Protection from Pneumonic Plague by the Induction of Heme Oxygenase-1

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by

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Protection from Pneumonic Plague by the

Induction of Heme Oxygenase-1

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Abbreviations

ACK	Ammonium Chloride Potassium
ANOVA	Analysis of variance
ARE	Anti-oxidant response element
ATP	Adenosine triphosphate
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
bp	Base pairs
BSA	Bovine serum albumin
BSL2	Biosafety level 2
BSL3	Biosafety level 3
C	Celsius
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CO	Carbon monoxide
CO ₂	Carbon dioxide
CoPP	Cobalt protoporphyrin IX
COX2	Cyclooxygenase-2
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DPI	Days post-infection
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte monocyte colony-stimulating factor
H&E	Hematoxylin and eosin
HIA	Heart infusion agar
HIB	Heart infusion broth
HIF-1 α	Hypoxic inducible factor-1 α
<i>hms</i>	Hemin storage locus
HPI	Hours post infection
HPT	Hours post treatment
HMWP	High molecular weight protein
IFN	Interferon
IL	Interleukin
in	Intranasal
IRF	Interferon regulatory factor
ip	Intraperitoneal
kb	Kilobase
LCR	Low calcium response
LD50	Mean lethal dose
LDH	Lactate dehydrogenase
LIDR	Laboratory of Infectious Diseases
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
mg	Milligram
mL	Milliliter
mM	Millimolar
μ g	Microgram
μ L	Microliter
μ m	Micron (micrometer)
MOI	Multiplicity of infection

MyD88	Myeloid differentiation primary response gene (88)
ng	Nanogram
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ns	Not significant
PaCO ₂	Partial pressure carbon dioxide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
pCD1	Plasmid denoting calcium dependence 1
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
<i>pgm</i>	Pigmentation locus
Pla	Plasminogen activator
PKC	Protein kinase C
pMT1	Plasmid encoding murine toxin 1
PMNs	Polymorphonuclear leukocytes (Neutrophils)
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
T3SS	Type 3 secretion system
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNFR-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferonβ
Ub	Ubiquitin
WT	wild type
ybt	yersiniabactin
YCV	Yersinia containing vacuole
Yop	Yersinia outer protein
ZnPP	Zinc Protoporphyrin IX

Abstract

Yersinia pestis is the etiological agent of the disease known as plague. The pathology of this disease is characterized by organ failure due to necrosis and hyperinflammation towards the end of the disease. However, *Y. pestis* has a type 3 secretion system (T3SS) with effector Yops that stifle inflammation, a *pgm* locus that enables better survival within the mammalian host, and a tetraacylated LPS that renders innate immune sensing by TLR4 anti-stimulatory. Based on the evidence of these virulence factors, added with the lack of inflammation that is present at the beginning of the infection we made the hypothesis that the inflammation may not be related to the pathogen, but to the damage the pathogen creates. This led our laboratory to investigate the damage to the host as the potential source of this hyperinflammation response. We found that the production of yersiniabactin was necessary to induce damage and establish *Y. pestis* colonies in the lung. This lung damage induced the cytoprotective enzyme heme oxygenase-1, which is an enzyme that catalyzes heme degradation into CO, biliverdin, and Fe²⁺. We show that by upregulating HO-1 activity by administering a compound, cobalt protoporphyrin IX, we were able to ameliorate the hyperinflammation, lessen tissue damage, and increase survival in animals infected with *Y. pestis*.

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Chapter 1. Introduction

Abstract

Yersinia pestis is the etiological agent of plague. It is characterized as a disease that onsets rapidly, leading to a widespread bacteremia, and death of the host within days. Plague pathology is characterized by organ failure due to necrosis and hyperinflammation towards the end of the disease. *Y. pestis* has a type 3 secretion system that stifles inflammation and induces cell-death by injecting effector Yops into host cells. Other virulence factors critical to the pathogenesis of plague within the mammalian host include: a pigmentation locus which enables better survival within the host by producing the siderophore yersiniabactin, and a tetraacylated lipopolysaccharide, expressed at 37°C that renders innate immune sensing by TLR4 anti-stimulatory. Due to these virulence factors and the rapid growth of bacteria within the host, an effective immune response cannot be mounted against the bacterial infection. Patients infected with *Y. pestis* that do not receive treatment, especially in the pneumonic and septicemic forms, nearly always succumb to the disease. Although many classes of antibiotics kill *Y. pestis in vitro*, there is a high rate of treatment failure, especially when antibiotics are initiated post-onset of symptoms. *Y. pestis* resides in a sylvatic reservoir and is endemic in most of the continents in the world. There will be a constant health risk to those that encounter animals infected with this pathogen and that eradication of the disease is difficult as the reservoir persists.

Yersinia pestis

Y. pestis is a gram-negative, non-motile coccobacillus that causes the disease known as plague. This facultative intracellular pathogen can grow between 4-40°C [1]. The lipopolysaccharide (LPS) of *Y. pestis* is categorized as rough, as it lacks an O-antigen. LPS found in most bacteria is hexaacylated and strongly induces inflammation through TLR4 signaling [2-4]. This molecule is important in the structure of the cellular membrane and bacteria deficient in LPS synthesis are more susceptible to antibiotics and host defenses [5, 6]. Due to the high virulence to humans and potential low infectious dose (~10 CFU via inhalation) *Y. pestis* is classified as a Tier 1 Select Agent and must be experimented with at biosafety level 3 (BSL3) if all virulence factors are present [1, 7].

Plague is a zoonotic disease affecting rodents with a flea vector. *Y. pestis* exists in a sylvatic reservoir on all of the continents, with the exception of Australia and Antarctica [1, 8-13]. Fleas are infected with *Y. pestis* after taking a blood meal from an infected animal. Over the course of 7-9 days, *Y. pestis* constructs a biofilm to block the proventriculus [1, 11-14]. This accomplishes two objectives: it allows for easier dissemination into animals that the flea feeds from and it increases the attempts the flea will make to feed because no blood meal can reach the midgut due to the biofilm obstruction. The flea vector then infects a new animal with *Y. pestis* and the cycle continues [14-16]. There are cases of rodent populations that serotype positive for plague exposure and it has been

hypothesized that in these zoonotic reservoirs, plague can persist in the environment [17-19].

Plague was first recorded as a bioweapon in 1347, where the Tartars catapulted plague-infected bodies into the city of Kaffa. During World War II, the Japanese dropped bomblets containing fleas infected with *Y. pestis* on Chinese cities. During the Cold War, the American and Soviet governments developed ways to aerosolize *Y. pestis* for air-dispersal [1, 9, 20, 21]. *Y. pestis* makes a prime candidate as a biothreat agent due to its ability to uptake and integrate new genes via lateral gene transfer [9, 22-24]. These abilities allow *Y. pestis* to easily integrate antibiotic-resistance genes. Other reasons that make *Y. pestis* a favorable bioterror agent is that it can be spread via aerosol and has a low infectious dose. In 2014, there was news of a laptop discovered in Syria. Within the files of the recovered laptop were directions on how to isolate *Y. pestis* from the environment and strategies to implement it as a bioweapon for acts of terrorism [25]. *Y. pestis* resides in a sylvatic reservoir, which means that total eradication of this threat is difficult, if not impossible. Constant surveillance of potential bioweapons manufacture is needed and contingency plans that include rapid treatment implementation are necessary to lessen the effects of *Y. pestis* being used as a bioweapon.

Plague manifests in three different disease-states: bubonic, septicemic, and pneumonic. Sudden onset of plague symptoms occur and are characterized by malaise and dizziness, with a high fever [26-28]. The rapid disease progression of plague as well as the virulence factors of *Y. pestis* neutralize the

immune response toward the infection, preventing resolution of the disease. If all plague types remain untreated, *Y. pestis* will ultimately be disseminated throughout the body causing a bacteremia with subsequent septic shock, widespread cellular death, and organ failure [1, 29-35]. The virulence factors that allow *Y. pestis* to evade the immune system include: a type 3 secretion system under the control of the low calcium response (LCR), and a pigmentation locus that encodes for the proteins that are required for the production of the siderophore yersiniabactin [1, 7, 36-38]. These virulence factors allow *Y. pestis* to establish a replicative niche within the host by dampening the inflammation response, inducing cellular death, and stealing resources from the host to allow for bacterial growth.

There are three biovars of *Y. pestis*: Antiqua, Medievalis, and Orientalis that are infectious to humans [1]. These biovars do exhibit some changes to virulence, such as the lack of plasminogen activator (Pla) in the biovar Antiqua [39]. Pla is a protein that can cleave fibrin which can actively resist the clotting mechanisms of the host [39-41]. However, disease pathology is typically conserved between Pla+ and Pla- strains and the biovars are distinguished by metabolic tests or genomic sequencing. *Y. pestis* evolved from *Y. pseudotuberculosis* about 10,000 years ago [16, 39, 42]. The first pandemic has been linked to the Antiqua biovar; with the Black Death strain of *Y. pestis* linked to the Medievalis biovar. The Orientalis biovar caused the 20th century pandemic in China and this biovar remains endemic to China and the United States to this day [1, 8, 24].

The History of *Y. pestis*

The first *Y. pestis* pandemic was known as the Justinian plague, named after the Byzantine emperor who ruled over most of the affected region. Lasting for three years the plague covered the Mediterranean region and Middle East until the epidemic ended. The bacteria receded into the environment within the sylvatic reservoir and the disease then cycled about every decade between 541 to 654 BCE [8]. During this time, Emperor Justinian I was leading a military campaign against the Ostrogoths to retake and reunite the two halves of the Roman Empire. He was able to wrest control over most of the Italian peninsula in 554 CE [8, 43]. Unfortunately, during this assault there was an outbreak of plague in the capital city of Constantinople which ended up killing up to 40% of the city's inhabitants. The resulting civil unrest and a massive drop in tax revenue halted the Empire's momentum in the Italian campaign. The epidemic prevented Justinian from sending reinforcements to aid in the occupation of Italy and in 568 the Lombards invaded the north of Italy and were able to displace the remaining Byzantine armies. By the 8th century, the losses due to plague were around 25% of the total population of the Byzantine Empire [8, 34, 43-46].

The second pandemic of plague is more well-known as the Black Death. This outbreak originated in Sicily in 1347 and possibly traveled along trade routes from the east. The initial epidemic of the Black Death lasted 5 years and killed up to 28 million people. This outbreak ultimately may have killed upwards of 100 million across Eurasia with cycles of epidemic plague that occurred every 2-5 years until 1480 [8, 34]. This led to tremendous depopulation in these areas

causing mass migrations of peasants. Draconian measures were taken by feudal lords to force workers to stay and work in the affected areas, to keep the economy and system of government functioning. Peasants began to break these laws to seek out a means to escape plague-infested areas or find more lucrative farming prospects under different feudal lords. Civil unrest followed which ultimately led to a breakdown in the feudalistic system of government that ruled Europe at the time. Higher wages were paid to peasants working in the fields due to the scarcity in labor because of the massive death toll. This influx of capital helped to create a middle class in Europe. This restructuring of wealth and the added desire to learn more about diseases and the human body, inspired by the effects of the plague pandemic, helped to usher in the Renaissance [47-49].

The third and most recent pandemic started in China in 1855. Spreading throughout southeastern Asia, it was particularly devastating in China and India. This recent pandemic lasted until 1918, with epidemics cropping up every 2-5 years throughout this time [1]. There are no accurate fatality numbers from China, but India estimates that 12.5 million people died during the pandemics. It was during this time that Alexandre Yersin and Shibasaburo Kitasato were independently trying to isolate the plague-causing bacterium [50]. Although initially Kitasato was credited with the discovery, the bacterium that Yersin isolated was identified as the agent of plague. In honor of his discovery, the bacterium was renamed *Yersinia pestis* from *Pasteurella pestis* in 1970 [1, 34, 50].

Clinical Manifestation of *Y. pestis*

The three manifestations of plague pathology are: bubonic, pneumonic, and septicemic. Bubonic plague is primarily contracted by an infected flea bite, but can also be contracted by bacterial contact with an open skin wound [1, 51]. Untreated bubonic plague fatality rates range from 40-60% [52]. The bubonic form of the disease is the most common, accounting for 80% of all plague infections in the United States between 1900 and 2012 [53]. Bubonic plague is the easiest to diagnose amongst the forms of plague. Along with fever and chills, the disease onsets with extremely tender lymph nodes known as buboes. These buboes can present 2-6 days post *Y. pestis* exposure [22, 26, 34, 50]. Discoloration and swelling of the buboes is common and toward the later stages of the infection, leakage of the bacteria into the blood causes secondary septicemia. If treated with antibiotics before the bacteria disseminate into the blood, most patients recover completely. However, if there are complications with the infection or if *Y. pestis* has entered the bloodstream, chances of survival are reduced [7, 54, 55].

Primary septicemia is distinguished from secondary septicemia by the lack of lymph node pathology [56]. It can be contracted from infected flea bites, infected animal bites, or laboratory accidents that allow the bacterium to gain access directly to the blood stream [28, 56, 57]. Symptoms of septicemic plague are like other gram-negative bacteremias, with patients presenting as febrile and having a high fever. This can potentially lead to misdiagnosis of disease and result in improper antibiotic treatment for the patient [58]. The mortality rate for

patients suffering from septicemic plague is 30-50%, even if they receive treatment [1, 55, 59-61]. Hallmarks of septicemic plague are high bacterial titers in the blood leading to colonization in the lungs, liver, and the spleen. If left untreated, this ultimately causes lesion formation and organ dysfunction. Sepsis and/or secondary lung colonization causes secondary pneumonic plague.

Primary pneumonic plague is typically spread by aerosolized respiratory droplets from an infected patient. This form of the disease is characterized by rapid colonization of the lungs which causes pneumonia [33, 58, 62]. The symptoms of this disease are flu-like with the addition of bloody sputum occurring with the onset of pneumonia. According to the Centers for Disease Control and Prevention (CDC), 7% of all plague cases in the United States over the last century have been pneumonic [53, 55]. Animal transmission is the prime source for acquiring pneumonic plague, with infected cats and dogs coming into contact with humans leading to most cases. Typically human to human transmission only occurs in this form of the disease, with outbreaks in China in 2009 and almost yearly outbreaks in Madagascar since 2013 [9, 63-65]. There is a high fatality rate in pneumonic plague cases and without antibiotics there is a 100% chance of lethality and with treatment there is a 30 to 60% chance of survival [32, 33, 66].

Post-exposure treatment of *Y. pestis* generally involves a 10-day course of streptomycin and gentamicin [27, 53, 67]. However, post-fever treatment with levofloxacin has been shown to have 100% survival rate in African Green macaques infected with pneumonic plague [68, 69]. The use of fluoroquinolones

like levofloxacin, is new to plague treatment strategies and may have to be implemented if antibiotic-resistant *Y. pestis* become problematic in endemic areas [70]. Ciprofloxacin is typically used as post-exposure prophylaxis [71].

