THE ROLE OF MATRIX METALLOPROTEINASES (MMPs) AND THEIR PROTEOLYTIC DEGRADATION OF CHEMOKINES IN THE LUNG

by

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Submitted in partial fulfillment of the requirements

For the degree of Master of Science

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May, 2010

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Acknowledgements

I would like to thank my soon to be husband, Scott Nolan, for his constant support and motivation with the completion of my coursework and this thesis. His advice and support along the way provided me with the confidence to complete this work. I would also like to thank Dr. Tracey Bonfield for her mentorship during the completion of this paper. Finally, I would like to thank my parents for the value they place on education and their pride in my educational goals over the years.

The Role of Matrix Metalloproteinases (MMPs) and their Proteolytic Degradation of Chemokines in the Lung

Abstract

by

MARY T. KOLOZE

Matrix metalloproteinases (MMPs) are critical for lung homeostasis including routine tissue maintenance, remodeling, and the immune response. Matrix metalloproteinases can also be detrimental to the lung environment when cleaving critical chemokines. Two important MMPs found commonly in lung conditions characterized by inflammation are MMP-2 and MMP-9. In pulmonary alveolar proteinosis (PAP), the lungs are filled with excess surfactant protein and impaired white blood cell recruitment from the periphery even with elevated cytokines and chemokines associated with inflammation. MMP-2 and the chemokine MCP-3 have increased levels of gene and protein expression in PAP. The granulocyte-macrophage colony-stimulating factor knockout (GM-KO) mouse develops PAP-like symptoms by 6-8 weeks of age. The GM-KO mouse also has excessive surfactant protein buildup in its lungs. We hypothesize that the GM-KO mouse should have increased MMP-2 and MCP-3 levels as seen in the human disease PAP. Furthermore, we propose that cleavage of MCP-3 by MMP-2 in the mouse results in an altered cellular differential and reduced inflammatory clearance. Conclusions: The GM-KO has elevated MCP-3 but not MMP levels in the context of a reduced cellular recruitment.

Introduction:

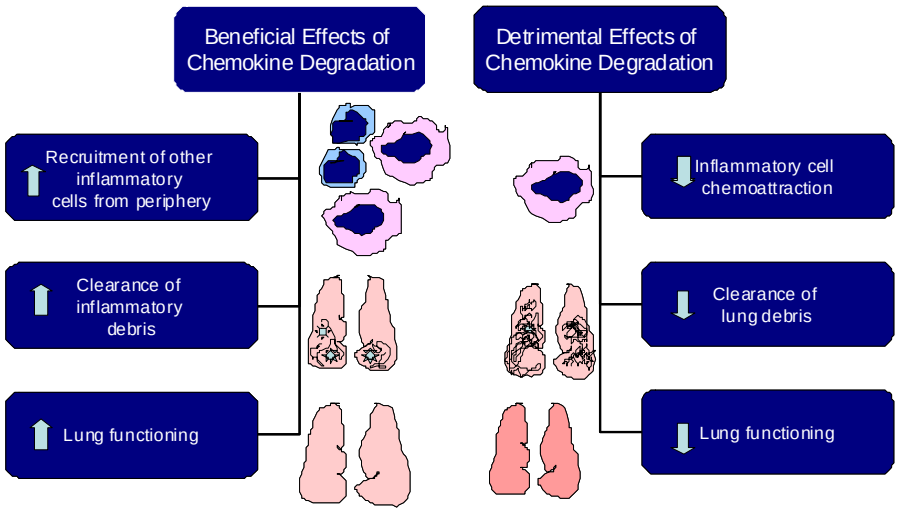
Matrix metalloproteinases (MMPs) are a diverse group of endopeptidases which carry out the degradation of the extracellular matrix (ECM) within the lung environment. In the inflamed lung, MMP activity serves many roles - beneficial as well as detrimental (See Figure 1). To study the roles of MMP activity within an inflammatory lung model, the granulocyte macrophage colony stimulating factor – knockout (GM-KO) mouse model is used since it portrays a model of MMP activity in lung disease. In the GM-KO lung, MMP gene and protein expression are increased. Furthermore, MMP endopeptidase activity is also increased. However, the role of MMPs in the GM-KO is unknown.

Matrix metalloproteinase degradation provides a protective effect to the lung tissue by helping to clear inflammatory debris as well as ECM turnover and remodeling. Matrix metalloproteinase activity also recruits inflammatory cells to sites of inflammation. During the degradation process, essential chemokines may also be degraded; as a result, less inflammatory cell chemoattraction and recruitment occurs to the inflamed site. Furthermore, inflammation clearance takes longer and consequently, lung functioning declines.

Lung inflammation is a very intricate process with many key components - MMPs being just one of them. However, MMPs provide a common underlying component to several lung conditions characterized by inflammation such as asthma, pulmonary alveolar proteinosis (PAP), chronic obstructive pulmonary disease (COPD), as well as cystic fibrosis. Studying the role of MMPs in the GM-KO lung, which provides an optimal chronic inflammation model, will help to provide universal applications

regarding possible treatments for additional lung conditions also characterized by inflammation.

Figure 1. MMP Degradation of Chemokines
MMPs serve to be beneficial as well as detrimental to the lung.



Goal of study:

The goal of this study is to investigate the role of MMPs in inflammation resolution and to evaluate their role in altering chemokine activity changing inflammation in the lung.

Background:

I. Lung inflammation

Lung inflammation is a characteristic feature found in many pulmonary conditions such as asthma, cystic fibrosis, chronic obstructive pulmonary disease (COPD), and pulmonary alveolar proteinosis (PAP). It is characterized by influx of inflammatory cells to the region of injury or to the foreign particle or bacteria taking up residence in the lung. These inflammatory cells arrive at the lung with a resolved purpose to attack and quickly eliminate any lung disturbance or inflammation in order to return the lung environment to its healthy homeostatic state. The cells that do this are primarily white blood cells such as monocytes/macrophages, neutrophils, and lymphocytes, that are recruited from the bloodstream via several chemokines and other factors already present in the lung (1). Acute inflammation is generally defined by enhanced recruitment of neutrophils. Chronic inflammation is generally defined by monocyte/macrophage recruitment. Lymphocytes can be part of both acute and chronic processes. The distribution of these inflammatory cells defines the unique nature of the inflammatory response (1).

Chemokines send out their chemoattractant signals to monocytes/macrophages, neutrophils, and lymphocytes requesting assistance in infection and inflammation clearance. After traveling through the bloodstream, the cells enter the lung by a process of extravasation through the endothelial lining of the blood vessels into the disturbed tissue site (See Figure 2). Here, the cells actively begin the process of attacking and phagocytosing the infection and proteolytically degrading the inflammatory debris (2). Chronic inflammation occurs when the immune system is unable to resolve the

inflammation following an infection or other insult. In this case, the immune response continues to actively recruit cells as if the body is responding to an active infection, even though the infection has been resolved (3). This buildup of inflammatory cells is the main component of chronic inflammation.

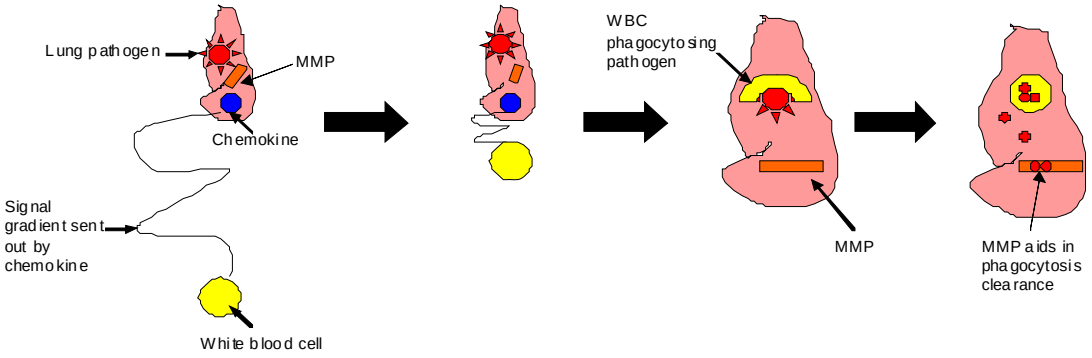


Figure 2. MMP and Chemokine Interaction in the Lung
In response to infection, chemokines send out signals via a gradient to inflammatory cells, or WBCs, in the periphery. WBCs travel to the site of infection and aid in the destruction of pathogens via phagocytosis. MMPs help to clear phagocytic debris.

Normally, the infection is cleared in a timely manner and lung functioning is only temporarily disturbed with minimal if any disturbance experienced by the individual. The process of inflammation is orchestrated similarly in all tissues; however, the mediators and original stimuli can differ greatly. Some of the most important mediators of inflammatory cell chemotaxis to the lung are called chemokines. As mentioned above, chemokines reside in any tissue and when inflammation is present, send out chemoattractant signals to inflammatory cells in the periphery. The interleukins (ILs) are an important and diverse group of chemokines that help to promote the maturation of the T and B cells and thus aid in the immune response specifically with immune cell maturation. One common interleukin in the inflamed human lung is interleukin-8 (IL-8), which helps to recruit mostly neutrophils from the periphery to sites of inflammation (4). The monocyte chemoattractant proteins (MCPs) are a group of chemokines responsible in

large part for the recruitment of most white blood cells to sites of inflammation. Macrophage inflammatory proteins (MIPs) also assist in chemotaxis of white blood cells; however, they also help to initiate white blood cell phagocytosis. Eotaxin proteins are another group of chemokines that assist in the recruitment and survival of eosinophils in the lung, specifically the asthmatic lung.

The prevalence of inflammation across most lung conditions is well characterized. However, each condition demonstrates some unique as well as overlapping characteristics of inflammatory mediators and chemokines. For example, during an asthmatic response, IgE antibodies on mast cells become activated to produce histamine and leukotrienes in response to an allergen, such as pollen, within the airways (5). Histamine and leukotrienes then stimulate blood vessel dilation allowing white blood cells to enter the area sooner. The lungs take on a hyper-reactive state where the blood vessels are dilated and airways are constricted which is consistent with the inflammation in the asthmatic lung.

Chronic obstructive pulmonary disease (COPD) is caused by long-term exposure to cigarette smoke, toxic gases, and particulate matter, leading to airflow limitation and pulmonary failure (6). The disease is characterized by a thickening of the airway walls and an accumulation of obstructing inflammatory cells and mucous. The alveoli are often destroyed and irreversible damage to the airway elasticity ensues. Emphysema and chronic bronchitis can associate with COPD in which the airways distal to the terminal bronchiole are slowly destroyed. It is also characterized by gradual destruction of the alveolae and capillary bed which prevents blood from being easily oxidized.

