Matrix Metalloproteinase 13a:
An early host protective factor during mycobacterial infection

Elizabeth Sachiko Seilie

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Reading Committee:
Lalita Ramakrishnan, Chair
William C. Parks
David R. Sherman

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Abstract

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Elizabeth Sachiko Seilie

Chair of the Supervisory Committee:
Dr. Lalita Ramakrishnan
Department of Microbiology

Tuberculosis (TB) is a bacterial disease caused by *Mycobacterium tuberculosis* (Mtb). Despite the availability of antibiotics, it remains an insidious problem worldwide, and the rise of drug resistant strains has threatened the ability to treat and eradicate the disease. We are looking to target host pathways as adjunct therapies to shorten drug treatment and improve survival. Our lab studies host pathways involved in immunity and pathogenesis of TB using the zebrafish-*M. marinum* (Mm) model of infection. Matrix metalloproteinases (MMPs), a class of proteases that are known to participate in inflammation and immunity, are upregulated during Mm and Mtb infection. Since MMPs are correlated to severity of infection, we wanted to what roles they play during tuberculosis. We have previously published that *mmp9* is subverted by mycobacteria to enhance granuloma formation and bacterial spread[1, 2]. Here, we have found that *mmp13a* is induced in response to infection with bacteria that express the ESX-1 virulence factor, similar to *mmp9*. However, unlike *mmp9*, *mmp13a* knockdown causes hypersusceptibility, indicating that it is a host-protective factor. Indeed, *mmp13a* expression enhances bactericidal activity by

macrophages. The knockdown phenotype for *mmp13a* is similar to that of *tnf* signaling deficiency. We show that MMP13a can cleave TNF *in vitro*, and that it may be an important TNF sheddase during mycobacterial infection. MMP13a may act by regulating the amount of bio-active TNF that is available during infection, and is a viable target for inhibition to treat hyper-inflammatory TB patients.

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List of Abbreviations:

Abbreviation	Full name
ADAM	A-Disintegrin-and-Metalloprotease
APMA	p-aminophenylmercuric acetate
BCG	Bacille Calmette Guerin
BSL	Bio-Safety Level
CFP-10	culture filtrate protein-10
CFU	Colony forming units
DIC	Differential Interference Contrast
dpf	days post fertilization
dpi	days post infection
ECM	extracellular matrix
ESAT-6	6-kDa early secreted antigenic target
ESX-1	ESAT-6 secretion system-1
GAG	glycosaminoglycan
hpf	hours post fertilization
hpi	hours post infection
HPX	hemopexin
IL	Interleukin
iNOS	Inducible nitrogen oxide synthase
KO	knockout
LRP-1	Low-density lipoprotein (LDL) receptor related protein-1
LTA4H	leukotriene-A-4-hydrolase
LTB4	Leukotriene B4
MBP	mannose-binding protein
MCP	Monocyte chemoattractant protein
memTNF	membrane bound TNF
Mm	*Mycobacterium marinum*
MMP	Matrix metalloproteinase
MO	Morpholino oligonucleotide
Mtb	*Mycobacterium tuberculosis*
NE	Neutrophil Elastase
NHBE	Normal Human Bronchial Epithelial
NLR	NOD-like receptor
RD1	Region of Difference-1
RECK	rich-protein-with-kazal-motifs
ROS	reactive oxygen species
RT-qPCR	Real time- quantitative PCR
SEM	Standard Error of Means
SNP	Single nucleotide polymorphism
sTNF	soluble TNF

TB	Tuberculosis (disease)
TGFβ	Tumor growth factor
TIMP	tissue inhibitor of metalloproteases
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
TNFR1	TNF receptor-1
WHO	World Health Organization
WT	Wildtype

Chapter 1:

Introduction

Infection by *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), is a leading cause of morbidity and mortality in the world, disproportionately affecting low- and middle-income countries. The World Health Organization (WHO) estimates that in 2013, 9 million people were diagnosed with TB and 1.5 million died of the disease, but over 95% of the cases and mortalities occurred in developing countries. TB is a treatable disease; however, difficulties in diagnosis and an extended treatment time of a minimum of 6 months have contributed to the slow eradication of the disease[3]. With the rise of multi-drug resistant strains of Mtb, better understanding of the immune responses during TB are of the utmost importance in order to find new drug and vaccine targets[4]. It is believed that about one-third of the world's population has been infected by Mtb, but remarkably, only 10% of this population has a lifetime risk of developing disease[3]. This discrepancy is an indication that the immune response can be effective in controlling Mtb and can potentially be harnessed to increase resistance to disease. The broad strokes of the immune response to Mtb are understood, but much remains unknown regarding specific factors that limit bacterial growth or mechanisms by which the bacterium escapes immune control.

Although Mtb can infect almost any part of the body, TB manifests primarily as a disease of the lungs since the bacterium is transmitted by aerosolized droplets[5]. Once inhaled, the droplets are deposited in alveoli deep in the lung, where they are phagocytosed by alveolar macrophages[6]. Mycobacteria can avoid intracellular killing through multiple mechanisms[7,

8]. Those that survive will continue to multiply while utilizing the macrophage to reach deeper tissues[5, 6]. Here, the infected macrophage aggregates with other uninfected leukocytes to form the granuloma, the hallmark pathology of TB. The granuloma continues to grow in size and eventually incorporates B and T lymphocytes as the adaptive immune response is activated. Granuloma progression typically involves necrosis at the core, forming a solid caseous center, and may lead to formation of cavities in the lung parenchyma. The engagement of the adaptive immune response is effective in limiting around 90% of infected cases[9], though exactly when this occurs remains unclear. In individuals who are unable to control infection, destruction of lung tissues will progress. Once the cavities reach airways, the granuloma may rupture, allowing Mtb to reach the airways and be aerosolized through coughing, starting the cycle again in a new host[5, 10].

The granuloma has traditionally been considered a host protective structure. Indeed, Mtb infection in individuals that are immunocompromised are characterized by widely disseminated disease and poor granuloma formation[9]. However, recent studies from the Ramakrishnan lab and other labs have challenged this notion. By developing a zebrafish larvae model of tuberculosis, we are able to observe the dynamic early immune responses to bacterial infection. For example, we have found that granuloma formation is actually driven by a bacterial virulence factor rather than by the host response to infection[1, 2, 11]. Interestingly, the bacterial virulence factor hijacks a host gene, matrix metalloproteinase-9 (*mmp9*), to induce macrophage aggregation, which facilitates bacterial replication and spread.

I am particularly interested in the processes involved in the innate immune response to infection. Many genes are known to be quickly upregulated by cells that encounter mycobacteria, however, their roles cannot not be studied in traditional animal models of tuberculosis due to the

inability to follow infection over multiple time points in a single organism. As a result, it was thought that the innate immune response was ineffective in mycobacterial control[9]. The zebrafish model of infection circumvents this problem since larvae are optically transparent, allowing real-time visualization of infection[12].

Many MMPs aside from *mmp9* are known to be upregulated during Mtb infection. Their roles in either immunity or pathogenesis of TB have not yet been explored. I will show that *mmp9* and *mmp13a* are induced specifically by virulent mycobacteria, and then demonstrate that the induction of *mmp13a*, unlike *mmp9*, is a host protective response by acting as a TNF converting enzyme during infection. We present data indicating that *mmp13a* is a target for pharmacologic control of hyper-inflammation in TB patients.

Chapter 2:

Background: Innate Immunity in Tuberculosis

2.1. Animal models of TB pathogenesis

The use of animal models has been vital to our understanding of TB pathogenesis. Studies in of TB in rabbits in the early to mid-1900s, for example, gave researchers an early understanding of the chronology of granuloma formation and requirements for cavity formation[10]. Lurie's TB susceptible and resistant rabbits confirmed the observation that susceptibility to TB in humans had a genetic component[5]. Despite the fact that rabbits show similar pathogenesis as humans during Mtb infection, this model is difficult to use owing to the size of the organism and the lack of genetic tools. Similarly, guinea pigs are exquisitely susceptible to TB infection and form granulomas similar to humans, but also lack genetic tools. By contrast, the mouse model is genetically tractable and replete with experimental tools by which the immune response can be manipulated and probed. These features have allowed researchers to investigate the role of specific genes such as *NRAMP1* and whole populations of immune cell types in establishing immunity to infection. Studies in mice have also confirmed the important role of inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β), in limiting bacterial growth[13, 14]. Despite the similarity in many aspects of immunity between mice and humans, there are a number of major drawbacks in this model that obscure understanding of human TB. For example, TB granulomas in mice do not take on the same architecture as those found in humans or rabbits: they are less organized and do not caseate or cavitate[10]. Experiments also require that the mice be culled to determine bacterial load or granuloma structures, making it difficult to study the dynamic nature of immunity and

understand the role of cell migration during infection. Finally, despite numerous studies in cell culture that show the importance of various innate immune factors on the control of mycobacterial growth in macrophages, these phenotypes are difficult to observe in mice. These phenotypes are not apparent until after 20 days post infection (dpi), by which time, adaptive immunity may have already begun to respond[9].

2.2. Modeling Tuberculosis infection in zebrafish

To address the earliest events during mycobacterial infection, our lab developed a model system utilizing zebrafish (*Danio rerio*) as a host to *M. marinum*, a close genetic relative of the Mtb complex.

2.2.1. The zebrafish as a model organism

The zebrafish is a small fresh water fish that has been an important model for developmental biologists. Their development, starting with the fertilization of the egg, are all viewable ex-utero, and live larvae are also transparent, allowing for observation of cell migration *in vivo* and in real-time[15]. Moreover, they are easy to breed and are fecund, each female laying up to 200 eggs per week. Their genome has been sequenced, allowing for both forward and reverse genetic approaches[16, 17]. Until recently, reverse genetics has been limited to temporary knock-down of gene translation through the use of morpholino oligonucleotides (MOs), which are 25-mer oligonucleotides with a modified backbone that can prevent proper processing of pre-mRNA or prevent translation binding machinery from binding the start site[18]. Targeted gene knockout strategies are available now, thanks to improvements in gene-editing technologies[19, 20]. MOs are still useful, since they offer the ability to investigate gene

function directly in injected larvae, rather than having to wait for breedable adults. Additionally, the small size and cheap cost of zebrafish housing allow researchers to relatively easily conduct forward genetic screens with randomly mutagenized populations to discover novel genes that produce measurable phenotypes[21]. Importantly, zebrafish share many important signaling pathways found in mammalian systems, allowing findings to be interpreted in the context of human disease[21].

2.2.2. Zebrafish immunology

The main components of the mammalian immune system and response are conserved in zebrafish and are observable early in development. Most classes of immune cells in humans are in adult zebrafish, including macrophages, neutrophils, eosinophils, mast cells/basophils, dendritic cells, B lymphocytes, T lymphocytes, and natural killer (NK) cells[22-24]. Many components of humoral immunity are present in the fish, including a well-developed complement system and antimicrobial proteins, such as lysozymes and defensins. Zebrafish orthologs of immune signaling components have also been discovered and appear to be similar to that of mammals, including pathogen recognition receptor families of toll-like receptors (TLRs) and NOD-like receptors (NLRs). In fact, zebrafish have more genes representing these components than what is present in mammalian systems, likely as a result of differences in the frequency and variety of microbes that fish encounter[25-27].

Owing to the transparency of zebrafish larvae, hematopoiesis is well understood in this system. The immune system of the developing embryo resembles that of embryonic mammals, and thus, the adaptive immune response is not fully functional until 4 weeks post fertilization[28]. The innate immune responses of zebrafish larvae, by contrast, are well

developed early in development, allowing for the isolated study of innate immune responses to various pathogens. Primitive macrophages in the zebrafish larvae arise by 22 hours post fertilization (hpf) and by 30hpf, are capable of detecting and responding to infection. Infection of larvae with non-pathogenic bacteria at this time will induce macrophage migration and phagocytosis, leading to bacterial killing[29]. In addition, macrophages are necessary to clear apoptotic cells that arise as a normal part of development, especially in the brain. Macrophage depletion results in the accumulation of apoptotic bodies in the brain of 3 day post fertilization (dpf) larvae, underscoring the homeostatic importance of early zebrafish macrophages[30]. Markers of neutrophils, such as myeloperoxidase (*mpx)* and lysozyme (*lyz),* are expressed in primitive macrophages through 32hpf, however, this changes when definitive hematopoiesis begins at 48hpf. At this time, *lyz* is specifically expressed by neutrophils, which now respond to signals distinct from that of macrophages, including interleukin-8 (IL-8) and leukotriene B_4 (LTB4)[31].

2.2.3. *Mycobacterium marinum* causes tuberculosis in zebrafish

One of the natural pathogens that infect zebrafish is *Mycobacterium marinum* (Mm)*,* a close genetic relative to the Mtb complex that has been used to model various aspects of Mtb biology. Unlike Mtb, which is a biosafety level 3 (BSL3) organism requiring special laboratory equipment and personal protective equipment, Mm is a BSL2 organism owing to the fact that its optimal growth temperature is between 25-35ºC and grows poorly at 37ºC. It is still capable of infecting mammals, however, and infection in humans is clinically recognized as ‘fish tank granulomas’, which are restricted to extremities[32]. Mm granulomas in humans are also pathologically indistinguishable from Mtb granulomas, pointing to the similarities in

mechanisms that Mm and Mtb use to cause disease[33]. Additionally, Mtb is also difficult to study since it doubles every 22 hours, and colonies take up to 3 weeks to develop[34]. By contrast, Mm doubles every 4-6 hours[33], and colonies are visible in 5-7 days, making genetic manipulations and *in vitro* assays easier to conduct.

Comparison of the Mm and Mtb genomes reveal that the two share genes that are attributed to virulence. The Mm genome, at 6.6 Mb, is about 1.5x larger than that of Mtb. Some of the genes missing in Mtb are thought to confer the ability of Mm to survive in environmental niches and appear to have been lost in Mtb as it adapted to survive exclusively within hosts[33]. Many of the genes shared by the two mycobacteria have roles in pathogenesis, which is not surprising given the similarities in the pathological features of infection. These include genes involved in intracellular survival (e.g., cell wall synthesis, efflux pumps) and virulence factor transport (e.g., the ESX secretion systems)[33, 34].

Mm granulomas in zebrafish are similar to that of humans. Infection of adult zebrafish with even 5 colony forming units (CFU) of Mm leads to progressive and chronic disease, characterized by the formation of well-structured granulomas[35]. Histological examination of these granulomas revealed that they are highly organized structures with an outer layer of epithelioid cells and an inner layer consisting of macrophages and some granulocytes and lymphocytes[36], similar in structure to what is seen in human granulomas. Over time, some granulomas form necrotic cores containing far greater numbers of bacteria than found in nonnecrotizing granulomas[35]. These features of the Mm granuloma in zebrafish are similar to human granulomas caused by Mtb, but in stark contrast to Mtb infection in mice, which do not result in the formation of organized or necrotizing granulomas[33, 35]. Unlike mammalian Mtb or Mm granulomas, which are characterized by an abundance of lymphocytes, zebrafish

granulomas contain very few lymphocytes. Regardless, lymphocytes play a protective role in zebrafish infection since *rag1* KO fish are more susceptible to Mm infection[35].

The similarity of the granuloma structure with humans indicate that the zebrafish is an ideal host to further probe the interaction between the host and mycobacteria during infection. The ability to observe cellular interactions and intracellular events *in vivo* will also help elucidate event in early infection that are difficult, if not impossible, to study using traditional mammalian models.

2.3. Innate immunity is critical in early restriction of bacterial growth

Studies of Mm pathogenesis in the larval zebrafish have allowed visual observation of early events during mycobacterial infection. Fluorescently-labeled Mm injected at 32hpf are rapidly taken up by macrophages, which do not clear infection. Phagocytosed Mm respond by expressing genes that induced during infection of human macrophages in culture, suggesting that the environment within the larval macrophage resembles that of human macrophages. By 3dpi, aggregates of infected macrophages appear, bearing features resembling epithelioid and multinucleated giant cells found in human TB granulomas[12, 37]. Notably, Mm genes that are induced in granulomas of adult frogs (*Rana pipiens*)[38] are also induced in zebrafish larvae granulomas. In all, since larvae are devoid of adaptive immunity, these indicate that innate immune factors are sufficient to drive granuloma formation during TB, contrary to popular belief at the time[9, 33, 39].

The significance of innate immunity in TB was unclear until recently. Although many genes involved in innate immunity were shown to be important to host resistance, studies in mice, rabbits, and guinea pigs also showed that bacteria replicated quickly for the first 20 days of

infection, only tapering off at the onset of the adaptive response, leading some to believe that the innate immune response was completely ineffectual[9, 40]. To further support this view, pathogenic mycobacteria are capable of growing in macrophages and are restricted only after activation of macrophages through the addition of interferon-γ (IFNγ) and TNF[41]. Other studies point to a protective role for innate immunity prior to the onset of the adaptive immune response. In one of the early studies of TB in animal models, Lurie observed that lines of rabbits susceptible to Mtb harbored 20-30 times greater numbers of bacteria relative to resistant rabbits at 7dpi. Furthermore, human epidemiological studies have linked polymorphisms in innate immune response genes, such as *IL-1β, VDR, NRAMP1*, to tuberculosis to susceptibility[5, 42-44].

The ease of macrophage depletion and observation of infection makes the zebrafish a prime candidate for asking questions regarding the complicated nature of innate immunity. Where as bacterial numbers are difficult to count in mammalian hosts, the transparent zebrafish larvae allows for observation of bacterial growth *in vivo* and in real time. When macrophages were knocked down in zebrafish larvae using a MO targeting a transcription factor essential to the development of the myeloid lineage[45], the larvae became exquisitely susceptible to Mm infection[46]. Even though bacterial replication appears out of control in the first three weeks of infection in mammalian models, complete loss of macrophages would likely result in more severe outcomes. In agreement with previous studies, these findings also also showed that macrophages quickly respond to infection by upregulating the inflammatory cytokines TNF and IL-1β, which are both implicated in bacterial killing in macrophages[46]. Bacterial mutants that survive poorly in macrophage cell cultures are rescued in macrophage-deficient larvae, confirming that macrophage activity is an important factor in bacterial restriction[46].

2.4. Balanced TNF expression is host protective

One of the earliest cytokines to be implicated in host protection during mycobacterial infection was TNF. It is highly upregulated in lungs of infected mice and in macrophages infected in culture[47] and treatment of macrophages with TNF and IFNγ increases their mycobactericidal capabilities[48]. The importance of TNF in human TB infection is apparent in patients treated with anti-TNF antibody therapies for autoimmune disorders, and were later diagnosed with disseminated tuberculosis, usually as a result of reactivation of a latent infection[49, 50]. Mice deficient for TNF or TNFR1, either through genetic means or treatment with anti-TNF antibodies, are hypersusceptible and succumb to infection as early as 20 dpi[13, 51, 52]. These early studies implicated loss of TNF with delayed formation of granulomas and failure of T-cell priming, both of which were considered to be critical for control of bacterial growth[52]. It was unclear from these studies if or how TNF played a role in bacterial control before granuloma formation.

Using the zebrafish model of infection allowed our lab to investigate the role of TNF in early responses to mycobacterial infection. The optical transparency of our model and ease of tracking bacterial growth at the single cell resolution lead to the discovery that TNF signaling in macrophages leads to the induction of intracellular bacterial killing[46, 53]. Later studies showed that bacterial killing was mediated through mitochondria produced reactive oxygen species (ROS) dependent mechanisms, as opposed to induction of iNOS, as macrophage culture studies indicated[54]. Rather than preventing granuloma formation, the accelerated bacterial growth in TNF-Receptor 1 (TNFR1) deficient larvae augmented granuloma formation. Despite this, the host quickly succumbs to infection as overburdened macrophages undergo necrosis and release bacteria in to extracellular space, where they can continue to grow unchecked[55]. Thus, TNF

influences immunity to TB at the earliest stages of infection, which are difficult to study in mammalian models of infection.

Not surprisingly, pathways that regulate the expression and activity of TNF are also important in TB in both zebrafish and humans. A forward genetic screen conducted in our lab identified leukotriene-A-4-hydrolase (*lta4h*) as a critical gene upstream of TNF gene expression[56]. Fish deficient in *lta4h* fail to produce sufficient levels of TNF, and infection phenocopies TNF deficient larvae. Importantly, genetic polymorphisms in this gene exist in human populations, and patients homozygous for the low expressing allele are more succumb to infection during TB meningitis[56]. A surprising finding from studying this patient population, was that while patients heterozygous for the allele were protected, homozygotes of the high-expressing *LTA4H* allele were also highly susceptible to death. Patients and zebrafish expressing high levels of *LTA4H* were also overexpressing TNF. This decreases bacterial growth in single macrophages through the overproduction of mitochondrial ROS. This paradoxically activates necroptosis, or programmed necrosis, of macrophages, and uncontrolled extracellular bacterial growth[53, 54]. Thus, a combination of studies in human patients and zebrafish lead to the discovery that TNF levels must be carefully controlled to enhance bacterial killing while preventing host damage.

2.5. ESX-1 promotes granuloma formation

Among the virulence factors shared by Mtb and Mm is the ESAT-6 secretion system-1 (ESX-1). This is a type VII secretion system that is encoded within the Region of Difference-1 (RD1) locus, named since it is missing in all *M. bovis* BCG vaccine strains, which are highly attenuated. The structure and function of components of this secretion system are still being

investigated, but the most characterized of its substrates are 6-kDa early secreted antigenic target (ESAT-6) and its chaperone, culture filtrate protein-10 (CFP-10)[11]. ΔRD1 mutants of both Mm and Mtb are defective for growth in cultured macrophages and during infection of zebrafish , frogs, and mice. Notably, granulomas of ΔRD1 mutants are smaller[11, 57, 58]. Unlike some mycobacterial mutants, such as Δ*kasb* and Δ*erp*, ΔRD1 do not exhibit a defect in intracellular growth; instead, the mutants fail to spread into neighboring macrophages[57, 59]. Dynamic visualization of in larvae revealed that where as macrophages infected with WT Mm aggregate soon after inoculation, ΔRD1 infected macrophages do not, often remaining as single infected cells through out the individual[11]. Moreover, once in aggregates, WT Mm induces increased apoptosis of its macrophage host, apparently to induce nearby uninfected macrophages to take up infected particles through the process of efferocytosis[2, 11]. In other words, Mm induce granuloma formation to facilitate spread into additional macrophages, contrary to the dogma that granulomas were solely host protective.

To dissect the host factors responding to the ESX-1 secretion system, the Ramakrishnan lab compared zebrafish gene expression during WT infection and ΔRD1 infection. As expected, many immune response genes were induced by infection, such as *tnf* and *il17a*[1]. Only two genes were induced higher during WT infection, which were *matrix metalloproteinase-9* (*mmp9*), a zinc dependent protease, and one of its endogenous inhibitors, *tissue inhibitor of metalloproteases-1* (*timp2b*). Knockdown of *mmp9* resulted in reduced growth of WT bacteria, as well as reduced formation of granulomas, reproducing the phenotype seen in ΔRD1 infected wild-type zebrafish[1]. This phenotype is reproduced in mouse models of TB infection, where *mmp9* KO mice, as well as mice treated with the broad-spectrum MMP inhibitor, BB-94, develop smaller granulomas and had reduced bacterial loads[60, 61]. Although *mmp9* expression

is generally associated with leukocytes, ΔRD1-dependent induction of *mmp9* occurred in epidermal cells near infected macrophages[1]. This highlights a possible role of the epithelium in host immunity to just as a barrier, but also as a coordinator of host immune responses, albeit in this case it is to the host's detriment.