Virulence Factors of *Y. pestis*

The type 3 secretion system is encoded on the virulence plasmid pCD1. T3SS genes are under transcriptional regulation by the low calcium response (LCR) and are expressed at the ambient vertebrate host temperature which is around 37°C. Effector Yops, or *Yersinia* outer proteins, are expressed and injected into host cells upon contact [1, 72-77]. The T3SS is essential to virulence and is necessary to cause mortality in all three forms of plague [77]. These Yops all function to inhibit the innate immune system, either through inducing cellular death in immune cells or disrupting pro-inflammatory signaling [73, 76, 78]. YopJ is a cysteine protease that has also been shown to act as an acetyltransferase which disrupts NF- κ B and IRF3 signaling [79-82]. YopJ activity has been shown to inhibit caspase 1 cleavage, inhibit TNF α production, and induce apoptosis in macrophages [72, 83-86]. Another Yop effector, YopM, inhibits pyrin inflammasome formation and modulates neutrophil recruitment/activity [87-90]. Counter to the action of YopM, YopE stimulates pyrin, as well as depolymerizes actin which induces apoptosis and leads to a reduction in phagocytosis by host cells [72, 88, 91]. There is evidence that YopE reduces reactive oxygen species (ROS) generation in neutrophils by inhibiting RhoG activity on NADPH oxidase [91, 92]. LcrV which is a *Yersinia* protein that partially makes up the tip complex of the T3SS has been shown to interact with TLR2 and CD14 on host immune

cells to induce IL-10 secretion [93]. Taken together the secreted proteins of the T3SS act to disrupt pro-inflammatory signaling, limit intracellular killing of bacteria, and induce cellular death in host cells. This allows *Y. pestis* to proliferate unrestricted by the immune system until later stages of the infection in which there are massive amounts of TNF α and IFN γ in the serum [35, 94]. This dysregulation of pro-inflammatory cytokines, along with the subsequent damage caused by the bacteria, negatively affects patient immunopathology and ultimately causes death [32].

Y. pestis produces a hexaacylated LPS at temperatures below 28°C, but when introduced to a 37°C environment *Y. pestis* synthesizes a tetraacylated LPS [1, 95]. This type of LPS has been found to be anti-stimulatory in dendritic cells and macrophages through not only TLR4, but also TLR2 and TLR9 [96]. This modified LPS is another method by which *Y. pestis* inhibits innate immune responses to evade immune detection [96, 97].

The pigmentation (*pgm*) locus is a 102-kb segment of the *Y. pestis* genome that consists of 87 genes and two main parts: a high pathogenicity island encoding for iron acquisition and the pigmentation segment [37, 38, 98]. *Y. pestis* bacteria containing the pigmentation segment can be distinguished by their ability to uptake Congo Red dye which has a three-dimensional configuration resembling heme. This uptake is mediated by the *hms* (Hemin storage) locus. The *hms* locus is an important virulence factor mediating the biofilm blockage in the flea proventriculus and transport of heme [1, 12, 99-101]. Colonies containing the *pgm* locus present as red due to their ability to internalize

Congo Red, instead of pale white. This is an important distinguishing tool as the *pgm* locus is flanked by repeated copies of an insertion sequence named IS100. These insertion sequences cause the *pgm* locus to undergo spontaneous homologous recombination and become excised from the chromosome at a frequency of $1:10^5$ [98, 102, 103].

Iron is important for bacterial growth and the acquisition of iron is essential for pathogen survival. *Y. pestis* bacteria have multiple systems for acquiring iron, and all of them are under Fur (Ferric uptake regulator) regulation [104]. The *hms* locus acquires iron, from heme in mammalian hosts, but it is not required for virulence [100]. The Hms system cannot take iron from any other host storage systems which makes it less optimal than other *Y. pestis* iron-scavenging molecules [100, 105]. The Yfe and Feo iron acquisition systems have been shown to primarily take up ferrous iron and are hypothesized to be important in acquiring iron while *Y. pestis* is inside macrophages [106-108].

The most important siderophore for mammalian pathogenesis that is produced by *Y. pestis* is yersiniabactin (ybt). Ybt has a formation affinity with ferric iron that is $4 \times 10^{36} \text{ M}^{-1}$. Ybt binds ferric iron with greater affinity than host molecules that sequester iron: lactoferrin ($\sim 10^{22} \text{ M}^{-1}$) or transferrin ($\sim 10^{20}\text{-}10^{21} \text{ M}^{-1}$) [109, 110]. Yersiniabactin is synthesized from salicylate by the bacterial proteins: HMWP1 and HMWP2 encoded by *irp1* and *irp2* respectively. All of the genes that synthesize ybt are under Fur control and are located within the *pgm* locus [103, 111, 112]. Other genes such as *ybtS* which converts chorismate into salicylate, and *ybtT*, *ybtE*, and *ybtU*, have also been shown to be necessary for

ybt synthesis [112-114]. Yersiniabactin-bound iron is actively transported into the bacteria by the pesticin receptor which is encoded by the *psn* gene [114, 115]. Mutants lacking the *psn* gene can synthesize ybt and are able to export it. However, these mutants lack the mechanism to uptake ybt-bound iron effectively sequestering the iron from the host and the bacteria. Other genes that control the active transport of ybt are *ybtP* and *ybtQ*, which code for putative inner-membrane permeases [116]. Deletion of these two genes presented a similar growth defect as observed in the Δpsn mutant, when grown at 37°C, in iron-deficient media. These genes are all under YbtA control, an AraC-like transcriptional regulator [117].

The secretion of ybt has not been fully elucidated; however, the gene *ybtX* which codes for an inner membrane protein has been implicated in ybt efflux based on its predicted structure. Mutants lacking *ybtX* showed no lack of ybt secretion, ybt-bound iron uptake, or growth defects in iron-restrictive media [114]. It is unknown if ybt is able to bypass the cellular membrane of eukaryotic cells, and steal intracellular iron from the host [114]. *Y. pestis* mutants lacking *irp1* or *irp2* cannot synthesize yersiniabactin, but only suffer growth defects when cultured in iron-deficient media. *Y. pestis* Δpsn bacteria present a significantly greater growth defect in iron-deficient media in comparison to $\Delta irp2$, possibly due to constantly producing ybt [118]. Despite this growth defect in iron-limited environments, intranasally infected *Y. pestis* Δpsn has been found to be more virulent than $\Delta irp2$ in Swiss-Webster mice. With the observed LD50 of Δpsn *Y. pestis* recorded to be 17,050 CFU, whereas $\Delta irp2$ had an LD50 of 268,000 CFU.

This shows that the presence of ybt in the host confers a benefit to the bacteria beyond iron acquisition for bacterial growth [118].

Like the other virulence factors of *Y. pestis*, the innate immune system cannot offer an effective defense against ybt. Lipocalin 2 is a protein secreted primarily by neutrophils that has the ability to bind and sequester siderophores such as enterobactin, but not yersiniabactin [119-122]. *Klebsiella pneumoniae* bacteria that produced ybt and enterobactin exhibited significantly lower bacterial titers in wild-type mice lungs, when compared to bacteria that produced only enterobactin [123, 124]. This evasion of the immune system in the lung was attributed to lipocalin 2 being unable to bind and sequester ybt that was being produced by *Klebsiella* bacteria.

Yersiniabactin also acts as a superoxide dismutase mimic when bound to copper. This allows a new function of ybt, which would be to catalyze reactive oxygen species conversion into H_2O_2 and O_2 , providing another layer of bacterial protection from the host [125-127]. In *K. pneumoniae* infections, ybt also increases inflammation in the lung by stabilizing HIF-1 α (Hypoxia Inducible Factor 1- α). This protein is a sensor for hypoxia within cells, but can be activated in an oxygen-independent manner [128]. The presence of siderophores in the host environment will lead to anemic or low-iron responses which will increase HIF-1 α stability and upregulate its transcription. Bacterial pathogen-associated molecular patterns (PAMPs) such as LPS can stimulate TLR4 and can also stabilize HIF-1 α [129-131]. In bacterial infections, HIF-1 α stabilization has been linked to increased intracellular killing of bacteria and promoting inflammation in

Pseudomonas aeruginosa infections [132]. However, in cases of sepsis caused by cecal ligation puncture (CLP) or in *K. pneumoniae* infection, the presence of HIF-1 α causes a detrimental effect to the host [128, 133, 134]. These other pathogenic factors allow yersiniabactin to confer more benefit to the bacteria than just iron-scavenging for bacterial growth.

Host Response to *Y. pestis*

The host response to *Y. pestis* is dysregulated and non-effective as plague generally develops so rapidly that mounting an effective immune response cannot occur [36, 135]. With bubonic plague killing the host within 7-10 days after exposure and pneumonic models known to kill within 72-96 hours, there is very little time for the immune system to coordinate and produce a protective response [32, 33, 36].

The cells most likely to initially encounter *Y. pestis* upon infection of the host are myeloid cells. Macrophages and neutrophils are able to phagocytose *Y. pestis* and it has been shown that neutrophils are necessary for clearance of *Y. pestis* [136, 137]. However, YopM has been shown to improve neutrophil survival and to change the chemokine profile of infected lungs [90, 138, 139]. This leads to neutrophil recruitment to the exterior of plague lesions and not to the plague foci. These pieces of data taken together could indicate that *Y. pestis* directs neutrophil function to create additional tissue damage in the lung, and prevent bacterial phagocytosis by misdirection of neutrophils [90]. YopJ has been shown to inhibit production of TNF α and IFN γ in macrophages, thus preventing

activation of nearby immune cells [80, 140-142]. TNF α and IFN γ are important pro-inflammatory cytokines that are critical to the clearance of plague [94].

Y. pestis bacteria have been shown, that if internalized by macrophages, they have the ability to replicate inside the phagolysosome [36, 86, 143, 144]. During *Y. pestis* internalization, Rab1b, a host factor that regulates vesicular protein transport and fusion, is recruited to the phagolysosome. Knockdown of Rab1b increased vacuole acidification and association with LAMP1, leading to lower bacterial intracellular survival within the *Yersinia* Containing Vacuole (YCV) [145]. This suggests that *Y. pestis* uses the host factor Rab1b to prevent phagosome maturation and improve intracellular survival.

Genes from the *pgm* locus linked to intracellular survival, *ripA* and *ripB*, were found to decrease nitric oxide production in IFN γ -stimulated macrophages, thereby preventing bacterial destruction [146]. The *phoPQ* genes also facilitate intracellular growth and replication inside of macrophages and neutrophils by allowing resistance to low pH and oxidative stress [147, 148]. A commonly held hypothesis is that macrophages can become a vehicle that *Y. pestis* uses to disseminate throughout the body. This idea has been recently expanded to encapsulate neutrophils and dendritic cells (DCs), which might also be possible dissemination candidates [149, 150]. However, *Y. pestis* is not contingent on spreading throughout the body using immune cells. By compromising the host organ's integrity between itself and the blood, *Y. pestis* can gain access to the blood and traffic throughout the body [33, 41, 151].

Among the innate immune cells involved with *Y. pestis* infection, DCs have been studied the least. DCs are integral to long-term immunity from bacterial pathogens as they destroy the pathogens in the phagosome and present antigens from those pathogens to T and B cells [152]. *Y. pestis* has been shown to downregulate MHCII in DCs, but does not change the chemokine profile; this allows the DCs to traffic to the lymph nodes aiding in potential dissemination [153-155]. This lack of activation in the DCs is due to two factors: the type 3 secretion system and the tetraacylated LPS [154, 156]. The T3SS paralyzes cytoskeletal rearrangement preventing antigen processing, and disrupts intracellular cell signaling to prevent activation [153]. The tetraacylated LPS has been shown to prevent DC activation by binding to TLR4 [96]. These factors that affect the activation and antigen presentation of *Y. pestis* epitopes, decrease the ability of the adaptive immune response to become involved in the resolution of the infection.

Recent reports of the mammalian inflammation profile during pneumonic plague have described it as a bi-phasic response [35, 143, 157]. During the first 24 hours post-infection (HPI), there is a slight increase in inflammation and recruitment of innate immune cells to the lungs. Between 24-36 HPI there is little cytokine production and no active immune cell recruitment; this is followed by a hyperinflammation response [7, 32, 33, 55, 69]. This ineffectual inflammation response typically leads to septic shock and patient death. Finding ways to correct the inflammation response to *Y. pestis* respiratory infection presents a potential opportunity to develop novel host therapies against the infection.

Heme oxygenase-1

Iron is important for cellular function because of its reversible oxidation state. This ability makes iron integral in maintaining intracellular redox balance, electron transfer reactions, and delivering oxygen to tissues [158, 159]. Heme is a molecule expressed all throughout the body; it is a tetrapyrrole ring with a Fe^{2+} ion at the center [160, 161]. Hemoglobin is a storage carrier of heme that facilitates the transport of dissolved gases throughout the body. Hemoglobin is comprised of four heme subunits with their own protein carrier chain [162, 163]. Should these carrier proteins be denatured or broken, this would produce free heme. The presence of free heme in aqueous environments can generate ROS which can damage lipids, proteins, and nucleic acids [164-167]. Free heme is a damage-associated molecular pattern (DAMP) that transduces through TLR4, CD14, and MyD88 leading to pro-inflammatory cytokine production [166-168].

Heme oxygenase is the enzyme which catabolizes heme into Fe^{2+} , carbon monoxide, and biliverdin [169]. There are three isoforms of the enzyme heme oxygenase, however, HO-3 is a poor catalyst. Heme Oxygenase 1 (HO-1) is inducible, whereas HO-2 is constitutively made by every cell in the body, especially in tissues (liver and spleen) in which erythrocytes are degraded [167, 169-172]. HO-1 can be induced through many outside stressors including tissue damage, hypoxia, anemia, and oxidative stress. These stress signals initiate cleavage of Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) from Keap1 (Kelch-like ECH-associated protein 1). Nrf2 then translocates to the nucleus and activates transcription of *Hmox1*, the gene that encodes for HO-1. BACH1 is a

repressor that blocks HO-1 transcription and must dissociate from the promoter region of HO-1, before Nrf2 can activate transcription of HO-1, ferritin, and other antioxidant-related genes [170, 173, 174]. BACH1 dissociates upstream from the antioxidant response element (ARE), when the cell is undergoing oxidative stress [175-177].

The Fe^{2+} that is produced from the catabolism of heme is oxidized to Fe^{3+} by ferroxidases in the cytoplasm [160, 165, 178]. This process controls for the potential oxidative effect of Fe^{2+} in an aqueous environment by limiting spurious catalyzation of the Fenton reaction and subsequent ROS generation. Fe^{3+} is stored in the heteropolymeric globular protein of ferritin that can store large amounts of iron ions [179, 180]. Ferritin can be an effective way of sequestering iron from extracellular bacterial pathogens as it is induced during bacterial infection, most noticeably in the liver [164, 178, 179].

Carbon monoxide (CO) is a cell-permeable, signaling molecule similar in mode of action to nitric oxide and hydrogen sulfide [181-183]. Carbon monoxide is typically anti-inflammatory in its effects and has been shown to be protective against sepsis in the CLP rodent model, by upregulating IL-10 production and repressing $\text{TNF}\alpha$ [184]. CO also downregulated TLR4/MD-2, by suppressing membrane trafficking of TLR4 to the cellular membrane by inhibiting its glycosylation which is required to allow interaction between TLR4 and MD-2. This lowers the chance that endotoxin and free heme will bind to TLR4/MD-2, and thus limits inflammation [185, 186]. There seems to be dual roles of CO in bacterial infections as there are reports that indicated that CO exposure to

macrophages increased phagocytosis and clearance of bacteria. This was due to CO increasing production of bacterial ATP and this action stimulated the NLRP3 inflammasome which significantly increased bacterial killing [187]. There is also evidence of CO upregulating autophagy and allowing epithelial cells to endure hyperoxic intracellular cell states [188]. This upregulation of autophagy could be a potential pathway in which CO increases bacterial killing. This increase in autophagy could be removing host tissue in the lung in a non-inflammatory manner, quelling sepsis by removing potential DAMPs [188-190]. More directly, CO has also been observed to negatively regulate NLRP3 activation and IL-18 secretion during sepsis [189]. These conflicting results with CO with regards to the inflammasome indicates that the role of CO and NLRP3 in the context of bacterial infections have yet to be fully elucidated.

Another product of HO-1 enzymatic activity is biliverdin which can be reduced to bilirubin [169, 191, 192]. Bilirubin and biliverdin provide cytoprotection against oxidative necrosis induced by NO, peroxide, or hypoxic stress. Exogenous biliverdin and bilirubin have protected animals against sepsis and works in concert with HO-1 and the products of heme catabolism to restore redox balance and lower inflammation [192, 193].