Inflammation in the CF lung is circular in nature and is commonly characterized by the role neutrophils play in the release of proinflammatory mediators, including reactive oxygen species and proteolytic enzymes (7). The CF lung is characterized by a persistent and excessive neutrophil infiltration. The neutrophils try to aid in the immune response; however, they are also contributing to the obstruction of the airways. Furthermore, in CF and COPD, after a pathogen or pollutant has been removed from the respiratory tract, scar tissue formation and a decline in respiratory health usually ensues (8, 9).

Pulmonary alveolar proteinosis is characterized by increased accumulation of surfactant, the lipid protein that aids in providing alveolar surface tension (10). The most common symptoms for patients are shortness of breath, cough, and weight loss. On a pathophysiological level, gas exchange between the lung capillaries and alveoli is strained and reduced resulting in labored breathing. Additionally, PAP individuals are prone to bacterial or fungal infections because of the surfactant buildup deep within the lung tissue (11).

Inflammation in the PAP lung tends to be complex in that there are multiple conflicting factors occurring simultaneously. For example, at disease onset, chemokines recruit peripheral inflammatory cells to the lung tissue with a purpose to clear inflammation. Surfactant proteins reside in the PAP lung and prevent certain proteases from cleaving the chemokines – keeping them active chemoattractants (12). Chemokines continue to recruit white blood cells from the periphery with no signal to stop recruiting. As a result, there is an overabundance of cells in the lung tissue trying to clear the inflammation and chronic inflammation persists. In another scenario, proteases cleave

chemokines rendering them inactive (13)**.** As a result, the lung in PAP is unique in that there is inflammation with cytokines and MMPs, but without robust cellular recruitment. Therefore, the elevated concentration of MMPs suggests a role in altering chemokine ability to recruit other cells.

Summary

Inflammation is a consistent finding within several lung conditions and involves overlapping as well as unique mediators of the inflammatory response. In certain lung conditions, such as PAP, the normal immune response is not sufficient to clear inflammation or accumulation of surfactant. Defects in surfactant protein production may lead to defects in inflammatory cell recruitment leaving the lung tissue in a unique chronically inflamed state. Research focused on this reduced cellular recruitment will contribute to possible therapeutic targets for individuals with PAP along with other conditions characterized by chronic states of inflammation.

II. Matrix Metalloproteinases (MMPs)

One group of mediators in inflammatory clearance is the matrix metalloproteinases (MMPs) (See Table 1). Matrix metalloproteinases are proteolytic enzymes implicated in many physiological and pathological processes including embryonic development, morphogenesis, reproductive processes, bone remodeling, wound healing, cancer, arthritis, as well as atherosclerosis (6). Matrix metalloproteinases tend to have multiple facets to their function as inflammatory mediators. For example, they can be beneficial or detrimental to the lung, especially when chronic inflammation is present. Matrix metalloproteinases are secreted to facilitate clearance of foreign and

noxious agents, but, when present in excess, can destroy the ECM environment, disrupt resident cells, and stimulate further inflammation (14).

Table 1. Matrix Metalloproteinases

Name	Classification name	Possible Substrates for MMP Groups	Structural Classification Based on Domain
MMP-1	Collagenase-1	Collagens I, II, III, VII, VIII, X, gelatin, proteoglycan link protein, aggrecan, veriscan, tenacin, and entactin, perlecan, fibronectin, osteonectin	Simple hemopexin
MMP-8	Collagenase-2		
MMP-13	Collagenase-3		
MMP-18	Collagenase-4		
MMP-2	Gelatinase-A	Collagens I, IV, V, VII, X, XI, XIV, gelatin, elastin, fibronectin, laminin, galectin, aggregan, decorin, entactin, proteoglycan link protein	Gelatin-binding
MMP-9	Gelatinase-B		
MMP-3	Stromelysin-1	Collagens III, IV, V, and IX, gelatin, aggrecan, versican, casein, elastin, proteoglycam link protein, laminin, fibronectin	Simple hemopexin
MMP-10	Stromelysin-2		
MMP-11	Stromelysin-3		
MMP-14	Membrane-type 1 MMP	Collagens I, II, III, casein, elastin, fibronectin, gelatin, laminin, vitronectin, large tenascin-C, entactin, proteoglycans, aggrecan, perlecan	Trans-membrane MMP-17, -25: GPI anchored
MMP-15	Membrane-type 2 MMP		
MMP-16	Membrane-type 3 MMP		
MMP-17	Membrane-type 4 MMP		
MMP-24	Membrane-type 5 MMP		
MMP-25	Membrane-type 6 MMP		
MMP-7	Matrilysin-2	Collagens IV, X, gelatin, aggrecan, decorin, proteoglycan link protein, fibronectin, laminin, insoluble fibronectin fibrils, entactin, large and small tenasci-C, beta 4-integrin, transferrin	Minimal, vitronectin-like
MMP-26	Matrilysin		
MMP-12	Metalloelastase	Collagen IV, gelatin, elastin, casein, laminin, enactin, vitronectin, fibronectin	Simple hemopexin
MMP-19		Gelatin	
MMP-20	Enamelysin	Amelogenin	Simple hemopexin
MMP-21		Unknown substrate	
MMP-23		Unknown substrate	Type II transmembrane
MMP-27		Unknown substrate	
MMP-28	Epilysin	Unknown substrate	Furin-activated
MMP-26	Endometase	Unknown substrate	Minimal

All reviewed by Chakraborti, 2003

They are a group of neutral endopeptidases that provide beneficial routine maintenance to the lung tissue via degradation of the extracellular matrix (ECM) during tissue turnover. They do this by attacking type IV collagen predominantly found in basement membranes (15). Expression of most MMPs is normally low in tissues and is induced when remodeling of ECM is required (16). Extracellular matrix turnover is critical for the homeostasis of the lung environment. Without periodic tissue turnover, the lung would continually fill with debris and inflammation would progress. Besides tissue turnover, MMPs also serve a critical function in the degradation of inflammatory debris resulting from alveolar macrophage phagocytosis.

Matrix metalloproteinases are produced and secreted from a variety of cells within the body. These enzymes along with their inhibitors are produced by structural cells of the bronchial tree and alveolae and by inflammatory cells upon stimulation (6). In healthy lung, fibroblasts constitutively produce MMP-2 and bronchial epithelial cells release MMP-2 and MMP-9 and their major inhibitor, TIMP-1 (6). Smooth muscle cells produce MMP-2 which controls their proliferation (17). Among inflammatory cells, neutrophils produce MMP-9, MMP-8 and TIMP-1 which are synthesized and released from their secondary granules upon activation (18). Matrix metalloproteinase-2 is synthesized by a wide variety of cells itself, including fibroblasts, endothelial cells, and alveolar epithelial cells (19). Matrix metalloproteinase-9 is produced mainly by inflammatory cells such as neutrophils, monocytes, macrophages, eosinophils, and lymphocytes (20). While most MMPs are produced and secreted upon activation from some outside source, such as tissue injury or an infectious agent, there are basal levels of constitutively present and active MMPs in each body tissue, including the lung.

MMP Structure

Matrix metalloproteinases contain several regions which are highly conserved, suggesting significant importance in maintaining metalloproteinase function. In general, MMP structure consists of a hemopexin domain and a catalytic domain which carries out the degradation. Hemopexin is known to be a plasma heme-binding and heme-transport protein. In MMP structure, there are two additional structures called the zinc-binding motif and the propeptide cysteine switch which are also highly conserved (See Figure 3). For the gelatinases, there is an additional component that is unique to its structure and ultimately its function as a gelatinase. This additional component contains three

fibronectin-like inserts in the catalytic domain of the gelatinase making it capable of enzymatic degradation of its primary substrate gelatin (21). These structures are schematized in Figure 3 and Figure 4 below.

Figure 3. Basic MMP Structure
A pre- and prodomain sit at the N-terminus of the MMP protein followed by the catalytic domain with its zinc-dependent active site. A hemopexin domain follows.

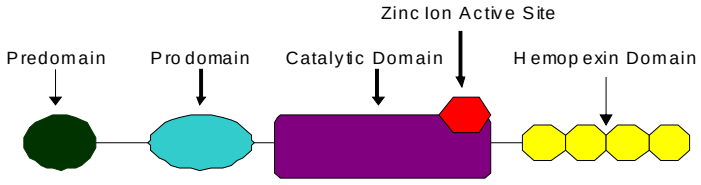
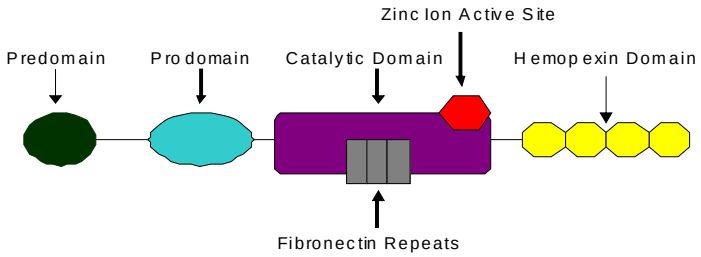


Figure 4. Gelatinase Structure
The gelatinases contain three additional fibronectin repeats involved in the degradation of gelatin.



MMP Substrates

Matrix metalloproteinases overlap cleavage properties with one another as well as cleave independently from one another. Therefore, one common classification system in which MMPs are classified is based on their optimal substrate specificities with similar structure and function such as collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs. Since their first description by Gross and Lapiere (1962) (22), twenty-eight MMPs have been described, twenty-four of which have been identified in vertebrates (6). The substrate specificities are described in the paragraphs below.

Interstitial collagenases include MMP-1, MMP-8, MMP-13, and MMP-18, are also called collagenases-1, -2, -3, and -4. Their substrates include collagens type I, II, III, VII, VIII, X, aggrecan, gelatin, and the pro-MMP-2 and pro-MMP-9 zymogens (16). This class functions to breakdown the peptide bonds within collagen.

Matrilysins include MMP-7, MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-26, MMP-27, and MMP-28 (16). Their major substrates include certain collagen types, elastin, tenascin, enamel, gelatin, aggrecan, fibronectin, and laminin (16). Matrix metalloproteinase-21, -23, -27, and -28 have unknown substrates to date. This group degrades the extracellular matrix (ECM) during tissue remodeling. This group also shares similarities with the stromelysins in specificity for substrates and share structural similarities with the interstitial collagenases. These MMPs do not contain the carboxy-terminal regions encoded by other MMP genes.

Stromelysins are a group of MMPs that consist of MMP-3, MMP-10, and MMP-11, also known as stromelysin-1, -2, and -3 respectively (16). Their primary substrates are fibronectin, laminin, gelatin, aggrecan, and collagens III, IV, and V (16). They function in roles of pathological turnover in connective tissues.

