MATRIX METALLOPROTEINASE 7 SUPPRESSES M1 MACROPHAGE POLARIZATION TO PROTECT AGAINST HELICOBACTER PYLORI-INDUCED GASTRIC INFLAMMATION

By

Michelle Stokes Krakowiak

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Richard Peek, MD
Harold Moses, MD

To Joseph Covert, Tina Davis and Ryan Fuller

“Hey, Stokes let’s go see Mr. Fuller.”

Who could have guessed that these seven simple words spoken eight years ago would have ultimately led to this.

I guess you three could.

Thanks for noticing and nurturing my potential.

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I also have deep gratitude for Elaine Ritter, my co-crazy cat lady partner in crime, for her endless friendship, support and proof-reading, and I will never be able to thank Chad and Carly Fair enough for opening their home to me so I could finish this work.

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LIST OF ABBREVIATIONS

Abl..... Abelson tyrosine kinase
Akt..... Akt murine thymoma viral oncogene homolog
BCA ..... bicinchoninic acid
BMDM..... bone marrow derived macrophages
Cag-PAI ..... Cytotoxin-Associated Gene Pathogenicity Island
CFU..... colony forming units
EBV.....Epstein Barr Virus
ECM..... extracellular matrix
EMT ..... epithelial to mesenchymal transition
EPIYA.....Glutamine Proline Isoleucine Tyrosine Alanine
ERK1/2 ..... Extracellular Regulated Kinases 1 and 2
FAK..... Focal Adhesion Kinase
FBS ..... fetal bovine serum
H. pylori ..... *Helicobacter pylori*
IFN-γ .....Interferon gamma
IL-1α ..... Interleukin 1 alpha
IL-1β ..... Interleukin 1 beta
IL-7 .....Interleukin 7
IL-12 .....Interleukin 12
IL-17 .....Interleukin 17
INS-GAS..... Insulin-Gastrin
IP-10.....Interferon-gamma-inducible Protein 10
M1 .....type 1 macrophages
M2 .....type 2 macrophages
Mreg.....regulatory macrophages
MACS ..... magnet assisted cell sorting
MMP .....matrix metalloproteinase
MMP7 .....Matrix Metalloproteinase 7
MMP10 .....Matrix Metalloproteinase 10
NCS..... neonatal calf serum
PBS ..... phosphate buffered saline
PGE_2.....Prostaglandin E_2
PMSS1 .....Pre-mouse Sydney Strain 1
PTEN.....Phosphatase and Tensin Homolog
PUMP1.....Punctuated Metalloproteinase 1
RANTES ..... Regulated on Activation Normal T Cell Expressed and Secreted
RPE .....R-Phycoerythrin
RPM ..... rotations per minute
RPMI.....Royal Park Memorial Institute
RT-qPCR.....reverse transcriptase quantitative polymerase chain reaction
Src ..... Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)

Th1 ..... T helper type 1
Th2 ..... T helper type 2
TNF-α..... Tumor Necrosis Factor alpha
TSA ..... tryptic soy agar
VacA ..... Vacuolating Cytotoxin
WHO ..... World Health Organization

CHAPTER I

INTRODUCTION

Gastric Cancer

Although gastric cancer is the second leading cause of cancer-death world-wide[1], incidence rates vary widely between geographic regions. The highest incidence rates are seen in Eastern Asia, Eastern Europe, and Central and South America[2]. Most often, incidence rates of gastric cancer are higher in developing countries[2] with the exception of Japan having the highest incidence rate in the world[3]. Approximately 10% of gastric cancers are non-Hodgkin's lymphomas and leimyosarcomas while 90% are adenocarcinomas[4].

There are two types of gastric adenocarcinoma that differ in their epidemiological and histopathological characteristics[4,5]. Diffuse-type gastric adenocarcinomas are typically experienced by younger individuals and affect men and women with equal incidence. Diffuse-type gastric adenocarcinomas are characterized by neoplastic cells infiltrating the stomach and a general thickening of the stomach wall without a discrete tumor mass. In contrast, intestinal-type gastric adenocarcinoma is more commonly seen in older individuals and presents more often in men than in women. Moreover, intestinal-type gastric adenocarcinomas develop following a well-defined series of histological steps, where gland-like structures ulcerate and form tumors. In the series of histological steps, normal tissue is initiated (most often by superficial gastritis) and progresses through stages of atrophic gastritis, metaplasia, dysplasia and finally adenocarcinoma.

In contrast to colon adenocarcinoma, which also proceeds through a series of discrete histological steps[6], there are no mutational events consistently associated with the initiation of gastric adenocarcinoma[5]. Rather, a bacterial pathogen, *Helicobacter pylori* (*H. pylori*), is widely considered to be the initiating agent for a significant percentage of gastric cancers[5]. However, the mechanisms by which *H. pylori* contributes to gastric carcinogenesis are not completely understood.

Helicobacter pylori

H. pylori is a Gram-negative curved-bacillus that selectively colonizes the stomach. *H. pylori* is able to survive the harsh acidic gastric environment by metabolizing urea to ammonia which creates a higher pH microenvironment immediately surrounding the bacterium[7]. Though the presence of *H. pylori* had been noted in pathological specimens as early as the 19th century, it was not classified until 1984 when Robin Warren and Barry Marshall successfully cultured the organism from gastric biopsies[8]. The World Health Organization (WHO) classified *H. pylori* as a class I carcinogen in 1994[5]. Subsequent studies have continued to demonstrate that *H. pylori* infection significantly augments gastric cancer risk[7], and *H. pylori*-induced gastritis is currently considered to be the highest risk factor for gastric cancer[5]. Although *H. pylori* colonizes 50% of the world's population, and most infected individuals develop chronic gastritis, only 1% of infected individuals develop gastric cancer[5]. It is widely believed that a patient's disease outcome is ultimately the result of interplay between environmental factors, bacterial virulence factors, and host factors.

One environmental factor that alters risk for *H. pylori*-induced gastric cancer is co-infection with other pathogens. For example, co-infection with helminth parasites can lower

risk for gastric cancer[7]. A potential explanation for this phenomenon is that co-infection with a helminth parasite could skew the immune response to a Th2 response rather than a pro-inflammatory Th1 response[9,10]. In contrast, co-infection with Epstein Barr Virus (EBV) can raise the risk for gastric cancer[4,11]. The mechanisms by which EBV contributes to gastric carcinogenesis are not completely understood, though one study has demonstrated that EBV can induce promoter methylation of a tumor suppressor gene, *Pten*[12]. This would be especially disadvantageous to the host during co-infection with *H. pylori* which can activate Akt[13].

Diet is another environmental factor that can affect the risk for gastric cancer, and one strong dietary risk factor is salt[7]. Epidemiological studies[14–16] as well as *in vivo* studies in gerbils[17–20] and mice[21] have demonstrated that diets high in salt increase the risk of gastric cancer in the context of *H. pylori* infection. Possible explanations for the increased risk of gastric cancer include damaging the mucosa directly to allow carcinogens into the tissue[7], upregulation of inflammatory cytokines[7] and increasing expression of *H. pylori* virulence factors[22,23].

There are several virulence factors, including those encoded by the cytotoxin-associated gene pathogenicity island (cag-PAI) which have been extensively shown to affect *H. pylori*-induced gastric cancer risk. The cag-PAI is a 27 gene locus, and persons infected with a cag-PAI positive strain are at higher risk for peptic ulcer disease, atrophic gastritis and distal gastric adenocarcinoma compared to persons colonized with cag-PAI negative strains[24–31]. The cag-PAI encodes genes whose products make a type IV secretion system which translocates the gene product of CagA as well as peptidoglycan into host cells[5]. Upon entering host cells, CagA can be phosphorylated on EPIYA motifs in its carboxyl terminus by Src and Abl kinases[5]. However, CagA can affect host cells in both phosphorylation-dependent and phosphorylation-independent manners. Phosphorylated CagA alters cell signaling by activating extracellular regulated kinases

1 and 2 (ERK1/2)[32] and by deactivating focal adhesion kinase (FAK) and Src[33,34]. Non-phosphorylated CagA induces β-catenin translocation into the nucleus[35] and has also been shown to induce a loss of cell polarity[36–39].

Another important *H. pylori* virulence factor is VacA[7]. Cellular consequences of binding VacA include disruption of epithelial cell barriers, changes in inflammatory response, disruption of late endosomes resulting in vacuoles, and release of Cytochrome c to induce apoptosis[40–46]. The gene locus for VacA is divided into four regions, the signal (s) region, the intermediate (i) region, the d region, and the middle (m) region[47,48]. There is diversity seen within the sequences of these regions, and varying degrees of activity of VacA are observed between strains. For example, s1m1 strains induce more vacuolation than s1m2 strains[49], and s1m1, i1 and d1 strains are associated with gastric ulcer disease and gastric cancer[48,50–54].