While *Y. pestis* is not shown to be hemolytic on blood agar plates, there is evidence of hemorrhage into the tissues during the course of the infection [22, 118]. Hemorrhaging into the tissues could lead to the release of free heme which could exacerbate damage by spontaneous formation of ROS or by inducing inflammation through TLR4 [167, 169, 194]. *Y. pestis* is also known to cause

cellular lysis throughout the course of an infection that could possibly result in free heme release. When combined, these occurrences could saturate the tissues with heme and overload the heme scavenger proteins haptoglobin and hemopoexin [164]. This could potentially lead to spontaneous ROS formation and subsequent oxidative damage to host cells, exacerbating inflammation.

Heme oxygenase in Bacterial Infections

The importance of HO-1 in bacterial infections has not been completely elucidated. It seems to have deleterious effects in the infection models it has been studied in, such as *Burkholderia pseudomallei* and *Salmonella enterica* Typhimurium [195, 196]. These effects are due to the anti-inflammatory byproducts (CO) and anti-oxidant properties of HO-1 enzymatic activity. However, it has been shown that silencing HO-1 expression induces further oxidative stress [171].

Burkholderia pseudomallei induced HO-1 expression in infected macrophages [195]. This led the researchers to investigate if this host factor was being manipulated to benefit the pathogen. Induction of HO-1 by Cobalt Protoporphyrin (CoPP) caused lowered production of proinflammatory cytokines, primarily IFN γ , and increased bacterial burdens within macrophages, and in subsequently infected tissues. These pro-disease effects were found to be primarily due to the carbon monoxide production from HO-1 enzymatic activity [195].

The presence of *Salmonella enterica* Typhimurium did not upregulate HO-1 production but does exploit its anti-inflammatory, enzymatic effects [196]. When HO-1 was silenced, *S. Typhimurium* bacterial burdens were lowered in macrophages and the gene *Ncf1* that encodes for an ROS-producing enzyme was upregulated. The authors postulated that lowered HO-1 activity led to less intracellular iron which stymied bacterial growth from within macrophages, but this was not directly shown. Overall this was another case in which HO-1 was found to be deleterious to host protection [196].

In the case for *P. aeruginosa* it has been found that HO-1 serves a protective role in host defense [197]. When HO-1 activity was upregulated, lungs of animals infected with *Pseudomonas aeruginosa* had lower neutrophilic lung inflammation. Researchers also found upregulation of Bcl-2, an anti-apoptotic factor linked to CO signaling. Both of these effects were found to increase the overall health of infected lungs [198]. *P. aeruginosa* has been shown to release a quinolone signal that induces reactive oxygen stress by downregulating HO-1 [197].

In the genus *Mycobacterium*, studies are somewhat conflicting. *Hmox1*^{-/-} mice infected with low dose intranasal inoculation of *Mycobacterium avium* succumbed significantly faster than their wild-type experimental counterparts [199]. However, in *Mycobacterium tuberculosis* infections it has been found that inhibition of HO-1 reduced proinflammatory cytokine production and reduced intracellular bacterial growth in human macrophages [200-202].

Considerations and Research Aims

Our laboratory has defined and investigated plague pathology through various mouse and rat models and time and again we have produced evidence of a dramatic immune response that has also been seen by our collaborators and peers [203-207]. However, given the virulence factors of *Y. pestis*, traditional stimulation through TLRs and other innate immune sensors should be quieted. The bi-phasic immune response is another key piece of evidence indicating that there is a dysregulated immune response toward *Y. pestis*. There is extensive tissue damage during the second inflammatory response to *Y. pestis* respiratory infection [32, 35, 62, 157]. Assuming the virulence factors of *Y. pestis* evade the immune response, a possible cause for this hyperinflammation toward the end of the bi-phasic response could be the damage to the host of the bacterial infection. We have shown that the *pgm* locus is required for tissue damage in the lung. The research described herein aimed to understand the link between the virulence factor yersiniabactin and lung tissue damage, and how the cytoprotective enzyme heme oxygenase-1 protects from pneumonic plague.

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Chapter 2. Materials and Methods

Ethics Statement. All animal procedures were in compliance with the Office of Laboratory Animal Welfare and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Missouri Animal Care and Use Committee.

Bacterial Strains. *Yersinia pestis* strains were routinely grown fresh from frozen stock by streaking for isolation onto Heart Infusion Agar (HIA) plates or HIA plates supplemented with 0.005% (w/v) Congo Red and 0.2% (w/v) galactose to screen for bacteria that retain the pigmentation locus [1]. Inocula for intranasal challenges were prepared from single colonies and incubated at 37°C shaken at 125 rpm for 16-20 hours in Heart Infusion Broth (HIB), supplemented with 2.5 mM CaCl₂. Bacterial concentration was measured by absorbance at OD₆₀₀, cultures were diluted to their appropriate inoculum in Phosphate Buffered Saline (PBS) and plated on HIA for CFU verification. For *in vitro* assays conducted at BSL2, single colony inocula were incubated at 26°C in HIB for 16-24 hours shaken at 125 rpm. Cultures were then diluted 1:20 in HIB supplemented with 2.5 mM CaCl₂ and incubated at 37°C and 125 rpm for 2.5 hours. All experiments using the bacterial strains that are noted as select agents were conducted at the University of Missouri's Laboratory for Infectious Disease Research (MU LIDR), a select agent-authorized biosafety level 3 laboratory.

Treatment Regimen for CoPP and ZnPP. Cobalt protoporphyrin (25 mg) (Enzo Life Sciences, Farmingdale, NY., USA) was dissolved in 10 mL of 0.1 M NaOH,

solution was then titrated with 1 M HCl, to physiological pH for mammals (pH 7.4), and then PBS was added to a final volume of 50 mL [2]. Zinc protoporphyrin (25 mg) (Millipore-Sigma, St. Louis, MO., USA) was dissolved in 1 mL of Dimethyl sulfoxide (DMSO) and titrated to a final volume of 50 mL [3]. Both CoPP and ZnPP were filter sterilized before use. Mice were pretreated 24 hours before infection with 5 mg/kg intraperitoneally of CoPP or ZnPP. A second treatment occurred after intranasal infection. Subsequent treatments occurred every 48 hours post- infection, until the final treatment was administered on day 8. Vehicle controls for CoPP treatments were equal volumes of PBS and vehicle controls for ZnPP were equal volumes of PBS and DMSO.

Bone Marrow-derived Macrophage (BMDM) and Dendritic Cell (BMDC)

Isolation. Bone marrow was isolated from C57BL/6 mice by flushing the femurs and tibias with cold PBS. The bone marrow was centrifuged at 1500 rpm for 5 minutes and the pellet underwent ACK (Ammonium-Chloride-Potassium) lysis for 5 mins. Cells were rescued by 5 volumes of medium and were centrifuged at 1500 rpm for 5 minutes. Cells were divided into bacterial culture dishes. Bone marrow cells that would make BMDMs were cultured for 7 days in Dulbecco's modified Eagle's medium (DMEM) containing 20 ng/mL M-CSF (Shenandoah Biotechnology, Warwick, PA., USA), 10% FBS (fetal bovine serum, eBiosciences, San Diego, CA., USA) [4]. BMDCs were cultured for 10 days in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 20 ng/mL GM-CSF (Shenandoah Biotechnology), 10% FBS, 1% sodium pyruvate, 1% Non-essential Amino Acids, and 50 μ M of β -mercaptoethanol [5]. After 10 days of

culture, cells (1×10^6) were seeded into 12-well plates in their respective growth media and incubated overnight prior to infection.

Western Blot. Cells were lysed in RIPA buffer (Cell Signaling Technologies, Danvers, MA., USA) supplemented with protease inhibitor cocktail for use with mammalian cell and tissue extracts (Goldbio, St. Louis, MO., USA) and phosphatase inhibitor cocktail according to manufacturer's guidelines (Goldbio). Immediately before analysis, samples were diluted 1:1 in 2X Sample Buffer (50 mM Tris HCl, pH 6.8, 1.6% (w/v) SDS, 7% (v/v) glycerol, 8 M urea, 4% β -mercaptoethanol, 0.016% (w/v) bromophenol blue) and boiled for 10 minutes. Samples were loaded into 1mm-thick 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Transfer buffer: 5.8% (w/v) Tris, 24.3% (w/v) glycine, 20% (v/v) methanol 0.1% (w/v) SDS), at 50 mA for 20 hours. Membranes were blotted for at least 1 hour in 1% bovine serum albumin (BSA) in 1X Tris-Buffered Saline (TBS) with 0.1% (v/v) Tween-20. Membranes were probed with primary antibodies overnight at 4°C, washed three times for 10 minutes each, and probed with secondary antibodies for 2 hours at room temperature. All incubations occurred with gentle agitation on a rocker. Membranes were washed and Immobilon Western Chemiluminescent HRP Substrate solution (Millipore-Sigma) was added. Visualization of bands was either done on blue radiography film or captured with a digital camera. Band intensities were quantified from the 16-bit digital image by densitometry in ImageJ and are shown normalized to the loading control lane for each target [6].

Histopathology. Lungs were perfused and fixed in 10% formalin; liver and spleen were also fixed in 10% formalin. Organs were further processed for paraffin embedment, blocked in wax, and cut into 5- μ m sections. Tissue sections were stained with hematoxylin and eosin, and stained slides were affixed with permanent coverslips at RADIL IDEXX (Columbia, MO., USA). Sample identities were single-blinded for analysis. For quantification, inflammatory lesions, necrosis, hemorrhage, edema and other lesions were scored 0-4, or 0-5 in lung samples, for increasing severity in size and/or frequency in at least 10 non-overlapping fields for each tissue. The scoring rubric used for these tissues was established and used in previous studies [7, 8].

- 0: Not infected
- 1: Small lesions present (0-10% tissue section affected), less than 5 lesions in the fields of view.
- 2: Larger lesions present (between 15-25% of the tissue affected) and more frequent lesions (5-15).
- 3: Presence of necrosis within the tissues with greater than 15 lesions present. At least 25% to 50% of the tissue affected with lesions or necrosis.
- 4: Greater than 50% of the tissues affected with lesions or necrosis. Large lesions present with necrosis.
- 5: (Lung only) Greater than 50% of the tissues affected with lesions or necrosis. Large lesions present with necrosis and large bacterial colonies occupying the alveolar space.

Imaging and scoring were conducted by Dr. Deborah Anderson.

Quantitative Polymerase Chain Reaction. RNA isolation was performed using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Germantown, MD., USA). Total RNA was treated with Turbo DNase (Ambion, Foster City, CA., USA) to remove genomic DNA contamination. First strand cDNA synthesis was carried out using MMLV-RT (Promega, Madison, WI., USA) on 2 µg of total RNA as per manufacturer's instructions. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA., USA) was used along with gene specific primers (Table 2.2) to detect the presence of amplified product. Results were analyzed using relative quantification on 7300 SDS software (Applied Biosystems). Data were normalized to the mouse gene *Ywhaz* [9].

ELISAs and Protein Assays. Blood was collected post-mortem by cardiac puncture and serum was separated via centrifugation at 10,000 rpm for 10 minutes. Lung were homogenized in sterile PBS and centrifuged at 10,000 rpm for 5 minutes to remove cellular debris. Serum and lung homogenate were treated with an antibiotic cocktail consisting of gentamicin, streptomycin, and penicillin to inactivate *Y. pestis* and stored at -80°C for future analysis. Antibiotic cocktail final concentrations were as follows: gentamicin (4 mg/mL), streptomycin (1,000 µg/mL), and penicillin (1,000 units/mL). For cytokine analysis, serum and lung homogenate were analyzed by Multiplex (Millipore-Sigma) for 5 cytokines, known to play a role in plague: IFN γ , TNF α , IL-1 β , IL-6, and IL-10. For testing individual cytokines as well as HO-1, ELISA was used: TNF α , IL-6, IFN γ , (R&D Systems, Minneapolis, MN., USA); IFN β (PBL Interferon, New Jersey

Piscataway, NJ., USA); HO-1 (Enzo Life Sciences). All ELISAs and Multiplex assays were conducted according to the manufacturer's instructions and were performed by Dr. Rachel Olson. Standard liver panel testing was conducted on serum (Comparative Clinical Pathology Services, Columbia, MO., USA); reference standards for C57BL/6 mice were obtained from Charles River Laboratories (www.criver.com). For total protein assays, lungs were perfused with PBS and lavage fluid was analyzed for total protein content by Bradford Assay (Bio-Rad, Hercules, CA., USA) [10].

FITC-Dextran Lung Permeability Assay. C57BL/6 mice that were infected with *Y. pestis* were sedated with isoflurane and intranasally instilled with filter-sterilized 5 µg of FITC-Dextran dissolved in 10 µL sterile PBS. 2 hours after FITC-Dextran instillation mice were euthanized and exsanguinated via cardiac puncture. Collecting a minimum of 500 µL of blood, it was then centrifuged at 10,000 rpm for 10 mins and a minimum of 200 µL of serum was collected. Serum was treated with an antibiotic cocktail containing: gentamicin, streptomycin, and penicillin to inactivate *Y. pestis*. To measure FITC-Dextran content in the blood, serum was then analyzed for fluorescence at 485 nm excitation and 528 nm emission. Fluorescent intensity values were normalized to fold change compared to naïve control [11].

Flow Cytometry. Lungs were excised from mice and the trachea was removed. The lobes of the lungs were cut into small pieces and were incubated in 3 mL of dissociation buffer (final concentration: 0.7 mg/mL of Collagenase A, 30 µg/mL of DNase A dissolved in Hank's Buffered Saline Solution) at 37°C agitated at 75

rpm [12, 13]. After 1 hour, the lung tissue was gently homogenized with a glass homogenizer. Homogenate then underwent ACK lysis for 5 minutes at room temperature; cells were rescued by adding 5 volumes of PBS and supernatant was decanted after centrifuging the cells at 1200 rpm for 5 minutes. The cells were resuspended in PBS and passed through a 70- μ m cell strainer and were then spun at 1200 rpm for 5 mins and buffer was decanted. Cells were incubated with 5 μ L Fc receptor blocking solution (BioLegend, San Diego, CA., USA) to eliminate non-specific binding for 30 min. After washing cells in PBS, cells were stained with titrated antibodies conjugated to fluorophores for 1 hour, after which the cells were washed with PBS and fixed by incubating with 4% paraformaldehyde overnight at 4°C. Cells were analyzed on a Beckman Coulter CyAn ADP (Beckman Coulter, Brea, CA., USA) using FlowJo 10 software (Flowjo LLC, Ashland, OR., USA).

Enumeration of Bacterial Titers and *in vivo* Cytokine Analysis. Immediately after euthanasia, blood was collected directly from the heart by cardiac puncture. Lungs, livers, and spleens were removed and homogenized in 1 mL of sterile PBS. Serial dilutions of homogenized tissues were then plated in triplicate onto HIA plates for quantification of bacterial titer (CFU/organ). For cytokine analysis, lung homogenate or serum was collected, treated with gentamicin, penicillin, and streptomycin, and stored at -80°C until analyzed.

Coinfection Study. C57BL/6 mice were intranasally infected with 5×10^3 CFU of KIM5+ and 5×10^3 CFU of KIM5- *Y. pestis*. Control mice were infected with 1×10^4 CFU of KIM5- to monitor *Y. pestis pgm*- infection progression. At 24, 48, 72 HPI,

lungs, livers, and spleens were homogenized in 1 mL of sterile PBS and serially diluted in sterile PBS. Dilutions were enumerated by plating on HIA with Congo Red and counting the colonies 3 days after plating. Colonies were distinguished as *pgm*⁺ by being able to uptake Congo Red dye and presenting as red colonies, and *pgm*⁻ bacteria were distinguished as white colonies.