Membrane-type MMPs consist of MMP-14, -15, -16, -17, -24, and -25; also referred to as MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, and MT6-MMP respectively. The major substrates are pro-MMP-2, gelatin, fibronectin, laminin, and aggrecan, with pro-MMP-2 being the most prevalent (16).

Gelatinase A and B, MMP-2 and MMP-9 respectively, fall within the gelatinase family of MMPs. The gelatinases serve to degrade extracellular matrix as well as specific substrates like gelatin commonly found in basement membranes, during tissue

inflammation and tissue repair (23). Cytokines and growth factors aid in the upregulation, activation, downregulation, and regulatory secretion and cleavage of these MMPs in order to accomplish their tasks within tissue. For example, the cytokine IL-1 is known to increase MMP-2 gene production in human cardiac endothelial cells (24). On the other hand, factors known to decrease MMP-2 function include transforming growth factor-beta (TGF-B), cycloheximide (CH), and protein kinase C in human pulp cell cultures (25).

Like most enzymes, gelatinases A and B consist of a latent and active form. Matrix metalloproteinase-2 consists of a pro (72 kD) and active (62 kD) form, while MMP-9 consists of a pro (92 kD) and active (82 kD) form (20). The pro forms compose the inactive zymogens that need to be cleaved by other substances in order to become functionally active within the lung environment. They are activated in response to cytokines or other environmental stimuli.

There are several theories as to how MMPs become activated. Matrix metalloproteinases are cleaved by several chemicals or growth factors such as other proteases not involved in the inflammatory response, organomercurials, oxidants, as well as some detergents (26). This conversion of pro-MMP into activated form can be performed in vitro by reactive oxygen species (ROS) or physical condition adjustments with pH or temperature (27). With regard to pro-MMP-2, cell surface receptors such as the membrane-type MMPs (MT-MMPs) have been shown to activate the proenzyme form. For example, MT1-MMP has the ability to initiate the activation of pro-MMP-2 along with MT2-MMP and MT3-MMP (16). Furthermore, mechanical stress on the lung tissue increases ROS in vascular smooth muscle cells and therefore could be responsible

for increased gene expression of MMP2 and increased release of pro-MMP-2 in this scenario (28).

Mechanism of Activation and Inhibition

Despite several initiators of the activation process, there is one basic and favored mechanism that occurs in order for the latent proenzyme to become activated and functional. This mechanism involves an orientation called the cysteine switch. Matrix metalloproteinases have a conserved PRCGVPDV sequence in which a cysteine residue maintains the latency of the zymogen by direct coordination with the active site zinc atom of the catalytic domain, blocking access of the catalytic site to the substrate (29). Aminophenylmercuric acetate (APMA) is a classic organomercurial that is a very common initiator of the latent activation step (26). It works by facilitating the loss of the MMP propeptide domain through an autolytic cleavage reaction of the cysteine switch (26). Removal of the propeptide containing the cysteine group by enzymatic cleavage or disruption of the zinc-cysteine interaction can result in activation of the latent enzyme (30).

However, not all MMPs are activated in such a way. For example, MMP-2 is unique in that it can be activated on the cell membrane. In this case, activation critically depends on the binding of pro-MMP-2 to MMP-14 along with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) (31). Therefore, a second active MMP-14 is allowed to cleave the prodomain of the enzyme and release the activated MMP-2 (32). Matrix metalloproteinases are diverse in that they can be secreted into the cytosol or they can remain embedded within the plasma membrane. Those that remain within the plasma membrane tend to be more resistant to inhibition by the four MMP inhibitors called the

tissue inhibitors of matrix metalloproteinases (TIMPs) (33). Therefore, MMP-2 could potentially be more resistant to TIMP inhibition if situated in the plasma membrane.

Gelatinase Functions

The gelatinases have many different as well as overlapping functions. For example, MMP-2 is considered a type IV collagenase because it degrades type IV collagen, the major structural component of basement membranes. This enzyme plays a role in endometrial menstrual breakdown, regulation of vascularization, and the induction or suppression of the inflammatory response (34). Matrix metalloproteinase-2 serves many important functions within the extracellular matrix as described above; however, there are also important functions that are carried out inside the cell. Matrix metalloproteinase-2 plays a role in lung inflammation through cell trafficking (35). It is responsible for directing important proteins or factors from the golgi to the plasma membrane for secretion by the cell during an inflammatory response.

Matrix metalloproteinase-9, on the other hand, is considered a type IV collagenase secreted as inactive proprotein and activated via cleavage by extracellular proteinases. It is a protease which is an enzyme that conducts protein catabolism via a hydrolysis reaction of the peptide bonds that link certain amino acids together within a protein. The enzyme secreted by this gene degrades type IV and V collagens within the extracellular matrix as well as in the basement membrane especially lining the airways and blood vessels in close proximity to those airways.

Furthermore, MMP-2 and MMP-9 have angiogenic capabilities suggesting potential roles in wound healing and cancer research. They are known to be upregulated in angiogenesis which often times accompanies or follows tumor growth and invasion.

For example, MMPs have been reported to be essential for the mobilization of vascular endothelial growth factor (VEGF) from the extracellular matrix (36). This is a critical factor involved in angiogenesis formation. Angiogenesis always plays an important role in metastasis via its direct role in expanding the vasculature. When tumor vasculature is expanded, additional growth factors and survival factors are brought in close proximity to the tumor to sustain and promote its growth. The presence of these additional growth factors and cytokines can initiate the degradative activity of MMPs residing there. As a result, angiogenesis is promoted further. Matrix metalloproteinases degrade in order to clear space for the growth of additional blood vessels. Therefore, the function of MMPs during metastasis becomes critical research when deciding upon treatment options and possible pharmaceutical targets.

Regulation of MMPs

The transcription of MMPs is regulated by a variety of pro- and anti-inflammatory cytokines. For example, tumor necrosis factor alpha (TNF-a) and IL-1β are known to induce the transcription of MMP genes (37); whereas, TGF-β represses MMP gene transcription. Therefore, depending on what cytokines are in a particular area or stage of inflammation results in the activation or repression of MMP transcription. As a result, MMP function ensues to maintain homeostasis, rebuild tissues after injury, or affect inflammation during the immune response.

Regulation of matrix metalloproteinases occurs at many levels including gene transcription, translation, activation, as well as inhibition via TIMPs. Regulation, therefore, can occur at any time point of MMP latency or activity. When an MMP becomes activated, the pro-peptide region of the molecule is removed (30); therefore,

removing the inhibitory component of the molecule allowing it to become activated. Tissue inhibitors of matrix metalloproteinases, or a molecule called alpha2-macroglobulin, can also inhibit MMPs. However, the gelatinases can sometimes form complexes with these TIMPs in their latent forms (38, 39). For example, pro-MMP-9 forms a complex with TIMP-1, with a function that is currently not known. Also, pro-MMP-2 forms a complex with TIMP-2 and facilitates the activation of pro-MMP-2 at the cell surface by MT1-MMP (MMP-14), a membrane anchored MMP.

Tissue inhibitors of matrix metalloproteinases are a specific class of proteases that inhibit activated MMPs. They block access of certain substrates to the catalytic site on MMPs by forming non-covalent interactions with the MMPs themselves. Their interaction is stoichiometric in a ratio of 1:1 with a specific MMP (40). There are four TIMP proteases that carry out similar functions, but inhibit different MMPs.

Tissue inhibitor of matrix metalloproteinase-1 is highly inducible in response to a variety of cytokines and even hormones. Tissue inhibitor of matrix metalloproteinase-2 functions as a metastasis suppressor with its ability to directly suppress the proliferation of endothelial cells which make up the vasculature and therefore the main component necessary for angiogenesis (41). It is also known to inhibit protease activity in tissue ECM being remodeled in which the MMPs are functionally active. Tissue inhibitor of matrix metalloproteinase-3 expression is induced in response to mitogenic stimulation. Tissue inhibitor of matrix metalloproteinase-3 is considered a netrin domain-containing protein that is always localized to the ECM. It is the only soluble or secreted TIMP. Finally, TIMP-4 is involved in the regulation of platelet aggregation and recruitment. It

may also play a critical role in hormonal regulation and endometrial tissue remodeling after endometrial breakdown during menstruation (42).

Alpha2-macroglobulin (a2M) is another molecule that helps to inhibit MMP function aside from the TIMPs. It does this via binding to and removing the active forms of the gelatinases A and B from the circulation via scavenger receptors on phagocytes. Alpha2-macroglobulin's structure is simple with four identical protein subunits linked together via cysteine bonds.

MMPs in Lung Disease

Matrix metalloproteinases are both beneficial and detrimental in the lung. They help to carry out essential functions such as lung remodeling. However, many MMPs reside in the lung with significant impact in diseases such as asthma, PAP, COPD, lung fibrosis, and CF (See Table 2). For instance, expression of several MMPs has been associated with asthma; increases in MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 have all been found in sputum and BAL from patients with asthma (43). In general, airway walls and their state of constriction or dilation are critical factors when studying the asthmatic lung since most patient discomfort results from airway wall constriction along with inflammation. The inflammation has been associated with Clara cell hypersecretion, bronchiole hyperplasia and excess mucus production (5). Increased smooth muscle mass is perhaps the most important component of the airway wall remodeling process in asthma (44).

It has been shown that MMP-2 contributes to the increased smooth muscle mass and inhibition of MMP-2 significantly reduces smooth muscle cell proliferation (17). Therefore, inhibitory targets of MMP-2 could potentially assist in the dilation of airway

walls. Further, it is thought that MMP-2 plays a role in the luminal clearance of inflammatory cells and deletion of MMP-2 causes inflammatory cell infiltration of the alveolae(6, 45). Therefore, MMP-2 not only contributes to the state of airway constriction or dilation but the overall presence of inflammatory cells in the airway lumen suggesting a central role of MMP-2 in the asthmatic lung environment. It is also important to mention that MMP-9 plays a role in clearing eosinophils from lung after inflammation which may contribute to asthma pathogenesis. Matrix metalloproteinase-9 expression correlates with tissue eosinophil number (46). Therefore, MMP-9 directly contributes to the asthmatic inflammatory cell profile.

