NEUTROPHIL COLLAGENASE
(MATRIX METALLOPROTEINASE-8):
REGULATORY ROLES IN INFLAMMATION AND AUTOIMMUNITY

by

Jennifer H. Cox

B.Sc., University of Victoria, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

October 2008

ABSTRACT

Inflammation is an essential process in wound healing and for the elimination of invading pathogens. However, unregulated inflammation can lead to numerous pathologies including autoimmunities, tumorigenesis, and atherosclerosis. Matrix metalloproteinases (MMPs), once thought to be only extracellular matrix degrading enzymes, are now known to be key regulators of inflammatory and immune responses through proteolysis of bioactive molecules. MMP-8, a neutrophil-specific MMP, is protective in skin cancer models where MMP-8 knockout mice have an initial delay in neutrophil infiltration followed by a massive accumulation at the site of treatment. We investigated this delay in a murine air pouch model of acute inflammation, where MMP-8 deficiency caused decreased neutrophil migration in response to LPS. This was attributed to MMP-8 processing and activation of LPS-inducible CXC chemokine (LIX), a murine neutrophil chemoattractant. Indeed, MMP-8 knockout mice had normal neutrophil infiltration in response to synthetic analogs of cleaved LIX. Furthermore, homologous pathways with human chemokines CXCL5 and CXCL8 were described. *In vivo*, an indirect interaction between MMP-8 and LIX also occurs, whereby MMP-8 processes and inactivates α1-proteinase inhibitor causing increased neutrophil elastase activity, which then efficiently cleaves and activates LIX. MMP-8 was protective in a model of rheumatoid arthritis where synovial tissues from MMP-8 deficient mice had an abundance of neutrophils. This prolonged neutrophil accumulation correlated with a loss of caspase-11 expression, consequent decreased caspase-3 activity and reduced apoptosis. MMP-8 shedding of TNF-α was also decreased in MMP-8 deficient leukocytes, potentially dampening a key apoptotic pathway in neutrophils. The role of MMPs in processing the Th1 cell CXCR3-binding chemokines CXCL9, CXCL10, and CXCL11 was investigated. The leukocytic MMPs -7, -8, -9, and -12 cleaved CXCL11 at both the amino and carboxy-terminus. N-terminal cleavage resulted in the conversion of a receptor agonist to antagonist whereas C-terminal cleavage by MMP-8 caused a significant loss in glycosaminoglycan binding, demonstrating for the first time that direct chemokine proteolysis can regulate the formation of haptotactic gradients. Therefore, MMP-8 is a pivotal regulator in the onset and termination of inflammation, and has multifaceted roles in innate and acquired immunity as well as the autoimmune disorder rheumatoid arthritis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α1-PI	α1-proteinase inhibitor
AEBSF	2-aminoethyl benzenesulfonyl fluoride hydrochloride
ADAM	a disintigrin and metalloproteinase
APMA	4-aminophenylmercuric acetate
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
CATG	cathepsin G
CD	circular dichroism
DMEM	dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
GRO	growth related oncogene
HBSS	Hank's balanced salt solution
ICE	interleukin-1 converting enzyme
IL	interleukin
IP-10	inducible protein 10
I-TAC	interferon-gamma inducible T cell alpha chemoattractant
LCD	linker hemopexin C domain
LIX	LPS-inducible CXC chemokine
LPS	lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MCP	monocyte chemoattractant protein
MEF	murine embryonic fibroblast
MIG	monokine induced by gamma interferon
MMP	matrix metalloproteinase
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MT	membrane type
NE	neutrophil elastase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
RPMI	Roswell Park Memorial Institute medium
SDF	stromal derived factor
SDS	sodium dodecyl sulfate
SLPI	secreted leukocyte protease inhibitor
TACE	TNF alpha converting enzyme
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Chris Overall for his optimism, encouragement, and genuine enthusiasm for science. I could not have imagined a more supportive supervisor, who always had confidence in me and truly gave me every opportunity to succeed. Also, I would like to acknowledge my supervisory committee for their involvement in this research: Dr. Francois Jean, Dr. Douglas Waterfield, and Dr. Leonard Foster. Furthermore, I would like to give a special thanks to collaborators Dr. Clive Roberts and Rendi Yan for their valuable contributions to this research.

The many members of the Overall lab, past and present, have made for an exceptional and memorable experience. Thanks especially to Amanda and Rich for so many insightful "chemokine" discussions; George, Charlotte, and Reini for great leadership and keeping the lab running smoothly; Yili for her purification skills and early morning cheer; Patrick and Oded for never turning down a coffee break; Alain and Caroline for mouse help; Verena for her contagious laugh; Anna, Ulrich, Oliver, and Olivier for sharing their proteomics expertise; and David and Magda for their latin energy. I have sincerely enjoyed my time as a graduate student, in large part because I had the opportunity to work with a friendly and talented group of scientists.

Most of all, I would like to thank my parents for their unrelenting support throughout all of my endeavors. They are not only excellent parents, but also great role models and friends. Thanks to Curtis for standing by my side during all the successes and failures that accompany a PhD. Finally, thanks to my big brother Jason for teaching me to be tough enough to endure all the challenges of life, whether it be a major earthquake or just a comprehensive exam.

CO-AUTHORSHIP STATEMENT

The chapters presented herein are all first-authored manuscripts prepared by the candidate with guidance from the graduate supervisor, Dr. Christopher Overall. Chapter 2 represents a manuscript that was a shared first authorship with Dr. Angus Tester, and the candidate would like to acknowledge Dr. Tester for his contribution of biochemical cleavage, chemotaxis, and air pouch experiments as noted in figure legends. Amanda Starr, Richard Dean, and Andrea Connor assisted with animal experiments for Chapter 2.

All of the data in Chapter 3 was acquired by the candidate and Dr. Richard Dean was a co-author for his contribution of recombinant MMP-12. Dr. Clive Roberts was acknowledged for the previous development of unpublished heparin binding techniques using fluorescence polarization. Dr. Alain Doucet contributed the representative model of CXCL11, as described in the manuscript.

The data for the manuscript in Chapter 4 was acquired by the candidate except for the TNF-α proteolysis experiment, which was performed by Dr. Christopher Overall, as noted in the figure legend. Amanda Starr is co-author for assistance with mouse experimentation, Dr. Reinhild Kappelhoff designed and developed the CLIP-CHIP^{TM} microarray, Rendi Yan assisted with mouse experiments and histological analysis, and Dr. Clive Roberts performed pathological grading of histological specimens.

All data in the Appendices was acquired by the candidate. Dr. Alain Doucet is acknowledged for obtaining the BALF fluid in Appendix A. Furthermore, Dr. Ulrich auf dem Keller, Dr. Anna Prudova, and Dr. Oliver Schilling are acknowledged for assistance in experimental design and data analysis in Appendix B.

CHAPTER 1. INTRODUCTION[1]

The Basics of Matrix Metalloproteinases

The first matrix metalloproteinase (MMP) was discovered in 1962 by Jerome Gross and Charles Lapiere upon the observation that tadpole tails undergoing metamorphosis had the unique ability to degrade fibrillar collagen (1). Similar activity was then demonstrated in human gingival tissues (2). It was not until the following decade that the first human collagenase, MMP-1, was isolated (3). Since then, the number of MMPs has grown to 23 in human and 24 in mice. This family of zinc-dependent endopeptidases is proposed to degrade all components of the extracellular matrix. As such, MMPs are thought to be involved in many normal physiological processes and have traditionally been associated with pathologies characterized by increased extracellular matrix degradation, most notably cancer and arthritis. However, recent substrate discovery efforts have revealed that MMPs specifically and efficiently cleave and modulate the activities of bioactive substrates including growth factors and their receptors, cytokines and chemokines, and integrins [reviewed in (4)]. Therefore, MMPs are key regulators of many processes such as angiogenesis, apoptosis, tumor metastasis and progression, and inflammation. The simplistic view of MMPs as "matrix bull-dozers" is outdated and drug-targeting strategies must account for the emerging pleiotropic roles of MMPs in disease and normal homeostatic processes.

MMPs are produced by a variety of cell types and are either secreted or membrane-bound. The general structure of mammalian MMPs consists of an auto-inhibitory pro-domain and a catalytic domain (Figure 1.1). The pro-domain acts through a cysteine switch mechanism where a conserved cysteine coordinates the active site zinc, thereby preventing catalysis. All MMPs except MMP-7, MMP-23, and MMP-26

---

[1] A version of the third section of Chapter 1 has been accepted for publication as a book chapter: Cox JH and Overall CM. Cytokine Substrates: MMP Regulation of Inflammatory Signalling Molecules. The Cancer Degradome - Proteases and Cancer Biology. Springer Science and Business Media. 2008 (In Press).
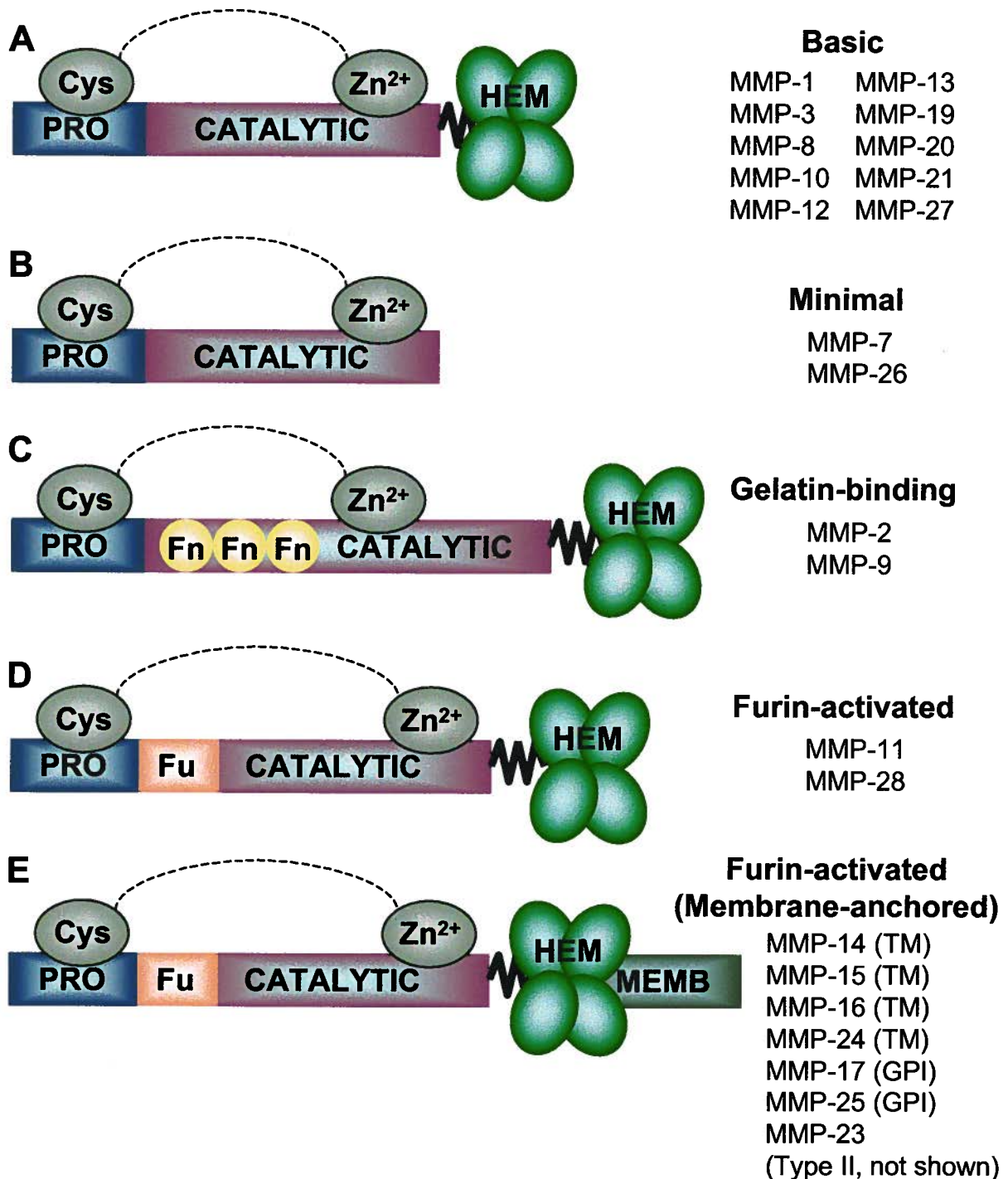
Figure 1.1. Schematic structure of MMPs. The domain compositions of the 23 human MMPs are shown. (A) The basic structure consists of a pro-domain (Pro), catalytic domain, and hemopexin C domain (Hem), attached by a linker domain. The pro-domain cysteine (Cys) associates with the catalytic Zn^{2+} to inhibit activity. (B) Minimal MMPs lack the hemopexin domain. (C) Gelatin-binding MMPs have 3 fibronectin-domain inserts (Fn) in the catalytic domain. (D) Furin-activated MMPs have a furin recognition domain (Fu) upstream of the catalytic domain. (E) Membrane-anchored MMPs are furin-activated and have C-terminal transmembrane (TM) domains with a cytoplasmic tail or alternatively are GPI-anchored. MMP-23 is a Type II transmembrane protein (not shown).

also contain a hemopexin C domain, which is a C-terminal four-bladed propeller structure important for protein-protein interactions and substrate recognition (5). The gelatinases, MMP-2 and MMP-9, have fibronectin type II repeats inserted into the catalytic domain to facilitate collagen binding (6). Of the six membrane-type (MT)-MMPs, MMP-14, -15, -16, and -24 have transmembrane domains and short cytoplasmic tails. In contrast, MMP-17 and MMP-25 have glycosylphosphatidylinositol (GPI) linkages and MMP-23 is a type II transmembrane protein.

MMPs have been attractive drug targets for the treatment of cancer and arthritis since their association with these diseases several decades ago. In the case of cancer, strong correlations between MMP overexpression and tumor progression prompted the development of many small molecule and peptidomimetic broad-spectrum MMP inhibitors, some of which advanced to phase III clinical trials. Universally, these trials were unsuccessful and has prompted re-evaluation of MMPs as therapeutic targets (7). In recent years with MMP-knockout mice, the individual functions of several MMPs have been examined in cancer, as well as other diseases, uncovering some unexpected host-protective roles. As such, several MMPs are now defined as drug anti-targets because inhibition would cause increased disease severity (8), giving some insight into the failure of broad-spectrum antagonists. The first such anti-target was MMP-8, the neutrophil-specific collagenase.

The Neutrophil Collagenase: MMP-8

Neutrophils, also called polymorphonuclear cells (PMNs), are often considered the front line of defense in innate immunity. Neutrophils express MMP-8, MMP-9, MMP-14, and MMP-25. MMP-8, which was the second MMP identified, is produced primarily by neutrophils and is released from specific granules at sites of inflammation (9, 10). Other cell types including macrophages (11), T lymphocytes (12), and synovial fibroblasts (13) have been reported to express low levels of MMP-

8. Along with MMP-1 and MMP-13, MMP-8 has traditionally been considered a collagenase for its potent ability to degrade collagen (14), in particular type I collagen which is a major component of bone and skin. In addition to proteolysis of structural components of the extracellular matrix, several bioactive substrates of MMP-8 are also known, including chemokines, adhesion molecules, and protease inhibitors, however physiological evidence was lacking (Table 1.1).

Table 1.1. Extracellular matrix and bioactive substrates of MMP-8

Known MMP-8 substrates	Reference
Extracellular matrix substrates	
Aggrecan	(15)
Collagen type I>type III>type II	(14)
Collagen VII	(16)
Collagen X	(16)
Fibrinogen	(17)
Fibronectin	(18)
Laminin-5	(19)
Nidogen	(18)
Proteoglycan core protein	(18)
Bioactive substrates	
Alpha-1 antitrypsin	(20)
Alpha-2 antiplasmin	(21)
Angiotensin	(22)
Bradykinin	(22)
C1 inhibitor	(23)
CCL2/MCP-1	(24)
mCXCL5/LIX	(25, 26)
CXCL6/GCP-2	(25)
CXCL10/IP-10	(27)
IGFBP-5	(28)
L-selectin	(18)
Tachykinin substrate P	(22)

Like other MMPs, MMP-8 is secreted as an inactive pro-enzyme and is converted to an active protease through the cysteine switch mechanism. This can occur by reactive oxidative species released from activated neutrophils (29), or proteolytically by cathepsin G and chymotrypsin (30), as well as a number of MMPs [reviewed in (31)]. However, it is unclear how MMP-8 becomes activated *in vivo* or how MMP-8 retains activity in the presence of endogenous inhibitors such as the tissue inhibitors

of metalloproteinases (TIMPs). Although MMP-8 is secreted through traditional pathways, it is now thought to function in a membrane-associated state which confers increased resistance to inhibition (32).

MMP-8 is often associated with the pathogenesis of diseases characterized by excessive neutrophil infiltration such as rheumatoid arthritis (33), cystic fibrosis (34), and periodontal disease (35), but the biological role of MMP-8 remains uncertain. The MMP-8 knockout mouse, generated by Balbin et al., exhibits normal development and fertility but surprisingly has increased susceptibility to skin cancer (26), thus defining MMP-8 as the first host-protective MMP in cancer. In MMP-8 deficient mice, there was an initial delay in neutrophil influx to the site of chemical carcinogen treatment followed by a massive neutrophil accumulation that persisted. Other phenotypes with the MMP-8 knockout mouse (summarized in Table 1.2, Page 8) include increased PMNs in LPS and allergen-induced airway inflammation (32, 36), resistance to TNF-induced lethal hepatitis (37), delayed wound healing (38), and less severe experimental autoimmune encephalomyelitis (EAE) (39). It is becoming evident that MMP-8 has pleiotropic roles in various diseases and inflammatory situations yet the specific mechanisms underlying these phenotypes remain poorly understood.

Cytokine Substrates: MMP Regulation of Inflammatory Signaling Molecules

Perspective

Inflammation is a highly regulated process involving tissue parenchyma, vascular and connective tissues, as well as host defense and immune cells, and is an essential component of both innate and acquired immunity. MMPs were traditionally considered to assist in the inflammatory response through the breakdown of extracellular matrix molecules, providing an environment condusive to leukocyte infiltration, edema, wound healing and tissue repair with continued upregulation contributing to the onset of chronic inflammation. It is now evident, however, that the repertoire of MMP substrates, the substrate degradome, extends far beyond the extracellular matrix and includes many key bioactive molecules, the processing of

which results in altered actions. In the context of inflammation, several MMPs have been shown biochemically to efficiently and selectively process cytokines and chemokines, as well as associated molecules, thereby changing their bioactive properties. Many of these substrates have been validated physiologically in genetic models of inflammatory disease, where a remarkable array of inflammatory phenotypes has been observed. As a whole, research involving MMPs in inflammation has demonstrated their integral role in orchestrating both the initiation and termination of inflammatory processes, rather than only tissue degradation.

MMPs and Inflammation

Inflammation is an essential process for the elimination of invading pathogens and in wound healing. Inflamed tissue is a dynamic interactive multi-cellular and multi-tissue system embedded in normal stroma. A wide variety of signaling networks operate in multi-directional communication between the component cells and tissues—the connective tissue cells and epithelium, vascular endothelium, reactive peripheral stroma, and host defense cells—that orchestrate innate immunity, inflammation, resolution and healing. Cytokine pathways that are perturbed in unresolved acute inflammation drive tissue destruction and inflammatory/immune cell responses in chronic inflammatory disease. Dysregulated inflammation has been linked to numerous pathologies including autoimmune diseases (40), tumorigenesis (41), and atherosclerosis (42). Stringent regulation of the inflammatory process is critical for both resolution and to buffer against pathological destruction. Proteolytic enzymes are now regarded as key effectors and regulators in inflammation and immunity, through not only protein degradation and turnover but also precise and efficient cleavage events leading to altered bioactivity of inflammatory mediators (43, 44).

Matrix metalloproteinases (MMPs) were historically thought to be exclusively involved in turnover of extracellular matrix components. Accordingly, attractive hypotheses evolved that involved matrix degradation in normal development and

6

physiological turnover as well as in many of the steps in inflammation that appeared to be dependent on extracellular matrix turnover. However, with the exception of MMP-14, none of the MMP-deficient mice generated thus far exhibit severe defects in matrix turnover for normal development or homeostasis. In addition, drug trials with broad-spectrum MMP inhibitors have not demonstrated significant abnormalities in extracellular matrix although a small number of patients did develop joint strictures (45). In contrast, MMP-deficient mice display a remarkable array of inflammatory and immune phenotypes (Table 1.2). Although MMPs have generally been considered pro-inflammatory molecules, promoting leukocyte migration through the breakdown of basement membrane and extracellular matrix, the observed phenotypes clearly show that MMPs can also function in an anti-inflammatory capacity and the mechanisms underlying these phenotypes are now beginning to emerge. As such, it is now evident that matrix metalloproteinases have a multitude of pleotropic functions in regulating inflammation.

In recent years, substrate discovery efforts have uncovered a plethora of novel bioactive substrates that link MMPs with both the onset and the resolution of inflammation (4, 46). Notably, MMPs are now known to have pro- and anti-target effects in cancer (8), and analogous opposing roles appear to exist in inflammation as well. As such, several authors have proposed that MMPs, by playing critical roles in regulating the activity of numerous inflammatory mediators, are homeostatic regulators and controllers of inflammation. Here we describe the conclusions from genetic models of inflammation as well as biochemical and proteomic evidence of the direct proteolytic actions of MMPs on cytokines and chemokines, and also indirect effects of MMPs on the inflammatory response.

Genetic Models of Inflammation

Murine models of inflammation and innate immunity have demonstrated diverse and unexpected functions of MMPs in the inflammatory context (Table 1.2). Currently, knockout mice have been generated for at least fifteen MMPs: MMP-2, 3, 7, 8, 9, 10,

11, 12, 13, 14, 19, 20, 23, 24, and 28 (47). Of these, MMP-2, 3, 7, 8, 9, 10, 12, and 28 have been reported to have altered inflammatory phenotypes. Due to the significant skeletal defects and short life-span of the MMP-14-null mice, inflammatory studies using these mice are not feasible, however organ transplantation allows for tissue specific evaluation of MMP-14 deficiency.

Table 1.2. Inflammatory and immune phenotypes of MMP-deficient mice

Mouse	Phenotypes relating to inflammatory processes	References
Mmp2$^{-/-}$	More severe antibody induced arthritis	(48)
	Diminished egress of lung inflammatory cells	(49)
	Earlier and more severe EAE due to increased MMP-9	(50)
	Prolonged cardiac allograft survival and lower cellular infiltration	(51)
	More severe myocardial inflammation and dysfunction	(52)
	Delay in inflammation associated corneal neovascularization	(53)
	Exacerbated experimental colitis	(54)
Mmp3$^{-/-}$	Markedly impaired contact hypersensitivity	(55)
	Reduced disc resorption and generation of macrophage chemoattractant	(56)
	Reduced PMN recruitment in acute lung injury	(57)
	Reduced macrophages in atherosclerotic plaques	(58)
	Impaired clearance of intestinal bacteria	(59)
	Delayed macrophage and lymphocyte recruitment in cardiac remodelling	(60)
	Marked resistance to S. typhimurium infection	(61)
Mmp7$^{-/-}$	Increased bacterial survival, lack of intestinal α-defensins	(62)
	Decreased epithelial cell apoptosis due to reduced FasL shedding	(63)
	Reduced macrophages in herniated discs, loss of TNFα shedding	(64)
	Reduced PMNs in lung due to altered chemokine gradients	(65)
	Less severe septic arthritis	(66)
Mmp8$^{-/-}$	Delayed PMN infiltration then accumulation in skin carcinogenesis model	(26)
	Increased PMN infiltration in intratracheal LPS model	(32)
	Increased PMN infiltration in allergen induced airway inflammation	(36)
	Resistant to TNF-induced lethal hepatitis	(37)
	Decreased PMN infiltration in LPS-treated air pouch	(67)
	Increased inflammation delays wound healing	(38)
	Decreased susceptibility in experimental autoimmune encephalomyelitis	(39)
	Decreased PMN infiltration in LPS-treated corneas	(68)
Mmp9$^{-/-}$	More persistant inflammation in contact hypersensitivity	(55)
	Less susceptible to experimental autoimmune encephalomyelitis	(69)
	Resistant to subepidermal blisters with reduced PMN recruitment	(70)
	Reduced macrophages in experimental cardiac infarction	(71)
	Impaired hematopoietic stem cell motility due to reduced sKitL	(72)
	Less severe antibody-induced arthritis	(48)
	Impaired cellular infiltration in airway inflammation	(73)
	Impaired defense in bacterial meningitis	(74)
	Reduced hematopoietic stem cell mobilization	(75)
	Impaired dendritic cell migration through tracheal epithelium	(76)
	Enhanced allergen-induced airway inflammation	(77)
	Inflammatory cell accumulation in allergic lung model	(78)
	Attenuated dextran sulphate-induced colitis	(79)
	Increased severity of septic arthritis	(80)
	Reduced resistance against E. coli peritonitis	(81)

Table 1.2. (continued)

Mouse	Phenotypes relating to inflammatory processes	References
Mmp10^{-/-}	Increased inflammation in models of infection and wound healing	(82)
Mmp12^{-/-}	Reduced macrophages in smoke-induced emphysema	(83)
	Less TNFα shedding in smoke-induced inflammation	(84)
	Improved recovery after spinal cord injury, reduced inflammation	(85)
	Reduction in OVA-induced airway eosinophilia	(86)
	Reduction in antigen-induced airway inflammation	(87)
	Reduced infiltrating macrophages in aortic aneurysms	(88)
	Less experimental autoimmune encephalomyelitis, higher Th1/Th2 ratio	(89)
	Reduced macrophages in atherosclerotic plaques	(90)
	Decreased macrophages in ligament healing	(91)
	No change in bleomycin-induced lung fibrosis	(92)
	BALF lacks chemotactic elastin fragments	(93)
Mmp28^{-/-}	Increased inflammatory response	(W.C. Parks, Unpublished)

Individual MMPs show remarkably divergent physiological functions in inflammatory disease models. For instance, Mmp2-null mice have increased inflammation and consequent disease severity in models of arthritis (48), experimental autoimmune encephalomyelitis (50) and experimental colitis (54), suggesting a general anti-inflammatory role for the enzyme. This protective role of MMP-2 is consistent with its near-constitutive expression, where a pro-inflammatory role would be very destructive. In contrast, MMP-7 deficient mice exhibit reduced inflammation in models of septic arthritis (66), herniated discs (64) and acute lung injury (65), demonstrating a pro-inflammatory function for MMP-7. MMP-3 and MMP-12 can also be generalized as pro-inflammatory effector molecules as these knockouts are characterized by decreased infiltrating leukocytes in several disease models (summarized in Table 1.2).

The functions of certain MMPs cannot be classified so easily. For example, the neutrophil protease MMP-8 seems to have opposing effects in acute and chronic inflammatory situations. In acute models such as the LPS-treated air pouch (67) and TNF-induced hepatitis (37), MMP-8 promotes neutrophil infiltration, at least in part due to ELR[+] chemokine processing and activation. In more chronic models of skin carcinogenesis (26) and wound healing (38), MMP-8 is involved in terminating and

9

resolving inflammation. MMP-9 also demonstrates complex regulatory properties. For instance, *Mmp9*-null mice have reduced PMN recruitment in subepidermal blisters (70) and decreased macrophages in response to cardiac infarction (71), but conversely show enhanced allergen-induced airway inflammation (78) and increased severity in a model of septic arthritis (80). Therefore both MMP-8 and MMP-9 can exert differential regulatory effects depending on the particular stimulus and the temporal status. However, in the neutrophil MMP-8 dominates as its absence leads to decreased neutrophil infiltration despite normal or elevated MMP-9 levels.

MMP Regulation of Cytokines

Cytokines have a central role straddling both innate and adaptive immunity, functioning in both pro-inflammatory and anti-inflammatory manners. Cytokines are produced by a variety of cell types and signal either *in cis* or *in trans* to nearby cells or throughout the organism. Through the binding of specific cell-surface receptors, cytokines trigger signalling cascades resulting in altered gene and protein expression during an inflammatory reaction. Therefore, precise control of cytokine activity is essential for the accurate control of inflammation. Selective proteolysis of cytokines provides a rapid and effective means to modulate the initiation and termination of an inflammatory response.

Interleukin-1β

Interleukin-1β is an early and potent pro-inflammatory cytokine that has been linked to fever, leukocytosis, anemia, and elevated acute phase proteins (94). IL-1β, along with TNF-α, are classical inducers of MMPs in numerous cell types. The production of IL-1β is primarily in macrophages, monocytes and PMNs, where secretion occurs through non-classical pathways as IL-1β lacks a secretory signal peptide (95). It is translated as an inactive 33-kDa precursor, some of which seems to be secreted through specialized lysosomes. Caspase-1, also known as IL-1β-converting enzyme/ICE, is an intracellular cysteine protease that cleaves latent IL-1β at Asp^{116}-Ala^{117} releasing the N-terminus to generate the fully active 17-kDa molecule (96).

Although caspase-1 is thought to play a dominant role in IL-1β activation, studies of local inflammation with caspase-1 deficient mice suggest that alternate mechanisms exist (97).

IL-1β processing by MMPs has been described *in vitro*, generating biologically active 17-kDa forms (98). Activation occurs with the following MMPs in order of increasing efficiency: MMP-2, MMP-3, and MMP-9, whereas MMP-1 lacks the ability to activate latent IL-1β (Table 1.3). With prolonged exposure and high enzyme concentrations, MMP-1, -2, -3, and -9 degrade IL-1β yielding a loss in activity (98, 99). Hence, the physiological role of MMPs in the regulation of IL-1β activity remains to be determined, but biochemical studies appear to show the potential for both pro-inflammatory and anti-inflammatory effects *in vivo*. The important caveat that biochemical cleavage does not equate to a physiologically relevant substrate must be recognized, and in the case of proIL-1β, which is normally processed intracellularly, substrate-enzyme colocalization must be considered. Similarly, high enzyme-substrate ratios reflect extreme parameters that may not be met *in vivo*. Regardless, IL-1β induced expression of MMPs possibly involves feed-forward or feed-back loops by which MMPs might further activate the cytokine, enhancing inflammation, or degrade IL-1β in an inhibitory loop to dampen or terminate the response.

Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine with pro-inflammatory and immunomodulatory roles (100). Overproduction of TNF-α has been implicated in inflammatory conditions such as rheumatoid arthritis, multiple sclerosis, ankylosing spondylitis and psoriasis (101). TNF-α is produced as a 26-kDa membrane-associated inactive precursor (proTNF-α) that is activated upon proteolytic shedding, thus releasing the soluble form. This cleavage was first attributed to metalloproteinases based on broad-spectrum metalloprotease inhibitors

Table 1.3. Bioactive MMP substrates: The pro-inflammatory and anti-inflammatory proteolysis events by MMPs

Substrate	MMPs	Cleavage site	References
Pro-inflammatory cleavages			
Interleukin-1β	2, 3, 9	ND	(98)
proTNF-α	1, 2, 3, 7, 9	ND	(102)
	12	^{68}SLISPLA↓QA↓VRSS	(103)
	14, 15	^{68}SL↓ISP↓LA↓QA↓VR	(104, 105)
	17	^{68}SLISPLA↓QAVR	(106)
CXCL5	1	^{4}AAAVLR↓ELRC	(25, 67)
	8, 9	^{4}AA↓A↓V↓LRELRC	
CXCL8	1, 8, 9, 13, 14	^{1}AVLPR↓SAKE	(67, 105, 107)
CX$_3$CL1	2	^{68}QAAA↓LTKN	(108)
LIX (mCXCL5)	1, 2, 8, 9, 12, 13	^{1}APSS↓VIAA	(25, 26, 67, 109)
Anti-inflammatory cleavages			
Interleukin-1β	1, 2, 3, 9	Degradation	(99)
			(98)
proTNF-α	14, 15	Degradation	(104)
Latent TGF-β	2, 3, 9, 13, 14	ND	(110-113)
CCL2	1, 3, 8, 12	^{1}QPDA↓INAP	(24, 109)
CCL7	1, 2, 3, 12, 13, 14	^{1}QPVG↓INTS	(24, 109, 114)
CCL8	3, 12	^{1}QPDS↓VSIP	(24, 109)
CCL13	1, 3, 12	^{1}QPDA↓LNVP	(24, 109)
CXCL1	9	Degradation	(107)
	12	^{3}VATE↓LRCQ	(109)
CXCL2	12	^{3}LATE↓LRCQ	(109)
CXCL3	12	^{3}VVTE↓LRCQ	(109)
CXCL4	9	Degradation	(107)
CXCL5	12	^{7}VLRE↓LRCV	(109)
CXCL6	12	^{11}RCTC↓LRVT	(109)
CXCL7	9	Degradation	(107)
CXCL8	12	^{7}AKEL↓RCQC	(109)
CXCL12	1, 2, 3, 9, 13, 14	^{1}KPVS↓LSYR	(115)
CX$_3$CL1	2	^{1}QHLG↓MRKC	(108)
mCXCL1	12	^{3}IANE↓LRCQ	(109)
mCXCL2	12	^{3}VASE↓LRCQ	(109)
mCXCL3	9, 12	^{3}VASE↓LRCQ	(109)
Unaltered bioactivity cleavages			
CXCL6	8, 9	^{1}GPVS↓A↓V↓LTELR	(25)
CXCL9	9	^{89}VLK↓VRK↓S↓QRSR	(27)
CXCL10	8, 9	^{66}KAV↓SKE↓MS↓KRSP	(27)
LIX (mCXCL5)	8	^{76}KKAK↓RNAL	(67)
	1, 9, 12	^{81}NALA↓VERT	(109)

preventing the shedding of TNF-α in cultured leukocytes and endotoxemia models (116-118). However, cloning and purification studies revealed that the major enzyme responsible is the metalloproteinase disintegrin ADAM-17, also called TNF-α converting enzyme (TACE) (119, 120).

Although ADAM-17 is considered to be the major generator of soluble TNF-α, studies with MMP-7 and MMP-12 knockout mice in models of herniated disc resorption (64) and cigarette smoke-induced inflammation (84), respectively, support a role for MMPs in the physiological release of TNF-α. In the case of MMP-7, intervertebral discs cocultured with peritoneal macrophages from Mmp7-null mice had a defect in macrophage influx. This was attributed to a decrease in soluble TNF-α, which was found to be necessary for MMP-3 induction and the consequent generation of a macrophage chemoattractant. In cigarette smoke-induced inflammation, MMP-12 knockout mice have decreased levels of soluble TNF-α and diminished ability to shed TNF-α from cultured alveolar macrophages in response to smoke exposure. Further, MMP-12 mediated shedding of TNF-α was found to promote endothelial activation, PMN infiltration and the proteolytic tissue damage associated with emphysema.

Biochemically, several MMPs can cleave proTNF-α to generate the active form *in vitro* (Figure 1.2). For example, purified MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 can cleave recombinant GST-TNF-α fusion protein (102, 118). In addition, MMP-12 (103), MMP-14 and MMP-15 (104), and MMP-17 (106) are capable of cleaving proTNF-α. In a proteomic screen evaluating breast cancer cells overexpressing MT1-MMP, TNF-α was increased in the conditioned medium in the MMP-14 transfected cells compared to vector control and biochemically MMP-14 was shown to cleave and generate the active form (105). The MMP cleavage sites in proTNF-α are variable, but lie either at or slightly upstream of the ADAM-17 cleavage site of Ala^{76}-Val^{77} (Table 1.3). Notably, ADAM-17 is stimulated by lipopolysaccharide or experimentally by phorbol myristyl acetate, where its role in

Figure 1.2. MMP proteolysis of cytokines and chemokines to precisely control the induction (left) and termination (right) of inflammation

Initiation of an inflammatory response (left) is triggered by injury or an infectious agent resulting in the rapid local release of neutrophil chemoattractants and proteases, including MMPs. Circulating neutrophils roll on the endothelial surface through interactions with selectins (not shown). Engagement with chemokines, immobilized by glycosaminoglycan binding, causes firm adhesion to the endothelium through integrins and subsequent extravasation into the underlying tissue. Once in the tissue, neutrophils are activated and degranulate releasing MMP-8 and MMP-9. MMP-8 processes and activates CXCL5 and CXCL8, resulting in additional PMN recruitment. Monocyte chemoattractant proteins (CCL2, 7, 8, 13) and CXCL12 promote the influx of monocytes and lymphocytes, respectively. The membrane-bound chemokine CX₃CL1 is shed by fibroblast-derived MMP-2 to generate a soluble chemoattractant. Macrophage-derived MMP-7 and MMP-12 are involved in shedding TNF-α, in cooperation with ADAM-17, yielding a soluble pro-inflammatory cytokine. TNF-α is also shed by MMP-14, depicted here on fibroblast cells.

Once the initiating stimulus is removed the pro-inflammatory signals are no longer generated (right). Local chemokine production is decreased and neutrophils no longer enter the tissue. Furthermore, some neutrophil chemokines (CXCL1, CXCL4, CXCL7) are degraded by MMP-9 resulting in inactivation. MMP-2, as well as other MMPs, is involved in dampening the response through the generation of chemokine antagonists. Shown here are CCL2, CCL7, CCL8, CCL13, and CX₃CL1 as substrates of MMPs, forming potent receptor antagonists and inhibiting chemotactic migration. Also, CXCL12 is processed by MMP-2 resulting in a switch in receptor specificity. MMP-14 is potentially involved in down-regulating TNF-α through degradation. Furthermore, MMP-2 and MMP-9 (not shown) cleave the latency-associated protein from TGF-β to liberate the active TGF-β homodimer with immunosuppressive properties. Active TGF-β induces MMP-2 and MMP-9 expression and extracellular matrix formation in a feedback mechanism.

Initiation

Termination

Neutrophil

CXCR1/2

GAGs

CXCL5
CXCL8

Endothelial layer

Macrophage

Active
CXCL5
CXCL8

MMP-8
MMP-9

Active
TNFα

MMP-7
MMP-12

CCR1

proTNFα

CCL2 CCL7
CCL8 CCL13

ADAM-17

CX₃CR

MMP-2

CX₃CL1

MMP-14

CXCR4

CXCL12

Fibroblasts

Lymphocyte

Latent
TGFβ

Neutrophil

CXCR1/2

GAGs

Endothelial layer

Macrophage

Degraded
CXCL1
CXCL4
CXCL7

MMP-8
MMP-9

MMP-7
MMP-12

Degraded
TNFα

CCR1

Antagonist
CCL2 CCL7
CCL8 CCL13

ADAM-17

CX₃CR

Antagonist
CX₃CL1

MMPs

MMP-2

MMP-14

CXCR4

Loss of agonist
CXCL12

Fibroblasts

Lymphocyte

MMP-2

Active
TGFβ

15

TNF-α shedding is well established. Conversely, in the absence of LPS stimulation such as non-infective situations, MMP-mediated shedding of TNF-α may be important and as such a variety of experimental approaches at different levels of complexity are needed to determine the relative contibutions of various proteases (121). It is likely that ADAM-17 and several MMPs have complementary roles in the rapid shedding and activation of proTNF-α in response to varying stimuli.

Transforming growth factor-β1

Transforming growth factor-β1 (TGF-β1) is generally considered an immunosuppressive cytokine but encompasses a multitude of functions that cannot be defined so simply. TGF-β1 is known for its ability to instigate and maintain immune tolerance (122, 123). Under normal circumstances, TGF-β1 is secreted in a latent form where its furin-cleaved 80-kDa N-terminus, also called the latency-associated peptide, remains non-covalently associated and requires proteolytic, conformational, or acidic conditions for its removal (124). MMP proteolysis, amongst other mechanisms, has been proposed to release the active 25-kDa cytokine homodimer from the latency-associated peptide. In chondrocyte cultures, MMP-3 (111) and MMP-13 (113) have been found to activate TGF-β1 and likewise MMP-14 expression in osteoblasts causes activation of the latent cytokine and thereby promotes osteoblast survival (110). In the context of tumor biology, the gelatinases MMP-2 and MMP-9 were found to activate TGF-β, potentially promoting tumor invasion and angiogenesis (112). Notably, TGF-β1 induces the expression of MMP-2 (125, 126) and MMP-9 (127), perhaps contributing to the potentiation of TGF-β1 activity in a feed-forward manner. Furthermore, TGF-β1 stimulates production of the extracellular matrix, a critical event in wound healing, and through increased collagen deposition leads to induction (128) and activation of MMP-2 (129) and consequent processing of monocyte chemoattractants (CCL2, 7, 8, and 13) to generate potent anti-inflammatory molecules, thereby terminating macrophage influx and promoting healing (24). Although the link between MMPs and TGF-β has yet to be clearly determined in an inflammatory setting, MMP activation of TGF-β could

provide a novel anti-inflammatory mechanism by which MMPs function to dampen an immune response.

MMP Regulation of Chemokines

Chemokines are a super-family of low molecular weight chemotactic cytokines that function in directing the migration of cells in normal leukocyte trafficking and the inflammatory response (130). *In vivo*, chemokines form gradients through interactions with proteoglycan glycosaminoglycans and signal through 7-transmembrane G protein-coupled receptors to induce a chemotactic response. Several chemokines are known to be regulated by proteolysis, many of which are MMP-mediated cleavages, resulting in a multitude of functional consequences including either enhanced or decreased receptor binding, conversion to a receptor antagonist, changing receptor specificity, and shedding of membrane-anchored chemokines.

Chemokines are characterized by the spacing of conserved cysteine residues that function in the formation of disulphide bonds. Although the sequence similarity amongst chemokines is quite low, ranging from 20-50% homology, the tertiary structure of these proteins is remarkably conserved (131). The general structure consists of an undefined N-terminus, three central anti-parallel beta-sheets, and a C-terminal alpha-helix. The N-terminal portion of chemokines appears to be most susceptible to proteolysis and cleavage often results in significant changes in activity. In contrast, some C-terminal processing events have been described but no functional changes have been ascribed to these truncations (27, 67, 132).

CC Chemokines

The CC family of chemokines is defined by adjacent cysteine residues in the N-terminus and its members typically induce the migration of monocytes, dendritic cells and natural killer cells. The monocyte chemoattractant proteins (MCPs), also known as CCL2, 7, 8, 13, were amongst the first chemokines to be identified as MMP

17

substrates. In a yeast genetic screen with the hemopexin domain of MMP-2, CCL7 was found to have a strong interaction with the exosite and was processed efficiently at Gly^4-Ile^5 with recombinant enzyme and by monocytes in culture and *in vivo* (114, 133). The truncated chemokine, CCL7 (5-76) still binds its cognate CC receptors but no longer promotes chemotaxis and instead functions as a receptor antagonist with potent anti-inflammatory properties *in vivo*, as determined in mouse models of subcutaneous and peritoneal inflammation. CCL7 is also processed by MMP-1, 3, 13, and 14 (24) and the cleaved form has been detected in rheumatoid synovial fluid (114).

All of the MCPs have been identified as MMP substrates (Table 1.3) with characteristic cleavage between residues 4-5 in the N-terminus, causing diminished activity and the generation of potent receptor antagonists (24). In addition to MMP-2, MMP-1, -3, -8, -13, and -14 cleave MCPs with different profiles of substrate preference, revealing specificity in this interaction (Table 1.3). Notably, this work was the first to demonstrate an unexpected dampening of inflammation by MMPs, traditionally considered to be pro-inflammatory proteases.

CXC Chemokines

The CXC chemokines predominantly influence the migration of polymorphonuclear neutrophils and T-lymphocytes. A subset of CXC chemokines are characterized by an ELR (glutamic acid-leucine-arginine) sequence proximal to the conserved CXC motif and act exclusively on CXCR1 and CXCR2 receptors, thereby promoting neutrophil chemotaxis. CXCL8, the most potent of the ELR^+ chemokines, is processed by MMP-1, -8, -9, -13, and -14 at Arg^5-Ser^6, causing a 10-fold increase in receptor binding affinity and chemotactic activity (67, 105, 107). In the cellular context, MMP-14-overexpressing cells were discovered to have decreased levels of CXCL8-derived peptides in the conditioned medium compared to vector controls, confirmed biochemically to be due to proteolysis (105).

Activation of PMN chemoattractants also applies to the human ELR$^+$ chemokine CXCL5 as well as the murine chemokine LIX (25, 26, 67). LIX, also called mCXCL5, is thought to be the mouse orthologue to human CXCL8 in that it is the most potent and abundant of the ELR$^+$ chemokines. LIX is efficiently cleaved at Ser^4-Val^5 by the neutrophil enzyme MMP-9 *in vitro*, resulting in a significant increase in chemoattractant activity (25). The generation of the MMP-8 deficient mouse revealed decreased processing of LIX in LPS-stimulated bronchoalveolar lavage fluid, suggesting a specific role for MMP-8 in the activation of LIX (26). This observation is coupled with delayed neutrophil infiltration to the site of challenge in a skin carcinogenesis model in *Mmp8*-null mice, followed by a massive accumulation of neutrophils. Furthermore, in an LPS-treated subcutaneous air pouch model of acute inflammation, *Mmp8*-null mice have a significantly decreased neutrophil influx compared to wild type controls (67). This difference is lost when mice are treated with the truncated chemokines LIX (5-92) or CXCL8 (6-77), demonstrating that LIX and CXCL8 are physiologically relevant substrates of MMP-8 and that the normal cell migration machinery and the ability of neutrophils to migrate through basement membrane and connective tissue is intact in the absence of MMP-8. Interestingly, several MMPs in addition to MMP-8 cleave LIX *in vitro*, yet animal studies indicate a lack of physiological redundancy in this pathway (67). This result also questions the importance of MMP-8 in collagen degradation in these matrices, a role traditionally assumed to be essential for neutrophil chemotaxis and extravasation.

There is mounting evidence to support the notion that MMPs are involved in promoting PMN infiltration via the proteolytic activation of neutrophil chemoattractants and recent data suggests an opposing role for MMPs in the inactivation of ELR$^+$ chemokines. The activation mechanism for ELR$^+$ chemokines differs from the majority of chemokines, where N-terminal truncation usually results in the loss of agonism coupled with the generation of potent receptor antagonists. MMP-12 cleaves multiple murine and human ELR$^+$ chemokines within the ELR motif (Table 1.3), thereby decreasing activity (109). This cleavage was reflected

physiologically by increased neutrophil accumulation in LPS-treated lungs of MMP-12 knockout mice suggesting that macrophage-derived MMP-12 is involved in terminating neutrophil infiltration. Likewise, MMP-9 has been reported to slowly degrade the ELR$^+$ chemokines CXCL1, CXCL4, and CXCL7 *in vitro*, but physiological evidence of this interaction is lacking (107).

CXCL12, also known as SDF-1, is a multifunctional chemokine with a critical role in development as well as a potent chemoattractant of T lymphocytes, monocytes, and CD34$^+$ stem cells. Proteolysis of CXCL12 occurs at Ser^4-Leu^5 by MMP-1, -2, -3, -9, -13, and -14, resulting in a loss of binding to the cognate receptors CXCR4 (115) and CXCR7 (134) and therefore loss of HIV protection and decreased chemotactic properties. Interestingly, the cleavage product, CXCL12 (5-67), is a potent and specific neurotoxin (135), an effect shown to be mediated through CXCR3 binding and signalling (136), demonstrating for the first time that proteolysis of a chemokine can cause a shift in receptor specificity.

Membrane-Bound Chemokines

CX$_3$CL1, also known as fractalkine, is unique amongst the chemokine superfamily in that it can exist in both membrane-anchored and soluble forms, giving it the dual functionality of an adhesion molecule and a chemoattractant (137). When shed from the membrane, CX$_3$CL1 is a potent chemoattractant for T cells and monocytes. Disintegrin-like metalloproteinases ADAM-10 and ADAM-17 have been reported to shed CX$_3$CL1 by cleaving adjacent to the transmembrane region, hence regulating CX$_3$CL1-mediated cell-cell adhesion and chemoattractant activity (138, 139). A proteomic approach recently identified CX$_3$CL1 as an MMP-2 substrate, where endogenous CX$_3$CL1 was found to be increased in the conditioned medium of MMP-2 expressing fibroblasts compared to *Mmp2*-null control cells (108). Tandem mass spectrometry coupled with peptide mapping enabled the identification of two cleavage sites: Ala^{71}-Leu^{72} in the C-terminus of the chemokine ectodomain, yielding a soluble chemoattractant, and Gly^4-Met^5, a truncation known to result in potent

antagonism of the receptor CX_3CR (140). Hence, it appears that MMP-2 can both activate and inactivate a single chemokine, allowing for precise biphasic regulation of CX_3CL1 activity in the conversion of a cell surface adhesion molecule/agonist to a soluble agonist and antagonist depending on the site of cleavage. The only other membrane-anchored chemokine, CXCL16, is also shed in a metalloproteinase-dependent manner. Solubilization of CXCL16 is inducible by TNF-α and significantly decreased upon treatment with GM6001, a broad-spectrum MMP and ADAM inhibitor, but the specific proteases involved are yet to be identified (141). Notably, in genetic and inhibitor studies, ADAM-10 and gamma-secretases were found to be major components of proteolytic cascades leading to CX_3CL1 and CXCL16 shedding (142).

Proteoglycan-mediated chemokine regulation

Direct processing of chemokines themselves is not the only way by which MMPs regulate chemokine activity. Many chemokines are known to require binding to the glycosaminoglycan side chains of proteoglycans for the formation of gradients and chemotactic activity *in vivo* (143, 144). As such, altering the proteolglycans themselves can affect chemokine gradients and physiological functions. The clearest example of such an interaction came from a study showing that MMP-7 deficient mice have altered neutrophil infiltration in lung inflammation, where neutrophils remain confined to the interstitium without advancing to the alveolar space (65). This defect was attributed to MMP-7 dependent shedding of the syndecan-1 ectodomain complexed with mCXCL1/KC, a murine ELR+ chemokine, a process required to direct and confine neutrophils to the site of injury. Hence, in the absence of MMP-7, neutrophils are unable to enter the lumen of the lung because the appropriate KC-syndecan-1 gradient has not been generated. Notably, sydecan-1 is also shed by MMP-14 and MMP-16 resulting in enhanced migration *in vitro* (145), potentially representing analogous mechanisms involving other MMPs.

Additional MMPs have also been suggested to affect chemokine activity through altering interactions with the extracellular matrix. In a model of TNF-induced acute hepatitis, MMP-8 deficient mice show improved survival coupled with defective neutrophil recruitment at 6 hours post-treatment, compared to wildtype controls (37). This phenotype was proposed to be due to the indirect effect of MMP-8 dependent release of LIX from the extracellular matrix, although direct processing of LIX and modulation of activity has also been observed (25, 26, 67). Furthermore, MMP-8 processing of LIX does not alter glycosaminoglycan binding, as determined with heparin sulphate affinity assays (67). Regardless, potential regulation of chemokine interactions with proteoglycans in the formation of gradients should be appreciated when considering chemokine bioactivity *in vivo*.

Future perspectives

Deciphering the individual functions of MMPs in the regulation of inflammatory processes is an overwhelming task but the importance of the field is highlighted by failed clinical trials with MMP inhibitors and the coinciding unpredicted side effects (7, 146-149). It is now evident that the physiological roles of MMPs and their respective underlying mechanisms need to be further defined before attempting to inhibit individual MMPs for clinical applications. It is critical that only well established targets are inhibited and anti-targets are completely avoided (8). With this approach the detrimental actions of MMPs are blocked while still preserving the beneficial anti-target MMP activities. As such, animal model and cell-based approaches complemented by biochemical and proteomic studies will be instrumental in achieving this objective.

Current methods of MMP substrate discovery are predominantly hypothesis-driven, which is a very time-consuming approach. However, more systematic approaches to uncover novel MMP substrates and downstream effects of MMP activity are beginning to emerge. For instance, two-dimensional gel electrophoresis was recently used in the analysis of bronchoalveolar lavage fluid of allergen-challenged

Mmp2 -/-/Mmp9 -/- mice, leading to the identification of Ym1, S100A8, and S100A9 as novel MMP substrates (150). Furthermore, in the cellular context, MMP-14 and MMP-2 transfected cells have been used to identify a variety of new bioactive substrates utilizing mass spectrometry-based proteomic analysis (105, 108, 151). This work has been extended with the use of MMP inhibitors, thus uncovering additional substrates with the potential to identify adverse inhibitor effects (152).