Cell Death Determination. Levels of supernatant LDH were determined 6 hours post infection using the CytoTox 96 non-radioactive cytotoxicity assay (Promega) according to the manufacturer's instructions. LDH release was calculated as % max lysis control. Maximum lysis was determined by adding 10 μ L of lysis solution to control wells 10 minutes prior to harvest. Percent max was calculated as (Experimental – background) / (Max lysis – background) x 100. Uninfected controls were used to normalize experiments.

Yersiniabactin Treatment. Yersiniabactin was purchased from Genaxxon (Genaxxon, Ulm, Germany). Yersiniabactin was dissolved in 100% methanol and stored at -20°C. Treatments of 25 μ g of yersiniabactin were titrated to a final volume of 10 μ L in sterile PBS and intranasally instilled into mice or directly added into cell medium.

Intracellular Survival Assay. Overnight cultures of *Y. pestis* CO92 were incubated at 37°C in HIB with 2.5 mM CaCl₂. KIMD27, KIM6⁺, KIM6⁻ were incubated at 26°C overnight and then diluted 1:20 and incubated at 37°C for 2.5 hours. Macrophages and dendritic cells were infected at a multiplicity of infection (MOI) of 50 and the plates were centrifuged at 500 rpm for 5 minutes before incubating at 37°C, 5% CO₂. Following 30 min, cells were washed with sterile

PBS and incubated in cell culture medium containing 50 µg/mL gentamicin and the infection continued for an additional 1.5 or 7.5 hours. For bacterial titers, media was aspirated, and wells were washed once with sterile PBS. Infected macrophages and dendritic cells were lysed with 300 µL of 0.1% (v/v) Triton X-100 in PBS, cells were then rescued with 700 µL of HIB, then serially diluted and plated in triplicate on HIA for enumeration of colony forming units. To ensure that only intracellular bacteria had been enumerated, 10 µL of aspirated media was plated on HIA for each well and no bacterial growth was recovered in these samples.

Statistical Evaluation. Data from all trials were analyzed for statistical significance using GraphPad Prism software (San Diego, CA., USA). Significance was concluded when $p < 0.05$. Biological replicates within experiments were pooled after verification of controls and visual analysis of data sets. Replicate analysis was also used to prove there were no significant differences between independent experiments. The D'Agostino-Pearson normality test was used to ensure normal distribution of data.

Table 2.1. Strains used in this work.

Strain	Genotype	Common Name
<i>Yersinia pestis</i>		
Orientalis		
CO92	<i>pgm+</i> , T3SS+	CO92 (Select agent)
Medievalis		
KIM5+	<i>pgm+</i> , T3SS+	KIM5+ (Select agent)
KIM D27	<i>pgm-</i> , T3SS-	KIMD27
KIM5+ $\Delta irp2$	<i>pgm+</i> , T3SS-	$\Delta irp2$
KIM5+ $\Delta irp2$ pCD1AP	<i>pgm+</i> , T3SS+, Amp ^r	$\Delta irp2$ pCD1AP
KIM5+ Δpsn	<i>pgm+</i> , T3SS-	(Select agent)
KIM5+ Δpsn pCD1AP	<i>pgm+</i> , T3SS+, Amp ^r	Δpsn pCD1AP
		(Select agent)
KIM6+	<i>pgm+</i> , T3SS-	<i>Y. pestis pgm+</i> (<i>pgm+</i>)
KIM6-	<i>pgm-</i> , T3SS-	<i>Y. pestis pgm-</i> (<i>pgm-</i>)

Table 2.2. Primer sequences used for quantitative PCR.

Gene target	Sequence
<i>Hmox1</i>	F: 5' cacgcatatacccgctacct 3' R: 5' aaggcgggtcttagcctcttc 3'
<i>Ifnβ</i>	F: 5' caagatccctatggagatga 3' R: 5' aagaaagacattctggagca 3'
<i>IL1β</i>	F: 5' gatccacactctccagctgca 3' R: 5' caaccaacaagtgatattctccat 3'
<i>Vegfa</i>	F: 5' gccctgagtcaagaggacag 3' R: 5' ggggtaaggagaggacgaag 3'
<i>Tnfa</i>	F: 5' ccccaaagggatgagaagtt 3' R: 5' cacttggtggttgacctacga 3'
<i>Ywhaz</i>	F: 5' cacagcctcccctcatcct 3' R: 5' gggagacggtgacagaccat 3'
<i>Ncf1</i>	F: 5' gtcaaaccaccccataccac 3' R: 5' accggagttacaggcaaatg 3'
<i>Ifng</i>	F: 5' ctgatgggaggagatgtcta 3' R: 5' agcctgttactacctgacaca 3'

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Chapter 3. Yersiniabactin creates a permissive environment in the lungs, promoting pneumonic plague.

Abstract

Yersinia pestis is the etiological agent of plague. This disease is characterized by a rapidly established infection that leads to a widespread bacteremia and death of the host within days. The pigmentation (*pgm*) locus is a 102-kb region of the chromosome that encodes for most of the iron-sequestering genes in *Y. pestis* including those for the siderophore yersiniabactin (ybt). It has been previously shown that loss of the *pgm* locus removes the ability of *Y. pestis* to establish a pneumonic infection and there is little to no inflammation present in the lungs of *pgm*⁻ *Y. pestis* infected animals. Previous research has determined that bacteria that produce ybt have increased virulence and this suggests that ybt could be the invasive factor leading to pneumonia. We found that the presence of *pgm*⁺ bacteria conferred a growth advantage to *pgm*⁻ in the lungs when coinfecting. This suggested that yersiniabactin could be altering the lungs to facilitate a growth niche leading to increased lung damage and permeability. We determined that this damage was not related to ybt causing hypoxic-induced damage, but that ybt allows for bacterial growth in the airspace. However, we found that a hypoxic-controlled factor, heme oxygenase-1 could be induced during ybt-induced damage.

Introduction

Yersinia pestis is the bacterium that causes the disease plague [1-6]. The disease presents in one of three ways: bubonic, septicemic, and pneumonic. Bubonic plague typically develops from a bite from a flea that is infected with *Y. pestis*. The bacteria enter the skin and are carried to the nearest draining lymph node, causing inflammation and necrosis [2, 7-11]. The swollen and discolored lymph node that results is called a bubo. Septicemic plague occurs when *Y. pestis* enters the bloodstream, quickly spreads to tissues all throughout the body, and establishes colonies within these peripheral tissues [1, 6, 8, 12-14]. Presence of *Y. pestis* in the bloodstream as well as the damage to the tissues causes sepsis in the infected host, leading to multi-organ failure and death [15-17]. Primary pneumonic plague is caused by transmission of *Y. pestis* via aerosolized droplets infecting the lung [2, 18, 19]. Secondary pneumonic plague starts as a septicemic infection that then infects the lung, which could lead to aerosolized spread of *Y. pestis* to others [20].

Pneumonic plague is characterized by high titers of bacteria, tissue damage and inflammation in the lungs. Patients either die from acute respiratory distress or congested organ failure from bacteria [1, 21, 22]. Those who have died from pneumonic plague often show signs of pleural effusions and hemorrhages with bloody or fibrinous exudate within the lungs [23]. High amounts of immune cells, mostly neutrophils are within the lungs near or at the time of death [24, 25]. This adds to the inflammation caused by the bacteria and increases the permeability of the vasculature due to mass extravasation and pro-

inflammatory cytokines. Damage combined with hyperinflammation in the lungs leads to a breakdown between the lung barrier and the blood [26-28].

Dissemination into the rest of the body is swift and colony formation is rapid within the tissues, occurring mostly in the spleen and liver.

Y. pestis has several virulence factors that allow the bacteria to proliferate within the host. These include a type 3 secretion system under the control of the low calcium response (LCR). The T3SS disrupts pro-inflammatory cytokine production and facilitates cellular death in the host by injecting effector Yops that disrupt intracellular signaling [29-35]. The bacterium also has the *pgm* locus that encodes for the proteins that are required for the production of the siderophore yersiniabactin [2, 16, 36]. This siderophore is necessary to promote bacterial growth of *Y. pestis* in iron-limited environments within the host, such as the lung [37, 38].

Previously published data from our laboratory established that mice intranasally challenged with *pgm*- bacteria do not contract pneumonic plague [39]. *Y. pestis pgm*- respiratory infections had lower bacterial titers and less inflammation observed by histology in the lung, when compared to *Y. pestis pgm*+ infections [39, 40]. Research from the Perry laboratory indicated that a mutant *Y. pestis* strain (Δpsn), which can only produce ybt but was not able to utilize the iron gained by yersiniabactin, showed increased virulence in intranasally-infected Swiss-Webster mice when compared to a strain unable to produce ybt ($\Delta irp2$) [41]. This led us to postulate that ybt, could be playing a role

in the establishment of pneumonia within the host and that this was unrelated to its acquisition of iron for the bacteria.

Yersiniabactin (ybt) is a siderophore produced by *Y. pestis* that has a very strong affinity to Fe^{3+} [42]. The *pgm* locus encodes most of the iron-sequestering genes for *Y. pestis*, including the proteins that encode for ybt synthesis: High Molecular Weight Protein1 (HMWP1) and High Molecular Weight Protein 2 (HMWP2) [43]. The genes that encode for the HMWPs are *irp1* and *irp2*. These HMWPs convert salicylate into yersiniabactin [44, 45]. Other genes such as *ybtS* which encodes for the protein that converts chorismate into salicylate, as well as *ybtT*, *ybtE*, and *ybtU* have been shown to be necessary for ybt synthesis [46, 47]. These genes are under Fur repression and without these genes yersiniabactin cannot be produced by the bacteria [47-50]. These genes are also under the control of YbtA, which encodes for an AraC-like transcriptional regulator [51]. Ybt-bound iron is actively transported into the bacteria by the pesticin receptor, encoded by the *psn* gene [52, 53]. Other genes that encode for the proteins necessary for the active transport of ybt are *ybtP* and *ybtQ*, which are putative inner-membrane permeases [54]. Deletion of these two genes presented a similar growth defect to the Δpsn mutant, when grown at 37°C in iron-deficient media. However, it is still unclear how these proteins interact with the pesticin receptor.

Lipocalin 2, also known as siderocalin, is secreted by neutrophils and binds to catecholate-type siderophores to inhibit their iron-sequestering activity [55-57]. Ybt has a mixed-type siderophore structure, meaning that it can evade

lipocalin 2 [58]. Evidence of this evasion can be found in *Klebsiella pneumoniae* bacteria that are able to produce ybt, as they have been shown to grow unimpeded in the lung with the presence of lipocalin 2. While in comparison, *K. pneumoniae* bacteria that produced the siderophores salmochelin or enterobactin had reduced bacterial titers in the lung when lipocalin 2 is present [59].

Another way that yersiniabactin allows for evasion of the innate immune response is by reducing the ability of myeloid cells to produce reactive oxygen species (ROS). The siderophore accomplishes this in two ways. It reduces the iron availability in the environment thus lessening the immune cell's ability to catalyze the Haber-Weiss reaction which produces hydroxyl radicals [60, 61]. The other way is that yersiniabactin can bind to Cu^{2+} and function as a superoxide dismutase mimic, thereby converting the ROS into O_2 and H_2O_2 [62, 63].

Myeloid cells including macrophages and dendritic cells are sentinel cells that would first encounter *Y. pestis* bacteria in the lower respiratory tract upon respiratory infection [24, 64]. Macrophages have been studied by others in the field, and they have established that the presence of the *pgm* locus increases survivability of *Y. pestis* within the phagolysosome [65-68].

Due to these methods that yersiniabactin uses to evade the host immune response and the observed virulence phenotype we hypothesized that ybt may be the *pgm*-encoded factor that permits alveolar growth. In this work, we tested this hypothesis and found a potential role for ybt in invasion and growth in the alveoli.

Results

Our previous observations showed that the *pgm* locus was required for lung inflammation and to establish primary pneumonic plague. We wanted to know if the presence of the *pgm* locus affected intracellular survival of the bacteria in dendritic cells, some of the first responders to infection within the lung. We therefore tested the effects the *pgm* locus had on uptake and survival within dendritic cells (DCs), as they are some of the ways that *Y. pestis* can evade and potentially disseminate throughout the body. Since dendritic cells traffic to the nearest lymphoid organ upon phagocytosis of foreign material, the *pgm* locus could aid in dissemination by allowing *Y. pestis* to survive longer within dendritic cells [65, 69]. To test this, dendritic cells (BMDCs) were cultured from mouse bone marrow for 10 days with GM-CSF, and then infected with *Y. pestis* at an MOI of 50. Gentamicin protection assays were conducted and at 2 HPI, there was a significant decrease in dendritic cells' ability to take up *pgm*⁺ *Y. pestis* bacteria. However, a gentamicin protection assay that was conducted to 8 HPI, observed no significant change in intracellular survival between the bacteria. In contrast to observations seen in macrophages, there was no significant increase to survival of *Y. pestis* bacteria when the *pgm* locus was present (Fig. 3-1A) [65].

We then hypothesized that the *pgm* locus increased DC-dependent pro-inflammatory transcription within DCs, which could lead to increased inflammation as evidenced in *pgm*⁺ *Y. pestis*-infected mice lungs. We have previously observed that IFN β was deleterious during *Y. pestis* infection [70-73]. Since DCs are major producers of IFN β , we wanted to monitor *Ifnb1* transcription

levels to determine if the *pgm* locus increased production of IFN β which could potentially explain the decrease in virulence observed in the *pgm*⁻ strains [70, 72, 73]. Since TNF α is important for the clearance of *Y. pestis*, *Tnfa* transcripts were also measured [74, 75]. BMDCs were infected with *pgm*⁻ or *pgm*⁺ *Y. pestis* for 2 hours. Quantitative PCR was then performed, samples were normalized to the housekeeping gene *Ywhaz* and uninfected controls. We found no significant change in the transcription of *Tnfa* or *Ifnb1* (Fig. 3-1B).

Since the presence of the *pgm* locus did not seem to be influencing aberrant cytokine expression, we hypothesized that the *pgm* locus would increase the killing capabilities of *Y. pestis*. This could increase the inflammation present in the lung and potentially allow *Y. pestis* to setup a replicative niche. Lactate dehydrogenase concentrations were assayed from supernatants of BMDCs infected with *Y. pestis* for 8 hours to observe *pgm*-mediated cell death. There was no significant increase of killing in BMDCs infected with *Y. pestis* containing the *pgm* locus compared to *pgm*⁻ *Y. pestis* (Fig. 3-1C).

The *pgm* locus was not directly affecting cytokine production or cytotoxicity in dendritic cells, however, it was still required to produce pneumonia [39]. Since the *pgm* locus carries most of the iron-scavenging genes in *Y. pestis*, it is important for bacterial growth within the host [52, 53, 76]. We needed to determine if the *pgm* locus was creating a permissive environment within the lungs or if its presence was just providing a growth advantage due to increased iron scavenging for the bacteria. To monitor this, we intranasally infected C57BL/6 mice with 5x10³ CFU (colony-forming units) of *pgm*⁺ wild-type *Y. pestis*

and 5×10^3 CFU of isogenic *pgm*⁻ *Y. pestis*. To establish a control for *pgm*⁻ growth in the lungs without *pgm*⁺ bacteria, mice were also intranasally infected with 1×10^4 *pgm*⁻ *Y. pestis* to establish a control for *pgm*⁻ growth.

At 24, 48, and 72 HPI, mouse lung homogenate was plated on HIA with Congo Red and white colonies were enumerated for CFU. Bacteria lacking the *pgm* locus, which contains the hemin storage locus (*hms*) cannot uptake Congo Red dye and thus present as white colonies, compared to wild-type *Y. pestis* that makes red colonies [77]. At all time points measured, there was a significant increase in the coinfecting *pgm*⁻ CFUs when compared to the *pgm*⁻ solo infection (Fig. 3-2). The *pgm*⁻ bacteria could colonize the lungs in higher numbers while growing with *pgm*⁺ bacteria. This indicated that the *pgm* locus conferred a growth advantage to *Y. pestis* in the lung that was not related to its ability to scavenge iron. Also, that *Y. pestis* did not need to possess the *pgm* locus to benefit from its presence in the infection; this indicated that a secreted factor could be responsible for this permissive environment within the lung.