Table 2. Lung Disease Associated with Gelatinase Activity

Protease	Disease	Function/Mechanism	Reference
MMP-2	***PAP***	MMP-2 mRNA and protein are increased in BAL from PAP patients. MMP processing of chemokines leads to lack of cellular migration to the lungs.	McQuibban (2002) Bonfield (2006) Greenlee (2007)
	Asthma	Increases in MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 expression are all found in sputum and BAL from patients with asthma. IGF-1 upregulates MMP-2 causing reduced smooth muscle cell proliferation.	Demedts et al (2005) Johnson (1999)
	COPD and emphysema	Dysregulated expression of MMPs. Association is not clear. Destruction of small bronchi and alveolae.	Greenlee (2007) Gueders (2006)
	Cystic Fibrosis	IL-8 induces increased expression of MMP-2 in CF patient BAL. Host defense against pathogens in the lung.	Sagel (2005) Greenlee (2007)
	IPF	TGF-β stimulation increases MMP-2 activity. Abnormal functioning of MMP-2 results in no cleavage of chemokines. This leads to an imbalance in the chemokine/chemokine receptors ratio. Impaired tissue-induced increased expression.	Greenlee (2007) Pignatti (2006) Gueders et al. (2006)
MMP-9	***PAP***	MMP-9 mRNA and protein are increased in BAL from PAP patients. MMP processing of chemokines leads to lack of cellular migration to the lungs.	Bonfield (2006) Greenlee (2007)
	Asthma	Increased MMP-9 activity in the subepithelial basement membrane accompanied by higher TGF-β and neutrophil count. Clears eosinophils from lung tissue after inflammation. MMP-9 presence correlates with tissue eosinophil number.	Wenzel et al (2003) Han (2003)
	COPD and emphysema	Dysregulated expression of MMPs. Implicated in lung tissue destruction. Destruction of small bronchi and alveolae. Increased expression of MMP9 in emphysematous lungs.	Greenlee (2007) Molet (2005) Gueders et al. (2006) Finlay et al. (1997b)
	Cystic Fibrosis	IL-8 induces increased expression of MMP-9 in CF patient BAL. Significant inverse relationship between MMP-9 and lung function. Host defense against pathogens in the lung.	Sagel (2005) Greenlee (2007)
	IPF	Imbalance in chemokine/chemokine receptors within the lung environment. Reduced or absent chemokine signaling and chemoattraction of inflammatory cells. Inflammation-induced increased expression.	Pignatti (2006) Gueders et al. (2006)

IPF= Idiopathic Pulmonary Fibrosis
COPD= Chronic Obstructive Pulmonary Disease
PAP= Pulmonary Alveolar Proteinosis

The PAP lung presents a very complicated tissue phenotype. It is characterized by many immature alveolar macrophages and surfactant accumulation within the airways

ultimately compromising respiration. Patients with PAP have chronic inflammation in the lung along with elevated levels of monocyte chemotactic proteins (31). Matrix metalloproteinase-2 and -9 mRNA and protein are increased in bronchoalveolar lavage (BAL) from PAP patients (47). Furthermore, MMP-2 is shown to cleave CCR2 (the receptor for many MCPs), preventing chemotaxis in vitro (13). As a result, PAP lungs demonstrate a reduced number of inflammatory cells from the periphery due to the ineffective function of chemokines within the lungs. The ineffective function of chemokines is mainly due to cleavage by MMPs as well as surfactant obstruction.

As mentioned above, COPD and emphysema are caused by many environmental factors, including prolonged cigarette smoke. Extensive tissue remodeling in COPD causes destruction of small bronchi and alveolae, leading to emphysema and involves many members of the MMP family (6). Increased tissue destruction and inflammatory mediator activation leading to the pathology that occurs during COPD is a result of increased macrophage numbers, the up-regulation of MMPs, and their co-release with other stored proteinases (48). As compared with healthy subjects, patients with COPD have marked increase in expression and activity of MMP-2, MMP-9 and MT1-MMP in their lung parenchyma and increased gelatinolytic activity linked to MMP-2 and MMP-9 in their sputum (49). The most well-studied MMPs in human COPD and emphysema are MMP-1, MMP-8, MMP-9, and MMP-12, which have all been implicated in tissue destruction in human COPD and emphysema (50).

Idiopathic pulmonary fibrosis (IPF) is a lung disorder in which fibrotic tissue invades the lung matrix causing impaired oxygen transfer and alveolae destruction and collapse. In this condition, there is an unknown imbalance in the synthesis and

degradation of matrix degradation of lung matrix. The condition is exacerbated by an influx of additional inflammatory cells such as macrophages, neutrophils, and lymphocytes, to the site of degradation imbalance. Matrix metalloproteinase-2 and MMP-9 have increased expression in IPF; however, they are initiated and function at different stages in the disease. For example, MMP-9 is linked to inflammation-induced tissue remodeling early on, while MMP-2 is associated with an impaired tissue remodeling system leading to pathological collagen deposition and interstitial fibrosis later (6).

Cystic fibrosis (CF) is a disease in which chronic inflammation exists. Patients experience a dysfunctional chloride channel in which chronic bacterial infections and inflammation result. It has been shown that patients with CF have increased levels of MMP-2, MMP-8, and MMP-9 in their BAL (51). With regard to MMP-9 and in children with stable CF, there is a significant inverse relationship between MMP-9 and lung function, as measured by forced expiratory volume (FEV) (31). For example, when MMP-9 levels are remarkably high, lung function is very poor. The role of MMPs in CF is unclear; however, their upregulation suggests they are potential targets for therapeutic intervention in the treatment of this lung disease.

Summary

Matrix metalloproteinases are critical for lung homeostasis including tissue turnover. They are present during routine tissue maintenance, remodeling, as well as during the immune response. Matrix metalloproteinases can also be detrimental to the lung environment if interfering with proper immune clearance and function. The gelatinases are two important MMPs found commonly in lung conditions characterized

by chronic inflammation including asthma, COPD, emphysema, IPF, CF, and PAP. The study of MMP function, specifically gelatinase function, in chronic inflammation can lead to possible therapeutic treatments for individuals with these conditions.

III. Chemokines

Chemokines are small cytokine proteins that resemble each other in basic structure. They are small in size, roughly 8 to 10kD each, or 70-130 amino acids. They are produced by a variety of cells including lymphocytes, monocytes/macrophages, smooth muscle cells, epithelial cells, endothelial cells, and fibroblasts (52). They consist of intramolecular disulfide bonds joining their cysteine residues. All are generally produced as pro-peptides with an N terminal signal peptide. This signal peptide usually runs about 20 amino acids long and is cleaved to produce the active protein. This cleavage occurs while the pro-peptide is secreted from the cell upon its release, maturation, and activation.

They serve many functions within the lung environment (See Table 3). In general, they carry out specific chemoattractant functions along with other white blood cells during an immune response or in response to growth factors, hormones, and other environmental stimulants. Commonly, inflammatory chemokines are released as a result of pro-inflammatory cytokines such as IL-1 and alveolar type I-like cells or type II cells which secrete chemokines in direct response to IL-1 exposure in humans. Their inhibition dramatically slows the immune response suggesting they serve critical roles through white blood cell chemotaxis. Proteolysis of chemokines may provide a mechanism for loss of chemoattractant signaling (13). For example, in recent research,

MMP-2 was shown to process the N-terminus of MCP-3 and stromal cell-derived factor-1α (SDF-1α) and β in humans, reducing their chemotaxic capabilities (53, 54).

Table 3. Chemokines and Some Functions in the Lung

Name	Receptor	Function in the Lung	Reference
IL-8 (CXCL8)	CXCR1, CXCR2	Recruit neutrophils from periphery to inflamed lung tissue.	Mantovani (2006)
MCP-1 (CCL2)	CCR2	Proteins 1-3 chemoattract monocytes and sometimes T cells. MCP-1 also recruits basophils and mast cells to sites of inflammation. MCP-4 is more of a locally expressed biomarker found in asthmatic lungs.	Taub (1995), Mantovani (2006)
MCP-4 (CCL13)	CCR1, CCR2		
MCP-3 (CCL7)	CCR1, CCR2, CCR3		
MCP-2 (CCL8)	CCR2, CCR5		
MIP-1β (CCL4)	CCR5	Overlapping functions include leukocyte (neutrophil) chemotaxis; specifically monocytes to sites of inflammation. Help to initiate phagocytosis at these sites.	Cook (1996)
MIP-1α (CCL3)	CCR1, CCR5		
MIP-3α (CCL20)	CCR6		
RANTES(CCL5)	CCR1, CCR3, CCR5	T cell, eosinophils, basophil chemotaxis, activate NK cells to form CHAK cells	Mantovani (2006)
Eotaxin (CCL11)	CCR3	Increased in asthmatic lung tissue. Eosinophil survival promotion, activation, and recruitment to airways.	Vliagoftis et al (2000) Gueders et al. (2006)
Eotaxin-2 (CCL24)	CCR3		
Eotaxin-3 (CCL26)	CCR3		

MIP= Macrophage Inflammatory Protein
RANTES= Regulated on Activation, Normal T Cell Expressed and Secreted
NK = Natural Killer
CHAK = CC-Chemokine activated killer cells
IL= Interleukin
MCP= Monocyte Chemoattractant Protein

Chemokine functioning relies greatly on G-protein-linked transmembrane receptors found on their target white blood cells (55). A signal cascade results through the white blood cell cytosol activating it to respond to this chemoattractant gradient. Once recruited by these receptors, the target cells follow an increasing signal concentration sent out from the chemokine to the area in which the chemokine resides, in this case, the lung (See Figure 2).

There are four classifications of chemokines based upon their positioning of the first two cysteine amino acids – CC, CXC, C, and CXXXC chemokines. The first group, the CC chemokines, is also called the β-chemokines and contains their two cysteines near the N terminus. They are responsible for the migration of natural killer cells, dendritic cells, as well as monocytes to the area of inflammation/injury (4). One common CC

chemokine is monocyte chemoattractant protein-1 (MCP-1) which attracts circulating monocytes (4).

The second group of chemokines is called the CXC chemokines. The first two cysteines are divided by one amino acid. This group of chemokines is broken down into two additional subgroups. One group contains a conserved motif of glutamic acid-leucine-arginine (ELR) positioned before the first cysteine residue while the second subgroup does not contain this ELR motif (56). Those containing the motif are specific for inducing the migration of neutrophils while those without the motif induce the migration of lymphocytes (56). Interleukin-8 falls within the subgroup containing the ELR motif; therefore, it aids in the induction of neutrophil migration to the site of injury or inflammation (4).

The third group, the C chemokines, is sometimes referred to as the γ-chemokines. This group is unique because the structure consists of two cysteine residues instead of the normal four and is positioned near the N terminus while the other presents itself further downstream. The C chemokines are known to induce migration of T cell precursors to the thymus during development (57). There are two well-known C chemokines that do this – lymphotactin-α (XCL1) and lymphotactin-β (XCL2) (57).

The fourth group, the CXXXC chemokines, contain three amino acids between the first two cysteine molecules. They are sometimes referred to as the Δ-chemokines. There is only one main CXXXC chemokine that is known to date, fractalkine (CXXXCL1) (58). Although its function is not well understood, it is thought to aid in cellular adhesion.