The remarkable progress in proteomic approaches, such as those listed above, will undoubtedly have far-reaching implications in the field of MMP research, as well as that concerning all proteases (153). Applying such techniques to *in vivo* models of inflammation will enable rapid and unbiased identification of physiologically relevant substrates and hence a deeper understanding of the numerous inflammation-related phenotypes described here. Regardless of the specific mechanisms, it is undeniable that MMPs are key pleiotropic regulators of inflammation and immunity, demonstrating a wide array of pro-inflammatory and anti-inflammatory functions, orchestrating both the onset and termination of inflammation. Hence, the former view that the major role of MMPs is to cleave extracellular matrix is no longer tenable. Rather, MMPs are key signalling proteases involved in normal homeostasis and initiating and terminating inflammation through efficient and specific processing of inflammatory mediators.

Theme and Hypotheses

The broad focus of the research presented herein is the role of MMPs, predominantly the neutrophil collagenase MMP-8, in regulating inflammation and immunity. At the time this thesis research began, the MMP-8 knockout mouse had been generated and was more susceptible to skin carcinogenesis, making MMP-8 the first MMP anti-target in cancer (26). MMP-8 deficient mice had an initial delay in neutrophil infiltration followed by a massive accumulation over time, presumably providing a microenvironment conducive to tumorigenesis (41). The following themes and hypotheses are addressed in this thesis.

23

i) In Chapter 2 we hypothesized that the mechanism of delayed neutrophil infiltration in MMP-8 deficient mice involved LIX/mCXCL5 chemokine proteolysis and activation. We tested this hypothesis using acute inflammatory models with MMP-8 deficient mice and these findings were published in *PLoS ONE.*

ii) Next, in Chapter 3 we hypothesized that MMPs would also process interferon-γ inducible CXCR3-binding chemokines and provide proteolytic regulation of T cell chemoattraction. This was evaluated with recombinant MMPs and synthetic chemokines, where we discovered that MMPs cleave both the N- and C-terminus of CXCL11, resulting in a switch from a receptor agonist to antagonist and altered glycosaminoglycan binding, respectively. This study was published in the *Journal of Biological Chemistry.*

iii) In Chapter 4, we hypothesized that MMP-8 would be protective in a murine model of arthritis. To accomplish this objective, an MMP-8 deficient mouse strain naturally susceptible to arthritis and other autoimmunities was created by backcross breeding. We also examined the underlying mechanisms by gene microarray analysis. Here we found that neutrophils from MMP-8 deficient mice undergo delayed apoptosis, which we propose is due to abolished caspase-11 levels and decreased TNF-α shedding. The resulting manuscript has been submitted for publication.

Appendix A describes unpublished results relating to indirect MMP-8 regulation of LIX/mCXCL5 and Appendix B reports cleavage specificity of mouse and human MMP-8 and identification of MMP-8 substrates. Taken together, this research has advanced our knowledge of the function and mechanism by which MMP-8 regulates both the onset and resolution of inflammation.

References

1. Gross, J., and C. M. Lapiere. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A* 48:1014-1022.

2. Bennick, A., and A. M. Hunt. 1967. Collagenolytic activity in oral tissues. *Arch Oral Biol* 12:1-10.

3. Bauer, E. A., A. Z. Eisen, and J. J. Jeffrey. 1970. Immunologic relationship of a purified human skin collagenase to other human and animal collagenases. *Biochim Biophys Acta* 206:152-160.

4. McCawley, L. J., and L. M. Matrisian. 2001. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 13:534-540.

5. Overall, C. M. 2002. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol Biotechnol* 22:51-86.

6. Steffensen, B., U. M. Wallon, and C. M. Overall. 1995. Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen. *J Biol Chem* 270:11555-11566.

7. Coussens, L. M., B. Fingleton, and L. M. Matrisian. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295:2387-2392.

8. Overall, C. M., and O. Kleifeld. 2006. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 6:227-239.

9. Lazarus, G. S., R. S. Brown, J. R. Daniels, and H. M. Fullmer. 1968. Human granulocyte collagenase. *Science* 159:1483-1485.

10. Murphy, G., J. J. Reynolds, U. Bretz, and M. Baggiolini. 1977. Collagenase is a component of the specific granules of human neutrophil leucocytes. *Biochem J* 162:195-197.

11. Prikk, K., P. Maisi, E. Pirila, R. Sepper, T. Salo, J. Wahlgren, and T. Sorsa. 2001. In vivo collagenase-2 (MMP-8) expression by human bronchial epithelial cells and monocytes/macrophages in bronchiectasis. *J Pathol* 194:232-238.

12. Toft-Hansen, H., R. K. Nuttall, D. R. Edwards, and T. Owens. 2004. Key metalloproteinases are expressed by specific cell types in experimental autoimmune encephalomyelitis. *J Immunol* 173:5209-5218.

13. Hanemaaijer, R., T. Sorsa, Y. T. Konttinen, Y. Ding, M. Sutinen, H. Visser, V. W. van Hinsbergh, T. Helaakoski, T. Kainulainen, H. Ronka, H. Tschesche, and T. Salo. 1997. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. *J Biol Chem* 272:31504-31509.

14. Hasty, K. A., J. J. Jeffrey, M. S. Hibbs, and H. G. Welgus. 1987. The collagen substrate specificity of human neutrophil collagenase. *J Biol Chem* 262:10048-10052.

15. Arner, E. C., C. P. Decicco, R. Cherney, and M. D. Tortorella. 1997. Cleavage of native cartilage aggrecan by neutrophil collagenase (MMP-8) is distinct from endogenous cleavage by aggrecanase. *J Biol Chem* 272:9294-9299.

16. Schmid, T. M., R. Mayne, J. J. Jeffrey, and T. F. Linsenmayer. 1986. Type X collagen contains two cleavage sites for a vertebrate collagenase. *J Biol Chem* 261:4184-4189.

17. Hiller, O., A. Lichte, A. Oberpichler, A. Kocourek, and H. Tschesche. 2000. Matrix metalloproteinases collagenase-2, macrophage elastase, collagenase-3, and membrane type

1-matrix metalloproteinase impair clotting by degradation of fibrinogen and factor XII. *J Biol Chem* 275:33008-33013.

18. Shapiro, S. D. 1998. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 10:602-608.

19. Pirila, E., A. Sharabi, T. Salo, V. Quaranta, H. Tu, R. Heljasvaara, N. Koshikawa, T. Sorsa, and P. Maisi. 2003. Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration. *Biochem Biophys Res Commun* 303:1012-1017.

20. Michaelis, J., M. C. Vissers, and C. C. Winterbourn. 1990. Human neutrophil collagenase cleaves alpha 1-antitrypsin. *Biochem J* 270:809-814.

21. Knauper, V., A. Osthues, Y. A. DeClerck, K. E. Langley, J. Blaser, and H. Tschesche. 1993. Fragmentation of human polymorphonuclear-leucocyte collagenase. *Biochem J* 291 (Pt 3):847-854.

22. Diekmann, O., and H. Tschesche. 1994. Degradation of kinins, angiotensins and substance P by polymorphonuclear matrix metalloproteinases MMP 8 and MMP 9. *Braz J Med Biol Res* 27:1865-1876.

23. Knauper, V., S. Triebel, H. Reinke, and H. Tschesche. 1991. Inactivation of human plasma C1-inhibitor by human PMN leucocyte matrix metalloproteinases. *FEBS Lett* 290:99-102.

24. McQuibban, G. A., J. H. Gong, J. P. Wong, J. L. Wallace, I. Clark-Lewis, and C. M. Overall. 2002. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 100:1160-1167.

25. Van Den Steen, P. E., A. Wuyts, S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* 270:3739-3749.

26. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35:252-257.

27. Van den Steen, P. E., S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Carboxyterminal cleavage of the chemokines MIG and IP-10 by gelatinase B and neutrophil collagenase. *Biochem Biophys Res Commun* 310:889-896.

28. Hemers, E., C. Duval, C. McCaig, M. Handley, G. J. Dockray, and A. Varro. 2005. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. *Cancer Res* 65:7363-7369.

29. Okamoto, T., T. Akaike, T. Nagano, S. Miyajima, M. Suga, M. Ando, K. Ichimori, and H. Maeda. 1997. Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Arch Biochem Biophys* 342:261-274.

30. Knauper, V., S. Kramer, H. Reinke, and H. Tschesche. 1990. Characterization and activation of procollagenase from human polymorphonuclear leucocytes. N-terminal sequence determination of the proenzyme and various proteolytically activated forms. *Eur J Biochem* 189:295-300.

31. Van Lint, P., and C. Libert. 2006. Matrix metalloproteinase-8: cleavage can be decisive. *Cytokine Growth Factor Rev* 17:217-223.

32. Owen, C. A., Z. Hu, C. Lopez-Otin, and S. D. Shapiro. 2004. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol* 172:7791-7803.

33. Matsuki, H., N. Fujimoto, K. Iwata, V. Knauper, Y. Okada, and T. Hayakawa. 1996. A one-step sandwich enzyme immunoassay for human matrix metalloproteinase 8 (neutrophil collagenase) using monoclonal antibodies. *Clin Chim Acta* 244:129-143.

34. Power, C., C. M. O'Connor, D. MacFarlane, S. O'Mahoney, K. Gaffney, J. Hayes, and M. X. FitzGerald. 1994. Neutrophil collagenase in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 150:818-822.

35. Lee, W., S. Aitken, J. Sodek, and C. A. McCulloch. 1995. Evidence of a direct relationship between neutrophil collagenase activity and periodontal tissue destruction in vivo: role of active enzyme in human periodontitis. *J Periodontal Res* 30:23-33.

36. Gueders, M. M., M. Balbin, N. Rocks, J. M. Foidart, P. Gosset, R. Louis, S. Shapiro, C. Lopez-Otin, A. Noel, and D. D. Cataldo. 2005. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J Immunol* 175:2589-2597.

37. Van Lint, P., B. Wielockx, L. Puimege, A. Noel, C. Lopez-Otin, and C. Libert. 2005. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol* 175:7642-7649.

38. Gutierrez-Fernandez, A., M. Inada, M. Balbin, A. Fueyo, A. S. Pitiot, A. Astudillo, K. Hirose, M. Hirata, S. D. Shapiro, A. Noel, Z. Werb, S. M. Krane, C. Lopez-Otin, and X. S. Puente. 2007. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *Faseb J.*

39. Folgueras, A. R., A. Fueyo, O. Garcia-Suarez, J. Cox, A. Astudillo, P. Tortorella, C. Campestre, A. Gutierrez-Fernandez, M. Fanjul-Fernandez, C. J. Pennington, D. R. Edwards, C. M. Overall, and C. Lopez-Otin. 2008. Collagenase-2 deficiency or inhibition impairs experimental autoimmune encephalomyelitis in mice. *J Biol Chem* 283:9465-9474.

40. Wucherpfennig, K. W. 2001. Mechanisms for the induction of autoimmunity by infectious agents. *J Clin Invest* 108:1097-1104.

41. Dvorak, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315:1650-1659.

42. Hansson, G. K., and P. Libby. 2006. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* 6:508-519.

43. Overall, C. M. 2004. Dilating the degradome: matrix metalloproteinase 2 (MMP-2) cuts to the heart of the matter. *Biochem J* 383:e5-7.

44. Turk, B. 2006. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov* 5:785-799.

45. Bramhall, S. R., M. T. Hallissey, J. Whiting, J. Scholefield, G. Tierney, R. C. Stuart, R. E. Hawkins, P. McCulloch, T. Maughan, P. D. Brown, M. Baillet, and J. W. Fielding. 2002. Marimastat as maintenance therapy for patients with advanced gastric cancer: a randomised trial. *Br J Cancer* 86:1864-1870.

46. Parks, W. C., C. L. Wilson, and Y. S. Lopez-Boado. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* 4:617-629.

47. Page-McCaw, A., A. J. Ewald, and Z. Werb. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 8:221-233.

48. Itoh, T., H. Matsuda, M. Tanioka, K. Kuwabara, S. Itohara, and R. Suzuki. 2002. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J Immunol* 169:2643-2647.

49. Corry, D. B., K. Rishi, J. Kanellis, A. Kiss, L. Z. Song Lz, J. Xu, L. Feng, Z. Werb, and F. Kheradmand. 2002. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat Immunol* 3:347-353.

50.   Esparza, J., M. Kruse, J. Lee, M. Michaud, and J. A. Madri. 2004. MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity. *Faseb J* 18:1682-1691.

51.   Campbell, L. G., S. Ramachandran, W. Liu, J. M. Shipley, S. Itohara, J. G. Rogers, N. Moazami, R. M. Senior, and A. Jaramillo. 2005. Different roles for matrix metalloproteinase-2 and matrix metalloproteinase-9 in the pathogenesis of cardiac allograft rejection. *Am J Transplant* 5:517-528.

52.   Matsusaka, H., M. Ikeuchi, S. Matsushima, T. Ide, T. Kubota, A. M. Feldman, A. Takeshita, K. Sunagawa, and H. Tsutsui. 2005. Selective disruption of MMP-2 gene exacerbates myocardial inflammation and dysfunction in mice with cytokine-induced cardiomyopathy. *Am J Physiol Heart Circ Physiol* 289:H1858-1864.

53.   Samolov, B., B. Steen, S. Seregard, I. van der Ploeg, P. Montan, and A. Kvanta. 2005. Delayed inflammation-associated corneal neovascularization in MMP-2-deficient mice. *Exp Eye Res* 80:159-166.

54.   Garg, P., M. Rojas, A. Ravi, K. Bockbrader, S. Epstein, M. Vijay-Kumar, A. T. Gewirtz, D. Merlin, and S. V. Sitaraman. 2006. Selective ablation of matrix metalloproteinase-2 exacerbates experimental colitis: contrasting role of gelatinases in the pathogenesis of colitis. *J Immunol* 177:4103-4112.

55.   Wang, M., X. Qin, J. S. Mudgett, T. A. Ferguson, R. M. Senior, and H. G. Welgus. 1999. Matrix metalloproteinase deficiencies affect contact hypersensitivity: stromelysin-1 deficiency prevents the response and gelatinase B deficiency prolongs the response. *Proc Natl Acad Sci U S A* 96:6885-6889.

56.   Haro, H., H. C. Crawford, B. Fingleton, J. R. MacDougall, K. Shinomiya, D. M. Spengler, and L. M. Matrisian. 2000. Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. *J Clin Invest* 105:133-141.

57.   Warner, R. L., L. Beltran, E. M. Younkin, C. S. Lewis, S. J. Weiss, J. Varani, and K. J. Johnson. 2001. Role of stromelysin 1 and gelatinase B in experimental acute lung injury. *Am J Respir Cell Mol Biol* 24:537-544.

58.   Silence, J., F. Lupu, D. Collen, and H. R. Lijnen. 2001. Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. *Arterioscler Thromb Vasc Biol* 21:1440-1445.

59.   Li, C. K., S. L. Pender, K. M. Pickard, V. Chance, J. A. Holloway, A. Huett, N. S. Goncalves, J. S. Mudgett, G. Dougan, G. Frankel, and T. T. MacDonald. 2004. Impaired immunity to intestinal bacterial infection in stromelysin-1 (matrix metalloproteinase-3)-deficient mice. *J Immunol* 173:5171-5179.

60.   Mukherjee, R., J. A. Bruce, D. M. McClister, Jr., C. M. Allen, S. E. Sweterlitsch, and J. P. Saul. 2005. Time-dependent changes in myocardial structure following discrete injury in mice deficient of matrix metalloproteinase-3. *J Mol Cell Cardiol* 39:259-268.

61.   Handley, S. A., and V. L. Miller. 2007. General and specific host responses to bacterial infection in Peyer's patches: a role for stromelysin-1 (matrix metalloproteinase-3) during *Salmonella enterica* infection. *Mol Microbiol* 64:94-110.

62.   Wilson, C. L., A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. Lopez-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks. 1999. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286:113-117.

63.   Powell, W. C., B. Fingleton, C. L. Wilson, M. Boothby, and L. M. Matrisian. 1999. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr Biol* 9:1441-1447.

64.   Haro, H., H. C. Crawford, B. Fingleton, K. Shinomiya, D. M. Spengler, and L. M. Matrisian. 2000. Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption. *J Clin Invest* 105:143-150.

65.   Li, Q., P. W. Park, C. L. Wilson, and W. C. Parks. 2002. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 111:635-646.

66.   Gjertsson, I., M. Innocenti, L. M. Matrisian, and A. Tarkowski. 2005. Metalloproteinase-7 contributes to joint destruction in Staphylococcus aureus induced arthritis. *Microb Pathog* 38:97-105.

67.   Tester, A. M., J. H. Cox, A. R. Connor, A. E. Starr, R. A. Dean, X. S. Puente, C. Lopez-Otin, and C. M. Overall. 2007. LPS Responsiveness and Neutrophil Chemotaxis In Vivo Require PMN MMP-8 Activity. *PLoS ONE* 2:e312.

68.   Lin, M., P. Jackson, A. M. Tester, E. Diaconu, C. M. Overall, J. E. Blalock, and E. Pearlman. 2008. Matrix Metalloproteinase-8 Facilitates Neutrophil Migration through the Corneal Stromal Matrix by Collagen Degradation and Production of the Chemotactic Peptide Pro-Gly-Pro. *Am J Pathol*.

69.   Dubois, B., S. Masure, U. Hurtenbach, L. Paemen, H. Heremans, J. van den Oord, R. Sciot, T. Meinhardt, G. Hammerling, G. Opdenakker, and B. Arnold. 1999. Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions. *J Clin Invest* 104:1507-1515.

70.   Liu, Z., X. Zhou, S. D. Shapiro, J. M. Shipley, S. S. Twining, L. A. Diaz, R. M. Senior, and Z. Werb. 2000. The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. *Cell* 102:647-655.

71.   Ducharme, A., S. Frantz, M. Aikawa, E. Rabkin, M. Lindsey, L. E. Rohde, F. J. Schoen, R. A. Kelly, Z. Werb, P. Libby, and R. T. Lee. 2000. Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest* 106:55-62.

72.   Heissig, B., K. Hattori, S. Dias, M. Friedrich, B. Ferris, N. R. Hackett, R. G. Crystal, P. Besmer, D. Lyden, M. A. Moore, Z. Werb, and S. Rafii. 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109:625-637.

73.   Cataldo, D. D., K. G. Tournoy, K. Vermaelen, C. Munaut, J. M. Foidart, R. Louis, A. Noel, and R. A. Pauwels. 2002. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol* 161:491-498.

74.   Bottcher, T., A. Spreer, I. Azeh, R. Nau, and J. Gerber. 2003. Matrix metalloproteinase-9 deficiency impairs host defense mechanisms against Streptococcus pneumoniae in a mouse model of bacterial meningitis. *Neurosci Lett* 338:201-204.

75.   Pelus, L. M., H. Bian, A. G. King, and S. Fukuda. 2004. Neutrophil-derived MMP-9 mediates synergistic mobilization of hematopoietic stem and progenitor cells by the combination of G-CSF and the chemokines GRObeta/CXCL2 and GRObetaT/CXCL2delta4. *Blood* 103:110-119.

76.   Ichiyasu, H., J. M. McCormack, K. M. McCarthy, D. Dombkowski, F. I. Preffer, and E. E. Schneeberger. 2004. Matrix metalloproteinase-9-deficient dendritic cells have impaired migration through tracheal epithelial tight junctions. *Am J Respir Cell Mol Biol* 30:761-770.

77.   McMillan, S. J., J. Kearley, J. D. Campbell, X. W. Zhu, K. Y. Larbi, J. M. Shipley, R. M. Senior, S. Nourshargh, and C. M. Lloyd. 2004. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J Immunol* 172:2586-2594.

78. Corry, D. B., A. Kiss, L. Z. Song, L. Song, J. Xu, S. H. Lee, Z. Werb, and F. Kheradmand. 2004. Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. *Faseb J* 18:995-997.

79. Santana, A., C. Medina, M. C. Paz-Cabrera, F. Diaz-Gonzalez, E. Farre, A. Salas, M. W. Radomski, and E. Quintero. 2006. Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. *World J Gastroenterol* 12:6464-6472.

80. Calander, A. M., S. Starckx, G. Opdenakker, P. Bergin, M. Quiding-Jarbrink, and A. Tarkowski. 2006. Matrix metalloproteinase-9 (gelatinase B) deficiency leads to increased severity of Staphylococcus aureus-triggered septic arthritis. *Microbes Infect* 8:1434-1439.

81. Renckens, R., J. J. Roelofs, S. Florquin, A. F. de Vos, H. R. Lijnen, C. van't Veer, and T. van der Poll. 2006. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. *J Immunol* 176:3735-3741.

82. Kassim, S. Y., S. A. Gharib, B. H. Mecham, T. P. Birkland, W. C. Parks, and J. K. McGuire. 2007. Individual matrix metalloproteinases control distinct transcriptional responses in airway epithelial cells infected with Pseudomonas aeruginosa. *Infect Immun* 75:5640-5650.

83. Hautamaki, R. D., D. K. Kobayashi, R. M. Senior, and S. D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 277:2002-2004.

84. Churg, A., R. D. Wang, H. Tai, X. Wang, C. Xie, J. Dai, S. D. Shapiro, and J. L. Wright. 2003. Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *Am J Respir Crit Care Med* 167:1083-1089.

85. Wells, J. E., T. K. Rice, R. K. Nuttall, D. R. Edwards, H. Zekki, S. Rivest, and V. W. Yong. 2003. An adverse role for matrix metalloproteinase 12 after spinal cord injury in mice. *J Neurosci* 23:10107-10115.

86. Pouladi, M. A., C. S. Robbins, F. K. Swirski, M. Cundall, A. N. McKenzie, M. Jordana, S. D. Shapiro, and M. R. Stampfli. 2004. Interleukin-13-dependent expression of matrix metalloproteinase-12 is required for the development of airway eosinophilia in mice. *Am J Respir Cell Mol Biol* 30:84-90.

87. Warner, R. L., N. W. Lukacs, S. D. Shapiro, N. Bhagarvathula, K. C. Nerusu, J. Varani, and K. J. Johnson. 2004. Role of metalloelastase in a model of allergic lung responses induced by cockroach allergen. *Am J Pathol* 165:1921-1930.

88. Longo, G. M., S. J. Buda, N. Fiotta, W. Xiong, T. Griener, S. Shapiro, and B. T. Baxter. 2005. MMP-12 has a role in abdominal aortic aneurysms in mice. *Surgery* 137:457-462.

89. Weaver, A., A. Goncalves da Silva, R. K. Nuttall, D. R. Edwards, S. D. Shapiro, S. Rivest, and V. W. Yong. 2005. An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization. *Faseb J* 19:1668-1670.

90. Johnson, J. L., S. J. George, A. C. Newby, and C. L. Jackson. 2005. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A* 102:15575-15580.

91. Wright, R. W., T. Allen, H. B. El-Zawawy, M. D. Brodt, M. J. Silva, C. S. Gill, and L. J. Sandell. 2006. Medial collateral ligament healing in macrophage metalloelastase (MMP-12)-deficient mice. *J Orthop Res* 24:2106-2113.

92. Manoury, B., S. Nenan, I. Guenon, E. Boichot, J. M. Planquois, C. P. Bertrand, and V. Lagente. 2006. Macrophage metalloelastase (MMP-12) deficiency does not alter bleomycin-induced pulmonary fibrosis in mice. *J Inflamm (Lond)* 3:2.

93. Houghton, A. M., P. A. Quintero, D. L. Perkins, D. K. Kobayashi, D. G. Kelley, L. A. Marconcini, R. P. Mecham, R. M. Senior, and S. D. Shapiro. 2006. Elastin fragments drive disease progression in a murine model of emphysema. *J Clin Invest* 116:753-759.

94. Dinarello, C. A. 2005. Blocking IL-1 in systemic inflammation. *J Exp Med* 201:1355-1359.

95. Andrei, C., C. Dazzi, L. Lotti, M. R. Torrisi, G. Chimini, and A. Rubartelli. 1999. The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* 10:1463-1475.

96. Kostura, M. J., M. J. Tocci, G. Limjuco, J. Chin, P. Cameron, A. G. Hillman, N. A. Chartrain, and J. A. Schmidt. 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc Natl Acad Sci U S A* 86:5227-5231.

97. Fantuzzi, G., G. Ku, M. W. Harding, D. J. Livingston, J. D. Sipe, K. Kuida, R. A. Flavell, and C. A. Dinarello. 1997. Response to local inflammation of IL-1 beta-converting enzyme-deficient mice. *J Immunol* 158:1818-1824.

98. Schonbeck, U., F. Mach, and P. Libby. 1998. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 161:3340-3346.

99. Ito, A., A. Mukaiyama, Y. Itoh, H. Nagase, I. B. Thogersen, J. J. Enghild, Y. Sasaguri, and Y. Mori. 1996. Degradation of interleukin 1beta by matrix metalloproteinases. *J Biol Chem* 271:14657-14660.

100. Kodama, S., M. Davis, and D. L. Faustman. 2005. The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis. *Cell Mol Life Sci* 62:1850-1862.

101. Taylor, P. C., R. O. Williams, and M. Feldmann. 2004. Tumour necrosis factor alpha as a therapeutic target for immune-mediated inflammatory diseases. *Curr Opin Biotechnol* 15:557-563.

102. Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. M. Clements, M. Crimmin, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, and et al. 1995. Matrix metalloproteinases and processing of pro-TNF-alpha. *J Leukoc Biol* 57:774-777.

103. Chandler, S., J. Cossins, J. Lury, and G. Wells. 1996. Macrophage metalloelastase degrades matrix and myelin proteins and processes a tumour necrosis factor-alpha fusion protein. *Biochem Biophys Res Commun* 228:421-429.

104. d'Ortho, M. P., H. Will, S. Atkinson, G. Butler, A. Messent, J. Gavrilovic, B. Smith, R. Timpl, L. Zardi, and G. Murphy. 1997. Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. *Eur J Biochem* 250:751-757.

105. Tam, E. M., C. J. Morrison, Y. I. Wu, M. S. Stack, and C. M. Overall. 2004. Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 101:6917-6922.

106. English, W. R., X. S. Puente, J. M. Freije, V. Knauper, A. Amour, A. Merryweather, C. Lopez-Otin, and G. Murphy. 2000. Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2. *J Biol Chem* 275:14046-14055.

107. Van den Steen, P. E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.

108. Dean, R. A., and C. M. Overall. 2007. Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome. *Mol Cell Proteomics* 6:611-623.

109. Dean, R. A., J. H. Cox, C. L. Bellac, A. Doucet, A. E. Starr, and C. M. Overall. In Press. Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC

chemokines and generates CCL2, 7, 8, and 13 antagonists: Potential role of the macrophage in terminating PMN influx. *Blood*:In Press.

110. Karsdal, M. A., L. Larsen, M. T. Engsig, H. Lou, M. Ferreras, A. Lochter, J. M. Delaisse, and N. T. Foged. 2002. Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. *J Biol Chem* 277:44061-44067.

111. Maeda, S., D. D. Dean, I. Gay, Z. Schwartz, and B. D. Boyan. 2001. Activation of latent transforming growth factor beta1 by stromelysin 1 in extracts of growth plate chondrocyte-derived matrix vesicles. *J Bone Miner Res* 16:1281-1290.

112. Yu, Q., and I. Stamenkovic. 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14:163-176.

113. D'Angelo, M., P. C. Billings, M. Pacifici, P. S. Leboy, and T. Kirsch. 2001. Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. *J Biol Chem* 276:11347-11353.

114. McQuibban, G. A., J. H. Gong, E. M. Tam, C. A. McCulloch, I. Clark-Lewis, and C. M. Overall. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 289:1202-1206.

115. McQuibban, G. A., G. S. Butler, J. H. Gong, L. Bendall, C. Power, I. Clark-Lewis, and C. M. Overall. 2001. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 276:43503-43508.

116. Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, and et al. 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* 370:218-220.

117. McGeehan, G. M., J. D. Becherer, R. C. Bast, Jr., C. M. Boyer, B. Champion, K. M. Connolly, J. G. Conway, P. Furdon, S. Karp, S. Kidao, and et al. 1994. Regulation of tumour necrosis factor-alpha processing by a metalloproteinase inhibitor. *Nature* 370:558-561.

118. Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, and et al. 1994. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* 370:555-557.

119. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385:729-733.

120. Moss, M. L., S. L. Jin, M. E. Milla, D. M. Bickett, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R. Didsbury, D. Hassler, C. R. Hoffman, T. A. Kost, M. H. Lambert, M. A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L. K. Overton, F. Schoenen, T. Seaton, J. L. Su, J. D. Becherer, and et al. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 385:733-736.

121. Overall, C. M., and C. P. Blobel. 2007. In search of partners: linking extracellular proteases to substrates. *Nat Rev Mol Cell Biol* 8:245-257.

122. Wahl, S. M., J. Wen, and N. Moutsopoulos. 2006. TGF-beta: a mobile purveyor of immune privilege. *Immunol Rev* 213:213-227.

123. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, and et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359:693-699.

124. Khalil, N. 1999. TGF-beta: from latent to active. *Microbes Infect* 1:1255-1263.

125. Overall, C. M., J. L. Wrana, and J. Sodek. 1989. Transforming growth factor-beta regulation of collagenase, 72 kDa-progelatinase, TIMP and PAI-1 expression in rat bone cell populations and human fibroblasts. *Connect Tissue Res* 20:289-294.

126. Overall, C. M., J. L. Wrana, and J. Sodek. 1991. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* 266:14064-14071.

127. Zhou, H., E. J. Bernhard, F. E. Fox, and P. C. Billings. 1993. Induction of metalloproteinase activity in human T-lymphocytes. *Biochim Biophys Acta* 1177:174-178.

128. Ellerbroek, S. M., Y. I. Wu, C. M. Overall, and M. S. Stack. 2001. Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. *J Biol Chem* 276:24833-24842.

129. Tam, E. M., T. R. Moore, G. S. Butler, and C. M. Overall. 2004. Characterization of the distinct collagen binding, helicase and cleavage mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): the differential roles of the MMP hemopexin c domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities. *J Biol Chem* 279:43336-43344.

130. Moser, B., M. Wolf, A. Walz, and P. Loetscher. 2004. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 25:75-84.

131. Fernandez, E. J., and E. Lolis. 2002. Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol* 42:469-499.

132. Hensbergen, P. J., D. Verzijl, C. I. Balog, R. Dijkman, R. C. van der Schors, E. M. van der Raaij-Helmer, M. J. van der Plas, R. Leurs, A. M. Deelder, M. J. Smit, and C. P. Tensen. 2004. Furin is a chemokine-modifying enzyme: in vitro and in vivo processing of CXCL10 generates a C-terminally truncated chemokine retaining full activity. *J Biol Chem* 279:13402-13411.

133. Overall, C. M., G. A. McQuibban, and I. Clark-Lewis. 2002. Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol Chem* 383:1059-1066.

134. Balabanian, K., B. Lagane, S. Infantino, K. Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 280:35760-35766.

135. Zhang, K., G. A. McQuibban, C. Silva, G. S. Butler, J. B. Johnston, J. Holden, I. Clark-Lewis, C. M. Overall, and C. Power. 2003. HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat Neurosci* 6:1064-1071.

136. Vergote, D., G. S. Butler, M. Ooms, J. H. Cox, C. Silva, M. D. Hollenberg, J. H. Jhamandas, C. M. Overall, and C. Power. 2006. Proteolytic processing of SDF-1alpha reveals a change in receptor specificity mediating HIV-associated neurodegeneration. *Proc Natl Acad Sci U S A* 103:19182-19187.

137. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 385:640-644.

138. Garton, K. J., P. J. Gough, C. P. Blobel, G. Murphy, D. R. Greaves, P. J. Dempsey, and E. W. Raines. 2001. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J Biol Chem* 276:37993-38001.

139. Hundhausen, C., D. Misztela, T. A. Berkhout, N. Broadway, P. Saftig, K. Reiss, D. Hartmann, F. Fahrenholz, R. Postina, V. Matthews, K. J. Kallen, S. Rose-John, and A. Ludwig. 2003. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood* 102:1186-1195.

140. Inoue, A., H. Hasegawa, M. Kohno, M. R. Ito, M. Terada, T. Imai, O. Yoshie, M. Nose, and S. Fujita. 2005. Antagonist of fractalkine (CX3CL1) delays the initiation and ameliorates the progression of lupus nephritis in MRL/lpr mice. *Arthritis Rheum* 52:1522-1533.

141. Hara, T., T. Katakai, J. H. Lee, Y. Nambu, N. Nakajima-Nagata, H. Gonda, M. Sugai, and A. Shimizu. 2006. A transmembrane chemokine, CXC chemokine ligand 16, expressed by lymph node fibroblastic reticular cells has the potential to regulate T cell migration and adhesion. *Int Immunol* 18:301-311.

142. Schulte, A., B. Schulz, M. G. Andrzejewski, C. Hundhausen, S. Mletzko, J. Achilles, K. Reiss, K. Paliga, C. Weber, S. R. John, and A. Ludwig. 2007. Sequential processing of the transmembrane chemokines CX3CL1 and CXCL16 by alpha- and gamma-secretases. *Biochem Biophys Res Commun* 358:233-240.

143. Handel, T. M., Z. Johnson, S. E. Crown, E. K. Lau, and A. E. Proudfoot. 2005. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annu Rev Biochem* 74:385-410.

144. Proudfoot, A. E., T. M. Handel, Z. Johnson, E. K. Lau, P. LiWang, I. Clark-Lewis, F. Borlat, T. N. Wells, and M. H. Kosco-Vilbois. 2003. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* 100:1885-1890.

145. Endo, K., T. Takino, H. Miyamori, H. Kinsen, T. Yoshizaki, M. Furukawa, and H. Sato. 2003. Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration. *J Biol Chem* 278:40764-40770.

146. Puente, X. S., L. M. Sanchez, C. M. Overall, and C. Lopez-Otin. 2003. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 4:544-558.

147. Pavlaki, M., and S. Zucker. 2003. Matrix metalloproteinase inhibitors (MMPIs): the beginning of phase I or the termination of phase III clinical trials. *Cancer Metastasis Rev* 22:177-203.

148. Overall, C. M., and C. Lopez-Otin. 2002. Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2:657-672.

149. Zucker, S., J. Cao, and W. T. Chen. 2000. Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. *Oncogene* 19:6642-6650.

150. Greenlee, K. J., D. B. Corry, D. A. Engler, R. K. Matsunami, P. Tessier, R. G. Cook, Z. Werb, and F. Kheradmand. 2006. Proteomic identification of in vivo substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. *J Immunol* 177:7312-7321.

151. Dean, R. A., G. S. Butler, Y. Hamma-Kourbali, J. Delbe, D. R. Brigstock, J. Courty, and C. M. Overall. 2007. Identification of candidate angiogenic inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based proteomic screens: disruption of vascular endothelial growth factor (VEGF)/heparin affin regulatory peptide (pleiotrophin) and VEGF/Connective tissue growth factor angiogenic inhibitory complexes by MMP-2 proteolysis. *Mol Cell Biol* 27:8454-8465.

152. Butler, G. S., R. A. Dean, E. M. Tam, and C. M. Overall. 2008. Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: Dynamics of matrix metalloproteinase-14 (MT1-MMP) mediated membrane protein shedding. *Mol Cell Biol*.

153. Schilling, O., and C. M. Overall. 2007. Proteomic discovery of protease substrates. *Curr Opin Chem Biol* 11:36-45.

CHAPTER 2. LPS RESPONSIVENESS AND NEUTROPHIL CHEMOTAXIS IN VIVO REQUIRE PMN MMP-8 ACTIVITY[2]

Perspective

We identify matrix metalloproteinase (MMP)-8, the polymorphonuclear (PMN) leukocyte collagenase, as a critical mediator initiating lipopolysaccharide (LPS)-responsiveness *in vivo*. PMN infiltration towards LPS is abrogated in *Mmp8*-null mice. MMP-8 cleaves LPS-induced CXC chemokine (LIX) at Ser^4-Val^5 and Lys^{79}-Arg^{80}. LIX bioactivity is increased upon N-terminal cleavage, enhancing intracellular calcium mobilization and chemotaxis upon binding its cognate receptor, CXCR2. As there is no difference in PMN chemotaxis in *Mmp8*-null mice compared with wild-type mice towards synthetic analogues of MMP-8-cleaved LIX, MMP-8 is not essential for extravasation or cell migration in collagenous matrices *in vivo*. However, with biochemical redundancy between MMPs 1, 2, 9, and 13, which also cleave LIX at position 4-5, it was surprising to observe such a markedly reduced PMN infiltration towards LPS and LIX in *Mmp8* -/- mice. This lack of physiological redundancy *in vivo* identifies MMP-8 as a key mediator in the regulation of innate immunity. Comparable results were found with CXCL8/IL-8 and CXCL5/ENA-78, the human orthologues of LIX. MMP-8 cleaves CXCL8 at Arg^5-Ser^6 and at Val^7-Leu^8 in CXCL5 to activate respective chemokines. Hence, rather than collagen, these PMN chemoattractants are important MMP-8 substrates *in vivo*; PMN-derived MMP-8 cleaves and activates LIX to execute an *in cis* PMN-controlled feed-forward mechanism to orchestrate the initial inflammatory response and promote LPS responsiveness in tissue.

---

[2] A version of this chapter has been published. Tester AM*, Cox JH*, Connor AR, Starr AE, Dean RA, Puente XS, Lopez-Otin C, and Overall CM. (2007) LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity. *PLoS ONE*. 2(3):e312. (* equal contribution). This topic has been examined further and is presented in Appendix A (unpublished data).

Introduction

Polymorphonuclear neutrophils (PMNs) are crucial inflammatory leukocytes in host protection from infection, where their primary role is in phagocytosis and killing of bacteria, fungi and protozoa, and in wound debridement and healing (1, 2). Given these critical roles of PMNs, it has long been recognised that neutropenic patients are at greater risk of infection (3), and that is often observed after intensive cancer chemotherapy (4, 5). Proteolysis of phagosome contents and damaged extracellular matrix are key PMN actions in inflammation. Cell migration, crossing basement membrane and connective tissue matrix barriers are other aspects of PMN function traditionally thought to require proteolytic activity (6). Additionally, PMNs are a source of chemotactic factors that guide the recruitment of specific and non-specific immune effector cells (7) and so these first line defence cells play key roles in innate and acquired immunity.

Of the two major chemokine subfamilies that provide directional cues for leukocyte migration and activation (8), the CXC chemokines predominantly influence PMNs and T-lymphocytes whereas the CC chemokines are active on monocytes, basophils and eosinophils (9). The expression of CXC chemokines is rapidly upregulated during acute inflammatory responses, such as that initiated by the endotoxin lipopolysaccaride (LPS) (10-13). A subset of the CXC chemokines are characterised by an ELR (glutamic acid-leucine-arginine) sequence proximal to the conserved CXC motif. ELR is essential for binding CXC-receptors (CXCR) 1 and 2 (14) leading to PMN activation, degranulation and release of proteases (15). The murine ELR$^+$ CXC chemokines act through a single receptor that is homologous to human CXCR2 (16). In humans there are seven ELR$^+$ CXC chemokines; CXCL8/interleukin-8 (IL-8); CXCL7/neutrophil-activating peptide-2 (NAP-2); CXCL6/granulocyte chemotactic protein-2 (GCP-2); CXCL5/epithelial cell-derived neutrophil activating peptide-78 (ENA-78); and CXCL1, -2 and -3 (also known as growth-related oncogenes (GRO) α, -β, and -γ). Only CXCL8/IL-8, the most potent of these chemokines, and CXCL6/GCP-2 bind CXCR1, whereas all members signal through the closely related receptor CXCR2 (14). Mice lack a homologue of CXCL8/IL-8 having only four ELR$^+$

CXC chemokines: LPS-induced CXC chemokine (LIX), the most abundant and potent of the murine chemokines and regarded as the orthologue of CXCL8 (17); keratinocyte-derived chemokine (KC); macrophage inflammatory protein-2 (MIP-2); and dendritic cell inflammatory protein-1 (DCIP-1). Physiological N-terminal cleavage of chemokines modifies their bioactivity—either enhancing activity of the ELR$^+$ CXC chemokines (15) or generating potent receptor antagonists from the CC chemokines CCL2, -7, -8 and -13 (also known as macrophage chemotactic proteins 1 to 4) (18, 19). Although several candidate proteases are proposed for ELR+ CXCL proteolytic activation, none have been validated *in vivo*.

Matrix metalloproteinases (MMPs) are traditionally associated with extracellular matrix protein degradation in many physiological and pathological processes, including inflammation, bacterial infection, wound healing, and cancer cell invasiveness (reviewed in ref (20)). However, it is now clear that MMPs mediate homeostasis of the extracellular environment (21) by modulating the biological activity of many bioactive molecules involved in cell function (22, 23), innate immunity (24) including chemokines (18, 19, 23, 25-27), TNF-α (22, 28), α-defensin (29), and mannose binding lectin (30), and in tumour initiation and progression. Inflammation in cancer, particularly macrophage infiltration and MMP-9 release, generates a microenvironment advantageous to neoplastic progression (31), with recent evidence indicating that a PMN source of MMP-9 can also promote tumorigenesis (32).

MMP-8 (human: P22894, mouse: CAA73786, rat: AJ007288), the neutrophil collagenase, is produced primarily by PMNs and is released from the specific granules at sites of inflammation (33). *Mmp8*-null mice have no overt phenotype, with normal embryonic development, fertility, and long-term survival (34). In contrast to other MMP deficient mice (35), *Mmp8*-null mice challenged with carcinogens showed a markedly increased susceptibility to tumorigenesis (34), but this only occurred in male mice. This was the first report of a MMP having a protective role in tumorigenesis, so validating MMP-8 as an anti-target in cancer therapy (35).

As a potent type I collagenase (36, 37) it had been predicted that mice lacking the *Mmp8* gene would show reduced PMN migration through collagenous matrices (34). Indeed, at the tumor stromal interface an abnormal inflammatory response is observed, characterised by an initially delayed and then a more diffuse PMN influx in the *Mmp8*-null mice (34). However, once established there was a prolonged chronic accumulation of PMNs that did not dissipate. Overall, this phenotype is reversed following transplantation of wild type bone marrow, confirming that the absence of MMP-8 produced by PMNs and not a tissue or tumour source resulted in the higher incidence of tumors. In a model of TNF-induced acute hepatitis, MMP-8 deficient mice showed dampened levels of PMN infiltration into the liver that was postulated to result from reduced LIX mobilization from an unidentified binding protein in the matrix (38). Together, these studies suggest a coordinating role for MMP-8 in physiological leukocyte trafficking both in acute and chronic inflammation, either through cleavage of collagen or chemokine binding proteins (38), or by processing of bioactive molecules, such as LIX (34) to control PMN migration or longevity.

Here we have investigated the role of MMP-8 in PMN recruitment during acute inflammation using LPS responsiveness as a trigger; the PMN influx was abrogated in the *Mmp8* -/- mouse. LIX is identified as a key inflammatory substrate of MMP-8 where N-terminal processing by MMP-8 activates the chemokine and so increases PMN chemotaxis and LPS responsiveness *in vitro* and *in vivo*. Although these activities are reduced in the *Mmp8*-null mouse, the loss of neutrophil collagenolytic activity did not alter *in vitro* PMN chemokinesis or *in vivo* chemotaxis when challenged with truncated LIX or truncated CXCL8/IL-8 chemokines. Hence, these data reveal a new auto-regulatory mechanism of PMN chemotaxis that is initiated by MMP-8 release from PMNs and executed, directly or indirectly, by the proteolytic activation of LIX in mice and CXCL8 and CXCL5/ENA-78 in man. This drives further PMN migration in a novel feed-forward mechanism that, remarkably, is a major determinant of LPS responsiveness.

Materials and Methods

Animals. Mice deficient in *Mmp8* on a C57BL6/J x 129 S background were provided by Dr. S. Shapiro (Boston, USA). Wild type C57BL6/J x 129 S mice were purchased from the Jackson Laboratory. Animal breeding and experimental procedures were approved by the Animal Care Committee of the University of British Columbia (Appendix C). 6-8 week old mice, segregated according to sex, were used for all experiments.

In vivo **PMN chemotaxis.** The air pouch model of PMN chemotaxis was used as described previously (59). Sterile air (3 mL) was injected under the dorsal skin of mice, two days later the air pouch was reinflated with 2 mL of sterile air. On day five, 1 µg LPS (Sigma) in phosphate buffered saline or 5 µg of chemokine in 1 mL 0.5% carboxymethylcellulose was injected into the air pouch. After 0, 4, 8, or 12 h the mice were sacrificed and air pouches lavaged with 2 mL of sterile PBS. The resulting cell suspensions were lysed with 0.1% Triton-X100 and freeze-thawed. The PMN content was determined by myeloperoxidase activity (60) using isolated PMN cells as a standard. Cell lysate aliquots were separated by 7.5% SDS-PAGE and MMP-8 was detected by western blot with 1:10,000 rabbit IgG against recombinant mouse MMP-8 (34).

Chemokines and proteinases. LIX (1-92), LIX (5-92), LIX (5-79), MIP-2, KC, DCIP-1, CXCL8, CXCL8 (6-77), CXCL5 and CXCL5 (8-78) were chemically synthesized and purified (61). Recombinant human MMP-1, -2, -8, -9, -13, -14, rodent MMP-8, -13, and recombinant human MMP-8 hemopexin C-domain were expressed and purified (18, 19). The synthetic hydroxamate MMP inhibitor Batimastat (BB94) was from British Biotech (Oxford, UK).

Chemokine cleavage assays. Analysis of chemokine cleavage by MMPs was performed at enzyme/chemokine ratios from 1:1000 up to 1:10 (w/w), at 37 °C in assay buffer (150 mM NaCl, 20 mM Tris, 5 mM $CaCl_2$, pH 7.5) in the presence of 1 mM APMA to activate proMMPs for 16-22 h. Digests were terminated by adding 5

µM EDTA. Recombinant MMP-8 hemopexin C-domain, EDTA or BB94 were added to cleavage assays as indicated. Reaction products were analyzed by 15% Tris-Tricine SDS-PAGE and stained with Coomassie Brilliant Blue R250. The [M+H]$^{+}$/z of each cleavage product was determined by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry on a Voyager-DE™ STR Biospectrometry Workstation (ABI). Mass spectrometry data was deconvoluted to identify the substrate cleavage sites and confirmed by Edman sequencing.

Heparin binding. To assess the effect of MMP truncations of LIX on heparin binding, 0.5 mL of 4 µM chemokine in 10 mM potassium phosphate, pH 7.5 was loaded onto a 1 mL Hitrap™ heparin-Sepharose column (GE Healthcare). Bound LIX and synthetic analogues of MMP-cleaved LIX were eluted using a linear gradient of 0 to 1.0 M NaCl over 20 min at a flow rate of 1.0 mL/min and monitored by in-line absorbance at 215 nm.

Cells. Murine PMNs were isolated from bone marrow as previously described (46) except PMNs were recovered from a density gradient comprised of Histopaque 1077 layered on top of Histopaque 1119 according to manufacture's instructions (Sigma, St Louis, USA). The murine pre-B 300-19 cell line stably expressing human CXCR2 was supplied by Dr. B. Moser (Bern, Switzerland). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 1% glutamine and 5 x 10^{-5} M β-mercaptoethanol (Sigma) under puromycin (1.5 µg/mL) selection.

Measurement of intracellular calcium mobilization. Murine PMNs or CXCR2-transfected B300-19 cells (1 x 10^{7}/mL in RPMI-1640 media supplemented with 1% serum) were incubated with 2 µM Fluo-4-acetoxymethyl ester (Molecular Probes), for 30 min, 37 °C. Cells were washed to remove unincorporated agent and resuspended at 1 x 10^{6} cells/mL in Hanks Balanced Salt Solution (Gibco), 20 mM HEPES, 2.5 mM probenecid (Sigma). The cells were allowed to equilibrate at 37 °C for 5 min prior to addition of ligand as indicated. Calcium concentration was monitored by excitation at 485 nm and emission of 520 nm with a PerkinElmer

LS50B spectrophotometer. Calibration was performed by addition of 5 μM ionomycin (Sigma) and 1 mM MnCl₂ (Fisher Biotech).

In vitro chemotaxis assays. Murine PMNs or CXCR2-transfected murine pre-B cells were plated (1 x 10⁶/well in αMEM, 0.1% BSA) in transwell inserts (pre-coated with 10% fetal bovine serum) containing 3 μm or 8 μm pores respectively (Costar). Chemokines were added to the lower chamber and the plates incubated at 37 °C. PMN chemotaxis assays were performed for 1 h. The migrated cells were then fixed with 4% paraformaldehyde prior to counting. The murine pre-B cell transfectants were assayed for 4 h after which the migrated cells were counted.

Results

LPS induced PMN response in mice

To ascertain the role of MMP-8 in PMN cell migration and LPS responsiveness *in vivo* we compared *Mmp8 -/-* with *Mmp8 +/+* mice. The PMN influx was significantly reduced ($P \leq 0.005$) when LPS was injected in air pouches formed under the dorsal skin of the MMP-8 knockout mice compared with wild type mice (Figure 2.1A). This was observed in both male and female mice, although the PMN infiltrate was generally greater in the females towards both LPS and the PBS control. Hence, the reduced PMN migration and accumulation in *Mmp8 -/-* mice reveals a critical role for this PMN-specific protease in neutrophil function in acute inflammation. Notably, MMP-8 was only detected in cell lysates from LPS-treated air pouches of wild type mice, revealing both the pro and active forms of the enzyme at 85 and 65 kDa, respectively (Figure 2.1B). A 30-kDa inactive degradation product of MMP-8 was also detected, as observed previously (39).

MMP-8 processing of murine ELR⁺ CXC chemokines

We investigated the potential role of MMP-8 to modulate the activity of the cognate ELR⁺ ligands of CXCR2 that may underlie the defect in LPS-induced PMN migration. The four murine ELR⁺ CXC chemokines LIX, KC, MIP-2, and DCIP-1 were incubated
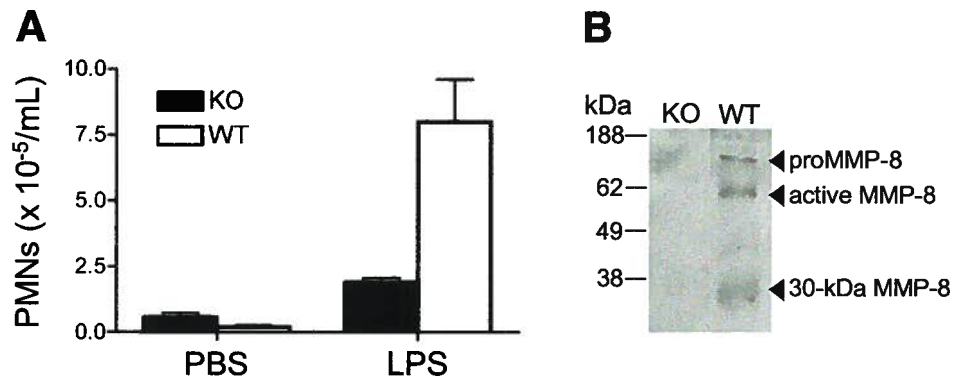
Figure 2.1. Impaired PMN responsiveness to LPS in MMP-8 deficient mice.
(A) Infiltration of PMNs *in vivo* in response 1 μg of LPS (n = 8) or phosphate buffered saline control (n = 4) injected into the air pouch of male *Mmp8 -/-* (black bar) and wild type mice (white bar) was assessed 8 h post-injection. The PMN influx was quantitated by myeloperoxidase activity. Error bars, standard error. (B) Western blot analysis of murine MMP-8 in LPS-treated air pouch PMN lysates corresponding to 50,000 cells per lane.

with recombinant MMP-8. Of these, LIX was the only chemokine susceptible to proteolytic processing (Figure 2.2A) — KC, MIP-2, and DCIP-1 were MMP-8-resistant even at high enzyme:substrate ratios (1:10) and after prolonged incubation times revealing protease substrate specificity. MALDI-TOF mass spectrometry analysis of the LIX cleavage products showed that MMP-8 processed the chemokine at two sites. The major product of 9,511 Da represents a deletion of the first four NH_2-terminal amino acid residues. Edman sequencing confirmed the deconvoluted MALDI-TOF data (Figure 2.2B). Hence, MMP-8 cleaves the 92-amino acid residue LIX between Ser-Val at position 4-5 to generate a new NH_2-terminus at Val^5 that we designate LIX (5-92). With a measured mass of 8,113 Da, the second cleavage product was processed near the COOH-terminus after Lys^{79}, resulting in the removal of 13 amino acid residues and generating the truncated form of LIX designated LIX (5-79) (Figure 2.2B). LIX (1-79) was never detected. Edman sequencing confirmed the deconvoluted mass spectrometry analysis of the Ser^4-Val^5 amino-terminal cleavage site of LIX (5-79).