Finally, in addition to environmental factors and bacterial virulence factors, there are also host factors that alter risk for gastric cancer[7]. Arguably the most critical host factors that augment the risk for gastric cancer are involved in the host inflammatory response. In addition to promoting inflammation by activating cyclooxygenase enzymes to synthesize PGE_2[7], a potent stimulator of inflammation in the gastrointestinal system, *H. pylori* also induces a strong and persistent inflammatory immune response largely mediated by Th1 cells[55]. Macrophages are recruited to the stomach early during infection[56,57] and are critical for initiating the inflammatory response to *H. pylori*[58]. Macrophages contribute to gastric carcinogenesis risk in at least two ways. First, they are required to establish the inflammatory response that acts as an initiating event[58]. Second, macrophages produce reactive oxygen and nitrogen species, which can induce DNA damage[59].

Matrix Metalloproteinase 7 and Gastric Cancer

The matrix metalloproteinase (MMP) family is comprised of 25 proteins which use zinc ions to cleave their substrates. Collectively, MMPs modulate the activity of several microenvironment proteins including extracellular matrix (ECM) proteins, growth factors, cytokines, adhesion molecules and protease inhibitors[60-61,62]. The majority of MMPs have four domains: a signal domain for secretion, a pro-domain that keeps the protein in an inactive form, a zinc-binding domain for catalytic activity, and a hemopexin-like domain that confers substrate specificity[61]. MMPs are important for normal physiological processes, but also play an important role in many pathologic processes, including cancer[61,63]. In addition to having their expression upregulated relative to normal tissue in almost all cancers[62], MMPs have been shown to participate in many of the hallmarks of cancer[64], namely cell growth, differentiation, apoptosis, migration, invasion, and angiogenesis[62]. One MMP that is important in gastric cancer specifically is matrix metalloproteinase 7 (MMP7).

MMP7, also called Matrilysin and PUMP1, was first isolated from rat uterus[65], and has since been identified in other tissues including skin, salivary glands, pancreas, liver, breast, intestine, urogenital tract, lungs and stomach[66]. While most MMPs are secreted from stromal or mesenchymal cells, MMP7 is characteristically produced by epithelial cells[67], though its expression has also been seen in macrophages and keratinocytes[66]. MMP7 has broad substrate specificity because it is a minimal-domain MMP[62] and lacks the hemopexin domain which dictates substrate specificity[61]. MMP7 substrates include many ECM components including elastin, type IV collagen, fibronectin, vitronectin, aggrecan, and proteoglycans, and also non-ECM components such as TNF-α, FAS ligand, HB-EGF, E-cadherin, and B_4-integrin[68–75].

Increased expression of MMP7 has been observed in premalignant and malignant lesions in many organs including colon, pancreas and stomach[76,77–81], and expression levels of MMP7 are directly related to tumor staging[77,82–85]. In a mouse model of breast cancer, over expression of MMP7 leads to hyperproliferation and increased cancer susceptibility[86]. Moreover, cell lines that overexpress MMP7 possess enhanced tumorigenic potential[87]. Finally, mice that are genetically predisposed to intestinal tumors have decreased intestinal tumorigenesis when bred onto a MMP7 null background[88]. Together, these observations suggest that MMP7 promotes carcinogenesis.

MMP7 and *H. pylori*

MMP7, like other MMPs, is generally expressed at low levels until it is induced by an extracellular signal. MMP7 expression is stimulated by a variety of signals, however bacterial contact is among the most potent inducers of MMP7 expression[89]. Given this observation, and that MMP7 has pro-tumorigenic activities, and that MMP7 is over expressed in pre-malignant lesions associated with *H. pylori* infection, studies were conducted to determine whether *H. pylori* induces MMP7 expression in gastric epithelial cells and whether MMP7 plays a role in *H. pylori*-induced gastric carcinogenesis[89]. Not only does *H. pylori* infection induce expression of MMP7 both *in vitro* and *in vivo*[67,89,90], but induction in gastric epithelial cells seems to be *H. pylori* specific[89]. Induction of MMP7 is Cag-PAI and ERK1/2 dependent[67,89], and two mechanisms of induction have been reported. It has been shown that MMP7 is induced by increased levels of gastrin[91], and it has also been shown that *H. pylori* induces dephosphorylation and mislocalization of p120 from the cell membrane into the nucleus. There, p120 relieves KAISO-mediated repression of the MMP7 gene promoter[92].

H. pylori-induced expression of MMP7 may contribute to gastric carcinogenesis by augmenting cell proliferation as well as cell migration and invasion. MMP7 contributes to both epithelial and mesenchymal cell proliferation by increasing the bioavailability of IGF-II[93], and MMP7 contributes to epithelial cell invasion and migration by cleaving proteins in the extracellular matrix[67]. MMP7 also contributes to cell invasion and migration by promoting epithelial to mesenchymal transition (EMT) by indirectly increasing levels of soluble HB-EGF[94].

Given all of the previous data regarding MMP7 in gastric and other cancers, it was predicted that knocking out MMP7 would protect against injury in the context of *H. pylori* infection. Surprisingly, MMP7-/- mice infected with *H. pylori* actually exhibit higher inflammation and injury than infected wild-type mice[95]. This suggests MMP7 may have an important role in modulating the inflammatory response to *H. pylori* infection.

Summary and Objective

In summary, the following findings have shaped the rational for this thesis. (1) MMP7 is thought to play a role early in carcinogenesis[77–81]. (2) *H. pylori*-induced gastritis is an initiating event for gastric adenocarcinoma[5]. (3) MMP7-/- mice have increased inflammation following challenge with *H. pylori*[95]. (4) Macrophages are critical to inducing *H. pylori*-induced gastritis[58]. (5) Activated macrophage phenotypes are heavily dependent on the microenvironment[96] and alterations in macrophage polarization can significantly contribute to diseases including cancers[96,97]. (6) MMP7 could alter the microenvironment as it is able to cleave components of the ECM as well as growth factors and cytokines[60–62]. Taking these observations together, we sought to further elucidate whether MMP7 contributes to or protects the host from *H. pylori*-

induced injury. Specifically this thesis strives to establish whether MMP7 alters macrophage polarization during *H. pylori* infection.

CHAPTER II

MATERIALS AND METHODS

H. pylori Strains

H. pylori strains 60190, 7.13 and PMSS1 were grown on tryptic soy agar (TSA) plates supplemented with 5% sheep blood (BD BBL) in an atmosphere of 5% CO_2 at 37° Celsius. Strains were passed every 24-48 hours for no more than 10 passages. To prepare strains for co-culture with mammalian cells, the strains were harvested from 24 hour old plates and grown in *Brucella* broth supplemented with 10% heat-inactivated neonatal calf serum (NCS) and 20μg/ml vancomycin and agitated in an atmosphere of 5% CO_2 at 37° Celsius for 18 hours. The strains were then harvested by centrifugation at 4000 RPM for 5 minutes at room temperature, washed once in phosphate buffered saline (PBS), and resuspended in Royal Park Memorial Institute (RPMI) 1640 cell media (Mediatech, Inc). Bacterial strains were added to mammalian cells at a multiplicity of infection of 1:100.

Cell Culture and Reagents

MKN28 human gastric epithelial cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine and HEPES in an atmosphere of 5% CO_2 at 37° Celsius. Cells were grown for no more than 20 passages.

Bicinchoninic Acid (BCA) Protein Assay

To determine the concentration of protein in cell lysates, Pierce BCA assays (Thermo Scientific 23225) were performed following manufacturer's instructions. Briefly, protein samples were diluted 1:10 in water and incubated with 2 mL working reagent at 37° Celsius for 30 minutes. Room temperature samples were read with a spectrophotometer at 562 nm.

qRT-PCR

RNA was isolated from MKN28 gastric epithelial cells or bone marrow derived macrophages (BMDM) using the Qiagen rnEASY kit (74104) according to manufacturer's instructions. qRT-PCR was performed using Applied Biosystems High Capacity cDNA Reverse Transcription kit (4368814) followed by real-time PCR using Applied Biosystems Taqman gene expression assays using a 7300 real-time PCR system (Applied Biosystems). Mouse specific gene expression assays used include Nos2 (Mm00440502_m1), Arg1 (Mm00475988_m1), Tgfb1 (Mm01178820_m1), Il10 (Mm00439614_m1), Chi3I3 (Mm00657889_mH), Tnfsf14 (Mm00444567_m1), Il1b (Mm00434228_m1), Il12a (Mm00434165_m1), and Retnla (Mm00445109_m1). Expression levels of target genes were normalized to expression levels of GAPDH (Mm99999915_g1).