Monocyte chemoattractant proteins (MCPs) are specifically chemotactic for monocytes and lymphocytes (59). Monocyte chemoattractant protein-3 (MCP-3) in particular, also referred to as CCL7, is a chemokine included in the CC chemokine group; therefore, it contains two adjacent cysteines at the N terminal end of the small protein. Monocyte chemoattractant protein-3 attracts monocytes to the site of injury and also aids in macrophage cell function (4). The chemoattractant protein is synthesized by macrophages as well as specific tumor cells. It is similar in function to MCP-1, or CCL2.

Interleukin-8 (IL-8 or CXCL8), also referred to as KC (CXCL1) in the mouse, is a part of the CXC subfamily of chemokines and is an important mediator of the inflammatory response. It is present at the inflammation site and aids in the extravasation of neutrophils from the blood vessels to the site of injury (4). In the lung, IL-8 is produced from alveolar macrophages and is therefore in close proximity to the inflamed site should it be needed. These alveolar macrophages release IL-8 to promote neutrophil recruitment to help clear infection or injury. Epithelial cells are also known to release IL-8 in response to inflammation. Furthermore, it is well-known that this chemokine has angiogenic capabilities suggesting a possible target in cancer therapeutics.

To date, there are nineteen chemokine receptors that signal intracellular pathways of activation. It is well-known that chemokines function through G-protein coupled transmembrane receptors (55). The chemokine signal interacts with the G protein via binding to the extracellular N terminal domain. A cascade of subsequent events allows the interaction of the G protein to phospholipase C (PLC). Well-known second messenger proteins known as inositol triphosphate (IP3) and diacylglycerol (DAG) carry out the functions of intracellular signaling and their respective pathways of calcium

release or protein kinase C (PKC) activation. From these events, additional pathways are activated, including the MAP kinase pathway. The MAP kinase pathway is associated with chemotaxis which is important regarding cellular recruitment to sites of inflammation (60). As a side note, the MAP kinase pathway also directs cellular degranulation, superoxide anion release, and cell adhesion via integrins (60, 16).

As mentioned above, MMPs are unique in that they serve several beneficial functions in homeostasis alongside roles in pulmonary disease pathogenesis. In the human, MMPs are known to cleave tissue chemokines and thereby prevent chemoattractant signals from reaching potentially helpful inflammatory cells from the periphery (61, 53). When this happens, inflammation is allowed to progress and tissue function declines. This represents a general case when MMPs prove to be detrimental to the normal immune response. Under normal conditions, when an infection or foreign invader is recognized, chemokines signal to inflammatory cells in the periphery and bloodstream to home to the site of injury or infection. When MMPs cleave these necessary chemokines, chemotaxis of white blood cells is reduced or absent completely. This is one main way in which inflammation is established and remains in the lung tissue.

Summary

Chemokines play a vital role in the normal functioning of the lung in response to infection and inflammation. Their function and recruitment of inflammatory cells is needed at sites of inflammation in order to effectively clear phagocytic debris as well as invading pathogens. Since chemokines are the mediators of this critical inflammatory response, any protease, such as MMPs, or other impediment which inhibits or reduces their function is detrimental to the tissue undergoing an immune response. Since

chemokines cover a vast array of cellular recruitment pathways in all tissues, they represent exciting therapeutic targets in which inflammation can be manipulated and possibly corrected on a broader scale among tissues.

IV. MMPs and Chemokines

The studies described here focus on active MMP and chemokine interaction in the inflamed lung. Matrix metalloproteinases play a universal role in routine tissue maintenance and remodeling. While some MMPs are constitutively active in remodeling tissue, other MMPs are cleaved into their active enzyme forms by surrounding factors within a tissue such as oxidants, SDS, and organomercurials (26). Since MMPs tend to be secreted from cells situated near inflammation or a site of injury, they act upon tissue in close proximity to where they were initially activated. They are critical for the homeostasis of the lung in order to clear the ECM from obstructing phagocytic fragments remaining in the airways. These proteases can also directly affect the immune response by proteolytically degrading the foreign invader or infectious agent themselves (62). For example, MMP-12 is known to demonstrate direct bactericidal activity inside the alveolar macrophage (62).

On the other hand, chemokines tend to have a more indirect role in the clearance of inflammation. They are often initiated by antigen; however, hormones and other factors can initiate their production and function. They are indirect mediators of inflammatory cells from the periphery sending out chemoattractant signals requesting inflammation clearance and degradation.

In the normal lung, MMPs and chemokines work efficiently to carry out their functions ensuring inflammation is cleared. Matrix metalloproteinases assist in the

degradation directly creating cellular debris which initiates chemokines to begin the cellular recruitment. Chemokines then attract white blood cells from the periphery to the site of inflammation. White blood cells carry out their designed functions of phagocytosis and lysosomal degradation. In turn, this creates more cellular debris which the MMPs assist in proteolytically degrading. Alongside one another, they function thoroughly to resolve inflammation in an efficient manner.

Summary

Matrix metalloproteinases and chemokines contribute to the homeostasis of the lung tissue by resolving inflammation efficiently and thoroughly - MMPs degrading phagocytic fragments and obstructing debris and chemokines recruiting white blood cell assistance. In states of chronic inflammation, MMP and chemokine gene and protein levels are increased. In the PAP lung, an unchanged cellular recruitment is often seen despite increased levels of chemokines.

V. Focal point – MMP-2 and MCP-3

In order to study the relationship between MMPs and chemokines in chronic lung inflammation, MMP-2 and the chemokine MCP-3 were the primary research focus simply because in humans, MMP-2 is known to cleave MCP-3 in the lung condition PAP (13). Pulmonary alveolar proteinosis is characterized by chronic lung inflammation in which the lung is in a perpetual state of surfactant accumulation and lipid buildup. In addition, PAP was shown to have increased MMP-2 and MCP-3 levels in the lung environment (47). In PAP, although there are elevated MMP-2 and MCP-3 levels, cellular recruitment is noticeably unchanged (47). This suggests somewhere along the way, MCP-3 is being cleaved into an inactive truncated protein. Current research has

discovered that indeed human MCP-3 is cleaved into the truncated protein by MMP-2 at the amino acid sequence G-I (13). When comparing to a normal patient cellular profile, there is an increased cellular recruitment found in the BAL fluid in response to inflammation, suggesting MCP-3 is unaltered and healthy white blood cell recruitment.

Summary

Matrix metalloproteinase-2 and MCP-3 gene and protein levels are increased in the PAP lung. Matrix metalloproteinase-2 is shown to cleave MCP-3 in the PAP lung decreasing peripheral inflammatory cell recruitment.

VI. Model

Pulmonary alveolar proteinosis is a rare disease characterized by the presence of immature alveolar macrophages and an accumulation of surfactant in the lung (63). Pulmonary alveolar proteinosis can arise from a congenital mutation in either the surfactant protein B (SP-B) or SP-C (64). It can also be acquired by secondary means which includes particulate exposure such as silicosis, titanium dioxide, cellulose fibers, as well as dust (64). Pulmonary alveolar proteinosis can also have an idiopathic origin in which a cause in unknown. This spontaneous arrival of PAP is especially frustrating for clinicians since diagnosis is often overlooked or missed altogether.

Morphologically, PAP lungs are filled with accumulated surfactant phospholipids as well as excess mucus in the alveoli (47). As a result, a state of inflammation results and persists. Similarly, the GM-KO mouse lung environment also consists of surfactant protein buildup. These mice develop a PAP-like lung disease after 6-8 weeks with seemingly no hematopoietic abnormalities (65, 66). Granulocyte-macrophage colony-stimulating factor knockout mice are absent of GMCSF only in their lungs, unlike PAP

lungs which have autoantibodies against their GMCSF (67). Although the cause of the inflamed lung is different between the PAP patient and GM-KO mouse, both lung environments experience nonfunctional GMCSF protein. Therefore, the GM-KO mouse lung provides an almost identical model of chronic lung inflammation in which to study MMPs and chemokines, specifically MMP-2 and its cleavage of MCP-3. Through the GM-KO mouse model, many other lung diseases characterized by chronic lung inflammation, or the immune response in general, can be studied more effectively.

Matrix metalloproteinase and chemokine levels prove to be elevated in the PAP lung; therefore, MMP-2 and MCP-3 levels should also be elevated in the GM-KO mouse lung as well as other conditions where inflammation is present. This is applicable not only to the lung environment, but other tissues experiencing inflammation.

Summary

The PAP lung is a chronically inflamed tissue producing excess surfactant and phospholipids reducing overall pulmonary function. The GM-KO mouse lung develops PAP-like symptoms by 6-8 weeks of age. The GM-KO lungs are also filled with excess surfactant proteins serving as an excellent model for studying chronic inflammation in the lung. Matrix metalloproteinases and chemokine levels are increased in the PAP lung environment; therefore, the GM-KO mouse lung should also demonstrate increased MMP and chemokine expression.

Hypothesis

The GM-KO mouse lung, which resembles the PAP lung, should have increased MMP-2 and MCP-3 levels. Proposed cleavage of MCP-3 by MMP-2 in the mouse should result in an unchanged cellular differential and reduced inflammatory clearance as seen in the PAP lung.

Materials and Methods:

Cell Culture

To manipulate MMPs in vitro, a mouse alveolar macrophage cell line was used called RAW264.7 (American Type Culture Collection, Manassas, VA). To do this, RAW264.7 cells were maintained in Complete DMEM media (Mediatech, Inc, Cleveland, OH) supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine (Invitrogen, Carlsbad, CA). Cells were stimulated with Lipopolysaccharide (LPS) from E.Coli (Sigma Adlrich, Inc, St. Louis, MO) in order to initiate production of MMP-2 and -9. A timecourse study of 24, 48, and 72 hours was used to study the effectiveness of LPS in eliciting a response over time. RAW264.7 cells were plated on polystyrene culture plates and incubated at 37 ̊C. The conditions included unstimulated cells and cells stimulated with LPS at 0.5µg/mL for a period of 24, 48, and 72 hours. The wells were then collected and processed for gene expression. Cell samples were run on a RT-PCR/Taqman 7300 System (Applied Biosystems Inc, Foster City, CA) to measure the gene expression of MMP-2, MMP-9, and GAPDH.

Mouse Experiments

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC). Wild-type, C57BL/6, mice from JAX (Jackson Laboratories, Bar

Harbor, ME) were used as comparison controls for the GM-KO mice. The GM-KO mice were obtained from Dr. Robert Paine (University of Michigan, Ann Arbor, MI) with permission of Dr. Glenn Dranoff (Dana-Farber Institute, Boston, MA), the person who generated the mice. The mice have been backcrossed eight generations to C57Bl/6 wild-type mice which were obtained from the Jackson Laboratory (Bar Harbor, ME).