As determined by MALDI-TOF mass spectrometry, the NH_2- and COOH-terminal processing of LIX was efficient; the cleavage products were first detected by mass spectrometry after 1 h with no full-length chemokine remaining at 18 h (Figure 2.2C). The COOH-terminal cleavage product was not detected in the absence of NH_2-terminal processing at any time point indicating it occurs subsequent to NH_2-terminal cleavage. Although the C-terminal cleavage was at an unusual highly cationic site ^{75}KKKAK-RNALA^{84} it was confirmed to be MMP dependent as two MMP inhibitors, EDTA and the synthetic hydroxamate small molecule chemical inhibitor BB94, blocked all LIX processing with no cleavage products detected by MALDI-TOF mass spectrometry or Tris-tricine SDS-PAGE (Figure 2.2D). Chemokine cleavage is often enhanced by MMP exosite interactions (40). For instance, the hemopexin C domain of MMP-2 greatly increases the catalytic rate constants of CCL2, -7, -8 and -13 cleavage (19). When molar excess of recombinant MMP-8 hemopexin C-domain was added in the cleavage assays, we likewise found that all LIX processing by MMP-8 was abrogated revealing that a binding site for LIX was present on the

A

DCIP-1 KC LIX MIP-2

MMP-8 + - + - + - + - +

7912 7912 7811 7811 9850 9511, 8113 7845 7845

B

| | m/z [M+H]$^+$ | | N-Terminal |
	Measured	Predicted	Sequence
LIX (1-92)	9,850	9,850	A-P-S-S-V-I
LIX (5-92)	9,511	9,507	V-I-A-A-T-E
LIX (5-79)	8,113	8,111	V-I-A-A-T-E
DCIP (1-72)	7,912	7,911	n.d.
KC (1-72)	7,811	7,812	A-P-I-A-N-E
MIP-2 (1-73)	7,845	7,847	n.d.

C

Time 0 .5 1 2 3 4 5 6 18 -

1-92 1-92 1-92, 5-92, 5-79 5-92, 5-79 1-92

D

MMP-8 CD EDTA BB94

MMP-8 - + + + +

1-92 5-92, 5-79 1-92 1-92 1-92

E
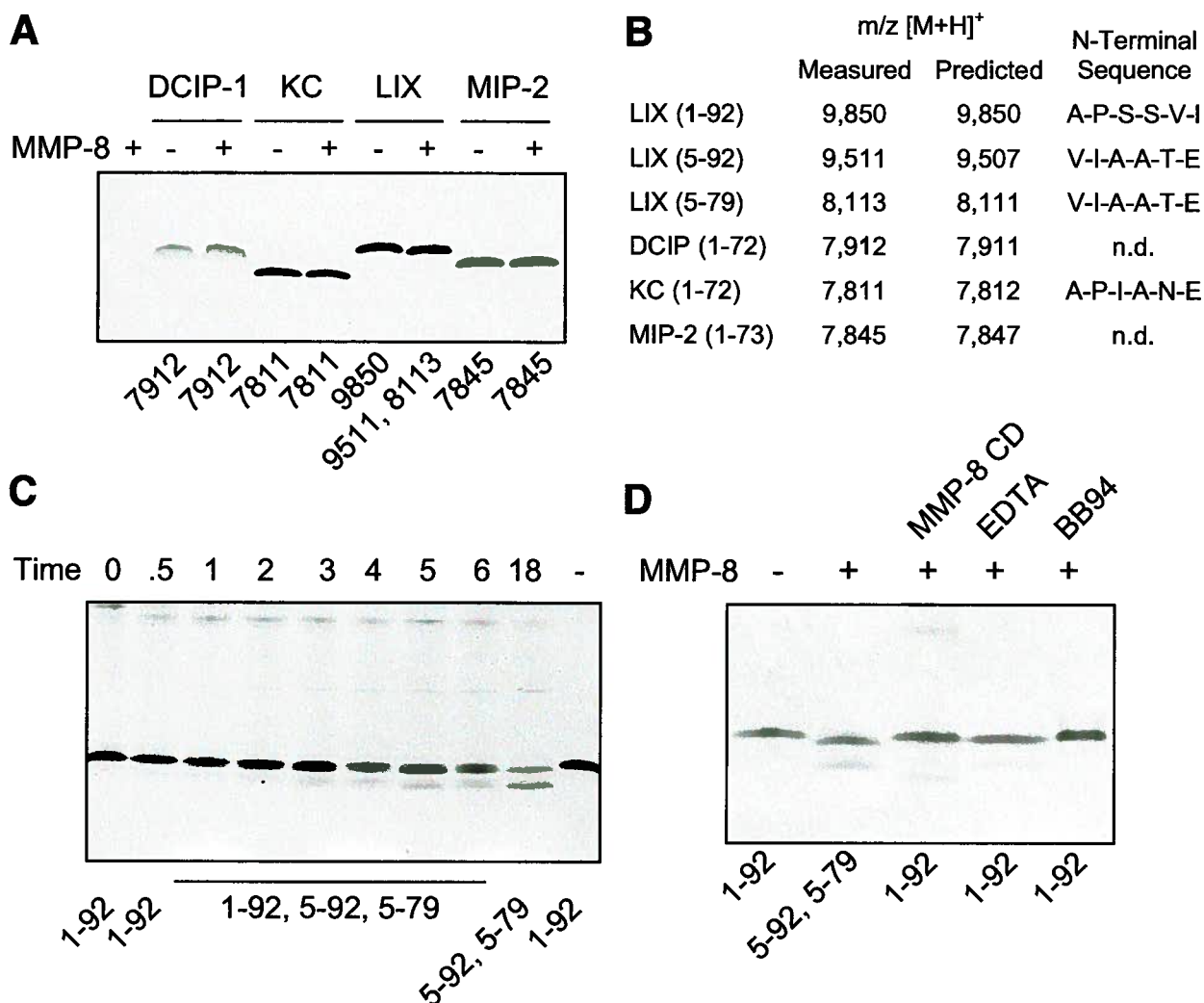
Figure 2.2. MMP-8 Cleavage of LIX. (A) Tris-tricine 15% SDS-PAGE gel analysis of MMP-8 cleavage of murine CXCR2-binding chemokines DCIP-1, KC, LIX and MIP-2. The m/z [M+H]$^+$ of reaction products are as shown. (B) Identification of cleavage products by MALDI-TOF mass spectrometry and NH$_2$-terminal Edman sequencing. n.d., not determined. (C) Tris-tricine 15% SDS-PAGE and MALDI-TOF analysis of LIX cleavage products generated over time (h) by MMP-8. (D) Tris-tricine 15% SDS-PAGE and MALDI-TOF analysis of LIX cleavage by MMP-8 in the presence of 10-fold molar excess of recombinant hemopexin C-domain (*MMP-8 CD*), 10 μM EDTA, or 10 μM BB94. (E) Heparin Sepharose AKTA chromatograms of synthetic analogues of MMP-8 cleavage products of LIX eluted with a linear gradient of NaCl as indicated. (Panels C&D contributed by Dr. Angus Tester)
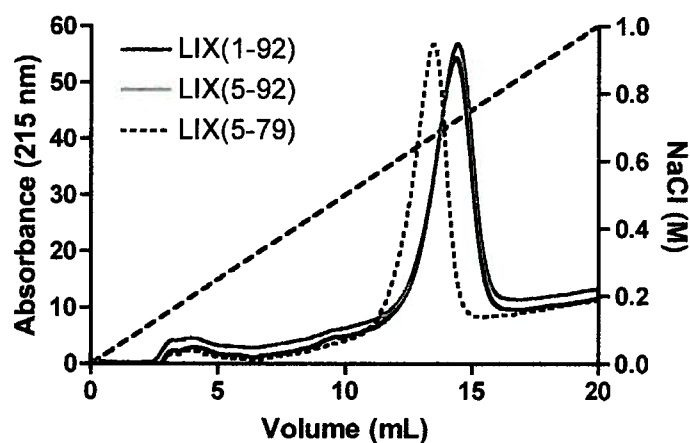
hemopexin C domain of MMP-8 (Figure 2.2D). The C-terminal cleavage at Lys^{79}-Arg^{80} only slightly reduced heparin affinity (Figure 2.2E) consistent with the removal of just two basic residues in the cleaved peptide $^{80}RNALAVERTASVQ^{92}$ while the N-terminal truncation had no effect on heparin binding.

Processing of LIX by other MMPs

Proteolytic screening of LIX cleavage by several MMPs showed that MMP-9, a prominent PMN MMP, processed LIX at Ser^4-Val^5 (Figure 2.3). In addition to MMP-9, other important stromal, endothelial and leukocytic MMPs 1, -2, and -13 could also process LIX at Ser^4-Val^5 (Figure 2.3), but like MMP-8, did not cleave KC, MIP-2, or DCIP-1 (not shown). Similar redundancy has been shown with MMPs for CXCL12 (also know as SDF-1) (25) and for CCL2, -7, -8 and -13 (19). Protease selectivity was also shown with MMP-14, the RNA of which is also expressed by PMNs (data not shown), but was incapable of processing LIX at any position (Figure 2.3A). Further, only MMP-8 could process the C-terminus of LIX at Lys^{79}-Arg^{80}.

Effect of LIX processing on in vitro biological activity

Upon binding to the receptor CXCR2, LIX mobilizes intracellular Ca^{++} ion stores. As measured in recombinant CXCR2-expressing murine pre-B 300-19 cells, synthetic analogues of the MMP-truncated forms of LIX (5-92) and LIX (5-79) both induced an ~2-fold greater intracellular Ca^{++} ion release compared to full-length LIX (1-92) (Figure 2.4A). This was confirmed using murine Mmp8 -/- PMNs where an even greater stimulation in Ca^{++} ion release was observed upon binding LIX (5-92) compared with full-length LIX (Figure 2.4B). Functionally, this translated into enhanced chemoattraction for the CXCR2-expressing pre-B 300-19 cells by both of the truncated forms of LIX versus the full-length chemokine (Figure 2.4C), and in purified PMNs from Mmp8 +/+ mice compared to the unprocessed LIX (1-92) (Figure 2.4D). Notably, PMNs isolated from Mmp8 -/- mice migrated towards LIX (5-92) in a comparable manner to the Mmp8 +/+ PMNs (Figure 2.4D and data not shown) indicating that the locomotor functions of PMNs isolated from both wild type and

45

A

MMP   -   8   8   2   9   13   13   14   1

LIX(1-92)
LIX(5-92)
LIX(5-79)

1-92
5-92, 5-79
5-92, 5-79
5-92
5-92
5-92
5-92
1-92
5-92

B

MMP-1, 2, 8, 9, 13

```
1          10            20
APSS↓VIAATELRCVCLTVTPKINPKLIAN

30        40           50
LEVIPAGPQCPTVEVIAKLKNQKEVCLDPE

60          70              80            90
APVIKKIIQKILGSDKKKAK↑RNALAVERTASVQ
                     MMP-8
```
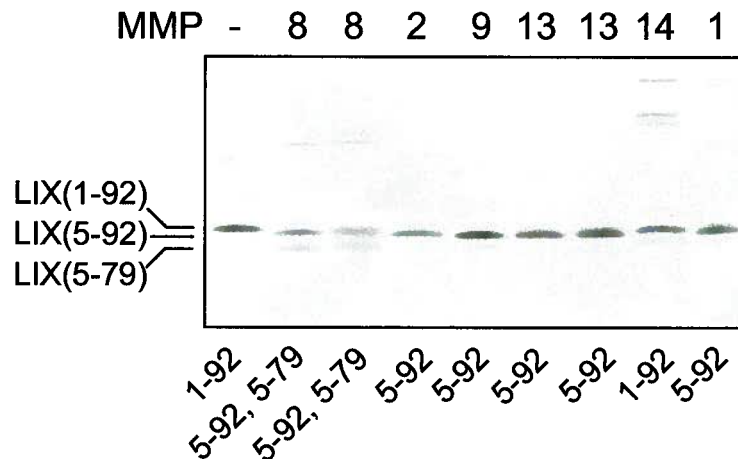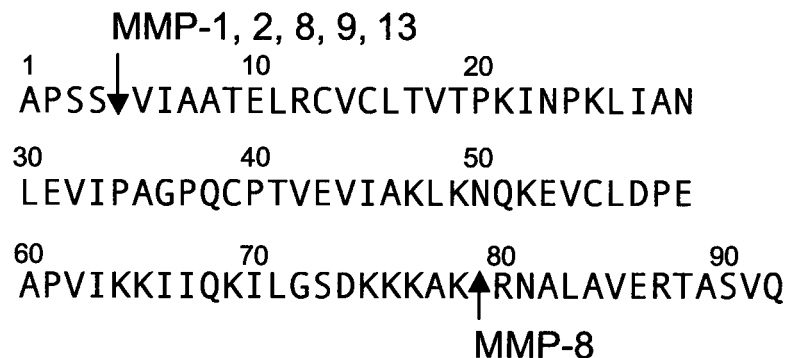
Figure 2.3. LIX is selectively cleaved by multiple MMPs. (A) Tris-tricine SDS-PAGE and MALDI-TOF mass spectrometry analysis of MMP processing of LIX (enzyme:substrate ratio of 1:100 (w:w)) showing that MMPs 1, 2, 8, 9 and 13 cleave LIX at position 4-5, whereas MMP-14 does not, and that only MMP-8 also cleaves at position 79~80. Cleavage assays of rodent MMP-8 and MMP-13 are shown in the second *8* and *13* lanes. (B) Cleavage data are summarized using the full-length sequence of LIX. Experiment was performed by Dr. Angus Tester.
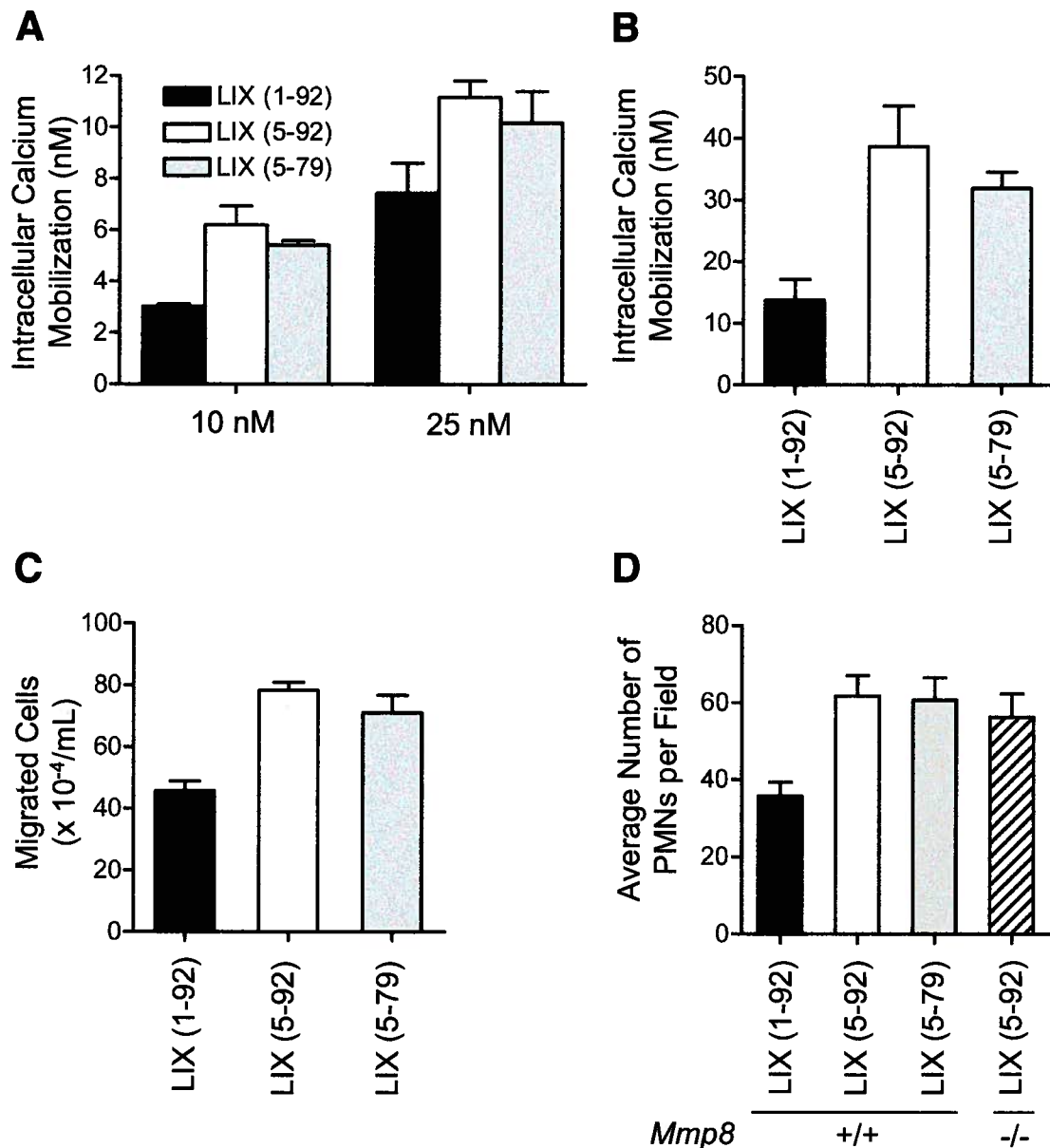
Figure 2.4. *In vitro* cellular responses to MMP-8 cleaved LIX. (A) Enhanced intracellular calcium mobilization was induced by LIX (5-92) and LIX (5-79) compared to full-length LIX (1-92) in recombinant CXCR2-expressing B300-19 cells and (B) PMNs isolated from *Mmp8* -/- mice (100 nM chemokine). (C) By transwell cell migration assay, both LIX (5-92) and LIX (5-79) truncated forms are more potent chemoattractants compared with the full-length LIX (1-92) for both CXCR2-expressing B300-19 cell transfectants and (D) murine PMNs isolated from either *Mmp8* +/+ or *Mmp8* -/- mice, all at 10 nM chemokine concentration. (Panels C&D contributed by Dr. Angus Tester)

MMP-8 knock out mice were equivalent *in vitro* and unaffected by the presence or absence of MMP-8.

In vivo PMN chemotaxis towards LIX

Infiltration of PMNs towards LIX (1-92) injected in a dorsal skin air pouch of *Mmp8*-/- mice was impaired at all time points compared to PMN infiltration in wild type mice, with an ~2-fold lower number of PMNs seen at 8 and 12 h in knock out compared with wild type mice (Figure 2.5). In contrast, when LIX (5-92) or LIX (5-79) were used as chemoattractants there was no significant difference in PMN infiltration into the air pouches of wild type and mice lacking MMP-8 (Figure 2.5). This indicates that MMP-8 activity is not essential for blood vessel extravasation and PMN cell migration *in vivo* and that there is little physiological redundancy by PMN MMP-9, or from tissue MMPs that we found competent in cleaving and activating LIX in the biochemical context *in vitro*.

MMP-8 processes and activates IL-8 and ENA-78

Our experimental data suggest that upon LPS-induced release of LIX and resultant PMN chemoattraction a feed-forward PMN activation mechanism operates *in vivo*. MMP-8 released from degranulating PMNs at the site of challenge fully activates LIX in the tissue to further enhance PMN migration towards the LPS stimulus. To ascertain whether a similar autologous CXCR2 ligand activation mechanism occurs in man we assessed every human CXCR2 ligand for MMP-8 cleavage. Of the seven ELR$^+$ CXC chemokines (CXCL8/IL-8, CXCL7/NAP-2, CXCL6/GCP-2, CXCL5/ENA-78, CXCL3/GROγ, CXCL2/GROβ and CXCL1/GROα) only CXCL8 and CXCL5 were processed by MMP-8. By MALDI-TOF MS and confirmation by Edman sequencing, CXCL8 was NH$_2$-terminally processed by MMP-8 at Arg^5-Ser^6 to generate CXCL8 (6-77) (Figure 2.6A, C) whereas CXCL5 was cleaved at Val^7-Leu^8 to generate CXCL5 (8-78) (Figure 2.6C). There were no COOH-terminal cleavages detected. Another difference from the murine system was that CXCL8 cleavage was not hemopexin C domain dependent — proteolysis was not inhibited in the presence of a molar excess of hemopexin C domain (Figure 2.6B).
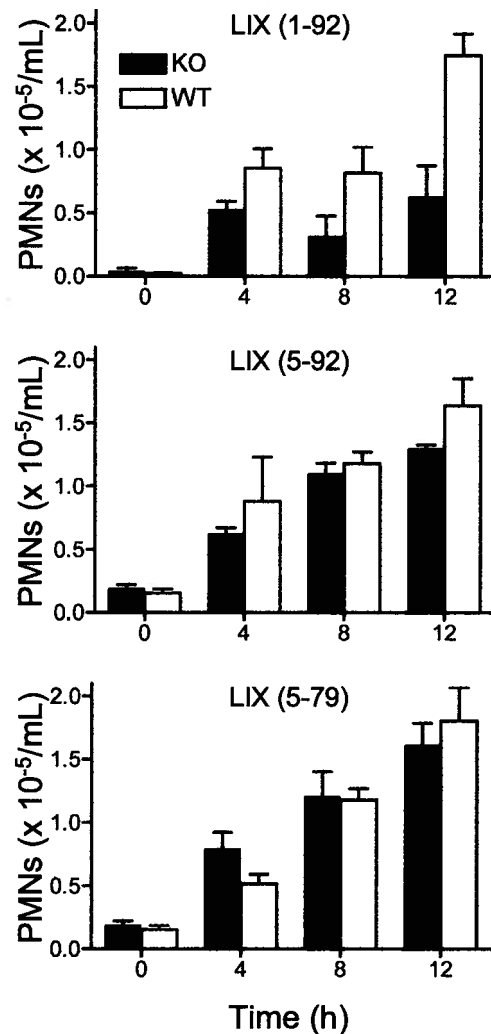
Figure 2.5. MMP-8 is required for PMN chemotaxis towards LIX *in vivo*, but is not required for PMN cell migration. PMN infiltration was greatly reduced in response to full-length LIX (1-92) injected into the dorsal skin air pouch of *Mmp8 -/-* mice (black bars) compared to wild type mice (white bars). PMN numbers were calculated from myeloperoxidase assay after sacrifice at 0, 4, 8 and 12 h following injection of chemokine (n = 4). Unaltered PMN cell migration into air pouches of *Mmp8 -/-* mice (black bars) compared to wild type mice (white bars) injected with MMP-cleaved analogues of LIX (5-92 or 5-79) reveals no intrinsic cell kinesis defects or chemotactic ability in the PMNs of the knock out mice. (Experiment performed by Dr. Angus Tester)

Figure 2.6. MMP processing of CXCL8 and CXCL5. (A) Tris-tricine 15% SDS-PAGE gel and MALDI-TOF mass spectrometry analysis of CXCL8 cleavage products after assay with the indicated human MMPs at an enzyme to substrate ratio of 1:100. Cleavage assays of rodent MMP-8 are shown in the second *8* lane. The NH$_2$-terminus of the CXCL8 truncated forms were deconvoluted from the mass spectrometry data and confirmed by Edman sequencing (as shown in C) with the CXCL8 forms identified shown below the corresponding gel lanes. *1-77*, full-length CXCL8. (B) The effect of recombinant MMP-8 hemopexin C domain (*MMP-8 CD*), 10 μM EDTA, or 10 μM BB94 on CXCL8 cleavage by human MMP-8 as determined by Tris-tricine 15% SDS-PAGE and MALDI-TOF mass spectrometry. The m/z [M+H]$^+$ of reaction products is as shown. (C) Identification of the NH$_2$-termini of MMP cleavage products of CXCL8 and CXCL5 by MALDI-TOF mass spectrometry and NH$_2$-terminal Edman sequencing. *n.d.*, not determined. (D) Location of the various MMP cleavage sites on the CXCL8 and CXCL5 sequences. (Experiment performed by Dr. Angus Tester)

A

MMP - 8 8 2 9 13 14 1

CXCL8

1-77
1-77, 6-77
1-77, 6-77
1-77
1-77, 7-77
1-77, 6-77
1-77, 6-77
1-77, 7-77

B

MMP-8 CD   BB94   EDTA

MMP-8 - + + + +

CXCL8

8919
8919, 8387
8919, 8387
8919
8919
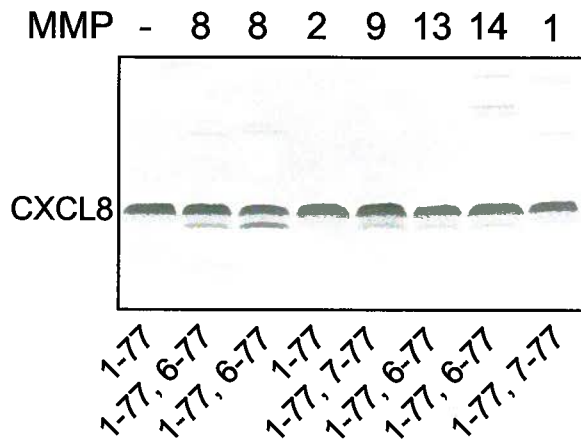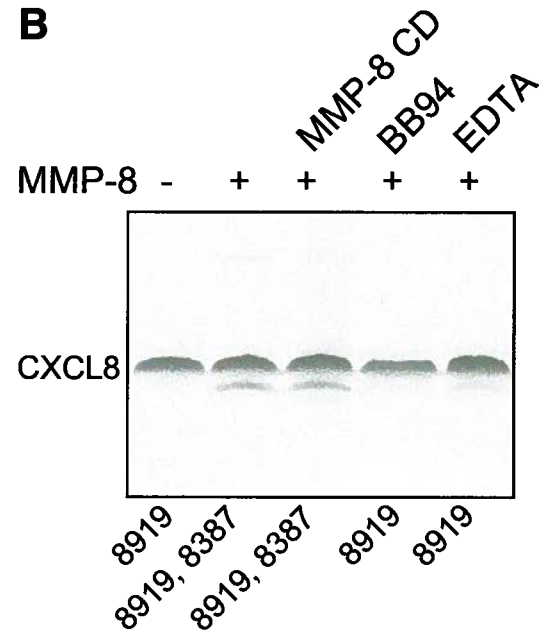
C

| | m/z [M+H]+ | | |
	Measured	Predicted	N-terminus
CXCL8 (1-77)	8,919	8,919	A-V-L-P-R-S
CXCL8 (6-77)	8,387	8,383	S-A-K-E-L-R
CXCL8 (7-77)	8,295	8,296	n.d.
CXCL5 (1-78)	8,402	8,399	n.d.
CXCL5 (8-78)	7,860	7,861	n.d.
CXCL5 (10-78)	7,589	7,592	n.d.

D

MMP-8, 13, 14, r8

1   10   20
CXCL8 AVLPR↓S↑AKELRCQCIKTYSK...

MMP-1, 9

MMP-8

1   10   20
CXCL5 AGPAAAV↓LR↑ELRCVCLQTTQ...

MMP-1, r8

51
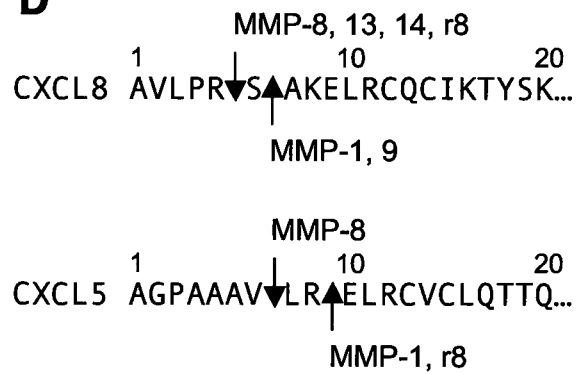
To determine protease specificity, other MMPs were screened for cleavage of CXCL5 and CXCL8. Only MMP-1 and MMP-8 cleaved CXCL5, with MMPs 1, 8, 9, 13 and 14 cleaving CXCL8. Some differences in cleavage site specificity were identified, but all cleavages were NH_2-terminal to the ELR motif (Figure 2.6D) and none cut in the C-terminal α-helix, as occurs for LIX. Consistent with previous studies (15, 26, 27) and our results with MMP-8 cleavage of LIX, MMP-8 processing of CXCL8 markedly activated the chemokine, with CXCL8 (6-77) leading to increased intracellular Ca^{++} mobilization (Figure 2.7A) and commensurate cell migration in transwells *in vitro* (Figure 2.7B). Despite several MMPs biochemically characterised to cleave and activate CXCL8, the critical importance of MMP-8 in CXCL8 activation was shown by injecting full-length CXCL8 in the air pouches of *Mmp8 -/-* mice (Figure 2.7C). Here, the early PMN migration was decreased by >50% at 4 h compared with wild type mice. At later time points, there was less of a difference, but still it was always depressed in the knock out mice. *Mmp8 -/-* PMN responsiveness and cell migratory behaviour *in vivo* was also shown to be unaffected by the absence of MMP-8 when challenged with the synthetic analogue of MMP-8-cleaved CXCL8 (6-77) (Figure 2.7C). This reconfirms the critical role of MMP-8 in directing chemotaxis by chemokine processing rather than cleavage of other molecules such as those in the blood vessel wall or extracellular matrix. Similar results were obtained using synthetic analogues of MMP-8-cleaved CXCL5 (8-78) compared with full-length CXCL5 (1-78) in the air pouch model (data not shown). In using human chemokines in a murine setting, it was important to show that rodent MMP-8 did cleave CXCL8 at the same site as human MMP-8 (Figure 2.6A, D). However, rodent MMP-8 cleaved human CXCL5 at Arg^9-Glu^{10} with no cleavage detected at Val^7-Leu^8 (Figure 2.6D). Hence, these *in vitro* and *in vivo* studies indicate that similar to PMN migration mechanisms towards LIX in mice, human PMN chemoattraction in response to CXCL8 and CXCL5 also exhibits a unique MMP-8 dependent feed-forward activation mechanism.
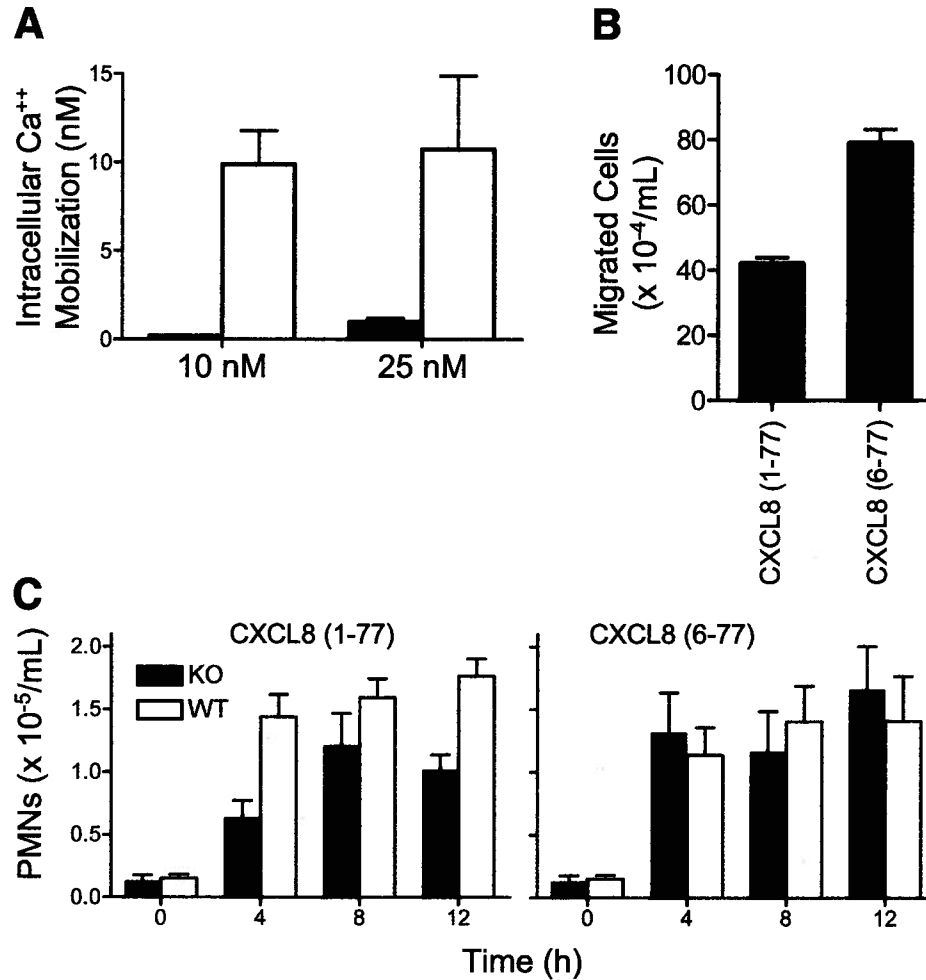
Figure 2.7. Enhanced bioactivity of MMP-truncated CXCL8 *in vitro* and *in vivo*. (A) Ca^{++} ion mobilization with 10 nM CXCL8 and (B) chemotaxis of CXCR2-transfected B300-19 cells stimulated with 10 nM full-length CXCL8 (1-77) and CXCL8 (6-77) synthetic analogue of MMP-8-cleaved CXCL8. (C) Time course of air pouch PMN influx in response to CXCL8 (1-77) and CXCL8 (6-77) in *Mmp8 -/-* mice (black) and wild type (white) mice as quantitated by myeloperoxidase assay. (Panels B & C contributed by Dr. Angus Tester)

Discussion

We have addressed the role of MMPs in the activation of ELR$^+$ CXC chemokines *in vivo*. Using *Mmp8* -/- mice, the essential role of PMN MMP-8 was shown in the activation pathway of the murine CXC chemokine LIX and human CXCL5 and CXCL8. Reflecting this, the absence of MMP-8 led to a profoundly defective PMN infiltration response *in vivo* to LPS or to full-length LIX, CXCL8, and CXCL5. This occurred despite the biochemical redundancy in chemokine activation by several commonly expressed MMPs including 1, 2, 9, 13, and 14 in a chemokine specific manner. PMN MMP-8 proteolysis leads to the activation of selected ELR$^+$ CXC chemokines responsible for directing PMN cell migration and activation *in vivo*. With MMP-8 being primarily expressed by PMNs, our study identifies MMP-8 as an essential mediator of an interesting and unique activation mechanism of PMNs in innate immunity. This highlights an unexpectedly important role of the PMN itself in the integration of stimuli for the appropriate release of MMP-8 for LIX, CXCL8, and CXCL5 activation and so reveals an autologous cellular activation mechanism that acts in a feed-forward manner to orchestrate the PMN influx and LPS responsiveness.

Precise control of innate immunity is key for the development of successful inflammatory responses, resolution of infection, tissue healing, and ultimately host survival. As first line defence cells, the rapid recruitment and efficient activation of PMNs is of critical importance. It has long been known that the ELR$^+$ CXCR2 cognate ligands that direct PMN chemotaxis and activation are expressed as relatively inactive ligands requiring proteolytic truncation of up to eight amino acid residues from the amino terminus for full activity, providing that the essential ELR motif remains intact (15). However, relatively little attention has been focused on identifying the responsible enzymes *in vivo* and the location of chemokine activation — key questions for understanding the control of PMN function and innate immunity mechanisms. Naturally occurring NH$_2$-terminal truncated forms of ELR$^+$ CXC chemokines including LIX (17, 41), CXCL8 (42), CXCL1, 3 and 5 (43), have been reported but the proteases involved have not been identified *in vivo*. Mice deficient in

MMP-9 (44), neutrophil elastase (45), both MMP-9 and neutrophil elastase (46), or cathepsin G (47) exhibit a normal PMN chemotactic response, indicating that PMNs neither require these enzymes for CXC chemokine activation, nor for migration and efficient chemotaxis *in vivo*. Hence, the deficient LPS and PMN migratory responses in the Mmp8 -/- mouse are unique and reveal the singular importance of MMP-8 in these processes. However, we do not discount the possibility that *in vivo* other proteases downstream of MMP-8 activity may also activate LIX.

Consistent with previous observations (15, 34, 41) was our demonstration through the use of in vitro calcium flux and chemotaxis assays that the MMP-8-processed chemokine products LIX (5-92), LIX (5-79), CXCL8 (6-77), and CXCL5 (8-78) were more potent than the full-length chemokines. Notably, LIX (5-92) and (5-79) were equally potent chemokines indicating that the C-terminal cleavage at position Lys^{79}-Arg^{80} did not influence chemotactic activity. Chemokines interact with the highly negatively charged glycosaminoglycan chains of proteoglycans resulting in immobilization and the generation of a haptotactic gradient within the extracellular matrix that is responsible for directing leukocyte migration (48). This has been confirmed physiologically for CC type chemokines (49) and murine ELR^+ CXC chemokines (50). Binding is typically through a heparin binding consensus sequence BBXB where B is a basic residue (51). The MMP-8 C-terminal cleavage of LIX only removes two basic amino acids, both arginines, in the truncated product and so only slightly reduces heparin affinity. The biological significance of this cleavage is therefore unknown.

None of the MMPs analysed could cleave any of the other murine ELR^+ chemokines (data not shown) revealing the specificity of MMP chemokine cleavage. This susceptibility of LIX to MMP processing is likely due to an extended N-terminus before the ELR motif, as compared with KC, MIP-2, and DCIP-1. Interestingly, rodent MMP-8 cleaved CXCL5 at Val^7-Leu^8 and not at the same site as human MMP-8, Arg^9-Glu^{10}. The active site of murine and rat MMP-8 differs from that of human MMP-8 by the presence of Lys^{187} at S_3' instead of Ala^{187} as found in human MMP-8. Charge repulsion between Lys^{187} in rodent MMP-8 at S_3' with the P_3' Arg^{12}

in CXCL5 may preclude CXCL5 cleavage at Arg^9-Glu^{10} and instead favour cleavage at Val^7-Leu^8. Here Glu^{10} of CXCL5 would be the P_3' residue and so likely forms a salt bridge with Lys^{187}. Although LIX shows structural homology to CXCL5 and CXCL6 (52), MMP-8 exclusively cleaved CXCL5 and CXCL8 amongst all seven human ELR^+ CXC chemokines. The activation of CXCL5 and CXCL8 were shown by *in vitro* calcium flux and chemotaxis assays, with the singular importance of MMP-8 in activating these human chemokines *in vivo* shown from studies comparing the *Mmp8 -/-* mice with the wild type controls.

Our results reveal that chemokine processing is one of the most important functions of MMP-8 *in vivo* and casts into doubt the importance of MMP-8 in collagen degradation, a role that has long been assumed to be of particular importance for PMN cell migration and chemotaxis. Indeed, using air pouch models, no difference in PMN cell migration and infiltration was observed in response to synthetic analogues of the MMP-8 cleaved LIX, CXCL8, and CXCL5. This shows that the PMN cell migration machinery to chemotactic agents does not require MMP-8 activity for responsiveness and that MMP-8 proteolysis of blood vessel basement membrane and interstitial extracellular matrix components is not essential for effective cell migration *in vivo*. Although MMPs have traditionally been thought to cleave extracellular matrix components and so disrupt extracellular matrix contacts with the tumour and potentiate tumour cell spread and metastasis, other biological roles for MMPs in cancer are now known (20, 53). Potentially related to this, sustained inflammatory responses maintain a microenvironment advantageous to tumour growth (54). Indeed, MMP-8 modulates the innate immune response induced by carcinogens leading to a protective role in preventing tumour progression (34). Mice lacking MMP-8 exhibited an abnormal inflammatory response upon application of carcinogen, with a delayed and more diffuse PMN influx to the site of the host challenge. Once established though, the inflammatory response was sustained and the mechanism for this is under investigation in our laboratory. With MMP-8 being the first MMP reported to have a protective role in tumorigenesis (34), the recognition of further MMP anti-targets in cancer continues (35). MMP-3 has a protective role in squamous cell carcinoma (55) and macrophage MMP-12 is an anti-

target in lung carcinoma (56). In these cases the proteases were reported to alter leukocyte infiltration, although the mechanism and substrates were not elucidated. In view of the considerable number of chemokines now known to be processed by MMPs, chemokines are strong candidate substrates to phenotypically explain cancer anti-target activity in these MMP genetic knockout mice.

The role of MMPs in LPS responsiveness and PMN migration differs from that found for macrophages and CXCR4-displaying leukocytes. Instead of promoting cell migration, MMP cleavage of CC chemokines CCL2, -7, -8 and -13 results in the loss of agonist activity and the generation of potent *in vitro* and *in vivo* CCR antagonists (18, 19). Interestingly, the MMP-2 cleavage and inactivation of CXCL12 (25) in the brain generates a potent and selective neurotoxin implicated in HIV dementia (57, 58). We also recently found that MMP-2 induces the shedding of the integral plasma membrane chemokine CX_3CL1 (fractalkine) by release of the chemokine domain from the stalk at Ala^{71}-Leu^{72} (23). Further, the cell surface agonist activity of CX3CL1 was converted to a soluble antagonist due to processing at Gly^4-Met^5. Hence, MMPs dynamically regulate the biological activity of chemokines and inflammatory and immune cell function in pleiotropic ways. Our present studies suggest that an *in cis* feed-forward activation mechanism occurs in which the PMN integrates the tissue signalling milieu leading to controlled release of MMP-8 that either directly or indirectly activates LIX in the mouse, and CXCL8 and CXCL5 in man for further PMN migration.

References

1. Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317-1327.

2. Park, J. E., and A. Barbul. 2004. Understanding the role of immune regulation in wound healing. *Am J Surg* 187:11S-16S.

3. Bodey, G. P., M. Buckley, Y. S. Sathe, and E. J. Freireich. 1966. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Ann Intern Med* 64:328-340.

4. Pizzo, P. A. 1993. Management of fever in patients with cancer and treatment-induced neutropenia. *N Engl J Med* 328:1323-1332.

5. Hughes, W. T., D. Armstrong, G. P. Bodey, E. J. Bow, A. E. Brown, T. Calandra, R. Feld, P. A. Pizzo, K. V. Rolston, J. L. Shenep, and L. S. Young. 2002. 2002 guidelines for the use of antimicrobial agents in neutropenic patients with cancer. *Clin Infect Dis* 34:730-751.

6. Madri, J. A., and D. Graesser. 2000. Cell migration in the immune system: the evolving inter-related roles of adhesion molecules and proteinases. *Dev Immunol* 7:103-116.

7. Lillard, J. W., Jr., P. N. Boyaka, O. Chertov, J. J. Oppenheim, and J. R. McGhee. 1999. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc Natl Acad Sci U S A* 96:651-656.

8. Foxman, E. F., J. J. Campbell, and E. C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol* 139:1349-1360.

9. Clark-Lewis, I., K. S. Kim, K. Rajarathnam, J. H. Gong, B. Dewald, B. Moser, M. Baggiolini, and B. D. Sykes. 1995. Structure-activity relationships of chemokines. *J Leukoc Biol* 57:703-711.

10. Utgaard, J. O., F. L. Jahnsen, A. Bakka, P. Brandtzaeg, and G. Haraldsen. 1998. Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells. *J Exp Med* 188:1751-1756.

11. Olszyna, D. P., E. De Jonge, P. E. Dekkers, S. J. van Deventer, and T. van der Poll. 2001. Induction of cell-associated chemokines after endotoxin administration to healthy humans. *Infect Immun* 69:2736-2738.

12. Arcaroli, J., J. Kupfner, H. K. Yum, R. Shenkar, J. S. Park, and E. Abraham. 2002. Lipopolysaccharide-induced neutrophil gene expression under in vivo or in vitro conditions. *Chest* 121:76S.

13. Malcolm, K. C., P. G. Arndt, E. J. Manos, D. A. Jones, and G. S. Worthen. 2003. Microarray analysis of lipopolysaccharide-treated human neutrophils. *Am J Physiol Lung Cell Mol Physiol* 284:L663-670.

14. Wuyts, A., P. Proost, J. P. Lenaerts, A. Ben-Baruch, J. Van Damme, and J. M. Wang. 1998. Differential usage of the CXC chemokine receptors 1 and 2 by interleukin-8, granulocyte chemotactic protein-2 and epithelial-cell-derived neutrophil attractant-78. *Eur J Biochem* 255:67-73.

15. Clark-Lewis, I., C. Schumacher, M. Baggiolini, and B. Moser. 1991. Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *J Biol Chem* 266:23128-23134.

16. Lee, J., G. Cacalano, T. Camerato, K. Toy, M. W. Moore, and W. I. Wood. 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J Immunol* 155:2158-2164.

17. Wuyts, A., A. Haelens, P. Proost, J. P. Lenaerts, R. Conings, G. Opdenakker, and J. Van Damme. 1996. Identification of mouse granulocyte chemotactic protein-2 from fibroblasts and epithelial cells. Functional comparison with natural KC and macrophage inflammatory protein-2. *J Immunol* 157:1736-1743.

18. McQuibban, G. A., J. H. Gong, E. M. Tam, C. A. McCulloch, I. Clark-Lewis, and C. M. Overall. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 289:1202-1206.

19. McQuibban, G. A., J. H. Gong, J. P. Wong, J. L. Wallace, I. Clark-Lewis, and C. M. Overall. 2002. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 100:1160-1167.

20. Egeblad, M., and Z. Werb. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2:161-174.

21. Overall, C. M. 2004. Dilating the degradome: matrix metalloproteinase 2 (MMP-2) cuts to the heart of the matter. *Biochem J* 383:e5-7.

22. Tam, E. M., C. J. Morrison, Y. I. Wu, M. S. Stack, and C. M. Overall. 2004. Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 101:6917-6922.

23. Dean, R. A., and C. M. Overall. 2007. Proteomic discovery of metalloproteinase substrates in the cellular context by iTRAQ0 labeling reveals a diverse MMP-2 substrate degradome. *Mol Cell Proteomics*.

24. Parks, W. C., C. L. Wilson, and Y. S. Lopez-Boado. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* 4:617-629.

25. McQuibban, G. A., G. S. Butler, J.-H. Gong, L. Bendall, C. Power, I. Clark-Lewis, and C. M. Overall. 2001. Matrix Metalloproteinase Activity Inactivates the CXC Chemokine Stromal Cell-derived Factor-1. *J. Biol. Chem.* 276:43503-43508.

26. Van den Steen, P. E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.

27. Van Den Steen, P. E., A. Wuyts, S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* 270:3739-3749.

28. Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, and J. L. Gordon. 1994. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* 370:555-557.

29. Wilson, C. L., A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. Lopez-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks. 1999. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286:113-117.

30. Butler, G. S., D. Sim, E. Tam, D. Devine, and C. M. Overall. 2002. Mannose-binding lectin (MBL) mutants are susceptible to matrix metalloproteinase proteolysis: potential role in human MBL deficiency. *J Biol Chem* 277:17511-17519.

31. Coussens, L. M., and Z. Werb. 2002. Inflammation and cancer. *Nature* 420:860-867.

32. Acuff, H. B., K. J. Carter, B. Fingleton, D. L. Gorden, and L. M. Matrisian. 2006. Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment. *Cancer Res* 66:259-266.

33. Weiss, S. J., G. Peppin, X. Ortiz, C. Ragsdale, and S. T. Test. 1985. Oxidative autoactivation of latent collagenase by human neutrophils. *Science* 227:747-749.

34. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35:252-257.

35. Overall, C. M., and O. Kleifeld. 2006. Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 6:227-239.

36.   Hasty, K. A., J. J. Jeffrey, M. S. Hibbs, and H. G. Welgus. 1987. The collagen substrate specificity of human neutrophil collagenase. *J Biol Chem* 262:10048-10052.

37.   Pelman, G. R., C. J. Morrison, and C. M. Overall. 2005. Pivotal molecular determinants of peptidic and collagen triple helicase activities reside in the S3' subsite of matrix metalloproteinase 8 (MMP-8): the role of hydrogen bonding potential of ASN188 and TYR189 and the connecting cis bond. *J Biol Chem* 280:2370-2377.

38.   Van Lint, P., B. Wielockx, L. Puimege, A. Noel, C. Lopez-Otin, and C. Libert. 2005. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol* 175:7642-7649.

39.   Owen, C. A., Z. Hu, C. Lopez-Otin, and S. D. Shapiro. 2004. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol* 172:7791-7803.

40.   Overall, C. M., G. A. McQuibban, and I. Clark-Lewis. 2002. Discovery of Chemokine Substrates for Matrix Metalloproteinases by Exosite Scanning: A New Tool for Degradomics. *Biol Chem* 383:105-1066.

41.   Wuyts, A., A. D'Haese, V. Cremers, P. Menten, J. P. Lenaerts, A. De Loof, H. Heremans, P. Proost, and J. Van Damme. 1999. NH2- and COOH-terminal truncations of murine granulocyte chemotactic protein-2 augment the in vitro and in vivo neutrophil chemotactic potency. *J Immunol* 163:6155-6163.

42.   Van Damme, J., M. Rampart, R. Conings, B. Decock, N. Van Osselaer, J. Willems, and A. Billiau. 1990. The neutrophil-activating proteins interleukin 8 and beta-thromboglobulin: in vitro and in vivo comparison of NH2-terminally processed forms. *Eur J Immunol* 20:2113-2118.

43.   Wuyts, A., C. Govaerts, S. Struyf, J. P. Lenaerts, W. Put, R. Conings, P. Proost, and J. Van Damme. 1999. Isolation of the CXC chemokines ENA-78, GRO alpha and GRO gamma from tumor cells and leukocytes reveals NH2-terminal heterogeneity. Functional comparison of different natural isoforms. *Eur J Biochem* 260:421-429.

44.   Betsuyaku, T., J. M. Shipley, Z. Liu, and R. M. Senior. 1999. Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B. *Am J Respir Cell Mol Biol* 20:1303-1309.

45.   Belaaouaj, A., R. McCarthy, M. Baumann, Z. Gao, T. J. Ley, S. N. Abraham, and S. D. Shapiro. 1998. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med* 4:615-618.

46.   Allport, J. R., Y. C. Lim, J. M. Shipley, R. M. Senior, S. D. Shapiro, N. Matsuyoshi, D. Vestweber, and F. W. Luscinskas. 2002. Neutrophils from MMP-9- or neutrophil elastase-deficient mice show no defect in transendothelial migration under flow in vitro. *J Leukoc Biol* 71:821-828.

47.   MacIvor, D. M., S. D. Shapiro, C. T. Pham, A. Belaaouaj, S. N. Abraham, and T. J. Ley. 1999. Normal neutrophil function in cathepsin G-deficient mice. *Blood* 94:4282-4293.

48.   Rot, A. 1993. Neutrophil attractant/activation protein-1 (interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism. *Eur J Immunol* 23:303-306.

49.   Proudfoot, A. E., T. M. Handel, Z. Johnson, E. K. Lau, P. LiWang, I. Clark-Lewis, F. Borlat, T. N. Wells, and M. H. Kosco-Vilbois. 2003. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* 100:1885-1890.

50.   Li, Q., P. W. Park, C. L. Wilson, and W. C. Parks. 2002. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 111:635-646.

51.   Perrimon, N., and M. Bernfield. 2000. Specificities of heparan sulphate proteoglycans in developmental processes. *Nature* 404:725-728.