Mice and Experimental Infections

All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. INS-GAS MMP7-/- mice were obtained by breeding commercially available C57BL/6 MMP-/- and INS-GAS mice together (Jackson laboratories). INS-GAS MMP7-/- progeny were backcrossed with INS-GAS mice for 12 generations. Wild-type C57BL/6 mice

were commercially purchased through Jackson laboratories. All mice were specific pathogen free.

6-8 week old wild-type and MMP7-/- C57BL/6 mice as well as wild-type and MMP7-/- INS-GAS mice were inoculated with 500μl *Brucella* broth containing $5.0x10^9$ colony forming units (CFU) *H. pylori* or *Brucella* broth alone via orogastric gavage. Inoculation occurred twice separated by 24 hours as described previously[35]. Mice were euthanized at 12 weeks post challenge. At necropsy, one half of the stomach was reserved for magnet assisted cell sorting, and the remaining half of the stomach was divided into three tissue strips. One strip was fixed in 10% neutral buffered formalin and paraffin embedded by the Vanderbilt Human Tissue Acquisition and Pathology Shared Resource. One strip was homogenized, plated on TSA plates supplemented with 5% sheep blood and 2μg/mL amphotericin, 30μg/mLBacitracin, 10μg/mL nalidixic acid and 20μg/mL vancomycin, and incubated under microaerobic conditions at 37° Celsius. After 7 days CFU were counted and standardized to gram of tissue as described previously[35]. The remaining strip was frozen before its use in the Milliplex cytokine/chemokine magnetic bead panel (Millipore MCYTOMAG-70-PMX).

Bone Marrow-Derived Macrophages

Femurs were dissected from 8 week old wild-type and MMP7-/- C57BL/6 mice and flushed with PBS to harvest the bone marrow. The harvested marrow was homogenized using a 21 gauge needle and strained through a 70μM cell strainer. Red blood cells were lysed by resuspending the marrow in 900μL of sterilized water for 20 seconds and immediately adding 100μl of 10X sterile PBS. To differentiate the cells into macrophages, remaining cells were plated at $1x10^6$ cells in 6 well tissue culture dishes in R20/30 Differentiation Media (RPMI 1640,

20% FBS, 30% L929 Cell Conditioned Media, 100U/mL penicillin, 100µg/mL Streptomycin, 2mM L-glutamine). After 7 days, cells were washed, and the media was replaced with R10/5 Differentiation Media (RPMI 1640, 10% FBS, 5% L929 Cell Conditioned Media, 2mM L-glutamine) for 24 hours prior to co-culture with *H. pylori* strain PMSS1 for 24 hours.

Magnet Assisted Cell Sorting

One half of the stomach tissue harvested as described above was brought back to the lab in 5mL ice cold RPMI 1640. The tissue was mechanically disrupted with sharp tip dissecting scissors and enzymatically digested with solution D (0.05% w/v collagenase, 2% w/v dispase in RPMI 1640) for 20 minutes with agitation at 37° Celsius. Following digestion, cells were filtered through a 70µM nylon cell strainer, added to 5mL of fresh complete RPMI and centrifuged at 2000 RPM for 10 minutes at 4° Celsius. The resulting pellet was resuspended in 100µL of biotin-conjugated F4/80 antibody (Caltag MF48015-3) diluted 1:250 in flow cytometry staining buffer solution (eBioscience 00-42222-26) and kept on ice for 20 minutes. Stained cells were washed once with in flow cytometry staining buffer solution and centrifuged as before. The resulting pellet was resuspended in 100µL of streptavidin conjugated to micrometal beads (BDImag PUS557812) diluted 1:2 in flow cytometry staining buffer solution (eBioscience 00-42222-26) and kept on ice for 20 minutes. The stained pellets were washed three times as follows. 1 mL of flow cytometry staining buffer solution was added to pellets, vortexed, and placed inside the magnetic apparatus (BD IMagnet 552311) for 5 minutes. Once the magnetic particles were visibly lined up against the magnet, the staining buffer was removed by pipette. Sorted cells were lysed with 200µL of PerfectPure Lysis Solution (5PRIME 2302650). Cell lysates were frozen and later, RNA was isolated using the PerfectPure RNA 96 cell CS kit

(5prime 2302530) according to manufacturer's instructions. Reverse transcriptase and real-time PCR was performed as above.

Luminex-based Multiplex Assay

Tissue from wild-type and MMP7-/- C57BL/6 mice stomachs was homogenized in 200μL of CelLytic MW Mammalian Tissue Lysis/Extraction Reagent (Sigma C3228-50ML) supplemented with protease inhibitor cocktails 2 and 3 (Sigma P0044-5ML and P5726-1ML) using a Qiagen TissueLyser (85300) set at 50 oscillations per second for 3 minutes. Protein extracts rested on ice for 10 minutes and were centrifuged for 10 minutes at 14,800 RPM at 4° Celsius. Cytokine and chemokine concentrations were determined using a Millipore Multiplex kit (MCYTOMAG-70K-PMX) according to manufacturer's protocol. Briefly, 25μL of tissue sample, standard sample and quality controls were incubated with 25μL of antibody coated beads overnight at 4^{o} Celsius with agitation. Plates were washed twice, and incubated with 25μL of secondary antibody at room temperature for 1 hour at room temperature with agitation. 25μL of streptavidin-RPE was added to each well and incubated at room temperature for 30 minutes with agitation. Plates were washed twice before adding 150μL of sheath fluid. After 5 minutes of agitation the plates were read using Luminex xPONENT software and a FlexMap3D multiplex machine. Data were acquired according to manufacturer's instructions using MILLIPLEX Analyst software, and analyzed with Microsoft Excel and graphed with Prism6 Graphpad software.

Tissue Staining

To score inflammation in mouse gastric tissue, 5 μM paraffin-embedded sections were stained with hemotoxylin and eosin by the Vanderbilt Human Tissue Acquisition and Pathology Shared Resource. A blinded pathologist scored acute and chronic inflammation, hyperplasia, and dysplasia in the antrum and corpus using an ordinal scale from 0-3. The scores from antrum and corpus were combined for a final score as described previously[95].

Statistical Analysis

Statistical significance was tested with student's T test using GraphPad Prism 6 software. Significance was defined as $P \leq 0.05$.

CHAPTER III

RESULTS

Loss of MMP7 Increases Inflammation In C57BL/6 and INS-GAS Mice

Increased MMP7 expression has been observed in gastric carcinomas[76,77] as well as premalignant lesions[77,78,81], and levels of MMP7 are directly related to tumor staging[77,82–85]. Additionally, mice which are genetically predisposed to intestinal cancer experience decreased tumorigenesis when MMP7 is knocked out[88]. Together, these observations suggest that MMP7 promotes gastric carcinogenesis. Therefore, it is surprising that in the context of *H. pylori* infection MMP7-/- mice have been shown to have more inflammation and injury than infected wild-type mice[95] when inflammation is an initiating event for *H. pylori*-induced gastric carcinoma.

Because it has been shown that the genetic background of mouse models can alter disease risk[98], we sought to determine if the increased inflammation seen in *H. pylori* infected MMP7-/- mice is an artifact of the C57BL/6 background. To determine this, wild-type and MMP-/- C57BL/6 mice as well as wild-type and MMP-/- INS-GAS mice were challenged with *H. pylori* strain PMSS1 or *Brucella* broth control. Twelve weeks post challenge the mice were euthanized, and the stomachs were harvested for histological analysis (**Figure 1**). MMP7-/- mice had higher inflammation scores than wild-type mice for both the C57BL/6 (**Figure 2** top panels) and INS-GAS backgrounds (**Figure 2** bottom panels). Representative images are shown in F**igure 3.**