Chapter 3:

Matrix Metalloproteinases as modulators of immunity and inflammation

3.1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases belonging to the metzincin clan of proteases thought to cleave components of the extracellular matrix (ECM). In humans, this family consists of 24 members, which were initially named and grouped according to their ECM substrate preference and structure (e.g. gelatinase, collagenase, elastase)[62]. Despite what their names suggest, MMPs have a diverse range of substrates beyond components of the ECM and are recognized to cleave a range of extracellular substrates, such as cytokines and chemokines, and are believed to also have some intercellular functions. Their role is particularly well studied in processes involving tissue remodeling, such as developmental processes, wound healing, and bone growth[63], but there is an increasing body of literature showing the MMPs roles in non-remodeling processes, such as cell signaling, immune cell migration, and transcriptional regulation, to name a few[64-66].

Of the MMPs, the interstitial collagenases (MMP1, -8, -13) represent a subfamily that is of particular interest for their roles in inflammation and immunity. In fact, the collagenases were the first MMPs to be discovered, when Gross and Lapiere (1962) identified the enzyme in the tail of tadpoles undergoing metamorphosis that was capable of digesting fibrillar collagens at a neutral pH. Now known as MMP18, this tadpole collagenase kicked off the search for similar enzymes in humans and led to the cloning of the first human ortholog, MMP1 (Collagenase-1)[67]. Initially named for the ability to cleave native fibrillar collagens, the collagenases have been primarily studied for their role in ECM remodeling conditions, such as arthritis (both

rheumatoid arthritis and osteoarthritis), wound healing, and bone development[67-70]. Non-ECM substrates of the collagenases have been identified through *in vitro* assays and proteomics starting in the 1990s, however, the implications of collagenase activity on these substrates are only beginning to be understood now[71, 72].

3.2 MMP: Structure

Although MMPs are functionally diverse as a family, four domains are present in every MMP: (1) a signal peptide, directing the extracellular localization of the protein; (2) the pro-domain, limiting the activity of the enzyme; (3) a hinge domain, which links the pro-domain to the (4) the catalytic domain. The catalytic domain contains a conserved zinc-binding motif, HEXXHXXGXXH, which is also a signature that is used to differentiate MMPs from other members of the metzincin clan[73]. The three histidine residues coordinate a zinc ion, and the glutamate residue is the nucleophile required for the hydrolysis of peptide bonds[64]. The N-terminal pro-domain of the MMP is required to limit enzymatic activity and contains a consensus sequence, PRCXXPD[64], where the cysteine residue blocks catalytic activity of the enzyme by directly interacting with the zinc-ion. With the exceptions of MMP7 and MMP26, MMPs have additional domains that are thought to confer substrate specificity and localization, such as the hemopexin-like (HPX) domain[64]. A subset of MMPs have a GPI- anchoring domain or a transmembrane domain, thus limiting their localization to cell surfaces[62, 64].

The HPX of collagenases are unique in that it is not only responsible for binding substrates such as collagen, but it is specifically required for relaxing fibrillar collagen, which is composed of three collagen subunits that are tightly associated in a triple-helix. Unwinding activity by the collagenase HPX exposes single chains that can fit into the active site of the MMP

and subsequently be proteolyzed[74, 75].

3.3 Regulation of MMPs

3.3.1 Transcriptional regulation of MMPs

Like other proteases, MMP expression and activity is limited to specific cell types and specific conditions [63]. With the exception of MMP2, most MMPs are spatially and temporally regulated at the transcriptional level. Thus, although MMPs are not normally expressed by healthy tissues, they can be found in almost all inflammatory or remodeling processes, in some cases leading to pathology[63, 64]. Signals that regulate MMP transcription are broad and include cytokines, hormones, ECM products, integrin-derived signals, cell stress, cell shape, and GTPases[62-64]. These diverse signals must be integrated to control the regulatory elements present in the promoters of the MMPs.

In a review on transcriptional regulation of MMPs, Yan and Boyd (2007) classify the MMPs in to three groups according to the composition of their promoters: (1) those containing a TATA-box at -30bp and an AP-1 binding site around -70bp; (2) those containing a TATA-box at -30bp but lacking an AP-1 site at -70bp; (3) those lacking TATA-boxes entirely. Although MMPs with group 1 promoters (e.g. MMP1, -7, -9, -12, -13) contain many elements aside from AP-1 binding site, their expression pattern is nonetheless dominated by activating protein-1 (AP-1) transcription factors. AP-1 transcription factors consist of homodimers and heterodimers of Jun, Fos, ATF, and JDP protein families, and are activated by the MAPK signaling pathway. This can be initiated by cytokine signals such as TNFα, TGFβ, EGF, VEGF, interleukins, and interferons[76, 77]. As a result, MMPs in this group are highly responsive to inflammation and are often implicated in the immune response and autoimmunity. Conversely, MMPs with group 3

promoters (MMP2, -14, -28) are largely under the control of Sp-1 binding sites, which are associated with important cellular events such as growth and apoptosis. Thus, the expression of these MMPs are more constitutive and less influenced by inflammatory signaling[76, 78]. Finally, members with group 2 promoters (MMP8, -11, -21) are also not inducible by inflammatory signals, but are influenced by unique elements. For example, MMP11 is the only member with a retinoic acid receptor element (RARE)[76].

This divergence of promoters among highly similar MMPs helps to specify the function and role of the MMP within the organism. For example, MMP2 and MMP9 are both gelatinases and share many substrates; however, MMP2 has a group 3 promoter and acts more as a "house-keeping" gene, where as MMP9 has a group 1 promoter and plays a role during inflammation[78]. Similarly, MMP1 and MMP8 are both collagenases that have diverged relatively recently[79]. MMP1 has a group 1 promoter and is most frequently associated with inflammation in multiple cell types including epithelial cells and macrophages, whereas MMP8 expression primarily occurs in neutrophils. Rather than increasing expression when necessary, MMP8 in neutrophils are stored as an inactive enzyme in granules and released if needed[78].

3.3.2 Regulation of MMP activation

MMPs are initially translated as proteolytically inactive pro-peptides, or zymogens (this form of an MMP will be referred to as a ProMMP). The pro-domain of the MMP folds over the catalytic domain allowing the thiol of the conserved cysteine to act as a fourth ligand to the active site zinc ion, rendering the ProMMP latent. Activation of the protease requires the disruption of this interaction in a process called the "cysteine switch", allowing the zinc ion to bind to a water molecule necessary for hydrolysis of MMP substrates[63, 64]. This disruption

can occur in several ways: (1) by the proteolysis of the pro-domain; (2) by the reduction of the cysteine residue; (3) by the allosteric disruption of the pro-domain[80]. Some MMPs contain a consensus sequence for cleavage by furins (RXKR or RRKR) between the pro- and catalytic domains. Since furins are proteases present in the trans-Golgi network, MMPs with this motif are activated within the secretory pathway[63, 80]. Other MMPs are activated extracellularly, likely by other proteases; for example, MMP2 can be activated through a multi-protein mechanism involving MMP14. Reactive oxygen species, such as those produced by activated leukocytes, including hypochlorous acid and hydroxyl radicals, can activate MMPs *in vitro* by disrupting the cysteine thiol long enough to allow for autolysis, either through inter- or intra-molecular proteolysis. However, there are limited *in vivo* data that show that this indeed occurs. Likewise, allosteric disruption of the pro-domain has been demonstrated to stabilize an "open", intermediate conformation of the pro- and catalytic domains to allow autolysis[80]. Activation of MMPs for experimental purposes takes advantage of this mechanism by adding p-aminophenylmercuric acetate (APMA) to ProMMPs, which interacts with the prodomain directly[81]. Biological molecules may be able to do this *in vivo*, since glycosaminoglycans (GAGs) can directly interact with MMPs to both activate and enhance MMP activity[82].

3.3.4 Compartmentalization

The localization of active MMPs post-translation is an important control mechanism that determines its ability to interact with potential substrates. This not only refers to the subcellular localization of MMP containing granules and the regulation of its secretion, but also to localization of the MMP in the pericellular or extracellular space. While some MMPs have domains that directly tether them to the cell surface (transmembrane or GPI anchoring domains)

secreted MMPs are bound to specific surfaces through interactions with other macromolecules[80]. For example MMP9 is localized to cell surfaces through its interaction with CD44, which is thought to increase the invasiveness of tumor cells[83]. Secreted MMP1, which is necessary for keratinocyte migration, binds $\alpha 2\beta 1$ integrin on cell surfaces to concentrate proteolytic activity[84, 85]. Proteoglycans appear to bind MMPs to specific extracellular space, while also enhancing proteolytic activity. MMP7, which is secreted by the endometrial epithelium, localizes close to the mucosal surface through its interaction with heparin sulfate[86]. Thus, compartmentalization of MMPs may serve to both concentrate proteolysis near biologically relevant substrates while also enhancing its catalytic capabilities.

3.3.5 Endogenous inhibition of MMPs

Like other proteases, many proteins are co-expressed with MMPs to limit proteolysis. These include general protease inhibitors such as $\alpha 2$-macroglobulin and the more specific tissue inhibitors of metalloproteases (TIMPs) and cell surface tethered reversion-inducing-cysteine-rich-protein-with-kazal-motifs (RECK), which bind MMP active sites at a 1:1 ratio[73]. Binding of MMPs to cell surfaces through receptors can also result in endocytosis to remove it from the environment, as demonstrated with Low-density lipoprotein (LDL) receptor related protein-1 (LRP-1), which reduces extracellular MMPs -2, -9, and -13[87, 88].

3.4 Discovery of MMP substrates

Many *in vitro* approaches have been taken to discover substrates of MMPs, however, validating these substrates *in vivo* continues to be a challenge. Due to the similarities in the

structure of MMPs, it is not uncommon for multiple MMPs to be able to process the same substrates *in vitro*. For example, multiple proteases can cleave stromal cell-derived factor-1α (SDF1α), a chemokine that plays a role in HIV-related dementia, however only MMP2 seems to be the relevant protease *in vivo*[89, 90]. Thus, the degradome, or the list of possible substrates for a given protease, must be validated through studies conducted in the context of the biological process *in vivo*.

3.4.1 Structural determinants of substrate preference

Unlike proteases such as furins, MMPs do not have strict consensus sequences that direct activity, making it impossible to scan sequenced genomes for substrates[91]. Rather, MMPs exhibit loose preference for certain peptide sequences to fit into the catalytic groove of the enzyme, the most important being the identity of the amino acid immediately following the siccile bond (P1'). This is determined by the characteristics and size of the corresponding surface of the MMP, called the S1' specificity pocket[92]. For example, the S1' of MMP1 is relatively small, thus the P1' of the substrate is limited to small hydrophobic residues[93]. By contrast, the S1' of MMP8 and MMP13 are large and open, allowing the accommodation more diverse amino acids[92]. This preference merely narrows possible substrates without identifying specific ones.

Exosites, or locations out side of the catalytic groove, play an important role in determining substrate binding and specificity. These sites can be located on the catalytic domain itself or within additional domains such as the HPX domain. One successful unbiased approach to identifying MMP2 substrates utilized its HPX domain as bait in a yeast-2-hybrid screen. This resulted in the identification of a chemokine, monocyte chemoattractant protein-3 (MCP-3), as a novel MMP2 substrate[94, 95]. This has lead to further candidate based search for chemokine

substrates of other MMPs, expanding the substrate repertoire and known functions for the MMPs[89, 96].

3.4.2 Biochemical approaches to substrate identification

Traditionally, MMP substrates have been identified through a candidate based approach. Potential substrate and protease lists can be created from what is known about the biological process, and systematically tested *in vitro* by incubating recombinant MMPs with the protein of interest. Combined with peptide sequencing, this provided initial data regarding ECM substrate preferences of the MMPs[63]. This approach, however, is slow and cumbersome, especially if no particularly good substrates have been identified for the specific condition. This necessitates the use of unbiased approaches that take advantage of proteomics to identify a larger pool of candidate substrates.

Proteomics based unbiased approaches have been refined and popularized over the past decade. Details of each methodology is beyond the scope of this thesis, but in essence, these methods apply active protease to a pool of substrates (collected from cell culture or tissue lysates) then attempt to amplify signal from new peptides that appear as a result of proteolysis. Novel labeling techniques amplify these signals by tagging new peptides and concentrating them prior to mass spectrometry, as seen in TAILS (terminal amine isotypic labeling of substrates)[97, 98].

3.4.3 Validation of substrates in *in vivo* systems

Validation of *in vitro* substrates should take advantage of animal models. Showing interaction of the MMP with the substrate via co-localization or immunoprecipitation is one way

of doing this[64, 98]. A more robust way is to use transgenic or knock out organisms to show, for example, that the loss of MMP function results in accumulation of uncleaved substrate, or that mutation of the cleavage site prevents its proteolysis[64]. Even using these approaches have lead to confusing results, as demonstrated by studies in mice to discover the proteases involved in bullous pemphigoid, an autoimmune condition that causes sever blistering[63]. Here, both MMP9 and a serine protease, Neutrophil Elastase (NE), were upregulated in blisters and capable of cleaving collagen XVII, the key substrate involved in blister formation, but knockouts of either gene was sufficient to prevent blisters. Experimentation in the animal model lead to the discovery that MMP9 was actually acting upstream of NE, which then directly processed collagen XVII to initiate pathology[63, 99].

To summarize, the identification of physiologically relevant MMP substrates starts with the identification of its degradome through biochemical approaches, but must be validated in animal models and through genetic approaches.

3.5 MMPs in the zebrafish

Twenty-five MMPs have been identified in the zebrafish genome to date, representing all but one of the six sub-groups of MMPs found in humans[79]. Although it is tempting to compare human MMPs to their orthologs in zebrafish, especially since similar numbers of MMP genes exist in the two species, phylogenetic analyses show that the MMP family expanded in teleosts and in mammals separately (Fig. 5-2). For example, all four members of the human transmembrane MMPs are represented in the zebrafish; however, three (*mmp14, -15,* and *-16*) are present as duplicates in the zebrafish, whereas *mmp24* only has one ortholog. The branch of MMPs containing the interstitial collagenases indicates duplication and further specialization of

MMPs in the mammalian lineages. Whereas this is limited to 2 genes in the zebrafish (*mmp13a* and *13b*), this has expanded to include 7 genes in humans (*MMP1, -3, -8, -10, -12, -13,* and *-27*). Interestingly, unlike the mouse model where knockouts of individual MMPs do not result in obvious developmental defects (with the exceptions of *MMP13, -14,* and *-16*), knockdowns of MMPs in the zebrafish often result in severe phenotypes, suggesting that MMPs in the fish have distinct substrates, and that MMPs cannot compensate for one another. Alternatively, MMP knockdowns using MOs may result in non-specific toxicity; therefore all studies must be carefully interpreted.

Like the mammalian systems, MMPs in zebrafish play important roles in cell migration and ECM remodeling. The fish ortholog of *MMP14*, the only MMP that is indispensable development in mouse models, is critical for cell migration during gastrulation and proper skeletal development[100, 101]. Its role in ECM degradation is also required in the proper development of the gut later in development[102]. Similar to mammalian models of wound healing, a number of MMPs are upregulated in response to tail fin amputation, including *mmp2, -9, and -14a*. Treatment of tail fins with a broad-spectrum MMP inhibitor, GM6001, results in the failure to regenerate the fin[103]. Finally, MMP activity has also been implicated in immune responses in the zebrafish, since *mmp9* and *mmp13a* are both upregulated during inflammation, and knockdown of the genes result in reduced leukocyte migration[1, 104, 105].

3.6 MMPs in immunity and inflammation

Until recently, MMPs have been described as drivers of pathology during inflammation by enhancing inflammation or by destroying tissue. It is now clear that MMPs play many other roles

during inflammatory responses, whether it is a host protective response to injury or infection, or mediation in resolution of inflammation.

3.6.1 Leukocyte migration

MMPs are traditionally thought to enable cell migration through the proteolysis of the ECM. Although this may be true, since inhibition of collagenases prevents various cell types from migrating through 3-D matrices[106, 107], MMP activity on cytokines and chemokines has been shown to be increasingly important for controlling leukocyte migration and activity. Cytokines are capable of inducing MMP expression in many different cell types, but some are also targets of MMP proteolysis. For example, multiple MMPs can release soluble TNF from the cell membrane, a process that is required for uncontrolled inflammation during endotoxin shock and models of arthritis[72, 108]. Transforming growth factor β (TGFβ) is an anti-inflammatory cytokine that is sequestered as a latent complex that can be released by MMP2, -3, -9, and -14 and is a potential mechanism for the regulation of anti-inflammatory processes[109].

Chemokine proteolysis by MMPs can directly regulate cell migration through modulation of the chemokine gradient. For example, SDF-1 can be deactivated through proteolysis by numerous MMPs[89]. Proteolysis of the N-terminus of all four monocyte chemoattractant proteins (MCP) by MMPs creates antagonists for their respective receptors[94, 96]. Interestingly, different MMPs can convert chemokine activity in opposite directions; whereas MCP-3 (CCL7) activity is enhanced by MMP9 proteolysis[110], MMP13 proteolysis at another location turns it into an antagonist[96]. It is unknown if MMP9 and MMP13 both regulate MCP3 *in vivo*, but this raises an interesting possibility for a method of chemokine regulation. Additionally, proteoglycans normally tether chemokines to the ECM or cell surfaces through their

glycosaminoglycan (GAG) chains[111, 112]. MMPs can process proteoglycans to release chemokines,[113, 114], resulting in the diffusion of the gradient to reach distal locations while simultaneously removing the chemotactic signal locally[115, 116]. This can result in chemokine release into the airway or the gut lumen to direct leukocyte egress into these areas[117, 118].

MMPs also affect cell fate once they have arrived at the site of inflammation. MMP expression is normally associated with M1 polarization of macrophages, typically described as an inflammatory response associated with increased bacterial killing. MMP28 does the opposite, skewing macrophages towards M2 polarization, which is associated with resolution of inflammation and fibrosis[119]. Neutrophils of MMP8 KO mice, unlike their WT counterparts, fail to undergo apoptosis after migrating to the site of inflammation. Overabundance of highly active neutrophils results in injury to host, demonstrated by increased tumorigenesis in a chemical skin injury model, as well as aggravated airway inflammation and arthritis[120-122].

3.6.2 Microbicidal effects

In addition to activating macrophages, MMPs have been implicated in bacterial killing through more direct mechanisms. For example, murine MMP7 is indispensable for the proteolytic activation of Paneth cell antimicrobial α-defensins, called cryptidins. As a result, MMP7 KO mice have an impaired ability bacterial growth in their gut and are hypersusceptible to orally administered *Salmonella typhimurium*[123]. Unlike the other MMPs, whose bactericidal effects are mediated by a substrate, MMP12 exhibits intrinsic bactericidal activity. MMP12 KO mice are hypersusceptible to *Staphylococcus aureus* infection, which could not be attributed to macrophage migration. Instead, MMP12 was responsible for direct bacterial killing in macrophages through a 20aa peptide that protrudes from the HPX domain. This peptide sequence

is unique to MMP12 and conserved in all orthologs. Accordingly, recombinant protein from both mice and humans are capable of killing multiple species of gram-negative and gram-positive bacteria[124].

3.6.3 MMPs as coordinators of inflammation

A recent study implicates MMP12 in a broader immune regulatory role as a transcription factor. The catalytic domain of MMP12 can directly bind DNA to inhibit transcription, ultimately leading to increased IFNα secretion from leukocytes to confer host resistance to viral infection. The transcriptional effects of MMP12 extend beyond the cells that express it; nearby cells take up MMP12 *in trans* where it traffics to the nucleus. Remarkably, the authors also found that the same genes regulated by MMP12 were also cleaved by MMP12. In other words, MMP12 can limit a gene's activity at the transcriptional and post-translational levels[125]. Direct transcriptional regulation may be a widespread feature of MMPs, since similar observations have been published for MMP14 and MMP3[126, 127]. Together, these studies point to a greater role for MMPs as a general coordinator of the immune response to invading pathogens.

3.7 MMPs in tuberculosis

The ECM degrading ability of MMPs was of particular interest in early tuberculosis research, as cavity formation in the TB infected lungs is known to correlate with higher bacterial burdens and increased rates of transmission[10]. By 1978, Dannenberg and colleagues had identified increased collagenolytic activity in rabbit dermal lesions caused by BCG inoculation[128]. Studies on human TB patients have identified numerous induced MMPs: MMP1, -2, -3, -7, -8, -9, -10, -12, -13, and -14[129-132].

Not only are MMPs highly expressed in the sputum of TB patients, but those with cavitation had higher concentrations of MMP1 and MMP2 than those without cavitation[129, 133]. Concentrations of these MMPs (but none others) also correlate positively with worse chest radiographic scores, and specifically, MMP1 concentrations correlate with increased bacterial numbers in sputum[129]. Furthermore, TB patients co-infected with HIV have lower MMP1, -2, -8, and -9 concentrations relative to TB only controls, correlating to the fact that these patients do not develop lung pathologies despite carrying higher bacterial burdens[129].

Genetic studies corroborate the important role of MMPs on tuberculosis disease severity. In the *MMP1* promoter, an additional *G* is inserted at -1607bp (*2G* allele) resulting in the creation of an additional Ets transcription factor-binding site adjacent to an AP-1 site. Because Ets transcription factors can coordinate and enhance AP-1 activity, this SNP results in increased *MMP1* expression. As a result, the *2G* allele potentiates MMP1 secretion in response to inflammatory signaling. Central and South Americans who are homozygous for the *2G* allele in addition to being homozygous for a high-expressing *MCP-1* allele were at higher risk for developing TB (OR=3.59-3.9)[134]. This combination of SNPs also increased risk for worse clinical symptoms of TB that was more difficult to treat (as measured by time to sputum conversion)[135]. Accordingly, patients with the high *MCP-1/MMP1* genotype had increased serum levels of MCP-1 and plasma levels of MMP1[135]. Another group also found that higher MMP1 expression amongst Taiwanese patients correlated with endobronchial tuberculosis and enhanced fibrosis in patients. Curiously, this study found that the *1G*, not the *2G*, allele had higher *MMP1* expression in this population, raising the possibility that other regulatory mechanisms unrelated to the *G* insertion could be at play[136, 137].

3.8 More studies are necessary to understand MMP roles in TB

While there is clearly a link between MMP expression and TB outcome, these correlative studies do not conclusively show a cause. While it is possible that MMP expression results in increased pathology, it is also possible that the opposite is true. A third factor such as bacterial burden may also drive both pathology and MMP expression. To address this, experiments must be done examining the effect of MMP inhibition on TB pathology and outcomes. Furthermore, studies in cell culture models show that cells infected with mycobacteria quickly upregulate MMP expression, but none of these studies address the role of MMPs in early infection. While such studies are difficult to do in a mouse model, the optical transparency of the zebrafish will allow me to visually follow the course of infection to determine if modifying MMPs has any effect on immunity to TB.

Chapter 4:

Materials and Methods

Bacterial strains

Mycobacterium marinum wild-type strain M (ATCC #BAA-535), ΔRD1 [11], or *Δerp* were transformed with plasmids expressing tdTomato or Wasabi under a constitutive Mm promoter (*msp12*) developed as previously described [138]. Bacteria were grown on Middlebrook 7H10 agar (Difco) supplemented with 0.5% bovine serum albumin, 0.005% oleic acid, 0.2% glucose, 0.2% glycerol, and 0.085% sodium chloride (OADS) or in Middlebrook 7H9 media (Difco) supplemented with OADS and 0.05% Tween 80. Media was supplemented with 50μg of Hygromycin when appropriate. Mm used in larval experiments were grown in Middlebrook 7H9 OADS until mid-log growth phase at 30ºC, then passed through a 5μm filter (Pall) to obtain single cell suspensions, which were then aliquotted and frozen for multiple uses. Aliquots are quantified by serial dilution and plating on 7H10 agar.