This led us to investigate the main secreted factor synthesized from proteins encoded on the *pgm* locus: yersiniabactin. This was an excellent candidate to start with as the presence of ybt had been shown to increase virulence and establish a pneumonic infection [41]. To begin assaying the effect ybt had on pneumonia we started with two mutants: $\Delta irp2$ pCD1Ap, which could not synthesize ybt and Δpsn pCD1Ap which could synthesize ybt but could not actively transport the ybt-bound iron back into the bacteria. This allowed us to

assay the effects that ybt had on the host without removing all the other genes in the *pgm* locus.

To confirm Perry's observation that Δpsn pCD1Ap was significantly more virulent than $\Delta irp2$ pCD1Ap was also true in our model, we compared the two strains of *Y. pestis* by assaying survival. [52]. C57BL/6 mice were intranasally infected with 1×10^5 CFU of each bacterial strain and survival was monitored for 14 days. At this challenge dose, C57BL/6 mice were significantly more susceptible to Δpsn pCD1Ap than to $\Delta irp2$ pCD1Ap. (Fig. 3-3A). The presence of ybt in the lungs increased the virulence of *Y. pestis*.

Our next hypothesis was that the secretion of ybt was leading to increased lung damage. To assay lung damage, we conducted an intranasal infection of 1×10^5 CFU of either *Y. pestis* Δpsn pCD1Ap or *Y. pestis* $\Delta irp2$ pCD1Ap and then 2 hours prior to euthanasia intranasally instilled 5 μ g of FITC-Dextran dissolved in 10 μ L of sterile PBS. Serum fluorescence was measured and compared to naïve mice. While there were indications that there was more vascular permeability in the Δpsn pCD1Ap infected mice, it was overall not significant (Fig. 3-3B).

Other researchers have found that the presence of ybt during an infection increases pro-inflammatory cytokine production [78]. To measure this, we infected BMDMs for 2 hours with *Y. pestis* $\Delta irp2$ and Δpsn lacking the T3SS to conduct this experiment at BSL2. To control for the effects that the T3SS has on pro-inflammatory cytokine production, the BMDMs were infected with a T3SS+ and *pgm* *Y. pestis* strain (KIMD27). There was no difference between the $\Delta irp2$

and Δpsn in the pro-inflammatory cytokine genes assayed that are generally affected by *Y. pestis* infection. The T3SS+ *Y. pestis*-infected BMDMs showed the only reduction in pro-inflammatory transcripts (Fig. 3-3C).

Since we could not find evidence of ybt contributing to damage or modulation of pro-inflammatory cytokine expression, we then hypothesized that the presence of ybt allowed for the establishment of pneumonia in the lungs. To assay this, histology was taken from moribund or recently dead mice that had been infected with 1×10^5 CFU of $\Delta irp2$ pCD1Ap or Δpsn pCD1Ap. The Δpsn pCD1Ap infection exhibited an increase of inflammatory cell infiltration along with filled and collapsed airspaces when compared to the $\Delta irp2$ pCD1Ap infection (Fig. 3-4C-D). This further supports our hypothesis that ybt is important in establishment of pneumonia. In contrast, there were more incidences of bacterial colony presence and breakdown of cellular architecture caused by the $\Delta irp2$ pCD1Ap mutant within the liver and the spleen (Fig. 3-4). This indicated that septicemic plague was the cause of pathology within the mice infected with $\Delta irp2$ pCD1Ap.

To determine if direct ybt exposure without *Y. pestis* bacteria could cause lung damage, we decided to look at the effects of exogenous ybt treatment on lung tissue. Yersiniabactin (25 μ g) was dissolved in methanol and delivered intranasally to BALB/C mice. After 6 hours, bronchoalveolar lavage fluid (BALF) was taken from the treated mice, and protein concentration of the BALF was measured by Bradford assay. This test has been used to measure alveolar damage by measuring protein content of BALF as potential debris [79]. Again, as

seen in the permeability assay, there was an increase in protein concentration in the BALF of ybt-treated mice in comparison to the vehicle. However, it was not significantly different (Fig. 3-5A).

Since ybt alone was not sufficient to cause alveolar damage, we sought to determine if ybt could promote growth of *Y. pestis* bacteria in the lung. Using the observations, we found in the coinfection experiment, we decided to observe CFUs at 24 HPI as this was the earliest time point in which a significant increase in bacterial numbers was seen. To replicate the *Y. pestis* *pgm*⁻ bacteria that was used in the coinfection, *Y. pestis* KIMD27 was selected as this strain would have no known ability to use ybt-bound iron for growth. We also introduced ybt into a *Δirp2* infection, to measure the effect ybt had on *Y. pestis* growth of a strain that possessed the ability to actively transport ybt-bound iron. Mice were intranasally treated with 25 µg of ybt dissolved in methanol and infected with 1x10⁶ CFU of *Y. pestis*. There was no change seen in bacterial growth observed in either treatment group at 24 hours (Fig. 3-5B). Ybt treatment could not replicate the increase of *pgm*⁻ *Y. pestis* CFUs as observed in the coinfection experiment.

Yersiniabactin has been shown to induce HIF-1α (Hypoxic Inducible Factor 1α) in cells in a hypoxic-independent manner [78, 80, 81]. It appears the presence of siderophores stabilizes this transcription factor, by limiting the iron available to host cells. Upon stabilization, HIF-1α translocates to the nucleus and activates transcription on hypoxic response elements (HREs). These genes encode for proteins that are cytoprotective and correct redox intracellular imbalances [82-84]. Some of the genes under transcriptional control are *Vegfa*,

Adm, and *Hmox1*; all of these genes produce proteins that are anti-inflammatory or pro-healing in nature [83, 85-87].

We hypothesized that ybt was stabilizing HIF-1 α and inducing a localized anti-inflammatory environment around the bacteria to allow permissive growth [36, 88, 89]. Western blot was used to measure the effect of ybt on HIF-1 α stabilization in macrophages. BMDMs were infected at an MOI of 50, for 6 hours and protein lysate was analyzed for HIF-1 α stabilization (Fig. 3-6A-B). Exposure to ybt with no bacteria was also monitored to control for the presence of bacterial components that could instigate pleiotropic HIF-1 α stabilization. We expected to see an increase in HIF-1 α stabilization in macrophages infected with *Y. pestis* that were able to produce ybt (*pgm*⁺ and Δ *psn*) compared to *Y. pestis* strains that were unable to synthesize ybt (*pgm*⁻ and Δ *irp2*). However, what we observed was that *pgm*⁺ and Δ *irp2* had the highest stabilization of HIF-1 α in macrophages infected with *Y. pestis*. There was an increase in HIF-1 α stabilization over vehicle (methanol) when macrophages were treated with ybt (Fig. 3-6C). These data indicate that ybt could be stabilizing HIF-1 α , but that under the context of direct bacterial infections the effect is unclear.

We then hypothesized that other elements that were under HIF-1 α transcriptional control could be affected by ybt. Upon researching genes under HIF-1 α control we found a gene that encoded a protein known as heme oxygenase-1 that formed a complex with interferon regulatory factor 3 (IRF3) in myeloid cells [90]. HO-1 is an enzyme that degrades heme into Fe²⁺, carbon monoxide, and biliverdin and is encoded by the gene *Hmox1*. Transcription of

Hmox1 is controlled by HIF-1 α and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [91-96]. This enzyme plays a critical role in maintaining intracellular redox balance and its byproducts are anti-inflammatory and cytoprotective [97-99]. Previous research from our laboratory has shown that bacteria harboring the *pgm* locus can evade IRF3-mediated protection during *Y. pestis* respiratory infection [72]. To measure if the presence of the *pgm* locus or ybt influenced *Hmox1* transcription, we observed the change in expression within *Y. pestis*-infected BMDMs by quantitative PCR. BMDMs were infected for 2 hours with an MOI of 50 of *Y. pestis*. The presence of the *pgm* locus induced a significant increase in expression of *Hmox1* (Fig. 3-7A).

While there was significant induction of *Hmox1* transcription from the *pgm*⁺ *Y. pestis*-infection, the Δpsn mutant was unable to generate a significant increase in *Hmox1* transcription by comparison. However, BMDMs infected with the Δpsn mutant showed elevated *Hmox1* transcripts in comparison to $\Delta irp2$ and *pgm*⁻. This lack of significance could be due to the fact that Δpsn mutants suffer a growth disadvantage in comparison to *pgm*⁺ strains of *Y. pestis* [41]. Since HO-1 can be induced during times of cytotoxic stress and we had indicated that the presence of ybt had increased damage to the lung, we wanted to determine if HO-1 could promote the permissive growth state by its anti-inflammatory activity.

To determine if HO-1 could promote lung damage during infection, zinc protoporphyrin IX (ZnPP), which is a competitive inhibitor of HO-1, was used to inhibit HO-1 during *Y. pestis* infection [99-103]. Our hypothesis was that if we inhibited HO-1, then the damage that ybt inflicted would illicit an inflammatory

response faster than a wild-type infection and ZnPP treatment might prevent the establishment of a permissive environment by *pgm+* *Y. pestis*. By doing this, the disease would proceed as a *pgm-* infection leading to an increase in survival of the treatment group with no pneumonia present. Mice were then intraperitoneally treated with 5 mg/kg of ZnPP and intranasally infected with 500 CFU of CO92, wild-type *Y. pestis*. During 2 trials there was no significant difference between the treatment group and vehicle-treated mice. This indicated that the effect ybt had on upregulating HO-1 during pneumonic plague was dispensable for overall virulence (Fig. 3-7B).

Next, we wanted to test the reverse, if inducing HO-1 allows a permissive environment to establish in the lung during a *pgm-* infection and leads to less mice surviving intranasal challenge. Cobalt protoporphyrin IX (CoPP) is a compound that upregulates HO-1 protein expression, but is not a competitive inhibitor like ZnPP, effectively acting as a HO-1 inducer [104-108]. Mice were treated intraperitoneally with CoPP, to induce HO-1 activity and then intranasally infected with 1×10^5 CFU of *pgm-* *Y. pestis*. We used KIMD27, instead of a CO92 strain for this infection as it is not a Tier 1 Select Agent, and thus the experiment could be conducted at BSL2. Our hypothesis was that with the induction of HO-1, during a *pgm-* *Y. pestis* intranasal infection will behave similarly to a *pgm+* *Y. pestis* infection and establish pneumonia. There were no deaths in the CoPP-treated mice during two trials showing that induction of HO-1 during a *Y. pestis* septicemic infection is protective to the host, rather than promoting pneumonia (Fig. 3-7C). HO-1 activity is known to be cytoprotective and our experiments

could indicate that ybt induces lung damage. It could be that CoPP treatment is ameliorating the damage caused by ybt.

Discussion

We have established that ybt contributes to pneumonia by allowing the bacteria to establish a growth niche in the lungs. The presence of the *pgm* locus did not change the cytokine transcription associated with *Y. pestis* in BMDCs, nor any changes to BMDC cell death. There was a lowering of bacterial uptake in the BMDCs when the *pgm* locus was present, and while this may indicate a lack of bacterial clearance in the lung, it is not known if this is the reason for establishment of pneumonia. Perhaps this is not the correct cell type to be measuring the effect that *pgm* is having on the host. In future experiments, transitioning to a pneumocyte model to better replicate the epithelial tissue of the lung could yield more definitive results as to whether the *pgm* locus induces direct damage to lung cells.

Evidence of the proliferation of *pgm*⁻ bacteria while coinfecting with *pgm*⁺ also indicates that the ability to establish bacterial growth in the lung is not a growth-mediated benefit being conferred to the bacteria. This is due to the inability of *pgm*⁻ bacteria to uptake ybt-bound iron during the coinfection. There are other factors within the *pgm* locus that may promote this permissive environment. However, the *Y. pestis* strain that exhibited the same pneumonic phenotype seen in the *pgm*⁺ infection was the Δpsn mutant. The presence of ybt allowed *Y. pestis* colonization of alveoli and led to more potential damage to the lung.

In the exogenous ybt study there were many complications that did not make it an ideal model of what would have been occurring during the coinfection. Methanol was used to dissolve ybt, and during intranasal instillation ybt may have been too readily absorbed in the upper respiratory tract and might not have reached the lower parts of the lungs. Perhaps to establish the replicative niche a high concentration of ybt is needed in a localized area around the bacteria. Going forward a coinfection of Δpsn and *pgm*- *Y. pestis* would better elucidate if this phenomenon is occurring. A potential hurdle may be that *pgm*- bacteria may not have a chance to establish colonies in the permissive environment before being cleared by the immune system. This would be due to the slow growth of Δpsn *Y. pestis* not establishing a permissive environment fast enough to improve *pgm*- *Y. pestis* growth.

The yersiniabactin virulence phenotype was observed in the lungs; however, for some *in vitro* assays BMDMs were used to assay the effect ybt had on *Y. pestis* virulence. These macrophages more resemble systemic or inflammatory macrophages that are recruited to the site of infection [109]. BMDMs that come into contact with bacterial PAMPs can induce a robust inflammation response [110]. This is in contrast to alveolar macrophages which produce a more measured inflammatory response, as excess inflammation within the lung would harm the host [64, 111, 112]. Macrophages exposed to LPS strongly activate HIF-1 α , and alveolar macrophages may not respond with as robust of a response from a bacterial factor such as ybt [113]. However, alveolar macrophages can stabilize and induce HIF-1 α in hypoxic conditions [114].

Further testing should be done in alveolar macrophages to observe if ybt can stabilize HIF-1 α in these macrophages.

It remains unclear how the permissive environment is established. It could be due to hyperinflammation from the disease/damage or that the damaged and dying cells are providing access to nutrients for bacteria to grow. In truth, it is probably a mixture of these two occurrences. In the Δpsn infection there was a tremendous amount of neutrophil infiltrate into the lungs. This is also characteristic of the *pgm+* *Y. pestis* infection. To further indicate that the permissive environment could be damage-oriented, we showed that the *pgm+* infection induces HO-1 transcription. HO-1 is a pro-healing enzyme with anti-inflammatory activity that is induced during cell stress or damage. More testing would be required to show if the induction of HO-1 is due to the oxidative damage or iron deprivation in the tissues.

Inhibiting HO-1 had no effect during a *pgm+* wild-type *Y. pestis* infection with CO92, meaning that HO-1 activity is not deleterious to the host during the infection. In fact, inducing HO-1 with CoPP during secondary septicemic plague, as modeled by *pgm-* *Y. pestis* respiratory infection, showed complete protection. This further indicates that induction of HO-1 during *Y. pestis* infections is in response to the damage from the bacteria and overall is beneficial to the host.

Figure 3-1. Presence of the *pgm* locus does not affect inflammation or cytotoxicity of

BMDCs during *Y. pestis* infection. (A) Phagocytosis activity of BMDCs during *Y. pestis*

infection. BMDCs were exposed to 50 MOI *Y. pestis* for 30 minutes and then 50 μ g/mL

gentamicin (Final conc.) was added to media. BMDC lysate was harvested at 2 and 8 HPI and

was plated on HIA. Data were collected from 3 independent experiments, $n=10$, and compared

using Mann Whitney test, $*p<0.05$, ns denotes not significant. **(B)** BMDCs were exposed to 50

MOI of *Y. pestis*. RNA was collected at 2 HPI and analyzed by RT-PCR. Fold change was

normalized to the *Ywhaz* gene. Data were collected from 3 independent experiments, $n=9$,

and compared using Two-way ANOVA and Sidak's Multiple Comparisons test, $*p<0.05$. **(C)**

Cellular membrane integrity assay of BMDCs during *Y. pestis* infection. BMDCs were exposed

to 50 MOI *Y. pestis* for 8 hours and LDH was measured. Data were collected from 3

independent experiments 2 biological replicates/experiment, $n=6$ and compared using One-

way ANOVA and Tukey's multiple comparisons test, $*p<0.05$.