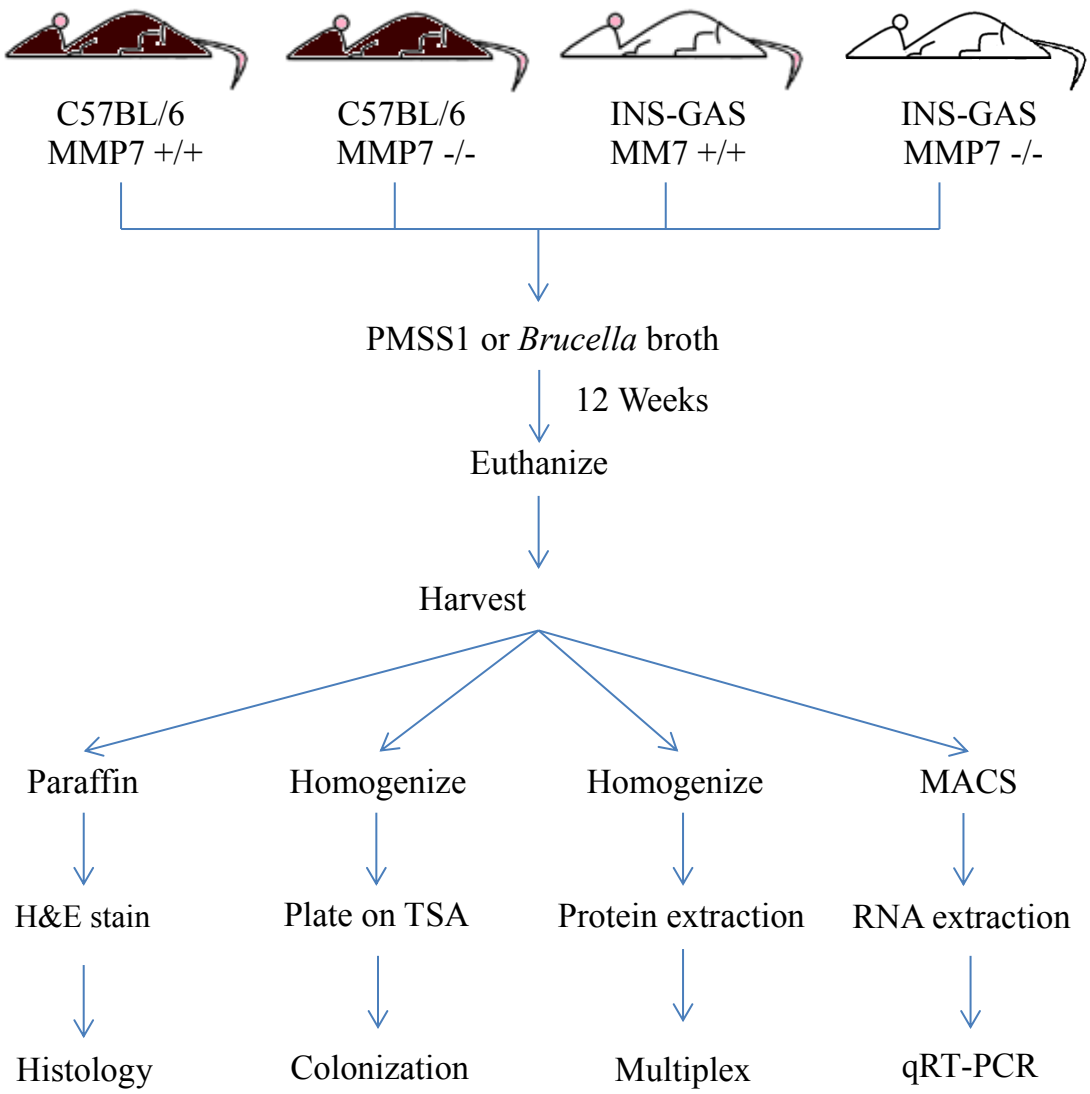

Figure 1 Experimental design 8 week old male mice were challenged with *H. pylori* strain PMSS1 or *Brucella* broth control. 12 weeks after challenge stomach tissue was harvested from euthanized mice for analysis. One half of the stomach was divided into three strips. One strip was embedded in paraffin for histological analysis. One strip was homogenized and plated on TSA to confirm colonization of the animals. The final strip was homogenized and lysed for protein extract used in a multiplex cytokine analysis. RNA extracted from macrophages isolated from the remaining stomach tissue via magnet assisted cell sorting (MACS) was analyzed by qRT-PCR.

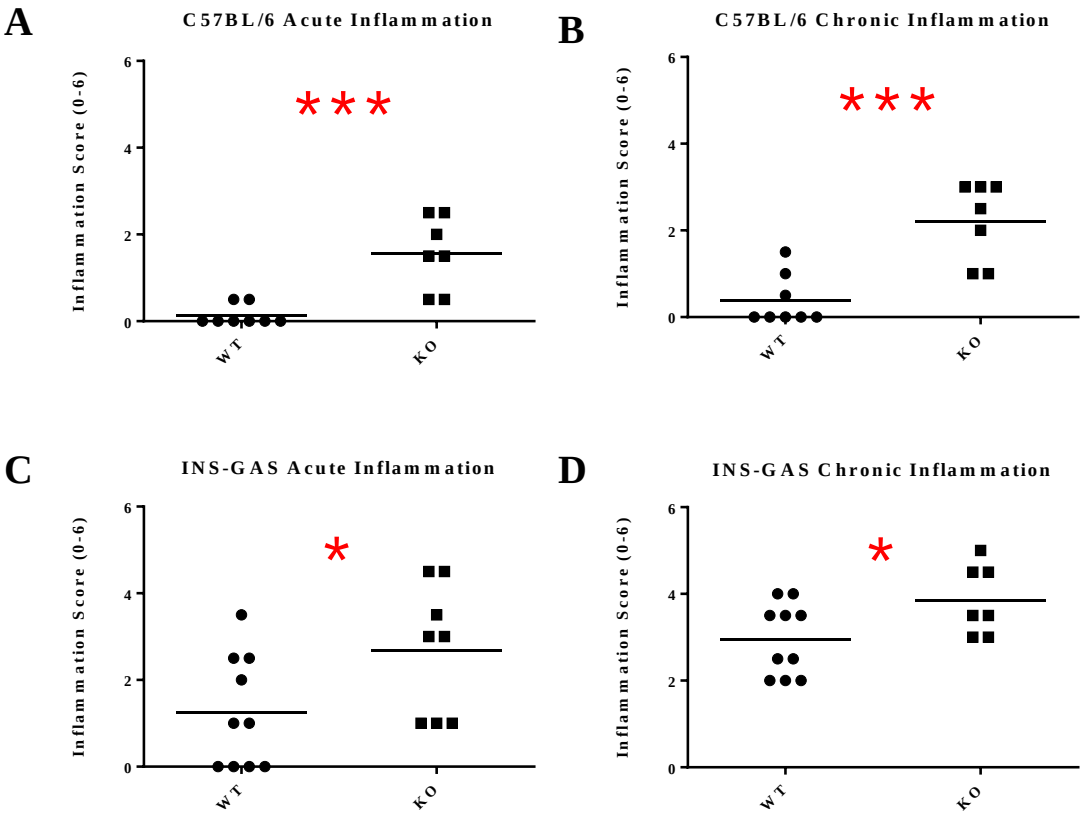

Figure 2 Increased inflammation in MMP7-/- mice Paraffin embedded sections of gastric tissue from *H. pylori* infected mice were stained with hemotoxylin and eosin. A blinded pathologist scored chronic and acute inflammation separately in the antrum and corpus using an ordinal scale (0-3) to represent no, mild, moderate and marked inflammation based on the presence of inflammatory infiltrate. The two scores from antrum and corpus were combined for a final inflammation score (0-6). Sections missing antrum (7 mice total) were excluded from this analysis. Red asterisk signifies statistical significance.
* $P \leq 0.05$ *** $P \leq 0.001$