52. Smith, J. B., L. E. Rovai, and H. R. Herschman. 1997. Sequence similarities of a subgroup of CXC chemokines related to murine LIX: implications for the interpretation of evolutionary relationships among chemokines. *J Leukoc Biol* 62:598-603.

53. Coussens, L. M., and Z. Werb. 1996. Matrix metalloproteinases and the development of cancer. *Chem Biol* 3:895-904.

54. Dvorak, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315:1650-1659.

55. McCawley, L. J., H. C. Crawford, L. E. King, Jr., J. Mudgett, and L. M. Matrisian. 2004. A protective role for matrix metalloproteinase-3 in squamous cell carcinoma. *Cancer Res* 64:6965-6972.

56. Houghton, A. M., J. L. Grisolano, M. L. Baumann, D. K. Kobayashi, R. D. Hautamaki, L. C. Nehring, L. A. Cornelius, and S. D. Shapiro. 2006. Macrophage elastase (matrix metalloproteinase-12) suppresses growth of lung metastases. *Cancer Res* 66:6149-6155.

57. Zhang, K., G. S. McQuibban, C. Silva, G. S. Butler, J. B. Johnston, I. Clark-Lewis, J. Holden, C. M. Overall, and C. Power. 2003. Metalloproteinase Cleavage of the Chemokine SDF-1α Induces Neuronal Apoptosis in HIV Encephalitis. *Nature Neuroscience* 6:1064-1071.

58. Vergote, D., G. S. Butler, M. Ooms, J. H. Cox, C. Silva, M. D. Hollenberg, J. H. Jhamandas, C. M. Overall, and C. Power. 2006. Proteolytic processing of SDF-1alpha reveals a change in receptor specificity mediating HIV-associated neurodegeneration. *Proc Natl Acad Sci U S A* 103:19182-19187.

59. Edwards, J. C., A. D. Sedgwick, and D. A. Willoughby. 1981. The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. *J Pathol* 134:147-156.

60. Tateda, K., T. A. Moore, M. W. Newstead, W. C. Tsai, X. Zeng, J. C. Deng, G. Chen, R. Reddy, K. Yamaguchi, and T. J. Standiford. 2001. Chemokine-dependent neutrophil recruitment in a murine model of Legionella pneumonia: potential role of neutrophils as immunoregulatory cells. *Infect Immun* 69:2017-2024.

61. Clark-Lewis, I., L. Vo, P. Owen, and J. Anderson. 1997. Chemical synthesis, purification, and folding of C-X-C and C-C chemokines. *Methods Enzymol* 287:233-250.

CHAPTER 3. MATRIX METALLOPROTEINASE PROCESSING OF CXCL11/I-TAC RESULTS IN LOSS OF CHEMOATTRACTANT ACTIVITY AND ALTERED GLYCOSAMINOGLYCAN BINDING[3]

Perspective

The CXCR3 chemokine receptor regulates the migration of Th1 lymphocytes and responds to three ligands: CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC. We screened for potential regulation of T cell responses by matrix metalloproteinase (MMP) processing of these important chemokines. The most potent of the CXCR3 ligands, CXCL11, was identified by MALDI-TOF MS as a substrate of the PMN-specific MMP-8, macrophage-specific MMP-12, and the general leukocyte MMP-9. The 73-amino acid residue CXCL11 is processed at both the amino and carboxy-termini to generate CXCL11 (5-73), (5-63), and (5-58) forms. N-terminal truncation results in loss of agonistic properties, as shown in calcium mobilization and chemotaxis experiments using CXCR3-transfectants and human T lymphocytes. Moreover, CXCL11 (5-73) is a CXCR3 antagonist and interestingly shows enhanced affinity to heparin. However, upon C-terminal truncation to position 58 there is loss of antagonist activity and heparin binding. Together this highlights an unexpected site for receptor interaction and that the carboxy terminus is critical for glycosaminoglycan binding, an essential function for the formation of chemokine gradients *in vivo*. Hence, MMP activity might regulate CXCL11 tissue gradients in two ways. First, the potential of CXCL11 (5-73) to compete active CXCL11 from glycosaminoglycans might lead to the formation of an antagonistic haptotactic chemokine gradient. Second, upon further truncation, MMPs disperse the CXCL11 gradients by proteolytic loss of a C-terminal GAG binding site. Hence, these results reveal potential new roles in downregulating Th1 lymphocyte chemoattraction through MMP processing of CXCL11.

Introduction

Chemokines are a super-family of low molecular weight chemotactic cytokines that function in directing the migration of leukocytes and other cell types in a multitude of processes including development, lymphocyte homing, inflammation and wound repair (1). Chemokines form haptotactic gradients *in vivo* through associations with proteoglycan glycosaminoglycans (2). Upon interaction with 7-transmembrane G protein-coupled receptors, chemokines induce a chemotactic response. The expression and secretion of inducible chemokines is stimulated during infection or injury to promote rapid and efficient inflammatory and immune responses. Conversely, dampening of inflammation, a critical event in allowing tissue repair to continue unimpeded and in preventing excessive tissue damage and autoimmunity, is known to involve coordinated down-regulation of chemokine expression (3), receptor internalization (4), scavenger receptors (5), and proteolytic mechanisms of inactivation and conversion to antagonists (6).

Specific and limited proteolysis, termed processing (7), of chemokines results in altered bioactivity with functional consequences such as increased or decreased receptor binding (8), conversion of an agonist to an antagonist (6, 9), shedding of membrane-anchored chemokines (10, 11), and changing receptor specificity (12). Cleavage of proteoglycan core protein also disrupts chemokine gradients formed by chemokine binding to the glycosaminoglycan side chains (13). A variety of proteases are thought to be involved in chemokine cleavage including CD13, also known as aminopeptidase N (14), CD26, also known as dipeptidylpeptidase-IV (15, 16), dipeptidylpeptidase-8 (16), neutrophil elastase (17), cathepsin G (18), cathepsins B and D (19), and the matrix metalloproteinases (MMPs) [Reviewed in (20-22)]. Several chemokines are important physiological substrates of MMPs, a family of proteases once thought to be only associated with extracellular matrix turnover (23), including the monocyte chemoattractant proteins CCL2, 7, 8, and 13 (6, 9), the ELR$^+$ chemokines CXCL5/ENA-78, CXCL8/IL-8 and murine LIX/mCXCL5 (24-27), CXCL9 and CXCL10 (28), CXCL12/SDF-1α and β (8, 29), and CX$_3$CL1/Fractalkine (10).

MMPs are zinc-dependent extracellular endopeptidases, with most having a carboxy-terminal hemopexin domain containing exosites for substrate binding (30). MMPs are upregulated in many pathologies including rheumatoid arthritis (31), multiple sclerosis (32, 33), and tumorigenesis (7, 34), all of which have a chronic inflammatory component. The expression of numerous MMPs by immune cells is commonly thought to promote cellular migration through extracellular matrix degradation and hence removal of physical barriers (23). However, this concept is outmoded following T lymphocyte migration studies in 3D extracellular matrices in the presence of potent protease inhibitors (35), MMP-8 knockout mice, where deficiency in this neutrophil collagenase did not reduce PMN cell migration in response to activated chemokines (25), and recognition of a multitude of bioactive substrates of MMPs through genetic (23) and proteomic analyses (10, 36, 37).

Interestingly, MMPs are produced by cells of both the innate and adaptive arms of immunity, suggesting pleiotropic roles coordinating rapid host defense and more specific or specialized acquired immunity. Examples include the neutrophil-specific MMP-8, the macrophage-specific MMP-12 (also known as metalloelastase), and MMP-9 (also known as gelatinase B), which is expressed by a variety of leukocytes. Interstitial collagenase (MMP-1) and MMP-7 (also known as matrilysin), an endothelial/epithelial-derived protease, are also expressed by macrophages (20). CD4$^+$ T helper lymphocytes (Th1) are involved in managing humoral and cell-mediated responses through cytokine release and subsequent activation of effector cells. Notably, the gelatinases MMP-2 (also known as gelatinase A) and MMP-9 are preferentially expressed by the Th1 subset and CD4$^+$ migration is reduced in the presence of MMP inhibitors (38). Furthermore, membrane type-1 MMP (MMP-14) is the major physiological activator of MMP-2 (39) and is expressed by monocytes and dendritic cells (40, 41).

The CXCR3 chemokine receptor is preferentially expressed on Th1 cells and has three known ligands: CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC. These chemokines are induced by the pro-inflammatory cytokine interferon-gamma in

several cell types including neutrophils, monocytes, macrophages, T cells, astrocytes, fibroblasts, and endothelial cells (1). The CXCR3 receptor and its cognate chemokines have been implicated in inflammatory disorders such as multiple sclerosis, rheumatoid arthritis, and allograft rejection, as they are upregulated in these pathological states, presumably causing enhanced infiltration of CXCR3-expressing Th1 cells (42-44). As such, precise regulation of the CXCR3 ligands is key in preventing excessive Th1 recruitment and the resulting pathology. Previous reports have shown that CXCL10 is cleaved at the carboxy-terminus by furin in the cellular context (45), and that CXCL9 and CXCL10 can be truncated at the carboxy-terminus by MMP-8 and MMP-9 (28), however no functional changes were found for any of these cleavages. Like many chemokines, CXCL11 is cleaved at the amino-terminus after proline-2 by dipeptidylpeptidase IV (46, 47) and aminopeptidase N (14), resulting in loss of agonism, but the propensity of MMPs in processing this chemokine are unknown. Here, we have biochemically evaluated CXCL9, CXCL10, and CXCL11 as leukocytic-MMP substrates and report that CXCL11 is processed by several MMPs resulting in altered receptor binding and glycosaminoglycan affinity. In particular, CXCL11 is first converted to a CXCR3 antagonist by N-terminal processing at position 4-5, but this is lost upon C-terminal truncation revealing an unexpected site for receptor binding. Therefore, with proteolytic loss of a C-terminal glycosaminoglycan-binding site this might result in dispersal of chemotactic gradients *in vivo* and so contribute to regulation of Th1 cell recruitment and cell accumulation.

Materials and Methods

Chemokine cleavage assays. All chemokines and analogues of MMP-cleaved chemokines were synthesized using tBoc (tertiary butyloxycarbonyl) solid phase chemistry as described previously (48). Recombinant human MMP-1, 2, 8, 9, 12, and soluble MMP-14 (lacking the transmembrane domain) were expressed and purified using standard techniques (49, 50). Human MMP-7 was purchased from

USB Corporation. Enzyme activity was followed by quenched fluorescence synthetic peptide cleavage assay with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (51).

Chemokine cleavage assays were performed in 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.5 with initial screening at an enzyme to substrate ratio of 1:10 (w:w) for 16 h at 37 °C, then assessed at lower enzyme concentrations to establish kinetic parameters, biological efficacy and relevance. Unlike the other MMPs assayed, MMP-12 showed significant loss of activity through autodegradation in 16 h incubations, therefore kinetic analysis was performed after 2 h. Inhibition experiments were done in the presence of 10 μM EDTA, 10 μM Batimastat, or 10-fold molar excess MMP-8 hemopexin C domain. Assay results were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix and confirmed by 15% Tris-Tricine SDS-polyacrylamide gel electrophoresis (PAGE).

Hemopexin C domain expression and binding. MMP-8 linker and hemopexin C domain with an NH₂-terminal His-tag was cloned into the pGYMX vector and expressed in *E. coli* BL21 gold cells. Cell lysates were solubilized and protein purified by Ni^{2+}-chelate chromatography followed by Superdex™ 75 gel filtration chromatography (GE Healthcare) yielding a pure 26-kDa product, confirmed to be MMP-8 linker and hemopexin C domain by western blotting with antibody against the MMP-8 C domain (Chemicon). Chemokines were immobilized in 96-well plates (Falcon) at 500 ng/well in phosphate buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) overnight at 4 °C. Wells were blocked for 1 h with PBS containing 2.5% bovine serum albumin (BSA). Binding of recombinant N-terminal His-tagged MMP-8 hemopexin C domain to immobilized chemokines was performed for 1 h in PBS at room temperature and was assessed by direct ELISA with alkaline phosphatase detection (Sigma). Washes were with PBS containing 0.05% Tween-20.

Transfected and isolated cells. Human CXCR3-transfected B300-19 cells (52) were kindly provided by B. Moser (Bern, Switzerland) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μM β-mercaptoethanol, and 1.0 mg/mL G-418. Human T cells were isolated from healthy volunteers as approved by the University of British Columbia Clinical Ethics Review Board (Appendix C). Peripheral blood was drawn into EDTA-treated vaccutainers and layered on Ficoll-Paque Plus (Amersham Biosciences), as per manufacturers protocol. Monocytes were removed by adhesion to flask surfaces following 3 h incubation at 37 °C, 5% CO_2. Isolated lymphocytes were cultured in RPMI-1640, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were activated by the addition of 5 μg/mL phytohemagglutinin-P (Sigma) for three days followed by six to twelve days in the presence of 100 U/mL Interleukin-2 (Peprotech). Media was changed every three days. Expression of CXCR3 was confirmed by flow cytometry (Beckman Coulter EPICS XL-MCL) using mouse anti-hCXCR3 (R&D Systems, clone 49801).

Calcium mobilization. CXCR3-transfected B300-19 cells were resuspended at 1 x 10^7 cells/mL in RPMI-1640 media supplemented with 1% FBS and incubated with 2 μM Fluo-4-acetoxymethyl ester (Molecular Probes) for 30 min at 37 °C. Cells were washed to remove unincorporated agent and resuspended at 1 x 10^6 cells/mL in Hanks Balanced Salt Solution (without calcium or magnesium, Sigma), 20 mM HEPES, 2.5 mM probenecid (Sigma). 3.5 x 10^5 cells were plated per well in 96-well clear bottom fluorescent plates (Nunc) and centrifuged at 1,000 x g for 5 min without braking. Cells were allowed to equilibrate at 37 °C for 15 min prior to addition of ligand. Calcium concentration was monitored by excitation of 485 nm and emission of 520 nm with a Molecular Devices Flexstation II as described previously (53). Calibration was performed by addition of 5 μM ionomycin (Sigma) followed by 1 mM $MnCl_2$ (Fisher Biotech) to determine F_{max} and F_{min}, resepctively. Absolute Ca^{2+} concentrations were calculated as K_d x $[(F-F_{min})/(F_{max}-F)]$ (54) where the K_d of Fluo-4 was 345 nM, as reported by Molecular Probes. Antagonist experiments were performed by preincubating cells in the presence of CXCL11 (5-73) or CXCL11 (5-

58), synthetic analogues of MMP-processed CXCL11 at position 4-5 (CXCL11 (5-73)) and 58-59 (CXCL11 (5-58)), for 120 s prior to the addition of 3 nM full-length CXCL11, designated CXCL11 (1-73).

CXCR3 Internalization. Internalization of CXCR3 on CXCR3-transfected B300-19 cells was measured following the incubation of 5 x 10^5 cells with 10 nM and 100 nM full-length or truncated CXCL11 for 30 min at 37 °C. Cells were washed three times with ice cold PBS containing 0.5% BSA, incubated for 45 min at 4 °C with 2 µg/mL mouse anti-CXCR3 (R&D Systems, Clone 49801) in PBS, 0.5% BSA. Cells were washed three times then incubated for 45 min at 4 °C with 1:200 diluted sheep anti-mouse IgG-FITC (Sigma) followed by 3 final washes and resuspension in 0.5 mL PBS. Staining was analyzed by flow cytometry (Beckman Coulter EPICS XL-MCL) and internalization was calculated from the mean fluorescence intensity values relative to untreated cells.

Chemotaxis. Chemotactic migration of CXCR3-transfectants and activated T lymphocytes was performed in a 48-well Boyden chamber across a 5 µm pore size polycarbonate membrane (Neuroprobe). Cells and chemokine were diluted in RPMI containing 20 mM HEPES and 1% BSA. To the upper chamber, 2.5 x 10^5 cells were added. The chamber was incubated for 3 h at 37 °C, 5% CO_2, then the upper chamber was aspirated and washed twice with distilled water. Inhibition experiments were performed as above except the lower chamber contained 10 nM CXCL11 (1-73) in combination with up to 10 µM of the truncated CXCL11 forms. Following incubation, the contents of the lower chamber were transferred to a MaxiSorp 96-well plate (Nunc) and frozen at -80 °C for at least 2 hours. Cell content was determined by CyQUANT analysis, a fluorescent nucleic acid-based cellular quantitation assay, by comparison with a standard curve, according to manufacturers protocol (Molecular Probes). Chemotactic index was calculated as a ratio of cells migrating in response to chemokine compared to the buffer control.

Circular dichroism. CD spectroscopy measurements were made in the far-ultraviolet range from 190-260 nm using a JASCO J-810 spectropolarimeter. Full-length and truncated CXCL11 forms, and full-length CXCL8, were resuspended at 10 µM in nanopure water and measured in a quartz cuvette of 0.1 cm path length at 25 °C. Data are reported as the average of 4 scans with a 1 nm band width. When possible, secondary structure composition was predicted with the SOMCD algorithm (55).

Glycosaminoglycan binding. CXCL11 binding to fluorescein-heparin (Molecular Probes) was determined using fluorescence polarization analysis on the Polarstar Optima 96-well fluorimeter (BMG) essentially as described previously for other binding proteins (56). Incubations contained 0.1 µM heparin-FITC and chemokines at concentrations of 0 to 2 µM, performed in 100 mM Tris, 150 mM NaCl, pH 7.4 for 1 h at 37 °C. To further assess the effect of MMP truncations of CXCL11 on heparin binding, 0.5 mL of 4 µM chemokine in 10 mM potassium phosphate, pH 7.5 was loaded onto 1 mL Hitrap™ heparin-Sepharose and cation exchange Sepharose columns (GE Healthcare) as described previously (57). Bound CXCL11 and synthetic analogues of MMP-cleaved CXCL11 were eluted using a linear gradient of 0 to 1.5 M NaCl over 30 min at a flow rate of 1.0 mL/min and monitored by in-line absorbance at 215 nm on an AKTA Purifier (Amersham Pharmacia Biotech). The effect of soluble glycosaminoglycans on CXCL11 cleavage by MMP-8 was assessed in 16 h reactions at 37 °C containing a chemokine to GAG ratio of 1:5 (w:w) and an enzyme to substrate ration of 1:10 (w:w). Heparan sulfate and chondroitin sulfate A, B, and C were obtained from Seikagaku and Healon hyaluronate was from Pharmacia.

Results

Selective processing of CXCL9 and CXCL10 by MMPs.

To screen for potential regulation of CXCR3 ligands by MMPs, recombinant MMP-1, 2, 7, 8, 9, 12, and 14 were incubated *in vitro* with CXCL9 and CXCL10 at an enzyme-substrate ratio of 1:10 (w:w). Of these MMPs, MMP-7 and 12 had the broadest activity, either cleaving or degrading both chemokines (Figure 3.1A, B). In the case of CXCL9, MMP-7 proteolysis generated a single product with a m/z of 10136 Da ([M+H]$^+$), determined by MALDI-TOF mass spectrometry. Upon deconvolution, this corresponds to a truncation of CXCL9 at position 90-91 with the cleavage site [88]VLK-VRK[93] (Figure 3.1D). The same C-terminal truncation of CXCL9 was observed with MMP-12 incubation, but this cleavage was not as efficient as with MMP-7, as some CXCL9 remained intact.

CXCL10 was partially processed by MMP-12, resulting in truncated forms of 8150 and 7931 Da ([M+H]$^+$), corresponding to CXCL10 (1-73) and CXCL10 (1-71), respectively (Figure 3.1B). The cleavage sites in CXCL10 were [69]SKE-MSK[74] and [71]EMS-KRS[76]. Although MMP-7 did not generate stable cleavage products of CXCL10, reproducibly reduced chemokine band intensity indicates that degradation occurred due to MMP-7 activity. Interestingly, proteolysis of CXCL9 and CXCL10 was exclusively in the carboxy-terminal region, as determined by MALDI-TOF mass spectrometry (Figure 3.1D). The cleavage products CXCL9 (1-90), CXCL10 (1-71), and CXCL10 (1-73) have been identified previously with MMP-8 and MMP-9 (28) and the latter was found to retain full activity when compared to full-length CXCL10 (45). Hence, the present results expand the repertoire of MMPs that process these CXCR3 chemokines. However, under our assay conditions, and even with high enzyme-substrate ratios, we did not detect cleavage of CXCL9 and CXCL10 by MMP-8 or MMP-9 as previously reported (28).

Figure 3.1. MMP processing of CXCR3 ligands. Recombinant MMP-1, 2, 7, 8, 9, 12, and 14 were incubated with CXCL9 (A), CXCL10 (B), and CXCL11 (C) at a 1:10 ratio (w:w) for 16 h at 37 °C and proteolysis was visualized on 15% Tris-tricine gels, silver-stained. (D) MALDI-TOF mass spectrometry and deconvolution enabled the assignment of cleavage fragments with mass to charge ratios indicated (m/z) with +1 charge ionization ([M+H]$^+$). Chemokine m/z values are corrected for disulfide bond formation (-4) and +1 charge ionization. Lanes with * show substrate degradation as observed by loss of band intensity. Note that CXCL11 incubation with MMP-8 resulted in complete conversion represented by slight shift in mass of band.

A

MMP	-	1	2	7	8	9	12	14	
									CXCL9

11721   10136   11721   11721,10136   11721   [M+H]⁺

B

MMP	-	1	2	7	8	9	12	14	
									CXCL10

8617 *   8617,8150,7931   8617   [M+H]⁺

C

MMP	-	1	2	7	8	9	12	14	
									CXCL11

8303   6451   7779,6524,5928   8303,6451,5928   7779,6451,5928 *   8303   [M+H]⁺

D
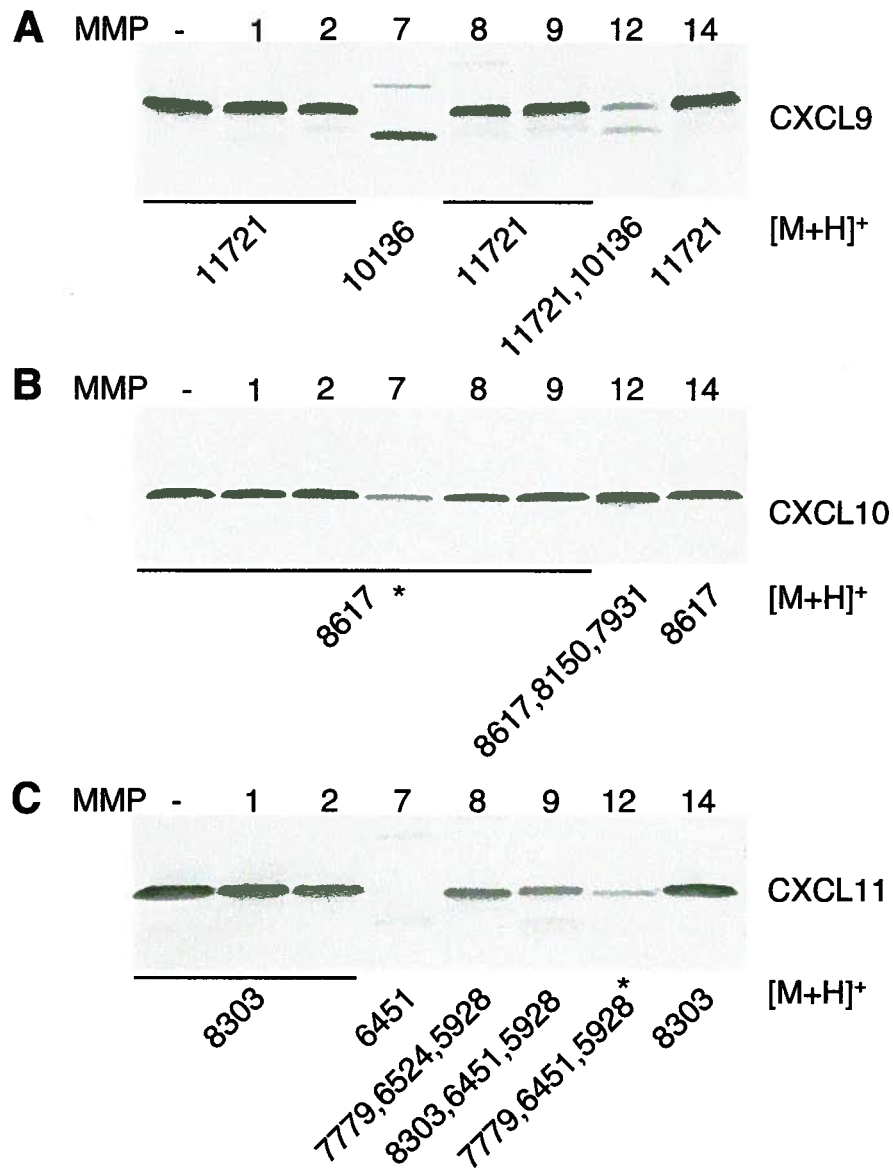
	m/z [M+H]⁺	
Chemokine	Measured	Predicted
CXCL9 (1-103)	11721	11722
CXCL9 (1-90)	10136	10137
CXCL10 (1-77)	8617	8618
CXCL10 (1-73)	8150	8150
CXCL10 (1-71)	7931	7931
CXCL11 (1-73)	8303	8304
CXCL11 (5-73)	7779	7781
CXCL11 (5-63)	6524	6525
CXCL11 (1-58)	6451	6451
CXCL11 (5-58)	5928	5928

Selective processing of CXCL11 by MMPs

Of the CXCR3-binding chemokines, CXCL11 was the most susceptible to MMP cleavage, being significantly processed by MMP-7, 8, 9, and 12 (Figure 3.1C). Although MMP-7 appeared to almost entirely degrade CXCL11, one peptide ion peak at 6451 Da ([M+H]$^+$) was detected by MALDI-TOF MS following cleavage at ^{56}PKS-KQA^{61} representing a transient cleavage product, CXCL11 (1-58) (Figure 3.1D). MMP-12 also degraded CXCL11, but three transient truncations were found generating peptides with m/z of 7779, 6451, and 5928 Da ([M+H]$^+$) corresponding to CXCL11 (5-73), (1-58), and (5-58) with the N-terminal cleavage at ^{2}PMF-KRG^7. The neutrophil-specific protease MMP-8 specifically cleaved CXCL11 generating stable 7779, 6524, and 5928 Da ([M+H]$^+$) truncations corresponding to CXCL11 (5-73), (5-63), and (5-58), respectively. MMP-9 processed the chemokine, generating two cleavage products of 6451 and 5928 Da ([M+H]$^+$), which represent CXCL11 (1-58) and (5-58). Hence, it is evident that several MMPs cleave CXCL11 at residues 4-5 and 58-59, while MMP-8 is unique in its cleavage of the 63-64 bond at ^{61}ARL-IIK^{66} (Figure 3.3).

Processing of mCXCL11

Murine CXCL11, which is 68% identical to the human form but lacks a proline at position 2, was also examined as a MMP substrate. MMP-1, 7, 8, and 12 cleaved the chemokine (Figure 3.2A). Several products were detected by MALDI-TOF mass spectrometry and upon deconvolution were determined to represent N-terminal cleavage at ^{1}FLM-FKQ^6, and C-terminal cleavages at ^{61}ARL-IMQ^{66} and ^{71}KNF-LRR^{76} (Figure 3.2B). MMP-8 cleaved mCXCL11 at all three sites, while MMP-7 and MMP-1 cleaved exclusively at the N-terminus and C-terminus, respectively (Figure 3.3). Notably, no stable truncation products were detected with MMP-12, as substrate degradation was evident.

A

MMP   -   1   2   7   8   9   12   14

mCXCL11

9112

9112, 8312, 7108

9112

9112, 8719

9112, 8719, 8312,
7921, 7108, 6717

9112

*

9112 $[M+H]^+$

B
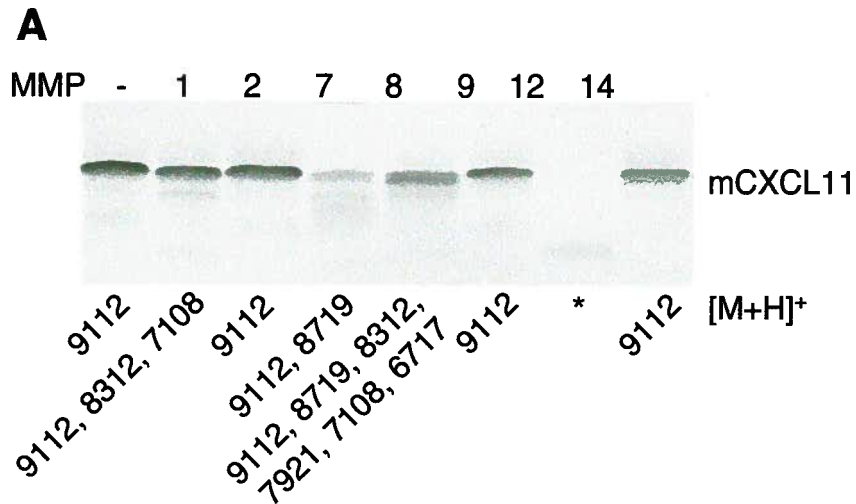
Chemokine	m/z $[M+H]^+$	
	Measured	Predicted
mCXCL11 (1-79)	9112	9110
mCXCL11 (4-79)	8719	8718
mCXCL11 (1-73)	8312	8311
mCXCL11 (4-73)	7921	7920
mCXCL11 (1-63)	7108	7108
mCXCL11 (4-63)	6717	6716

Figure 3.2. MMP processing of murine CXCL11. (A) Recombinant MMP-1, 2, 7, 8, 9, 12, and 14 were incubated with murine CXCL11 for 16 h at a 1:10 ratio (w:w) at 37 °C, visualized on 15% Tris-tricine gels, silver-stained. (B) Cleavage products were assigned by MALDI-TOF mass spectrometry by comparing measured and predicted mass to charge ratios (m/z) with +1 charge ionization ($[M+H]^+$). Lane with * shows substrate degradation as observed by loss of band intensity.

CXCL9

1   10   80   90   100
TPVVRKGRCS...KQKNGKKHQKKKVLK▲VRKSQRSRQKKTT

MMP-7, 12

CXCL10

1   10   60   70
VPLSRTVRCT...CLNPESKAIKNLLKAVSKE▲MS▲KRSP

MMP-12

CXCL11

MMP-8 ,9, 12

1   10   60   70
FPMF▼KRGRCL...GQRCLNPKS▲KQARL▲IIKKVERKNF

MMP-7, 8, 9, 12   MMP-8

mCXCL11

MMP-7, 8

1   10   60   70
FLM▼FKQGRCL...DPRSKQARL▲IMQAIEKKNF▲LRRQNM

MMP-1, 8   MMP-1, 8

Figure 3.3. MMP cleavage sites in CXCL9, CXCL10, CXCL11, and murine CXCL11. Carboxy-terminal processing of CXCL9 by MMP-7 and MMP-12 at Lys^{90}-Val^{91} yields CXCL9 (1-90). CXCL10 cleavage by MMP-12 generates C-terminal truncations CXCL10 (1-71) and (1-73) at Glu^{71}-Met^{72} and Ser^{73}-Lys^{74}. MMP-8, 9, and 12 cleave CXCL11 at two sites to generate CXCL11 (5-73) and (5-58) whereas MMP-7 only cleaves at Ser^{58}-Lys^{59} to give CXCL11 (1-58). MMP-8 alone cleaves CXCL11 at Leu^{63}-Ile^{64}. Murine CXCL11 is cleaved by MMP-7 and 8 at Met^{3}-Phe^{4} and by MMP-1 and 8 at Leu^{63}-Ile^{64} and Phe^{73}-Leu^{74}.

Kinetic analysis of CXCL11 processing

We first screened for cleavage at high enzyme-substrate ratios. Since cleavage alone does not indicate a biologically relevant substrate (7), we assessed the enzyme kinetics of MMP cleavage of CXCL11 (Figure 3.4). Based on densitometry analysis, where $k_{cat}/K_M = (\ln 2)/(E)(t_{1/2})$, the k_{cat}/K_M of CXCL11 processing was calculated to be in the range from 96-190 $M^{-1}s^{-1}$ for MMP-7, 8, 9, and 12 (Figure 3.4E). Notably, MMP-7 partially cleaved CXCL11 at the lowest enzyme concentration tested, 16 nM, which corresponds to an enzyme-substrate ratio of 1:800 (mol:mol) (Figure 3.4A). However, degradation is apparent at concentrations of 62.5 nM MMP-7 and greater, as was also observed for MMP-12, at concentrations of 1000 nM (Figure 3.4D). In control incubations MMP activity against fluorogenic substrate was maintained at 16 h except for MMP-12, which exhibited loss of activity due to autodegradation. Hence, MMP-12 was analyzed at higher concentrations for only 2 hours due to instability observed in longer incubations. Both MMP-8 and MMP-9, which are expressed and secreted by neutrophils, efficiently and precisely cleaved CXCL11 at enzyme concentrations of 125 nM and greater, resulting in both amino and carboxy-terminal truncations (Figure 3.4B, C; See also Figure 3.8A), so indicating the potential for Th1 lymphocyte regulation by neutrophil-derived proteases.

Interaction of the MMP-8 hemopexin C domain with CXCL11

It is well established that MMPs contain exosites, which are substrate-binding sites outside of the catalytic domain important in dictating substrate specificity and efficiency in cleavage (30, 58). In particular, the hemopexin C domains of several MMPs interact with chemokines to promote proteolysis (6, 25, 58). In the case of MMP-8 processing of CXCL11, the addition of 10-fold molar excess MMP-8 hemopexin C domain inhibited the formation of truncation products indicating that CXCL11 cleavage is hemopexin C domain-dependent (Figure 3.5A). As positive controls, the hydroxamate MMP inhibitor Batimastat (BB94) and the general metalloprotease inhibitor EDTA also blocked proteolysis. Interestingly MMP-7 and -12 can process several chemokines yet MMP-7 does not have a hemopexin C

A

MMP-7 (nM)   0   16   32   62.5 125 250 500

CXCL11→
(1-73)
                 ←CXCL11 (1-62)
                 ←CXCL11 (1-58)

B

MMP-8 (nM)   0   16   32   62.5 125 250 500

CXCL11→
(1-73)
                 ←CXCL11 (5-73)
                 ←CXCL11 (5-63)
                 ←CXCL11 (5-58)

C

MMP-9 (nM)   0   16   32   62.5 125 250 500

CXCL11→
(1-73)
                 ←CXCL11 (5-73)
                 ←CXCL11 (5-58)

D

MMP-12 (nM) 0   125 250 500 1000 2000 4000

CXCL11→
(1-73)
                 ←CXCL11 (5-73)
                 ←CXCL11 (5-58)

E

Enzyme	k_{cat}/K_M $(M^{-1}s^{-1})$
MMP-7	1.9×10^2
MMP-8	1.3×10^2
MMP-9	9.6×10^1
MMP-12	9.6×10^1
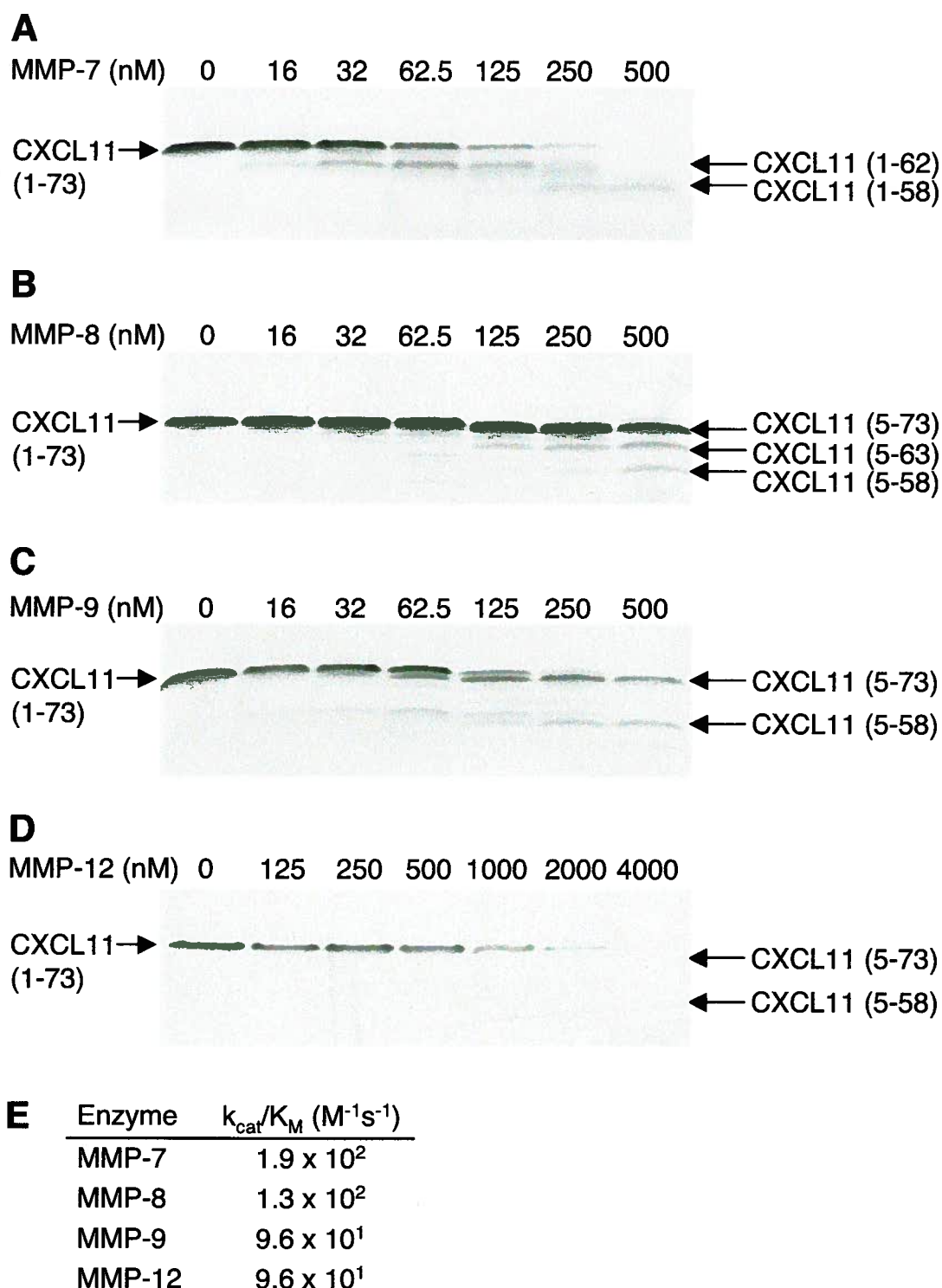
Figure 3.4. Titration of MMP processing of CXCL11. Recombinant MMP-7 (A), MMP-8 (B), MMP-9 (C) were incubated with 10 μM CXCL11 at concentrations ranging from 16 - 500 nM for 16 h at 37 °C. (D) Due to decreased enzyme stability, 125 - 4000 nM MMP-12 was incubated with 10 μM CXCL11 for 2 h at 37 °C. Cleavage products were visualized on 15% Tris-tricine gels and were assigned by MALDI-TOF mass spectrometry. (E) Specificity constant (k_{cat}/K_M) values were determined by densitometry from gels.

domain and MMP-12 autolytically removes this during synthesis. Interestingly the hemopexin C domain of MMP-12 has significant antibacterial actions (59), which also likely contributes to the beneficial activities of MMP-12 in innate immunity.

To investigate the molecular basis for the selective interaction of MMP-8 with CXCL11 compared to CXCL9 and CXCL10, where there was no cleavage, the affinity of the CXCR3 ligands for the MMP-8 hemopexin C domain was assessed. ELISA experiments demonstrated that CXCL11 has a significantly higher affinity for MMP-8 hemopexin C domain compared with CXCL9 and CXCL10, which only bound at or below background controls (Figure 3.5B). The CXCL11 MMP-truncated forms CXCL11 (5-73) and CXCL11 (5-58) bound with similar affinity to the MMP-8 hemopexin C domain as full-length CXCL11 (data not shown), suggesting that the interaction occurs with the central region of CXCL11, distant to the cleavage sites.

Calcium mobilization of CXCR3-transfected B300-19 cells

Synthetic analogues of MMP-truncated CXCL11 were tested for activity with human CXCR3-transfected B300-19 cells. At a concentration of 800 nM chemokine, there was a clear loss of activity in the MMP-truncated forms of CXCL11, where calcium mobilization is measured as a burst in relative fluorescence (Figure 3.6A). Full-length CXCL11 (1-73) caused a dose-dependent increase in intracellular calcium mobilization with an EC_{50} of 1 nM, consistent with previous reports (60) (Figure 3.6B). In contrast, the truncated forms of CXCL11 had no detectable agonist activity at any concentration tested. Since MMP-cleaved CCL2, 7, 8, and 13 (9) and CX_3CL1 (10) are receptor antagonists, we assessed the CXCL11 cleaved analogues as receptor antagonists by calcium mobilization. A two-minute pre-incubation, shown previously to be sufficient time for receptor interaction (61, 62), with CXCL11 (5-73) resulted in significant inhibition of full-length CXCL11 with a moderate IC_{50} of 500 nM (Figure 3.6C), as observed previously (61). However, the CXCL11 (5-58) truncation had reduced inhibition of calcium mobilization, suggesting that the C-terminus is involved in binding the CXCR3 receptor. Neither CXCL11 (5-73) nor CXCL11 (5-58) caused CXCR3 receptor internalization as measured by flow
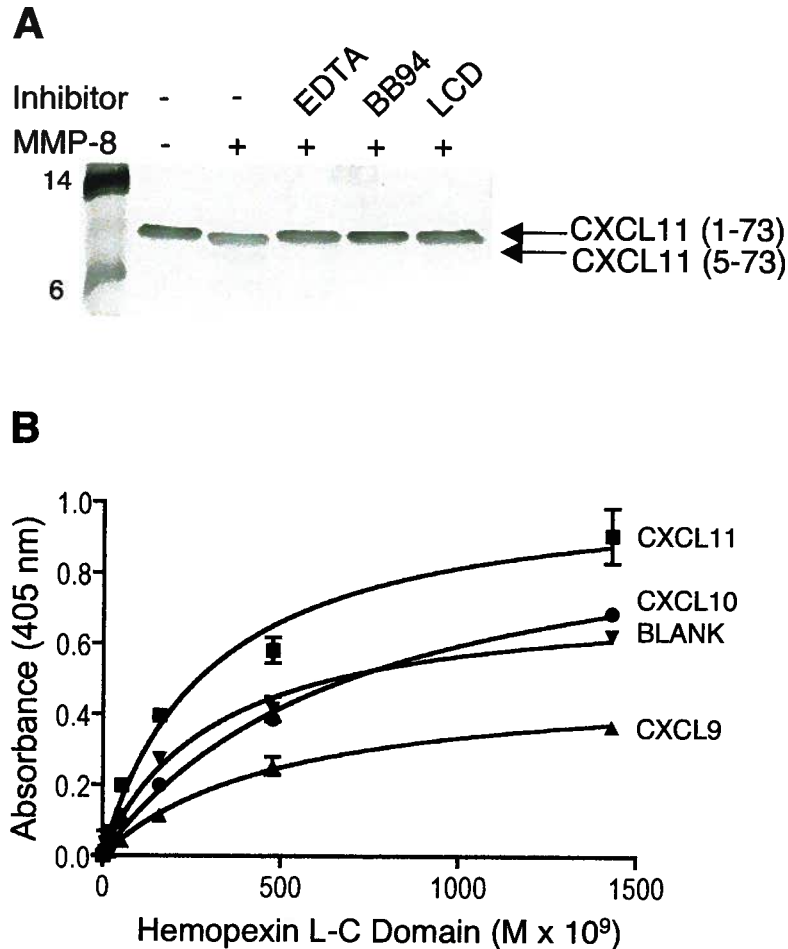
Figure 3.5. Exosite interactions with CXCL11. (A) MMP-8 cleavage of CXCL11 (16 h at 37 °C at 1:10 enzyme to substrate ratio (w:w)) was inhibited in the presence of EDTA, Batimastat (BB94), and 10-fold molar excess of the MMP-8 linker and hemopexin C domain (LCD). (B) ELISA analysis of MMP-8 hemopexin linker-C (L-C) domain binding to the CXCR3 chemokines, immobilized in 96-well plate format. The order of hemopexin C domain binding affinity was CXCL11>CXCL10>CXCL9.
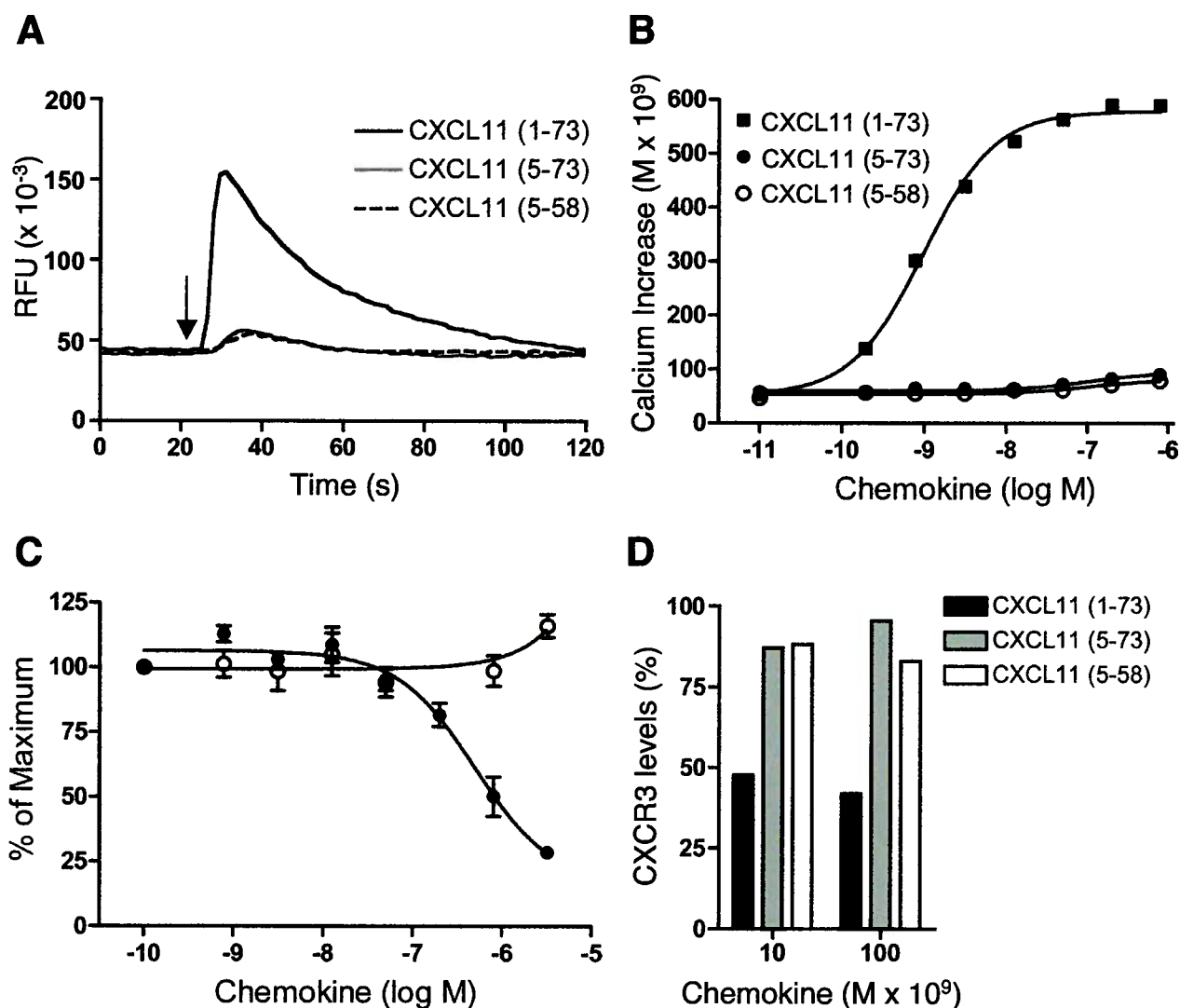
Figure 3.6. MMP-mediated cleavages of CXCL11 result in loss of agonism and the generation of antagonist activity in calcium mobilization assays. (A) Representative trace of calcium flux, represented by a burst in relative fluorescence units (RFU) following addition of 800 nM CXCL11 in CXCR3-transfected B300-19 cells loaded with Fluo-4 calcium indicator reagent. Arrow indicates time of CXCL11 addition. (B) Dose response of calcium mobilization in CXCR3-transfected B300-19 cells in response to CXCL11 (1-73), (5-73), and (5-58). Calcium concentrations were calculated from relative fluorescence based on calibration with ionomycin and $MnCl_2$. (C) Antagonism of calcium mobilization in CXCR3-transfected B300-19 cells by CXCL11 (5-73) and (5-58). Cells were preincubated with antagonists for two minutes prior to the addition 3 nM full-length CXCL11. Activity values are reported as a percentage relative to that of uninhibited control cells. (D) CXCR3 internalization of CXCR3-transfected B300-19 cells following 30 minute incubation of 10 and 100 nM CXCL11 at 37 °C. Detection of cell surface levels of CXCR3 was performed by flow cytometry with anti-CXCR3 monoclonal antibody and mean fluorescence intensities were compared to untreated controls and reported as percentage CXCR3 levels.

cytometry (Figure 3.6D), confirming the loss of agonistic activity, whereas 100 nM CXCL11 (1-73) caused approximately 60% internalization, consistent with previous reports (63).

Chemotactic migration of CXCR3-transfected B300-19 cells and human T lymphocytes

To confirm the calcium mobilization results, CXCR3-transfected cells and activated T lymphocytes were evaluated for chemotaxis toward full-length and truncated forms of CXCL11.   In both cell types CXCL11 (1-73) promoted dose-dependent chemotactic migration as expected (Figure 3.7A, C). However, both CXCL11 (5-73) and CXCL11 (5-58) had minimal activity, even at concentrations as high as 100 nM. Again, the N-terminal truncated CXCL11 (5-73) had antagonistic activity with IC_{50} values of 100 nM and 1000 nM for CXCR3-transfected B300-19 cells and T lymphocytes, respectively (Figure 3.7B, D).   Also, the CXCL11 (5-58) truncated chemokine was only a very weak antagonist, with an IC_{50} of greater that 10 μM in both cell types. However, it is important to note that this represents a concentration that is biologically unattainable to achieve antagonism.