Δ*esat6* Mm mutants were acquired from F. Chu and S. Phillips.

Zebrafish infection and husbandry

All zebrafish experiments conducted conform to the Public Health Service Policy on Humane Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee of the University of Washington. WT zebrafish are of the *AB line originally purchased with ZIRC (Eugene, OR) and maintained at the UW as described [139]. Larval zebrafish were maintained at 29ºC and infected via caudal vein 36-48hpf with Mm or Mock (7H9 OADS) using 0.5% phenol red as a visual marker of injection [12]. Innocula of

injection was determined by plating the same volume on 7H10 OADS agar and enumerating colonies 7 days later. Bacterial burdens of larvae infected with fluorescent Mm were measured as fluorescent pixel counts (FPC), which enumerates pixels brighter than a background threshold determined by uninfected larvae. FPC was determined using a custom made image analysis macro designed for ImageJ (NIH) [140, 141].

Cell Culture and Infection

HEK293a cells (Life Technologies) were obtained from members of the John Scott lab and maintained in High-glucose DMEM with L-glutamine, 10%FBS, and Pen/Strep (Life Technologies). Normal human bronchial epithelial cells (Lonza, #CC-2540) were passaged according to manufacturer's instructions and maintained in BEGM media supplemented with the bullet kit (Lonza) at 37ºC with 5% CO_2. $5x10^5$ NHBE cells were infected at an MOI of 20 of either M or Δesat6 Mm strains for 6 hours at 33ºC prior to harvesting for RNA.

Morpholino and RNA injections

Morpholinos were obtained from Gene-tools (Eugene, OR). We utilized three *mmp13a* specific MOs as listed in Table 4-1. ATG1 and ATG2 were used in combination at 0.015mM and 0.06mM, respectively. The splice blocker e4i4 was used at 0.35mM alone or 0.25mM in combination with ATG2 (0.06mM). Efficacy of the MMP13a e4i4 MO was determined by RT-PCR using primers spanning the MO target site (F: 5'-GCTGACCTACAGGATTGAGAACTAC-3'; R: 5'-GTGTTTGGGCCATAAAGTGACTGGA-3').

pu.1 MO used to ablate macrophage populations were designed against the translation start site and the exon 4-intron 4 boundary, and used at final concentrations of 0.375 mM and 0.025 mM, respectively [46].

mmp13b MO was designed to target the exon 3-intron 3 boundary and used at 0.75mM. Efficacy of the MO inhibition was assessed via RT-PCR (F:5'-GTTTTATGGCATGCCGGCTGGTCT-3', R:F'- GTCGTCCTCGGAGAGAGGGAAACC-3')

Zebrafish *lta4h* mRNA was transcribed using mMessage Machine T7 transcription kit (Life Technologies) and poly-adenylated using the Poly(A)tailing kit (Life Technologies). 200ng of mRNA was injected in to embryos to create the LTA4H high phenotype [53].

Prior to use, MO or mRNA were diluted into embryo injection buffer (0.5x tango buffer, 0.5% phenol red solution) and ~5nl of this final solution was injected into the yolk of embryos at the 1-4 cell stage.

Quantitative RT-PCR

Total RNA from either larvae or cell cultures were obtained by extraction with TRIzol Reagent (Life Technologies, 15596) and treated with TURBO DNA-free kit (Life Technologies), then used as template for generating cDNA using reverse transcription with PrimeScript Reagent kit (TaKaRa; using oligo dT_{18} primers). All RT-qPCR was conducted on an ABI Prism 7300 Real Time PCR system (Applied Biosystems/Life Technologies) in technical triplicates with 20μL reaction volumes. SYBR green assays were conducted with Fast SYBR Green Master Mix (Life technologies, 4385612) using 250nM of gene specific primers or β-actin primers, which was used as house keeping gene primers (Table 4-2). TaqMan qRT-PCR assays for zebrafish larvae were conducted in TaqMan® Universal Master Mix II with UNG (Life Technologies)

with custom-made primers and FAMDHQ-probe sets ordered from Life Technologies. Primers were used at a final concentration of 100nM and probes at 250nM (Table 4-3). qRT-PCR for human samples was conducted using TaqMan primer/probe sets that were ordered as predesigned assays, with final primer concentration at 900nM and probes at 250nM (Table 4-4). C_T values for target genes were normalized to β-actin C_T prior to comparison against different conditions ($\Delta\Delta C_T$ analysis).

Recombinant TNF injection

His_6 tagged recombinant zebrafish soluble TNF (rsTNF) was produced via affinity purification on a TALON column and frozen in 10% glycerol (50 mM Tris-HCl, 150 mM NaCl, pH 7.8) as described in[142]. 0.5ng of rsTNF or vehicle (with 0.25% phenol red) was microinjected into the caudal vein of infected larvae 16hpi. [53]

Whole-mount fluorescent *in situ* hybridization

mRNA was detected in whole infected larvae as described in Clay and Ramakrishnan (2004). Probes: Fluorescein tagged anti-sense RNA probes were transcribed from zebrafish cDNA cloned into a pCRII-TOPO vector using T7 polymerase (Roche) and Fluorescein labeling NTP kit (Roche). As a negative control, the vector was transcribed for a sense probe from the SP6 promoter. Probe quality was confirmed by denaturing RNA gel.

Samples: Larvae were mock or WT infected at 36hpf, then fixed at 4dpi in 4% PFA in PBS overnight at 4ºC. Fixed larvae were dehydrated in MeOH at -20ºC for at least one night prior to starting the staining protocol. Larvae were progressively rehydrated into PBS-Tween (PBST, 0.01% Tween-20), then permeabilized in 40µg/mL for 30min at 37ºC, then refixed in 4%PFA-PBST. Samples were then incubated in pre-hybridization buffer (50µg/mL Heparin, 500µg/mL

tRNA, 50% Formamide, 5x SSC, 0.1% Tween-20, Citric acid to pH6.0) for 6h at 65ºC then hybridized with antisense or sense probe over two nights at 65ºC. Unbound probe was washed out in stringent SSC washes at 65ºC-70ºC prior to blocking for antibody staining using 1x Roche Western blocking reagent. Primary Sheep anti-FLU antibodies (Roche), then secondary HRP-anti-Sheep antibodies were used to detect probes. TSA staining was performed overnight with AlexaFluor488 substrate (Life Technologies).

Microscopy

Microscopy was performed on a Nikon E600 equipped with DIC optics, a Nikon D-FL-E fluorescence unit with 100W Mercury lamp and MFC-1000 z-step controller (Applied Scientific Instrumentation) or, for whole animal images, a motorized Nikon inverted Ti-E microscope. Objectives used included 2x Apo Objective 0.1 NA, 10x Plan Fluor 0.3 NA, 40x Plan Fluor 0.75 NA and 60x Oil Plan Apo, 1.4 NA. Widefield fluorescence were captured on a CoolSnap HQ or CoolSnap CF CCD camera (Photometrics) using Meta- Morph 7.1 (Molecular Devices) and Elements AR software [53].

Expression constructs and recombinant protein production

MMP13a: I cloned MMP13a into a mammalian expression vector to ensure proper zymogen folding and disulfide bond formation (See Results Fig 5-12). Full-length zebrafish cDNA (accession BC159192; OpenBiosystems/GE Healthcare, clone 8154682) was PCR amplified using Pfu Ultra polymerase. In order to facilitate purification and detection, a StrepTagII sequence was inserted between a human signal peptide (for secretion) and the pro-domain of MMP13a (indicated with underline), using primers F:5'-

GATCGGTACCATGAAGACCTGCTTCAGGCTGTGTGTTTT CGTTACTCTGCTGTTTTCAGGCCACAGCAACCCGgattacaaggatgacgacgataagtctgttgctggtC CGCTTGCTGGAGGAGAC-3' and R:5'-ATGTCAGCGGCCCGCTTATTTGCACGGCA GGAAA-3'. The PCR product was inserted into a pCEP4 vector (Life Technologies) through the KpnI and NotI sites. The pCEP4 vector carries a CMV promoter in addition to a Hyg selection cassette, allowing successfully transfected mammalian cells to be selected and maintained as a cell line. To collect recombinant proteins, transfected cells were grown in DMEM supplemented with 1x Lactalbumin enzymatic hydrolysate and Hyg. Conditioned media from transfected cells were collected, concentrated using centrifugal concentrators 10 fold (10kMWCO, Pall) prior to adding to Strep-Tactin sepharose beads (IBA, Germany). Bound protein was washed with 5 x5 bed volumes of buffer (100mM Tris-HCl, pH8.0, 150mM NaCl) then eluted with 2.5mM Desthiobiotin in wash buffer. Pooled fractions were desalted on a PD-10 column (GE Healthcare) into 100mM Tris-HCl, pH8.0, 150mM NaCl, then frozen at -80ºC.

MBP-TNF: The transmembrane domain of zebrafish TNF was predicted as amino acids 35-57 using TMHMM Transmembrane prediction tool (CBS, Denmark; http://www.cbs.dtu.dk/services/TMHMM-2.0/). The extracellular portion of TNF (AA58-234) was PCR amplified with PfuUltra (primers: F-5'aaccaaggatccCATAAGACCCAGGGCAATCAAC-3'; R-5' agtcagaattcCAAACCAAACACCCCAAAGAAG-3') using zfproTNF-V5/His6 [143] as a template. The PCR product was then ligated into the bacterial expression vector pMALc5x (NEB), using BamHI and EcoRI in order to create an expression construct for an MBP-TNF fusion protein. BL21DE3 *E. coli* cells transformed with this construct was grown to OD=0.6

(37ºC, 220rpm) prior to adding IPTG to 0.1mM to induce protein production. Cells were collected 3 hours after induction, pelleted, and frozen at -20ºC. Cells were lysed in **BUFFER**, and debris cleared using centrifugation. Cleared lysates were applied on an AKTA platform outfitted with MBPTrapHP column (GE Healthcare), which contains Dextrin Sepharose beads. The column was washed with 12 column volumes of column buffer (20mM Tris-HCl pH7.4, 200mM NaCl), then eluted in 50mL elution buffer (10mM Maltose, 20mM Tris-HCl pH7.4, 200mM NaCl). Fractions were pooled applied on a Superdex 16/600 S200 gel filtration column with buffer (100mM Tris-HCl, pH8.0, 150mM NaCl). Peak fractions were pooled and frozen in -80ºC.

Identity of protein fractions and final purified product was confirmed using Mini-PROTEAN TGX Stain-free gels or Western blotting.

Western blots

Samples were boiled in loading buffer (0.05% Bromophenol blue, 10% Glycerol, 2% SDS, 1% β-mercaptoethanol) for 10 min prior to loading on TGX 4-15% precast gels (BioRad). Gels were transferred on to nitrocellulose blotting paper (Pall) at 100v for 60 min. Blots were incubated in 5% milk in TBS +0.1% Tween-20 for 1h, prior to o/n incubation (4ºC) with 1/200 dilution of rabbit-anti-zebrafish MMP13a antibody (Anaspec), 1/200 dilution of rabbit-anti-zebrafish TNF antibody (Anaspec), or 1/2000 rabbit-anti-β actin antibody (Sigma). Primary antibodies were diluted in 1% FBS, 0.1% NaN_3, TBST. Blots were washed 3x5m in TBST on a shaker, incubated in 1/5000 goat-anti-rabbit HRP antibody (Southern Biotech), and visualized with ECL solution.

rMMP13a activation

300µg/mL rMMP13a was incubated with 1mM APMA in 100mM Tris-HCl, pH8.0, 150mM NaCl for 2h at 37ºC.

Collagenase assay

Collagenase activity of whole larvae or purified proteins was determined using EnzCheck Gelatinase/Collagenase assay kit, using DQ Collagen I as the substrate (LifeTechnologies). Collagen I is known to be the preferred substrate of MMP13.

ProTNF cleavage assay

Activated rMMP13a was mixed with MBP-TNF at a 1:5 molar ratio in 100mM Tris-HCl, pH8.0, 150mM NaCl at 37ºC for up to 2h. Collected samples were immediately mixed with loading buffer on ice to inhibit the reaction prior to running it on a gel for Western blotting.

Phylogenetic analysis

Primary amino acid sequences were downloaded from the ensemble web server (www.ensembl.org) and aligned into the PHYLIP format using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Sequences were then analyzed using PROML, PROTPARS, PROTDIST, and KITSCH from the PHYLIP package using default settings

Statistical Analysis

Statistical analysis was performed with Prism (Graphpad Software) and is detailed in figure legends.

Table 4- 1. Sequences of MO used for experiments.

Gene	Target	Name	Sequence (5'-3')
mmp13a	Translation start	ATG1	CAGGTCTTCATGTTTGCTTTCCTTC
mmp13a	Translation start	ATG2	GCTTTCCTTCTCCTTCAAGAAACTG
mmp13a	Exon 4- intron 4	e4i4	GACTTTTCCTCTGTGCTTACATTGA-3'
pu.1	Translation start	ATG	CCTCCATTCTGTACGGATGCAGCAT
pu.1	Exon 4- intron 4	e4i4	GGTCTTTCTCCTTACCATGCTCTCC
mmp13b	Exon 3-intron 3	e3i3	AATGACACAAACGGTGGTACCTTTT

Table 4- 2. Zebrafish RT-qPCR primers used in SYBR assays.

Gene	Other names	F (5'-3')	R (5'-3')
MMP2	Gelatinase A	ACCTCTCAGGGTCGAGATGATGGG	AAGGGGCTCCATCTGCATTTCCAC
MMP9	Gelatinase B	GTGAGGGCCGCAATGATGGAAAAC	GCATGTCCAAACTCATGAGCAGCC
MMP11a	Stromelysin-3	GTGCCAGACTATCCCGAACAGAGA	GAAAATCCGCCTCACTTTATCCTCA
MMP11b	Stromelysin-3	AACAGTAAAGGCGGCAGAGAGACG	CTCGCCTCGGATCATGGATACAGC
MMP13a	Collagenase-3	AACAGCTTCTTCTGGCGCAGTT	TCTTCACATTTTTCCGCAGACG
MMP13b	Collagenase-3	GTCTTCAGCGACCGGGAAAGT	CGGCTCTGTCCACAACCTTCTT
MMP14a	MT1 MMP	GCCATTTTCTACACACCCACTGGC	ATCTGGTACTCCTTGCCATACACCG
MMP14b	MT1 MMP	TGGATGCAGCTCTTCTCTACACGC	TGGATCCACTGAGTGCGTGTCTTC
MMP15	MT2-MMP	CAATTAGTGTGTGGGGCACTTCTG	GCTCGCTTGTCATTCTGTGATTGT
MMP16a	MT3-MMP	CACCGAGGACAAAAACGAGGTCA	TGTAGATGAGCACCAGCAGACACA
MMP16b	MT3-MMP	TGGGAAGACGTGGCCAAAACCTAC	CGAATGCTCCCTGTGGAGAGTCTG
MMP17a	MT4-MMP	CCTTCAATAACAGCAGAGCAGATGG	GAGCAGCGAGGGGTCTTCATTA
MMP17b	MT4-MMP	CGCCATAGACATCACAGACTCACA	TAGGAACAACCCTGACTGACACCA
MMP19		CGATACCCCAAACCACTGAAAAGA	GTCCCACTGCCAATAGCCTGA
MMP20a		TGCGTCATTTCTACCACCTGAAAA	TTGCTCTGATCTTGTCCTTCATGC
MMP20b		CCAAAGCCAGTCCTCTCACATTCT	ATTGTAGCCAGCCGTCCATACTTC
MMP23bb		GGAAATCACCAACACCAGCAAAAG	TCACCGTTCAGTCCATCAAAACAC
MMP24	MT5-MMP	ATCAGACCTGCGATCGGAGAAAGC	GTGATCTGGAACTCCACACCGAGG
MMP25a	MT6-MMP	TGTGTTCTTCTCTGCGGTTCATTC	GAGTTTGCCTGTTTCCTTGAGTCC
MMP25b	MT6-MMP	CAGGAGAGGTAAAAACAAGGTGATGA	GGAGAGTAGTGGAAACGCAGAGGT
MMP28	Epilysin	CACAGCTGTCTGTCACCATTGAAGC	CACTGCTCCTCTTGGGGAATCCAG
MMP30		CGAGATGACCGACTTGGGGTTCAG	CAAGTCACGCTTGCATGGTTCTGG
ADAM17a		CACAAAAGTTGCCCCTGGAGAG	GGCAGAGTCCTCCTAATCCTGGT
ADAM17b		CGGCTGCTTGTGTATCGTTCAG	AGTTGAGGGTGGTGCTCTCCTC
TNF		AGGCAATTTCACTTCCAAGG	CAAGCCACCTGAAGAAAAGG
β-actin		ACCTGACAGACTACCTGATG	TGAAGGTGGTCTCATGGATAC

Table 4- 3. Zebrafish RT-qPCR primers and probes used for Taqman assays.

Gene	F (5'-3')	R (5'-3')	Probe (5'-3')
b-actin	CTGAATCCCAAAGCCAACAGAGA	GCCTGGATGGCCACATACAT	CATGATCTGTGTCATCTTC
mmp9	CATCACTGAAATCCAGAAGGAGCTT	GTTCACCATTGCCTGAGATCTTC	AAGGACGGGCGCTACT
mmp13	CTGGAATGACCGGGAAGGT	TGGAGCCAAACTCAAGCATCTTT	CCAGTACAGAGGTTTAAAC
b-actin	CTGAATCCCAAAGCCAACAGAGA	GCCTGGATGGCCACATACAT	CATGATCTGTGTCATCTTC

Table 4- 4. Human RT-qPCR primer/probe sets ordered from LifeTechnologies.

Gene	Catalogue#
b-actin	ACTB endogenous control: 4352935
MMP1	Hs00899658_m1
MMP8	Hs01029057_m1
MMP9	Hs00234579_m1
MMP13	Hs00233992_m1

Chapter 5:

Results.

5.1. MMP13a and MMP9 are specifically induced during infection with Mm

We previously conducted a microarray assessing gene expression during infection with virulent Mm, and found that *mmp9* was upregulated, in addition to numerous immune response genes, such as *tnf* and complement pathway genes[1]. However, the zebrafish genome has been updated since the original microarray, and more MMPs have been identified[79].Therefore, we wanted to do a more detailed assessment of MMP transcription using the newly updated annotations. We infected zebrafish larvae with ~100 CFU WT Mm and collected RNA from whole larvae at 5dpi to conduct RT-qPCR to compare transcription between infected and mock infected larvae (n=3, biological replicates). *mmp9* and *tnf* were both highly induced during infection with 11.4 ± 2.9 fold (SEM; p=0.0112) and 18.9 ± 4.0 fold (SEM; p=0.0066) relative to mock injected larvae, respectively, in agreement with previously published data (Fig. 5-1a)[1]. Only one other MMP, *mmp13a*, was induced during infection, at 9.4±1.4 fold (SEM; p=0.00115) over baseline expression levels. We also assessed transcription levels for A-Disintegrin-and-Metalloprotease (ADAM) genes, a closely related class of metalloproteases; however, none were induced over baseline at this time point (Fig. 5-1b).

MMP13a belongs to a subset of MMPs known as the interstitial collagenases, known for their ability to cleave fibrillar collagens. Collagenases in humans are associated with inflammatory conditions such as arthritis and atherosclerosis[64, 113]. *MMP9* expression in human TB is associated with severe disease, and studies from our lab and others revealed that it

recruits additional macrophages to expand granulomas[1, 2, 61]. Like *MMP9*, collagenase expression during human TB has been associated with worse outcomes and cavity formation in human TB patients[129, 131]. It is still unknown what roles collagenases may play in immunity and pathology to TB.

There are three collagenase genes in humans: *MMP1*, *MMP8*, and *MMP13*. To better understand the relationship of the zebrafish collagenases to the mammalian ones, we conducted phylogenetic analyses with amino acid sequences of all zebrafish and human MMPs, as well as mouse, rat, and medaka collagenases (Fig. 5-2). Our analysis produced results similar to published findings[79]. There are two collagenases in the zebrafish genome, *mmp13a* and *mmp13b,* which share 50% and 52% identity to human MMP13 protein, respectively. Despite what the names suggest, *mmp13a* and *mmp13b* are distinct genes that are only 45% identical to each other. Whereas *mmp13b* clades closely with mammalian *MMP13*, *mmp13a* appears to be a teleost specific collagenase that diverged earlier. Unlike *mmp13a*, *mmp13b* was not induced in infection (Fig. 5-1a). Other studies in zebrafish larvae have shown *mmp13a* gene induction in response to infection with *S. typhimurium* and wounding[104, 144], and suggest a role for *mmp13a* in leukocyte migration towards tail wounds[104]. Aside from phylogenetic analyses, nothing is known about *mmp13b.*

5.2. MMP13a is induced in an RD1-dependent manner

We wondered if *mmp13a* shared other aspects of expression with *mmp9*. First, we assessed if its expression was dependent on the bacterial virulence factor ESX-1, which enhances the induction of *mmp9*. This is a type VII secretion system that is encoded by the RD1 locus, and mutants defective in secretion are highly attenuated, resulting from the inability of the bacterium

to form granulomas and thereby infect new macrophages[2, 11, 59]. We infected larvae with either ~150 CFU WT Mm or ~450 CFU ΔRD1 Mm. At 4dpi, larvae were assessed for bacterial burdens using FPC to ensure that both groups had roughly equal bacterial loads (Fig. 5-3a). These, along with mock-infected larvae were compared for *mmp13a* and *mmp9* expression using RT-qPCR (n=6, biological replicates). Loss of ESX-1 impairs the induction of *mmp13a* (WT induces 2.0 fold over ΔRD1; p=0.0051) as with *mmp9* (4.8 fold over ΔRD1, p=0.0012)(Fig. 5-3b).

5.3. MMP13a has a similar distribution to MMP9, expressed in neutrophils and induced in epithelial cells.

To further characterize the nature of MMP13a expression in infected larvae, we conducted fluorescent *in situ* hybridization (FISH) on whole larvae to localize MMP13a induction. In uninfected and infected samples, single cells stained positively throughout the larvae (Fig. 5-4a,b). Using Differential Interference Contrast (DIC) microscopy, we identified these cells as neutrophils by the presence of granules within the cells[145]. In infected samples, there were additional clusters of cells that stained for *mmp13a* mRNA near granulomas (Fig. 5-4c, d). These cells were not in the granuloma, but on the surface of the larvae. With DIC microscopy, we identified the cells as epidermal cells which also express *mmp9*[1].

mmp9, in the context of ESX-1 competent infection, is induced regardless of macrophage presence. We next assessed if *mmp13a* expression was also independent of macrophage signaling. To do this, we used a morpholino targeting the *pu.1* transcription factor, which is required for development of the myeloid lineage[45]. Thus, *pu.1* morphants (MO) are highly susceptible to infection, as Mm grows unchecked in the absence of macrophages[46]. We

infected control and *pu.1* MO with various doses of WT Mm, such that by 3dpi both groups had similar bacterial burdens (Fig. 5-5a). There was no significant difference in induction of *mmp13a* with or without macrophages, indicating that macrophage signaling was unnecessary for induction (Fig. 5-5b). Additionally, this indicates that macrophages are not the primary source of *mmp13a* during infection. Thus, both *mmp13a* and *mmp9* are induced in zebrafish larvae through direct interaction of cells with Mm.