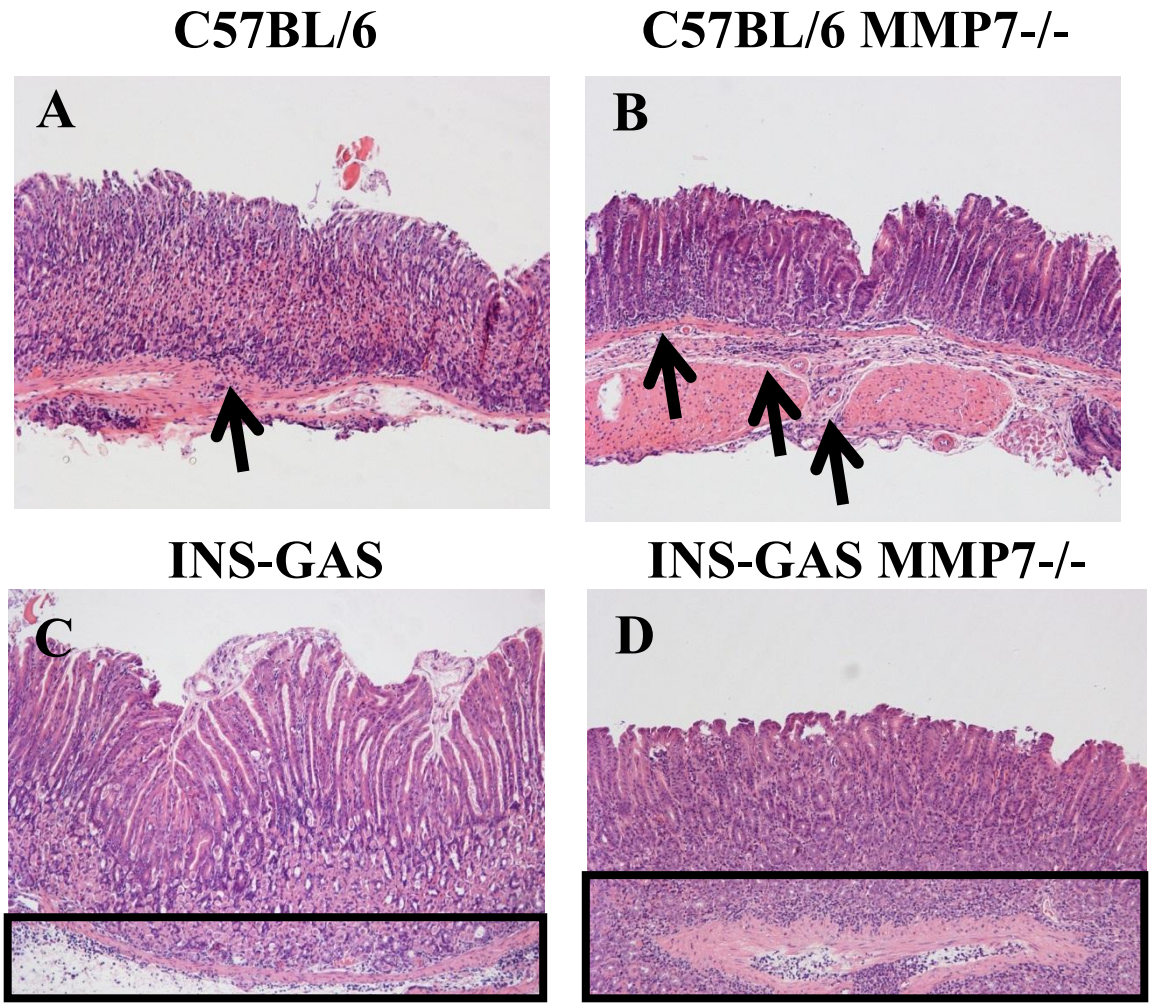

Figure 3 Representative images of increased inflammation in MMP7-/- mice Paraffin embedded sections of gastric tissue from *H. pylori* infected C57BL/6 (A) C57BL/6 MMP7-/- (B) INS-GAS (C) and INS-GAS MMP7-/- mice (D) were stained with hemotoxylin and eosin. Black boxes and arrows indicate areas with inflammatory infiltrate. All panels are 10X magnification.

C57BL/6 mice rarely show any evidence of pre-malignant or malignant lesions prior to 15 months of infection with *H. pylori*[99], but INS-GAS mice can exhibit premalignant lesions as early as 6 weeks post challenge with *H. pylori*[100]. In this experiment, both the INS-GAS and MMP7-/- INS-GAS mice that were infected with *H. pylori* displayed pit abscesses and premalignant lesions. However, the MMP7-/- INS-GAS mice had worse inflammation-induced injury than wild-type INS-GAS mice. Representative histological images of inflammation and injury are shown in **Figure 5** respectively. MMP7-/- INS-GAS mice had increased hyperplasia evidenced by having a thicker mucosa than wild-type mice (**Figure 5A and B**). Wild-type INS-GAS mice exhibited indefinite dysplasia with some enlarged, darkened nuclei (**Figure 5C box 1**). Wild-type mice also had slightly irregularly shaped glands though the nuclei remained uncrowded (**Figure 5C box 2**). INS-GAS MMP7-/- mice displayed dysplasia with highly irregular, angular glands (**Figure 5D box 1)** and hyperchromatic nuclei in crowded cells demonstrating pseudo-stratification (**Figure 5D box 2).** Both wild-type and MMP7-/- INS-GAS mice displayed abscesses. However, lesions in MMP7-/- mice were larger and more numerous (**Figure 5E and F)**.

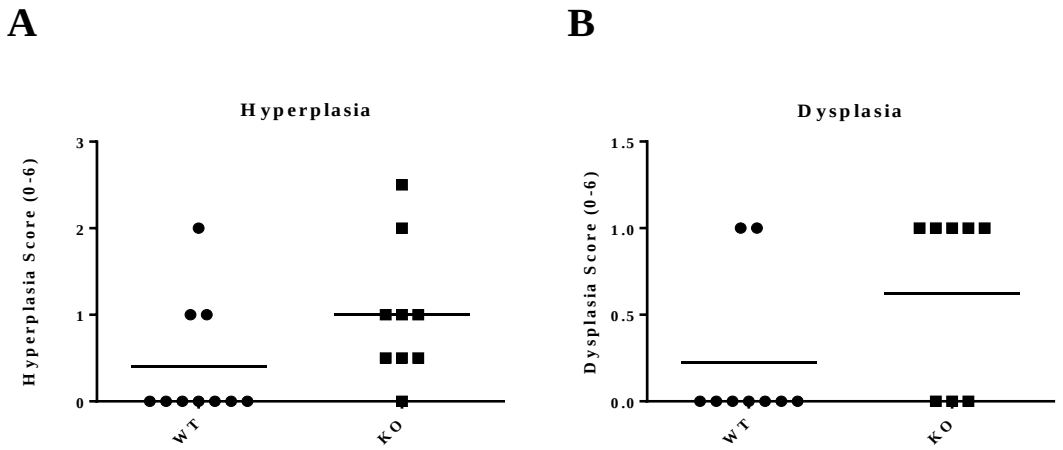

Figure 4 Increased premalignant lesions in MMP7-/- mice Paraffin embedded sections of gastric tissue from *H. pylori* infected mice were stained with hemotoxylin and eosin. A blinded pathologist scored hyperplasia and dysplasia separately in the antrum and corpus using an ordinal scale (0-3) to represent no, mild, moderate and marked characteristics. Hyperplasia was scored on the thickness of the mucosa. Dysplasia was scored on physical appearance of cells and the architecture of the glands. The two scores from antrum and corpus were combined for final scores (0-6). Sections missing antrum (1 mouse) were excluded from this analysis.