Structural analysis of truncated CXCL11

MMP processing of CXCL11 results in the removal of 4 N-terminal amino acids and either 10 or 15 C-terminal amino acids (Figure 3.8A). The carboxy terminal α-helix removed by MMP cleavage is markedly cationic, supporting a role in GAG interactions, as shown in this structural representation based on the published NMR structure (Protein Databank ID 1RJT) (64). Notably, none of the MMP truncations affect the cysteine residues involved in disulfide formation, hence tertiary protein structure is not expected to be adversely affected. Circular dichroism comparing full length and truncated CXCL11 suggests that the structural integrity remains intact (Figure 3.8B). Interestingly, the overall CD profile of CXCL11 resembles that of CC chemokines with a high percentage of random structure, as indicated by strong negativity at 200 nm (65). CXCL8 was analyzed for comparison with another CXC chemokine and this spectrum of a highly ordered protein correlates with published
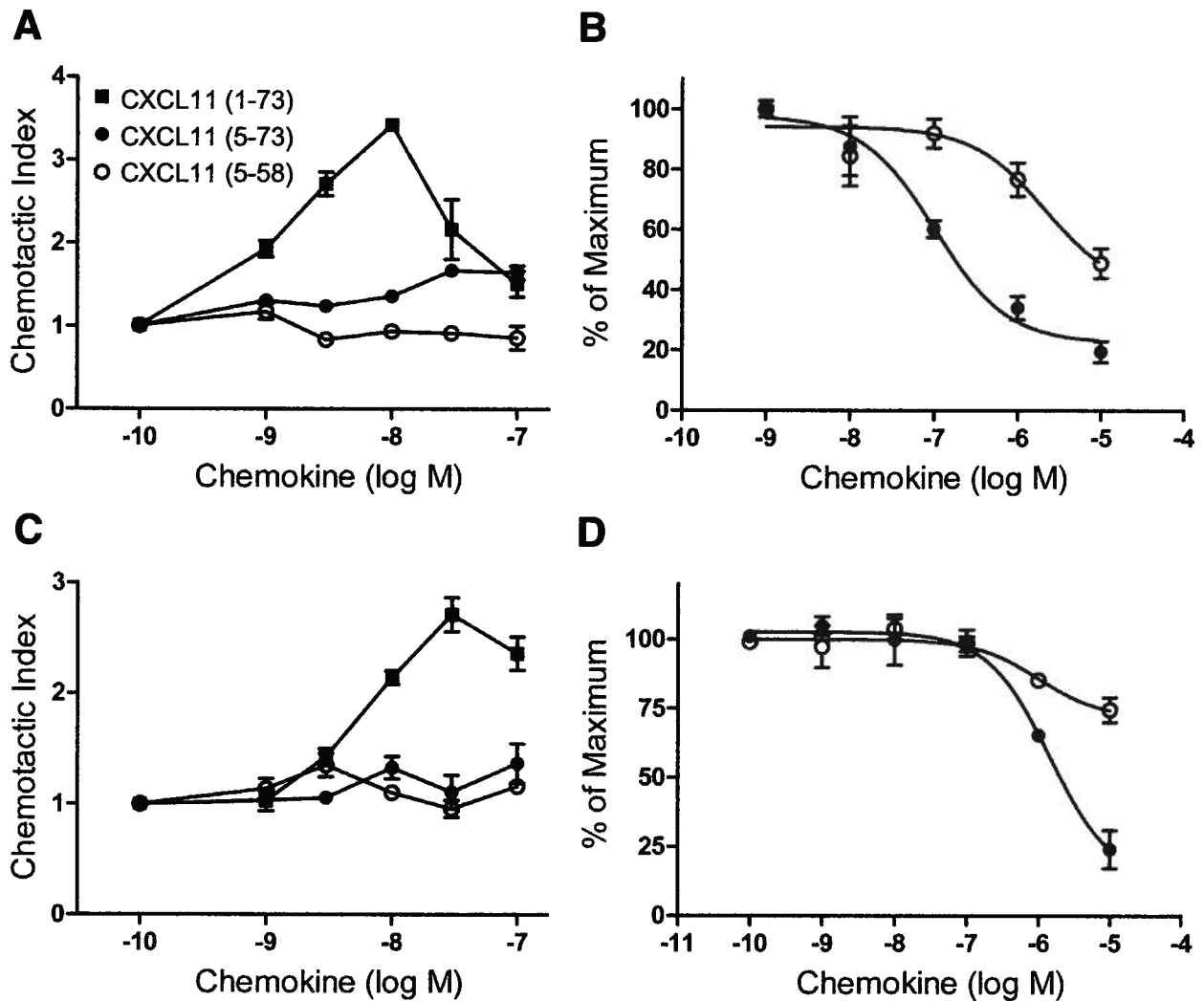
Figure 3.7. Chemotactic migration is decreased and antagonized in response to CXCL11 (5-73) and (5-58). Chemotaxis of CXCR3-transfected B300-19 cells (A) and isolated T lymphocytes (C) is abolished in response to CXCL11 (5-73) and (5-58). Chemotaxis was measured across a 5 μm pore size for 3 hours in response to 1-100 nM chemokine. Migrated cells were quantitated by CyQUANT assay and a chemotactix index was calculated, defined as the ratio of cells migrating in response to stimulus compared to the buffer control. Migration is inhibited moderately by CXCL11 (5-73) in CXCR3-transfected cells (B) and T cells (D) whereas CXCL11 (5-58) is a weak inhibitor. Chemotaxis was performed for 3 hours in response to 10 nM to 10 μM truncated CXCL11 co-incubated with 10 nM full length CXCL11. Percentage of maximum was calculated relative to uninhibited samples.
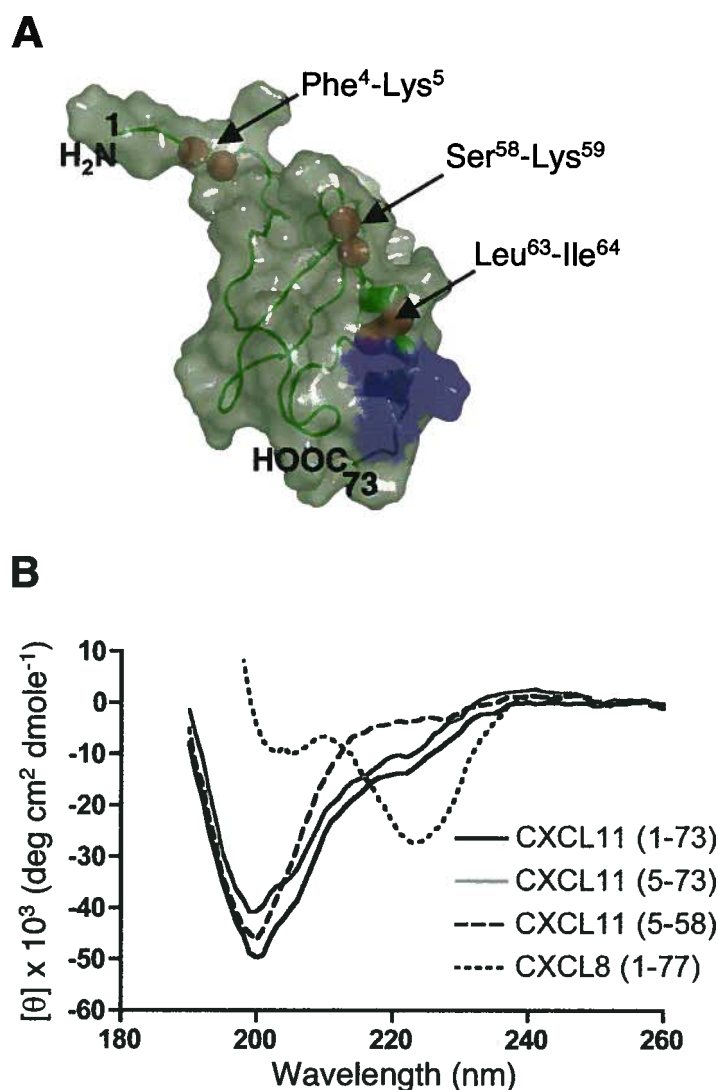
Figure 3.8. Structural analysis of full length and truncated CXCL11. (A) Structural representation of full--length CXCL11 demonstrating MMP cleavage sites (arrows) at position 4-5 in the N-terminus and positions 58-59 and 63-64 in the C-terminus. The blue coloring highlights the C-terminal cationic GAG-binding region removed by MMP proteolysis. The amino terminus (NH_2, 1) and caroxy terminus (COOH, 73) are shown. I acknowledge Dr. Alain Doucet for his contribution of this figure. (B) Circular dichroism of CXCL11 (1-73), (5-73), (5-58) and CXCL8 (1-77) in the far-ultraviolet wavelengths ranging from 190-260 nm. Strong negativity at 200 nm illustrates a high degree of disorder for all three CXCL11 peptides. Ellipticity at 220 nm represents α-helical content and this signal is lost upon C-terminal truncation.

data (65). The higher degree of disorder in CXCL11, in comparison with other CXC chemokines, has been reported previously by NMR analysis (64). Here the CD spectra for CXCL11 (1-73) and (5-73) are super-imposable and SOMCD analysis predicts 17.9-19.4% α-helix, 27.3-29.9% β-sheets, 11.8-15.6% turns, and 39-39.3% random structure (55). In contrast, the CXCL11 (5-58) spectrum reveals reduced ellipticity at 220 nm, suggesting a loss in α-helical structure as expected, but otherwise is comparable to the spectrum for full length CXCL11. The potential role of MMP disruption of the haptotactic CXCL11 gradient on proteoglycan glycosaminoglycans is depicted (Figure 3.10).

Heparin binding affinity of CXCL11

The abundance of positively charged amino acids in the carboxy-terminal α-helix of CXCL11 (Figure 3.8A) prompted the prediction that C-terminal truncation would result in reduced glycosaminoglycan binding. In a heparin-binding assay K_Ds were measured by fluorescence polarization spectroscopy. CXCL11 (1-73) and CXCL11 (5-73) bound to heparin with similar affinity, with binding constants ranging from 0.4 to 0.6 μM (Figure 3.9A). In contrast, CXCL11 (5-58) had weakened heparin binding, and as important, showed significantly reduced levels of bound chemokine upon saturable heparin binding. Together this indicates loss of a C-terminal glycosaminoglycan-binding site that reduced the overall affinity and amounts of chemokine binding. Consistent with this, CXCL11 (5-58) was also shown to have impaired binding to chondroitin sulfate A (data not shown).

Heparin-Sepharose chromatography was used to refine the characterization of the heparin binding properties of the cleaved forms of CXCL11 in relation to the full-length chemokine. Here again the C-terminal truncation had significantly reduced affinity (Figure 3.9B), confirming the assessment by fluorescence polarization. CXCL11 (1-73) eluted at 0.74 M NaCl whereas CXCL11 (5-58) eluted at 0.52 M NaCl. Surprisingly, the N-terminal truncation caused enhanced affinity as CXCL11 (5-73) eluted at 0.81 M NaCl, significantly higher than the full-length counterpart.

Figure 3.9. Heparin binding is altered upon CXCL11 cleavage. (A) Fluorescence polarization with heparin-FITC reveals a loss in affinity for CXCL11 (5-58). Chemokines were incubated for 1 hour with 0.1 μM heparin-FITC at 37 °C prior to analysis. (B) Elution profile of CXCL11 and cleaved analogues from heparin-Sepharose column (top) and strong cation exchange (SCX) Sepharose column (bottom). Elution was with a gradient reaching 1.5 M NaCl and absorbance was measured at 215 nm. (C) MMP-8 processing of CXCL11 is unaffected in the presence of 5-fold excess of heparan sulfate (HS), chrondroitin sulfate A (CSA), chondroitin sulfate B (CSB), chondroitin sulfate C (CSC), and hyaluronic acid (HA). The enzyme to substrate ratio was 1:10 (w:w) and the reaction was 16 h at 37 °C and products were visualized on a 15% Tris-tricine gel, silver stained. Note that MALDI-TOF analysis confirmed the presence of CXCL11 (5-73), (5-63) and (5-58) in all MMP-8 treated samples.

A

B

C
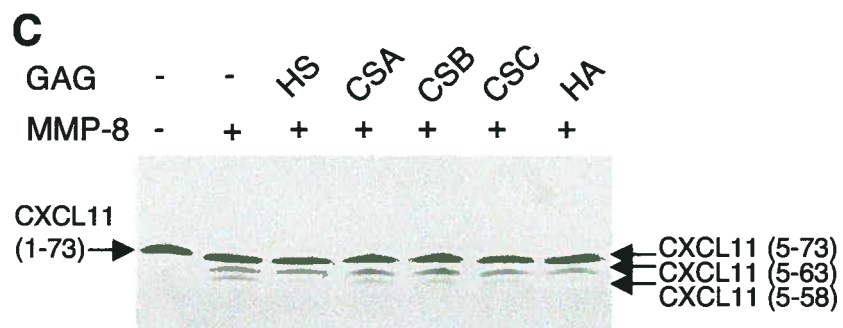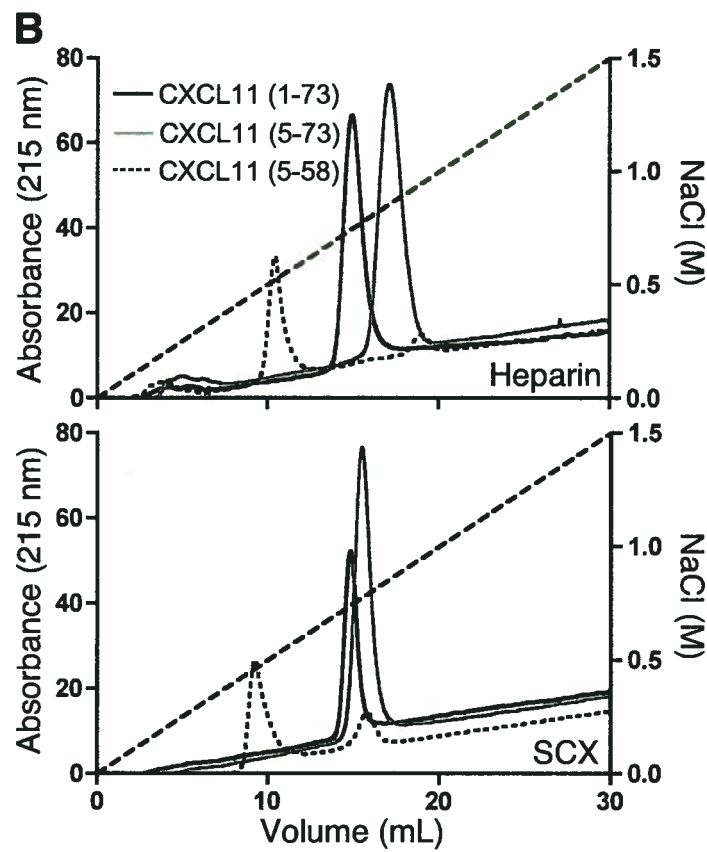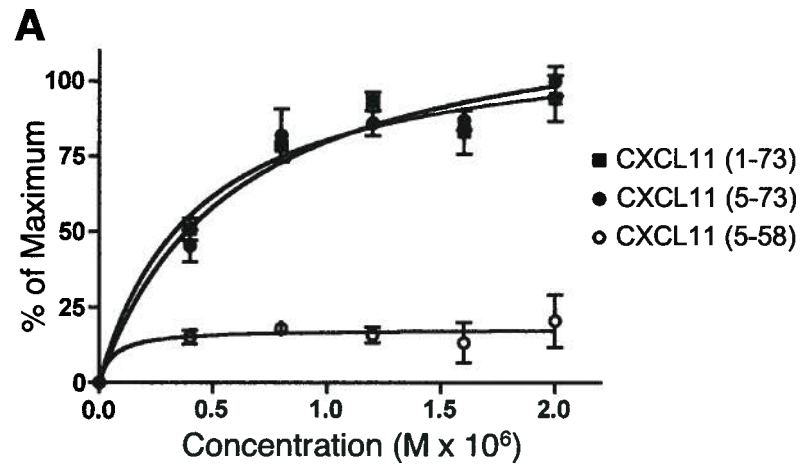
To assess the effect of specificity of interaction versus charge effects alone on the chemokine binding properties, an equivalent experiment was performed with a strong cation exchange column. The elutions of CXCL11 (1-73), (5-73), and (5-58) occurred at 0.74, 0.77, and 0.46 M NaCl, respectively. Loss of the cationic C-terminal α-helix in CXCL11 (5-58) yielded a similar reduction in binding to both the heparin and strong cation exchange columns, in comparison with CXCL11 (1-73), suggesting that the removal of charge is responsible for decreased glycosaminoglycan binding. However, the N-terminal truncation in CXCL11 (5-73) appears to enhance specific heparin interactions because the elution shift relative to full-length CXCL11 is greater on the heparin column compared to the strong cation exchange column. The glycosaminoglycan interaction with the cationic residues of the C-terminal peptide [59]KQARLIIKKVERKNF[73], forming an α-helix in the chemokine, might block MMP cleavage. The addition of excess heparan sulfate, chondroitin sulfate, and hyaluronic acid did not affect MMP-8 processing of CXCL11 at position 58-59, confirmed by MALDI-TOF MS analysis (Figure 3.9C). Hence, these data demonstrate that CXCL11 can be processed by MMPs in the presence of glycosaminoglycans and that the resulting cleavages alter binding affinity.

Discussion

Presented herein is the first instance of chemokine cleavage directly affecting glycosaminoglycan-binding properties. Previously, the murine chemokine mCXCL1/KC was shown to be mobilized by MMP-7 cleavage of the core protein of syndecan-1, leading to altered neutrophil chemotaxis, but the chemokine itself was not directly processed (13). Hence, the cleavage of the cationic C-terminal peptide of CXCL11 and resultant loss of a glycosaminoglycan-binding site reported in our biochemical study represents a novel mechanism of chemokine regulation. We suggest that the neutrophil MMPs 8 and 9, and macrophage MMPs 7, 9 and 12 mobilize CXCL11 from proteoglycans with disruption of the haptotactic chemokine gradient predicted (Figure 3.10). This would serve to enhance the direct effects on chemokine activity resulting from proteolysis of CXCL11 by these leukocytic MMPs.
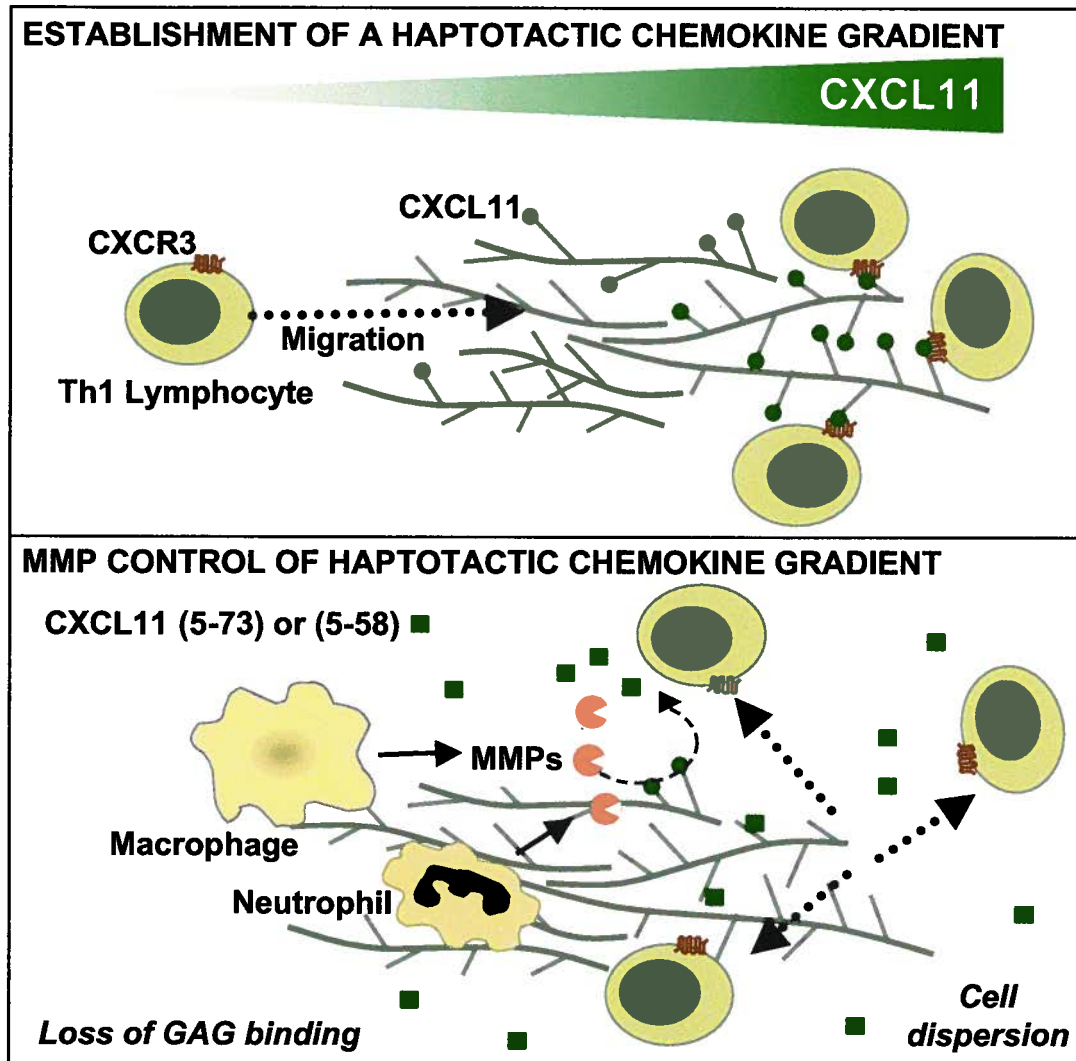
Figure 3.10. Model representing the potential role of leukocytic MMPs 7, 8, 9 and 12 in disrupting the haptotactic gradient of CXCL11. The establishment of a full-length CXCL11 gradient through glycosaminoglycan interactions is depicted in the top panel. This gradient is modified upon CXCL11 processing by neutrophil and macrophage-derived MMPs (bottom panel), resulting in the formation of CXCL11 (5-73) and (5-58) and leading to potential dispersion of Th1 lymphocytes.

Amino-terminal processing at Phe^4-Lys^5 resulted in loss of agonist activity in calcium flux and chemotactic migration assays with CXCR3-transfectants and isolated T lymphocytes. However, CXCL11 (5-73) retained receptor affinity, as it was a moderate antagonist against full-length CXCL11. Interestingly, the C-terminal truncation at Ser^{58}-Lys^{59} caused a reduction in receptor binding as indicated by decreased antagonistic properties so revealing a new CXCR3 binding site on the chemokine. Together, MMP cleavage potentially dampens T cell migration by loss of agonist activity, generation of a CXCR3 antagonist, and disruption of the chemokine gradient (Figure 3.10). The *in vivo* analysis of these effects are now in progress.

Chemokine activity is modulated at several levels including regulated expression and secretion, proteolysis, and the formation of haptotactic gradients in tissue. High affinity interactions with the glycosaminoglycan side-chains of proteoglycans are critical for *in vivo* activity of many chemokines as an immobilization mechanism for cell-surface retention and possibly also for presentation to circulating leukocytes (2). MMP-mediated proteolysis has been shown previously to indirectly influence chemokine gradients, but by a different mechanism. In the case of MMP-7, knockout mice demonstrate altered neutrophil infiltration in lung inflammation where neutrophils remain in the interstitium without advancing to the alveolar space (13). This phenotype was attributed to MMP-7–dependent shedding of the syndecan-1 ectodomain complexed with the murine ELR^+ chemokine mCXCL1/KC, a process required to direct neutrophils to the site of injury. However, proteolysis of KC did not occur. This mechanism contrasts the potential mobilization of CXCL11, where we demonstrate direct MMP cleavage of the chemokine and loss of a heparin-binding site that also has the potential to disrupt the chemokine gradient.

The basic residues lysine and arginine direct glycosaminoglycan affinity through electrostatic interactions; in CCL5 a BBXB motif has been identified (66) whereas CXCL8 contains several key residues at the C-terminus (67). Notably, the heparin-binding cationic cradle is rarely a simple consensus sequence, but rather a more complex combination of remote positively charged residues that are clustered in the

folded conformation, as illustrated with fibronectin module III-13 (68). We hypothesized that the glycosaminoglycan binding-site of CXCL11 is located in the C-terminus due to an abundance of basic amino acids and indeed the heparin binding interaction was strongly reduced in CXCL11 (5-58). However, residual binding suggests additional sites of heparin interaction independent of the C-terminus. Surprisingly, removal of the N-terminal tetrapeptide FPMF in CXCL11 increased heparin-Sepharose binding, perhaps due to the removal of steric hinderance providing stronger interactions with key basic residues. The enhanced affinity of CXCL11 (5-73) is not completely charge-dependent as the equivalent comparison on a strong cation exchange column showed only a minimal increase.

Despite the location of a glycosaminoglycan-binding site near the site of MMP cleavage between positions 58-59, MMP cleavage of CXCL11 was unaltered when interacting with soluble heparan sulfate, chondroitin sulfate, and hyaluronic acid. Hence, these findings show for the first time for any protease that direct proteolysis of a chemokine can alter its glycosaminoglycan-binding properties. The resulting enhanced and reduced affinities, depending on the cleavage site, reveal biphasic regulation of chemokine binding to glycosaminoglycans and hence potential complex modulation of *in vivo* localization and chemotactic activity that is currently under investigation.

Unlike many other proteases, the cleavage site specificity of MMPs remains largely unpredictable, likely due to less restricted substrate amino acid binding pockets (in particular S3-S3') and the complex role of exosite binding in substrate recognition (58). Peptide substrate libraries have demonstrated an MMP preference for proline at P3 (69), and notably the majority of chemokines contain a proline at residue 2 in the N-terminus. Therefore this proline appears to dictate MMP proteolysis of many chemokines at the characteristic residues 4-5 in the N-terminus. However, examples at other N-terminal cleavage sites suggest that specificity is not so simple. For instance CXCL5, which has a proline at position 3, is cleaved by MMPs at Val^7-Leu^8 and Arg^9-Glu^{10} (25, 27). Interestingly, CXCL9, CXCL10, and CXCL11 share a proline

at position two and are approximately 40% identical (60), yet only CXCL11 is susceptible to N-terminal processing by MMPs, illustrating that additional elements are important, such as exosite interactions. Indeed, only CXCL11 bound the hemopexin C domain of MMP-8, but neither CXCL9 or CXCL10 did and these were not cleaved. Furthermore, murine CXCL11, which has high sequence identity to the human homolog, contains a leucine in the place of proline at position 2 and is processed efficiently at position Met^3-Phe^4 rather than Phe^4-Lys^5 by MMP-7 and -8. Nonetheless, the predominant C-terminal cleavage of CXCL11 at residues 58-59 also contains a proline at P3. Hence, this data suggests that Pro^2 is not essential for CXCL11 processing, but rather that it directs cleavage to position 4-5 in the N-terminus. Interestingly, all three cleavage sites on CXCL11 lie on the same face of the protein in a line indicating potential processivity of cleavage upon chemokine tethering to the hemopexin C domain.

Of the MMPs evaluated, MMP-1, -2, and -14 had no activity on any of the human CXCR3 ligands revealing specificity of the main leukocytic MMPs for chemokine cleavage and hence an immune cell-specific feedback mechanism regulating chemokine activity. In contrast to the specific and selective cleavages by MMP-8 and MMP-9, processing or degradation of the three chemokines by MMP-7 and MMP-12 represents broad specificity. Interestingly, MMP-7 and MMP-12 share structural similarities in that MMP-7 does not encode a hemopexin C domain (70) and MMP-12 loses the domain upon activation (71). In the case of the CXCR3 ligands, it is probable that MMP-7 and MMP-12 have weak specificity, but high activity, resulting in substrate degradation in contrast to the precise processing by other MMPs. Therefore, it seems likely that exosite interactions of the hemopexin C domain contribute greatly to selective cleavage of substrates.

Amino-terminal truncation of chemokines has been reported to result in conversion of receptor agonists to antagonists (6, 9). CXCL11 truncations have been previously characterized and CXCL11 (4-73) was a potent receptor antagonist whereas CXCL11 (5-73) had moderate inhibitory properties (61). In addition, CD26-mediated

cleavage of CXCL11 generates CXCL11 (3-73), another receptor antagonist (47). Antagonist studies with CXCL11 (5-73) are consistent with previous findings, where moderate IC_{50}s of 100-1000 nM were observed. Surprisingly, the MMP-mediated C-terminal truncation of CXCL11 at residues 58-59 significantly decreased the inhibitory properties of this molecule with IC_{50}s greater than 10 μM, concentrations that are unlikely to be reached *in vivo*. The C-terminus of chemokines is not traditionally associated with receptor binding properties. Rather, the N-loop following the first two cysteines is the major receptor binding site that confers receptor specificity. Electrostatic interactions in the 30s loop, the region connected the first two beta-strands, are also thought to contribute to receptor affinity (72). Therefore, we have demonstrated an unexpected role for the C-terminus of CXCL11 in receptor binding.

Amino-terminal CXCL11 processing has been described previously for CD13 (14) and CD26 (46, 47), however the physiological relevance of these interactions remains unclear, particularly given the clinical use of CD26 inhibitors for diabetes (73). In contrast, the role of MMPs in chemokine processing *in vivo* is well established with evidence from genetic mouse models and neo-epitope antibodies detecting the MMP-processed forms in human biological samples (6, 25, 29). The murine homolog of CXCL11 lacks a proline at position 2 and hence MMP processing of mCXCL11 is altered, restricting the use of genetic models to further explore this mechanism in animal models. Interestingly, MMPs are the only enzymes known to cleave the C-terminus of CXCL11, indicating potential synergism between multiple proteases in the precise regulation of this potent T-cell chemoattractant.

Specific and limited proteolysis is emerging as a widespread mechanism of rapid and efficient modulation of both cytokine and chemokine activity [see reviews (20, 21). To date, MMPs regulate chemokine function by activation, inactivation, generation of antagonists, shedding from the cell membrane, and cleavage of binding proteins. Our present data demonstrates a new mechanism whereby direct cleavage of a chemokine leads to loss of proteoglycan binding predicted to result in

dispersion of the chemokine gradient. Here we have biochemically characterized MMP processing of the CXCR3 ligands, uncovering cleavages that alter receptor activation and binding as well as glycosaminoglycan interactions. These cleavage events have the potential to modulate Th1 cell migration and hence disorders such as multiple sclerosis and rheumatoid arthritis, which are now under study in our laboratory. Neutrophils are implicated in mediating tissue damage in the context of chronic inflammation. However, the abundance of neutrophil-derived MMP-8 and MMP-9 may enable regulation of the adaptive immune response through the fine-tuning of Th1 chemoattraction. Furthermore, macrophage proteases MMP-7 and MMP-12 have the potential to dampen the Th1 response through chemokine degradation. The ensuing challenge is to assess these mechanisms in the context of homeostatic and pathological states to decipher the physiological consequences of altering these chemotactic signaling pathways in T cell chemotaxis and immune regulation.

References

1. Moser, B., M. Wolf, A. Walz, and P. Loetscher. 2004. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 25:75-84.

2. Handel, T. M., Z. Johnson, S. E. Crown, E. K. Lau, and A. E. Proudfoot. 2005. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annu Rev Biochem* 74:385-410.

3. Hamilton, T. A., M. Novotny, S. Datta, P. Mandal, J. Hartupee, J. Tebo, and X. Li. 2007. Chemokine and chemoattractant receptor expression: post-transcriptional regulation. *J Leukoc Biol* 82:213-219.

4. Neel, N. F., E. Schutyser, J. Sai, G. H. Fan, and A. Richmond. 2005. Chemokine receptor internalization and intracellular trafficking. *Cytokine Growth Factor Rev* 16:637-658.

5. Locati, M., Y. M. Torre, E. Galliera, R. Bonecchi, H. Bodduluri, G. Vago, A. Vecchi, and A. Mantovani. 2005. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine Growth Factor Rev* 16:679-686.

6. McQuibban, G. A., J. H. Gong, E. M. Tam, C. A. McCulloch, I. Clark-Lewis, and C. M. Overall. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 289:1202-1206.

7. Overall, C. M., and O. Kleifeld. 2006. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 6:227-239.

8. McQuibban, G. A., G. S. Butler, J. H. Gong, L. Bendall, C. Power, I. Clark-Lewis, and C. M. Overall. 2001. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 276:43503-43508.

9. McQuibban, G. A., J. H. Gong, J. P. Wong, J. L. Wallace, I. Clark-Lewis, and C. M. Overall. 2002. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 100:1160-1167.

10. Dean, R. A., and C. M. Overall. 2007. Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome. *Mol Cell Proteomics* 6:611-623.

11. Schulte, A., B. Schulz, M. G. Andrzejewski, C. Hundhausen, S. Mletzko, J. Achilles, K. Reiss, K. Paliga, C. Weber, S. R. John, and A. Ludwig. 2007. Sequential processing of the transmembrane chemokines CX3CL1 and CXCL16 by alpha- and gamma-secretases. *Biochem Biophys Res Commun* 358:233-240.

12. Vergote, D., G. S. Butler, M. Ooms, J. H. Cox, C. Silva, M. D. Hollenberg, J. H. Jhamandas, C. M. Overall, and C. Power. 2006. Proteolytic processing of SDF-1alpha reveals a change in receptor specificity mediating HIV-associated neurodegeneration. *Proc Natl Acad Sci U S A* 103:19182-19187.

13. Li, Q., P. W. Park, C. L. Wilson, and W. C. Parks. 2002. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 111:635-646.

14. Proost, P., A. Mortier, T. Loos, J. Vandercappellen, M. Gouwy, I. Ronsse, E. Schutyser, W. Put, M. Parmentier, S. Struyf, and J. Van Damme. 2007. Proteolytic processing of CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. *Blood* 110:37-44.

15. Van Damme, J., S. Struyf, A. Wuyts, E. Van Coillie, P. Menten, D. Schols, S. Sozzani, I. De Meester, and P. Proost. 1999. The role of CD26/DPP IV in chemokine processing. *Chem Immunol* 72:42-56.

16. Ajami, K., M. R. Pitman, C. H. Wilson, J. Park, R. I. Menz, A. E. Starr, J. H. Cox, C. A. Abbott, C. M. Overall, and M. D. Gorrell. 2008. Stromal cell-derived factors 1alpha and 1beta, inflammatory protein-10 and interferon-inducible T cell chemo-attractant are novel substrates of dipeptidyl peptidase 8. *FEBS Lett* 582:819-825.

17. Petit, I., M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R. S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 3:687-694.

18. Lim, J. K., W. Lu, O. Hartley, and A. L. DeVico. 2006. N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4-68 variant. *J Leukoc Biol* 80:1395-1404.

19. Hasan, L., L. Mazzucchelli, M. Liebi, M. Lis, R. E. Hunger, A. Tester, C. M. Overall, and M. Wolf. 2006. Function of liver activation-regulated chemokine/CC chemokine ligand 20 is differently affected by cathepsin B and cathepsin D processing. *J Immunol* 176:6512-6522.

20. Parks, W. C., C. L. Wilson, and Y. S. Lopez-Boado. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* 4:617-629.

21. Cox, J. H., and C. M. Overall. In press. Cytokine Substrates: MMP Regulation of Inflammatory Signalling Molecules. *The Cancer Degradome - Proteases and Cancer Biology. Springer Science and Business Media.*

22. Cauwe, B., P. E. Van den Steen, and G. Opdenakker. 2007. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit Rev Biochem Mol Biol* 42:113-185.

23. Page-McCaw, A., A. J. Ewald, and Z. Werb. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 8:221-233.

24. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35:252-257.

25. Tester, A. M., J. H. Cox, A. R. Connor, A. E. Starr, R. A. Dean, X. S. Puente, C. Lopez-Otin, and C. M. Overall. 2007. LPS Responsiveness and Neutrophil Chemotaxis In Vivo Require PMN MMP-8 Activity. *PLoS ONE* 2:e312.

26. Van den Steen, P. E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.

27. Van Den Steen, P. E., A. Wuyts, S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* 270:3739-3749.

28. Van den Steen, P. E., S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Carboxyterminal cleavage of the chemokines MIG and IP-10 by gelatinase B and neutrophil collagenase. *Biochem Biophys Res Commun* 310:889-896.

29. Zhang, K., G. A. McQuibban, C. Silva, G. S. Butler, J. B. Johnston, J. Holden, I. Clark-Lewis, C. M. Overall, and C. Power. 2003. HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat Neurosci* 6:1064-1071.

30.   Overall, C. M. 2002. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol Biotechnol* 22:51-86.

31.   Burrage, P. S., K. S. Mix, and C. E. Brinckerhoff. 2006. Matrix metalloproteinases: role in arthritis. *Front Biosci* 11:529-543.

32.   Bar-Or, A., R. K. Nuttall, M. Duddy, A. Alter, H. J. Kim, I. Ifergan, C. J. Pennington, P. Bourgoin, D. R. Edwards, and V. W. Yong. 2003. Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis. *Brain* 126:2738-2749.

33.   Kouwenhoven, M., V. Ozenci, A. Gomes, D. Yarilin, V. Giedraitis, R. Press, and H. Link. 2001. Multiple sclerosis: elevated expression of matrix metalloproteinases in blood monocytes. *J Autoimmun* 16:463-470.

34.   Lopez-Otin, C., and L. M. Matrisian. 2007. Emerging roles of proteases in tumour suppression. *Nat Rev Cancer* 7:800-808.

35.   Wolf, K., R. Muller, S. Borgmann, E. B. Brocker, and P. Friedl. 2003. Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. *Blood* 102:3262-3269.

36.   Dean, R. A., G. S. Butler, Y. Hamma-Kourbali, J. Delbe, D. R. Brigstock, J. Courty, and C. M. Overall. 2007. Identification of candidate angiogenic inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based proteomic screens: disruption of vascular endothelial growth factor (VEGF)/heparin affin regulatory peptide (pleiotrophin) and VEGF/Connective tissue growth factor angiogenic inhibitory complexes by MMP-2 proteolysis. *Mol Cell Biol* 27:8454-8465.

37.   Tam, E. M., C. J. Morrison, Y. I. Wu, M. S. Stack, and C. M. Overall. 2004. Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 101:6917-6922.

38.   Abraham, M., S. Shapiro, A. Karni, H. L. Weiner, and A. Miller. 2005. Gelatinases (MMP-2 and MMP-9) are preferentially expressed by Th1 vs. Th2 cells. *J Neuroimmunol* 163:157-164.

39.   Will, H., S. J. Atkinson, G. S. Butler, B. Smith, and G. Murphy. 1996. The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem* 271:17119-17123.

40.   Matias-Roman, S., B. G. Galvez, L. Genis, M. Yanez-Mo, G. de la Rosa, P. Sanchez-Mateos, F. Sanchez-Madrid, and A. G. Arroyo. 2005. Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. *Blood* 105:3956-3964.

41.   Yang, M. X., X. Qu, B. H. Kong, Q. L. Lam, Q. Q. Shao, B. P. Deng, K. H. Ko, and L. Lu. 2006. Membrane type 1-matrix metalloproteinase is involved in the migration of human monocyte-derived dendritic cells. *Immunol Cell Biol* 84:557-562.

42.   Gerard, C., and B. J. Rollins. 2001. Chemokines and disease. *Nat Immunol* 2:108-115.

43.   Hancock, W. W., L. Wang, Q. Ye, R. Han, and I. Lee. 2003. Chemokines and their receptors as markers of allograft rejection and targets for immunosuppression. *Curr Opin Immunol* 15:479-486.

44.   Sorensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, J. L. Frederiksen, and R. M. Ransohoff. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* 103:807-815.

45. Hensbergen, P. J., D. Verzijl, C. I. Balog, R. Dijkman, R. C. van der Schors, E. M. van der Raaij-Helmer, M. J. van der Plas, R. Leurs, A. M. Deelder, M. J. Smit, and C. P. Tensen. 2004. Furin is a chemokine-modifying enzyme: in vitro and in vivo processing of CXCL10 generates a C-terminally truncated chemokine retaining full activity. *J Biol Chem* 279:13402-13411.

46. Ludwig, A., F. Schiemann, R. Mentlein, B. Lindner, and E. Brandt. 2002. Dipeptidyl peptidase IV (CD26) on T cells cleaves the CXC chemokine CXCL11 (I-TAC) and abolishes the stimulating but not the desensitizing potential of the chemokine. *J Leukoc Biol* 72:183-191.

47. Proost, P., E. Schutyser, P. Menten, S. Struyf, A. Wuyts, G. Opdenakker, M. Detheux, M. Parmentier, C. Durinx, A. M. Lambeir, J. Neyts, S. Liekens, P. C. Maudgal, A. Billiau, and J. Van Damme. 2001. Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. *Blood* 98:3554-3561.

48. Clark-Lewis, I., L. Vo, P. Owen, and J. Anderson. 1997. Chemical synthesis, purification, and folding of C-X-C and C-C chemokines. *Methods Enzymol* 287:233-250.

49. Butler, G. S., E. M. Tam, and C. M. Overall. 2004. The canonical methionine 392 of matrix metalloproteinase 2 (gelatinase A) is not required for catalytic efficiency or structural integrity: probing the role of the methionine-turn in the metzincin metalloprotease superfamily. *J Biol Chem* 279:15615-15620.

50. Tam, E. M., T. R. Moore, G. S. Butler, and C. M. Overall. 2004. Characterization of the distinct collagen binding, helicase and cleavage mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): the differential roles of the MMP hemopexin c domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities. *J Biol Chem* 279:43336-43344.

51. Knight, C. G., F. Willenbrock, and G. Murphy. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett* 296:263-266.

52. Loetscher, M., B. Gerber, P. Loetscher, S. A. Jones, L. Piali, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1996. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med* 184:963-969.

53. Fricker, S. P., V. Anastassov, J. Cox, M. C. Darkes, O. Grujic, S. R. Idzan, J. Labrecque, G. Lau, R. M. Mosi, K. L. Nelson, L. Qin, Z. Santucci, and R. S. Wong. 2006. Characterization of the molecular pharmacology of AMD3100: a specific antagonist of the G-protein coupled chemokine receptor, CXCR4. *Biochem Pharmacol* 72:588-596.

54. Takahashi, A., P. Camacho, J. D. Lechleiter, and B. Herman. 1999. Measurement of intracellular calcium. *Physiol Rev* 79:1089-1125.

55. Unneberg, P., J. J. Merelo, P. Chacon, and F. Moran. 2001. SOMCD: method for evaluating protein secondary structure from UV circular dichroism spectra. *Proteins* 42:460-470.

56. Kakehi, K., Y. Oda, and M. Kinoshita. 2001. Fluorescence polarization: analysis of carbohydrate-protein interaction. *Anal Biochem* 297:111-116.

57. Lau, E. K., C. D. Paavola, Z. Johnson, J. P. Gaudry, E. Geretti, F. Borlat, A. J. Kungl, A. E. Proudfoot, and T. M. Handel. 2004. Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1: implications for structure and function in vivo. *J Biol Chem* 279:22294-22305.

58. Overall, C. M., G. A. McQuibban, and I. Clark-Lewis. 2002. Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol Chem* 383:1059-1066.

59. Shapiro, S. D. 2003. Proteolysis in the lung. *Eur Respir J Suppl* 44:30s-32s.

60. Cole, K. E., C. A. Strick, T. J. Paradis, K. T. Ogborne, M. Loetscher, R. P. Gladue, W. Lin, J. G. Boyd, B. Moser, D. E. Wood, B. G. Sahagan, and K. Neote. 1998. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* 187:2009-2021.

61. Clark-Lewis, I., I. Mattioli, J. H. Gong, and P. Loetscher. 2003. Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. *J Biol Chem* 278:289-295.

62. Ogilvie, P., G. Bardi, I. Clark-Lewis, M. Baggiolini, and M. Uguccioni. 2001. Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5. *Blood* 97:1920-1924.

63. Sauty, A., R. A. Colvin, L. Wagner, S. Rochat, F. Spertini, and A. D. Luster. 2001. CXCR3 internalization following T cell-endothelial cell contact: preferential role of IFN-inducible T cell alpha chemoattractant (CXCL11). *J Immunol* 167:7084-7093.

64. Booth, V., I. Clark-Lewis, and B. D. Sykes. 2004. NMR structure of CXCR3 binding chemokine CXCL11 (ITAC). *Protein Sci* 13:2022-2028.

65. Lusti-Narasimhan, M., A. Chollet, C. A. Power, B. Allet, A. E. Proudfoot, and T. N. Wells. 1996. A molecular switch of chemokine receptor selectivity. Chemical modification of the interleukin-8 Leu25 --> Cys mutant. *J Biol Chem* 271:3148-3153.

66. Proudfoot, A. E., S. Fritchley, F. Borlat, J. P. Shaw, F. Vilbois, C. Zwahlen, A. Trkola, D. Marchant, P. R. Clapham, and T. N. Wells. 2001. The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. *J Biol Chem* 276:10620-10626.

67. Kuschert, G. S., A. J. Hoogewerf, A. E. Proudfoot, C. W. Chung, R. M. Cooke, R. E. Hubbard, T. N. Wells, and P. N. Sanderson. 1998. Identification of a glycosaminoglycan binding surface on human interleukin-8. *Biochemistry* 37:11193-11201.

68. Busby, T. F., W. S. Argraves, S. A. Brew, I. Pechik, G. L. Gilliland, and K. C. Ingham. 1995. Heparin binding by fibronectin module III-13 involves six discontinuous basic residues brought together to form a cationic cradle. *J Biol Chem* 270:18558-18562.

69. Turk, B. E., L. L. Huang, E. T. Piro, and L. C. Cantley. 2001. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat Biotechnol* 19:661-667.

70. Quantin, B., G. Murphy, and R. Breathnach. 1989. Pump-1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. *Biochemistry* 28:5327-5334.

71. Shapiro, S. D., D. K. Kobayashi, and T. J. Ley. 1993. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. *J Biol Chem* 268:23824-23829.

72. Fernandez, E. J., and E. Lolis. 2002. Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol* 42:469-499.

73. Herman, G. A., P. P. Stein, N. A. Thornberry, and J. A. Wagner. 2007. Dipeptidyl peptidase-4 inhibitors for the treatment of type 2 diabetes: focus on sitagliptin. *Clin Pharmacol Ther* 81:761-767.

CHAPTER 4. MMP-8 DEFICIENCY DELAYS NEUTROPHIL APOPTOSIS AND EXACERBATES RHEUMATOID ARTHRITIS[4]

Perspective

Neutrophil accumulation in inflammation is determined by cell infiltration, apoptosis and clearance. We identify an important new mechanism regulating neutrophil apoptosis that is orchestrated by the neutrophil-specific matrix metalloproteinase-8 (MMP-8) or collagenase-2. *Mmp8 -/-* mice were backcrossed onto the MRL/*lpr* background, characterized by a defective Fas receptor that results in systemic autoimmunity including spontaneous rheumatoid arthritis. Surprisingly, male MMP-8 deficient mice had earlier and more severe joint inflammation coupled with a massive accumulation of neutrophils in synovial tissues. CLIP-CHIP^{TM} microarray analysis revealed abolished expression of caspase-11 in neutrophils from MMP-8 knockout mice. This was confirmed at the protein level in unstimulated or LPS, IFN-γ and TNF-α-treated MMP-8 -/- neutrophils. Caspase-11 is an activator of caspase-3 and in *Mmp8 -/-* neutrophils caspase-3 activity was significantly reduced as was constitutive apoptosis. MMP-8 sheds TNF-α, which induces neutrophil apoptosis. In unstimulated MMP-8 deficient neutrophils, TNF-α shedding was reduced. Therefore, MMP-8 is required for expression of caspase-11 and downstream activation of caspase-3, constitutive TNF-α shedding and neutrophil apoptosis in the resolution of inflammation. In autoimmune diseases such as rheumatoid arthritis, MMP-8 deficiency leads to an exaggerated accumulation of neutrophil infiltrates with concurrent pathological changes including earlier onset, increased incidence and more severe disease. Hence MMP-8 is an anti-target in the treatment of arthritis.

---

[4] A version of this chapter has been submitted for publication. Cox JH, Starr AE, Kappelhoff R, Yan R, Roberts CR, and Overall CM. Matrix metalloproteinase-8 deficiency exacerbates rheumatoid arthritis through delayed neutrophil apoptosis and reduced caspase-11 expression. 2008. Manuscript currently under review at *The Journal of Immunology*.

Introduction

Dysregulation of the cellular components of the innate and adaptive immune responses can result in autoimmune diseases such as rheumatoid arthritis. Neutrophils, typically considered acute inflammatory cells, are also present in a variety of chronic inflammatory diseases including arthritis, autoimmune colitis, and psoriasis (1, 2). In rheumatoid arthritis, neutrophils degrade cartilage components and are essential for the initiation and maintenance of inflammation in murine models of arthritis (3-5). Matrix metalloproteinases (MMPs) have long been considered promising drug targets in arthritis treatment due to their ability to degrade collagen and proteoglycans (6), major components of cartilage and bone, and MMPs are upregulated in the synovial fluid and tissues of affected joints (7). MMP-8 (also known as collagenase-2) is produced primarily by neutrophils and is released from the specific granules at sites of inflammation and some reports indicate that low levels of MMP-8 mRNA can be induced in synovial cells (8). MMPs have more recently been shown to precisely process and regulate inflammatory mediators such as chemokines (9, 10), tumor necrosis factor-α (TNF-α) (11), and cytokine binding proteins that otherwise mask bioactivity (12), identifying pleiotrophic roles for MMPs in both promoting and dampening inflammation. This has complicated MMP drug-targeting strategies that must now consider additional roles other than simply extracellular matrix degradation (13).

The pro-inflammatory cytokines interleukin-1β (IL-1β), TNF-α, and interferon-γ (IFN-γ) are central molecules in the pathogenesis of chronic inflammation, acting in signaling cascades with chemokines, initiating the recruitment of inflammatory cells, and enabling the self-perpetuation of inflammation. In auto-inflammatory disorders such as rheumatoid arthritis, blocking IL-1β and TNF-α activity with receptor antagonists or neutralizing antibodies has proven beneficial, however animal models have shown that IL-1β is more important in joint damage (14, 15). Furthermore, deletion of caspase-1, also known as interleukin-1 converting enzyme (ICE), prevents maturation of IL-1β and improves inflammatory diseases (16). Caspase-11,

which is an activator of both caspase-1 and the pro-apoptotic caspase-3 (17), could also represent a potential drug target to inhibit chronic inflammation, being involved in neutrophil apoptosis and hence clearance of cellular infiltrate.

MMP-8 deficient mice have no overt phenotype, with normal embryonic development, fertility, and long-term survival (18) but they have diminished LPS responsiveness in a subcutaneous air pouch model (19). In skin carcinogenesis studies, an abnormal inflammatory response was observed at the tumor-stromal interface in MMP-8 knockout mice that was characterized by an initial delay in neutrophil infiltration followed by a prolonged accumulation that did not dissipate, resulting in increased tumorigenesis (18). We recently investigated the mechanism of this initial lag finding that MMP-8 cleaves and activates murine lipopolysaccharide-induced CXC chemokine, human CXCL5/ENA-78, and human CXCL8/IL-8, all potent neutrophil chemoattractants, in a novel *in cis* feed-forward mechanism that is lost upon MMP-8 gene deletion (19). Hence, MMP-8 plays a coordinating role in leukocyte trafficking with clear implications for disease pathogenesis. However, its role in chronic inflammatory disease is unknown as is the mechanism leading to chronic accumulation of neutrophils in the absence of MMP-8. Elucidating this mechanism will reveal important new aspects of the control of neutrophil function in innate immunity and pathology.

The MRL/*lpr* lymphoproliferative mouse is characterized by systemic autoimmune disease including inflammation of the joints, skin, salivary glands, and brain and marked lymphoid hyperplasia, glomerulonephritis, and vasculitis, resulting in significant mortality in the first 6 months of life (20). The *lpr* mutation has been localized to the *fas* gene and causes faulty expression of Fas receptor (CD95/Apo-1), leading to defective Fas-dependent lymphocyte apoptosis and impaired deletion of autoreactive lymphocytes (21, 22). The MRL/*lpr* strain provides a mild, age-dependent, naturally susceptible model for arthritis and other autoimmune diseases. As such, it is considered a good mimic of human disease compared to acute and severe models such as collagen-induced arthritis. Here we examine the role of

MMP-8 in MRL/*lpr* autoimmune arthritis. We show a protective role for the enzyme, as MMP-8 deficient mice have an accumulation of neutrophils in the joints. Moreover, MMP-8-deficient cells were found to have no expression of caspase-11, reduced caspase-3 activity and hence downstream reduction in neutrophil apoptosis. TNF-α shedding from MMP-8 deficient neutrophils was also dampened. We conclude that MMP-8 has anti-inflammatory functions in the chronic inflammatory setting, in part due to regulation of caspase-11 expression and consequent caspase-3 activity, thereby promoting neutrophil apoptosis. Hence, the neutrophil MMP-8 is a new anti-target in chronic inflammation.

Materials and Methods

Generation of *Mmp8* -/- mice. MMP-8 deficient mice on a C57BL/6 x 129 background were kindly provided by Dr. Steven Shapiro (Harvard School of Medicine, USA) and backcrossed for 8 generations onto the MRL/*lpr* lymphoproliferative strain (Jackson Labs 006825), after which wild-type and MMP-8 deficient mice were maintained as homozygous lines. Genotyping was performed by Hot Shot DNA isolation (23) followed by three-primer PCR with wild-type primers TGT CGG GTC CTG GTT TAC ATT CTG (forward) and AAG GTC AGG GGC GAT GCT ACA (reverse) and knockout primer CGC CTT CTT GAC GAG TTC TTC TGA (reverse). The PCR products were 1100 bp for wild-type and 550 bp for knockout. All animal breeding and experimental procedures were approved by the University of British Columbia Animal Care Committee (Appendix C).