5.4. Normal Human Bronchial Epithelial cells also induce MMP9 and collagenases in the presence of ESX-1 competent Mm.

To corroborate our zebrafish findings that macrophage signaling is dispensable for epithelial induction of *mmp9* and *mmp13a*, we turned to a cell culture model of infection. Normal Human Bronchial Epithelial (NHBE) cells, a primary cell line harvested from healthy human subjects, express *MMP1* and *MMP9* when exposed to conditioned media of Mtb infected but not uninfected macrophages[133, 146]. We asked if NHBE cells would express MMPs when in direct contact with Mm. To do this, NHBE cells were infected at an MOI of 20 with either WT or ΔesxA Mm, then harvested at 6hpi for RT-qPCR. *MMP9, MMP1,* and *MMP13* were induced at both time points by WT, but not by ΔesxA Mm (Fig. 5-6). *MMP8*, however, was undetectable in all conditions. This agrees with previous reports that *MMP8* is primarily a neutrophil collagenase[69].

5.5. MMP13a morphants are hypersusceptible to infection.

Given that collagenases are also highly upregulated in human TB, we wanted to know what role, if any, *mmp13a* played in innate immunity to mycobacterial infection. We utilized

MOs targeting either the translation start site (ATG1 and ATG2) or a splice acceptor site (e4i4) to transiently knockdown *mmp13a* expression in zebrafish larvae. Both MOs reduced collagenase activity in whole larvae (Fig. 5-7a,b), as determined by in a fluorimetric *in vitro* collagenase activity assay using fluorescein-conjugated collagen I. Morphants were then infected and assessed for bacterial burdens at 4dpi. ATG1/2 or e4i4 injected morphants had higher bacterial burdens relative to control morphants (2 fold, p=0.003; 1.8 fold, p=0.047)(Fig. 5-7c,d). These findings indicate that *mmp13a* is a host protective factor, unlike *mmp9*, which is a host susceptibility factor. In contrast, *mmp13b* morphants were not hypersusceptible consistent with their lack of induction in response to infection (Fig. 5-8a,b).

5.6. MMP13a morphants are deficient in control of Mm growth within macrophages.

We next detailed the infection phenotype to identify the specific step(s) during the immune response that was defective in MMP13a morphants. We investigated the ability of individual macrophages to control bacterial growth early in infection by assessing macrophage bacterial burden at 2dpi. Although collagenases have not been directly linked to bacterial killing, other MMPs have been shown to do so, either through direct microbicidal activity through a non-catalytic domain[124], indirectly through activation of antimicrobial peptides[123], or through macrophage activation by altering signaling pathways[147]. To test this, we utilized the Δ*erp* mutant of Mm, which is specifically susceptible to intramacrophage killing, giving us additional sensitivity in detecting host defects in this step[138]. Morphants had higher bacterial burden per macrophage relative to controls (p=0.0013; Fig. 5-9), indicating that MMP13a was necessary for bacterial killing within individual macrophages.

We were struck by the similarity of the MMP13a morpholino phenotype to that of deficiency of TNF signaling[53, 55, 56]. The loss of TNF or its receptor, TNFR1, results in the inability of macrophages to control bacterial growth, resulting in the necrosis of macrophages and ultimately, unchecked extracellular bacterial growth. Extracellular growth of Mm is visible as "cording", or masses of bacterial growth that take on a serpentine structure. By contrast, growth in macrophages, even in a granuloma, is characterized as distinct packets of infection[56]. Indeed, we found that the granulomas of MMP13a MO were more likely to cord by 3dpi ($p=0.0001$)(Fig. 5-10).

5.7. Treatment with recombinant sTNF rescues bacterial killing in MMP13a deficient macrophages.

We hypothesized that MMP13a deficiency resulted in the reduction of TNF signaling. We first asked if this was the result of insufficient *tnf* transcription, as was the case in zebrafish mutants of LeukotrieneA-4-hydrolase (*lta4h*)[56]. However, *mmp13a* MO did not have a reduction in *tnf* induction relative to control morphants (Fig. 5-11).

Another important aspect of TNF regulation occurs post-translationally. Initially TNF is synthesized as a 27kDa transmembrane protein that forms a trimer with two other TNF proteins (memTNF). This latent homotrimer is then enzymatically cleaved, releasing trimeric soluble TNF (sTNF) consisting of three 17kDa chains[108]. Although ADAM17 is thought to be the primary protease responsible for this processing in mammals, other metalloproteases including the collagenases have also been shown to have TNF processing capabilities in vitro[122, 148-153]. We hypothesized that in the context of tuberculous infection, MMP13a was acting as an

ancillary TNF converting enzyme, boosting local sTNF concentrations to enhance microbicidal activity by macrophages.

In zebrafish lacking sufficient TNF expression, the hypersusceptibility phenotype can be rescued by reintroducing TNF in larvae by injection with recombinant soluble zebrafish TNF (rsTNF). If *mmp13a* MO are deficient in TNF production, rsTNF treatment of infected morphants should rescue the hypersusceptibility phenotype. To test this, *mmp13a* MO were infected as in other experiments, and at 1dpi were injected with 0.5ng of rsTNF or vehicle. *mmp13a* MO treated with rsTNF had reduced intramacrophage bacterial growth, down to WT levels (Fig. 5-12). In addition to supporting our hypothesis that TNF levels are insufficient in *mmp13a* MO, these results suggest that TNF receptor signaling is intact in the morphants.

5.8. TNF is an MMP13a substrate.

Although human recombinant collagenases have TNF converting activity, it was important to show that this activity is retained in the zebrafish enzyme, as zMMP13a is only 48% identical to any of the human collagenases. To address this, we cloned full length zMMP13a into a mammalian expression construct with an N-terminal Strep-tag-II to facilitate purification and detection. The tag was placed before the pro-domain so that it would not interfere with protein function once MMP13a is activated (Fig. 5-13a). As a control, we also cloned a catalytic mutant by alanine substitution of a critical glutatate residue at position 224 (E224A) via site directed mutagenesis (Fig. 5-13b). Both WT and E224A MMP13a were expressed and purified from culture medium of HEK293A cells. We first tested their ability to activate and cleave collagen I, a classic substrate of human MMP13. As expected, WT zMMP13a was able to shed its pro-domain, transforming it from a 55kDa protein to a 42kDa form, whereas the catalytic mutant

failed to do so (Fig. 5-14a). Activated rMMP13a was then mixed with a fluorescently-quenched collagen I substrate in an *in vitro* assay to determine enzymatic activity. WT activated MMP13a was able to cleave collagen I (Fig. 5-14b). As expected, the E224A showed no enzymatic activity, confirming that it was catalytically inhibited.

We produced recombinant zebrafish TNF by replacing the predicted (N-terminal) intracellular and transmembrane domains with mannose-binding protein (MBP) to allow for maximum solubility and purification. Although the cleavage recognition sequence of mammalian TNF is not conserved in zTNF, we preserved the entirety of the extracellular domain including the region predicted to be homologous to the 'juxtamembrane domain' where the cleavage site resides (Fig. 5-13c). To test if TNF is a possible substrate of MMP13a, we combined MBP-TNF with activated WT zMMP13a at 37degC at a 5:1 molar ratio. In less than 5 minutes, MBP-TNF was cleaved into a number of smaller fragments that were identified as MBP (42kDa) or TNF (17kDa) via Western blotting (Fig. 5-15a,b). This was abrogated by the addition of O-phenanthroline, a metal chelating inhibitor of metalloproteases. In addition to the 17kDa-fragment corresponding to sTNF, there were 3 smaller fragments which all bound to anti-zTNF antibody, possibly indicating further processing of TNF. This phenomenon has also been noted for the processing of human TNF by human MMP13[152]. The E224A mutant of zMMP13a failed to cleave any MBP-TNF, indicating that catalytic activity of MMP13a is required for cleavage of TNF (Fig. 5-15c,d).

5.9. MMP13a modulates sTNF bioavailability and is a target for treatment in high TNF conditions.

If MMP13a is a TNF converting enzyme, and sTNF is required for enhanced microbicidal activity in macrophages, we reasoned that increasing unprocessed native TNF expression should not rescue *mmp13a* MO. We previously published that overexpression of LTA4H through the injection of mRNA increased the baseline transcription of TNF[53]. We co-injected the *lta4h* mRNA with *mmp13a* MO and conducted the intramacrophage bacterial killing assay. While *lta4h* mRNA had the expected effect of reducing bacterial burden within macrophages in WT animals, it did not rescue the increased bacterial burdens of *mmp13a* MO (Fig. 5-16a).

We also asked if this was the case for overall bacterial burdens after infection with WT Mm. Although increased TNF expression initially favors the host by enhancing bacterial killing, the same mechanism also leads to ROS-mediated necroptosis of the macrophage, leading to extracellular release of bacteria and uncontrolled infection[53, 54]. Thus, although intramacrophage bacterial numbers are reduced in the first couple days of infection, by 3dpi, both rsTNF treated larvae and *lta4h* mRNA injected larvae have higher overall bacterial burdens relative to controls[53]. We were able to also show that *lta4h* mRNA injected larvae are hypersusceptible to WT infection (Fig. 5-16b). Consistent with our earlier findings, larvae co-injected with *mmp13a* MO and *lta4h* mRNA were as hypersusceptible as larvae injected with one or the other, confirming that increased TNF transcription does not rescue MMP13a deficiency. Since *lta4h* mRNA injected larvae are replete with memTNF, we concluded that sTNF produced by MMP13a sheddase activity is the form of TNF that is responsible for increased bactericidal activity.

We concluded from these experiments that MMP13a is a checkpoint for TNF activation during Mm infection and acts as a rheostat for modulating bio-active TNF levels. If no processing of memTNF occurs as in *mmp13a* MO, then no sTNF is available to enhance macrophage activation. Conversely, we predict that in the context of high-TNF conditions, such as those induced by *lta4h* overexpression, sufficient MMP13a exists in the larvae to convert most memTNF into sTNF, leading to hyper-inflammatory cell death.

To test this prediction, we co-injected *lta4h* mRNA with doses of *mmp13a* MO ranging from none to the maximum, which is the dose required for hypersusceptibility. Again, *lta4h* mRNA alone and *mmp13a* MO alone both resulted in hypersusceptibility relative to WT controls. When the maximum dose of *mmp13a* MO was co-injected with *lta4h* mRNA, the larvae remained hypersusceptible. However, when we reduced the *mmp13a* MO concentration to 33% of the maximal dose, the larvae were no longer hypersusceptible, exhibiting similar bacterial burdens as WT controls (Fig. 5-17a). At this dose, the extracellular bacterial growth caused by *lta4h* overexpression is also reduced to WT levels (Fig. 5-17b). This indicates that tuning MMP13a levels can prevent macrophage necrosis, likely through controlling sTNF levels in infected larvae.

5.10. Figures and figure legends.

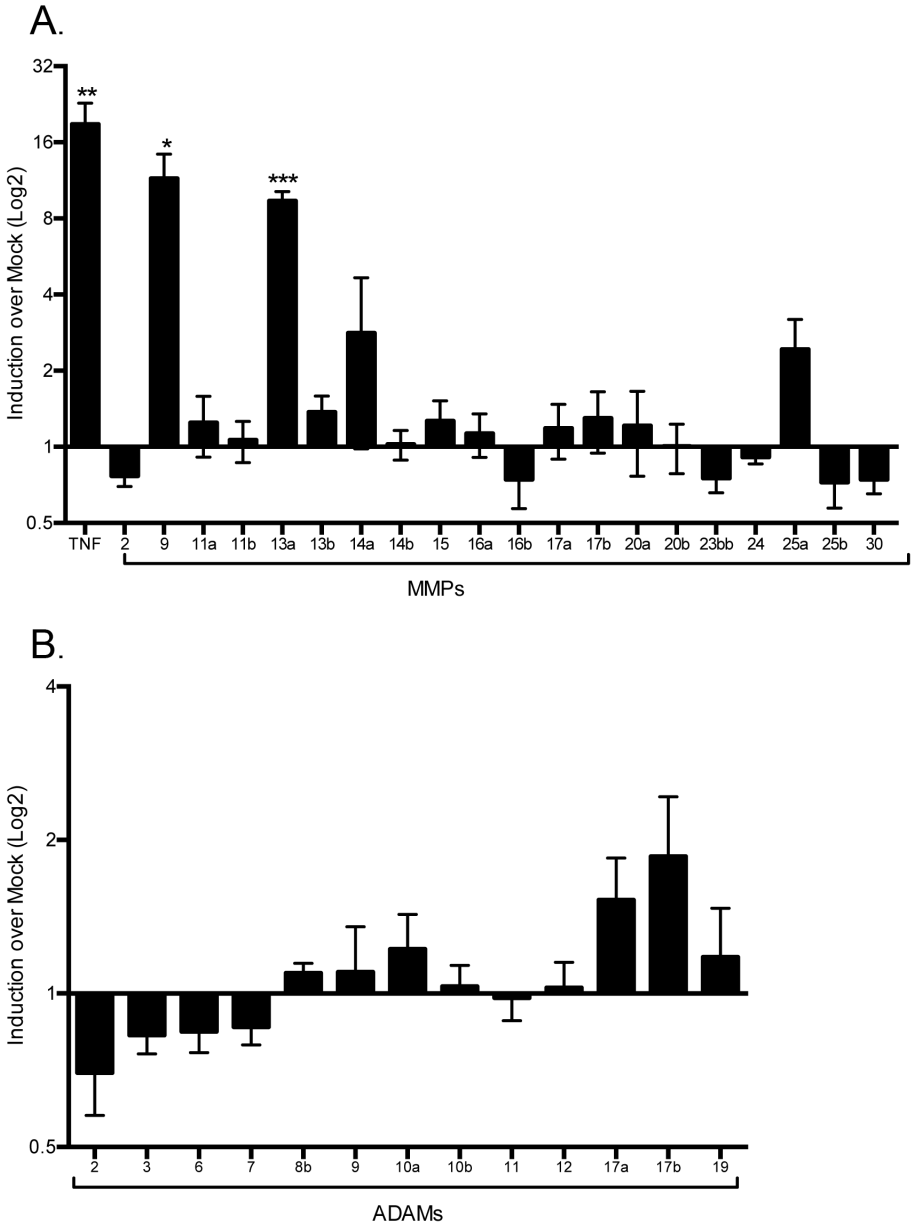

Figure 5- 1. *mmp9* and *mmp13a* are the only metalloproteases induced during infection.

Relative induction of (A) MMP genes and (B) ADAM genes. Larvae infected with ~150cfu Mm were collected at 5dpi for RT-qPCR. Gene expression was normalized to *β-actin* expression, then compared to expression in mock controls (ΔΔCt method). *tnf* was used as a positive control for infection induced expression. Error bars indicate SEM. n=3 biological replicates. Results were $\log_{10}$ transformed prior to analysis with one-sample t-test against a hypothetical value of 0.0. *p=0.011 **p=0.007 ***p=0.0015.

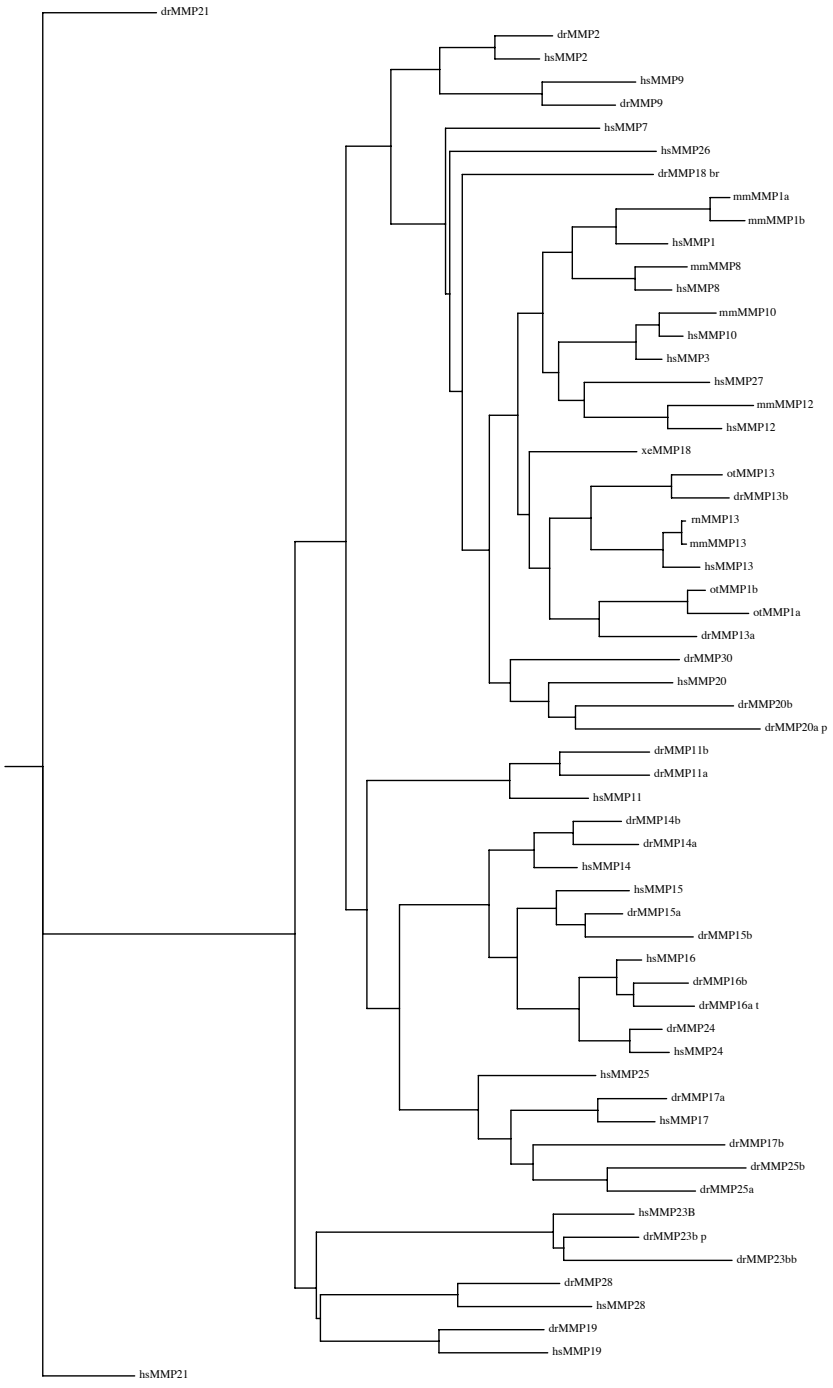

Figure 5- 2. Dendrogram comparing human MMPs to zebrafish MMPs.

Figure. 5-2. Dendragram comparing human MMPs to zebrafish MMPs. Trees were calculated with Protein Most Likelihood (protml) algorithm of PHYLIP package of programs, displayed with Drawgram.

dr= Zebrafish
hs= Human
mm= Mouse
rn= Rat
ot= medaka

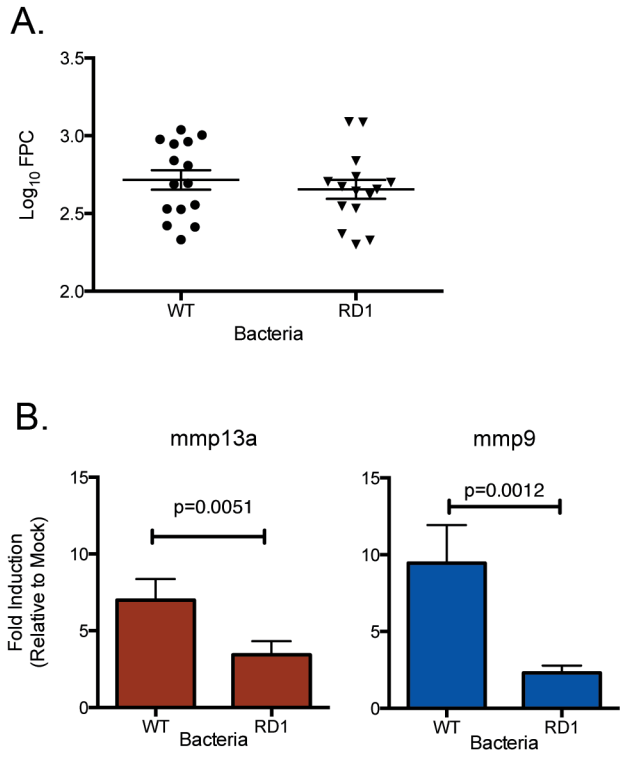

Figure 5- 3. *mmp13a* and *mmp9* expression are dependent on ESX-1 competence.

Since ΔRD1 Mm are highly attenuated, infecting doses were adjusted to having matching end doses. (A) Representative bacterial loads after 4dpi, prior to collection of larvae for RT-qPCR. Larvae were infected with ~100 cfu WT or ~450 cfu ΔRD1 Mm, then imaged at 4dpi for FPC. Error bars indicate SEM. n.s. by Student's T-test. (B) Induction of *mmp13a* and *mmp9* by WT or RD1 bacteria, relative to mock. Error bars indicate SEM. *n*=7 biological replicates. p-values were generated by Student's paired t-test.

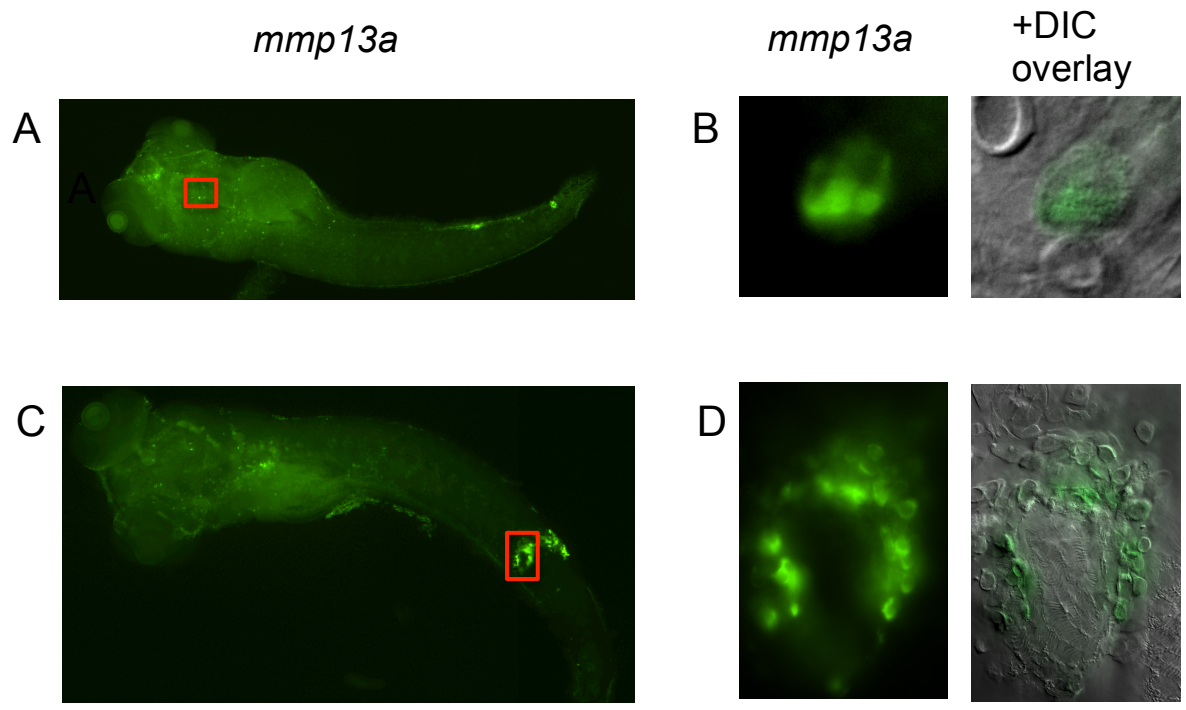

Figure 5- 4. *mmp13a* is expressed by neutrophils and induced in epithelial cells near the granuloma.