Figure 3-2. The presence of *pgm*⁺ *Y. pestis* establishes a permissive environment leading to greater *pgm*⁻ bacterial growth during coinfection. Mice were intranasally infected with 5x10³ CFU of KIM5+ (*pgm*⁺) and 5x10³ CFU of KIM5- (*pgm*⁻) or were infected with 1x10⁴ CFU of KIM5- (*pgm*⁻). At 24, 48, and 72 HPI mice were sacrificed and their lungs were homogenized. Homogenate was serially diluted and plated on HIA. Plates were incubated at 26°C and counted 3 days later. Data were pooled from 2 independent experiments, n=8. Mann-Whitney test was used to compare data for every time point; **p*<0.05, ****p*<0.0001.

Figure 3-3. Production of ybt increases virulence in *Y. pestis* in C57BL/6 mice. (A) Mice were infected intranasally with 1×10^5 CFU KIM5+ $\Delta irp2$ pCD1Ap or KIM5+ Δpsn pCD1Ap, 2 experiments; $n=20$, $*p<0.05$. Survival changes were analyzed by Gehan-Breslow log rank test. **(B)** Mice were infected intranasally with 1×10^5 CFU of either KIM5+ $\Delta irp2$ pCD1Ap or KIM5+ Δpsn pCD1Ap. Lung permeability was assayed 72 HPI by measuring the amount of FITC-dextran was present in the serum 2 hours after intranasal instillation. Serum fluorescence was normalized to relative naïve and data were compared by Student's unpaired *t*-test. Results are combined from 3 independent experiments, $n=14$; $*p<0.05$. **(C)** Quantitative PCR of pro-inflammatory cytokine transcripts of BMDMs, 2 independent experiments, $n=4$; $*p<0.05$. Data were compared by Two-way ANOVA with Sidak's Multiple Comparisons test.

Figure 3-4. Presence of ybt allows for establishment of growth niche in alveoli. (A-L)

Groups of 5 C57BL/6 mice were intranasally infected with 1×10^5 CFU of KIM5+ $\Delta irp2$ pCD1Ap or KIM5+ Δpsn pCD1Ap. These tissues were taken from recently dead or moribund mice. **(A-D)** Lungs, black arrows from **C** and **D** indicate airspaces. **(E-H)** Liver, black arrows from **G** and **H** indicate break down of tissue architecture and inflammatory infiltrate. **(I-L)** Spleen, black arrows in **K** indicate bacterial colonies. The black bar indicates 50 μ m. All images were captured and analyzed by Dr. Deborah Anderson.

Figure 3-5. Exogenous ybt cannot establish the permissive environment in the lung required for growth. (A) Groups of 5 BALB/C mice were intranasally treated with 25 μ g of ybt dissolved in methanol and titrated in PBS to a final volume of 10 μ L. After 6 hours, bronchial lavage fluid (BALF) was taken from the mice, BALF was centrifuged at 10,000 rpm for 5 mins to remove debris. Protein was measured by Bradford Assay at OD₅₉₅. Absorbance was compared to standard curve to calculate total protein. Data were combined from 2 independent experiments, n=10, and analyzed by unpaired Student's *t*-test. **(B)** Groups of 4 BALB/C mice were intranasally dosed with 25 μ g of ybt and then infected with 1×10^6 CFU of either KIMD27 *Y. pestis* (*pgm-*) or *Y. pestis* Δ *irp2*. After 24 HPI, mice lungs were diluted and plated for enumeration. Medians were plotted, and data combined from 3 independent experiments, n=12. Data were analyzed by strain of *Y. pestis* via Mann-Whitney test.

Figure 3-6. Yersiniabactin may not cause hypoxia during *Y. pestis* infection. (A-B)

Western blots of BMDMs infected with *Y. pestis* strains at an MOI of 50 for 6 hours. Blots were probed for HIF-1 α and GAPDH was used a loading control. **(C)** Analysis of the western blots done by ImageJ. Relative increases normalized to GAPDH loading control, n=1. Relative fold was calculated by: Band intensity of HIF-1 α / Band intensity of GAPDH.

Figure 3-7. Heme oxygenase-1 is necessary for protection in *pgm-* *Y. pestis* infection.

(A) BMDMs were infected with 50 MOI of *Y. pestis* for 6 hours. RNA was extracted and qPCR was performed monitoring the fold change of *Hmox1* relative to *Ywhaz*. Data are combined from 2 independent experiments, $n=4$ in each group. Data were analyzed by One-way ANOVA and Tukey's multiple comparisons test, $*p<0.05$. **(B)** Groups of 10 C57BL/6 mice were treated ip with 5 mg/kg of ZnPP or equal volume of vehicle the day before challenge (-1); booster treatments were given on challenge day and then every other day throughout the study. Mice were then intranasally infected with 500 CFU of CO92 *Y. pestis*. Survival was monitored for 10 days and analyzed by Gehan-Breslow log-rank test. Data are combined from 2 independent experiments, $n=20$, $*p<0.05$. **(C)** Groups of 10 C57BL/6 mice were treated ip with 5 mg/kg of CoPP or equal volume of vehicle and then intranasally infected with 1×10^5 CFU of KIMD27 *Y. pestis* (*pgm-*). Survival was monitored for 10 days and analyzed by Gehan-Breslow log-rank test. Data are combined from 2 independent experiments, $n=20$, $*p<0.05$.

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Chapter 4. Induction of Heme oxygenase-1 protects against pneumonic plague.

Abstract

Pneumonic plague, caused by the gram-negative bacterium *Yersinia pestis*, is a devastating disease that progresses rapidly due to a toxic immune response that fails to control bacterial growth. Although there has not been a large-scale outbreak of human pneumonic plague since the early 20th century, cases are reported throughout the world each year with 20-70% mortality. Focal bronchopneumonia develops soon after inhalation of *Y. pestis* and the resulting damage to the alveoli and vasculature rapidly leads to sepsis. Consequently, pneumonic plague patients develop severe diseases associated with sepsis, including disseminated intravascular coagulation and systemic inflammatory response syndrome. The combined conditions of pneumonia and sepsis lead to poor outcome even when antibiotics are administered. In this work, we show that therapeutic induction of the cytoprotective enzyme heme oxygenase-1 (HO-1) can reduce pneumonic plague mortality in a murine model. Treatment of mice with the heme analog cobalt protoporphyrin IX (CoPP) induced an increase in HO-1 which was associated with an early reduction in bacterial growth in the lungs followed by sustained protection from alveolar damage with no significant anti-inflammatory effect. The data suggest that CoPP treatment provided multiple benefits to the host that resulted in a more effective inflammatory response to *Y.*

pestis. Heme oxygenase therapy may therefore be an effective treatment strategy that improves the outcome of human pneumonic plague.

Introduction

Y. pestis is a non-motile, gram-negative coccobacillus that causes the disease known as plague [1-4]. There are three manifestations of the disease: bubonic, septicemic, and pneumonic [1, 5, 6]. Pneumonic plague is contracted via inhalation of *Y. pestis* bacteria which rapidly develops into acute bronchopneumonia and secondary sepsis [7-9]. *Y. pestis* invades and colonizes the alveoli leading to cellular damage and ultimately collapse of the airways in the lung. This damage permits access to the bloodstream where *Y. pestis* spreads throughout the body infecting secondary tissues such as the liver and spleen [7, 10, 11]. Uncontrolled bacterial growth in these tissues correlates with progressing disease and the frequent development of severe sepsis [12-14].

Y. pestis can infect multiple tissues and lead to the death of the host by use of its many virulence factors. The type 3 secretion system (T3SS) is a virulence factor that is required for infection of the mammalian host [15, 16]. The needle complex and the effector Yops that are injected into host cells are encoded on the pCD1 plasmid. These Yops induce apoptosis, inhibit cytoskeletal rearrangement, and disrupt pro-inflammatory cytokine production [15, 17-20].

Yersinia pestis also contains a pigmentation locus (*pgm*) which contains the genes that encode for iron acquisition, namely yersiniabactin [21-28].

Yersiniabactin (ybt) is a siderophore with a high binding affinity to ferric iron: Fe³⁺. This attraction is strong enough to bind iron with higher affinity than host iron

scavenger/storage proteins: lactoferrin and transferrin [21, 23]. Ybt also evades host factor lipocalin 2, which is a siderophore-binding protein secreted by immune cells [29]. This method of evasion allows ybt to sequester iron from the host unabated. Previous work in Chapter 3 indicates that there may be a link between the presence of ybt-producing *Y. pestis* bacteria and heme oxygenase-1.

Heme oxygenase-1 is a host enzyme that catalyzes the catabolization of heme into biliverdin, carbon monoxide, and Fe^{2+} [30-33]. This enzymatic activity is anti-inflammatory as it removes a damage associated molecular pattern (DAMP) in heme and produces carbon monoxide which is a signaling chemical that induces the production of IL-10 and has been shown to reduce the inflammatory potency of heme [34-36]. Biliverdin also helps to balance intracellular redox homeostasis by acting as a potent anti-oxidant [32, 37, 38].

Cobalt protoporphyrin (CoPP) is a metalloprotoporphyrin that resembles heme constructed of a protoporphyrin IX ring with a Co^{3+} ion in the center [39, 40]. It stimulates the production of HO-1; however, it does not act as a competitive inhibitor of heme, like some other metalloprotoporphyrins: zinc and tin protoporphyrin [41, 42]. Therefore, it is an effective inducer of HO-1 expression and subsequent HO-1 activity. CoPP induces transcription of *Hmox1*, which encodes for HO-1, by binding and destabilizing BACH1 [39, 43, 44]. Inactivation of BACH1 allows the activator Nrf2 to bind DNA and stimulate transcription of *Hmox1*. In addition to HO-1, Nrf2 regulates expression of other genes which contribute to an anti-inflammatory environment during oxidative

stress [37, 38, 45-48]. The NLRP3 inflammasome and the transcription of inflammatory cytokines *Il6* and *Tnfa* are suppressed by Nrf2, and these effects are believed to temper the over-activation of the inflammatory response [49-54].

Induction of HO-1 produces an anti-inflammatory effect on the immune system. HO-1 activity has been shown to reduce inflammation and improve survival in CLP-models of sepsis [55, 56]. The anti-oxidant and cytoprotective effects of HO-1 activity improve and protect mice from acute lung injury [49, 57]. HO-1 activity has been shown to direct macrophages to a M2 profile [58, 59]. These macrophages are important for tissue repair and healing and are characterized by producing anti-inflammatory cytokines such as IL-10 and pro-healing growth factors such as VEGF [60-62].

The outcomes of what HO-1 activity does during bacterial infections have not been fully elucidated. During *Burkholderia pseudomallei* infection, HO-1-dependent CO reduced pro-inflammatory cytokines and increased intracellular burden resulting in an increase in susceptibility to severe disease [63]. During *Salmonella* Typhimurium infection, heme breakdown by HO-1 impaired neutrophil chemotaxis and oxidative burst responses which led to an increase in host susceptibility [64]. For *Mycobacteria spp.*, there are conflicting data on whether HO-1 restricts or promotes intracellular growth and mice lacking HO-1 were more sensitive to lethal infection [65-67]. This suggests that HO-1 generally contributes to host defense, yet HO-1-dependent CO can contribute to the pathogenesis cycle of *M. tuberculosis* by inducing a dormancy gene expression program that permits evasion from immunity and antibiotic treatment.

Pneumonic plague infections are described by the presence of damage and collapsing airways in the lung that leads to pneumonia and later bacteremia throughout the host. However, *Y. pestis* contains virulence factors such as the T3SS and a tetraacylated LPS that have been shown to evade the immune system and downregulate inflammation [68-70]. This posed a conundrum as toward the later stages of the lung infection there are massive amounts of immune cell infiltrate into the lungs, inferring that there was inflammation at the site of infection. If the inflammation is not being instigated by bacterial molecular patterns, perhaps the damage that the infection is inflicting on the host is the cause of the inflammation. We hypothesized that the damage incurred by the *Y. pestis* infection was the driving factor in mice mortality and that treating the damage by restoring healing factors (e.g. HO-1) to the host would improve survival.

Results

Previous work in the laboratory has shown in macrophages that *Y. pestis* containing the *pgm* locus induces *Hmox1* transcription. We initially thought that this was a way for *Y. pestis* to modulate the immune response to establish a permissive environment, but we now hypothesize that HO-1 is being induced due to the damage being caused by *Y. pestis*. We then set out to test whether therapeutic induction of HO-1 expression during pneumonic plague could lessen damage and improve survival.

Mice were treated intraperitoneally with 5 mg/kg of CoPP daily or every 48 hours and levels of HO-1 induction were measured in the lungs at 72 HPT. We

determined there was no difference in HO-1 induction between daily or every other day CoPP treatment (Supp. Fig. 4-1A). Using these data, we established our treatment schedule for the mice during the infection (Supp. Fig. 4-1B). Mice were treated with vehicle or CoPP and infected with 5×10^3 CFU (colony-forming units) of wild-type CO92. Survival was observed for 10 days post-infection. Intraperitoneal CoPP treatments significantly increased survival in C57BL/6 mice during pneumonic plague (Fig. 4-1A). To ensure that the CoPP treatment was inducing HO-1, lungs were taken from infected animals at 48 and 72 HPI and HO-1 protein was measured by ELISA (Fig. 4-1B-C). This showed that the production of HO-1 was significantly elevated at 48 HPI, but there was more induction of HO-1 at 72 HPI, due to the last treatment being 24 hours previous or a possible synergistic response to the infection.

Bacterial loads in the lung were enumerated at 48 and 72 HPI to observe the effect CoPP was having on the progression of the disease (Fig. 4-2A-B). At 48 HPI there was a significant decrease in the number of bacteria found in the CoPP-treated lungs, by 72 HPI there was no difference between the treatment groups. There were some deaths in the vehicle-treated group that were not assayed at 72 HPI. To find the reason why there were lower numbers of bacteria in the lungs at 48 HPI, we measured the potential antibacterial effect of CoPP on the growth rate of *Y. pestis*. KIM6- *Y. pestis* was used to measure growth at BSL2. KIM6+ and KIMD27 were also monitored for growth in CoPP-treated medium, but there were no significant differences between the *Y. pestis* strains (data not shown). Bacteria were grown overnight in HIB supplemented with CaCl_2

at 26°C then diluted to a final OD₆₀₀ of 0.1 in minimal medium (TMH), then incubated at 37°C over 24 hours [71]. The cultures were then supplemented with CoPP to a final concentration of 50 µM or an equal volume of vehicle. There was no significant decrease in growth and at some time points, there were significant increases of bacterial growth in the CoPP-treated cultures compared to the vehicle-treated bacteria, as measured by OD₆₀₀ (Fig. 4-2C). This indicated that the reduction in lung titers seen at 48 HPI was not a result of a potential antibacterial activity of CoPP treatment.

Ruling out the potential antimicrobial effects CoPP could have been having on the bacteria, we investigated to see if CoPP could increase phagocytosis of *Y. pestis*. For our *in vitro* assays we used MHS cells. These cells are a murine alveolar macrophage cell line and were used to model for the resident alveolar macrophages in the lung. We treated MHS cells with vehicle or 50 µM of CoPP overnight and infected them with an MOI of 50 of *Y. pestis* CO92 for 30 minutes before adding gentamicin to a final concentration of 50 µg/mL. There was no significant increase in phagocytosis between CoPP-treated and untreated cells at 2 HPI (Fig. 4-3A).