Western blot and immunohistochemistry. Murine bone marrow neutrophils were purified by flushing of freshly isolated femurs and tibias with Dulbecco's modified eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) followed by separation on a discontinuous Histopaque gradient (Sigma) (24). Neutrophil content and viability was consistently confirmed to be greater than 95% by Diff-Quick staining (IMEB Inc.) and trypan blue exclusion, respectively. Cell lysates were prepared by 15 min incubation with 0.2% Zwittergent 3-14 detergent

(Calbiochem) in PBS at 4 °C. MMP-8 and MMP-9 western blots were performed on 10% SDS-PAGE gels transferred to Immobilon-FL PVDF membrane (Millipore) and imaged on a LI-COR Odyssey using an Alexa-conjugated secondary antibody (Molecular Probes). Rabbit anti-mouse MMP-8 antibody was kindly provided by Dr. C. Lopez-Otin (University of Oviedo, Spain) (18), used at 1:10,000 and rabbit anti-mouse MMP-9 was used at 1:1,000 (Sigma). Rabbit anti-actin was used at 1:200 (Sigma).

Induction and measurement of arthritis. To synchronize and enhance the initiation of spontaneous arthritis, male and female mice at 13-14 weeks of age were injected intradermally at two inguinal sites with 0.05 mL of a 1:1 emulsion of water with complete Freund's adjuvant (Sigma) supplemented to 10 mg/mL heat-killed Mycobacterium tuberculosis (Difco). The width of both hind ankles was measured on 3 consecutive days before arthritis induction and every 2-3 days for 30 days following injection using an electronic digital caliper (Marathon). Baseline ankle width was calculated as the average of pre-injection measurements. Arthritis was considered to have occurred following a 10% increase in average ankle width. Statistical analysis was done with Prism 4.0 (Graphpad Software) using two-way ANOVA with Bonferroni post-tests.

Histological assessment of arthritis. At day 30 post-injection, mice were sacrificed by CO_2 asphyxiation, hind ankles were removed, skinned and formalin-fixed for 8-12 h. Ankles were then decalcified for 72 h in 10% formic acid, dehydrated and paraffin-embedded. Sections (3-4 μm) were stained with hematoxylin and eosin or toluidine blue. Tissue sections were coded and graded for inflammation by a blinded observer. Neutrophil immunostaining was performed with a 1:2,000 dilution of rat anti-mouse 7/4 antibody (Serotec) coupled with Vectastain ABC and 3,3'-diaminobenzidine (DAB) detection (Vector labs). Macrophages and T lymphocytes were stained with F4/80 antibody (Serotec) and anti-CD3 (Dako), respectively, both at a 1:50 dilution. Tissues were stained for collagenase cleavage sites in type II collagen using the COL2-3/4C$_{short}$ neoepitope antibody against the

peptide GPHypGPGG, kindly provided by Dr. John Mort (McGill University, Canada) (25). Aggrecan neoepitope antibodies generated against VDIPEN peptide (MMP cleavage) and NITEGE peptide (aggrecanase cleavage) were generously provided by Dr. Amanda Fosang (University of Melbourne, Australia) (26, 27).

CLIP-CHIP™ microarray analysis. Murine CLIP-CHIP™ analysis, a focused DNA microarray with probes for all murine proteases, their inhibitors, and inactive homologs was performed as described previously (28). Isolated bone marrow neutrophils from 2-3 mice were pooled and total RNA was purified using the Qiagen RNeasy protocol including an on-column DNaseI digest. Reverse transcription and amplification was done with MessageAmp II aRNA amplification kit (Ambion). Two-color labeling was performed with ULS aRNA fluorescent labeling kit reagents (Kreatech). All hybridizations were performed in quadruplicate including dye-swap controls. Microarray slides were scanned on a 428 Array Scanner (MWG) and images were analyzed with Imagene 6.1 (Biodiscovery). Data from biological replicates was analyzed with TM4 Microarray Software Suite including background subtraction and local LOWESS normalization (29).

Quantitative real-time PCR. Total RNA from bone marrow neutrophils was reverse transcribed with Ambion Arrayscript and 50 ng cDNA was used for Taqman analysis with the caspase-11 gene expression assay and 18S RNA as a control (Applied Biosystems). Real time PCR was performed on the BioRAD Opticon: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative quantification of transcript abundance was performed using the $\Delta\Delta$Ct method (30).

Murine MMP-8 Expression. The murine MMP-8 pro-enzyme was cloned with a FLAG-tag in the pGWIHG vector and expressed in Chinese hamster ovary cells. Protein from conditioned medium was purified on Reactive Green Agarose (Sigma) followed by FLAG affinity chromatography (Sigma). Before use, enzyme was activated for 1 h with 1 µM 4-aminophenymercuric acetate (Sigma) at 37 °C followed by desalting on a Zeba spin column (Pierce). Concentration was determined by

active site titration with murine tissue inhibitor of metalloproteinases-2 (TIMP-2).

Caspase-11 immunoblotting. Bone marrow neutrophils and mononuclear cells were isolated by Histopaque gradient as described above and treatments included 24 h incubation at 37 °C, 5% CO_2 with 100 ng/mL LPS (*E. coli* 0111:B4, Sigma), 10 µg/mL LPS, 10 ng/mL interferon-γ (Peprotech), 20 ng/mL murine TNF-α (Peprotech), or 1 µg/mL active murine MMP-8. Cell lysates for caspase-11 analysis were prepared by lysis in 50 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet-40, pH 7.4 containing EDTA-free protease inhibitor cocktail (Roche) for 30 min at 4 °C followed by 15 min centrifugation at 13,000 x g at 4 °C to remove insoluble material. Protein concentration was determined by Bradford assay (BioRAD). Proteins were separated on a 12% SDS-PAGE gel and transferred to Immobilon-P PVDF membrane (Millipore). Monoclonal rat anti-caspase-11 (Sigma) was used at 1:500 and secondary detection was with anti-rat-HRP (Sigma) followed by Enhanced Chemiluminescence (ECL) exposure (GE Healthcare) to X-omat blue film (Kodak).

Caspase activity and interleukin-1β ELISA. Caspase-1 and caspase-3 activity were measured following incubation of 2 x 10^6 cells/mL for 24 h at 37 °C, 5% CO_2 in DMEM containing 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin. Treatments included 100 ng/mL LPS (*E. coli* 0111:B4, Sigma), 10 µg/mL LPS, and 10 ng/mL interferon-γ (Peprotech). Lysates were prepared by incubation of 2 x 10^6 cells with 75 µL lysis buffer (Biovision) for 15 min at 4 °C followed by centrifugation at 13,000 x g to remove insoluble material. Protein content was determined by Bradford assay (BioRAD). Activity was measured in 20 mM HEPES, 10% glycerol, pH 7.5 containing 2 mM and 10 mM DTT for caspase-3 and caspase-1, respectively. Substrate concentrations were 50 µM Ac-YVAD-AMC (Calbiochem) for caspase-1 and 10 µM Ac-DEVD-AMC (Calbiochem) for caspase-3 and rates were measured at 390nm/460nm on a BMG Polarstar fluorescence plate reader. Rates were converted to pmoles/h per µg protein based on aminomethylcoumarin (Calbiochem) standard curves. Interleukin-1β was quantified in cell-free medium by ELISA quantikine kit (R&D Systems) as per manufacturers

protocol.

TNF-α cleavage and ELISA. Full length proTNF-α N-terminally fused to GST was expressed in *E. coli* and affinity-purified as described before (11) and incubated with human and rodent MMP-8 in 200 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij25, 50 mM Tris-HCl, pH 7.4. Incubation was at enzyme-substrate ratio of 1:100 (w:w) for 18 h at 37 °C. Synthetic hydroxymate inhibitor BB2116 was used at 20 μM as a negative control. Reaction mixes were analyzed on 6-15% gradient SDS-PAGE gels and protein bands in the gels were stained with coomassie G250 and in parallel gels were transferred to nitrocellulose for N-terminal Edman sequencing (5 cycles). TNF-α secretion from isolated neutrophils was quantified in cell-free medium by ELISA quantikine kit (R&D Systems) as per manufacturers protocol.

Neutrophil apoptosis. Spontaneous apoptosis of bone marrow-derived neutrophils was determined following 24 h incubation at 37 °C, 5% CO_2. Briefly, cytospins (Shandon) of 30,000 cells were prepared and stained with TUNEL (Roche) following manufacturers protocol with Hoechst nuclear staining. A minimum of four random fields were analyzed per slide and percentage of TUNEL positive cells was determined relative to Hoechst control.

Results

Generation of MMP-8 deficient MRL/lpr mice

To address the role of MMP-8 in inflammatory arthritis, *Mmp8*-null mice on a C57BL/6 x 129 background were backcrossed for 8 generations with the MRL/*lpr* lymphoproliferative strain. Genotype was determined by PCR with bands at 550 and 1100 bp for wild-type and knockout, respectively (Figure 4.1A), and was confirmed by western blotting (Figure 4.1B), showing an absence of MMP-8 in neutrophil lysates from MRL/*lpr* x *Mmp8* -/- mice. Mice lacking MMP-8 had normal development and fertility, comparable to wild-type controls. MMP-9, another MMP expressed in neutrophils, has been described previously to increase in the MMP-8 deficient
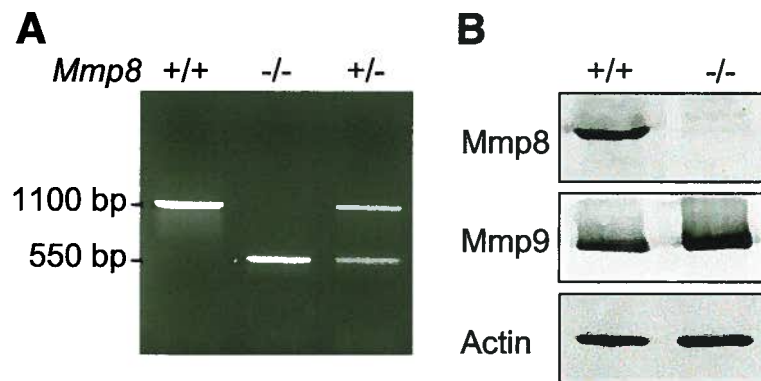
Figure 4.1. Generation and characterization of MRL/*lpr* x *Mmp8* -/- mice. (A) PCR genotyping results demonstrating wild-type and knockout products at 1100 and 550 base pairs, respectively. (B) Western blot of lysates of bone marrow neutrophils from *Mmp8* +/+ and *Mmp8* -/- mice (both on the MRL/*lpr* background) showing loss of MMP-8 and an increase in MMP-9 levels in MMP-8 deficient cells. Actin was analyzed as a loading control.

C57BL/6 x 129 strain (31). Here we also found a similar compensation on a different genetic background, confirming this to be an MMP-8 associated effect (Figure 4.1B).

More severe arthritis in male MMP-8 deficient MRL/lpr mice

The MRL/*lpr* model of arthritis is an age-dependent spontaneous inflammatory disease model that can be initiated and enhanced by injection of complete Freund's adjuvant (20). Therefore, the induction of arthritis was synchronized by injection at 13-14 weeks of age and MMP-8 wild-type and knockout mice on the MRL/*lpr* background were monitored for ankle swelling for 30 days, at which time humane endpoints were apparent and animals were sacrificed (*n*= 13 and 12 wild-type male and female, *n*= 11 and 8 knockout males and females). In the male group, MMP-8 knockout mice developed much earlier and more severe joint swelling than wild-type controls, with significant differences as early as 11 days post-treatment (Figure 4.2A). At day 30, average changes in ankle width were 0.25 +/- 0.02 and 0.56 +/- 0.06 mm for wild-type and knockout males, respectively. In contrast, female knockouts had no significant increase in swelling compared to wild-type females with final ankle width change of 0.32 +/- 0.03 and 0.36 +/- 0.07, respectively (Figure 4.2B).

Incidence of disease, characterized as a greater than or equal to 10% increase in ankle width for two consecutive measurements, reached 100% in knockout males but only 31% in wild-type males at 30 days, with a much earlier onset in knockout mice (Figure 4.2C). Female knockout mice had a slight shift toward earlier incidence, but this was due to one mouse only. MMP-8 knockout and wild-type females had similar penetrance (75% and 83%, respectively) (Figure 4.2D), consistent with previous reports studying arthritis in MRL/*lpr* mice (32). Severity of disease, as determined by maximal percent ankle width increase throughout the experiment, was greater in males deficient in MMP-8 (55% exhibiting severe swelling), and slightly more severe in MMP-8 deficient females (25% exhibiting severe swelling), whereas no wild-type mice had >20% increase in ankle width (Figure 4.2E, F). These results clearly display a protective effect of MMP-8 in the

Figure 4.2. Increased joint swelling in male MMP-8 deficient MRL/*lpr* mice. (A) Mice were monitored for changes in hind ankle width for 30 days following treatment with complete Freund's adjuvant and male MMP-8 deficient mice developed more severe swelling than wild-type controls (N=13 and 11 for WT and KO males, respectively) (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). (B) No significant change was detected in comparing female MMP-8 knockout mice with wild-type controls (N=12 and 8 for WT and KO females, respectively). (C, D) Disease incidence was defined as >10% increase in ankle width (relative to baseline width) for at least two consecutive measurements. (C) Male MMP-8 knockout mice reached 100% incidence at day 30 whereas only 31% of wild-type males displayed measurable disease. (D) Female wild-types and knockout mice had similar incidence with a slightly earlier onset in the knockout population. (E,F) Severity of disease was graded based on the maximal width increase (%) throughout the experiment: mild (0-10%), moderate (10-20%), and severe (>20%). (E) Male MMP-8 knockouts had much more severe disease with over 50% of mice having severe disease whereas female knockouts (F) had a modest shift toward increased severity.
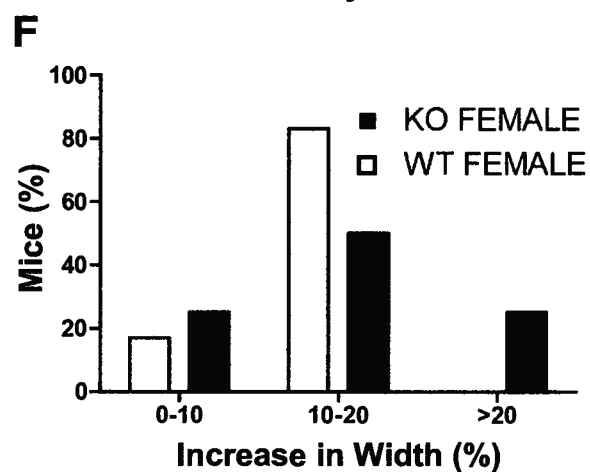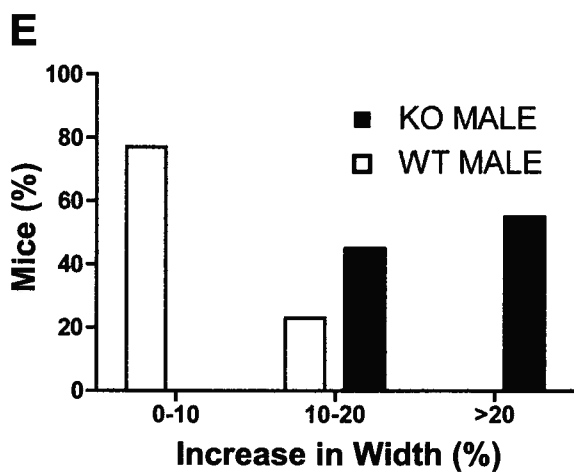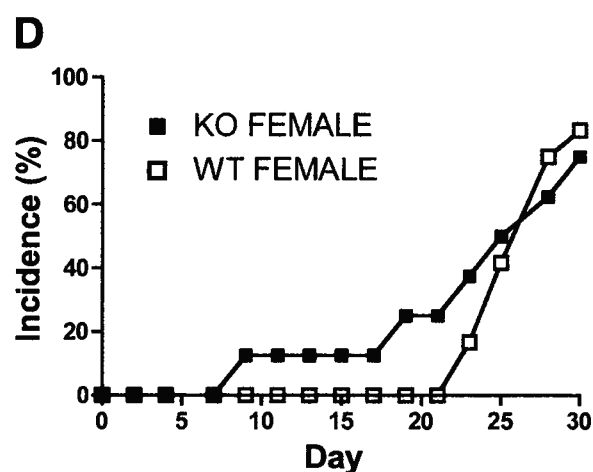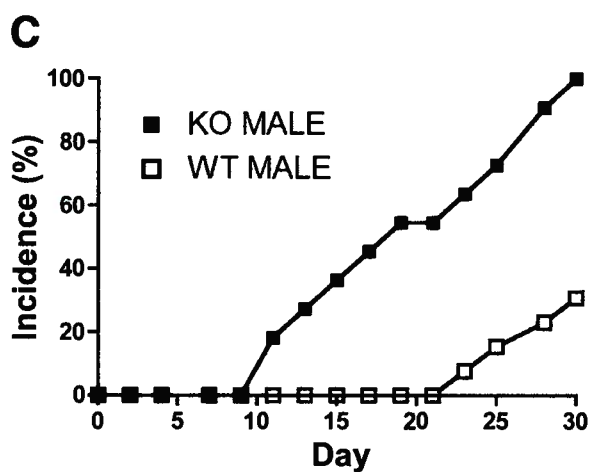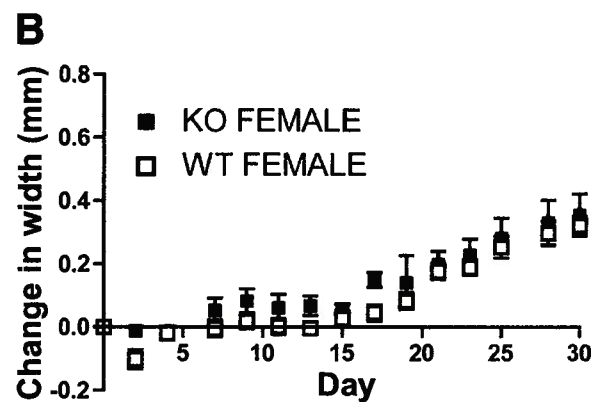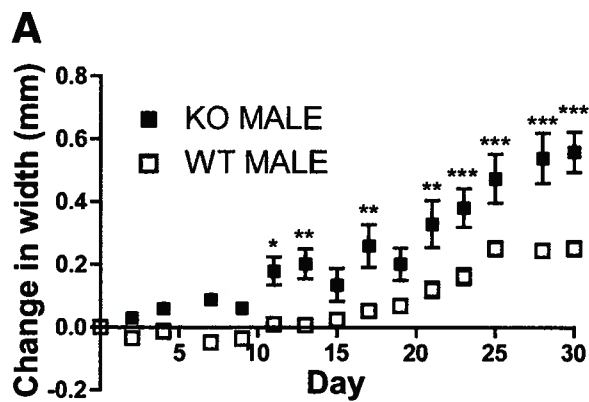
clinical manifestations of inflammatory arthritis, predominantly in male mice. Furthermore, this gender-based effect exemplifies the importance of studying both sexes in animal models of inflammatory disease.

Histological assessment of arthritis

Hematoxylin and eosin staining of sectioned joints revealed increased cellular infiltration in the MMP-8 knockout males, relative to wild-type males, with an abundance of inflammatory cells in the subsynovial tissue and some cellularity in the joint space (Figure 4.3A). In contrast, female wild-types and knockouts had similar levels of cellular infiltration (Figure 4.3B). Scoring of inflammatory content revealed a significant increase in knockout males ($p < 0.001$) with no change in females (Figure 4.3I, J). Toluidine blue staining for proteoglycans, a measure of cartilage integrity, displayed an absence of cartilage damage in all groups, a surprising finding given the extent of inflammation present in these tissues (Figure 4.3C and D). MMP-8 cleaves aggrecan and so genetic deficiency of MMP-8 might account for this despite the large amount of cellular infiltrate (33). However, use of neo-epitope antibodies to detect MMP-8 processed murine type II collagen and murine aggrecan resulted in undetectable amounts, supporting a lack of cartilage degradation in the wild-type or MMP-8 knockout mice in this model (data not shown). Immunostaining demonstrated a massive accumulation of neutrophils in the subsynovial tissue surrounding swollen joints and also in the joint space of joints from MMP-8 deficient male mice and all female synovial tissues (Figure 4.3E-H). Macrophages and T lymphocytes were also detected with levels correlating with overall inflammation (data not shown).

CLIP-CHIP™ microarray analysis of MMP-8 deficient neutrophils

The CLIP-CHIP™, a DNA microarray dedicated to 823 proteases, non-proteolytic homologs and protease inhibitors (28), was employed to examine if compensation by other MMPs or changes in the protease web occurred following deletion of MMP-8. By the criteria of greater than 1.5-fold change in expression with $p < 0.01$, only 8

Figure 4.3. Histological analysis of joints reveals increased neutrophil infiltration in male MMP-8 deficient mice. Hematoxylin and eosin staining of sections from wild-type and MMP-8 knockout male (A) and female (B) mice. A, articular surface; B, bone marrow; S, subsynovium. Toluidine blue staining for proteoglycan content and cartilage integrity in male (C) and female (D) ankle sections. (E-H) Neutrophil immunostaining showing an abundance of neutrophils in the subsynovium and joint space of sections from male MMP-8 deficient and all female joints. Scoring of cellular infiltration (I,J) by a blinded observer demonstrating significantly increase subsynovial inflammation in male *Mmp8*-null mice (p<0.001). Original magnifications x100 (A-F) and x400 (G-H). Scale bar is equivalent to 100 mm.

MALE

Mmp8 +/+   Mmp8 -/-
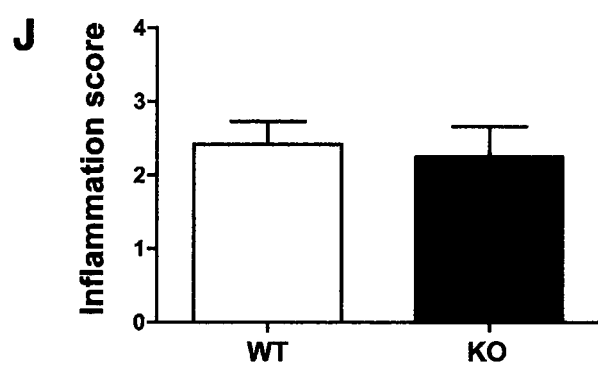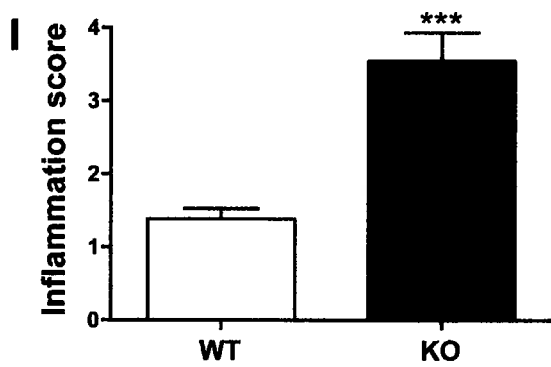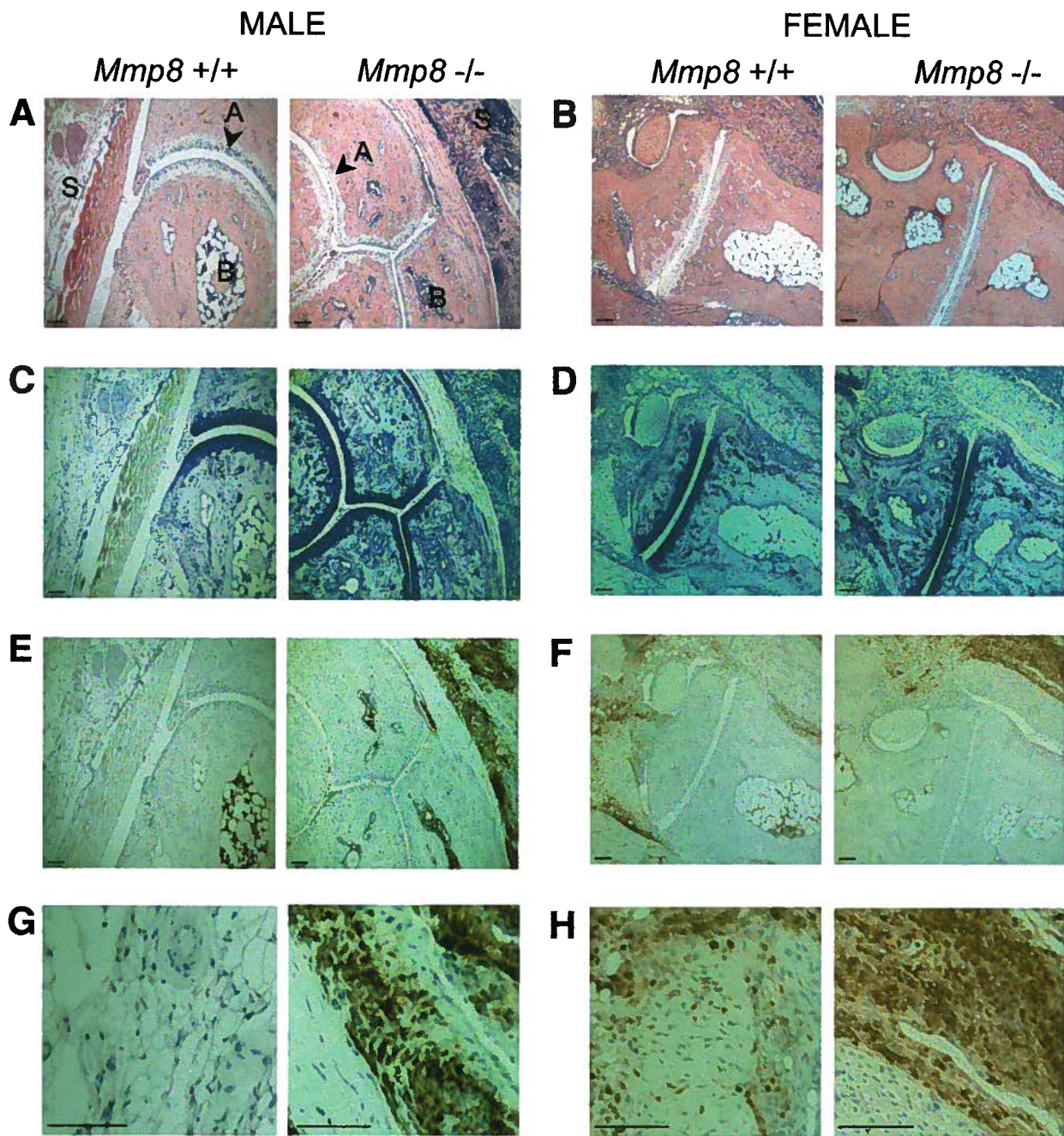
FEMALE

Mmp8 +/+   Mmp8 -/-

genes in male neutrophils and 9 in female neutrophils were differentially expressed between wild-type and knockout samples (Figure 4.4A). Hence, protease and inhibitor expression was essentially unaltered between wild-type and MMP-8 deficient bone marrow neutrophils, indicating minor homeostatic alterations in the protease web following loss of MMP-8 expression as demonstrated in a representative R-I (ratio versus intensity) plot (Figure 4.4B). These changes represent approximately 1% of all analyzed genes. Internal controls of the transcript analysis were MMP-8 and neomycin, which is the selection marker used in generating the *Mmp8*-null mice.

A notable common change to both male and female neutrophils was caspase-11, the mouse homolog of human caspases 4 and 5, which was unexpectedly downregulated in MMP-8 knockout samples. Moderate changes in male MMP-8 deficient neutrophils include decreased levels of stefin A1, stefin A2, kallikrein related peptidase-11, and cationic trypsin. In female MMP-8 knockout neutrophils the transcripts for ubiquitin specific protease (USP)-13, USP-31, tissue inhibitor of metalloproteinases (TIMP)-2, and complement factor I were downregulated. Other than neomycin, few genes were upregulated in the absence of MMP-8. Kallikrein related peptidase-9 was increased, however expression levels were very low, only slightly above background. In addition, carboxypeptidase A5 was increased 1.6-fold in female MMP-8 knockout neutrophils.

Caspase-11 is not expressed in MMP-8 deficient neutrophils

Caspase-11 was explored further due to its roles in caspase-3 and caspase-1 activation, with consequent implications in apoptosis and inflammation (17). Relative quantitation by real time PCR generated a DDCT value of 10.7, translating to a greater than 1600-fold change in caspase-11 transcript levels, showing that caspase-11 expression is absent in MMP-8 deficient neutrophils (Figure 4.5A). Notably, this differential caspase-11 expression was also observed in neutrophils

A

MALE (KO:WT)

Gene	Fold Change	P value
Neomycin	13.5 ↑	2E-8
Kallikrein-9	6.8 ↑	0.009
Stefin A1	1.5 ↓	0.003
Stefin A2	1.6 ↓	0.008
Kallikrein-11	1.7 ↓	0.003
Cationic Trypsin	1.8 ↓	4E-5
Caspase-11	8.3 ↓	8E-4
MMP-8	10.4 ↓	8E-8

FEMALE (KO:WT)

Gene	Fold Change	P value
Neomycin	14.2 ↑	1E-7
Kallikrein-9	6.7 ↑	4E-4
Carboxypept. A5	1.6 ↑	0.009
USP13	1.6 ↓	0.005
USP31	1.7 ↓	0.006
TIMP-2	1.9 ↓	0.004
Complem. factor I	2.5 ↓	0.007
Caspase-11	6.0 ↓	1E-5
MMP-8	14.2 ↓	4E-8

B

Not expressed ◄——► Expressed

R [log_2(KO/WT)]

Neomycin

Caspase-11

MMP-8

I [log_{10}(WT•KO)]

Figure 4.4. CLIP-CHIP™ analysis of bone marrow neutrophils from MMP-8 knockout and wild-type MRL/*lpr* mice. (A) Differential expression of proteases and inhibitors with p<0.01 in male and female bone marrow neutrophils. Changes are relative to MMP-8 knockout samples. USP, ubiquitin specific protease; TIMP, tissue inhibitor of metalloproteinases. (B) Representative R-I (ratio versus intensity) scatter plot of data following local LOWESS normalization. An intensity sum of 7 illustrates the boundary between expression levels and low signal. Neomycin, MMP-8, and Caspase-11 are highlighted.

following *ex vivo* stimulation with LPS, known to induce caspase-11 expression, and also in neutrophils isolated from the original C57BL/6 x 129 strain (Figure 4.5A).

Western blots confirmed that caspase-11 was absent in MMP-8 deficient neutrophil lysates and was not induced by treatment with interferon-γ or LPS, treatments that resulted in increased levels in wild-type neutrophils (Figure 4.5B). Surprisingly, caspase-11 was also absent and non-inducible in the mononuclear cell fraction (predominantly monocytes and T lymphocytes) from MMP-8 deficient mice, cells that do not produce MMP-8, suggesting potential paracrine activation of caspase-11 expression in development during hematopoiesis (Figure 4.5C). We attempted to induce caspase-11 levels in MMP-8 knockout neutrophils and mononuclear cells by rescuing with exogenous active murine MMP-8 or with cell-free bone marrow fluid isolated from wild-type mice. However, caspase-11 levels remained unchanged (Figure 4.5D and data not shown). Hence, MMP-8 regulation of caspase-11 gene expression may require factors from additional cell types or could occur upstream in hematopoietic differentiation.

Unaltered caspase-1 activity and interleukin-1β secretion in MMP-8 deficient neutrophils

Previous reports have defined caspase-11 as an essential activator of caspase-1, the interleukin-1 converting enzyme that is key for IL-1β maturation and release (34). In response to increasing concentrations of LPS, bone marrow derived MRL/*lpr* neutrophils had elevated caspase-1 activity, as measure by the fluorometric synthetic peptide substrate Ac-YVAD-AMC (Figure 4.6A). However, comparing wild-type and *Mmp8*-null neutrophils, there was no significant difference in caspase-1 activity, despite substantial differences in caspase-11 expression. LPS-mediated induction of caspase-1 activity was more pronounced in mononuclear cells, reaching an activity greater than 0.4 pmoles/h/µg (Figure 4.6B), compared to 0.1 pmol/h/µg in neutrophils. However, caspase-1 activities in wild-type and MMP-8 deficient mononuclear cells were also unchanged (Figure 4.6B). Consistent with unaffected

Figure 4.5. Neutrophils and mononuclear cells from MMP-8 deficient mice have abolished constitutive and inducible levels of caspase-11. (A) Relative quantification of caspase-11 transcript by real-time PCR with Taqman probes revealed loss of expression in *Mmp8* -/- neutrophils from either MRL/*lpr* or C57BL/6x129 mouse strains. This reduction was maintained following 1 h treatment of MRL/*lpr* neutrophils with 10 μg/mL LPS. Lysates (50 μg total protein) from bone marrow neutrophils (B) and mononuclear cells (C) untreated or incubated with 10 ng/mL IFN-γ, 0.1 μg/mL or 10 μg/mL LPS show a loss of caspase-11 protein levels in cells from male MMP-8 deficient mice. (D) Addition of 1 μg/mL active murine MMP-8 failed to rescue the caspase-11 expression defect. Actin intensity is used as a loading control.

A

SAMPLE	$\Delta\Delta CT$ (WT:KO)	$2^{\Delta\Delta CT}$ (WT:KO)
MRL/*lpr*	10.7 +/- 0.4	1660
MRL/*lpr* + LPS	9.2 +/- 0.2	590
C57BL/6x129	9.9 +/- 0.4	960

B

C

D
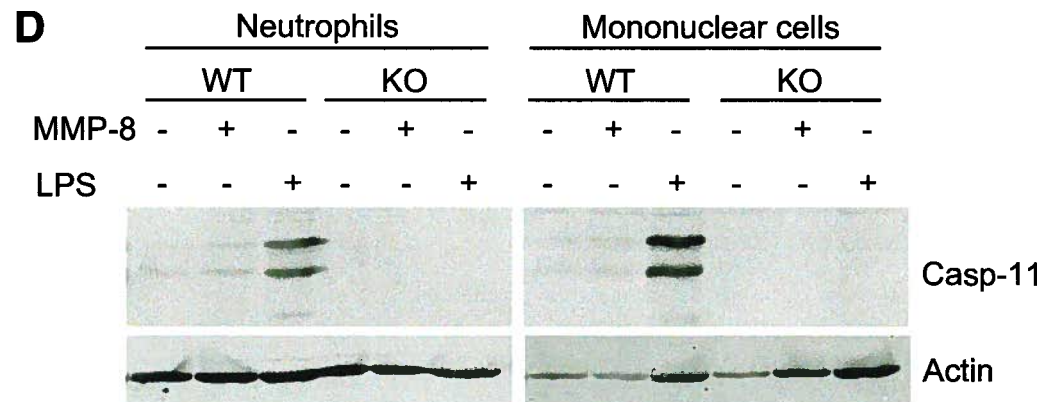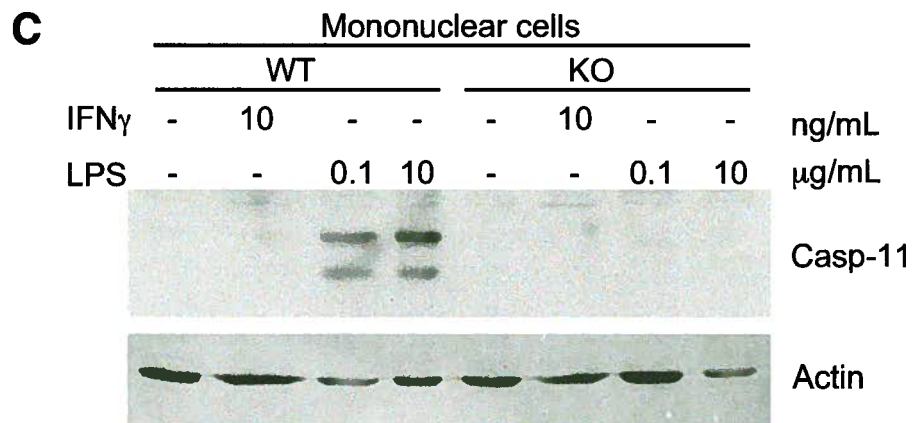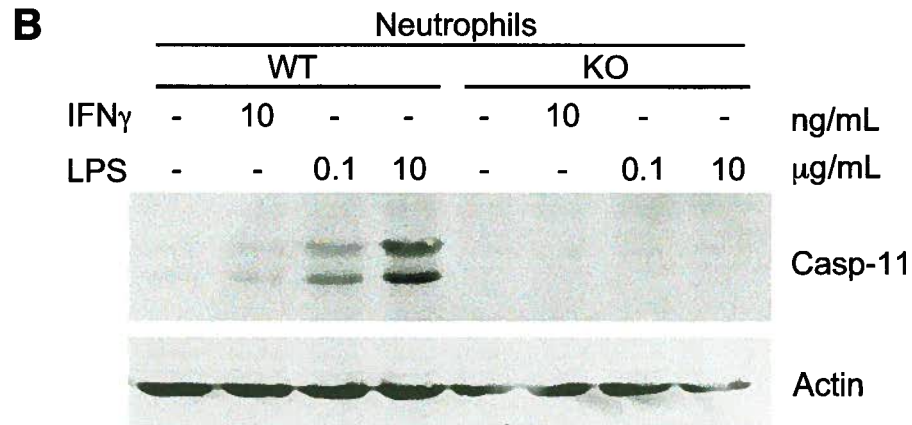
Figure 4.6. MMP-8 deficient neutrophils have unaltered caspase-1 activity and IL-1β secretion. Caspase-1 activity, measured by Ac-YVAD-AMC cleavage, was unchanged in untreated, IFN-γ, or LPS stimulated male MMP-8 deficient neutrophils (A) and mononuclear cells (B) compared to wild-type control cells (n=3). (C) IL-1β secretion (pg/mL) following 24 h culture of bone marrow neutrophils, as determined by ELISA (n=3). Histograms show the means and the SEM for samples in each group where each sample was from an independent mouse.

A Neutrophils

Caspase-1 Activity Ac-YVAD-AMC Cleavage (pmol/h/μg)

WT
KO

Untreated | IFN-γ (10 ng/mL) | LPS (0.1 μg/mL) | LPS (10 μg/mL)

B Mononuclear cells

Caspase-1 Activity Ac-YVAD-AMC Cleavage (pmol/h/μg)

WT
KO

Untreated | IFN-γ (10 ng/mL) | LPS (0.1 μg/mL) | LPS (10 μg/mL)

C

IL-1β (pg/mL)

WT
KO

Untreated | LPS (0.1 μg/mL) | LPS (10 μg/mL)

120
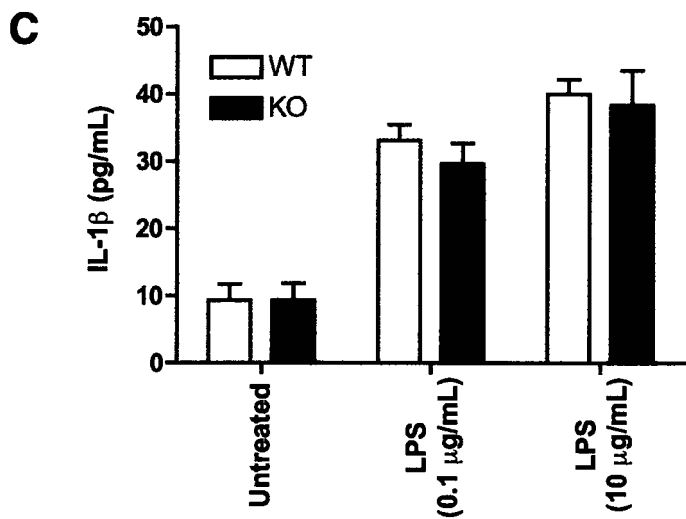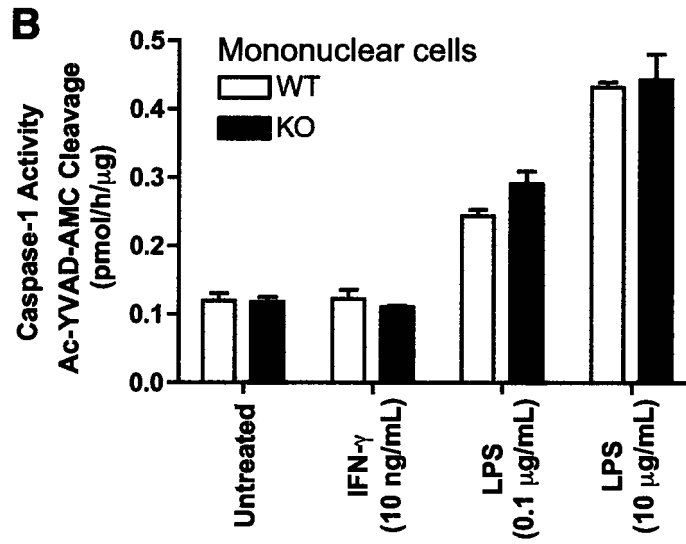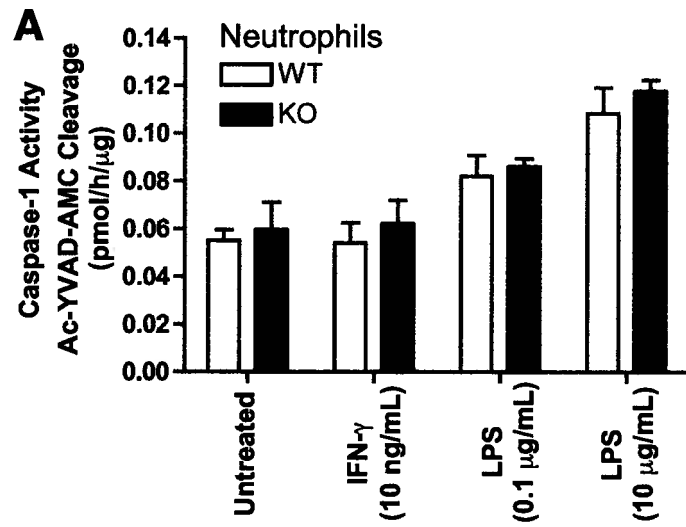
caspase-1 activity, secreted interleukin-1β concentrations from untreated and LPS-treated neutrophils were unaltered in MMP-8 deficient samples (Figure 4.6C).

Proteolytic cleavage and shedding of TNF-α by MMP-8

TNF-α is a central pro-inflammatory cytokine that is unique in that it can also induce apoptosis (35). TNF-α is produced as a membrane-associated inactive precursor that is solubilized upon proteolysis, an event that is required for normal inflammatory functions via TNFR1 (36). Previous work has demonstrated that a number of MMPs can process proTNF-α to generate the active soluble form, both biochemically (11) and in vivo (37, 38). In vitro incubation of a GST-proTNF-α fusion protein with human and rodent MMP-8 resulted in efficient and specific cleavage in the prodomain (Figure 4.7A). N-terminal sequencing defined the new N-terminus as VRSSS, which is the physiological terminus of soluble TNF-α. Bone marrow derived MMP-8 deficient neutrophils were examined for their ability to shed TNF-α (Figure 4.7B). In untreated MMP-8 knockout neutrophils, TNF-α shedding was absent and upon IFN-γ treatment, soluble TNF-α was reduced compared with wild-type neutrophils. However, after LPS treatment there was no difference, likely due to induction of ADAM-17, the predominant TNF-α converting enzyme in infection (39, 40). The effect of exogenous soluble TNF-α on caspase-11 expression was examined (Figure 4.7C). Wild-type neutrophils had significantly elevated caspase-11 expression in response to TNF-α but neutrophils from MMP-8 deficient mice had undetectable levels.

Caspase-3 activity and constitutive neutrophil apoptosis

Caspase-11 deficient mice have reduced apoptosis and cell death in pathological states due to decreased activation of caspase-3, which is known to be important in the execution phase of apoptosis (41-43). Therefore, caspase-3 activity was measured in leukocytes from wild-type and MMP-8 deficient mice using the
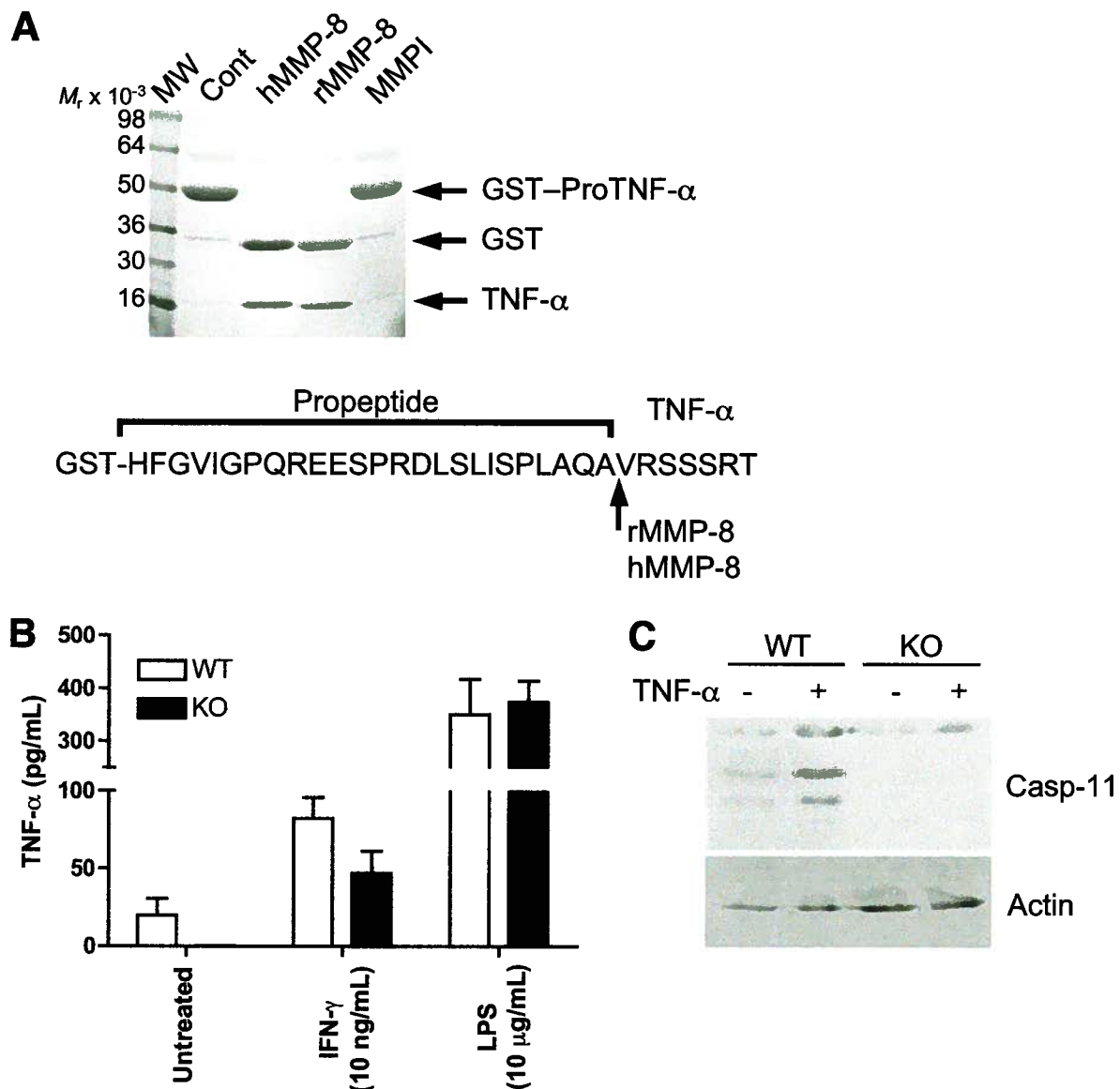
Figure 4.7. MMP-8 mediated cleavage of proTNF-α and shedding of TNF-α from neutrophils. (A) Full length proTNF-α N-terminally fused to GST was incubated with human (h) and rodent (r) MMP-8 resulting in cleavage in the propeptide. *MMPI*, assays performed in the presence of 20 µM BB2112 hydroxamate inhibitor. Proteins were analyzed on a 6-15% gradient SDS-PAGE, stained with Coomassie G250. Original gel contained other MMP reactions that were removed from figure for presentation. Cleavage products were analyzed by N-terminal sequencing to determine cleavage site. Experiment was performed by Dr. Christopher Overall. (B) TNF-α secretion from bone marrow neutrophils from wild-type and MMP-8 deficient mice (*n*=3, histogram shows SEM). Neutrophils were incubated for 24 h in media alone (untreated), 10 ng/mL IFN-γ, or 10 µg/mL LPS and cell-free supernatant was analyzed for TNF-α by ELISA. (C) Bone marrow neutrophils were stimulated for 24 h with 20 ng/mL murine TNF-α and the levels of caspase-11 were determined by western blot.

fluorometric synthetic peptide substrate Ac-DEVD-AMC (Figure 4.8). Both untreated and interferon-γ treated MMP-8 knockout neutrophils had significantly reduced caspase-3 activity compared to wild-type cells (p=0.005 and 0.016, respectively) (Figure 4.8A). This difference was abolished in the presence of LPS, an inhibitor of neutrophil apoptosis (44), resulting in reduced caspase-3 activity in both wild-type and knockout neutrophils. Treatment with TNF-α caused elevated caspase-3 activity in both wild-type and knockout neutrophils. In contrast, no significant change in Ac-DEVD-AMC activity was observed in parallel treatments of isolated mononuclear cells from wild-type and MMP-8 deficient mice (Figure 4.8B), suggesting a neutrophil-specific effect that is driven by MMP-8.