Images of 5dpf larvae, fixed and probed for *mmp13a* mRNA (green) with whole mount *in situ* hybridization. Red boxes indicate regions magnified in B and D. (A) Uninfected larvae (40x) express *mmp13a* in individual cells throughout the whole body, identified as (B) neutrophils with 600x magnification. Identification was conducted with aid of DIC microscopy, where granules are visible within the cell. (C) 4dpi infected larvae have additional cell types that express *mmp13a* near the granuloma. (D) They are identified as epithelial cells at 200x magnification.

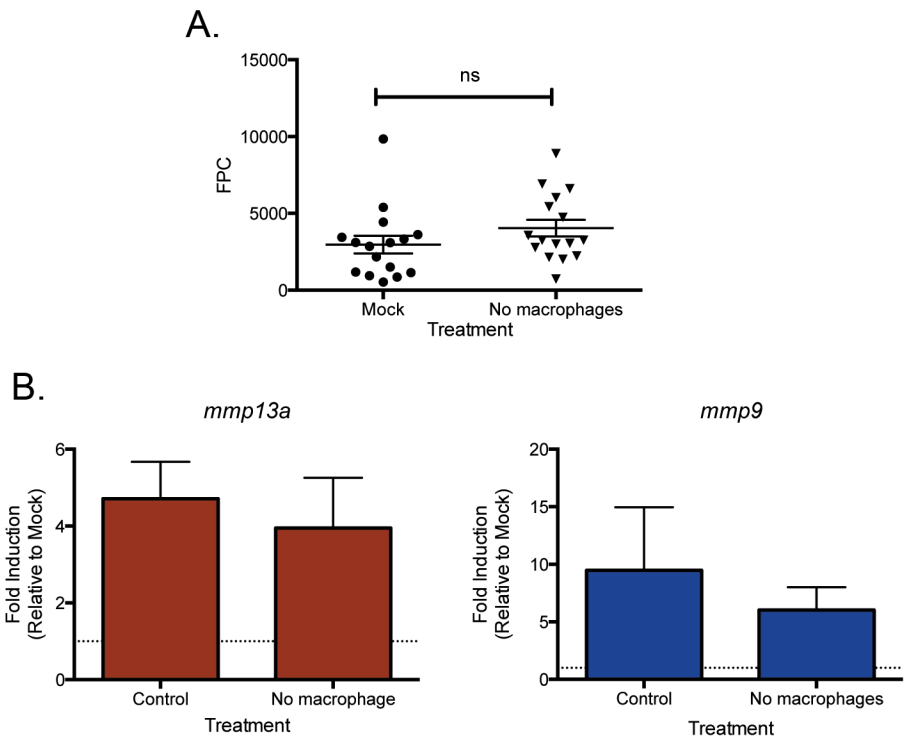

Figure 5- 5. *mmp13a* expression is independent of macrophage signaling.

Mock and *pu.1* morphants were infected to have similar end point bacterial loads prior to collection for RT-qPCR at 3dpi. (A) FPC graph, depicting a representative example endpoint of bacterial loads for collected samples. Mock morphants were infected with ~300cfu and *pu.1* morphants (No macrophages) were infected with ~100cfu, then imaged at 3dpi for FPC. Error bars indicate SEM. Student's t-test indicate n.s. (B) Relative induction of *mmp13a* and *mmp9* during infection in larvae with or without macrophages. Error bars indicate SEM. Student's t-test, n.s.

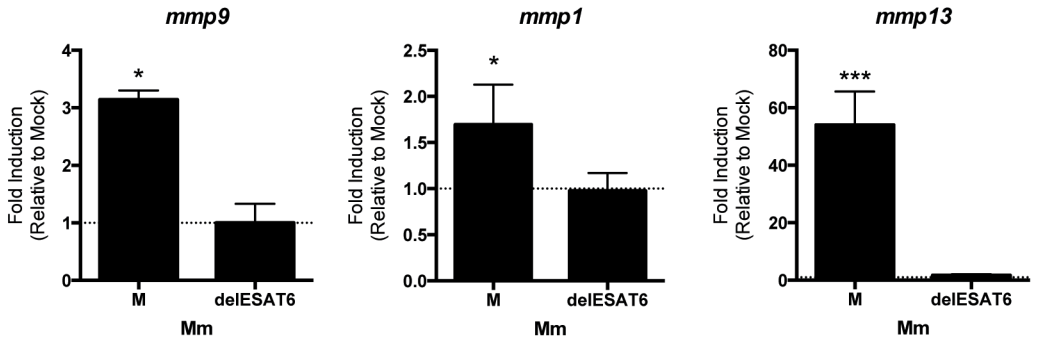

Figure 5- 6. NHBE cells express *MMP9*, *MMP1*, and *MMP13* when infected by ESX-1 competent Mm.

Relative induction of human MMPs by M strain (WT) or Δesat6 Mm, compared to mock infected controls. Despite higher MOI of Δesat6, MMPs are induced only when infected by ESX-1 competent bacteria. C_ts were normalized to β-actin. *MMP8* was below the limit of detection. NHBE cells were infected with M strain at an MOI of 20, Δesat6 at an MOI of 42. Error bars indicate SEM. p-values were generated with one-sample T-tests, with against a hypothetical value of 1. (*$p<0.05$, ***$p<0.0001$).

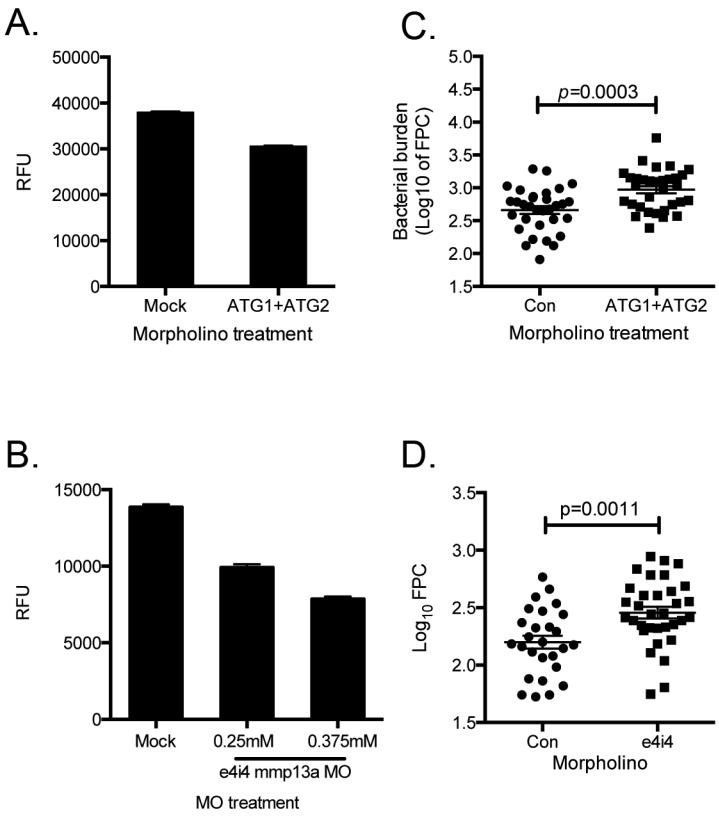

Figure 5- 7. *mmp13a* morphants are hypersusceptible to Mm.

(A)(B) Collagenase activity is reduced in larvae injected with (A) ATG1 + ATG2 MO and (B) e4i4 MO. Whole larval lysates were incubated with DQ Collagen I substrate for 4h at RT, then measured for fluorescence. Graphs are depicted as relative fluorescence units (RFU).
(C)(D) FPC graphs (Log_{10}) comparing mock morphants with (C) ATG1 + ATG2 or (D) e4i4 morphants. Larvae were infected with 120-150 cfu of WT Mm. Error bars indicate SEM. p-values generated by Student's t-test.

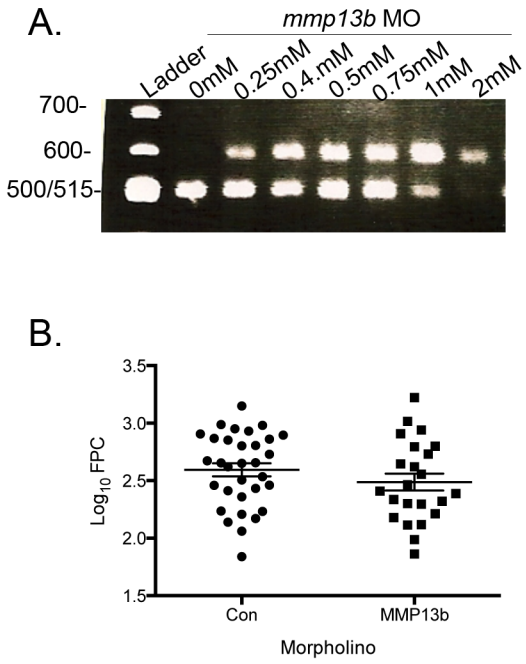

Figure 5- 8. *mmp13b* morphants are not hypersusceptible to Mm infection.

(A) Morpholino targeting exon3-intron3 junction results in insertion of 92bp intron. Series of MO dilutions were injected into eggs and collected at 4dpf for RT-qPCR. (B) FPC graphs (Log_{10}) comparing mock or *mmp13b* morphants (used at 0.75mM). Error bars indicate SEM. N.S. by Student's t-test.

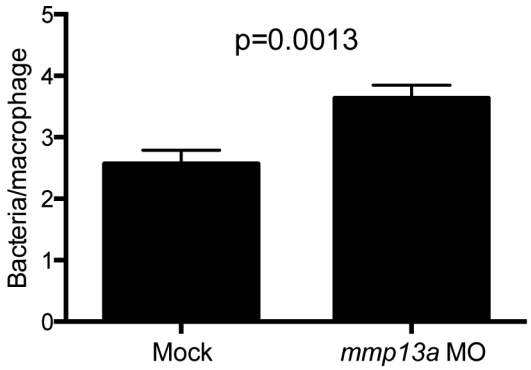

Figure 5- 9. *mmp13a* morphants are deficient in control of Mm within macrophages.

Mean (±SEM) number of bacteria growing per macrophage at 40-44hpi in mock or *mmp13a* MO. Larvae were infected with ~150 cfu Δ*erp* Mm. p-values generated by Student's t-test.

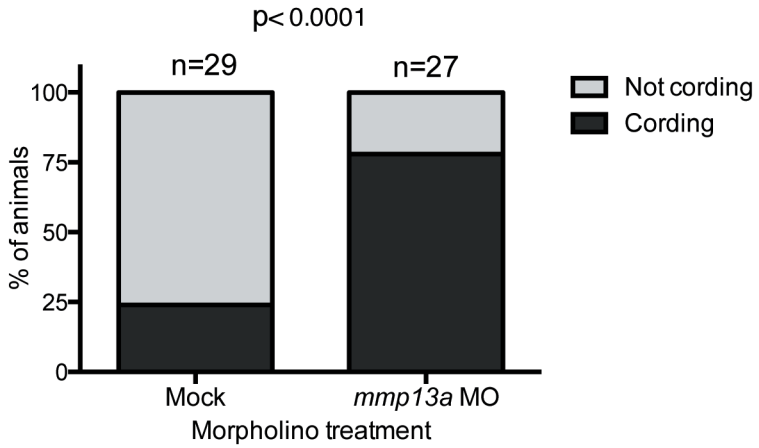

Figure 5- 10. *mmp13a* morphants cord at higher frequencies during infection with WT Mm.

Mock or *mmp13a* morphants were infected with 120-150 cfu of WT Mm, then assessed at 3dpi for cording. Number of fish analyzed shown above each bar. Representative of 4 independent experiments. Statistical analysis done with Fisher's exact test.

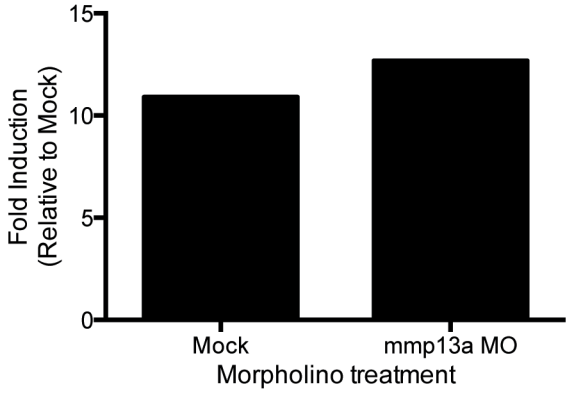

Figure 5- 11. *tnf* gene induction is not impaired in *mmp13a* morphants.

Mock or *mmp13a* morphants were either mock injected or infected with 200-250 cfu WT Mm and collected at 2dpi for RT-qPCR. Data was normalized to *β-actin* expression prior to comparison with uninfected controls (ΔΔCt method).

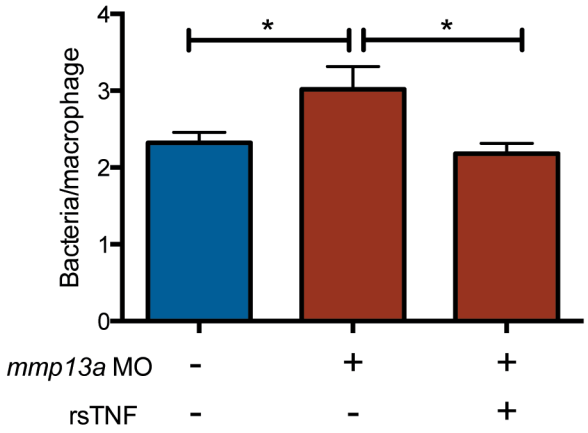

Figure 5- 12. rsTNF treatment rescues bacterial killing in *mmp13a* deficient macrophages.

Mean (±SEM) number of bacteria growing per macrophage at 40-44hpi. Morphants were infected with 150-200cfu Δ*erp* Mm, and at 16hpi, injected with 0.5ng rsTNF or vehicle. Error bars indicate SEM. p-values generated by ANOVA, with Dunnett's post-hoc test (*<0.05).

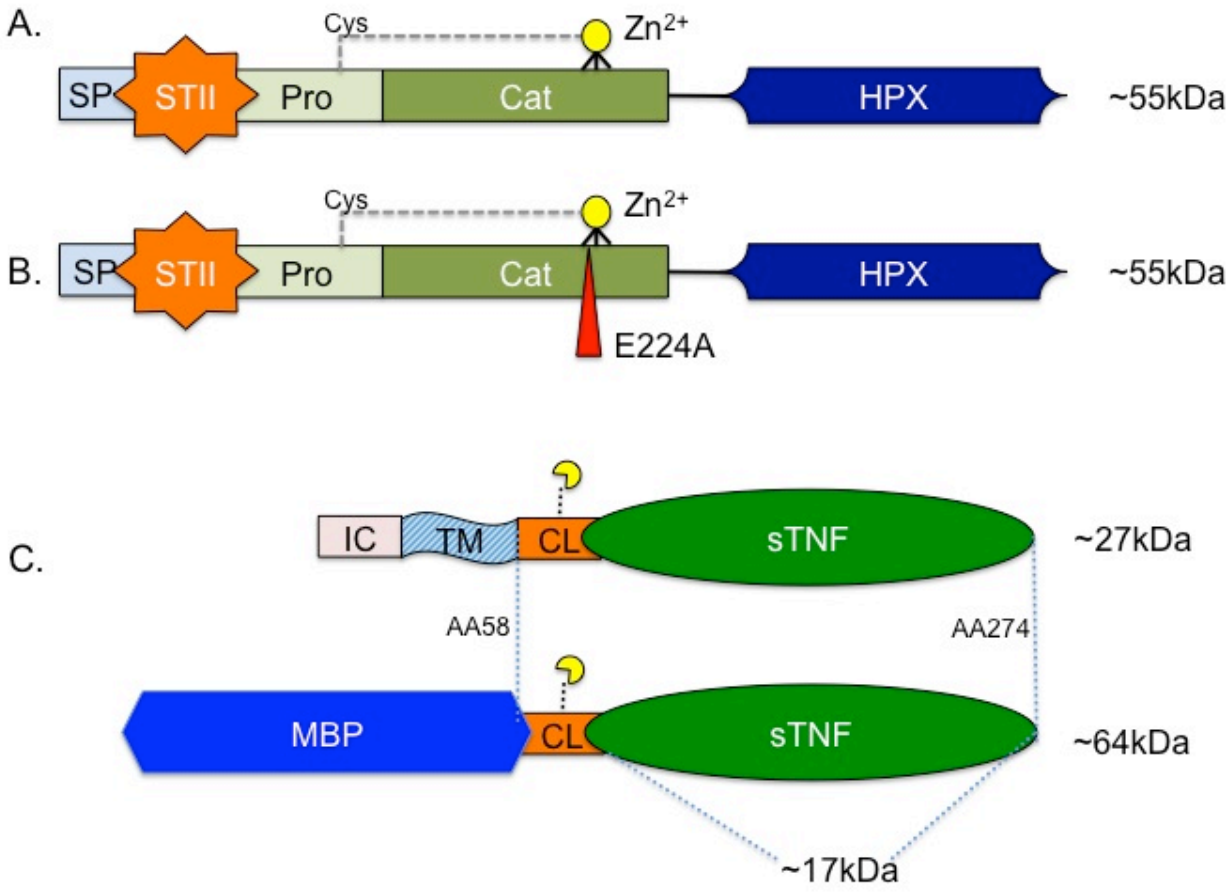

Figure 5- 13. Design of recombinant proteins used for *in vitro* assays.

(A) Recombinant StrepTagII-proMMP13a (WT). (B) Recombinant catalytically inhibited StrepTagII-proMMP13a. (E224A-rMMP13a). SP= signal peptide. STII= StrepTagII. Pro= pro-domain, contains cysteine switch. Cat= Catalytic domain, contains zinc ion. HPX= Hemopexin domain.
(C) Recombinant MBP-TNF was designed to exclude the intracellular and the transmembrane domains, but keeps the cleavage domain intact. sTNF is expected to be about 17kDa in size. IC=intracellular domain. TM=transmembrane. CL=cleavage domain intact.

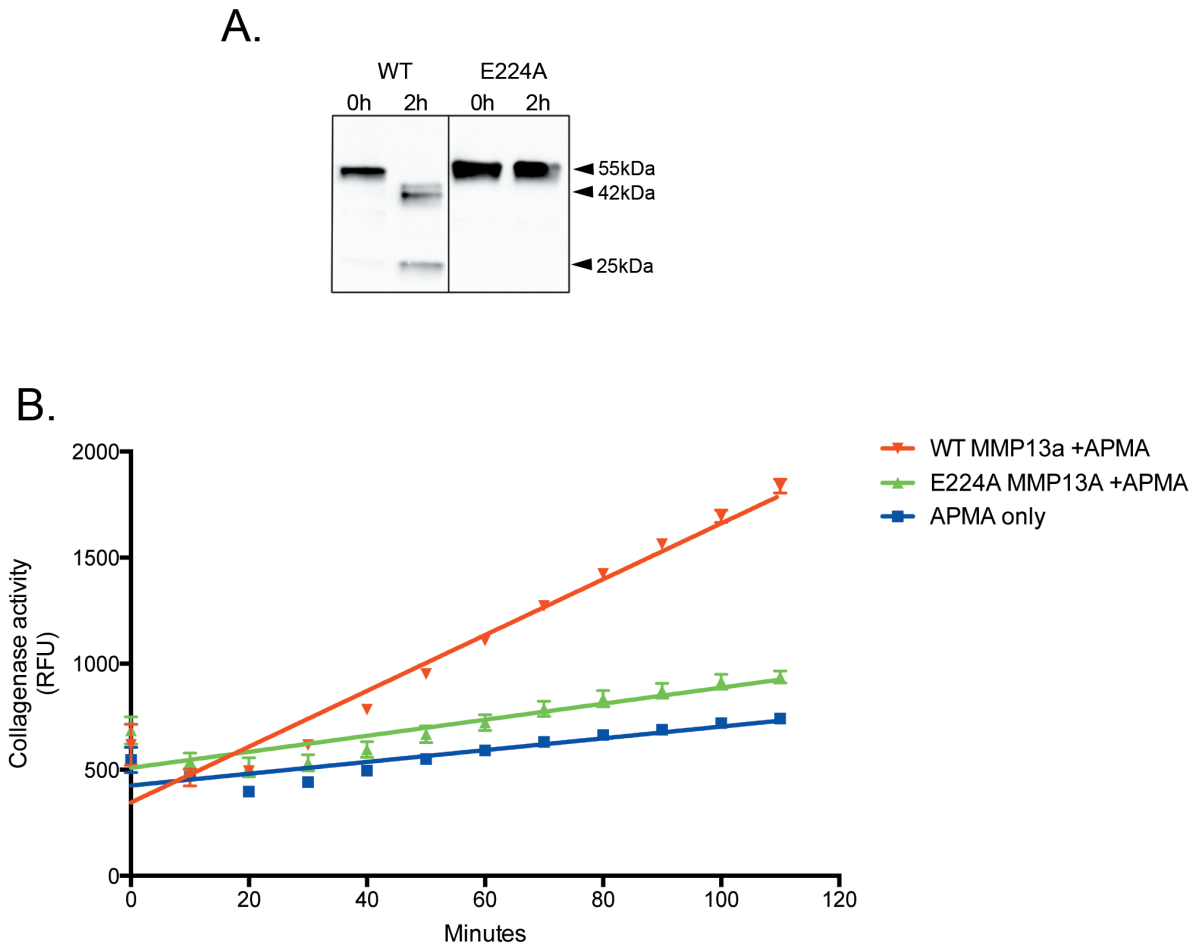

Figure 5- 14. WT, but not E224A, rMMP13a is catalytically active.

(A) Western blot of WT or E224A rMMP13a, probed with anti-zMMP13a antibody, after two hours of incubation with 1mM APMA at 37ºC. WT rMMP13a is able to autolyse the pro-domain, resulting in a 42kDa product corresponding to active protease, and a 25kDa product that appears to be a degradation product. (B) Fluorimetric collagenase assay shows that WT MMP13a, but not E224A MMP13a, is enzymatically active and capable of lysing fluorescein conjugated Collagen I. Recombinant proteases were activated in APMA for two hours prior to the assay.