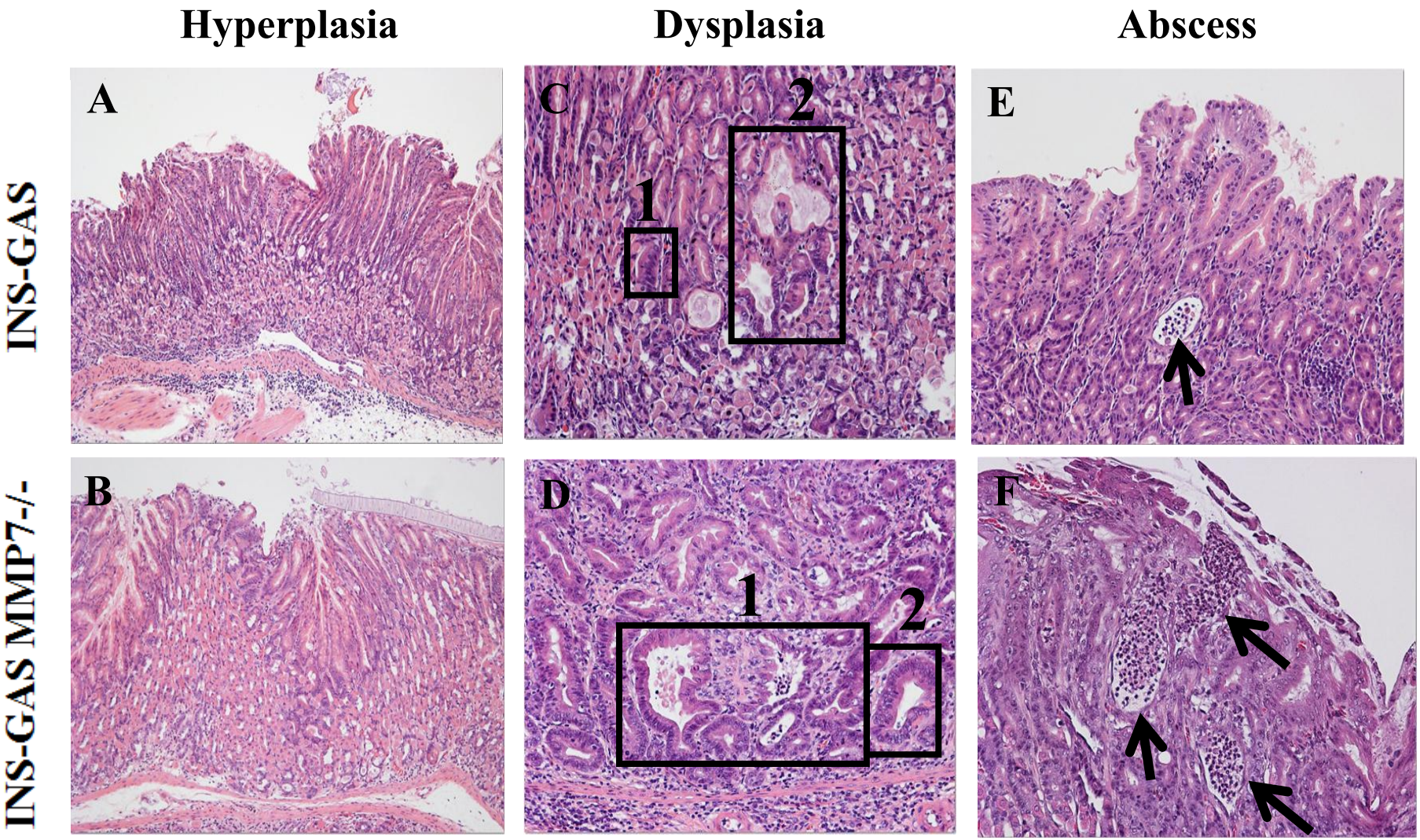

Figure 5 Representative images of increased injury in MMP7-/- mice Paraffin embedded sections of gastric tissue from *H. pylori* infected INS-GAS (top panels) and INS-GAS MMP7-/- mice (bottom panels) were stained with hemotoxylin and eosin. Black boxes indicate areas of dysplasia. Black arrows indicate abscesses. Panels A-D are 10X magnification. Panels E-F are 20X magnification.

Similar Colonization Is Seen In Wild-type and MMP7-/- Mice

It is possible that the MMP7-/- mice had more inflammation because they were colonized with more bacteria. Therefore, we sought to determine the colonization of each infected mouse. One strip of stomach tissue from each mouse was homogenized, and the homogenate was plated on TSA plates for 7 days (**Figure 1**). Colonization data for wild-type and MMP7-/- mice are graphed in **Figure 6**. There is no statistically significant difference in colonization between wild-type and MMP7-/- mice for either the C57BL/6 or INS-GAS backgrounds.

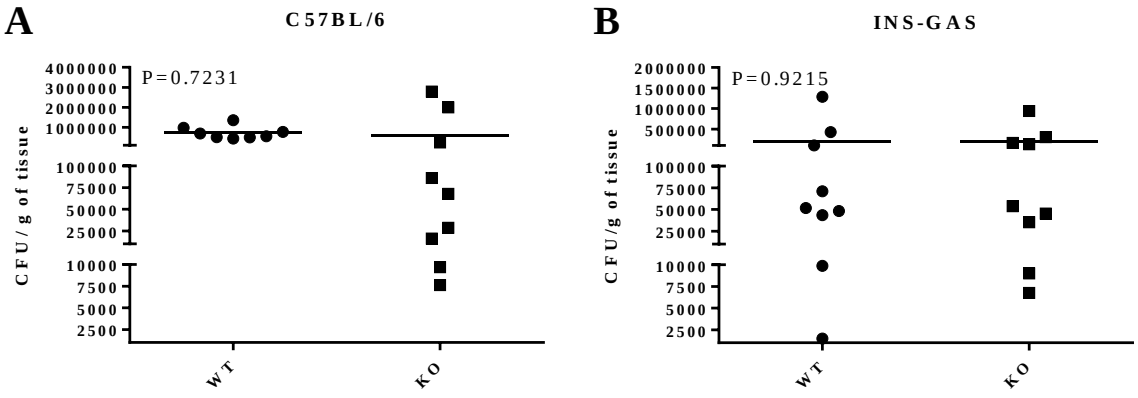

Figure 6 Similar colonization in wild-type and MMP-/- mice To assess colonization, gastric tissue from *H. pylori* infected mice was homogenized and plated on TSA agar for 7 days. Colony forming units (CFU) were standardized to grams of homogenized tissue.

Loss of MMP7 Increases Production of Inflammatory Cytokines

We sought to determine if MMP7-/- mice have a different cytokine profile from wild-type mice. To examine this, protein lysates of homogenized stomach tissue from *H. pylori* infected mice was were analyzed by multiplex assay (**Figure 1**). Of the twenty-five factors analyzed, one factor (IFN-γ) was induced in wild-type mice but not MMP7-/- mice (**Figure 7A**). Five factors (IL-1α, IL-1β, IL-7, MIP-1β, and TNF-α) were induced in MMP7-/- mice but not wild-type mice (**Figure 7B-7F**). Three factors (IL-17, IP-10 and RANTES) were induced in both wild-type and MMP7-/- mice, but had higher induction in the MMP7-/- mice compared to wild-type mice (**Figure 7G-7I**).

Loss of MMP7-/- Increases Expression of M1 Macrophage Markers

Macrophages are critical for establishing *H. pylori* induced gastritis[58]. Activated macrophage phenotypes are transient, and the process by which they acquire a phenotype is known as macrophage polarization[57–65]. The type of macrophages that exist in a tissue is heavily dependent on the type, concentration and half-life of the factors present in the microenvironment[96]. MMP7 is a promiscuous protease that cleaves many different factors. Here, we have shown that loss of MMP7 alters the cytokine/chemokine profile of *H. pylori*-infected mice. Given that MMP7 has been shown to control distinct transcriptional responses in airway epithelial cells infected with *Pseudomonas aeruginosa*[108], and that mice genetically engineered to lack another MMP, MMP10, have been shown to lack M2 macrophages in a chemically-induced colitis model[109], we hypothesized that MMP7 could induce transcriptional programs that alter macrophage polarization in response to *H. pylori*. To test this *in vitro*, we challenged bone-marrow derived macrophages from wild-type and MMP7-/- C57BL/6 mice