Bronchoalveolar lavage (BAL) was performed to extract lung macrophages by instilling sterile 1x Dulbecco's Phosphate Buffered Saline (1x D-PBS) (Invitrogen, Carlsbad, CA) into the lungs of both GM-KO and WT mice. Three instillations of 1mL total volume were instilled into the lungs via 1mL syringe and 20 gauge feeding needle to obtain an optimal return of alveolar macrophage cells for the studies. Baseline pellets were used from both WT and GM-KO to process for gene expression.

Resident peritoneal macrophages were extracted from WT mice in order to observe cellular recruitment via chemotaxis assays. To do this, the mouse peritoneum was slowly filled with 10mL sterile 1x D-PBS using a 10mL syringe and 20 gauge needle. The fluid was then collected from the peritoneum and the cells were counted using a hemacytometer. Cells were used for chemotaxis assays.

RT-PCR

Cultured RAW264.7 and baseline mouse cells were processed for gelatinase gene expression. To do this, RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was prepared from that using Superscript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA. Gene expression was measured using the 7300 Real-Time PCR Taqman System (Applied Biosystems, Foster City, CA). Murine MMP-2 and MMP-9 primers were compared to GAPDH primer as a housekeeping control.

Primers were obtained and validated from Applied Biosystems (Foster City, CA) and ΔCt values plus ΔΔCt values were analyzed using Prism 3.0 software.

Western Blots

To study the level of MMP-2 protein expression in the GM-KO and WT mouse lung. Western blots of the BAL were probed and run for MMP-2. A murine serum albumin (AbCam, Cambridge, MA) loading control at a dilution of 1:1000 was used.

To assemble the apparatus, an 8% resolving gel and a 5% stacking gel were prepared using 30% acrylamide solution (Sigma Aldrich, Inc, St. Louis, MO), 1.5M Tris (Sigma Aldrich, Inc, St. Louis, MO) (pH 8.8) for the resolving gel, 1.0M Tris (Sigma Aldrich, Inc, St. Louis, MO) (pH 6.8) for the stacking gel, 10% Sodium Dodecylsulfate (SDS) (Sigma Aldrich, Inc, St. Louis, MO), 10% ammonium persulfate (APS) (Sigma Aldrich, Inc, St. Louis, MO), and TEMED (Sigma Aldrich, Inc St. Louis, MO).

Bronchoalveolar lavage fluid was loaded at a concentration of 5ug protein/appropriate lane based on a basic BCA assay. The gel was run at 125V for approximately 1-2 hours in 1X Running Buffer (Sigma, Aldrich, Inc, St. Louis, MO). Gels were transferred for one hour at 77V in 1X Transfer Buffer (Sigma Aldrich, Inc, St. Louis, MO). Blots were blocked in a 5% nonfat Milk-TBS-T buffer for 2 hours at room temperature. An anti-mouse MMP-2 primary antibody (0.1mg/mL) (R&D Systems, Minneapolis, MN) was incubated overnight in 4°C followed by incubation in an anti-goat IgG HRP-conjugated secondary antibody (1:1000) (R&D Systems, Minneapolis, MN) for one hour at room temperature the next day.

Gelatin Zymography

In order to observe the functional proteolytic activity of MMP-2 in the lung, a gelatin zymography was performed. To do this, BAL fluid from WT and GM-KO mice were run at 10ug protein/lane on a gelatin-bound gel. A separating gel was prepared using 10% SDS, 10% APS, 30% acrylamide solution, 3mg/mL gelatin solution, TEMED, and a 4X separating gel buffer which includes Tris and SDS (pH 8.8). A stacking gel was prepared using 10% SDS, 10% APS, 30% acrylamide solution, TEMED, and a 4X stacking gel buffer which includes Tris and SDS (pH 6.8). An MMP-2 and MMP-9 control was run at a concentration of 0.1μg/μl (EMD Biosciences, Darmstadt, Germany).

The gel was run at 80V for 20minutes then at 125V for 2-2.5hours. The gel was placed in Renaturing Buffer (1x) (BioRad, Hercules, CA) for two incubations, then overnight in Development Buffer (1x) (BioRad, Hercules, CA) for 16-20 hours at 37℃. After the overnight incubation, the gel was placed in Gel Fixation Buffer consisting of methanol, acetic acid, and distilled water for 10 minutes. Coomassie Blue stain (Sigma Aldrich, Inc, St. Louis, MO) was used along with a destaining solution to clear bands.

The enzymatic activity of the MMPs was then assessed by observing the presence of white bands on a Coomassie Blue background stained gel under a basic light box.

Chemotactic Experiments

Resident wild-type peritoneal macrophage cells were extracted and used to measure the amount of chemotaxis demonstrated by WT and GM-KO BAL fluid. One to two million peritoneal macrophage cells were placed in the upper chamber of a sterile 12mm diameter, 0.4μm pore polyester membrane chemotaxis well (Corning, Corning, NY). Wild-type and GM-KO BAL fluid was placed in the bottom well of the chemotaxis

Mass Spectrometry

Protein was extracted from the WT and GM-KO BAL fluid via a methanol chloroform procedure. Bronchoalveolar lavage fluid was resuspended in a solvent mixture of chloroform, methanol, and saline in a ratio of 8:4:3. The methanol layer was removed after shaking for 20 minutes at room temperature and run on a mass spectrometer for the presence of the cleaved MCP-3 peptide. The proposed cleaved peptide consists of an N-terminal sequence of QPDG, with a charge mass ratio of 415.5.

Statistics

A couple types of statistical analyses were used to study the data. The first, Analysis of Variation (ANOVA), is used to study data with multiple variables (68). Here, it was used to study the RT-PCR values from the RAW264.7 timecourse study in order to compare the different timepoints with LPS stimulation for gene expression of MMP-2 and -9. One-way ANOVA is used when comparing multiple variables to one reference point. Our analysis used the one-way ANOVA since we were comparing multiple variables to one set reference point. The second type of analysis, the t-test, is used when comparing two groups/variables (69). Here, it was used to analyze the GM-KO RT-PCR values in order to compare two groups – the WT and GM-KO.

Results:

Cell culture

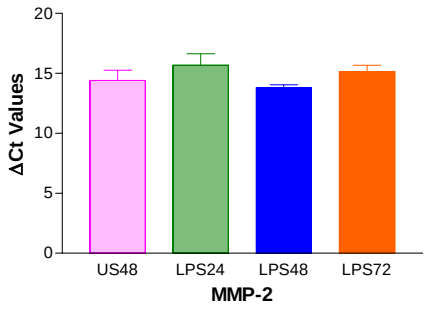
RAW 264.7 cells are a modified mouse macrophage cell line that can easily be manipulated with regard to activation or inhibition of cell functions. Lipopolysaccharide was used to elicit an inflammatory response among the RAW264.7 cells. Once an inflammatory response was initiated, MMP-2 and MMP-9 are released from the macrophages with the purpose of clearing or phagocytosing the invader. It seems that over a timecourse of 24, 48, and 72 hours, RAW cells expressed the most MMP-2 and MMP-9 expression at 48 hours (See Figure 5A-D).

Gene expression using the RT-PCR system uses cycle threshold (Ct) values that correspond to the number of copies of a particular gene in your sample. For each set of data, a threshold curve is generated. The smaller the Ct value, the faster the curve is reached and the more DNA the sample contains. The ΔCt values are the Ct values normalized to the control gene - GAPDH. A low ΔCt value means there is more gene expression in a sample. The ΔΔCt values are the normalized samples compared to the reference point – WT or unstimulated culture wells. A higher ΔΔCt value corresponds to more gene expression for a particular sample.

Figures 5A and 5C represent the ΔCt values for MMP-2 and -9 respectively when normalized to the GAPDH control gene. RAW264.7 cells stimulated with LPS for 48 hours did a better job of initiating MMP-2 and -9 production; however, the data is not statistically significant for MMP-2 when using one-way ANOVA analysis (Figure 5A $p=0.387$). The data is also not statistical for MMP-9 when using one-way ANOVA analysis (Figure 5C $p=0.312$). Figures 5B and 5D represent the ΔΔCt values for MMP-2

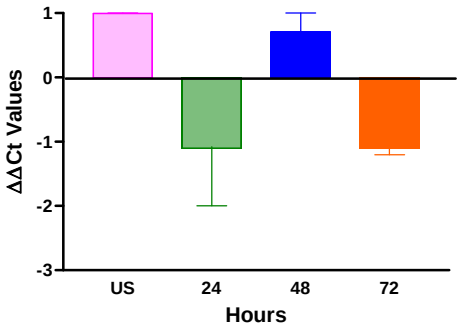
and MMP-9 relative to US. The data is not significant for MMP-2 when using one-way ANOVA analysis (Figure 5B p=0.06), as well as for MMP-9 (Figure 5D p=0.164). More studies need to be conducted to prove significance for MMP-2 and -9 production upon LPS stimulation.

Figure 5A. RAW264.7 ΔCt: LPS Timecourse for MMP-2



P=0.387 (one-way ANOVA, non-parametric)

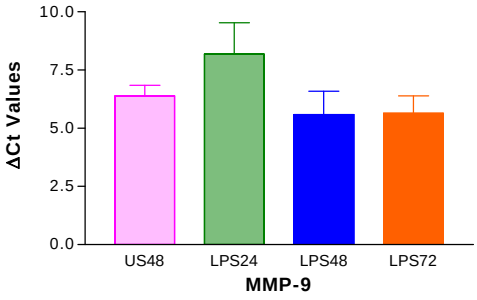
Figure 5B. ΔΔCt for MMP-2 Relative to US



P=0.06 (one-way ANOVA, non-parametric)

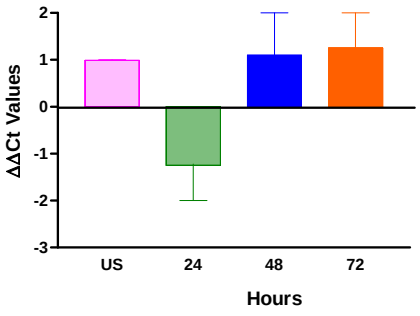
(Samples are normalized to US, which is set at 1.0)

Figure 5C. RAW264.7 ΔCt: LPS Timecourse for MMP-9



P=0.312 (one-way ANOVA, non-parametric)

Figure 5D. ΔΔCt for MMP-9 Relative to US



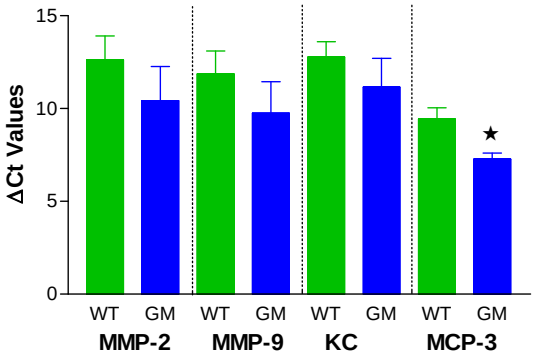
P=0.164 (one-way ANOVA, non-parametric)