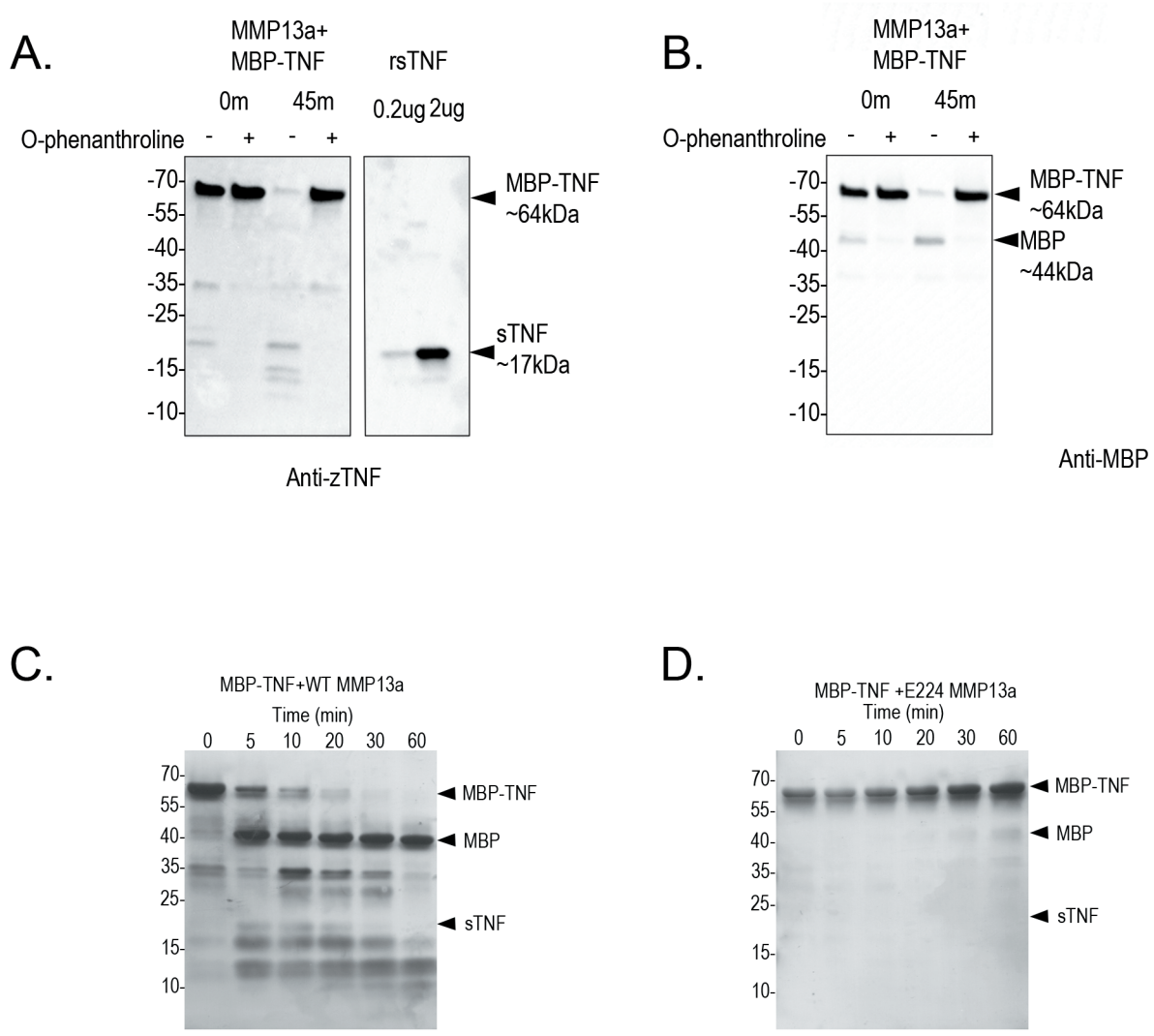

Figure 5- 15. MBP-TNF is a zMMP13a substrate.

(A)(B) MBP-TNF and activated WT zMMP13a were incubated at a 5:1 molar ratio for 45 minutes with or without 10µM O-phenanthroline. Western blot with (A) anti-TNF antibody with rsTNF as a control shows that sTNF is released from the fusion protein by MMP13a. (B) Western blot with anti-MBP antibody.
(C)(D) Silver stain of MBP-TNF and activated WT or E224A zMMP13a incubated at 5:1 molar ratio at 37ºC. Samples were collected at indicated times.

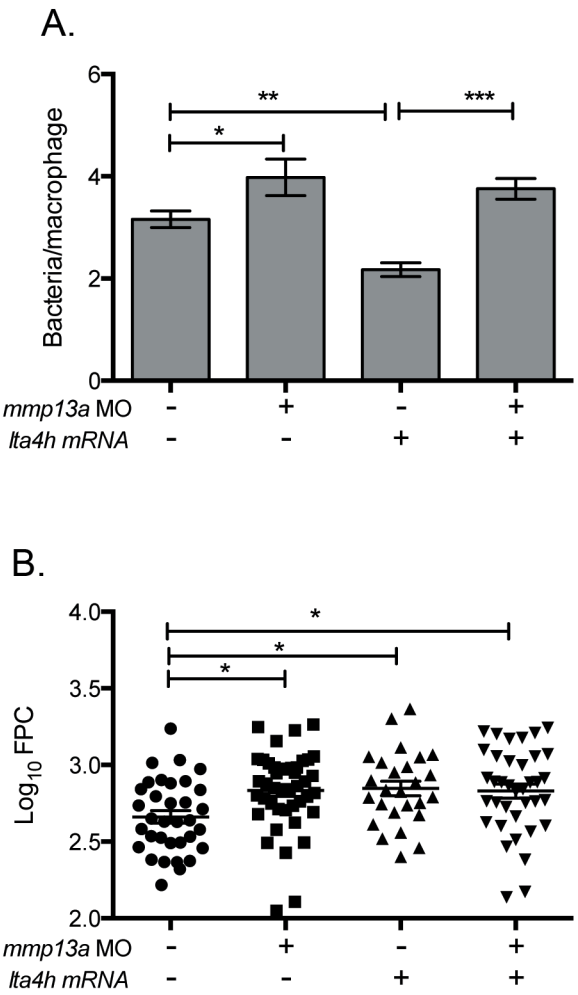

Figure 5- 16. *mmp13a* morphants are not rescued by increasing *tnf* transcription.

Eggs were injected with either *mmp13a* MO, *lta4h* mRNA, or a combination. (A) Average (±SEM) bacterial counts per macrophage at 44hpi. Larvae were infected with 120-150cfu Δ*erp* Mm at 2dpi. $p<0.0001$; ANOVA with Dunnett's post-hoc test. *$p<0.05$; **$p<0.01$; $p<0.001$.(B) Average FPC (±SEM) at 3dpi. Larvae were infected with 150-200cfu WT Mm. $p= 0.0088$; ANOVA with Dunnett's post-hoc test. *$p<0.05$.

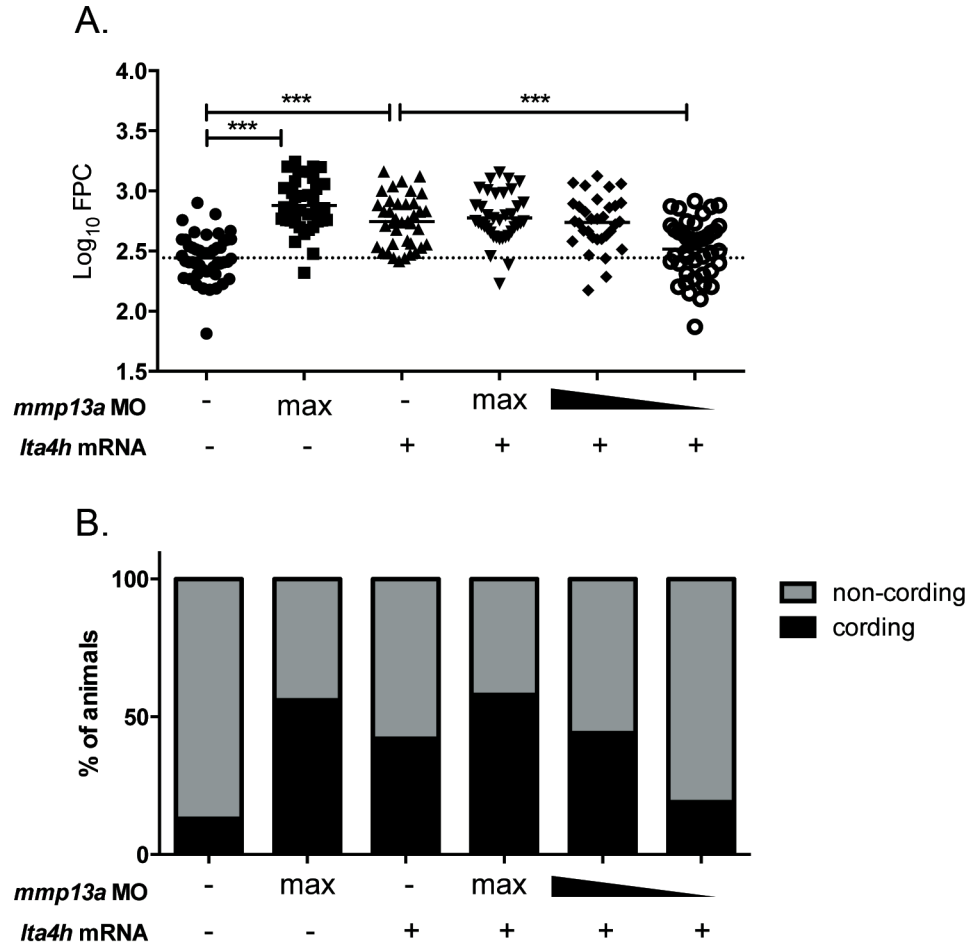

Figure 5- 17. Hypersusceptibility of *lta4h*-high larvae is rescued by partial knockdown of *mmp13a*.

Eggs were injected with *mmp13a* MO, *lta4h* mRNA, or *lta4h* mRNA and 100%, 66%, or 33% of *mmp13a* MO, then infected at 2dpi with 150-200cfu WT Mm. (A) FPC at 3dpi. $p<0.0001$; ANOVA with Bonferroni post-hoc test. ***p<0.0001. (B) Cording analysis at 3dpi. $p< 0.0001$; Fisher's exact test.

Chapter 6:

Discussion

High MMP expression during TB in human patients has been linked to poor outcomes. Their role during infection has been difficult to infer, owing to a lack of appropriate animal models and a difficulty of studying *in vivo* dynamics of granulomas. However, through the use of the transparent zebrafish larvae, we have shown that *mmp13a* is upregulated in an ESX-1-dependent manner and that its activity is necessary for TNF mediated bacterial killing in macrophages very early in infection. This is the first time a collagenase has been implicated in a host protective role during TB infection, and may explain why they are expressed early during infection. Moreover, this presents a novel target for the treatment of TB in patients overexpressing TNF as a result of underlying genetic causes[53]

6.1. Collagenases are induced during mycobacterial infection

We investigated gene expression of a comprehensive list of zebrafish metalloproteases during mycobacterial infection, and found that *mmp9* and *mmp13a* were the only MMPs upregulated. Both MMP9 and collagenases are represented in studies of gene induction and protein levels in TB infected lungs[131, 154]. In agreement with our data, *mmp13a* and *mmp9* were also induced in other gene expression studies conducted in Mm infected zebrafish[155, 156]. In contrast to these studies, we did not see upregulation of *mmp14a*, which is also upregulated in human and Rhesus macaque granulomas[154, 155, 157]. Since the other studies were conducted in adult organisms, this difference likely stems from developmental changes in immune cell repertoires.

Although phylogenetic analyses have shown that there are no exact orthologs of *mmp13a* in mammalian lineages, at least two of the three mammalian collagenases are known to be upregulated in TB infected lungs. Recent studies have also detected *MMP13* induction in Mtb infected mice and rabbits later during infection, though its role is unknown[158, 159]. *MMP1,* on the other hand, is well known to be upregulated during TB in humans, macaques, and rabbits, and correlates with cavity formation and worse outcomes[131, 157, 158]. Interestingly, other mammal-specific MMPs in this sub-clade have been shown to be induced during infection with Mtb, such as *MMP3, -7, -10,* and *-12*[130, 131, 158]. Response to inflammation or infection could be a general function for this group, and *mmp13a* may be a functional ortholog for these MMPs during mycobacterial infection.

We have found that both *mmp9* and *mmp13a* expression can be stimulated by cells through direct contact with bacteria, rather than through macrophage signaling. In accordance, infection of human lung epithelial cells with WT, but not ΔESAT6 Mm, induced *MMP9* and two collagenases, *MMP1* and *MMP13*. These findings agree with and extend previous work from our lab[1], suggesting that the signaling pathway induced in an ESX-1-dependent manner may not be *mmp9* specific, but a more general inflammatory pathway. This pathway includes *mmp13a*, and possibly other genes that we have yet to investigate, such as potential substrates for *mmp9*. Interestingly, infection of zebrafish larvae with ΔRD1 Mm still resulted in statistically significant induction of both *mmp9* and *mmp13a*, whereas infection of NHBE cells with ΔESAT-6 Mm did not induce any MMPs. We believe that epithelial cells are specifically expressing these MMPs in response to the ESX-1 secretion system and not to a general signal of infection. Indeed, Volkman *et al.* (2010) showed that *mmp9* was induced in both neutrophils and macrophages upon infection with either WT or ΔRD1 Mm. This is likely to be true for *mmp13a* as well, but needs to be

confirmed using multiplexed *in situ* hybridization to against probes for neutrophils, macrophages, and epithelial cells.

Our data that epithelial cells express MMPs during TB infection is supported by work from other labs. Histology of human TB patients shows expression of *mmp1* and *-9* in epithelial cells near the granuloma[133, 160], as well as in macrophages within the granuloma[146, 161]. MMP9 expression in both cell types has also been observed in mice granulomas[159]. The same studies showed that human lung epithelial cell cultures also secrete MMP1 and -9, but with a major difference: these studies were unable to induce MMP expression with direct infection with Mtb, but instead required conditioned medium from Mtb-infected macrophages[133, 160]. A key difference is that we have measured gene expression, which captures rapid responses to stimuli, whereas these studies measured secreted MMP activity in through gel zymography. MMP activity may be relatively stable as a result of post-translational control of MMP protein through endogenous inhibitors, degradation, or reuptake. Resolution of this discrepancy by repeating our gene expression experiment with Mtb would help to solidify the similarity in signaling mechanisms with Mm.

It is still unclear exactly what signals, from the pathogen or the host, are responsible for the induction of the two MMPs. In addition to ESAT-6 and CFP-10, ESX-1 substrates include ESX-1 secretion associated proteins (*esp* proteins) and a number of PE/PPE proteins, so named for containing conserved N-terminal motifs Pro-Glu or Pro-Glu-Glu[162, 163]. The functions of individual substrates are not yet elucidated, since many substrates are codependent for secretion[59, 164]. As a result, it is unknown which of the substrates are responsible for the pathogenic roles of ESX-1. Recent studies have discovered and utilized point mutants of EspA and ESAT-6 that isolate pathogenic effects of single substrates from their role in the

functionality of the secretion machinery[165, 166]. The point mutations appear to disrupt the interaction of the substrate with host proteins without disrupting interactions with secretion system proteins, thus solving problems caused by codependence of ESX-1 substrate secretion. Use of these and similar mutants in other ESX-1 substrates would aid in better understanding of how the bacterium induces MMP expression.

Notably, the ESX-1 secretion system has been implicated in the disruption of cell membranes leading to cytolysis[167-169]. This has been largely attributed to pore formation by ESAT-6, though the mechanisms remain unclear[170, 171]. It is possible that the cytolytic activity of ESX-1 is inducing wound-healing responses in nearby epidermal cells, even though there are no overt signs of wounding on the surface of the larvae. MMPs, including MMP9 and the collagenases, are known to be expressed in response to wounding. Under normal conditions, MMP activity aids in leukocyte migration towards the wound and eventual contraction of wound size through the migration of keratinocytes[68, 172-174]. A recent study using zebrafish points to a role for immune response gene 1-like (*irg1l*) upstream of epidermal *mmp9* induction in wound healing and in *Salmonella* infection[105]. In this model, whereas *Salmonella* infection upregulates *mmp9* via MyD88-dependent macrophage signals, wounding appears to directly stimulate *irg1l* through mitochondrially derived ROS, which then stimulates MMP9 secretion[105]. The latter mechanism maybe at play during ESX-1 dependent induction of MMPs. Mycobacteria may be taking advantage of this response to recruit macrophages to the granuloma through *mmp9*, but an unintended consequence of this may be the upregulation of *mmp13a*. Further dissection of these pathways will elucidate if indeed wound-healing pathways are necessary for MMP induction.

6.2. MMP13a enhances microbicidal activity of macrophages

Our study is the first to show that collagenase activity is host protective through increased bacterial killing. We have found that this occurs through the activity of MMP13a on latent TNF to increase the bioavailability of its soluble form, which can diffuse to infected macrophages to enhance microbicidal activity. Other studies have implicated MMPs in bacterial killing through intrinsic antimicrobial activity (MMP12)[124] or through activation of anti-microbial peptides (MMP7)[123]. Because of the known activity of MMPs on inflammatory cytokines such as IL-1β and TNF, increased bactericidal activity through enhanced signaling to macrophages has been theorized, but not definitively proven[64]. At least one other study has implicated MMPs in enhanced macrophage activity and host resistance. MMP2/9 double KO mice are hypersusceptible to *Streptococcus pneumoniae* infection as a result of reduced phagocytosis and intracellular killing of bacteria by neutrophils. The mechanisms of reduced killing were not dissected thoroughly, but they determined that MMP9 KO macrophages were deficient in ROS production[147]. Induction of microbicidal activity in macrophages is an important, but poorly understood function of MMPs involved in immunity to infection.

The finding that collagenase expression is host protective during mycobacterial infection is unexpected, since studies have linked MMP expression to worse outcomes in TB. Increased MMP9 secretion in human TB is liked to increased granuloma size[161, 175, 176] and animals deficient in MMP9 form fewer and smaller granulomas[1, 61]. Similarly, MMP1 expression is increased in TB infected humans, macaques, and rabbits[129, 131, 158, 177], and increased expression correlates to worse lung pathology[129, 135, 158]. Understanding the role of collagenases has been difficult since the mouse model does not express collagenases in the lung, and mice granulomas are not organized in the same way human granulomas are[8, 131]. One

study has addressed this issue by using transgenic mice that express human MMP1 under the control of the scavenger receptor A (SrA) promoter/enhancer, which is turned on in activated macrophages[178, 179]. Infection with virulent, but not ΔRD1, Mtb in these mice result in cavity formation within 130dpi, supporting the view that MMP1 expression in TB drives pathology. Interestingly, this study found that expression of MMP1 did not have an effect of bacterial burdens around the same time point[133]. Our data neither support nor refute this claim. It is possible that collagenase expression early during mycobacterial infection is host protective, but unchecked, it causes pathology. Similar mechanisms have been attributed to the dichotomous role for TNF during human TB, where having both extremes of TNF levels result in reduced host survival[53]. Notably, MMP1 expression has been found in both epithelia and macrophages of Mtb infected lungs. The transgenic mouse model addresses only the role of MMP1 from macrophages. MMP1 from epithelia may have a different role, since the substrates localized near the alveolar epithelium is distinct from those near granulomas and macrophages. Finally, SrA-driven MMP1 expression may not represent the course of natural infection, in which proteins necessary for localization or control of activity are not co-expressed. We argue that understanding collagenase activity during TB is better understood through models that express collagenases naturally, such as the zebrafish, rabbit, guinea pig, or macaque model. Of these models, only the zebrafish model allows for observation of intracellular events in early infection.

6.3. MMP13a is a TNF sheddase

The phenotype seen in *mmp13a* morphants was similar to that of TNF-deficient larvae, exhibiting increased bacterial load per macrophage, reduced cellularity of granulomas and extracellularization of mycobacteria, and ultimately increasing overall bacterial burden. Our

findings suggest that MMP13a is an important TNF sheddase, and deficiency of MMP13a results in reduced bioavailability of sTNF.

Conversion of memTNF to sTNF has been shown to be critical for signaling during inflammatory conditions. For example, in diseases of hyper-inflammation where TNFα signaling is known to be detrimental, such as sepsis and arthritis, mutations in the cleavage domain that prevent proteolysis also result in reduced morbidity and mortality[180, 181]. sTNF and memTNF also appear to have different roles during TB. Whereas mice TNF KO mice succumb to infection early (between 40-60dpi) and WT mice survive for over 300 days, mice with only memTNF have an intermediate susceptibility phenotype, succumbing to infection around 100-200 days[182-184]. Some studies observed reduced iNOS expression and activity, implying defects in macrophage activation and bacterial killing, consistent with our hypothesis that sTNF is the biologically important form of TNF during mycobacterial infection.

Currently, ADAM17 is believed to be the primary TNF sheddase. In zebrafish, there are two genes analogous to human *ADAM17*, *adam17a* and *adam17b* (58% and 59% identical to human *ADAM17*, respectively). Both genes are expressed in the larval zebrafish, however neither is induced by infection (Fig. 5-1b). Although *Adam17* KO mice die shortly after birth, conditional knockouts of *Adam17* in macrophage and neutrophil populations have drastically reduced sTNF shedding during endotoxin shock and survive LPS challenge[185]. However, it is also clear that many other proteases can cleave TNF *in vitro*, such as protease-3 (PR3), ADAM10, ADAM19, MMPs -1, -2, -7, -8, -9 and -13[122, 148-153]. Recent studies have begun to show their importance *in vivo.* For example, in addition to showing that MMP13 could process TNF *in vitro,* Vandenbroucke et al. (2013) demonstrated that *Mmp13* KO mice were also protected from endotoxin shock, similar to *Adam17* conditional KO, and that this was the result

of reduced TNF release. Another group showed that MMP8 knockdown in mice was sufficient to significantly reduce sTNF shedding during neuroinflammation induced by LPS[186]. We believe that our work contributes to the list of biologically relevant TNF sheddases, and should be investigated further.

A major caveat to these studies, including ours, is that they have yet to show that collagenases are directly cleaving TNF *in vivo*; after all, it is surprising that MMP8 or 13 do not seem to compensate for TNF cleavage in ADAM17 KO mice. A salient explanation is that ADAM17 is the only physiological TNF sheddase, but its activity is regulated by other MMPs. For example, MMP9 activates Neutrophil Elastase by degrading an endogenous reversible inhibitor[99]. Collagenases may regulate ADAM17 activity in a similar manner[187]. Another possibility is that ADAM17 and collagenases cooperate during TNF activation either in a proteolytic complex or a regulatory network, and that the catalytic activity of both proteases are required for efficient TNF shedding[72]. Even if MMPs do not have direct TNF cleavage activity *in vivo*, understanding the interaction between MMPs and ADAM17 would still be important since the regulation of ADAM17 activity is still not well understood[187]. We will address these questions in future experiments with our system first by testing the effect of ADAM17 knockdown during infection. If MMP13a is the only relevant TNF sheddase, then ADAM17 should not have the same phenotypes that we observed during infection in *mmp13a* MO. Second, we will confirm MMP13a sheddase activity *in vivo* by measuring protein expression of both soluble and membrane bound forms TNF in WT and MMP13a deficient larvae. We expect MMP13a deficient larvae to have reduced levels of sTNF relative to memTNF. Regardless of the outcome of these results, our data is indicative of an important role for MMP13a in mediating the

effects of TNF during infection, and should be further investigated in mammalian systems as a mediator of host protective immunity.

Because ADAM17 was shown to have a role in many autoimmune/inflammatory conditions, it has been an attractive target for inhibition. A number of inhibitors had gone into clinical trials[188], however, ADAM17 inhibition exhibited toxic side effects that were attributed to its role as a sheddase for over 70 other proteins. Sheddase activity on some of these substrates are critical, as exhibited by the lack of viability of ADAM17 KO mice[72, 185, 188]. Thus, the discovery that other MMPs can affect TNF release is of primary interest in developing more inhibitors that may have fewer toxic side effects. Already, one group has shown that an MMP inhibitor targeting the MMP1,-8,-9, and -13 was as effective as an ADAM17 specific inhibitor in limiting TNF shedding in an infant rat model of pneumococcal meningitis[189].