Previous phenotypes in MMP-8 deficient mice, including increased airway inflammation and delayed wound healing, were proposed to be due to an apoptotic defect (31, 45). Therefore, the accumulation of neutrophils in joints of MMP-8 deficient mice and the absence of caspase-11 in leukocytes from MMP-8 knockout mice prompted the examination of constitutive apoptosis in isolated neutrophils. A 34% decrease in TUNEL-positive cells in the knockout population (p=0.04), from 14.3% to 9.5%, was found (Figure 4.8C, D), and wild-type cell apoptotic staining was consistent with reported values (46). Therefore, we propose that impaired apoptosis in neutrophils from MMP-8 knockout mice involves two novel pathways: abolished caspase-11 expression and reduced TNF-α shedding from MMP-8 deficient neutrophils (Figure 4.9). These mechanisms potentially contribute to the accumulation of inflammatory cells in arthritic disease and other inflammatory disorders in MMP-8 deficient mice.
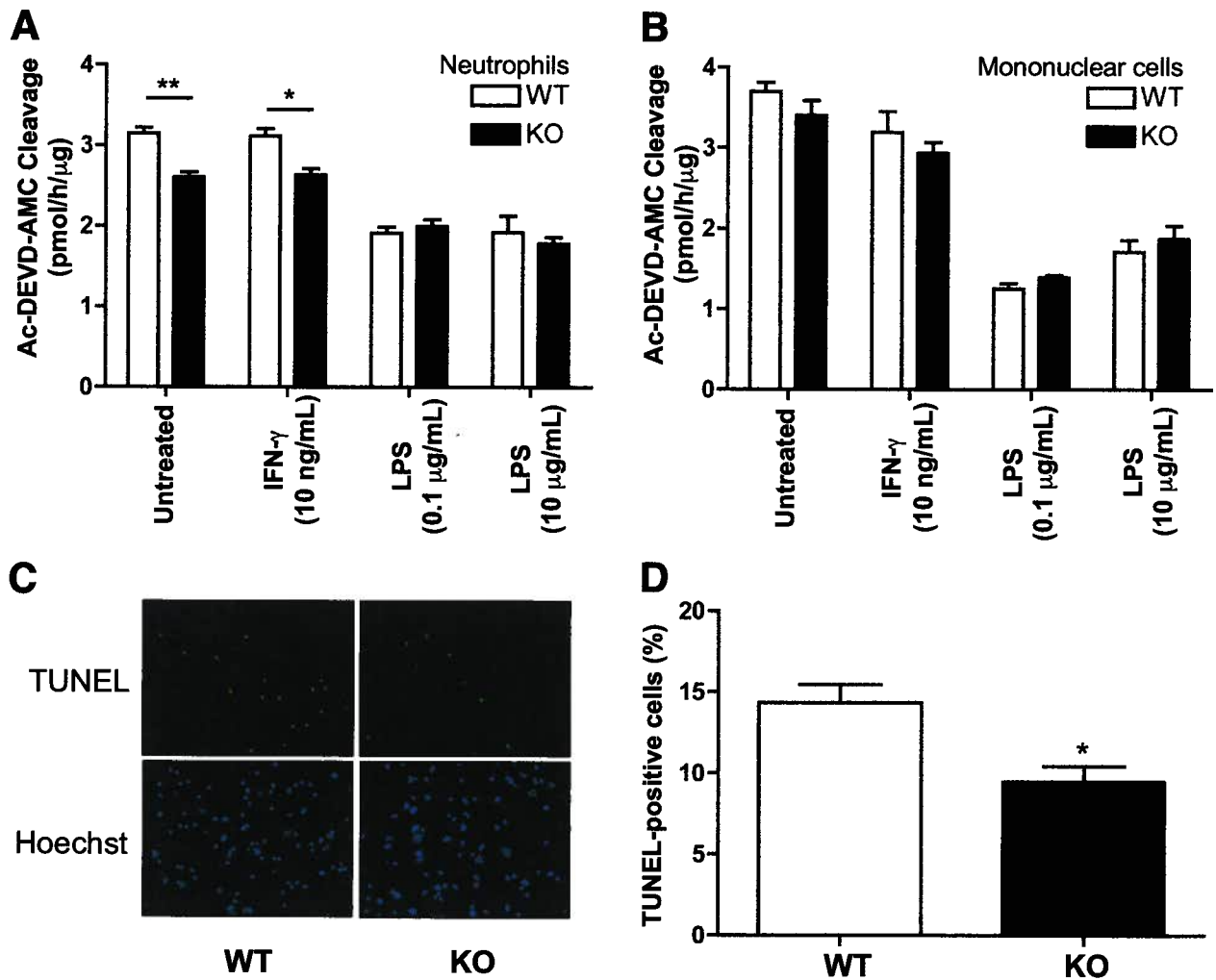
Figure 4.8. Reduced caspase-3 activity and TUNEL staining in MMP-8 knockout neutrophils. Activity assay for caspase-3 with fluorometric substrate Ac-DEVD-AMC with neutrophil (A) and mononuclear cell (B) lysates from wild-type and MMP-8 knockout male mice ($n=3$) (* $p<0.05$, ** $p<0.01$). Constitutive apoptosis of bone marrow neutrophils from MMP-8 wild-type and knockout mice. (C, D) Cells were cultured for 24 h and cytospins were analyzed for DNA fragmentation by TUNEL staining (C) and percentage of TUNEL-positive cells was calculated relative to Hoechst control with MMP-8 knockout cells having significantly less apoptosis ($p=0.04$, $n=3$) (D). All histograms show the means and the SEM.
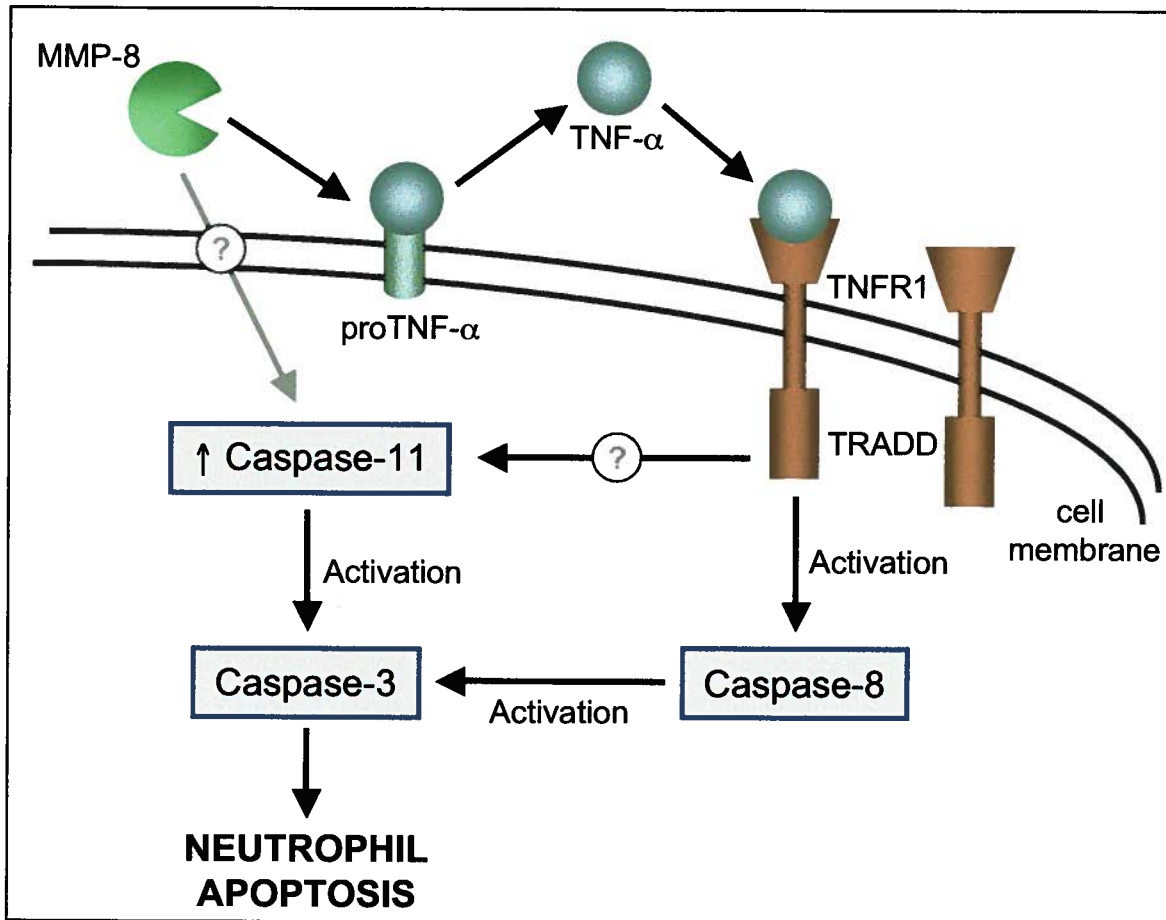
Figure 4.9. Proposed mechanisms for MMP-8 promotion of programmed cell death through induction of caspase-11 expression and shedding of TNF-α. MMP-8 expression and activity in the neutrophil enables basal caspase-11 expression and responsiveness to LPS and IFN-γ through an unknown pathway. MMP-8 also cleaves cell membrane proTNF-α to generate soluble TNF-α which signals through TNFR1 (TNF receptor 1) and TRADD (TNF receptor associated death domain) to induce caspase 11 and activate caspase-8. Caspase-8 activates caspase-3, leading to neutrophil apoptosis. Therefore, MMP-8 acts via multiple pathways that converge to execute apoptosis in the neutrophil.

Discussion

Our experiments reveal the surprising finding that the secreted neutrophil-specific protease MMP-8 is a key regulator in the expression of caspase-11, basal shedding of TNF-α, and the orchestration of neutrophil apoptosis. We reported previously that MMP-8, originally described as a collagenase, promotes neutrophil migration in acute inflammation not by collagen degradation, but by processing of the ELR$^+$ chemokine LIX in mouse and CXCL5/ENA-78 and CXCL8/IL-8 in human (19). In contrast, the resolution of inflammation requires the termination of neutrophil influx and clearance of infiltrated cells. Here we report that male leukocytes from MMP-8 deficient mice lack caspase-11 expression at the RNA transcript and protein level accompanied by reduced activity of caspase-3 and decreased constitutive apoptosis. Moreover, MMP-8 deficient neutrophils exhibited reduced TNF-α shedding. Biologically, this was reflected by an exaggerated accumulation of infiltrated neutrophils in a murine model of autoimmune rheumatoid arthritis, correlating with increased joint swelling, and so likely contributes to the disease pattern. Hence, MMP-8 is protective in a murine model of autoimmune arthritis by enabling clearance of neutrophils through normal levels of apoptosis, so defining MMP-8 as an anti-target for arthritis treatment.

Neutrophil apoptosis is a critical process in the resolution of inflammation. Upon removal of an inflammatory stimulus, neutrophils rapidly undergo programmed cell death through TNF-α, Fas-dependent, or constitutive intrinsic mechanisms (47). Several caspases are key effector molecules in apoptosis, forming the apoptosome, with caspase-3 functioning as an execution phase caspase (48). Levels of active caspase-3 correlate with increased apoptosis (49). Caspase-3 is expressed as a pro-enzyme and can be proteolytically activated by several caspases, including caspase-11 (42). Here we report a novel regulatory interaction between MMP-8 and caspase-11. Interestingly other MMPs have pro-apoptotic roles in non-immune cell types. For example, MMP-7 (matrilysin) promotes epithelial cell apoptosis through processing of FasL (50, 51).

By means of DNA microarray analysis, we discovered that MMP-8 deficiency abolishes expression of caspase-11, along with ten other alterations in the protease web. Importantly, complete loss of caspase-11 expression was also observed in neutrophils from MMP-8 deficient C57BL/6 x 129 mice, demonstrating a strain-independent association between MMP-8 and caspase-11. Isolation of bone marrow neutrophils and mononuclear cells from MMP-8 knockout mice confirmed an absence of caspase-11, even following treatment with LPS or interferon-γ, previously reported to induce caspase-11 expression through nuclear factor-κB and signal transducer and activator of transcription (STAT)-1, respectively (52). Interestingly, the genes for *Mmp8* and *Casp11* are both located on mouse chromosome 9 but are separated by over 2 million base pairs and are located at 7.56 and 5.31 megabases, respectively. Notably, the *Casp11* gene is adjacent to other inflammasome caspases (53), *Casp1* and *Casp12,* both of which had normal expression in MMP-8 deficient neutrophils. This further reduces the likelihood that genetic disruption of the capsase-11 gene by recombination during the knockout of *Mmp8* accounted for these results. Surprisingly, high concentrations of exogenous active murine MMP-8 had no effect on the expression of caspase-11 in either wild-type or MMP-8 knockout cells. This suggests that the regulatory role of MMP-8 requires factors derived from surrounding cell types or that an MMP-8 dependent regulatory event occurs upstream in hematopoietic differentiation that persists independent of the presence or absence of MMP-8 activity.

Caspase-11, the murine homolog of human caspases 4 and 5, is a cysteine protease involved in cytokine maturation and programmed cell death through the proteolytic activation of caspase-1 and caspase-3, respectively (17, 42). Furthermore, caspase-11 regulates cell migration through actin interacting protein-1, an activator of cofilin-mediated actin depolymerization (54). Nonetheless, we previously showed that the chemotactic ability of MMP-8 knockout neutrophils *in vivo* is no different than wild-type neutrophils (19). MMP-8 deficient bone marrow neutrophils have reduced caspase-3 activity compared to wild-type neutrophils. Reduced caspase-3 activity correlated with decreased constitutive apoptosis in

isolated bone marrow neutrophils from MMP-8 deficient mice. However, this difference was negated upon treatment with LPS where both wild-type and MMP-8 deficient neutrophils had reduced caspase-3 activity, consistent with the anti-apoptotic effect of LPS (44). Caspase-1 activity and IL-1β secretion were unchanged in neutrophils and mononuclear cells from MMP-8 deficient mice. Though caspase-11 has been described as an essential activator of caspase-1 (34), previous studies with caspase-11 deficient mice have revealed tissue-dependent phenotypes. For instance, intraperitoneal injection of LPS causes reduced IL-1β levels whereas intravenous administration yields normal secretion of IL-1β, relative to wild-type controls (55). As such, the tissue microenvironment plays a decisive role in caspase-11 activation of caspase-1 and consequent IL-1β secretion, suggesting that caspase-11-independent pathways exist. Our results reveal the possibility of such an alternate pathway in the neutrophil. Although our data show no change in IL-1β levels, we cannot discount the possibility that IL-1β levels are reduced in MMP-8 knockouts in other cellular microenvironments or disease contexts.

TNF-α plays a central role in the pathogenesis of rheumatoid arthritis through the regulation of cytokine, chemokine and MMP production, recruitment of inflammatory cells into joints, angiogenesis, and cell death (56). It was previously reported that MMP-7 and MMP-12 contribute to TNF-α shedding and activation in vivo (37, 38), although ADAM-17 is the major TNF-α converting enzyme in response to LPS stimulation or bacterial infection. Here we show for the first time that MMP-8 cleaves proTNF-a in vitro, specifically and efficiently generating the mature form detected physiologically. In comparison to other collagenolyic MMPs (-1, -2, -13, -14) only MMP-8 produced exclusively the correctly processed active form (data not shown). Unstimulated and interferon-γ treated MMP-8 deficient neutrophils showed decreased shedding of TNF-α, compared to wild-type neutrophils, whereas LPS treatment resulted in equal levels presumably due to induction of ADAM-17. However, LPS stimulation and the consequent role of ADAM-17 in TNF-α shedding is likely not relevant in the context of autoimmune disease. The reduction in soluble

TNF-α production in the absence of MMP-8 might reduce an autocrine TNF-α mediated apoptotic mechanism within the neutrophil, hence delaying apoptosis and resolution of inflammation. Decreased levels of soluble TNF-α in the context of arthritis could explain the lack of cartilage and bone damage observed in the MMP-8 knockout male mice, despite an abundance of infiltrated inflammatory cells. Notably, TNF-α is a potent inducer of MMP expression in arthritic chondrocytes and synovial fibroblasts (57). Therefore, less TNF-α shedding from MMP-8 deficient neutrophils in the joint should result in decreased production of other MMPs, namely MMP-13, now considered to be the predominant MMP drug target in treating arthritis (58).

Neutrophils are essential for the initiation and maintenance of chronic joint inflammation in rheumatoid arthritis (5). However, the roles of neutrophil-derived proteases are poorly defined in the onset and progression of arthritis. The creation of the novel MMP-8 deficient MRL/lpr mouse strain, a genetic background characterized by systemic autoimmunity including rheumatoid arthritis (59), enabled the evaluation of MMP-8 in inflammatory arthritis. Male MMP-8 knockout mice had much earlier and more severe arthritis as compared with wild-type controls, whereas female MMP-8 knockouts had unaltered clinical disease and histology, relative to female wild-type counterparts. The gender dependence of MMP-8 in chronic inflammation was observed previously in skin carcinogenesis where the protective role of MMP-8 only occurred in male mice and was rescued by bone marrow transplant (18). In the present study we have demonstrated the same gender effect in a different inflammatory disease and genetic background, thereby supporting the original finding. Despite the marked accumulation of neutrophils in MMP-8 deficient joints of male mice that we attribute to reduced apoptosis, there was a surprisingly low level of cartilage destruction. This is perhaps reflective of the slow progressive nature of this spontaneous model of chronic arthritis that can only be evaluated over a relatively short time span due to deteriorating health of the MRL/lpr mice beyond 18 weeks of age. More acute and aggressive arthritis models would be useful to clarify the role of MMP-8 in cartilage destruction, but these models are less predictive of chronic human rheumatoid arthritis.

129

Our data highlight the complex effects of different MMPs in arthritic disease. The roles of individual MMPs in arthritis remain poorly defined despite progress with genetic knockout mice, recently revealing contradictory roles of MMPs in disease progression and severity. In the case of the gelatinolytic MMPs, MMP-2 (gelatinase A) deficient mice had more severe clinical and histological arthritis compared with wild-types whereas the MMP-9 (gelatinase B) knockouts displayed milder arthritis (60). In contrast, MMP-3 deficient mice had no significant change in a collagen-induced arthritis model (61). The MMP-13 over-expressing transgenic mouse showed increased destruction of articular cartilage reminiscent of human osteoarthritis (62), but the role of MMP-13 at physiological levels is unknown. Global interpretation of these studies is difficult given the diverse nature of arthritis models that range from acute inflammation to spontaneous chronic inflammation as investigated here. Due to the short life span of mice, inflammatory changes are not always associated with the cartilage destruction that is typical in chronic human disease.

In regulating neutrophil apoptosis and clearance, MMP-8 exhibits a potent and novel anti-inflammatory role in chronic inflammation in the context of rheumatoid arthritis, thereby defining MMP-8 as an anti-target in the disease. A protective role of certain MMPs in joint inflammation is not unexpected in light of musculoskeletal side effects in clinical trials with broad-spectrum MMP inhibitors (63). Undoubtedly, it will be necessary to spare MMP-8 in the development of novel selective MMP inhibitors for treating chronic diseases requiring long-term dosing. MMP-8 has also emerged as an anti-target in cancer therapy (13, 18), but surprisingly has a detrimental effect in mouse models of multiple sclerosis where MMP-8 deficient mice have improved outcome (64). Clearly the disease and organ-dependent functions of MMP-8 need to be explored further, in combination with substrate discovery efforts, to decipher the physiological mechanisms and functions of this so-called collagenase, whose roles clearly extend beyond extracellular matrix degradation.

References

1. Duan, H., T. Koga, F. Kohda, H. Hara, K. Urabe, and M. Furue. 2001. Interleukin-8-positive neutrophils in psoriasis. *J. Dermatol. Sci.* 26:119-124.

2. Mitsuyama, K., A. Toyonaga, E. Sasaki, K. Watanabe, H. Tateishi, T. Nishiyama, T. Saiki, H. Ikeda, O. Tsuruta, and K. Tanikawa. 1994. IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin. Exp. Imm.* 96:432-436.

3. Chatham, W. W., R. Swaim, H. Frohsin, Jr., L. W. Heck, E. J. Miller, and W. D. Blackburn, Jr. 1993. Degradation of human articular cartilage by neutrophils in synovial fluid. *Arthritis Rheum.* 36:51-58.

4. Mohr, W., H. Westerhellweg, and D. Wessinghage. 1981. Polymorphonuclear granulocytes in rheumatic tissue destruction. III. an electron microscopic study of PMNs at the pannus-cartilage junction in rheumatoid arthritis. *Ann. Rheum. Dis.* 40:396-399.

5. Wipke, B. T., and P. M. Allen. 2001. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J. Immunol.* 167:1601-1608.

6. Murphy, G., and H. Nagase. 2008. Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat. Clin. Pract. Rheumatol.* 4:128-135.

7. Tchetverikov, I., H. K. Ronday, B. Van El, G. H. Kiers, N. Verzijl, J. M. TeKoppele, T. W. Huizinga, J. DeGroot, and R. Hanemaaijer. 2004. MMP profile in paired serum and synovial fluid samples of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 63:881-883.

8. Hanemaaijer, R., T. Sorsa, Y. T. Konttinen, Y. Ding, M. Sutinen, H. Visser, V. W. van Hinsbergh, T. Helaakoski, T. Kainulainen, H. Ronka, H. Tschesche, and T. Salo. 1997. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. *J. Biol. Chem.* 272:31504-31509.

9. Cox, J. H., and C. M. Overall. In press. Cytokine Substrates: MMP Regulation of Inflammatory Signalling Molecules. *The Cancer Degradome - Proteases and Cancer Biology. Springer Science and Business Media.*

10. Parks, W. C., C. L. Wilson, and Y. S. Lopez-Boado. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat. Rev. Immunol.* 4:617-629.

11. Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, and et al. 1994. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* 370:555-557.

12. Dean, R. A., G. S. Butler, Y. Hamma-Kourbali, J. Delbe, D. R. Brigstock, J. Courty, and C. M. Overall. 2007. Identification of candidate angiogenic inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based proteomic screens: disruption of vascular endothelial growth factor (VEGF)/heparin affin regulatory peptide (pleiotrophin) and VEGF/Connective tissue growth factor angiogenic inhibitory complexes by MMP-2 proteolysis. *Mol. Cell. Biol.* 27:8454-8465.

13. Overall, C. M., and O. Kleifeld. 2006. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat. Rev. Cancer* 6:227-239.

14. Christodoulou, C., and E. H. Choy. 2006. Joint inflammation and cytokine inhibition in rheumatoid arthritis. *Clin. Exp. Med.* 6:13-19.

15. Zwerina, J., K. Redlich, K. Polzer, L. Joosten, G. Kronke, J. Distler, A. Hess, N. Pundt, T. Pap, O. Hoffmann, J. Gasser, C. Scheinecker, J. S. Smolen, W. van den Berg, and G. Schett. 2007. TNF-induced structural joint damage is mediated by IL-1. *Proc. Natl. Acad. Sci. USA* 104:11742-11747.

16. Furlan, R., G. Martino, F. Galbiati, P. L. Poliani, S. Smiroldo, A. Bergami, G. Desina, G. Comi, R. Flavell, M. S. Su, and L. Adorini. 1999. Caspase-1 regulates the inflammatory process leading to autoimmune demyelination. *J. Immunol.* 163:2403-2409.

17. Kang, S. J., S. Wang, H. Hara, E. P. Peterson, S. Namura, S. Amin-Hanjani, Z. Huang, A. Srinivasan, K. J. Tomaselli, N. A. Thornberry, M. A. Moskowitz, and J. Yuan. 2000. Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J. Cell Biol.* 149:613-622.

18. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat. Genet.* 35:252-257.

19. Tester, A. M., J. H. Cox, A. R. Connor, A. E. Starr, R. A. Dean, X. S. Puente, C. Lopez-Otin, and C. M. Overall. 2007. LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity. *PLoS ONE* 2:e312.

20. Ratkay, L. G., L. Zhang, J. Tonzetich, and J. D. Waterfield. 1993. Complete Freund's adjuvant induces an earlier and more severe arthritis in MRL-lpr mice. *J. Immunol.* 151:5081-5087.

21. Singer, G. G., and A. K. Abbas. 1994. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity* 1:365-371.

22. Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314-317.

23. Truett, G. E., P. Heeger, R. L. Mynatt, A. A. Truett, J. A. Walker, and M. L. Warman. 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *BioTechniques* 29:52, 54.

24. English, D., and B. R. Andersen. 1974. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Methods* 5:249-252.

25. Billinghurst, R. C., L. Dahlberg, M. Ionescu, A. Reiner, R. Bourne, C. Rorabeck, P. Mitchell, J. Hambor, O. Diekmann, H. Tschesche, J. Chen, H. Van Wart, and A. R. Poole. 1997. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* 99:1534-1545.

26. Mort, J. S., and D. J. Buttle. 1999. The use of cleavage site specific antibodies to delineate protein processing and breakdown pathways. *Mol. Pathol.* 52:11-18.

27. Sztrolovics, R., M. Alini, P. J. Roughley, and J. S. Mort. 1997. Aggrecan degradation in human intervertebral disc and articular cartilage. *Biochem. J.* 326 (Pt 1):235-241.

28. Kappelhoff, R., and C. M. Overall. 2007. The CLIP-CHIP Oligonucleotide Microarray: Dedicated Array for Analysis of All Protease, Nonproteolytic Homolog, and Inhibitor Gene Transcripts in Human and Mouse. *Curr. Protoc. Protein Sci.* 49:21.19.21-21.19.16.

29. Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush. 2003. TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* 34:374-378.

30. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.

31. Gutierrez-Fernandez, A., M. Inada, M. Balbin, A. Fueyo, A. S. Pitiot, A. Astudillo, K. Hirose, M. Hirata, S. D. Shapiro, A. Noel, Z. Werb, S. M. Krane, C. Lopez-Otin, and X. S. Puente.

2007. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J.* 21:2580-2591.

32. Ratkay, L. G., B. Tait, J. Tonzetich, and J. D. Waterfield. 1994. Lpr and MRL background gene involvement in the control of adjuvant enhanced arthritis in MRL-lpr mice. *J. Autoimmun.* 7:561-573.

33. Fosang, A. J., K. Last, P. J. Neame, G. Murphy, V. Knauper, H. Tschesche, C. E. Hughes, B. Caterson, and T. E. Hardingham. 1994. Neutrophil collagenase (MMP-8) cleaves at the aggrecanase site E373-A374 in the interglobular domain of cartilage aggrecan. *Biochem. J.* 304 (Pt 2):347-351.

34. Wang, S., M. Miura, Y. K. Jung, H. Zhu, E. Li, and J. Yuan. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* 92:501-509.

35. Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* 72:3666-3670.

36. Ruuls, S. R., R. M. Hoek, V. N. Ngo, T. McNeil, L. A. Lucian, M. J. Janatpour, H. Korner, H. Scheerens, E. M. Hessel, J. G. Cyster, L. M. McEvoy, and J. D. Sedgwick. 2001. Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* 15:533-543.

37. Churg, A., R. D. Wang, H. Tai, X. Wang, C. Xie, J. Dai, S. D. Shapiro, and J. L. Wright. 2003. Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *Am. J. Respir. Crit. Care Med.* 167:1083-1089.

38. Haro, H., H. C. Crawford, B. Fingleton, K. Shinomiya, D. M. Spengler, and L. M. Matrisian. 2000. Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption. *J. Clin. Invest.* 105:143-150.

39. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385:729-733.

40. Moss, M. L., S. L. Jin, M. E. Milla, D. M. Bickett, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R. Didsbury, D. Hassler, C. R. Hoffman, T. A. Kost, M. H. Lambert, M. A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L. K. Overton, F. Schoenen, T. Seaton, J. L. Su, J. D. Becherer, and et al. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 385:733-736.

41. Hisahara, S., J. Yuan, T. Momoi, H. Okano, and M. Miura. 2001. Caspase-11 mediates oligodendrocyte cell death and pathogenesis of autoimmune-mediated demyelination. *J. Exp. Med.* 193:111-122.

42. Kang, S. J., S. Wang, K. Kuida, and J. Yuan. 2002. Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. *Cell Death Differ.* 9:1115-1125.

43. Lee, J., J. Hur, P. Lee, J. Y. Kim, N. Cho, S. Y. Kim, H. Kim, M. S. Lee, and K. Suk. 2001. Dual role of inflammatory stimuli in activation-induced cell death of mouse microglial cells. Initiation of two separate apoptotic pathways via induction of interferon regulatory factor-1 and caspase-11. *J. Biol. Chem.* 276:32956-32965.

44. Colotta, F., F. Re, N. Polentarutti, S. Sozzani, and A. Mantovani. 1992. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80:2012-2020.

45. Gueders, M. M., M. Balbin, N. Rocks, J. M. Foidart, P. Gosset, R. Louis, S. Shapiro, C. Lopez-Otin, A. Noel, and D. D. Cataldo. 2005. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J. Immunol.* 175:2589-2597.

46. Giudice, E. D., A. Ciaramella, N. Balestro, D. Neumann, P. G. Romano, M. P. Cesaroni, G. Maurizi, P. Ruggiero, D. Boraschi, and P. Bossu. 2001. Neutrophil apoptosis in autoimmune Fas-defective MRL lpr/lpr mice. *Eur. Cytokine. Netw.* 12:510-517.

47. O'Neill, A., M. C. Greenan, B. Doyle, J. M. Fitzpatrick, and R. W. Watson. 2004. Gene profiling of in vitro and in vivo models of delayed neutrophil apoptosis: a common pathway? *Biochem. Soc. Trans.* 32:470-473.

48. Riedl, S. J., and G. S. Salvesen. 2007. The apoptosome: signalling platform of cell death. *Nat. Rev. Mol. Cell Biol.* 8:405-413.

49. Pongracz, J., P. Webb, K. Wang, E. Deacon, O. J. Lunn, and J. M. Lord. 1999. Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta. *J. Biol. Chem.* 274:37329-37334.

50. Powell, W. C., B. Fingleton, C. L. Wilson, M. Boothby, and L. M. Matrisian. 1999. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr. Biol.* 9:1441-1447.

51. Fingleton, B., T. Vargo-Gogola, H. C. Crawford, and L. M. Matrisian. 2001. Matrilysin [MMP-7] expression selects for cells with reduced sensitivity to apoptosis. *Neoplasia* 3:459-468.

52. Schauvliege, R., J. Vanrobaeys, P. Schotte, and R. Beyaert. 2002. Caspase-11 gene expression in response to lipopolysaccharide and interferon-gamma requires nuclear factor-kappa B and signal transducer and activator of transcription (STAT) 1. *J. Biol. Chem.* 277:41624-41630.

53. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* 10:417-426.

54. Li, J., W. M. Brieher, M. L. Scimone, S. J. Kang, H. Zhu, H. Yin, U. H. von Andrian, T. Mitchison, and J. Yuan. 2007. Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization. *Nat. Cell Biol.* 9:276-286.

55. Mueller, N. J., R. A. Wilkinson, and J. A. Fishman. 2002. Listeria monocytogenes infection in caspase-11-deficient mice. *Infect. Immun.* 70:2657-2664.

56. Feldmann, M., and R. N. Maini. 2001. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu. Rev. Immunol.* 19:163-196.

57. Vincenti, M. P., and C. E. Brinckerhoff. 2002. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res.* 4:157-164.

58. Takaishi, H., T. Kimura, S. Dalal, Y. Okada, and J. D'Armiento. 2008. Joint diseases and matrix metalloproteinases: a role for MMP-13. *Curr Pharm Biotechnol* 9:47-54.

59. O'Sullivan, F. X., H. G. Fassbender, S. Gay, and W. J. Koopman. 1985. Etiopathogenesis of the rheumatoid arthritis-like disease in MRL/I mice. I. The histomorphologic basis of joint destruction. *Arthritis Rheum.* 28:529-536.

60. Itoh, T., H. Matsuda, M. Tanioka, K. Kuwabara, S. Itohara, and R. Suzuki. 2002. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J. Immunol.* 169:2643-2647.

61. Mudgett, J. S., N. I. Hutchinson, N. A. Chartrain, A. J. Forsyth, J. McDonnell, Singer, II, E. K. Bayne, J. Flanagan, D. Kawka, C. F. Shen, K. Stevens, H. Chen, M. Trumbauer, and D. M. Visco. 1998. Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction. *Arthritis Rheum.* 41:110-121.

62. Neuhold, L. A., L. Killar, W. Zhao, M. L. Sung, L. Warner, J. Kulik, J. Turner, W. Wu, C. Billinghurst, T. Meijers, A. R. Poole, P. Babij, and L. J. DeGennaro. 2001. Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J. Clin. Invest.* 107:35-44.

63. Coussens, L. M., B. Fingleton, and L. M. Matrisian. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295:2387-2392.

64. Folgueras, A. R., A. Fueyo, O. Garcia-Suarez, J. Cox, A. Astudillo, P. Tortorella, C. Campestre, A. Gutierrez-Fernandez, M. Fanjul-Fernandez, C. J. Pennington, D. R. Edwards, C. M. Overall, and C. Lopez-Otin. 2008. Collagenase-2 deficiency or inhibition impair experimental autoimmune encephalomyelitis in mice. *J. Biol. Chem.*

135

CHAPTER 5. CONCLUSIONS AND PERSPECTIVES
Dynamic Regulation of Inflammation and Immunity by MMP-8

The research presented in this thesis expands on our previous knowledge of the functions of MMPs and the mechanisms by which MMPs regulate inflammation and immunity, with a particular focus on MMP-8, the neutrophil collagenase. The most significant findings of this thesis research are (i) identifying for the first time and characterizing a feed-forward neutrophil recruiting mechanism by MMP-8 regulation of mouse and human neutrophil chemoattractants, (ii) biochemically identifying a novel MMP chemokine substrate, the T cell chemoattractant CXCL11, and evaluating the functional consequences of truncation including a novel proteolytic effect on glycosaminoglycan binding and hence dispersal of the chemokine gradient, and (iii) defining MMP-8 as an anti-target in arthritis and identifying potential mechanisms by which MMP-8 promotes neutrophil apoptosis and the resolution of inflammation. Taken together, this thesis highlights the diverse roles of a single protease in the context of different immunological processes, encompassing the innate, adaptive, and autoimmune scenarios.

Once considered to be simple matrix degrading enzymes, MMPs are now known to have pleiotropic roles in normal homeostasis and pathological processes by acting through a plethora of bioactive substrates. As such, defining suitable MMP drug targets has become a challenging task. In the case of MMP-8, the data from air pouch models suggest that MMP-8 inhibition could be beneficial in dampening acute inflammation. In collaboration work not presented in this thesis, we showed that MMP-8 was detrimental in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (1). In contrast, MMP-8 was found to be protective in rheumatoid arthritis, a chronic inflammatory disease. Furthermore, I propose that MMP-8, in conjuction with other MMPs, functions to downregulate Th1 cell chemoattraction and modulate acquired immunity. Therefore, the risks and benefits of targeting a multi-functional protease such as MMP-8 must be carefully assessed and balanced. Protective roles in cancer and arthritis define MMP-8 as an

anti-target in these diseases and hence MMP-8 must be spared in the design of novel MMP inhibitors for clinical use.

Despite the large number of MMP family members, which is 23 in human and 24 in mouse, there exists a remarkable lack of redundancy *in vivo*. Nearly every MMP-deficient mouse generated to date has one or more phenotypes relating to inflammatory or immunological processes, as reviewed in Chapter 1. The neutrophil alone produces MMP-8, MMP-9, MMP-14, and MMP-25. Although several MMPs, including MMP-9 and MMP-14, biochemically process and activate the neutrophil chemoattractant LIX, MMP-8 is the dominant physiological regulator of LIX activity. This shows that while there is biochemical redundancy, there is less physiological redundancy than expected. In additon, four MMPs cleaved the T lymphocyte chemokine CXCL11/I-TAC but only MMP-8 cleaved within the C-terminal alpha-helix, resulting in the loss of glycosaminoglycan affinity. In the absence of MMP-8, MMP-9 is considerably upregulated in the neutrophil, yet this does not compensate for the more severe arthritis observed in MMP-8 deficient male mice nor the delayed neutrophil apoptosis in the absence of MMP-8. Therefore, in spite of an apparently redundant system, the data presented herein supports a unique and complex role for MMP-8 as a pivotal regulator in inflammation and immunity.

This research has used a wide variety of techniques to explore the biological functions of MMP-8, including biochemical analyses with mass spectrometry, cell culture, gene microarrays, and mouse models of inflammation. This multifaceted approach has proven very successful in viewing MMPs from different perspectives, at both the molecular and physiological levels, and hence gaining valuable insight into both mechanism and function. Biochemical tools, such as the MALDI-TOF MS analysis that I performed, aided in the discovery of new MMP-8 substrates, specifically LIX, CXCL5, CXCL8, CXCL11, and TNF-α. Isolation and culture of primary cells enabled the characterization of functional consequences of proteolysis of bioactive substrates. Furthermore, gene microarrays revealed potential mechanisms by which MMP-8 controls neutrophil accumulation and resolution.

Finally, the MMP-8 knockout mouse and the novel MRL/*lpr* MMP-8 deficient strain that I generated by backcross breeding were instrumental in deciphering the physiological roles of MMP-8 in acute and chronic inflammation.

Bridging the gap between what happens in a test tube and what occurs in an animal is far from trivial. This is exemplified in Chapters 2 and 3 which describe biochemical cleavages that resulted in either chemokine activation or switching a receptor agonist to an antagonist, respectively. A direct link between MMP-8 mediated LIX activation and decreased neutrophil infiltration in MMP-8 deficient mice seemed obvious at first. However, some key pieces to the puzzle were missing. Most notably, the MMP processed forms of LIX were never detected in physiological samples although decreased cleavage was evident in MMP-8 deficient mice (2). More recent findings, as described in Appendix A, have prompted the hypothesis that MMP-8 is regulating LIX activation in an indirect manner. Demonstrating evidence of *in vivo* processing of CXCL11 has been unsuccessful to date, despite the raising of sensitive neo-epitope antibodies specific for the MMP processed forms of the chemokine. Many biochemical cleavage events are *in vitro* artefacts that do not occur physiologically, and must be considered when designing experiments. In hindsight, studying chemokine processing at both the biochemical and cellular level would provide a more relevant starting point for the identification of physiological substrates.

Animal models of disease are without doubt extremely useful research tools. However, no model is perfect in predicting human disease. This is especially true for mouse models of arthritis. There is no consensus on the best murine model of rheumatoid arthritis, which range from acute and severe collagen-induced arthritis, serum transfer methods, and the milder MRL/*lpr* systemic autoimmune model that was described in Chapter 4. With researchers using such an array of models, comparison across studies becomes increasingly difficult. The MRL/*lpr* model of autoimmunity provided a strong inflammatory response in the mouse joints, which was evident by clinical edema and histological analysis. However, this model did not

yield detectable cartilage damage, a hallmark of rheumatoid arthritis. As such, using a single model to study such a complex disease is a limitation and future arthritis studies should use multiple models to strengthen conclusions and assess different aspects of disease pathology.

Over the past five years, conflicting results regarding the role of MMP-8 in inflammatory disease have emerged (Table 1.2). In acute situations, such as the LPS-treated air pouch (Chapter 2), TNF-induced lethal hepatitis (3), and LPS-treated cornea (4), MMP-8 assumes a pro-inflammatory function with knockout mice exhibiting decreased levels of neutrophils. This phenotype is reversed in lung models of acute inflammation where MMP-8 deficient mice have elevated infiltration of neutrophils (5, 6), demonstrating tissue-dependent effects. In contrast, chronic inflammation such as that in skin carcinogenesis (2), wound healing (7), and rheumatoid arthritis (Chapter 4), reveal an anti-inflammatory role for MMP-8, where MMP-8 knockout mice are protected from disease.

Based on this research and that of others, I currently hypothesize that MMP-8 is acting through multiple pathways to promote neutrophil migration in acute inflammation and to resolve inflammation during the transition for acute to chronic inflammation (Figure 5.1). In acute inflammation, MMP-8 is released from stimulated neutrophils to cleave and inactivate α1-proteinase inhibitor, a serine protease inhibitor. This enhances the activity of neutrophil elastase which can efficiently cleave and activate LIX, a potent murine neutrophil chemoattractant. This mechanism is depicted in Figure A.7 (Appendix A). In the normal resolution of inflammation, I hypothesize that MMP-8 promotes neutrophil apoptosis and clearance through induction of caspase-11 expression and TNF-α shedding (Figure 4.9). Although adaptive immune processes have not been specifically studied in the MMP-8 knockout mouse, I propose that MMP-8 and other MMPs can process CXCL11 and convert it into a CXCR3 antagonist Th1 lymphocyte chemokine, alter its haptotactic gradient localization on glycosaminoglycans, and ultimately downregulate Th1 lymphocyte recruitment (Figure 3.10).
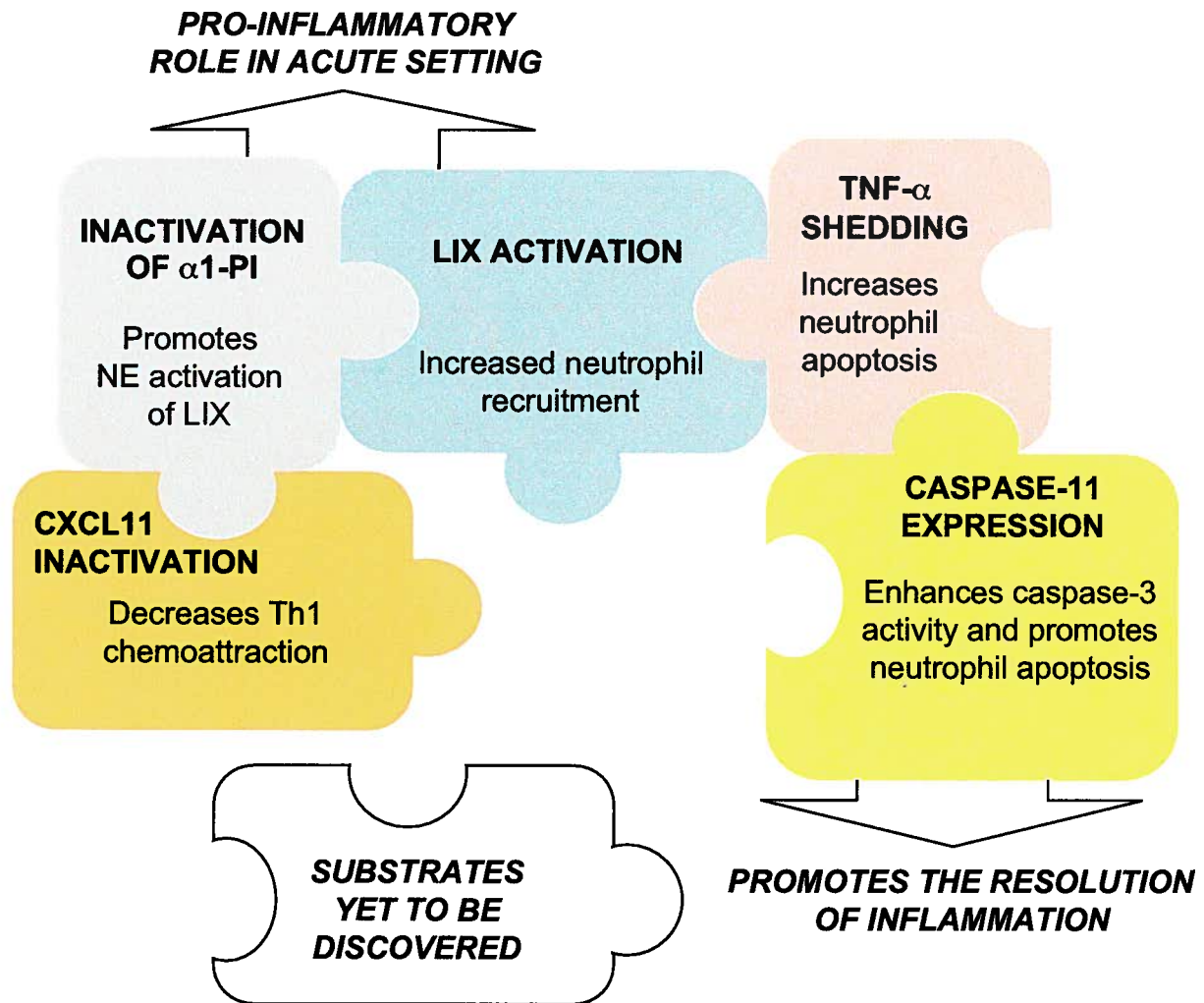
Figure 5.1. Solving the MMP-8 puzzle: diverse functions of MMP-8 in inflammation and acquired immunity. MMP-8 is released from specific granules of activated neutrophils resulting in many proteolytic functions. To promote neutrophil recruitment in acute inflammation, MMP-8 either activates the neutrophil chemoattractant LIX directly or increases neutrophil elastase (NE) activation of LIX via inactivation of α1-proteinase inhibitor (α1-PI). Alternatively, MMP-8 promotes the normal resolution of inflammation through TNF-α shedding and the regulation of caspase-11 expression, pathways proposed to converge and induce neutrophil apoptosis. TNF-α shedding results in increased MMP expression (not shown). MMP-8 also processes the Th1 chemoattractant CXCL11 yielding a receptor antagonist with predicted reduction of Th1 lymphocyte recruitment *in vivo*. The discovery of additional physiologically relevant substrates will be essential in fully understanding the complex and multifaceted functions of MMP-8 in neutrophil physiology and inflammation.

Future research stemming directly from these findings should include examination of MMP processing of CXCL11 *in vivo* as well as further characterizing the pro-apoptotic effect of MMP-8. CXCL11 processing could be compared in normal and diseased tissues using mass spectrometry and neo-epitope antibody-based detection. Specifically, due to an abundance of neutrophils and T lymphocytes, tissues from autoimmunities such as multiple sclerosis, psoriasis, and arthritis would be of particular interest. Experiments to decipher the mechanism for the regulatory interaction between MMP-8 and caspase-11 should focus on developmental aspects. Studying leukocytes from mice of various ages could provide insight into this mechanism. Furthermore, examining caspase-11 levels in hematopoietic stem cells and myeloid progenitor cells from MMP-8 deficient mice could reveal the basis of this regulatory effect. Ultimately, experiments should be aimed at discovering and validating substrates through which MMP-8 promotes neutrophil apoptosis.

The homeostatic and pathological functions of proteases are defined by the substrates they cleave and the biological consequences of these post-translational modifications. Until recently, substrate discovery efforts have been largely biased and limited by the classical hypothesis-driven approach. However, novel mass spectrometry based methods for substrate discovery are emerging with far-reaching potential to revolutionize protease research, providing systematic and unbiased routes to substrate identification (8). Although numerous bioactive MMP-8 substrates have been identified biochemically (Table 1.1), none have been validated *in vivo*, which is a challenging task due to the low abundance of many substrates such as chemokines. This highlights the necessity for multiple levels of substrate validation, which can be achieved by demonstrating biochemical, cellular, animal model, and ultimately human relevance (9). The use of MMP-8 knockout mice, primary neutrophils, or MMP-8 deficient cell systems in conjunction with proteomic substrate discovery techniques will validate proposed mechanisms and potentially uncover novel pathways by which MMP-8 coordinates both the initiation and resolution of inflammation. One such technique is described in Appendix B.

Data relating the effects of MMPs in mouse models with those in human disease are currently lacking. Fundamental differences between mouse and human MMPs are evident by the increased number of MMPs, and all proteases, in mice (10) and also from failed MMPI clinical trials, despite promising efficacy in animals (11). Such differences should be assessed at the molecular level by comparing cleavage site specificities (described in Appendix B), kinetics of substrate cleavage, and inhibition profiles for individual mouse and human proteases. This should then be expanded upon using human cell lines in conjunction with small interfering RNA and specific inhibitors, and ultimately correlation studies with disease severity should be examined in humans, as described recently for MMP-8 in cancer metastasis (12). The majority of homeostatic and pathogenic pathways appear to be conserved across species, although specific mediators may differ. However, these differences must be appreciated and clearly defined.

In conclusion, this thesis has demonstrated the pivotal role of MMP-8 in both the initiation and resolution of inflammation. Once thought to be simply a collagen-degrading protease, MMP-8 is emerging as a multifaceted regulator of neutrophil recruitment and clearance with protective roles in arthritis and cancer. MMPs are now known to act through a wide variety of bioactive molecules and function as key regulators in numerous physiological processes, including inflammation and immunity. As our knowledge of the mechanisms and physiological functions of MMPs continues to expand, there is the potential to uncover novel therapeutic strategies for the many diseases that involve dysregulated inflammation.

References

1. Folgueras, A. R., A. Fueyo, O. Garcia-Suarez, J. Cox, A. Astudillo, P. Tortorella, C. Campestre, A. Gutierrez-Fernandez, M. Fanjul-Fernandez, C. J. Pennington, D. R. Edwards, C. M. Overall, and C. Lopez-Otin. 2008. Collagenase-2 deficiency or inhibition impairs experimental autoimmune encephalomyelitis in mice. *J Biol Chem* 283:9465-9474.

2. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35:252-257.

3. Van Lint, P., B. Wielockx, L. Puimege, A. Noel, C. Lopez-Otin, and C. Libert. 2005. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol* 175:7642-7649.

4. Lin, M., P. Jackson, A. M. Tester, E. Diaconu, C. M. Overall, J. E. Blalock, and E. Pearlman. 2008. Matrix Metalloproteinase-8 Facilitates Neutrophil Migration through the Corneal Stromal Matrix by Collagen Degradation and Production of the Chemotactic Peptide Pro-Gly-Pro. *Am J Pathol.*

5. Gueders, M. M., M. Balbin, N. Rocks, J. M. Foidart, P. Gosset, R. Louis, S. Shapiro, C. Lopez-Otin, A. Noel, and D. D. Cataldo. 2005. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J Immunol* 175:2589-2597.

6. Owen, C. A., Z. Hu, C. Lopez-Otin, and S. D. Shapiro. 2004. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol* 172:7791-7803.

7. Gutierrez-Fernandez, A., M. Inada, M. Balbin, A. Fueyo, A. S. Pitiot, A. Astudillo, K. Hirose, M. Hirata, S. D. Shapiro, A. Noel, Z. Werb, S. M. Krane, C. Lopez-Otin, and X. S. Puente. 2007. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *Faseb J.*

8. Schilling, O., and C. M. Overall. 2007. Proteomic discovery of protease substrates. *Curr Opin Chem Biol* 11:36-45.

9. Overall, C. M., and C. P. Blobel. 2007. In search of partners: linking extracellular proteases to substrates. *Nat Rev Mol Cell Biol* 8:245-257.

10. Puente, X. S., L. M. Sanchez, C. M. Overall, and C. Lopez-Otin. 2003. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 4:544-558.

11. Coussens, L. M., B. Fingleton, and L. M. Matrisian. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295:2387-2392.

12. Gutierrez-Fernandez, A., A. Fueyo, A. R. Folgueras, C. Garabaya, C. J. Pennington, S. Pilgrim, D. R. Edwards, D. L. Holliday, J. L. Jones, P. N. Span, F. C. Sweep, X. S. Puente, and C. Lopez-Otin. 2008. Matrix metalloproteinase-8 functions as a metastasis suppressor through modulation of tumor cell adhesion and invasion. *Cancer Res* 68:2755-2763.

APPENDIX A. PROCESSING OF ELR$^+$ CHEMOKINES BY NEUTROPHIL SERINE PROTEASES[5]

Perspective

The manuscript in Chapter 2 entitled "LPS responsiveness and neutrophil chemotaxis *in vivo* require PMN MMP-8 activity" describes the pro-inflammatory role of MMP-8 in neutrophil infiltration in acute immunity. In a subcutaneous air pouch model, MMP-8 deficient mice had reduced neutrophil migration in response to LPS. This was attributed to MMP-8 processing and activation of the murine ELR$^+$ chemokine LIX. Indeed, air pouch experiments with the activated form of LIX showed equal migration of neutrophils in wild-type and MMP-8 knockout mice, validating LIX as a physiological MMP-8 dependent mechanism for promoting neutrophil infiltration. This demonstrated that there was no defect in the PMN chemotactic machinery and that MMP-8 cleavage of the extracellular matrix is not required for migration. Neo-epitope antibodies specific for the MMP-8 processed forms of LIX were generated, but these truncations were not detected in air pouch fluids, thereby prompting the examination of alternate MMP-8 dependent pathways for LIX activation and neutrophil recruitment. In this Appendix, efficient neutrophil elastase processing of LIX is demonstrated and MMP-8 cleavage and regulation of α1-PI, a potent neutrophil elastase inhibitor, is proposed. By this indirect pathway, MMP-8 might increase neutrophil elastase activity and hence increase the proteolytic activation of LIX, thereby recruiting neutrophils in a feed-forward mechanism.

---

[5] The studies presented in Appendix A are unpublished and expand on the published manuscript in Chapter 2.

Results

Processing of LIX by isolated bone marrow neutrophils

MMP-8 processing of LIX was studied in the cellular context with bone marrow neutrophils from wild-type and MMP-8 deficient mice. Cleavage was analyzed by Tris-tricine gel electrophoresis and MALDI-TOF mass spectrometry. Processing occurred after only a few hours with either wild-type or MMP-8 deficient neutrophils (Figure A.1A, B). Deconvolution of MALDI-TOF data identified cleavage products as LIX (9-92), LIX (1-78), and LIX (9-78) with mass to charge ratios (m/z) of 9150, 8325, and 7626 $[M+H]^+$, respectively (Figure A.1C). Therefore, neutrophil-mediated proteolysis of LIX occurs at ^{6}IAA-TEL^{11} and ^{76}KKA-KRN^{81} (Figure A.1D). These cleavage products are expected to have enhanced activity based on previous findings with ELR$^+$ chemokines (1, 2). Therefore, neutrophil proteases are able to process and activate LIX and this is not MMP-8 dependent in the cellular context.