(Samples are normalized to US, which is set at 1.0)

Mouse Experiments

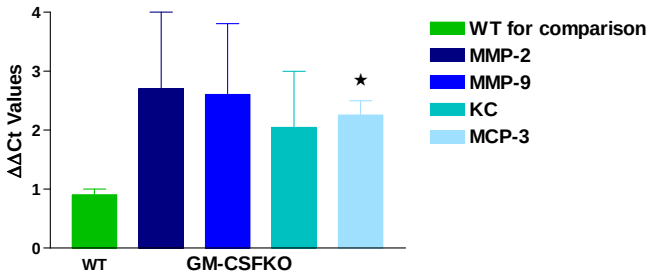
The GM-KO mouse was used to study the presence of MMP-2 and -9 in the lung. Wild-type, C57BL/6, mice were used as comparison controls. Figure 6A represents the ΔCt values for each gene for WT and GM-KO mice normalized to GAPDH. When assessing the presence of WT versus GM-KO BAL baseline cells, it was shown that MMP-2, MMP-9, and KC gene expression levels were slightly increased even at baseline; however the data is not significant when using t-tests for each gene (Figure 6A $p \geq 0.42$ for each gene). However, when using the t-test for MCP-3 expression between WT and GM-KO BAL cells, the data was significant (Figure 6A $p=0.02$). Figure 6B represents the ΔΔCt values when compared to WT, but the data is not significant, except for expression of MCP-3 in the GM-KO (Figure 6B $p=0.02$). This data suggests that there are possible trends towards increased MMP-2, MMP-9, and KC levels in inflamed lung tissue. Further, this data demonstrates statistically significant levels of MCP-3 in the GM-KO when compared to WT.

Figure 6A. WT v. GM-KO BAL ΔCt



P≥0.42 for MMP-2, MMP-9 and KC- Students t- test
★ P=0.02 for MCP-3

Figure 6B. ΔΔCt Values Relative to WT

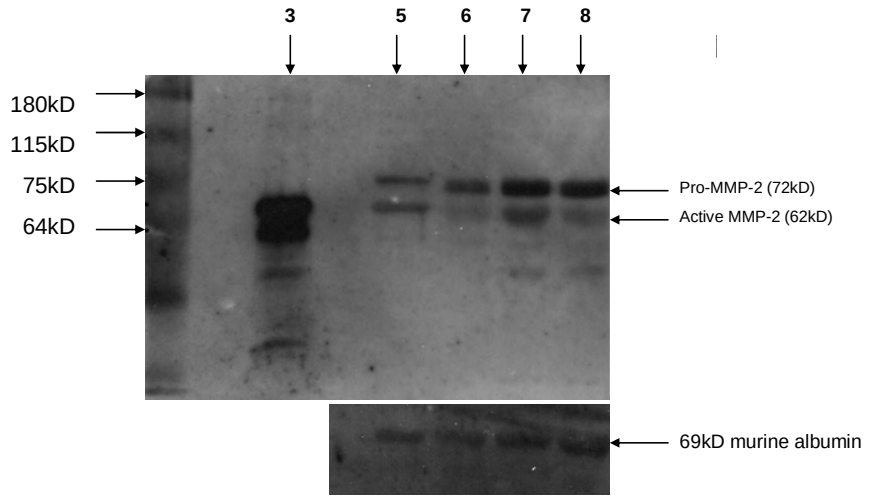


P≥0.3 for MMP-2, MMP-9 and KC, Students t-test
★ P=0.02, for MCP-3

Western Blots

The GM-KO mouse expressed more MMP-2 protein in the BAL fluid when compared to WT control BAL fluid. Both the pro- and active forms of the MMP-2 protein were increased in the GM-KO BAL fluid (See Figure 7). This suggests not only is the gene expression increased inside the alveolar macrophage cells, but also in the secreted products found in the surrounding alveolar fluid.

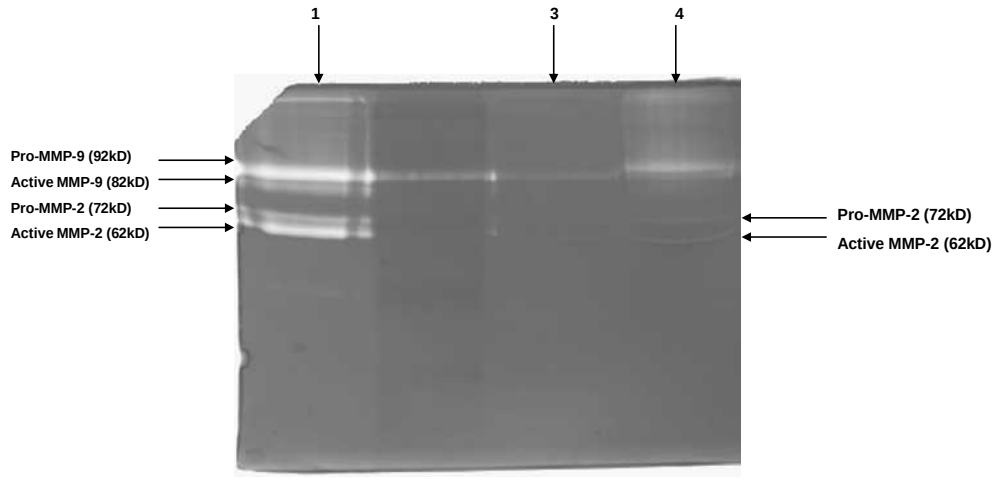
Figure 7. WT and GM-KO BAL probed for MMP-2
Murine MMP-2 control loaded in Lane 3. WT BAL (at 5µg/lane) was run in Lanes 5 & 6. GM-KO BAL (5µg/lane) was run in Lanes 7 & 8.



Gelatin Zymography

The enzymatic activity of the MMPs was then assessed by observing the presence of white bands on a Coomassie Blue background stained gel. Since the gelatinases degrade gelatin, which was incorporated into the gel matrix, as one of their primary substrates, gelatinase activity was correlated with the white bands in which the gel was degraded. The zymography showed increased intensity of the white bands in the GM-KO BAL fluid as compared to the WT BAL fluid (See Figure 8). This suggests there are elevated MMP-2 and MMP-9 enzymes actively degrading the gelatin matrix in the GM-KO BAL fluid.

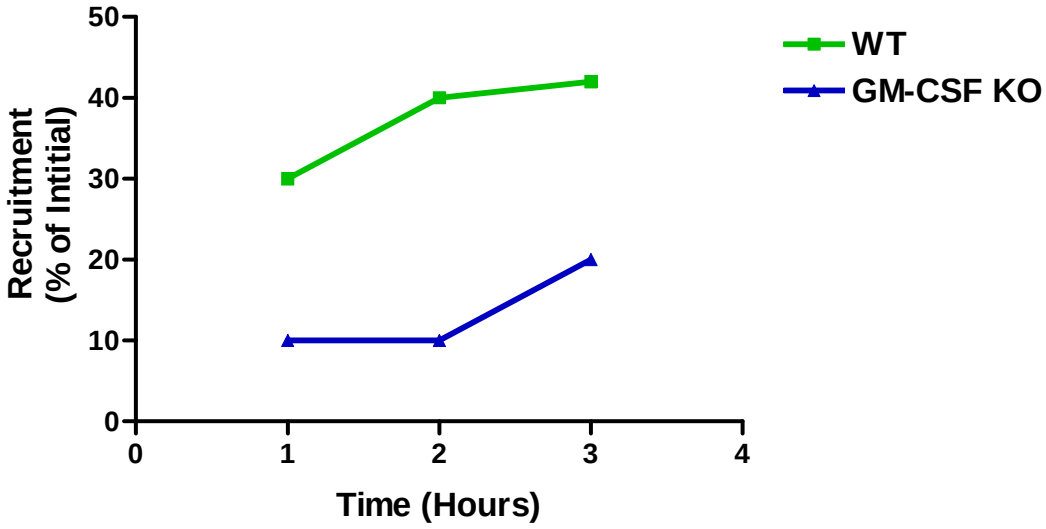
Figure 8. Gelatinase Enzymatic Activity is increased in the GM-KO BAL fluid. Lane 1 contains the MMP-2 and MMP-9 controls. Lane 3 contains 10µg WT BAL fluid. Lane 4 contains GM-KO BAL fluid.



Chemotactic Experiments

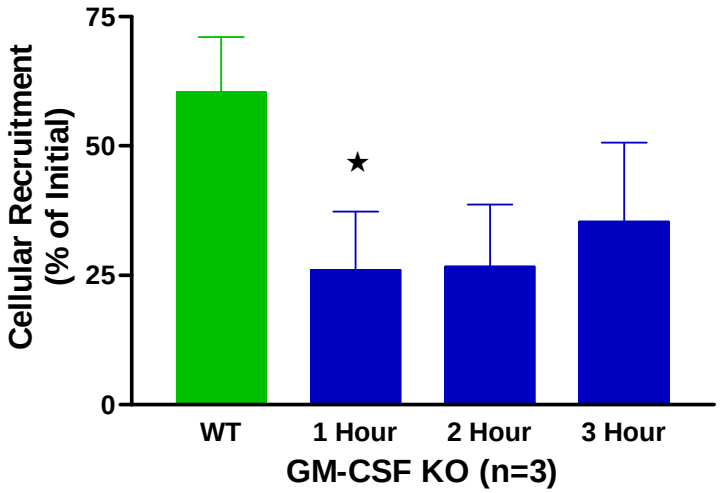
Wild-type peritoneal macrophage cells were used to measure the amount of chemotaxis demonstrated by WT and GM-KO BAL fluid. The WT mouse BAL fluid was expected to have a higher frequency of chemoattracting WT peritoneal macrophages from the top well to the bottom well over the three hour incubation period since there are thought to be no interfering excess of MMPs cleaving the chemoattractant proteins - rendering them inactive. Figure 9A represents a single experiment in which there seems to be an overall decrease in % recruitment of cells in the GM-KO mouse and the data is statistically significant when using the student t-test (Figure 9A $p=0.01$). When looking at Figure 9B, which represents an $n=3$, there is a reduced % cell recruitment over the entire three hours in the GM-KO mouse and the data is statistically significant also when using t-test analysis for the one hour timepoint (Figure 9B $p=0.06$ at one hour). Further, at hour three, there seems to be a "catch-up" or slight elevation in chemotaxis in the GM-KO - suggesting more than one phase of chemotactic activity.