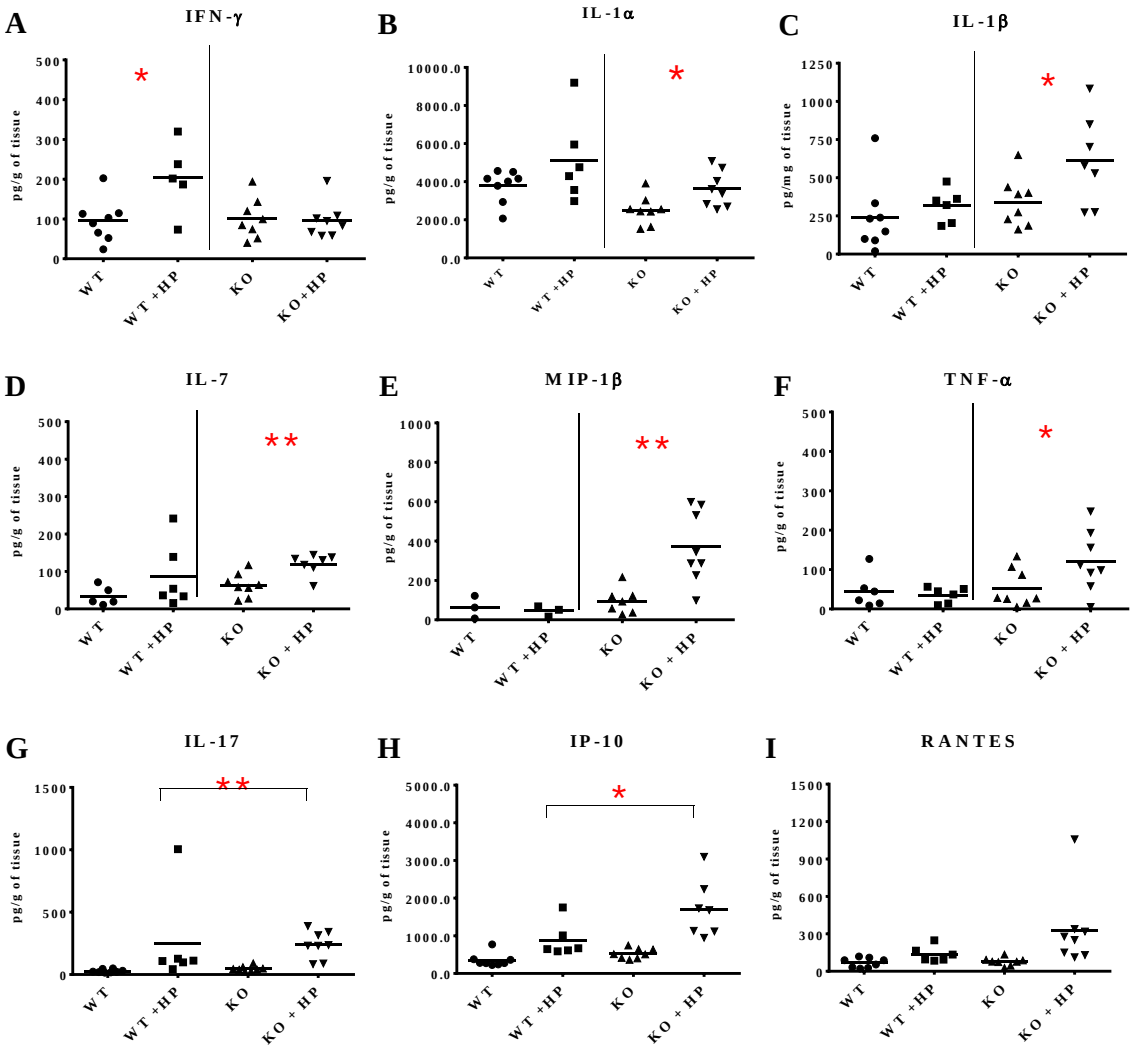

Figure 7 Cytokines induced by *H. pylori* 8 week old male wild-type and MMP7-/- C57BL6 mice were challenged with *H. pylori* strain PMSS1 or *Brucella* broth alone. 12 weeks after challenge with *H. pylori* mice were euthanized and stomach tissue was harvested for multiplex analysis. Samples were excluded from the analysis if the cytokine concentration fell outside of the standard curve. Red asterisk signifies statistical significance. *P ≤ 0.05 ** P ≤ 0.005

with *H. pylori* and analyzed expression of various M1, M2 and Mreg macrophage markers by qRT-PCR. Of the 9 markers analyzed two, IFN-γ and IL-1β, had statistically significant higher expression in MMP7-/- mice compared to wild-type mice (**Figure 8).**

To extend these findings *in vivo* we isolated macrophages from the stomachs of wild-type and MMP7-/- C57BL/6 mice infected with *H. pylori* and examined the expression of M1 markers by qRT-PCR (**Figure 1**). The results were similar to those from the bone marrow-derived macrophages with iNOS and IL-1β having higher expression in macrophages isolated from MMP7-/- mice compared to macrophages isolated from wild-type mice (**Figure 9)**.

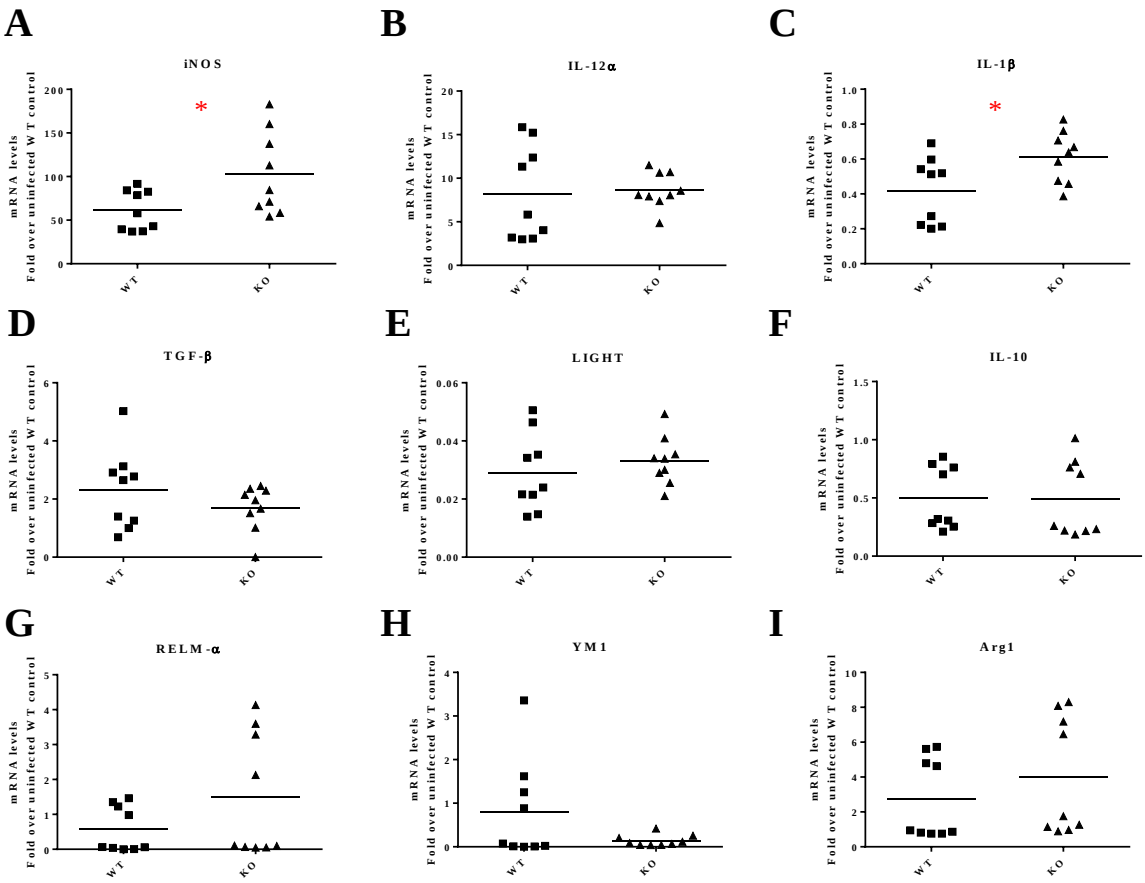

Figure 8 Increased expression of M1 markers in MMP7-/- bone marrow derived macrophages Bone marrow was harvested from the femurs of 8 week old male wild-type and MMP7-/- C57BL/6 mice. Red blood cells were lysed and the remaining harvested cells were differentiated to macrophages *in vitro*. Following 24 hour co-culture with *H. pylori* strain PMSS1 RNA was isolated and qRT-PCR was performed to analyze expression of markers of M1 (A-C) M2 (D-F) and Mreg (G-I) macrophages. Red asterisk signifies statistical significance. * $P \leq 0.05$

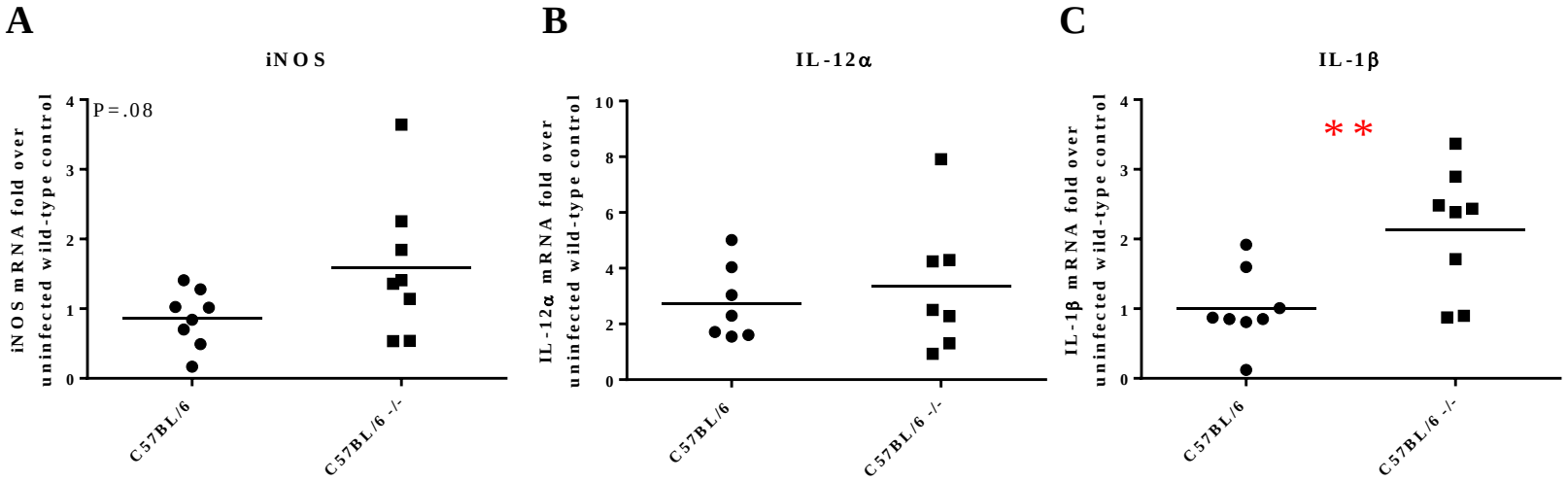

Figure 9 Increased expression of M1 markers in MMP7-/- macrophages isolated by MACS 8 week old male wild-type and MMP7-/- C57BL6 mice were challenged with *H. pylori* strain PMSS1 or *Brucella* broth control. 12 weeks after challenge the mice were euthanized and stomach tissue was harvested and digested. Macrophages were then isolated by magnet assisted cell sorting with an F4/80 antibody. RNA was isolated from the sorted cells and qRT-PCR was performed to analyze expression of M1 macrophage markers. Red asterisk signifies statistical significance. ** $P \leq 0.01$