Knowing now that CoPP did not increase phagocytosis we asked if the CoPP-treated macrophages were able to better survive contact with *Y. pestis*. CoPP treatment protected MHS cells from cellular death from the type 3 secretion system but did not have any effect on cells that produced ybt or contained the *pgm* locus. This suggests that CoPP treatment may help alveolar macrophages survive *Y. pestis*-killing in a ybt-independent manner (Fig. 4-3B). If

CoPP-treated alveolar macrophages are surviving T3SS-mediated death, that would mean there were more resident macrophages in the lung to potentially phagocytose *Y. pestis* bacteria in the lungs. To further define the effect the T3SS had on the production of HO-1, western blotting was done on the MHS cells infected in Fig. 4-3B. The T3SS downregulates the production of HO-1 in the untreated samples, but CoPP treatment overcomes the T3SS's ability to ablate HO-1 (Fig. 4-3C).

Previous research has shown that induction of HO-1 leads to a more M2-like macrophage profile. Both M1 and M2 macrophages have enhanced phagocytic profiles, but M1 macrophages have enhanced bacterial killing [61, 62, 72, 73]. The inflammatory profile of CoPP-treated macrophages was observed by qPCR during *Y. pestis* infection. *Il1 β* transcripts were significantly reduced and previous data from our laboratory has shown that ablation of type 1 interferon signaling is protective during *Y. pestis* infection [74]. However, there was no significant change in the pro-inflammatory transcripts *Il1b* and *Tnfa* and the growth factor *Vegfa* (Fig. 4-3D). *Ncf1* transcripts were lower in CoPP-treated macrophages. *Ncf1* is the gene that encodes for p47phox, a protein that is a subunit for NADPH oxidase, a complex that generates superoxide during respiratory burst. This did confirm some of our hypothesis that potential oxidative damage would be lower in CoPP-treated lungs.

If macrophages were protected from the T3SS of *Y. pestis*, it would then stand to reason that there would be more macrophages, and possibly other immune cells, present during the infection. This increase in innate immune cells

was most likely responsible for the decrease in bacteria seen at 48 HPI. To measure the change in myeloid cell populations that CoPP treatment had during *Y. pestis* infection, we conducted flow cytometry. All results were compared as percentages of leukocytes that were determined by gating on CD45. Alveolar macrophages were gated on CD45⁺ CD11c⁺ F4/80⁺, non-resident macrophages were CD45⁺ F4/80⁺, while neutrophils were CD45⁺ CD11b⁺ Ly6G⁺. Dendritic cells were gated on CD45⁺ CD11c⁺ (Fig. 4-4A). Comparing the resultant percentages of leukocytes to naïve controls over the course of 4 independent experiments, there were no significant increases or changes in population (Fig. 4-4B). This result ran counter to our hypothesis as we expected to see increases in the myeloid cell populations due to CoPP treatment protecting against T3SS-mediated death. The reduction of bacteria observed in the CoPP-treated lungs at 48 HPI was not being cleared by more immune cells present in the lung. Perhaps CoPP treatment was changing the inflammatory environment and that was aiding in the clearance of bacteria.

To determine if the lowered pro-inflammatory cytokine transcripts found in macrophages translated to a change in the cytokines within the lungs of mice treated with CoPP, we directly measured cytokines at 48 HPI. Due to the enzymatic activity of HO-1 being anti-inflammatory, we expected upregulation of anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines in comparison to the vehicle-treated mice [75-77]. We observed IFN γ and TNF α as those have been shown in the literature to be important during *Y. pestis* infection [78]. There was no significant change between CoPP-treated and vehicle in

either cytokine measured (Fig. 4-5A-B). There were more samples that had elevated TNF α in the CoPP treatment group, which ran counter to our hypothesis. Observing later time points might show some more dramatic differences as the disease progression continued.

Cytokines were measured from lung homogenate at 72 HPI. The proinflammatory cytokine TNF α did not significantly differ between vehicle and CoPP-treated lungs (Fig. 4-5C). This is consistent with the qPCR data gained from CoPP-treated infected macrophages. However contrary to our hypothesis which expected other pro-inflammatory cytokines to be reduced, we observed significant increases in IL-6 and IL-1 β levels in CoPP-treated lungs (Fig. 4-5D-E). Anti-inflammatory cytokine IL-10 which has been shown to be upregulated upon treatment with CoPP, was elevated in CoPP-treated mice, but was not overall significant (Fig. 4-5F). CoPP has been shown to induce production of VEGF, a growth factor important in wound healing. However, in CoPP-treated animals there was no detectable increase in VEGF cytokine in the lungs (Fig. 4-5G). Overall these data showed that CoPP treatment does affect lung pro-inflammatory cytokine production. However, it is still unclear if the effects of CoPP treatment were helpful in resolving the infection. Moving forward, we needed to visualize if these changes in inflammation affected tissue injury within CoPP-treated lungs.

We evaluated the lungs by scoring histopathology for the severity of alveolar damage (including edema, exudate, necrosis and hemorrhage) as well as inflammatory infiltrate. Overall, CoPP treatment appeared to result in

decreased alveolar damage despite an apparent increase in inflammatory infiltrate in these animals, albeit not significantly different (Fig. 4-6A-C). Inflammatory cells found in the lungs of both groups of animals were primarily neutrophils, but non-neutrophilic lesions were also observed. Of note, serum accumulation was commonly observed in blood vessels of CoPP-treated mice whereas this was essentially absent in all the vehicle-treated mice. To quantify the degree of tissue damage, we measured protein content in the alveoli by collecting bronchial alveolar lavage fluid (BALF) from vehicle and CoPP-treated mice at 72 HPI (Fig. 4-6D). Indeed, protein concentration in the BALF was significantly higher in infected vehicle-treated mice compared to CoPP-treated. This is consistent with the histology which showed reduced alveolar injury in CoPP-treated mice infected with *Y. pestis*. With less lung damage in the CoPP-treated animals, a new hypothesis was being put forth: that CoPP was protecting the lung from cellular death.

Discussion

Limiting damage from a bacterial infection is key in resolution of the infection. Overall, we have seen that HO-1 slowed the progression of the disease and increases survivability of mice to *Y. pestis* infection. Compared to vehicle-treated mice, the disease was protracted in CoPP-treated mice with most of the deaths occurring post-96 HPI. Further evidence of this slowing disease progression can be found in the CFU counts in the lungs at 48 HPI, with CoPP-treated animals having lower amounts of *Y. pestis* bacteria present. This difference in lung bacterial titers was lost at 72 HPI, but there were also dead

mice present in the vehicle group, while there were none in the CoPP-treated group, further indicating that the disease progression was slowed upon treatment with CoPP.

We observed resistance to T3SS-mediated cellular death in MHS cells and we expected this to translate to greater numbers of innate immune cells in CoPP-treated mice lungs at 48 HPI (Fig. 4-7). This would help to explain why there were less bacteria present at 48 HPI, as they were being cleared by greater numbers of immune cells. However, CoPP treatment did not change the innate immune cell populations in the lungs during the *Y. pestis* infection. We also observed no difference in the phagocytic capabilities of CoPP-treated MHS cells, indicating that the reduction of bacteria was not due to improved clearance capability of the immune system. A potential hypothesis is that CoPP treatment protects other cell types from cellular death, such as epithelial cells. (Fig. 4-7). In doing so this would lower the overall damage within the lung and could rob *Y. pestis* of the growth niche that it requires to establish rapid-onset pneumonia and subsequently delay the disease. This would also aid in maintaining lung integrity perhaps allowing for clearance by immune cells while *Y. pestis* struggles to proliferate and disseminate from the lungs.

Despite HO-1 activity being anti-inflammatory in nature, there was no overall decrease in pro-inflammatory cytokines in the lungs of CoPP-treated mice. In the case of the pro-inflammatory cytokines IL-6 and IL-1 β , there were increases seen at 72 HPI within CoPP-treated animals. However, this could be a potential mechanism by which HO-1 activity could correct inflammatory disruption

caused by *Y. pestis*. It is still unclear how HO-1 activity affects the inflammasome during *Y. pestis* respiratory infection. Previous research indicates that HO-1 inhibits inflammasome formation [49, 58, 79, 80]. Further investigation into how CoPP treatment affects the inflammasome during *Y. pestis* infection is needed to see if its effects are resolutive to the host.

Induction of HO-1 has been shown to have inhibitory effects on TNF α , and treatment with CoPP should have lowered this cytokine within the lung during the course of the infection [81-84]. However, this was not observed as generally there was more TNF α production in CoPP-treated animals. Further data in the literature showed that CoPP treatment during LPS-induced sepsis showed increased IL-6 production and mice entered into fever faster compared to untreated mice [85]. This raises a potential method by which CoPP is protecting the host by priming the immune response for infection (Fig. 4-7). Increased IL-10 levels were seen in CoPP-treated animals, but from the data observed in the lungs it is still unknown if this is how HO-1 induction is helping to limit inflammation.

From the data presented here, most of the evidence about how HO-1 is protecting the lungs is through the prevention of damage. There was less alveolar inflammation, with more neutrophils present, and less lung debris present in BALF of CoPP-treated mice. Elevated HO-1 activity is permitting more immune cells to infiltrate at 72 HPI without causing more damage to the host. *Y. pestis* has been shown to evade superoxide burst in macrophages as well as survive in neutrophils [86-88]. Perhaps the overwhelming presence of neutrophils

seen at the later stages of pneumonic plague is causing more damage to the host than clearance of the bacteria. Data from the CoPP-treated macrophage studies affirms this hypothesis as there were less *Ncf1* transcripts in the CoPP-treated group. This could mean that there could be lowered amounts of ROS production within the lung, potentially leading to less ROS-mediated damage to host cells. However, it is not known if HO-1 induction inhibits neutrophil clearance of *Y. pestis*. This is unlikely due to the necessity of neutrophilic-activity to resolve *Y. pestis* infection [89].

Previous research has established that damage is very important to the establishment of a growth niche of *Y. pestis* within the lung [12, 90, 91]. We have shown that there is less alveolar damage in CoPP-treated lungs. Host damage could be providing the bacteria with nutrients from the dead cells as well as breaking down the barrier to the bloodstream enabling dissemination throughout the body (Fig. 4-7). We believe that HO-1 is helping to limit this dissemination by maintaining lung integrity and preventing bacterial access to the bloodstream.

Figure 4-1. CoPP induces HO-1 protein expression in lungs and improves survival during *Y. pestis* infection. **(A)** Groups of 5-10 C57BL/6 mice were challenged by intranasal infection with 5,000 CFU *Y. pestis* CO92 and monitored for survival. CoPP (5 mg/kg) or vehicle treatment was administered via ip the day before challenge (-1); booster treatments were given on challenge day and then every other day throughout the study; data shown were combined from 3 independent trials and were analyzed by Gehan-Breslow log rank test; * $p < 0.05$ **(B-C)** Groups of 4-10 C57BL/6 mice were treated with CoPP or vehicle followed by infection with 5,000 CFU *Y. pestis* CO92. At 48 **(B)** and 72 HPI **(C)**, mice were euthanized, the lungs removed, and homogenized in sterile PBS. Filtered lung homogenates were analyzed by ELISA. **(A)** $n = 20$ per group, **(B)** $n = 14$ per group. **(C)** $n = 20-22$ per group. **(B-C)** Statistically analyzed by One-way ANOVA and Tukey's multiple comparisons test; * $p < 0.05$.

Figure 4-2. CoPP treatment reduces bacterial growth in the lungs (A) Groups of 3-10 C57BL/6 mice were treated with CoPP or vehicle followed by intranasal infection with 5,000 CFU *Y. pestis* CO92. Bacterial titer was determined by diluting and plating lung homogenate collected at **(A)** 48 (n=13 per group) and **(B)** 72 HPI (n=26-28 per group); data shown were combined from 3-5 independent trials and medians are shown. Data were analyzed for statistical significance by Mann-Whitney test, * $p < 0.05$; †: animal died prior to 72 HPI. **(C)** KIM6- *Y. pestis* was grown in TMH minimal medium with vehicle or CoPP (50 μ M, dotted line) added at time 0. OD₆₀₀ was measured at the indicated time points. Error bars show standard error. Data shown were pooled from 3 independent experiments, n=9. Analyzed for statistical significance using Two-way ANOVA with Sidak's Multiple Comparisons test, * $p < 0.05$.

Figure 4-3. CoPP treatment reduces T3SS-mediated death and restores HO-1

expression in MHS Cells. (A-C) MHS (murine alveolar macrophages) cells were seeded overnight with 50 μ M of CoPP or vehicle. **(A)** Gentamicin protection assay was performed on MHS cells infected at a MOI of 50 of *Y. pestis* CO92 for 2 hours. Analyzed for statistical significance by Mann Whitney test, data shown were combined from 2 independent trials, n=12. **(B)** Supernatant LDH was measured in MHS cells infected with *Y. pestis* at an MOI of 50 for 6 hours and compared to total cell lysis control. Analyzed for statistical significance by Two-way ANOVA and Sidak's Multiple Comparisons test, data shown were combined from 2 independent trials, n=10, * p <0.05. **(C)** Protein expression of HO-1 in MHS cells infected with *Y. pestis* for 6 hours was measured by western blot. **(D)** Inflammatory cytokine expression on BMDMs infected with CO92 *Y. pestis* measured by qPCR. Analyzed for statistical significance by Two-way ANOVA and Sidak's Multiple Comparisons test, data shown were combined from 3 independent trials, n=9, * p <0.05.

Figure 4-4. CoPP treatment does not change immune cell populations during *Y. pestis* infection. Following infection with 5×10^3 CFU of *Y. pestis* CO92, single cell-suspensions of pulmonary tissue (Vehicle or CoPP-treated) were collected at 48 HPI. After antibody staining, samples were run on Beckman Coulter Cyan ADP Flow Cytometer. **(A)** Gating strategy is shown. **(B)** Data were analyzed, and cell populations were graphed as percentages of total leukocytes. Each cell population was analyzed by Two-way ANOVA and Sidak's Multiple Comparisons test, $n=16$ for infected samples and $n=4$ for naïve samples, combined from 4 independent experiments.

Figure 4-5. CoPP does not lower inflammation in the lungs. (A-B) Groups of 5 C57BL/6 mice were treated with CoPP or vehicle followed by infection with 5,000 CFU *Y. pestis* CO92. At 48 HPI, mice were euthanized, lungs removed, and homogenized in sterile PBS. Filtered lung homogenates were analyzed by ELISA (n=14 per group): **(A)** TNF α ; **(B)** IFN γ ; Bars indicate medians. **(C-G)** 72 HPI Cytokines, 5 independent trials, n=20-22: **(C)** TNF α ; **(D)** IL-1 β ; **(E)** IL-6; **(F)** IL-10 **(G)** VEGF. All data shown were collected in 3-5 independent trials and were analyzed for statistical significance by Mann-Whitney test comparing vehicle-treated to CoPP-treated. IL-10 was analyzed by Two-way ANOVA and there was replicate effect but no treatment effect. * $p < 0.05$. All ELISAs were conducted by Dr. Rachel Olson.

Figure 4-6. CoPP prevents lung damage. (A-D) Lungs collected from animals at 72 HPI challenged with 5,000 CFU of *Y. pestis* CO92, **(A-C)** were processed for histopathology. **(A-B)** Representative images for lungs, followed by **(C)** severity scoring of alveolar damage (including edema, hemorrhage and transudate). AN: alveolar necrosis, H: hemorrhage, E: edema, S: serum, N/I: neutrophilic and/or inflammatory foci. Data were analyzed by Mann Whitney test, n=16-18; * $p < 0.05$. Scoring of images was conducted by Dr. Deborah Anderson. **(D)** Bronchial alveolar lavage was collected from *Y. pestis* CO92 infected animals at 72 HPI and measured for total protein content. Data were combined from 3 independent trials (n=12-15 per group) and analyzed by unpaired Student's *t*-test; * $p < 0.05$.

