 2006, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>267</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from November 2006. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 061107715 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.
UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on February 14, 2009. The Assurance number is FWA00005960.

Principal Investigator: CLANCY, JOHN PAUL
Co-Investigator(s):
Protocol Number: F020408013
Protocol Title: Matrix Metalloproteinases in Cystic Fibrosis Lung Disease

The IRB reviewed and approved the above named project on 1/10/2007. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received FULL COMMITTEE review.

IRB Approval Date: 1/10/2007
Date IRB Approval Issued: 01/10/2007
Identification Number: IRB00000196
HIPAA Waiver Approved?: No

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.
Federal regulations require IRB approval before implementing proposed changes. Please complete this form and attach the changed research documents. Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator’s Brochure, questionnaires, surveys, advertisements, etc.)

Principal Investigator: JP Clancy, MD  Date: September 5, 2003
Contact: Valerie Eubanks  Phone #: 939-5743  Campus Address: 616 ACC
Study/Protocol Title: Matrix Metalloproteins in Cystic Fibrosis Lung Disease
IRB Protocol #: F020408013

Current Status of Project: (check only one)
- Currently in Progress (# participants entered: 16)
- Study has not yet begun (no participants entered)
- Closed to participant enrollment (remains active); # participants on therapy/intervention_____; # participants in long-term follow-up only_____.

This submission changes the status of this study in the following manner:
(check all that apply)
- Protocol Revision
- Protocol Amendment
- Study Closed to participant entry
- Study Terminated
- Revised Consent Form
- Addendum (new) consent form
- Enrollment temporarily suspended by sponsor
- Other, (specify)_____.

1. Briefly describe, and explain the reason for, the revision or amendment. Include a copy of supportive documents with changes highlighted. Please highlight changes/revisions/additions to the consent form, protocol, research questionnaire, etc.

We wish to add Dr. Amit Gaggar to the consent document and human subjects protocol. We are also adding University Hospital and Kirklin clinic to the HIPAA form.

2. Does this revision/amendment revise or add a genetic or storage of samples component?  Yes  No
   If yes, please see the Guidebook to assist you in revising or preparing your submission documents or call the IRB office at 4-3789.

3. Does the change affect subject participation (e.g. procedures, risks, costs, etc.)?  Yes  No

4. Does the change affect the consent document?  Yes  No
   If yes, briefly discuss the changes.
   - New name is included on the consent document.

Include the revised consent form with the changes highlighted.
Will any participants need to be reconsented as a result of the changes?  Yes  No
If yes, when will participants be reconsented?_____.

Signature of Principal Investigator  

FOR IRB USE ONLY
Informed Consent - Controls

Title of Research: Matrix Metalloproteinases in Cystic Fibrosis Lung Disease

Principal Investigator: J.P. Clancy, M.D.

Other Investigators: Randall Young, M.D., Valerie Eubanks Tarn, M.S., R.D., Amit Gaggar, M.D., Heather R. Young, MAE, RRT., Yao Li, PhD., Steven Rowe, M.D., Ginger Reeves, BS, RRT

Sponsor: The Research Institute at Children's Hospital

For Minors (persons under 19 years of age) participating in the study, the use of the term "You" refers to "You or Your Child" and addresses both the participant and the parent or legally authorized representative.

INFORMED CONSENT

You have been asked to take part in a Research Study. The purpose of this consent form is to provide you with the information you need to consider in deciding whether to participate in this research study.

RIGHT TO WITHDRAW

You are participating on a voluntary basis and you may choose to withdraw from this study at any time. If you withdraw from this study this will in no way jeopardize your future care at the University of Alabama at Birmingham Hospital System or Children's Hospital, and will not be associated with any loss of benefits. Dr. J.P. Clancy, or any of the other investigators involved in this study have the right to stop your participation at any time. This could occur, for example, if you developed an unexpected problem or failed to follow directions.

PURPOSE

Mucus normally coats the surface of the airway tubes, and is cleared from the lungs by coughing. When this mucus is infected with bacteria, it attracts white blood cells to fight the infection. In certain diseases like cystic fibrosis, the mucus becomes very thick and full of bacteria and white blood cells. This combination damages the airway tubes and...
September 19, 2007

Amit Gaggar
UAB Department of Physiology/Biophysics
1918 University Blvd.
McCallum Room 898
Birmingham, AL  35294-0005

Dear Mr. Gaggar:

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Amit Gaggar, Yao Li, Nathaniel Weathington, Margaret Winkler, Michele Kong, Patricia Jackson, J. E. Blalock, and J. P. Clancy
   Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients

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Sincerely,

Margaret Reich

Ms. Margaret Reich
Director of Publications
The American Physiological Society

MR/pr
September 14, 2007

Amit Gaggar
University of Alabama-Birmingham
716 Crest Lane
Homewood, AL 35209
USA
Phone: 205-934-5400
Fax: 205-934-1721
Email: agaggar@uab.edu

Dear Dr. Gaggar:

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Gene G. Bailey
Senior Editorial Manager
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