CHAPTER IV

DISCUSSION

Collectively, several observations suggest that MMP7 promotes carcinogenesis. For example, increased expression of MMP7 has been observed in premalignant and malignant lesions in many organs including colon, pancreas and stomach[76,77–81], and expression levels of MMP7 are directly related to tumor staging[77,82–85]. Moreover, MMP7 contributes to the proliferation, tumorigenic potential, migration and invasion of cells[67,86,87], and mice that are genetically predisposed to intestinal tumors have decreased intestinal tumorigenesis when bred onto a MMP7-/- background[88]. Because MMP7 expression is induced by the carcinogenic bacteria *H. pylori* it was predicted that loss of MMP7 would be beneficial to a host infected with *H. pylori.* Thus, it was surprising that MMP7-/- mice experience increased inflammation in response to *H. pylori* infection[95], when gastritis is believed to be the initiating event for gastric cancers[5]. The objective of this thesis was to elucidate an explanation for the surprising increase in inflammation seen in *H. pylori*-infected MMP7-/- mice.

Increased Inflammation in MMP7-/- Mice Is Not an Artifact of the C57BL/6 Background

Because it has been shown that the genetic background of mouse models can alter disease risk[98] one possible explanation for the increased inflammation seen in MMP7-/- mice is that it is an artifact of the C57BL/6 background. An alternative explanation is that the previous results were an unfortunate type II statistical error. To address these two possibilities we repeated the initial experiment. We infected wild-type and MMP7-/- C57BL/6 mice with *H. pylori* strain

PMSS1 and examined inflammation 12 weeks post challenge. Additionally, wild-type and MMP7-/- INS-GAS mice (of the FVB genetic background) were included in the analysis (**Figure 1**). Again, there was increased inflammation seen in the MMP7-/- mice on both the C57BL6 and INS-GAS backgrounds (**Figure 2 and 3**). INS-GAS mice are more susceptible to malignant transformation in response to *H. pylori* infection than C57BL/6 mice[95]. Importantly, in our experiment, MMP7-/- INS-GAS mice had increased severity of pre-malignant lesions and tissue injury compared to wild-type INS-GAS mice (**Figure 3 and 4**).

To determine if the increased inflammation could have been a result of increased colonization, we determined the colonization of each infected mouse by counting CFU from stomach tissue homogenate plated on TSA plates (**Figure 1**). There was no statistically significant difference in colonization between wild-type and MMP7-/- mice for either the C57BL/6 or INS-GAS backgrounds (**Figure 5**). Collectively, these results suggest that the increase in inflammation seen in MMP7-/- mice is neither an artifact of the C57BL/6 background nor a type II statistical error. Therefore, we sought to elucidate a mechanism by which loss of MMP7 results in increased inflammation.

MMP7 suppresses M1 macrophage polarization during *H. pylori* infection

Gastritis is thought to be an initiating event for the majority of gastric carcinomas[5], and macrophages have been shown to be critical for initiating gastritis in response to *H. pylori*[58]. Because MMP7 is thought to play a role early in carcinogenesis[77–81], and we have shown that it plays a role in shaping the cytokine profile in response to *H. pylori*, we considered the possibility that MMP7 could play a role in the macrophage polarization during *H. pylori* infection.

For several decades, activated macrophages were thought to only play a role in clearing pathogens from the body[101]. However, recent research has demonstrated that there are different activated macrophage phenotypes which serve important roles in both normal and pathological processes[57–65]. M1 macrophages (also known as classically activated macrophages) are important for clearing pathogens from the body, and do so by intracellular microbiacidal activity[96] and by secreting inflammatory mediators. M1 macrophages promote a Th1 adaptive immune response while simultaneously dampening the Th2 type response[105,110]. M2 macrophages are important for wound healing and tissue remodeling[96]. In addition to secreting components of the extracellular matrix[96], M2 macrophages also secrete factors to promote a Th2 adaptive immune response while simultaneously dampening a Th1 response[105,110]. Regulatory macrophages (Mregs) like M2 macrophages are anti-inflammatory but unlike M2 macrophages do not deposit extracellular matrix[96].

Macrophage polarization is heavily influenced by the microenvironment as evidenced by the fact that macrophages that are identical other than their microenvironments will respond differently to identical stimuli[96]. Macrophage polarization is reversible, and is not only dependent on the type of stimulating factor, but also the concentration and half-life of the stimulating factors present in the microenvironment[96]. Proinflammatory, pathogen clearing M1 macrophages polarize in response to IFN-γ and TNF-α. Anti-inflammatory/wound-healing M2 macrophages polarize in response to IL-4 and IL-13. Anti-inflammatory regulatory Mregs polarize in response to IL-10. Given the wide variety of MMP7 substrates, and given that MMP7 has been shown to induce distinct transcriptional responses in epithelial cells[108] in addition to the belief that aberrations in macrophage polarization can contribute to cancer[96,97], we

hypothesized that loss of MMP7 could alter the cytokine profile of the microenvironment and thus alter macrophage polarization in response to *H. pylori*.

To test this hypothesis, we examined the cytokine profiles of stomach tissue from wild-type and MMP7-/- mice infected with *H. pylori.* Multiplex analysis revealed eight inflammatory cytokines that were expressed more highly in MMP7-/- mice compared to wild-type mice (**Figure 7**). Two of these factors include IL-1β, a cytokine effector secreted by M1 macrophages[102], and TNF-α, a potent inducer of M1 macrophage polarization[101]. We also examined expression of macrophage markers both from bone-marrow derived macrophages from wild-type and MMP7-/- mice challenged with *H. pylori in vitro* and from macrophages isolated from the stomachs of wild-type and MMP7-/- mice infected with *H. pylori in vivo*. Both analyses revealed an increase in expression of the M1 macrophage markers iNOS and IL-1β. Together, these observations suggest that loss of MMP7 results in increased M1 macrophage polarization. Therefore, in a MMP7+/+ host, MMP7 may suppress M1 macrophage polarization. Given that M1 macrophages can contribute to cancer development by releasing toxic oxygen and nitrogen intermediates that induce DNA damage and mutations[111], this would suggest that MMP7 normally plays an important role in protecting the host from *H. pylori*-induced gastric carcinogenesis.

Future Directions

Although *H. pylori* colonizes 50% of the world's population, and is the highest risk factor for developing gastric cancer, only 1% of infected individuals develop gastric cancer[5]. It is widely believed that a patient's disease outcome is ultimately the result of interplay between environmental factors, bacterial virulence factors, and host factors. Results from this study

suggest that MMP7 could be a host factor that determines gastric cancer risk. It is possible that differences in MMP7 polymorphisms could explain why some patients infected with *H. pylori* develop gastric cancer and others do not. For example, patients with a polymorphism that decreases MMP7 activity could be susceptible to increased M1 polarization in response to *H. pylori* infection, thus putting them at higher risk for developing gastric cancer. Therefore, an important follow up study is to analyze publicly available sequence data from human gastric biopsies from healthy patients and patients with gastric cancer to see if any differences in MMP7 gene sequence correlate with patient outcome.

One reason gastric cancer is so deadly is that often it is not diagnosed until it has already reached end stage disease. Having a genetic marker such as MMP7 could help identify patients infected with *H. pylori* that are at higher risk for cancer. Identified patients could be observed more closely for signs of malignant transformation, and earlier detection would save the lives of many gastric cancer patients. One argument against this approach is that this may not be currently practical given that the majority of gastric cancer patients live in developing countries. However, the cost of gene sequencing is decreasing significantly over time as technology improves, and therefore could be more achievable in the future.

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