LIX is processed by serine proteases neutrophil elastase and cathepsin G

To assess the neutrophil protease(s) responsible for LIX processing, protease inhibitors were added to the cleavage mixture. The serine protease inhibitor 2-aminoethyl benzenesulfonyl fluoride hydrochloride (AEBSF) was an effective inhibitor of LIX cleavage (Figure A.2A). This data indicates that LIX is processed by at least one of the three major serine proteases in neutrophils: proteinase-3, cathepsin G, and neutrophil elastase. The mechanism of serine protease mediated LIX cleavage was examined further using endogenous serine protease inhibitors (Figure A.2A). At concentrations as low as 100 nM, both α1-proteinase inhibitor (α1-PI) and secreted leukocyte protease inhibitor (SLPI) effectively inhibited LIX cleavage by neutrophils. It is known that SLPI does not inhibit proteinase-3 (3), whereas both α1-PI and SLPI inhibit neutrophil elastase and cathepsin G (4). Therefore, potential candidates for neutrophil-mediated LIX processing were neutrophil elastase and cathepsin G.

A

Time (h)	3	-	1	1	2	2	3	3
LIX	-	+	+	+	+	+	+	+
PMA (μM)	-	-	0.05	10	0.05	10	0.05	10

WT
PMNs

$[M+H]^+$ 9849   9849 9150 8325   9150 7626   7626

B

Time (h)	3	-	1	1	2	2	3	3
LIX	-	+	+	+	+	+	+	+
PMA (μM)	-	-	0.05	10	0.05	10	0.05	10

KO
PMNs

$[M+H]^+$ 9849   9849 9150 8325   9150 7626   7626

C

Chemokine	m/z $[M+H]^+$ Measured	Predicted
LIX (1-92)	9849	9850
LIX (9-92)	9150	9153
LIX (1-78)	8325	8325
LIX (9-78)	7626	7628

D

1　　　　　　10　　　　　　　　　　80　　　　　　90
APSSVIAA↑TELR...SDKKKA↑KRNALAVERTASVQ
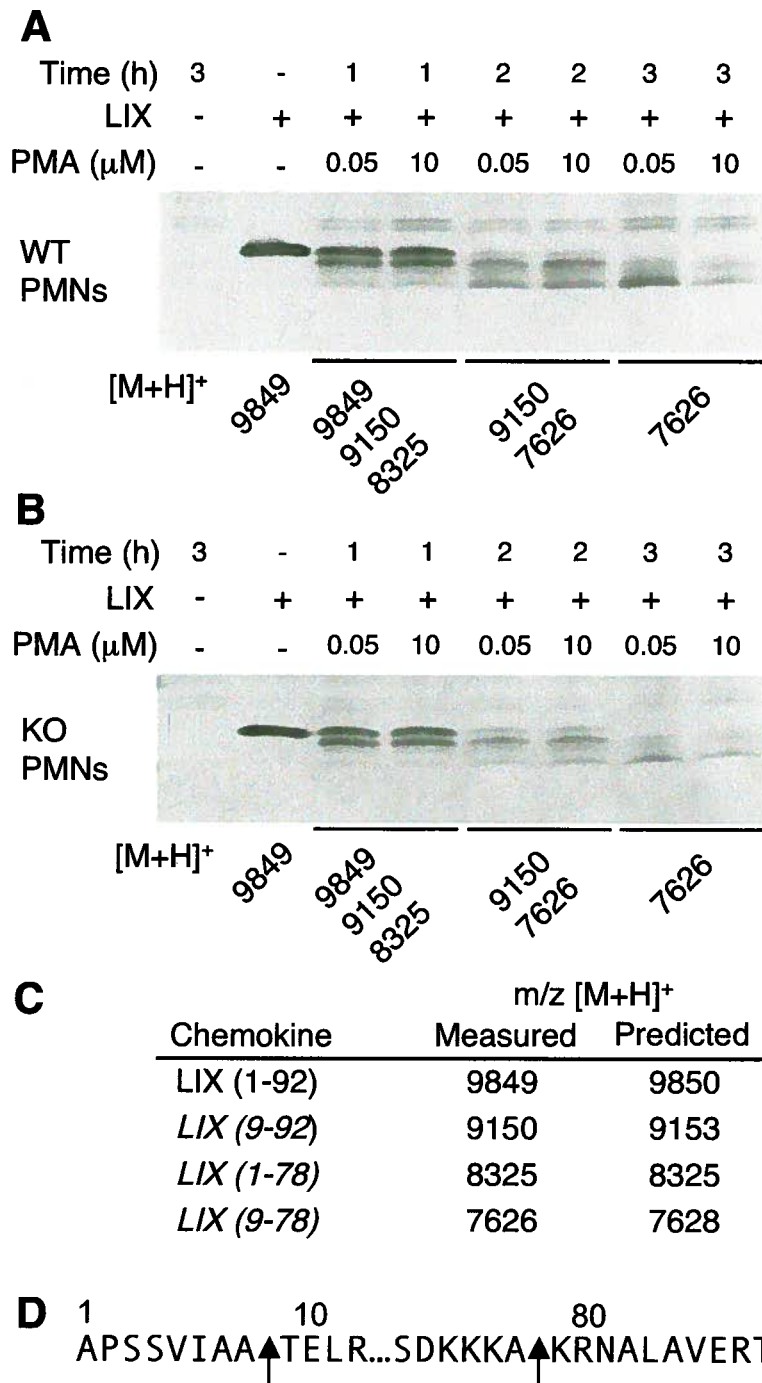
Figure A.1. **Neutrophil processing of LIX is independent of MMP-8.** Bone marrow neutrophils were isolated from wild-type and MMP-8 deficient mice then incubated with LIX. Reactions contained 1 x 10⁶ cells, activated with PMA as marked, and 10 μg LIX in RPMI and were incubated at 37 °C for up to 3 hours. Cleavage with wild-type (A) and MMP-8 knockout (B) neutrophils was visualized by 15% Tris-Tricine gel electrophoresis and analyzed by MALDI-TOF MS in sinapinic acid matrix. (C) Mass to charge ratios ($[M+H]^+$) were deconvoluted to determine truncation products (italics). (D) N-terminal cleavage at Ala⁸-Thr⁹ and the C-terminal cleavage at Ala⁷⁸-Lys⁷⁹ are depicted with arrows in the LIX sequence.

A

Inhib (μM)	-	-	AEBSF		α1-PI		SLPI	
			100	500	0.1	0.5	0.1	0.5
WT PMNs	-	+	+	+	+	+	+	+

LIX (1-92)→

LIX (9-92)
LIX (1-78)
LIX (9-78)

B

E:S — 1:10000 1:5000 1:1000 1:500 1:100 1:50

MMP-8

LIX (1-92)
LIX (5-92)
LIX (1-79)

CatG

←LIX (1-92)
←LIX (1-79)

NE

LIX (1-92)
LIX (7-92)
LIX (7-83)
LIX (7-78)

C

Enzyme	k_{cat}/K_M (M^{-1}s^{-1})
MMP-8	6.0×10^2
CatG	6.0×10^1
NE	1.2×10^4

D

MMP-8   NE
1   ↓   ↓   * 10
APSS↓VI↓AATELRCVC...

                    NE
        * 80   ↓   90
...GSDKKKA↑K↑RNAL↓AVERTASVQ
        NE MMP-8, CATG
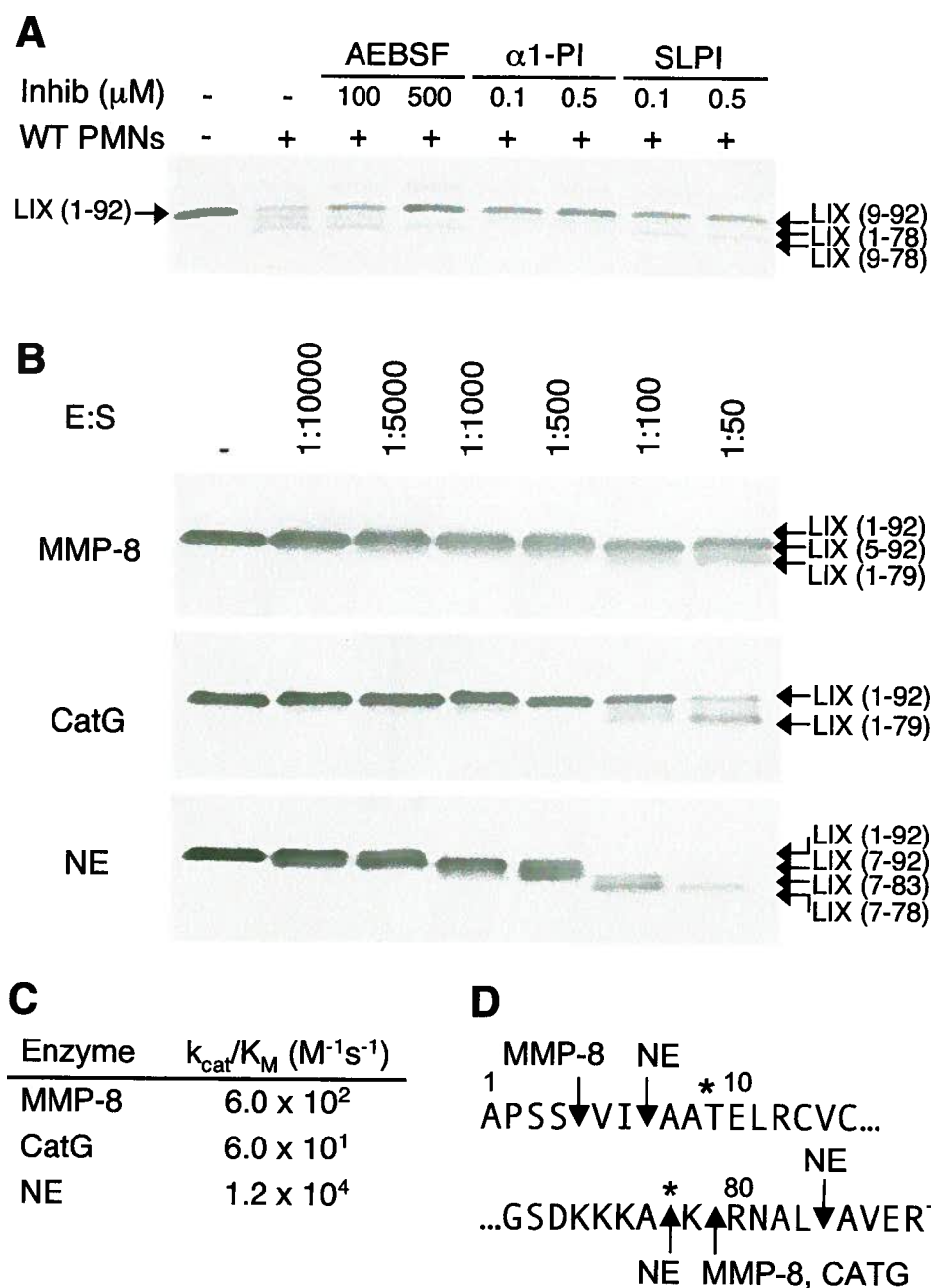
Figure A.2. Neutrophil-derived serine proteases cleave LIX. Bone marrow neutrophils were isolated from wild-type mice then incubated with LIX. 1 x 10⁶ cells were activated with 50 nM PMA and preincubated with AEBSF, α1-PI, or SLPI before the addition of 10 μg LIX. Reactions were incubated at 37 °C for 4 h. (A) Cell-free conditioned media was visualized on 15% Tris-Tricine SDS-PAGE and analyzed by MALDI-TOF MS in sinapinic acid matrix to determine truncation products. (B) Biochemical cleavage efficiencies of MMP-8, cathepsin G (CatG; Calbiochem) and neutrophil elastase (NE; Elastin Products) for LIX were assessed by titration. Enzyme-substrate ratios (E:S) are mol:mol. (C) Specificity constants (k_{cat}/K_M) were determined by densitometry. (D) MMP-8, CatG, and NE cleavage sites in LIX are depicted. Asterisks mark the sites of neutrophil-mediated cleavage.

Human neutrophil elastase and cathepsin G were tested *in vitro* for cleavage of LIX, and directly compared with MMP-8, which is known to cleave LIX biochemically (Figure A.2B). MMP-8 processed LIX at Ser^4-Val^5 and Lys^{79}-Arg^{80}, as determined previously, with cleavage starting at an enzyme concentration of 20 nM. Cathepsin G also cleaved LIX at Lys^{79}-Arg^{80}, but only at concentrations above 100 nM. In contrast, neutrophil elastase was an efficient processor of LIX at concentrations as low as 1 nM, resulting in cleavages at Ile^6-Ala^7, Ala^{78}-Lys^{79}, and Leu^{83}-Ala^{84}. By densitometry analysis as described previously (5), specificity constants (k_{cat}/K_M) of LIX cleavage were estimated to be 600, 60 and 1200 $M^{-1}s^{-1}$ for MMP-8, cathepsin G, and neutrophil elastase, respectively (Figure A.2C). Notably, only the C-terminal Ala^{78}-Lys^{79} cleavage by neutrophil elastase matches a neutrophil-mediated truncation of LIX (Figure A.2D). The N-terminal Ala^8-Thr^9 cleavage was not reproduced by any of the enzymes tested, however these were human enzymes and murine counterparts could have slightly shifted specificity. Nonetheless, based on the kinetic efficiency of neutrophil elastase cleavage of LIX and the inhibitio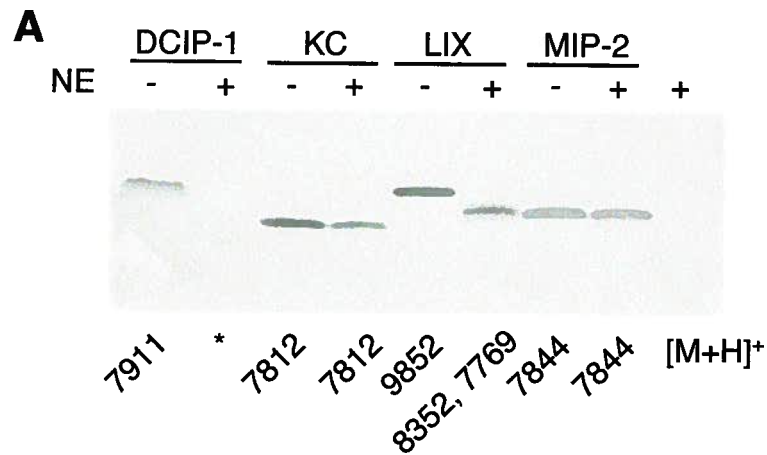n profile in neutrophil-mediated LIX cleavage, neutrophil elastase is the best candidate for LIX processing and activation *in vivo*.

In vitro *cleavage of mouse ELR$^+$ chemokines by neutrophil elastase*

We tested whether neutrophil elastase cleavage of LIX was a specific interaction or if other murine ELR$^+$ chemokines are processed. Incubations containing 20 nM enzyme and 10 μM chemokine (E:S of 1:500) revealed that KC and MIP-2 are resistant to cleavage and that DCIP-1 is degraded by neutrophil elastase (Figure A.3A). As expected, LIX was efficiently and specifically cleaved, generating LIX (7-83) and LIX (7-78), corresponding to $[M+H]^+$ values of 8352 and 7769, respectively. Therefore, LIX is the only murine neutrophil chemoattractant that is precisely cleaved and activated by neutrophil elastase.

In vitro *cleavage of human ELR+ chemokines by neutrophil elastase*

Human ELR$^+$ chemokines were investigated for neutrophil elastase cleavage to assess whether homologous chemokine activation mechanisms exist in human. At enzyme substrate ratios of 1:500 (mol:mol), neutrophil elastase cleaved GRO-γ at

A

DCIP-1 | KC | LIX | MIP-2

NE   -   +   -   +   -   +   -   +   +

7911   *   7812   7812   9852   7769   7844   7844   [M+H]$^+$
  8352,

B

Chemokine	m/z [M+H]$^+$	
	Measured	Predicted
DCIP-1 (1-73)	7911	7911
KC (1-72)	7812	7812
LIX (1-92)	9852	9850
LIX (7-83)	8352	8353
LIX (7-78)	7769	7770
MIP-2 (1-73)	7844	7847

Figure A.3. Neutrophil elastase processing of murine ELR$^+$ chemokines. Chemokines were incubated for 16 h at 37 °C in 50 mM Tris, 200 mM NaCl, 5 mM CaCl$_2$, pH 7.4. Enzyme to substrate ratio was 1:500 (mol:mol). (A) Reactions were visualized by 15% Tris-Tricine gel electrophoresis (silver stained) and analyzed by MALDI-TOF MS in sinapinic acid matrix. * signifiies loss of band intensity and protein degradation. (B) Mass to charge ratios ([M+H]$^+$) were deconvoluted to determine truncation products (italics).

position Val^4-Thr^5, but left GRO-α and GRO-β intact (Figure A.4A, B). Like DCIP-1, ENA-78 was also largely degraded by neutrophil elastase, but a peak at $[M+H]^+$ of 7862 was detected, which corresponds to a cleavage at Val^7-Leu^8 (Figure A.4A, B). NAP-2 was partially cleaved in the C-terminus at residues Val^{59}-Gln^{60} and IL-8 was resistant to cleavage at these conditions. The cleaved sequences reveal a strong preference for Valine in P1 (Figure A.4C), as reported previously in peptide library screens (6).

MMP-8 processing of α1-proteinase inhibitor

MMPs have been previously reported to cleave and inactivate α1-PI (7, 8) and modulate the activity of serine proteases physiologically through this mechanism (9). Other naturally occurring neutrophil elastase inhibitors SLPI and elafin are reported to be resistant to MMP-8 processing (10). MMP-8 efficiently cleaves the 54-kDa α1-PI to generate the 50-kDa inactive form (Figure A.5A), with processing evident at enzyme-substrate ratios as low as 1:5000 (w:w). A time course of MMP-8 proteolysis of α1-PI (Figure A.5B) enabled the estimation of a specificity constant (k_{cat}/K_M) of 7.7 x 10^3 $M^{-1}s^{-1}$ from densitometry analysis.

Altered processing of LIX in BALF of MMP-8 deficient mice

Several mouse tissues and fluids were examined for the presence of LIX and MMP-8 truncated LIX including air pouch fluid, corneal extracts, and arthritic joints with no success (data not shown). Previous work demonstrated decreased proteolysis in bronchoalveolar lavage fluid (BALF) from MMP-8 deficient mice and therefore processing was attributed to MMP-8 (11). Likewise, in the present work we observed reduced proteolysis of LIX in MMP-8 deficient lungs following 72-h treatment with LPS (Figure A.6A). However, these bands were not stained by neo-epitope antibodies specific for MMP-8 cleavage products of LIX (data not shown), suggesting that the cleavage sites are distinct from those produced by MMP-8. Western blots with the same BALF samples stained for α1-PI demonstrate increased levels of full-length α1-PI in knockout compared to wild-type lungs at both 48 and 72 h (Figure A.6B).

A

	GRO-α	GRO-β	GRO-γ	ENA-78	NAP-2	IL-8
NE	- +	- +	- +	- +	- +	- + +

7747 7747 7889 7889 7860 7505   8402 7862⁺ 7624 7624, 6481 8918 8918   [M+H]⁺

B

Chemokine	m/z [M+H]⁺ Measured	Predicted		Chemokine	m/z [M+H]⁺ Measured	Predicted
GRO-α (1-72)	7747	7748		ENA-78 (1-78)	8402	8403
GRO-β (1-73)	7889	7890		*ENA-78 (8-78)*	7862	7861
GRO-γ (1-73)	7860	7862		NAP-2 (1-70)	7624	7624
GRO-γ (5-73)	7505	7506		*NAP-2 (1-59)*	6481	6482
				IL-8 (1-77)	8918	8919

C

```
GRO-α    ASVATELRCQCLQTLQGIHP
GRO-β    APLATELRCQCLQTLQGIHL
GRO-γ    ASVVTELRCQCLQTLQGIHL
ENA-78   AGPAAAVLRELRCVCLQTTQGVHP
NAP-2         AELRCMCIKTTSGIHP... KKIVQKKLAGDESAD
IL-8     AVLPRSAKELRCQCIKTYSKPFH
```

Figure A.4. Neutrophil elastase processing of human ELR⁺ chemokines. Chemokines were incubated for 16 h at 37 °C in 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.4. Enzyme to substrate ratio was 1:500 (mol:mol). (A) Reactions were visualized by 15% Tris-Tricine gel electrophoresis and analyzed by MALDI-TOF MS in sinapinic acid matrix. (B) Mass to charge ratios ([M+H]⁺) were deconvoluted to determine truncation products (italics). (C) N-terminal cleavage sites in GRO-γ and ENA-78 and the C-terminal cleavage in NAP-2 are depicted in red. Sequences are aligned by the ELR motif, highlighted in gray.

151
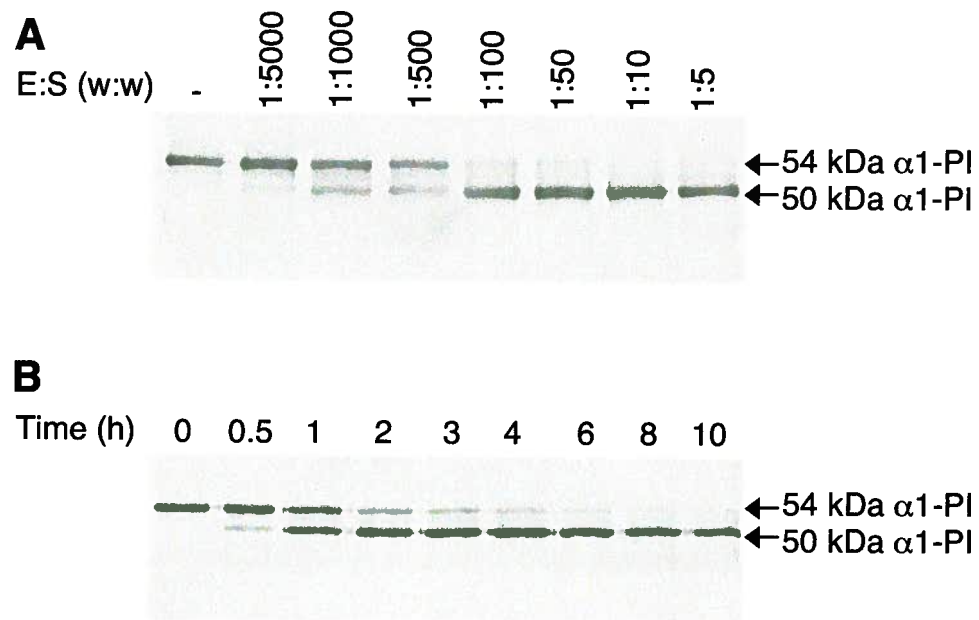
Figure A.5. MMP-8 cleavage of α1-proteinase inhibitor. (A) The serine protease inhibitor α1-PI was incubated with MMP-8 for 16 h at 37 °C in 50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, pH 7.4 containing 1 mM APMA. Enzyme to substrate ratio ranged from 1:5 to 1:5000 (w:w). Reactions were visualized on a 10% SDS-PAGE (silver stained). (B) Time course of MMP-8 cleavage of α1-PI at 1:50 (w:w) enzyme-substrate ratio.
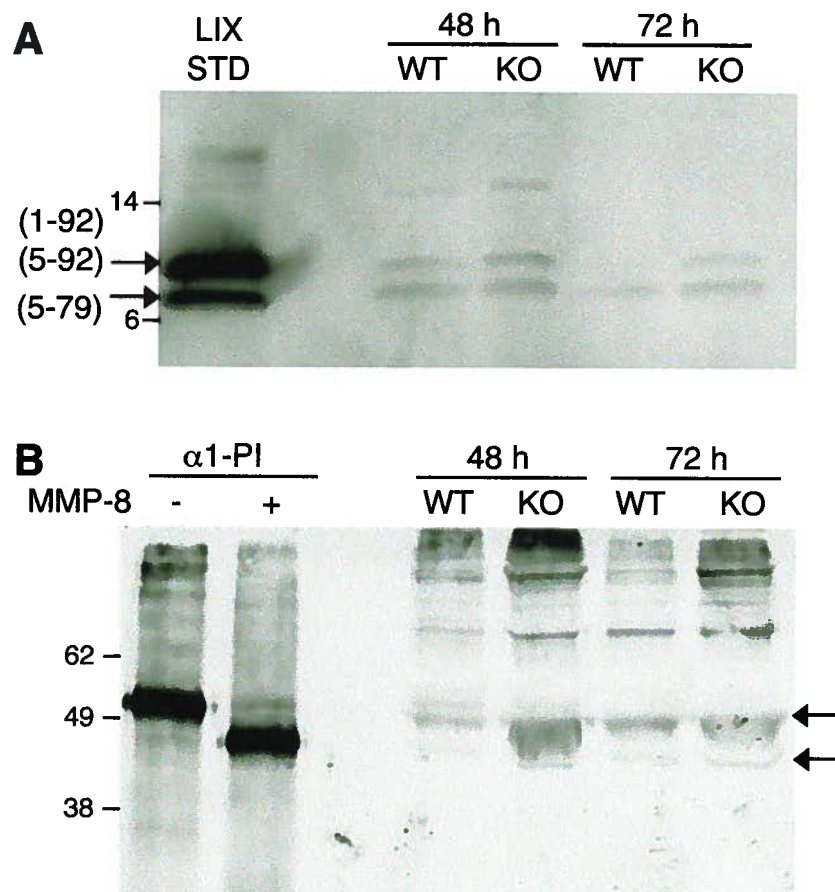
Figure A.6. Reduced LIX processing and increased full length α1-PI in BALF from MMP-8 deficient mice. Female wild-type and MMP-8 deficient mice had 2 μg LPS instilled in their lungs and mice were sacrificed and lungs lavaged with PBS at 48 and 72 h (Performed by Dr. Alain Doucet). Cell-free BALF from 3 mice was pooled and concentrated by acetone precipitation. (A) Western blot for LIX in BALF samples. Standard contains 5 ng each of LIX (1-92), (5-92), and (5-79). Antibody was polyclonal anti-LIX from R&D Systems and was used at 1:100. (B) Western blot for α1-PI in BALF. Standards were 50 ng α1-PI (uncleaved) and 50 ng α1-PI cleaved with MMP-8 (+MMP8). Antibody was anti-human antitrypsin (Sigma) used at 1:1000. Detection was with Alexa-conjugated antibodies (Molecular Probes) on the LiCOR Odyssey.

Summary and Future Experiments

The data presented herein supports our current hypothesis that MMP-8 processes and inactivates α1-PI, leading to increased neutrophil elastase activity and LIX activation, and thereby promoting neutrophil infiltration (Figure A.7). Conversely, in the MMP-8 knockout mouse, α1-PI is not inactivated and therefore neutrophil elastase remains inhibited. Notably, other neutrophil MMPs do not seem to compensate in the MMP-8 knockout mouse. As such, LIX processing and activation by neutrophil elastase is decreased in the absence of MMP-8, resulting in decreased neutrophil infiltration. However, some inconsistencies must be addressed. The N-terminal cleavage site generated by murine neutrophils (Ala^8-Thr^9) is different than that of human neutrophil elastase (Ile^6-Ala^7). This may simply reflect a slight difference in species specificity or could result from protease cooperativity in the context of the neutrophil, an effect that is absent in biochemical cleavage experiments. This could be clarified with murine neutrophil elastase, but this enzyme is not commercially available. Alternatively, LIX proteolysis and activation in lung inflammation could be studied in the presence of specific neutrophil elastase inhibitors to define the role of this serine protease in the physiological cleavage and activation of LIX.
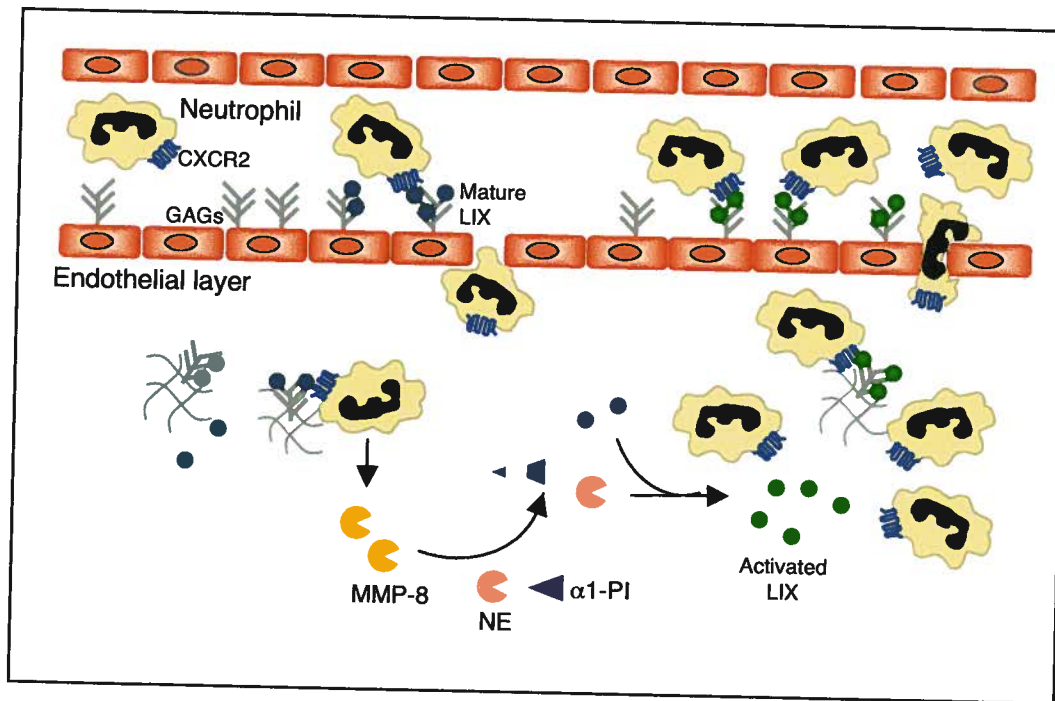
Figure A.7. Proposed mechanism for MMP-8 regulation of neutrophil elastase-mediated LIX activation. During infection or injury, tissue and endothelial cells secrete neutrophil chemoattractants including LIX/mCXCL5. In the mature form, LIX is a weak chemoattractant. Neutrophils that extravasate into the tissue are activated and degranulate, releasing MMP-8 and neutrophil elastase (NE). NE is inhibited by the irreversible serpin, α1-proteinase inhibitor (α1-PI). However, MMP-8 cleaves the C-terminus of α1-PI rendering it inactive and thereby increasing NE activity. Uninhibited NE cleaves the N-terminus of LIX resulting in increased chemoattraction of neutrophils, demonstrating an indirect feed-forward mechanism of neutrophil recruitment.

References

1. Tester, A. M., J. H. Cox, A. R. Connor, A. E. Starr, R. A. Dean, X. S. Puente, C. Lopez-Otin, and C. M. Overall. 2007. LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity. *PLoS ONE* 2:e312.

2. Van Den Steen, P. E., A. Wuyts, S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* 270:3739-3749.

3. Rao, N. V., B. C. Marshall, B. H. Gray, and J. R. Hoidal. 1993. Interaction of secretory leukocyte protease inhibitor with proteinase-3. *Am J Respir Cell Mol Biol* 8:612-616.

4. Wright, C. D., J. A. Kennedy, R. J. Zitnik, and M. A. Kashem. 1999. Inhibition of murine neutrophil serine proteinases by human and murine secretory leukocyte protease inhibitor. *Biochem Biophys Res Commun* 254:614-617.

5. McQuibban, G. A., J. H. Gong, E. M. Tam, C. A. McCulloch, I. Clark-Lewis, and C. M. Overall. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 289:1202-1206.

6. Schilling, O., and C. M. Overall. 2008. Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites. *Nat Biotechnol* 26:685-694.

7. Knauper, V., H. Reinke, and H. Tschesche. 1990. Inactivation of human plasma alpha 1-proteinase inhibitor by human PMN leucocyte collagenase. *FEBS Lett* 263:355-357.

8. Vissers, M. C., P. M. George, I. C. Bathurst, S. O. Brennan, and C. C. Winterbourn. 1988. Cleavage and inactivation of alpha 1-antitrypsin by metalloproteinases released from neutrophils. *J Clin Invest* 82:706-711.

9. Liu, Z., X. Zhou, S. D. Shapiro, J. M. Shipley, S. S. Twining, L. A. Diaz, R. M. Senior, and Z. Werb. 2000. The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. *Cell* 102:647-655.

10. Henry, M. T., K. McMahon, C. Costello, M. X. Fitzgerald, and C. M. O'Connor. 2002. Secretory leukocyte proteinase inhibitor and elafin are resistant to degradation by MMP-8. *Exp Lung Res* 28:85-97.

11. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35:252-257.

APPENDIX B. PROTEOMIC ANALYSIS OF MMP-8 CLEAVAGE SPECIFICITY AND SUBSTRATE IDENTIFICATION[6]

Perspective

There are fundamental differences between mouse and human MMPs, where humans have 23 and mice have 24 MMPs. It is very important to consider such differences when interpreting results from mouse models. Biochemical data with the ELR$^+$ chemokine CXCL5 shows a shift in cleavage specificity between human and rodent MMP-8, likely due to a lysine at S3' in the rodent enzyme in place of alanine in human MMP-8 (Chapter 2). Therefore, the cleavage specificities of human and mouse MMP-8 were compared to validate studies with the MMP-8 knockout mouse model. Traditional peptide-centric methods for profiling the cleavage site specificity of a protease are time consuming, expensive, and provide limited information [reviewed in (1)]. A novel technique that relies on proteome-derived, database-searchable peptide libraries for the identification of cleavage sites, termed PICS (2), was used to compare the sequence specificities of mouse and human MMP-8. Very minor differences in consensus cleavage sites were detected, suggesting that mouse and human MMP-8 act on similar substrates and have conserved physiological functions. To identify tissue-derived extracellular MMP-8 substrates in a systematic, non-biased manner, recombinant murine MMP-8 was added to immortalized embryonic fibroblasts that were isolated from MMP-8 knockout mice. The protein mixture in the conditioned media was then simplified by terminal amine isotopic labeling of substrates (termed TAILS), which enriches for N-terminal peptides (Kleifeld et al., manuscript in preparation). By comparing the intensities of isotopic labels in untreated and mMMP-8-treated samples, the levels of 20 peptides were significantly changed. Hence, the corresponding proteins represent potential mMMP-8 substrates. Future work will be aimed at validating potential substrates and using quantitative proteomics to compare neutrophils isolated from wild-type and MMP-8 knockout mice.

---

[6] The studies presented in Appendix B are unpublished.

Results

PICS analysis of MMP-8 cleavage specificity

The proteolytic cleavage specificities of murine and human MMP-8 were determined as described previously (2). Briefly, peptide libraries were prepared from the insoluble lysis pellet HEK 293 cells, digested with trypsin, and cysteines and amines were protected with iodoacetamide and formaldehyde treatment, respectively (library provided by Dr. Oliver Schilling). The library was treated with murine or human MMP-8 at a ratio of 1:100 (w:w) for 16 h at 37 °C. Neo-amino termini were conjugated to cleavable biotin and purified on immobilized streptavidin. Following LC-MS/MS on a QSTAR Pulsar (Applied Biosystems, Operated by the UBC Centre for Blood Research Mass Spectrometry Suite), peptides were identified by MASCOT in conjunction with PeptideProphet analysis. The C-terminal (non-prime) side of the original substrates was inferred from the human IPI database (Perl script by Dr. Oliver Schilling and Dr. Ulrich auf dem Keller).

PICS analysis of tryptic libraries with MMP-8 yielded 187 and 266 cleaved peptides with mouse and human enzymes, respectively. The resulting heat maps, generated with Pro FIT software, reveal a strong preference for leucine at P1', proline and alanine at P3, and glycine and alanine at P3' (Figure B.1). This specificity is comparable to that reported for MMP-2 (2). Notably, there were very few differences between the mouse and human cleavage preferences. Human MMP-8 had slightly elevated preference for glycine at P5, whereas mouse MMP-8 had increased preference for serine at P1 and leucine at P4 and P5'. Overall, the specificities look very similar, suggesting conserved cleavage specificities for human and mouse MMP-8 *in vivo*.
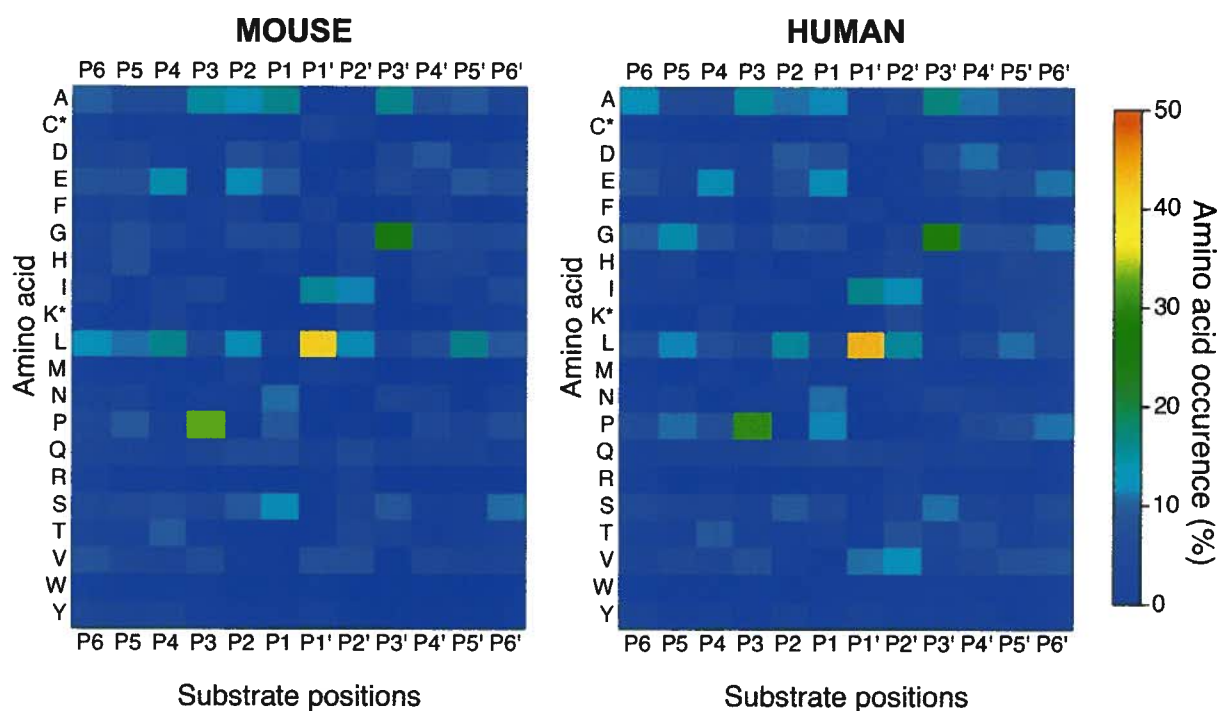
Figure B.1. Cleavage site specificities of murine and human MMP-8. PICS analysis of tryptic peptide libraries was performed at an enzyme-library ratio of 1:100 (w:w) for 16 h at 37 °C. Amino acid occurences as percentages from P6 to P6' are plotted. C, carboxyamidomethylated cysteine; K, dimethylated lysine.

MMP-8 substrate discovery with TAILS enrichment method

Over the past few years, numerous proteomic approaches to protease substrate discovery have been reported including 2-dimensional gel electrophoresis (3), isotope-coded affinity tags (ICAT) (4-6), and isobaric tags for relative and absolute quantitation (iTRAQ) (7). A novel method with the benefit of simplifying complex proteomes is called TAILS, for terminal amine isotopic labeling of substrates (Kleifeld et al., manuscript in preparation). Like iTRAQ, TAILS relies upon isotopic labeling of protein N-termini comparing untreated and protease-treated samples. However, following tryptic digestion of proteins, internal peptides are removed by coupling of free N-termini to a large polymer containing reactive aldehyde groups. The original N-termini in the sample are blocked with isotopic labels and therefore do not bind the polymer. As such, the sample is greatly enriched for original and protease-derived N-terminal peptides allowing for better detection of potential protease cleavage sites.

Embryonic fibroblasts from MMP-8 knockout mice were isolated and immortalized to provide a naive system for studying MMP-8 proteolysis of extracellular matrix and secreted tissue proteins. Briefly, embryos were isolated from time-mated MMP-8 knockout females on the C57BL/6 x 129 background at 13.5 days gestation. Notably, timed mating was most successful when females were placed in a male-scented cage 3 days prior to mating, thereby initiating the female estrus cycle. Embryonic heads and livers were removed and remaining tissue was homogenized in trypsin and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μM 2-mercaptoethanol. Cells were passaged every 3-5 days until immortalized and then subcloned.

Recombinant murine MMP-8 was added to *Mmp8-/-* fibroblast cultures in serum-free conditions for 24 h at a concentration of 0.4 μg/mL, as determined by active site titration with TIMP-1 and TIMP-2. Conditioned media was collected and

concentrated by precipitation with trichloroacetic acid, resuspended in 50 mM NaOH, then neutralized with 250 mM HEPES, pH 8.0. 500 μg each of untreated and MMP-8 treated proteomes were reduced, denatured, isotopically labeled with heavy or light label, combined, and trypsin digested. The resultant peptide mixture was then incubated with an excess of aldehyde-containing polymer to remove internal peptides. The remaining peptides were fractionated by strong cation exchange HPLC and analyzed by LC-MS/MS on a QSTAR Pulsar (Applied Biosystems, Operated by the UBC Centre for Blood Research Mass Spectrometry Suite). Peptides were identified by MASCOT assignment and relative abundance quantified by Trans Proteomic Pipeline (TPP, Institute for Systems Biology). The resulting data was analyzed and annotated by Dr. Ulrich auf dem Keller.

Following polymer enrichment, 432 unique peptides were identified. Of these, 76% had iTRAQ-labeled N-termini, 13% had acetylated N-termini, 10% had iTRAQ-labeled lysines, and 1% were completely unlabeled (Figure B.2A). Therefore, 11% of peptides had unlabeled N-termini. In contrast, 89% of peptides had blocked N-termini and hence represent either mature or proteolytically generated N-terminal peptides, demonstrating clear enrichment using the TAILS technique. The distribution of isotopic ratios relating MMP-8 treated to untreated controls is centered around natural log of 0, indicating a 1:1 labeling ratio (Figure B.2B). The median of this distribution was 0.96. Using a cut-off of a two fold change in isotopic intensity, 9 peptides were significantly upregulated in MMP-8 treated samples, including MMP-8 itself (Figure B.2C). The other increased peptides could represent direct MMP-8 substrates but these have yet to be validated. In contrast, 12 peptides were significantly downregulated in the presence of MMP-8. This could be the result of protein degradation by MMP-8, MMP-8 regulation of other proteases in the cellular context, or alternatively MMP-8 cleavage within the detected peptide. The latter of these possibilities is probable in several cases where a strong MMP consensus sequence of proline at P3 and leucine or isoleucine P1' are evident (shown in red), such as SPARC, shown previously to be cleaved by several MMPs at this site (8). Notably, 9 of the downregulated peptides contain RL and RV on the N-terminal side

of cleavage (P2 and P1). Potentially, these sites are cleaved by unknown fibroblast-derived protease(s), and hence these changes could result from MMP-8 downregulation of the activity of other proteases in the protease web.

Summary and Future Directions

The data presented here has demonstrated similar cleavage site specificities for murine and human MMP-8 with a consensus of leucine at P1', proline and alanine at P3, and glycine and alanine at P3'. However, it is known that exosites such as the hemopexin C domain are major determinants of substrate specificity, and exosite interactions are unlikely at the peptide level (9). Therefore, comparing human and mouse MMP-8 activities on protein substrates should be addressed in the future. The TAILS data shown here is really just the beginning of a project aimed at identifying MMP-8 substrates, through the use of immortalized cells as well as primary neutrophils from wild-type and MMP-8 knockout mice. Several potential substrates were identified and these will be confirmed with repeat experiments and validated both biochemically and physiologically. Quantitative proteomic techniques including TAILS have the potential to reveal the precise mechanisms through which MMPs such as MMP-8 are regulating fundamental cellular and physiological processes.

A Lysine 10% Unlabeled 1%

Acetylated 13%

iTRAQ-labeled N-termini 76%

B

Relative frequency

ln (MMP8:Control)

C

PROTEIN	SEQUENCE	MMP8:Control
Neutrophil collagenase	VPEH.LEEKNIKTAENYLR	> 20
Fibronectin	YLPN.VDEEVQIGHVPR	5.3
Beta 2 microglobulin	TGLY.AIQKTPQIQVYSR	4.8
Pfas Novel protein	QQLG.ASVVPLSPDHAR	3.6
Tyrosine protein kinase like 7	AGAQ.AAIVFIKEPSSQDALQGR	3.4
Extracellular matrix protein	ALAS.AASEGAFKASDQR	3.1
Nuclear pore complex-associated TPR	EGGD.GTDPGTETEESMGGAESHQR	2.6
Thrombospondin 2	RASA.GDHVKDTSFDLFSISNINR	2.5
Dystroglycan	AVAQ.AHWPSEPSEAVR	2.4
Periostin	KIID.GVPVEITEKQTR	0.49
Transitional ER ATPase	RVRL.GDVISIQPCPDVKYGKR	0.21
Protein disulfide isomerase A3	KKFI.SDKDASVVGFFR	0.07
PDZ and LIM domain protein 1	GFRL.VGGKDFEQPLAISR	0.07
Moesin	QKHL.KALTSELANAR	0.06
Heat shock cognate 71 kDa protein	TERL.IGDAAKNQVAMNPTNTVFDAKR	0.05
Actin cytoplasmic 1	ELRV.APEEHPVLLTEAPLNPKANR	0.05
Transketolase	INRL.GQSDPAPLQHQVDIYQKR	<0.05
Superoxide dismutase Cu Zn	EDRV.ISLSGEHSIIGR	<0.05
SPARC	EKRL.EAGDHPVELLAR	<0.05
Fibronectin	GYRV.EVLPVSLPGEHGQR	<0.05
Hspg2 perlecan	EARL.VSEDPINDGEWHR	<0.05

Figure B.2. CLIP-TAILS analysis of _Mmp8-/-_ MEF secretome in the presence and absence of murine MMP-8. (A) Distribution of 432 unique peptides identified by TAILS enrichment. (B) Relative frequency of ratios plotted as a function of ln (MMP8:control) ratios based on the intensity of heavy to light isotopic labels. The median of distribution is 0.96. (C) Isotopic intensities of several peptides were significantly altered and are listed with their corresponding proteins. Sequences in red indicate potential MMP cleavage sites within the identified sequence.

References

1. Schilling, O., and C. M. Overall. 2007. Proteomic discovery of protease substrates. *Curr Opin Chem Biol* 11:36-45.

2. Schilling, O., and C. M. Overall. 2008. Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites. *Nat Biotechnol* 26:685-694.

3. Greenlee, K. J., D. B. Corry, D. A. Engler, R. K. Matsunami, P. Tessier, R. G. Cook, Z. Werb, and F. Kheradmand. 2006. Proteomic identification of in vivo substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. *J Immunol* 177:7312-7321.

4. Tam, E. M., C. J. Morrison, Y. I. Wu, M. S. Stack, and C. M. Overall. 2004. Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 101:6917-6922.

5. Dean, R. A., G. S. Butler, Y. Hamma-Kourbali, J. Delbe, D. R. Brigstock, J. Courty, and C. M. Overall. 2007. Identification of candidate angiogenic inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based proteomic screens: disruption of vascular endothelial growth factor (VEGF)/heparin affin regulatory peptide (pleiotrophin) and VEGF/Connective tissue growth factor angiogenic inhibitory complexes by MMP-2 proteolysis. *Mol Cell Biol* 27:8454-8465.

6. Butler, G. S., R. A. Dean, E. M. Tam, and C. M. Overall. 2008. Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: Dynamics of matrix metalloproteinase-14 (MT1-MMP) mediated membrane protein shedding. *Mol Cell Biol*.

7. Dean, R. A., and C. M. Overall. 2007. Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome. *Mol Cell Proteomics* 6:611-623.

8. Sasaki, T., W. Gohring, K. Mann, P. Maurer, E. Hohenester, V. Knauper, G. Murphy, and R. Timpl. 1997. Limited cleavage of extracellular matrix protein BM-40 by matrix metalloproteinases increases its affinity for collagens. *J Biol Chem* 272:9237-9243.

9. Overall, C. M. 2002. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol Biotechnol* 22:51-86.

APPENDIX C. UBC RESEARCH ETHICS BOARD CERTIFICATES

This appendix contains the following approval certificates from the University of British Columbia.

1. Animal Care certificate of approval of mouse breeding (A06-0347)

2. Animal Care certificate of approval of air pouch model (A06-0303)

3. Animal Care certificate of approval of MRL/lpr arthritis experimentation (A06-0170)

4. Clinical Research Ethics Board approval of use of human blood in chemotaxis and proteolysis experiments (H6-00047)

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0347

Investigator or Course Director: Christopher Overall

Department: Oral Biological & Medical Sciences

Animals:

Mice 1500

Start Date:	June 24, 2006	**Approval Date:**	August 28, 2007

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Leukocyte matrix metalloproteinases (MMPs) in arthritis
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Functional proteomics and activation of cancer matrix metalloproteinases
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Neutrophil collagenase (MMP-8) in inflammation
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Proteomic and degradomic analysis of breat cancer metastases
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Metalloproteinase degradomics: systems biology of the protease web in periodontitis
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	CIHR Group in Matrix Dynamics

Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Molecular determinants of human MMP-2 substrate activity
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Functional proteomics and activation of cancer matrix metalloproteinases
Funding Agency:	UBC Operating Budget
Funding Title:	BREEDING: Production of MMP-2 and -8 deficient mice
Funding Agency:	Canadian Arthritis Network (CAN) - Networks of Centres of Excellence (NCE)
Funding Title:	Anti-inflammatory chemokine antagonists for treating arthritis
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	CIHR Group in Matrix Dynamics
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Proteomic and degradomic analysis of breast cancer metastases
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

UBC

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0303

Investigator or Course Director: Christopher Overall

Department: Oral Biological & Medical Sciences

Animals:

Mice 129 Sv/Ev MMP12 KO 100
Mice B6129SF2/J MMP8 KO 100
Mice 129 Sv/Ev 100
Mice B6129SF2/J 100

Start Date:	**June 25, 2006**	**Approval Date:**	**August 20, 2007**

Funding Sources:

Funding Agency:	Canadian Arthritis Network (CAN) - Networks of Centres of Excellence (NCE)
Funding Title:	Anti-inflammatory chemokine antagonists for treating arthritis

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

UBC

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0170

Investigator or Course Director: Christopher Overall

Department: Oral Biological & Medical Sciences

Animals:

Mice MRL/MpJ-Faslpr/J KOMMP8 60
Mice MRL/MpJ-Faslpr/J 60

Start Date:	**May 8, 2006**	**Approval Date:**	**August 3, 2007**

Funding Sources:

Funding Agency:	Canadian Arthritis Network (CAN) - Networks of Centres of Excellence (NCE)
Funding Title:	Anti-inflammatory chemokine antagonists for treating arthritis

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

UBC

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR: Christopher Overall	DEPARTMENT: UBC/Dentistry/Oral Biological & Medical Sciences	UBC CREB NUMBER: H06-00047

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:	
Institution	**Site**
UBC	Vancouver (excludes UBC Hospital)
Vancouver Coastal Health (VCHRI/VCHA)	UBC Hospital
Other locations where the research will be conducted: N/A	

CO-INVESTIGATOR(S):
Amanda Starr
Charlotte Morrison
Richard A Dean
Alain Doucet
Ulrich Auf dem Keller
Georgina S. Butler
Reinhild Kappelhoff
Jennifer Cox

SPONSORING AGENCIES:
Canadian Arthritis Network (CAN) - Networks of Centres of Excellence (NCE) - "Anti-inflammatory chemokine antagonists for treating arthritis"
Canadian Institutes of Health Research (CIHR) - "Substrate identification for matrix metalloproteinases (MMPs) involved in arthritis progression"
National Cancer Institute of Canada

PROJECT TITLE:
Migration and proteolytic activity of human immune cells

EXPIRY DATE OF THIS APPROVAL: April 7, 2009

APPROVAL DATE: April 7, 2008

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by:

Dr. Bonita Sawatzky, Associate Chair