Figure 9A: Chemotaxis: Exp #2



P=0.01; Student t-test

Figure 9B: Chemotaxis is not Efficient in GM-CSF KO



★ P=0.06 (1 hour); P=0.09 (2 hour); P= 0.1 (3 hour); Student T-test

Mass Spectrometry

The methanol layer contains the proteins of interest from the BAL of WT and GM-KO mice. Protein extraction was a necessary step in order to analyze the proteins without contaminating surfactant. An obvious peak was seen at 414.5, correlating to our suspected cleaved protein, QPDG, of mass 415.5 (See Figure 10). Figure 10A contains the data for the WT methanol layer with proteins and Figure 10B contains the GM-KO methanol layer. Both WT and GM-KO have the same peak suggesting there may not be specificity in the GM-KO disease for cleaved MCP-3. Further studies will immunoprecipitate the truncated MCP-3.

Figure 10A. Mass Spectrometry: WT Methanol Layer
A peak at 414.5 corresponds to murine cleaved MCP-3 protein.

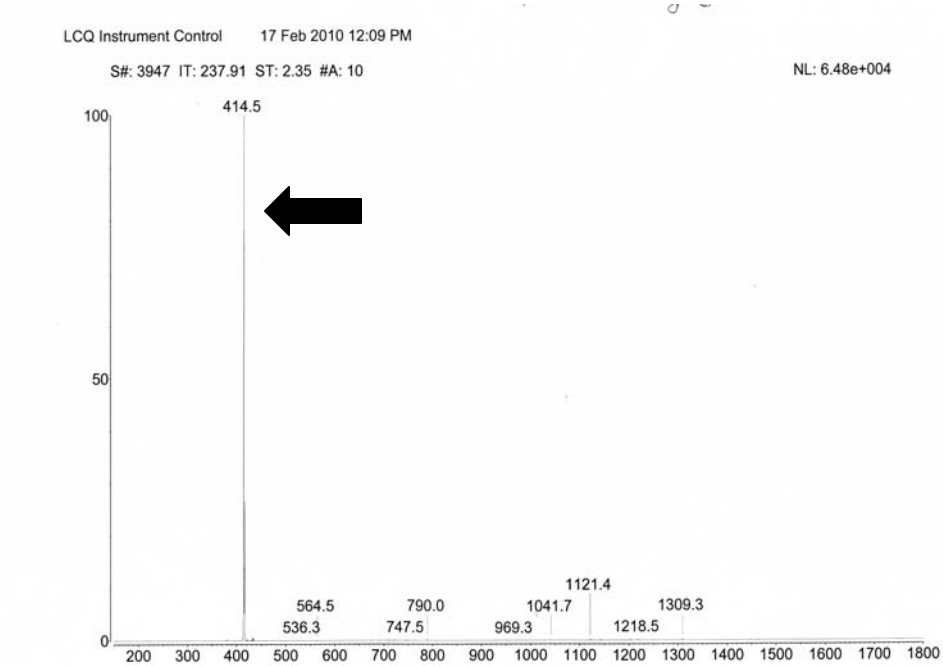


Figure 10B. Mass Spectrometry: GM-KO Methanol Layer
A peak at 414.5 corresponds to murine cleaved MCP-3 protein.

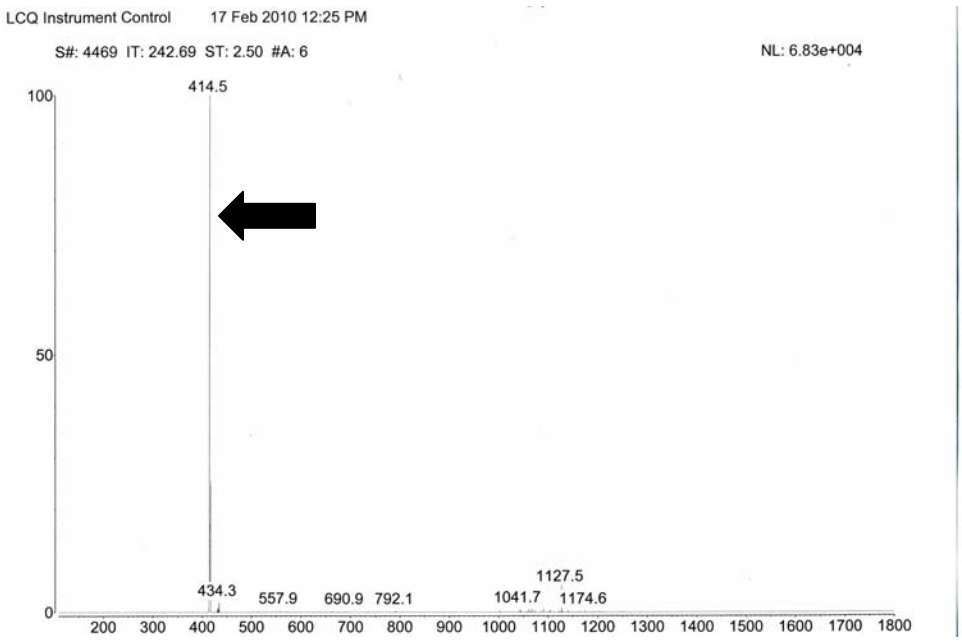


Table 4. Summary of Results

RT-PCR	**Gelatinase gene expression is only modestly increased in the GM-KO.**
Western Blot	**MMP-2 protein expression is increased in the GM-KO BAL fluid.**
Zymography	**Gelatinase enzymatic activity is increased in GM-KO BAL fluid.**
Chemotaxis	**Chemotaxis is reduced in the presence of GM-KO BAL fluid.**
Mass Spec	**Lack of specificity for the possible cleaved MCP-3**

Discussion:

Matrix metalloproteinases serve a beneficial role in the lung by assisting in the clearance of inflammatory products and sometimes the source of inflammation itself. Matrix metalloproteinases are also useful in tissue turnover and remodeling, a critical

component to healthy lung functioning. Matrix metalloproteinase activity also initiates chemokine recruitment of inflammatory cells to the lung tissue in order to clear the invading infection/injury. However, MMPs can exacerbate lung inflammation by indiscriminately degrading critical chemokines required for inflammatory cell recruitment and inflammation clearance. One example is MMP degradation of biologically relevant chemokines found in the lung, preventing essential peripheral inflammatory cell recruitment, specifically the MMP-2 cleavage of MCP-3.

In the GM-KO mouse lung, there is excessive pulmonary surfactant. In addition to surfactant effects on lung functioning, MMPs and chemokines are found to be expressed; however, there is reduced cell recruitment. In these studies, MMP-2 and MMP-9 gene expression levels were found to be slightly increased in the GM-KO BAL cells when compared to WT BAL cells although not significant. There is a trend toward increased expression but more studies are needed to reach a point of significance. Increased protein expression and enzymatic activity was seen in the BAL fluid from GM-KO mice when compared to WT. When studying the chemotactic activity of the GM-KO BAL fluid, a decreased recruitment was found over the WT BAL fluid. The overabundance of surfactant protein in the GM-KO BAL made processing the BAL cells for RT-PCR extremely cumbersome. Increased numbers of mice and studying a time-course may help to better define the role of MMPs in GM-KO lung pathology.

The protein expression of MMP-2 in the GM-KO BAL fluid compared to WT BAL fluid demonstrates a clear increase in expression. Two bands are clearly shown representing the latent (72kD) and active (62kD) states of the MMP-2 protein. Therefore, both the latent and active proteins are increased in the GM-KO BAL fluid. Matrix

metalloproteinases generated from cells other than the BAL cell pellet such as fibroblasts may contribute to the elevated MMPs with a trend toward increased gene expression. Possible future directions could investigate the role of these two additional protein forms and their potential impact on lung functioning. Additionally, we plan to use quantifiable methods such as ELISA or Luminex to determine the concentration of MMP-2 and MMP-9 as well as determine the cell source in the lung.

The enzymatic activity of the secreted gelatinases was found to be increased in the GM-KO BAL fluid versus WT when studied on a gelatin-based gel. Both MMP-2 and MMP-9 were found to be increased in the latent and active forms by zymography. Significant numbers of mice were used to obtain enough BAL fluid to run on the gels and although the bands are not well-defined, there is a clear difference in band intensity between the WT and GM-KO mouse. The additional white bands on the gel could potentially correspond to other MMPs in which gelatin is a substrate, especially since many MMPs overlap substrate specificities. Future directions could study the gelatinase function as a function of time in other models of inflammation using specific MMP inhibitors.

While studying the functionality of chemokine activity in the BAL fluid, a decrease in C57Bl/6 murine monocyte/macrophage chemotaxis was seen in the fluid obtained from the GM-KO. Individual studies showed significance but when grouped together the variability minimized the significance to trends. Since a primary cell line was used, in this case murine C57Bl/6 peritoneal monocytes, cellular coagulation might have clogged the filter membranes and prevented transmigration of some cell clusters into the lower chambers, thereby affecting total cell counts in top and bottom chambers

and adding variation assay to assay. Initially, at the one hour time point, recruitment seemed to be much lower in general for the GM-KO BAL fluid than in the WT. Around the three hour time point, the GM-KO BAL fluid seemed to attract cells from the top chamber. This suggests that over time, there is an adjustment allowing efficient chemotaxis and cell attraction. Future studies will look at MMP inhibitors that can be used to exclude other MMPs from cleaving the chemokines as a reason for decreased cell recruitment.

The mass spectrometry shown in this thesis was extremely preliminary. When interpreting the mass spectrometry data, it is important to keep in mind the samples used here were separated into lipid/protein layers but still crude. Further immunoprecipitation steps are necessary to purify the proteins. Since the proteins were not pure, additional peaks are seen. There seems to be no obvious difference in the amount of the cleaved MCP-3 protein between WT and GM-KO BAL, suggesting a lack of model specificity.

Aside from the additional MMP-2 and MCP-3 studies mentioned above, there are several future directions for this research. The work presented here has simply scratched the surface, especially since MMPs are involved in inflammation on such a broad scale. Since GM-KO mice do not have the GMCSF protein, BAL cells could be cultured with recombinant GMCSF to correct for the absent protein. When culturing BAL cells from the GM-KO mouse, supernatants could be run for secreted cytokines. Since IL-8 is known to be increased in the PAP lung, KC (IL-8 analog in mice) should also be increased as a secreted product in the mouse.

Another aspect not studied here involves macrophage-colony-stimulating factor (MCSF). In the PAP lung, MCSF is upregulated in the absence of GMCSF and as a

result, MMP-2 and MMP-9 are also known to be upregulated (47). The direct correlation of increased MCSF and MMP-2 and -9 could be studied and manipulated to better understand MMP involvement in lung inflammation in the GM-KO.

This research suggests that the murine model of PAP, the GM-KO, is not necessarily the best model to represent this part of the lung physiology observed in PAP. However, the observations highlighted in this work may provide opportunities to study MMP specificity between mouse and man and how this translates in MMP biology. Further, this research also assists in the study of pharmaceutical targets for MMPs.

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