6.4. TNF shedding as a target for treatment of TB patients with excessive TNF

The dual-nature of inflammatory responses during TB is increasingly being recognized as an important aspect in improving its clinical treatment. On one hand, hyper-inflammatory states can cause damage to host tissues, as we have demonstrated with TNF, and others have demonstrated with IL-1β signaling and neutrophillic influx[54, 190, 191]. Conversely, complete loss of components of the inflammatory response results in an inability to control bacterial growth[31, 192, 193]. A similar dichotomous pattern has been noted for anti-inflammatory molecules such as IL-10 and Lipoxins[56, 194, 195]. Our lab has been exploring methods for managing infection in patients who have a genetic predisposition for either too little, or too much TNF[53, 195]. We have already published methods for controlling hyper-inflammatory conditions with anti-inflammatory drugs or by blocking downstream mediators of TNF-induced

cell death, such as ROS-scavengers and necroptosis inhibitors[54, 196]. Here, we have identified another point of control for high inflammation states. As a TNF-sheddase, MMP13a is a rheostat that controls TNF bioavailability and activity. Reducing, but not completely abolishing, MMP13a activity decreases bacterial burdens and necrosis in LTA4H-high larvae.

While we have presented MMP13a inhibition as a potential mechanism for limiting TNF-high pathology, our study should also be taken as a cautionary tale against non-specific inhibition of MMPs in TB. Like cytokines, MMP activity during TB is dichotomous and must be carefully regulated. Inhibitors targeting MMP9 or MMPs involved in cavitation will be helpful adjunct therapeutics to antibiotics by blocking host pathways that enhance granuloma growth and lung pathology[129, 132]. However, accidental inhibition of MMP13a early in infection, or in patients who do not produce sufficient levels of TNF, may result in exacerbated disease. Epidemiological studies have already shown that treatment of patients who are TNF-low with dexamethasone, an anti-inflammatory glucocorticoid, results in drastically reduced survival[53]. It is also important to remember that there are numerous mammalian MMPs that are known to be upregulated during Mtb infection whose functions are not yet elucidated. The failure to recognize the diverse roles of MMPs has already led to disastrous results for clinical trials of MMP-inhibitors for the treatment of cancer in the 1990s. The main causes of the failure included the development of musculoskeletal symptoms and, in some cases, higher fatality rates among patients[197, 198]. Both of these are attributable to non-specific inhibition of MMPs and poor timing of treatment, highlighting the beneficial roles of metalloproteases[197]. Thus, MMP inhibition to treat TB should be designed with mind to the host's genetics, the stage of infection, and the specificity of the inhibitor to a limited number of MMPs. Further experimentation is

necessary to ensure that if MMP inhibitors are utilized, that they are specific for preventing cavity and granuloma formation, and do not interfere with protective immune responses.

6.5. Conclusion:

We have discovered that MMP13a is a host protective infection response gene whose expression is enhanced by the presence of a bacterial virulence factor. Our studies have given additional support for MMPs playing a beneficial role during bacterial infection by acting on macrophage activity, and the possibility that ADAM17 is not the only relevant TNF sheddase *in vivo*. Because controlling TNF sheddase activity is an important aspect of limiting hyperinflammatory conditions including but not limited to tuberculosis, we believe that further research should be done to determine the functional correlate of MMP13a in mammalian systems. Furthermore, MMP inhibition should be considered strongly as an adjunct therapy to prevent pathology during tuberculosis, however, researchers must be careful to target a specific gene at a specific stage of infection.

Notes:

For NHBE gene expression, F. Chu did the infections and total RNA preparations, and S. Phillips generated Δesat6 Mm mutants. All experiments involving *lta4h* mRNA injection into eggs or sTNF injection into larvae were conducted in partnership with or by F.J. Roca.

References

1. Volkman, H. E., Pozos, T. C., Zheng, J., Davis, J. M., Rawls, J. F., and Ramakrishnan, L. (2010). Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science *327*, 466–469.

2. Davis, J. M., and Ramakrishnan, L. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. CELL *136*, 37–49.

3. WHO ed. (2014). Tuberculosis (WHO) Available at: http://www.who.int/mediacentre/factsheets/fs104/en/ [Accessed December 1, 2014].

4. Zhang, Y. (2005). THE MAGIC BULLETS AND TUBERCULOSIS DRUG TARGETS. Annu Rev Pharmacol Toxicol *45*, 529–564.

5. van Crevel, R., Ottenhoff, T. H. M., and van der Meer, J. W. M. (2002). Innate immunity to Mycobacterium tuberculosis. Clinical Microbiology Reviews *15*, 294–309.

6. Dannenberg, A. M. (1993). Immunopathogenesis of pulmonary tuberculosis. Hosp Pract (Off Ed) *28*, 51–58.

7. Philips, J. A., and Ernst, J. D. (2012). Tuberculosis Pathogenesis and Immunity. Annu. Rev. Pathol. Mech. Dis. *7*, 353–384.

8. Cosma, C. L., Sherman, D. R., and Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. Annu Rev Microbiol *57*, 641–676.

9. North, R. J., and Jung, Y.-J. (2004). Immunity to tuberculosis. Annu Rev Immunol *22*, 599–623.

10. Helke, K. L., Mankowski, J. L., and Manabe, Y. C. (2006). Animal models of cavitation in pulmonary tuberculosis. Tuberculosis *86*, 337–348.

11. Volkman, H. E., Clay, H., Beery, D., Chang, J. C. W., Sherman, D. R., and Ramakrishnan, L. (2004). Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS Biol *2*, e367.

12. Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in

zebrafish embryos. Immunity *17*, 693–702.

13. Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W., and Bloom, B. R. (1995). Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity *2*, 561–572.

14. Mayer-Barber, K. D., Barber, D. L., Shenderov, K., White, S. D., Wilson, M. S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Núñez, G., et al. (2010). Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. The Journal of Immunology *184*, 3326–3330.

15. van der Sar, A. M., Appelmelk, B. J., Vandenbroucke-Grauls, C. M. J. E., and Bitter, W. (2004). A star with stripes: zebrafish as an infection model. Trends Microbiol *12*, 451–457.

16. Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., Mclaren, K., Matthews, L., et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. Nature, 1–6.

17. Kettleborough, R. N. W., Busch-Nentwich, E. M., Harvey, S. A., Dooley, C. M., Bruijn, E. de, Eeden, F. V., Sealy, I., White, R. J., Herd, C., Nijman, I. J., et al. (2013). A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature, 1–6.

18. Corey, D. R., and Abrams, J. M. (2001). Morpholino antisense oligonucleotides: tools for investigating vertebrate development. Genome Biol *2*, REVIEWS1015.

19. Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., Amora, R., Hocking, T. D., Zhang, L., Rebar, E. J., et al. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol *26*, 702–708.

20. Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Meng, X., Paschon, D. E., Leung, E., Hinkley, S. J., et al. (2011). A TALE nuclease architecture for efficient genome editing. Nat Biotechnol *29*, 143–148.

21. Carradice, D., and Lieschke, G. J. (2008). Zebrafish in hematology: sushi or

science? Blood *111*, 3331–3342.

22. Lugo-Villarino, G., Balla, K. M., Stachura, D. L., Bañuelos, K., Werneck, M. B. F., and Traver, D. (2010). Identification of dendritic antigen-presenting cells in the zebrafish. Proc Natl Acad Sci USA *107*, 15850–15855.

23. Dobson, J. T., Seibert, J., Teh, E. M., Da'as, S., Fraser, R. B., Paw, B. H., Lin, T.-J., and Berman, J. N. (2008). Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. Blood *112*, 2969–2972.

24. Crowhurst, M. O., Layton, J. E., and Lieschke, G. J. (2002). Developmental biology of zebrafish myeloid cells. Int J Dev Biol *46*, 483–492.

25. Lieschke, G. J., and Trede, N. S. (2009). Fish immunology. Curr Biol *19*, R678–82.

26. Stein, C., Caccamo, M., Laird, G., and Leptin, M. (2007). Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol *8*, R251.

27. Meijer, A. H., Krens, S. F. G., Medina Rodriguez, I. A., He, S., Bitter, W., Snaar-Jagalska, B. E., and Spaink, H. P. (2004). Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol *40*, 773–783.

28. Kanther, M., and Rawls, J. F. (2010). Host-microbe interactions in the developing zebrafish. Curr Opin Immunol *22*, 10–19.

29. Herbomel, P., Thisse, B., and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development *126*, 3735–3745.

30. Peri, F., and Nüsslein-Volhard, C. (2008). Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. CELL *133*, 916–927.

31. Yang, C.-T., Cambier, C. J., Davis, J. M., Hall, C. J., Crosier, P. S., and Ramakrishnan, L. (2012). Neutrophils Exert Protection in the Early Tuberculous Granuloma by Oxidative Killing of Mycobacteria Phagocytosed from Infected Macrophages. Cell Host Microbe *12*, 301–312.

32. Ramakrishnan, L., and Falkow, S. (1994). Mycobacterium marinum persists

in cultured mammalian cells in a temperature-restricted fashion. Infect Immun *62*, 3222–3229.

33. Tobin, D. M., and Ramakrishnan, L. (2008). Comparative pathogenesis of Mycobacterium marinum and Mycobacterium tuberculosis. Cell Microbiol *10*, 1027–1039.

34. Shiloh, M. U., and Digiuseppe Champion, P. A. (2009). To catch a killer. What can mycobacterial models teach us about Mycobacterium tuberculosis pathogenesis? Curr Opin Microbiol.

35. Swaim, L. E., Connolly, L. E., Volkman, H. E., Humbert, O., Born, D. E., and Ramakrishnan, L. (2006). Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun *74*, 6108–6117.

36. van der Sar, A. M., Abdallah, A. M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C. M. J. E., and Bitter, W. (2004). Mycobacterium marinum strains can be divided into two distinct types based on genetic diversity and virulence. Infect Immun *72*, 6306–6312.

37. Adams, D. O. (1976). The granulomatous inflammatory response. A review. Am J Pathol *84*, 164–192.

38. Chan, K., Knaak, T., Satkamp, L., Humbert, O., Falkow, S., and Ramakrishnan, L. (2002). Complex pattern of Mycobacterium marinum gene expression during long-term granulomatous infection. Proc Natl Acad Sci USA *99*, 3920–3925.

39. Saunders, B. M., and Cooper, A. M. (2000). Restraining mycobacteria: role of granulomas in mycobacterial infections. Immunol Cell Biol *78*, 334–341.

40. Flynn, J. L., and Chan, J. (2001). Immunology of tuberculosis. Annu Rev Immunol *19*, 93–129.

41. Lesley, R., and Ramakrishnan, L. (2008). Insights into early mycobacterial pathogenesis from the zebrafish. Curr Opin Microbiol *11*, 277–283.

42. Wilkinson, R. J., Patel, P., Llewelyn, M., Hirsch, C. S., Pasvol, G., Snounou, G., Davidson, R. N., and Toossi, Z. (1999). Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1beta on tuberculosis. J Exp Med *189*, 1863–1874.

43. Wilkinson, R. J., Llewelyn, M., Toossi, Z., Patel, P., Pasvol, G., Lalvani, A., Wright, D., Latif, M., and Davidson, R. N. (2000). Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. The Lancet *355*, 618–621.

44. Bellamy, R. R., Ruwende, C. C., Corrah, T. T., McAdam, K. P. K., Whittle, H. C. H., and Hill, A. V. A. (1998). Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. N Engl J Med *338*, 640–644.

45. Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, A. T., and Kanki, J. P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. Dev Cell *8*, 97–108.

46. Clay, H., Davis, J. M., Beery, D., Huttenlocher, A., Lyons, S. E., and Ramakrishnan, L. (2007). Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. Cell Host Microbe *2*, 29–39.

47. Rhoades, E. R., Cooper, A. M., and Orme, I. M. (1995). Chemokine response in mice infected with Mycobacterium tuberculosis. Infect Immun *63*, 3871–3877.

48. Flesch, I. E., and Kaufmann, S. H. (1990). Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect Immun *58*, 2675–2677.

49. Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., Siegel, J. N., and Braun, M. M. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med *345*, 1098–1104.

50. Harris, J., and Keane, J. (2010). How tumour necrosis factor blockers interfere with tuberculosis immunity. Clin Exp Immunol *161*, 1–9.

51. Denis, M. (1991). Involvement of cytokines in determining resistance and acquired immunity in murine tuberculosis. J Leukoc Biol *50*, 495–501.

52. Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D., and Britton, W. J. (1999). Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not

compensated for by lymphotoxin. J Immunol *162*, 3504–3511.

53. Tobin, D. M., Roca, F. J., Oh, S. F., Mcfarland, R., Vickery, T. W., Ray, J. P., Ko, D. C., Zou, Y., Bang, N. D., Chau, T. T. H., et al. (2012). Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. CELL *148*, 434–446.

54. Roca, F. J., and Ramakrishnan, L. (2013). TNF Dually Mediates Resistance and Susceptibility to Mycobacteria via Mitochondrial Reactive Oxygen Species. CELL, 1–14.

55. Clay, H., Volkman, H. E., and Ramakrishnan, L. (2008). Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity *29*, 283–294.

56. Tobin, D. M., Vary, J. C., Ray, J. P., Walsh, G. S., Dunstan, S. J., Bang, N. D., Hagge, D. A., Khadge, S., King, M.-C., Hawn, T. R., et al. (2010). The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. CELL *140*, 717–730.

57. Gao, L.-Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J. N., and Brown, E. J. (2004). A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol *53*, 1677–1693.

58. Lewis, K. N., Liao, R., Guinn, K. M., Hickey, M. J., Smith, S., Behr, M. A., and Sherman, D. R. (2003). Deletion of RD1 from Mycobacterium tuberculosis mimics bacille Calmette-Guérin attenuation. J INFECT DIS *187*, 117–123.

59. Guinn, K. M., Hickey, M. J., Mathur, S. K., Zakel, K. L., Grotzke, J. E., Lewinsohn, D. M., Smith, S., and Sherman, D. R. (2004). Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. Mol Microbiol *51*, 359–370.

60. Izzo, A., Izzo, L., Kasimos, J., and Majka, S. (2004). A matrix metalloproteinase inhibitor promotes granuloma formation during the early phase of pulmonary infection. Tuberculosis *84*, 387–396.

61. Taylor, J. L., Hattle, J. M., Dreitz, S. A., Troudt, J. M., Izzo, L. S., Basaraba, R. J., Orme, I. M., Matrisian, L. M., and Izzo, A. A. (2006). Role for Matrix Metalloproteinase 9 in Granuloma Formation during Pulmonary

Mycobacterium tuberculosis Infection. Infect Immun *74*, 6135–6144.

62. Greenlee, K. J., Werb, Z., and Kheradmand, F. (2007). Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiol Rev *87*, 69–98.

63. Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol *17*, 463–516.

64. Parks, W. C., Wilson, C. L., and López-Boado, Y. S. (2004). Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol *4*, 617–629.

65. Vandooren, J., Van Den Steen, P. E., and Opdenakker, G. (2013). Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. Crit Rev Biochem Mol Biol *48*, 222–272.

66. Cauwe, B., and Opdenakker, G. (2010). Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. Crit Rev Biochem Mol Biol *45*, 351–423.

67. Pardo, A., and Selman, M. (2005). MMP-1: the elder of the family. Int J Biochem Cell Biol *37*, 283–288.

68. Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'armiento, J., and Okada, Y. (2009). MMP-13 Plays a Role in Keratinocyte Migration, Angiogenesis, and Contraction in Mouse Skin Wound Healing. Am J Pathol.

69. Dejonckheere, E., Vandenbroucke, R. E., and Libert, C. (2011). Matrix metalloproteinase8 has a central role in inflammatory disorders and cancer progression. Cytokine and Growth Factor Reviews *22*, 73–81.

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

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

72. Becker-Pauly, C., and Rose-John, S. (2013). TNF cleavage beyond TACE/ADAM17: matrix metalloproteinase 13 is a potential therapeutic

target in sepsis and colitis. EMBO Mol Med.

73. Visse, R., and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res *92*, 827–839.

74. Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P., and Docherty, A. J. (1992). The role of the C-terminal domain in collagenase and stromelysin specificity. J Biol Chem *267*, 9612–9618.

75. Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J. L., Fields, G. B., Visse, R., and Nagase, H. (2004). Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. EMBO J *23*, 3020–3030.

76. Yan, C., and Boyd, D. D. (2007). Regulation of matrix metalloproteinase gene expression. J Cell Physiol *211*, 19–26.

77. Karin, M., Liu, Z. G., and Zandi, E. (1997). AP-1 function and regulation. Curr Opin Cell Biol *9*, 240–246.

78. Chakraborti, S., Mandal, M., Das, S., Mandal, A., and Chakraborti, T. (2003). Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem *253*, 269–285.

79. Wyatt, R. A., Keow, J. Y., Harris, N. D., Haché, C. A., Li, D. H., and Crawford, B. D. (2009). The zebrafish embryo: a powerful model system for investigating matrix remodeling. Zebrafish *6*, 347–354.

80. Ra, H.-J., and Parks, W. C. (2007). Control of matrix metalloproteinase catalytic activity. Matrix Biol *26*, 587–596.

81. Nagase, H., Enghild, J. J., Suzuki, K., and Salvesen, G. (1990). Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. Biochemistry *29*, 5783–5789.

82. Ra, H.-J., Harju-Baker, S., Zhang, F., Linhardt, R. J., Wilson, C. L., and Parks, W. C. (2009). Control of promatrilysin (MMP7) activation and substrate-specific activity by sulfated glycosaminoglycans. J Biol Chem *284*, 27924–27932.

83. Yu, Q., and Stamenkovic, I. (1999). Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-

mediated tumor invasion. Genes Dev *13*, 35–48.

84. Stricker, T. P., Dumin, J. A., Dickeson, S. K., Chung, L., Nagase, H., Parks, W. C., and Santoro, S. A. (2001). Structural analysis of the alpha(2) integrin I domain/procollagenase-1 (matrix metalloproteinase-1) interaction. J Biol Chem *276*, 29375–29381.

85. Dumin, J. A., Dickeson, S. K., Stricker, T. P., Bhattacharyya-Pakrasi, M., Roby, J. D., Santoro, S. A., and Parks, W. C. (2001). Pro-collagenase-1 (Matrix Metalloproteinase-1) Binds the 2 1 Integrin upon Release from Keratinocytes Migrating on Type I Collagen. Journal of Biological Chemistry *276*, 29368–29374.

86. Yu, W. H., and Woessner, J. F. (2000). Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7). J Biol Chem *275*, 4183–4191.

87. Barmina, O. Y., Walling, H. W., Fiacco, G. J., Freije, J. M., López-Otín, C., Jeffrey, J. J., and Partridge, N. C. (1999). Collagenase-3 binds to a specific receptor and requires the low density lipoprotein receptor-related protein for internalization. J Biol Chem *274*, 30087–30093.

88. Etique, N., Verzeaux, L., Dedieu, S., and Emonard, H. (2013). LRP-1: A Checkpoint for the Extracellular Matrix Proteolysis. BioMed Research International *2013*, 1–7.

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

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

91. Gomis-Rüth, F. X. (2003). Structural aspects of the metzincin clan of metalloendopeptidases. Mol Biotechnol *24*, 157–202.

92. Overall, C. M. (2001). Matrix metalloproteinase substrate binding domains, modules and exosites. Overview and experimental strategies. Methods Mol Biol *151*, 79–120.

93. Lovejoy, B., Welch, A. R., Carr, S., Luong, C., Broka, C., Hendricks, R. T., Campbell, J. A., Walker, K. A., Martin, R., van Wart, H., et al. (1999). Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. Nat Struct Biol *6*, 217–221.

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

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

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

97. Butler, G. S., and Overall, C. M. (2013). Matrix metalloproteinase processing of signaling molecules to regulate inflammation. Periodontol 2000 *63*, 123–148.

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

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

100. Coyle, R. C., Latimer, A., and Jessen, J. R. (2008). Membrane-type 1 matrix metalloproteinase regulates cell migration during zebrafish gastrulation: evidence for an interaction with non-canonical Wnt signaling. Exp Cell Res *314*, 2150–2162.

101. Zhang, J., Bai, S., Zhang, X., Nagase, H., and Sarras, M. P. (2003). The expression of novel membrane-type matrix metalloproteinase isoforms is required for normal development of zebrafish embryos. Matrix Biol *22*, 279–293.

102. Yin, C., Kikuchi, K., Hochgreb, T., Poss, K. D., and Stainier, D. Y. R.

(2010). Hand2 Regulates Extracellular Matrix Remodeling Essential for Gut-Looping Morphogenesis in Zebrafish. Dev Cell *18*, 973–984.

103. Bai, S., Thummel, R., Godwin, A. R., Nagase, H., Itoh, Y., Li, L., Evans, R., McDermott, J., Seiki, M., and Sarras, M. P. (2005). Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. Matrix Biol *24*, 247–260.

104. Zhang, Y., Bai, X.-T., Zhu, K.-Y., Jin, Y., Deng, M., Le, H.-Y., Fu, Y.-F., Chen, Y., Zhu, J., Look, A. T., et al. (2008). In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury. The Journal of Immunology *181*, 2155–2164.

105. Hall, C. J., Boyle, R. H., Sun, X., Wicker, S. M., Misa, J. P., Krissansen, G. W., Print, C. G., Crosier, K. E., and Crosier, P. S. (2014). Hall_Nat Comms_2014. Nature Communications *5*, 1–17.

106. Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., and Parks, W. C. (1997). The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J Cell Biol *137*, 1445–1457.

107. Hotary, K., Allen, E., Punturieri, A., Yana, I., and Weiss, S. J. (2000). Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. J Cell Biol *149*, 1309–1323.

108. Horiuchi, K. (2013). A Brief History of Tumor Necrosis Factor α – converting Enzyme: An Overview of Ectodomain Shedding. Keio J. Med. *62*, 29–36.

109. Van Lint, P., and Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J Leukoc Biol *82*, 1375–1381.

110. Greenlee, K. J., Corry, D. B., Engler, D. A., Matsunami, R. K., Tessier, P., Cook, R. G., Werb, Z., and Kheradmand, F. (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.

111. Mantovani, A., Bonecchi, R., and Locati, M. (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. Nat Rev Immunol *6*, 907–918.

112. Sarris, M., Masson, J.-B., Maurin, D., Van Der Aa, L. M., Boudinot, P., Lortat-Jacob, H., and Herbomel, P. (2012). Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. Curr Biol *22*, 2375–2382.

113. Rodríguez, D., Morrison, C. J., and Overall, C. M. (2010). Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta *1803*, 39–54.

114. Cox, J. H., Dean, R. A., Roberts, C. R., and Overall, C. M. (2008). Matrix Metalloproteinase Processing of CXCL11/I-TAC Results in Loss of Chemoattractant Activity and Altered Glycosaminoglycan Binding. Journal of Biological Chemistry *283*, 19389–19399.

115. Tanino, Y., Coombe, D. R., Gill, S. E., Kett, W. C., Kajikawa, O., Proudfoot, A. E. I., Wells, T. N. C., Parks, W. C., Wight, T. N., Martin, T. R., et al. (2010). Kinetics of Chemokine-Glycosaminoglycan Interactions Control Neutrophil Migration into the Airspaces of the Lungs. J Immunol.

116. Hayashida, K., Parks, W. C., and Park, P. W. (2009). Syndecan-1 shedding facilitates the resolution of neutrophilic inflammation by removing sequestered CXC chemokines. Blood *114*, 3033–3043.

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

118. Swee, M., Wilson, C. L., Wang, Y., McGuire, J. K., and Parks, W. C. (2008). Matrix metalloproteinase-7 (matrilysin) controls neutrophil egress by generating chemokine gradients. J Leukoc Biol *83*, 1404–1412.

119. Gharib, S. A., Johnston, L. K., Huizar, I., Birkland, T. P., Hanson, J., Wang, Y., Parks, W. C., and Manicone, A. M. (2014). MMP28 promotes macrophage polarization toward M2 cells and augments pulmonary fibrosis. J Leukoc Biol *95*, 9–18.

120. Balbín, M., Fueyo, A., Tester, A. M., Pendás, A. M., Pitiot, A. S., Astudillo, A., Overall, C. M., Shapiro, S. D., and López-Otín, C. (2003). Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet *35*, 252–257.

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

122. Cox, J. H., Starr, A. E., Kappelhoff, R., Yan, R., Roberts, C. R., and Overall, C. M. (2010). Matrix metalloproteinase 8 deficiency in mice exacerbates inflammatory arthritis through delayed neutrophil apoptosis and reduced caspase 11 expression. Arthritis Rheum *62*, 3645–3655.

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

124. Houghton, A. M., Hartzell, W. O., Robbins, C. S., Gomis-Rüth, F. X., and Shapiro, S. D. (2009). Macrophage elastase kills bacteria within murine macrophages. Nature *460*, 637–641.

125. Marchant, D. J., Bellac, C. L., Moraes, T. J., Wadsworth, S. J., Dufour, A., Butler, G. S., Bilawchuk, L. M., Hendry, R. G., Robertson, A. G., Cheung, C. T., et al. (2014). A new transcriptional role for matrix metalloproteinase-12 in antiviral immunity. Nat Med *20*, 499–508.

126. Eguchi, T., Kubota, S., Kawata, K., Mukudai, Y., Uehara, J., Ohgawara, T., Ibaragi, S., Sasaki, A., Kuboki, T., and Takigawa, M. (2008). Novel Transcription Factor-Like Function of Human Matrix Metalloproteinase 3 Regulating the CTGF/CCN2 Gene. Molecular and Cellular Biology *28*, 2391–2413.

127. Shimizu-Hirota, R., Xiong, W., Baxter, B. T., Kunkel, S. L., Maillard, I., Chen, X. W., Sabeh, F., Liu, R., Li, X.-Y., and Weiss, S. J. (2012). MT1-MMP regulates the PI3K {middle dot}Mi-2/NuRD-dependent control of macrophage immune function. Genes Dev *26*, 395–413.

128. Sugimoto, M., Dannenberg, A. M., Wahl, L. M., Ettinger, W. H., Hastie, A. T., Daniels, D. C., Thomas, C. R., and Demoulin-Brahy, L. (1978).

Extracellular hydrolytic enzymes of rabbit dermal tuberculous lesions and tuberculin reactions collected in skin chambers. Am J Pathol *90*, 583–607.

129. Walker, N. F., Clark, S. O., Oni, T., Andreu, N., Tezera, L., Singh, S., Saraiva, L., Pedersen, B., Kelly, D. L., Tree, J. A., et al. (2012). Doxycycline and HIV Infection Suppress Tuberculosis-induced Matrix Metalloproteinases. Am J Respir Crit Care Med *185*, 989–997.

130. Singh, S., Kubler, A., Singh, U. K., Singh, A., Gardiner, H., Prasad, R., Elkington, P. T., and Friedland, J. S. (2014). Antimycobacterial Drugs Modulate Immunopathogenic Matrix Metalloproteinases in a Cellular Model of Pulmonary Tuberculosis. Antimicrob Agents Chemother *58*, 4657–4665.

131. Elkington, P., Shiomi, T., Breen, R., Nuttall, R. K., Ugarte-Gil, C. A., Walker, N. F., Saraiva, L., Pedersen, B., Mauri, F., Lipman, M., et al. (2011). MMP-1 drives immunopathology in human tuberculosis and transgenic mice. J Clin Invest *121*, 1827–1833.

132. Ong, C. W. M., Elkington, P. T., and Friedland, J. S. (2014). Tuberculosis, Pulmonary Cavitation, and Matrix Metalloproteinases. Am J Respir Crit Care Med *190*, 9–18.

133. Elkington, P. T., Green, J. A., Emerson, J. E., Lopez-Pascua, L. D., Boyle, J. J., O'Kane, C. M., and Friedland, J. S. (2007). Synergistic up-regulation of epithelial cell matrix metalloproteinase-9 secretion in tuberculosis. Am J Respir Cell Mol Biol *37*, 431–437.

134. Ganachari, M., Ruiz-Morales, J. A., Gomez de la Torre Pretell, J. C., Dinh, J., Granados, J., and Flores-Villanueva, P. O. (2010). Joint effect of MCP-1 genotype GG and MMP-1 genotype 2G/2G increases the likelihood of developing pulmonary tuberculosis in BCG-vaccinated individuals. PLoS ONE *5*, e8881.

135. Ganachari, M., Guio, H., Zhao, N., and Flores-Villanueva, P. O. (2012). Host gene-encoded severe lung TB: from genes to the potential pathways. Genes Immun.

136. Kuo, H.-P., Wang, Y.-M., Wang, C.-H., He, C.-C., Lin, S.-M., Lin, H.-C., Liu, C.-Y., Huang, K.-H., Hsieh, L.-L., and Huang, C.-D. (2008). Matrix metalloproteinase-1 polymorphism in Taiwanese patients with endobronchial tuberculosis. Tuberculosis *88*, 262–267.

137. Wang, C.-H., Lin, H.-C., Lin, S.-M., Huang, C.-D., Liu, C.-Y., Huang, K.-H., Hsieh, L.-L., Chung, K. F., and Kuo, H.-P. (2010). MMP-1(-1607G) polymorphism as a risk factor for fibrosis after pulmonary tuberculosis in Taiwan. Int J Tuberc Lung Dis *14*, 627–634.

138. Cosma, C. L., Klein, K., Kim, R., Beery, D., and Ramakrishnan, L. (2006). Mycobacterium marinum Erp is a virulence determinant required for cell wall integrity and intracellular survival. Infect Immun *74*, 3125–3133.

139. Cosma, C. L., Swaim, L. E., Volkman, H., Ramakrishnan, L., and Davis, J. M. (2006). Zebrafish and frog models of Mycobacterium marinum infection. Current protocols in microbiology *Chapter 10*, Unit 10B.2.

140. Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., Edelstein, P. H., Cosma, C. L., and Ramakrishnan, L. (2011). Drug Tolerance in Replicating Mycobacteria Mediated by a Macrophage-Induced Efflux Mechanism. CELL, 1–25.

141. Takaki, K., Davis, J. M., Winglee, K., and Ramakrishnan, L. (2013). Evaluation of the pathogenesis and treatment ofMycobacterium marinum infection in zebrafish. Nat Protoc *8*, 1114–1124.

142. García-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegrín, P., Meseguer, J., and Mulero, V. (2004). The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci *61*, 1331–1340.

143. Roca, F. J., Mulero, I., López-Muñoz, A., Sepulcre, M. P., Renshaw, S. A., Meseguer, J., and Mulero, V. (2008). Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol *181*, 5071–5081.

144. Stockhammer, O. W., Zakrzewska, A., Hegedus, Z., Spaink, H. P., and Meijer, A. H. (2009). Transcriptome Profiling and Functional Analyses of the Zebrafish Embryonic Innate Immune Response to Salmonella Infection. The Journal of Immunology *182*, 5641–5653.

145. Le Guyader, D., Redd, M. J., Colucci-Guyon, E., Murayama, E., Kissa, K., Briolat, V., Mordelet, E., Zapata, A., Shinomiya, H., and Herbomel, P. (2008). Origins and unconventional behavior of neutrophils in developing

zebrafish. Blood *111*, 132–141.

146. Elkington, P. T. G., Nuttall, R. K., Boyle, J. J., O'Kane, C. M., Horncastle, D. E., Edwards, D. R., and Friedland, J. S. (2005). Mycobacterium tuberculosis, but not vaccine BCG, specifically upregulates matrix metalloproteinase-1. Am J Respir Crit Care Med *172*, 1596–1604.

147. Hong, J.-S., Greenlee, K. J., Pitchumani, R., Lee, S.-H., Song, L.-Z., Shan, M., Chang, S. H., Park, P. W., Dong, C., Werb, Z., et al. (2011). Dual protective mechanisms of matrix metalloproteinases 2 and 9 in immune defense against Streptococcus pneumoniae. The Journal of Immunology *186*, 6427–6436.

148. Mohan, M. J., Seaton, T., Mitchell, J., Howe, A., Blackburn, K., Burkhart, W., Moyer, M., Patel, I., Waitt, G. M., Becherer, J. D., et al. (2002). The tumor necrosis factor-alpha converting enzyme (TACE): a unique metalloproteinase with highly defined substrate selectivity. Biochemistry *41*, 9462–9469.

149. Chesneau, V., Becherer, J. D., Zheng, Y., Erdjument-Bromage, H., Tempst, P., and Blobel, C. P. (2003). Catalytic Properties of ADAM19. Journal of Biological Chemistry *278*, 22331–22340.

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

151. Arduise, C., Abache, T., Li, L., Billard, M., Chabanon, A., Ludwig, A., Mauduit, P., Boucheix, C., Rubinstein, E., and Le Naour, F. (2008). Tetraspanins Regulate ADAM10-Mediated Cleavage of TNF- and Epidermal Growth Factor. The Journal of Immunology *181*, 7002–7013.

152. Vandenbroucke, R. E., Dejonckheere, E., Van Hauwermeiren, F., Lodens, S., De Rycke, R., Van Wonterghem, E., Staes, A., Gevaert, K., López-Otin, C., and Libert, C. (2013). Matrix metalloproteinase 13 modulates intestinal epithelial barrier integrity in inflammatory diseases by activating TNF. EMBO Mol Med, n/a–n/a.

153. Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., and Gordon, J. L. (1994). Processing of tumour necrosis factor-alpha precursor

by metalloproteinases. Nature *370*, 555–557.

154. Kim, M.-J., Wainwright, H. C., Locketz, M., Bekker, L.-G., Walther, G. B., Dittrich, C., Visser, A., Wang, W., Hsu, F.-F., Wiehart, U., et al. (2010). Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. EMBO Mol Med *2*, 258–274.

155. Meijer, A. H., Verbeek, F. J., Salas-Vidal, E., Corredor-Adámez, M., Bussman, J., van der Sar, A. M., Otto, G. W., Geisler, R., and Spaink, H. P. (2005). Transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis due to Mycobacterium marinum infection. Mol Immunol *42*, 1185–1203.

156. van der Sar, A. M., Spaink, H. P., Zakrzewska, A., Bitter, W., and Meijer, A. H. (2009). Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. Mol Immunol *46*, 2317–2332.

157. Mishra, B. B., Moura-Alves, P., Sonawane, A., Hacohen, N., Griffiths, G., Moita, L. F., and Anes, E. (2010). Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol *12*, 1046–1063.

158. Kübler, A., Luna, B., Larsson, C., Ammerman, N. C., Andrade, B. B., Orandle, M., Bock, K. W., Xu, Z., Bagci, U., Molura, D. J., et al. (2014). Mycobacterium tuberculosisdysregulates MMP/TIMP balance to drive rapid cavitation and unrestrained bacterial proliferation. J Pathol, n/a–n/a.

159. Ramos-Martínez, A. G., Enciso-Moreno, J. A., Espinosa-Ayala, I., Mata-Espinoza, D., Rivas-Santiago, B., Trujillo-Paez, V., Monárrez-Espino, J., Hernandez-Pando, R., and Serrano, C. J. (2014). Expression kinetics of metalloproteinases and their tissue inhibitors in experimental murine pulmonary tuberculosis. Exp Lung Res.

160. Elkington, P. T. G., Emerson, J. E., Lopez-Pascua, L. D. C., O'Kane, C. M., Horncastle, D. E., Boyle, J. J., and Friedland, J. S. (2005). Mycobacterium tuberculosis up-regulates matrix metalloproteinase-1 secretion from human airway epithelial cells via a p38 MAPK switch. J Immunol *175*, 5333–5340.

161. Sheen, P., O'Kane, C. M., Chaudhary, K., Tovar, M., Santillan, C., Sosa, J., Caviedes, L., Gilman, R. H., Stamp, G., and Friedland, J. S. (2009). High MMP-9 activity characterises pleural tuberculosis correlating with

granuloma formation. European Respiratory Journal *33*, 134–141.

162. Bottai, D., Di Luca, M., Majlessi, L., Frigui, W., Simeone, R., Sayes, F., Bitter, W., Brennan, M. J., Leclerc, C., Batoni, G., et al. (2012). Disruption of the ESX-5 system of Mycobacterium tuberculosis causes loss of PPE protein secretion, reduction of cell wall integrity and strong attenuation. Mol Microbiol *83*, 1195–1209.

163. Champion, M. M., Williams, E. A., Pinapati, R. S., and Champion, P. A. D. (2014). Correlation of Phenotypic Profiles Using Targeted Proteomics Identifies Mycobacterial Esx-1 Substrates. J. Proteome Res. *13*, 5151–5164.

164. Brodin, P., Majlessi, L., Marsollier, L., de Jonge, M. I., Bottai, D., Demangel, C., Hinds, J., Neyrolles, O., Butcher, P. D., Leclerc, C., et al. (2006). Dissection of ESAT-6 system 1 of Mycobacterium tuberculosis and impact on immunogenicity and virulence. Infect Immun *74*, 88–98.

165. Brodin, P., de Jonge, M. I., Majlessi, L., Leclerc, C., Nilges, M., Cole, S. T., and Brosch, R. (2005). Functional Analysis of Early Secreted Antigenic Target-6, the Dominant T-cell Antigen of Mycobacterium tuberculosis, Reveals Key Residues Involved in Secretion, Complex Formation, Virulence, and Immunogenicity. Journal of Biological Chemistry *280*, 33953–33959.

166. Chen, J. M., Zhang, M., Rybniker, J., Basterra, L., Dhar, N., Tischler, A. D., Pojer, F., and Cole, S. T. (2013). Phenotypic profiling of Mycobacterium tuberculosis EspA point mutants reveals that blockage of ESAT-6 and CFP-10 secretion in vitro does not always correlate with attenuation of virulence. J Bacteriol *195*, 5421–5430.

167. Koo, I. C., Wang, C., Raghavan, S., Morisaki, J. H., Cox, J. S., and Brown, E. J. (2008). ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. Cell Microbiol *10*, 1866–1878.

168. Smith, J., Manoranjan, J., Pan, M., Bohsali, A., Xu, J., Liu, J., McDonald, K. L., Szyk, A., LaRonde-LeBlanc, N., and Gao, L.-Y. (2008). Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in Mycobacterium marinum escape from the vacuole. Infect Immun *76*, 5478–5487.

169. Rybniker, J., Chen, J. M., Sala, C., Hartkoorn, R. C., Vocat, A., Benjak, A.,

Boy-Röttger, S., Zhang, M., Székely, R., Greff, Z., et al. (2014). Anticytolytic Screen Identifies Inhibitors of Mycobacterial Virulence Protein Secretion. Cell Host Microbe *16*, 538–548.

170. de Jonge, M. I., Pehau-Arnaudet, G., Fretz, M. M., Romain, F., Bottai, D., Brodin, P., Honoré, N., Marchal, G., Jiskoot, W., England, P., et al. (2007). ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. J Bacteriol *189*, 6028–6034.

171. De Leon, J., Jiang, G., Ma, Y., Rubin, E., Fortune, S., and Sun, J. (2012). Mycobacterium tuberculosis ESAT-6 Exhibits a Unique Membrane-interacting Activity That Is Not Found in Its Ortholog from Non-pathogenic Mycobacterium smegmatis. Journal of Biological Chemistry *287*, 44184–44191.

172. Purwar, R., Kraus, M., Werfel, T., and Wittmann, M. (2007). Modulation of Keratinocyte-Derived MMP-9 by IL-13: A Possible Role for the Pathogenesis of Epidermal Inflammation. J Invest Dermatol *128*, 59–66.

173. Toriseva, M., Laato, M., Carpén, O., Ruohonen, S. T., Savontaus, E., Inada, M., Krane, S. M., and Kähäri, V.-M. (2012). MMP-13 Regulates Growth of Wound Granulation Tissue and Modulates Gene Expression Signatures Involved in Inflammation, Proteolysis, and Cell Viability. PLoS ONE *7*, e42596.

174. Pilcher, B. K., Sudbeck, B. D., Dumin, J. A., Welgus, H. G., and Parks, W. C. (1998). Collagenase-1 and collagen in epidermal repair. Arch Dermatol Res *290 Suppl*, S37–46.

175. Price, N. M., Farrar, J., Tran, T. T., Nguyen, T. H., Tran, T. H., and Friedland, J. S. (2001). Identification of a matrix-degrading phenotype in human tuberculosis in vitro and in vivo. J Immunol *166*, 4223–4230.

176. Park, K. J., Hwang, S. C., Sheen, S. S., Oh, Y. J., Han, J. H., and Lee, K. B. (2005). Expression of matrix metalloproteinase-9 in pleural effusions of tuberculosis and lung cancer. Respiration; international review of thoracic diseases *72*, 166–175.

177. Mehra, S., Pahar, B., Dutta, N. K., Conerly, C. N., Philippi-Falkenstein, K., Alvarez, X., and Kaushal, D. (2010). Transcriptional reprogramming in nonhuman primate (rhesus macaque) tuberculosis granulomas. PLoS ONE

5, e12266.

178. Lemaître, V., O'Byrne, T. K., Borczuk, A. C., Okada, Y., Tall, A. R., and D'armiento, J. (2001). ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. J Clin Invest *107*, 1227–1234.

179. Lemaître, V., O'Byrne, T. K., Dalal, S. S., Tall, A. R., and D'armiento, J. M. (1999). Macrophage-specific expression of human collagenase (MMP-1) in transgenic mice. Ann N Y Acad Sci *878*, 736–739.

180. Alexopoulou, L., Kranidioti, K., Xanthoulea, S., Denis, M., Kotanidou, A., Douni, E., Blackshear, P. J., Kontoyiannis, D. L., and Kollias, G. (2006). Transmembrane TNF protects mutant mice against intracellular bacterial infections, chronic inflammation and autoimmunity. Eur J Immunol *36*, 2768–2780.

181. Ruuls, S. R., Hoek, R. M., Ngo, V. N., McNeil, T., Lucian, L. A., Janatpour, M. J., Körner, H., Scheerens, H., Hessel, E. M., Cyster, J. G., et al. (2001). Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. Immunity *15*, 533–543.

182. Olleros, M. L., Vesin, D., Bisig, R., Santiago-Raber, M.-L., Schuepbach-Mallepell, S., Kollias, G., Gaide, O., and Garcia, I. (2012). Membrane-Bound TNF Induces Protective Immune Responses to M. bovis BCG Infection: Regulation of memTNF and TNF Receptors Comparing Two memTNF Molecules. PLoS ONE *7*, e31469.

183. Saunders, B. M., Tran, S., Ruuls, S., Sedgwick, J. D., Briscoe, H., and Britton, W. J. (2005). Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection. J Immunol *174*, 4852–4859.

184. Allie, N., Alexopoulou, L., Quesniaux, V. J. F., Fick, L., Kranidioti, K., Kollias, G., Ryffel, B., and Jacobs, M. (2008). Protective role of membrane tumour necrosis factor in the host's resistance to mycobacterial infection. Immunology *125*, 522–534.

185. Horiuchi, K., Kimura, T., Miyamoto, T., Takaishi, H., Okada, Y., Toyama, Y., and Blobel, C. P. (2007). Cutting edge: TNF-alpha-converting enzyme

(TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. J Immunol *179*, 2686–2689.

186. Lee, E. J., Han, J. E., Woo, M. S., Shin, J. A., Park, E. M., Kang, J. L., Moon, P. G., Baek, M. C., Son, W. S., Ko, Y. T., et al. (2014). Matrix Metalloproteinase-8 Plays a Pivotal Role in Neuroinflammation by Modulating TNF- Activation. The Journal of Immunology *193*, 2384–2393.

187. Le Gall, S. M., Maretzky, T., Issuree, P. D. A., Niu, X.-D., Reiss, K., Saftig, P., Khokha, R., Lundell, D., and Blobel, C. P. (2010). ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. J Cell Sci *123*, 3913–3922.

188. Moss, M. L., Sklair-Tavron, L., and Nudelman, R. (2008). Drug Insight: tumor necrosis factor-converting enzyme as a pharmaceutical target for rheumatoid arthritis. Nat Clin Pract Rheumatol *4*, 300–309.

189. Liechti, F. D., Grandgirard, D., Leppert, D., and Leib, S. L. (2014). Matrix metalloproteinase inhibition lowers mortality and brain injury in experimental pneumococcal meningitis. Infect Immun.

190. Berry, M. P. R., Graham, C. M., Mcnab, F. W., Xu, Z., Bloch, S. A. A., Oni, T., Wilkinson, K. A., Banchereau, R., Skinner, J., Wilkinson, R. J., et al. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature *466*, 973–977.

191. Mishra, B. B., Rathinam, V. A. K., Martens, G. W., Martinot, A. J., Kornfeld, H., Fitzgerald, K. A., and Sassetti, C. M. (2012). Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome–dependent processing of IL-1β. Nat Immunol *14*, 52–60.

192. Schoenen, H., Bodendorfer, B., Hitchens, K., Manzanero, S., Werninghaus, K., Nimmerjahn, F., Agger, E. M., Stenger, S., Andersen, P., Ruland, J., et al. (2010). Cutting Edge: Mincle Is Essential for Recognition and Adjuvanticity of the Mycobacterial Cord Factor and its Synthetic Analog Trehalose-Dibehenate. J Immunol.

193. Blomgran, R., Desvignes, L., Briken, V., and Ernst, J. D. (2012). Mycobacterium tuberculosis Inhibits Neutrophil Apoptosis, Leading to Delayed Activation of Naive CD4 T cells. Cell Host Microbe *11*, 81–90.

194. Dorhoi, A., and Kaufmann, S. H. E. (2014). Seminars in Immunology. Semin Immunol *26*, 533–542.

195. Tobin, D. M., Roca, F. J., Ray, J. P., Ko, D. C., and Ramakrishnan, L. (2013). An Enzyme That Inactivates the Inflammatory Mediator Leukotriene B4 Restricts Mycobacterial Infection. PLoS ONE *8*, e67828.

196. Tobin, D. M., and Ramakrishnan, L. (2013). TB: the Yin and Yang of lipid mediators. Current Opinion in Pharmacology, 1–5.

197. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002). Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science *295*, 2387–2392.

198. Ramnath, N., and Creaven, P. J. (2004). Matrix metalloproteinase inhibitors. Curr Oncol Rep *6*, 96–102.

Vita:

Sachiko Seilie was raised in Bellevue, Washington, and moved to South Bend, Indiana, to attend the University of Notre Dame du Lac. She graduated in 2008 with a Bachelor of Science in Biology and Anthropology, after developing in interest in infectious diseases of global importance. In 2008, she moved back to Seattle, Washington, to join the Molecular and Cellular Biology program at the University of Washington, where received her Doctor of Philosophy in 2014.