Figure 4-7. Model of CoPP-mediated protection during pneumonic plague. Effects of CoPP treatment in the lung. There were higher amounts of IL-6 and macrophages were more resistant to T3SS-mediated death. However, overall innate immune cell numbers were similar across treatment groups. According to our model CoPP-treated lungs have less damage.

Supplemental Figure 4-1. HO-1 induction with varying treatment regimens and final treatment schedule. (A) Lung induction of HO-1 measured by ELISA treatments at 72 hours post initial treatment. Treatments consisted of 5 mg/kg of CoPP, injected ip into mice starting at 24 prior to infection. Groups were compared by One-way ANOVA with Tukey's post-test, n=10-11, *p<0.05, ns denotes not significant. **(B)** Final dosing regimen scheme that indicates dosing treatment schedule to conduct the experiments.

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Chapter 5. Induction of Heme oxygenase-1 protects against sepsis during pneumonic plague.

Abstract

Pneumonic plague casualties are characterized by an established bronchopneumonia and bacterial dissemination throughout the body leading to multiple organ failure. *Y. pestis* dissemination from the lungs leads to septicemic plague in which the liver and the spleen are colonized. These organs suffer from large areas of necrosis, high-density bacterial colony formation, and inflammation. This dissemination and damage is known as secondary septicemia and it causes severe sepsis and septic shock. Heme oxygenase-1 (HO-1) has been known to aid in host toleration of sepsis and improve host survival. Our laboratory has previously observed that induction of HO-1 by cobalt protoporphyrin (CoPP) protected against pneumonic plague by inhibiting damage to the lung. This led us to investigate if CoPP treatment improved survival by protecting organ health during secondary septicemic plague. While CoPP treatment had no effect on the numbers of bacteria in the disseminated tissues, histopathology showed that CoPP-treated livers suffered from less necrotic lesions and inflammation. Liver enzyme panels indicated that CoPP treatment was not able to improve overall liver function during the course of the infection. Animals that were considered septic showed reduced pro-inflammatory cytokines. Altogether the data presented indicate that CoPP potentially reduced liver damage and aids in the host's ability to endure the inflammation during sepsis.

Introduction

Yersinia pestis is the bacterium that causes the disease plague [1-5]. The disease may onset in three ways: bubonic, septicemic, and pneumonic. Bubonic plague manifests by *Y. pestis* penetrating the skin, typically through an infected flea bite, and the bacteria infecting the nearest draining lymph node [2, 5-9]. Inflammation and necrosis due to the infection, result in the lymph node becoming swollen and discolored. Primary pneumonic plague is contracted by aerosolized droplets containing *Y. pestis* bacteria that infect and colonize the lungs [1, 10-13]. Dissemination from either the lymph nodes or the lungs allow the bacteria to spread into the bloodstream. This form of plague is called septicemic plague, and it allows the bacteria to quickly spread to tissues throughout the body and establish colonies in the liver, spleen, and lymph nodes [1, 5, 7, 14-16]. Presence of *Y. pestis* in the bloodstream as well as the damage to the tissues causes sepsis in the infected host leading to multiple organ failure [17].

The type 3 secretion system (T3SS) is a necessary virulence factor of *Y. pestis* that is required to infect a mammalian host. The T3SS of *Y. pestis* injects effector Yops (Yersinia Outer Proteins) into cells disrupting intracellular signaling, halting cytoskeletal rearrangement, and leading to cellular death [18-22]. Our laboratory has shown that intranasal infections of T3SS+, *pgm*- *Y. pestis* have low amounts of lung inflammation and no bronchopneumonia [23]. This is likely due to the absence of yersiniabactin production, encoded on the *pgm* locus, which would allow for establishment of pneumonia [24]. However, T3SS+, *pgm*-

Y. pestis still led to host death even with the lack of lung disease. Histology showed that moribund mice had bacterial colonies present in their spleens and livers. This indicated that morbidity was brought about by dissemination of *Y. pestis* into the bloodstream leading to secondary septicemia [23]. Causes of death in primary and secondary septicemic plague involve organ dysfunction with hypotension, suggesting that systemic infections of *Y. pestis* result in both septic shock and severe sepsis [1, 15, 16, 25, 26]. *Y. pestis* lacking the *pgm* locus created hypotension and fever in rats after developing non-pulmonary organ damage [23, 27].

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [28-31]. This is a broad term and in recent years there have been new definitions associated with sepsis to aid in the diagnosis, and determination of effective treatment options. Septic shock is defined as a subset of sepsis in which particularly acute circulatory, cellular, and metabolic abnormalities are profound enough to substantially increase mortality [28, 32, 33]. These metabolic abnormalities used to be specific to either hypotension or hyperlactatemia, but the definition has been changed to incorporate severe sepsis into the septic shock definition [32]. Severe sepsis is an older term that was specifically used to describe instances in which sepsis is complicated by acute organ dysfunction [30, 34-36]. A related disease-state to sepsis is SIRS (Systemic Inflammatory Response Syndrome) and it is defined as 2 or more of the following variables [37]:

- Fever of more than 38°C (100.4°F) or less than 36°C (96.8°F) – This is present in the pneumonic and septicemic *Y. pestis* infections with an average time to fever at 40 HPI in the pneumonic model. This fever was followed by a period of hypothermia that persisted until death [27, 38].
- Heart rate of more than 90 beats per minute – The heart rate during pneumonic infection was elevated, but not distinguishable from baseline in rat models. However, it has been documented in human and cynomolgus macaques that *Y. pestis* infection does significantly increase heart rate over baseline [39]. Increased heart rate was correlative with the onset of fever [40].
- Respiratory rate of more than 20 breaths per minute or arterial carbon dioxide tension (PaCO₂) of less than 32 mm Hg – Both pneumonic and septicemic *Y. pestis* infections present with elevated respiratory rates post-fever [27, 39].
- Abnormal white blood cell count (>12,000/μL or <4,000/μL) – Septicemic plague patients present with similar high leukocyte counts in the blood after presenting with fever symptoms, with the majority of the leukocytes being neutrophils [41].

These data suggest that the pneumonic or septicemic forms of plague induce SIRS and septic shock during the infection. Treating the symptoms of sepsis rapidly is necessary as systemic inflammation quickly becomes uncontrolled. Without rapid treatment in patients suffering from SIRS there is a mortality rate

ranging from 20-30% [42]. Plague cases are similar, in that they meet the same criteria as SIRS and that treatment must be administered soon after the onset of fever to prevent patient mortality [6, 43-47].

Fever is an important symptom in the clinical manifestations of plague and sepsis. The cytokine IL-6 is a part of the IL-6–COX2–PGE2 axis that regulates the fever response [32, 33]. IL-6 serum concentrations that are >1,000 pg/mL have been shown to be a biomarker for severe sepsis in humans [48]. Human and mouse responses to endotoxin injections at 2 hours post exposure showed that similar levels of IL-6 are produced in mice (about 1,000 pg/mL) [49].

IL-6 binds to homodimeric gp130 for signaling through the signal transducer and activator of transcription 3 (STAT3) pathway. To do so it must first complex with its primary receptor which exists as a transmembrane protein (IL-6R), or as a soluble secreted receptor (sIL-6R) consisting of a soluble form of the extracellular domain of IL-6R [50, 51]. Cis-signaling takes place when IL-6 binds to the transmembrane IL-6R and trans-signaling occurs when IL-6 binds to sIL-6R. Expression of transmembrane IL-6R is limited to a few cell types notably hepatocytes, lymphocytes, and microglia, thus these are the only cell types able to undergo cis-signaling [52]. Shedding of sIL-6R occurs during apoptosis of cells or secretion by activated neutrophils, and is regulated by protein kinase C (PKC) [53-56]. Since expression of gp130 is ubiquitous, every cell can undergo IL-6 trans-signaling. IL-6 cis-signaling has been seen to result in intestinal epithelial cell proliferation and inhibition of apoptosis [57]. IL-6 trans-signaling is pro-inflammatory and results in the upregulation of chemokines in damaged/infected

areas for trafficking of immune cells, and upregulation of differentiating growth factors for monocytes [50, 57]. IL-6 trans-signaling induces neutrophil apoptosis and resolves neutrophil inflammation through the activation of monocyte/macrophage recruitment to the infection site [51, 57-60].

Y. pestis has multiple virulence factors that can disrupt the effectiveness of the immune system, such as tetraacylated lipopolysaccharide (LPS) and the type 3 secretion system (T3SS). The tetraacylated LPS is expressed at 37°C and it has been shown to be anti-stimulatory to TLR4 [61, 62]. The T3SS injects effector Yops that disrupt cell signaling, induce apoptosis, and inhibit pro-inflammatory cytokine production [21, 63-66]. However, patients infected with *Y. pestis* present with a high fever and high amounts of inflammation within infected tissues during the infection. This is despite the virulence factors of *Y. pestis* that should allow for bacterial evasion from the host innate immune system. A potential hypothesis is that the inflammation generated from the infection could be stimulated by the damage inflicted by the bacteria upon host cells.

Evidence for this hypothesis can be seen in high morbidity rates during sepsis which are correlative to organ damage sustained. The organs that can be affected are: the cardiovascular system, lungs, liver, and kidneys [29]. The liver is of special importance as it functions as a lymphoid organ during sepsis, helping to establish tolerance during the infection [67, 68]. Also, liver dysfunction is a risk factor for multiple organ dysfunction and sepsis-induced death [67]. Protecting the liver during sepsis could be an effective way to ameliorate multiple organ damage and prevent patient death.

It has been shown that heme oxygenase-1 is effective at preventing liver damage by modulating TLR4 responses and inhibiting apoptosis [69-72]. Heme oxygenase-1 (HO-1) catalyzes the reaction of heme into Fe^{2+} , CO, and biliverdin [71, 73-78]. This activity has been shown to be anti-inflammatory and aids the host in tolerating the effects of sepsis. The byproducts of HO-1 activity are cytoprotective and lower the amount of free heme which can act as a damage associated molecular pattern (DAMP) [79-81]. Carbon monoxide provides anti-inflammatory activity by downregulating heme's host receptor: TLR4 [79, 81]. CO also stimulates the transcription of IL-10, which is a cytokine important for modulating inflammation during sepsis [82, 83]. Other anti-inflammatory actions of CO include the down-regulation of NLRP3-dependent inflammasome activation and IL-18 secretion [84]. Biliverdin is cytoprotective in that it is an anti-oxidant and has been shown to protect against sepsis by reducing the expression of MCP-1 and IL-6 [73, 80, 85].

Cobalt protoporphyrin (CoPP) treatment, which induces HO-1, has been shown to be effective against sepsis [86-89]. Previous data indicated that CoPP was protective against intranasal CO92 *Y. pestis* infection. In this work, we studied whether HO-1 also protected from secondary septicemic plague.

Results

Observing that CoPP treatment protected mice by maintaining the lung barrier in the pneumonic plague model, we looked at the effects that CoPP treatment had on secondary infected tissues and the ability of *Y. pestis* to disseminate from the lungs. Mice were infected with 5×10^3 CFU (colony-forming

units) of wild-type CO92 *Y. pestis* and either treated with 5 mg/kg of CoPP or an equal volume of vehicle following the established treatment schedule (Supp. Fig. 5-1B). Heme oxygenase-1 levels were measured in the liver to verify an increase conferred by intraperitoneal CoPP treatment (Supp. Fig. 5-1A). Approximately 3-5-fold increase in HO-1 was observed in the liver of CoPP treated mice.

At 72 HPI, liver and spleen homogenates were plated and CFUs were observed. There was no significant difference in the number of bacteria recovered between the treatment groups (Fig. 5-1A-B). This indicated that CoPP had little effect on systemic bacterial growth. However, it was observed that fewer CoPP-treated mice had bacterial dissemination into the liver and spleen. The percentage of mice with bacteria present in the liver and spleen was compared using Chi Square. However, this observation was not statistically significant (Fig. 5-1C).

While CoPP treatment may not have influenced the growth of bacteria that had disseminated from the lung, it has been known to lower inflammation. To see what effect HO-1 activity had on systemic inflammation, we measured the cytokines in the blood at 72 HPI. Both the vehicle and CoPP-treatment groups had mice with very high titers of pro-inflammatory cytokines, however CoPP-treated mice presented with significantly higher amounts of IL-6 compared to vehicle-treated (Fig. 5-2A). This ran counter to our hypothesis which anticipated that there would be lowered amounts of inflammation in the CoPP-treated group. To observe if progression of the lung infection correlated with higher amounts of IL-6 within the serum, lung CFUs were compared to serum IL-6 concentration

(Supp. Fig. 5-2). There was a strong correlation between these factors.

Therefore, we used >1,000 pg/mL of IL-6 as a biomarker for disease progression. This sepsis biomarker had been previously established in humans and mice and high levels of IL-6 in the serum correlated with pneumonic plague progression [48, 49].

There were 12 CoPP mice that had IL-6 levels that were higher than 1,000 pg/mL, whereas there were 6 mice in the vehicle group [48, 49]. This suggested that CoPP could be aiding in the endurance of sepsis or increasing sepsis severity. We separated the septic mice from the study and compared the effects CoPP treatment had on inflammatory cytokines during sepsis (Fig. 5-2A). To evaluate the severity of sepsis, we compared TNF α , IFN β , IL-1 β , IFN γ , and IL-10 levels in the serum of mice with elevated IL-6. A significant reduction in serum IL-1 β and IFN γ was observed in the CoPP-treated septic mice, suggesting a reduction in the severity of sepsis compared to the vehicle-treated mice with sepsis (Fig. 5-2D&F). Serum IL-10, TNF α , and IFN β were not significantly different although the median values for each of these cytokines were lower in the CoPP-treated group (Fig. 5-2B-C&E). Overall these data suggest that CoPP treatment did not prevent sepsis but reduced its progression and overall severity. This blunting of the pro-inflammatory cytokine response could be the reason why the CoPP-treated mice were able to endure the systemic infection and the subsequent sepsis. Furthermore, contrary to the LPS and CLP models, we did not see a HO-1 dependent increase in IL-10 [69, 80, 83, 90-92].

We asked if CoPP treatment also reduced the severity of damage to the liver. Histopathology analysis of vehicle-treated mice showed moderate inflammatory and necrotic lesions in the liver at 72 HPI (Fig. 5-3A-B). In contrast, these were reduced in livers of CoPP-treated mice, although not significantly different (Fig. 5-3C). To determine if these histological differences reflected a change in liver function at 72 HPI, liver profile testing was done on the serum (Fig. 5-3D). Alanine and aspartate aminotransferases are biomarkers of liver injury when both are elevated in the serum. However, both enzymes were within range of the reference standards for each treatment group suggesting relatively low levels of liver injury occurred at 72 HPI [www.criver.com]. Of the other parameters tested, only one of these enzymes, alkaline phosphatase was found to be different from the reference standards and was significantly reduced in the CoPP-treated group. The significance of this finding is unclear, since serum alkaline phosphatase is not specific to liver injury.

In vitro studies were done in RAW 264.7 macrophages to better understand how systemic macrophages reacted to CoPP treatment during a secondary septicemic infection. Since the severity scores in the CoPP-treated livers were lower, this could have been due to an increased phagocytic activity of CoPP-treated macrophages clearing bacteria and preventing lesion formation. RAW macrophages were treated with 50 μ M of CoPP overnight, and then infected with *Y. pestis* strains at an MOI of 50. Gentamicin assays were conducted to measure uptake differences of CO92 *Y. pestis* between CoPP-treated and untreated macrophages. There was no change in the number of

intracellular bacteria recovered from RAW macrophages in either treatment group at 2 hours (Fig. 5-4A). Supernatants from these experiments were assayed also for normally intracellular protein lactate dehydrogenase as a measure for loss of cell membrane integrity. CoPP treatment protected RAW macrophages against T3SS-mediated death compared to untreated (Fig. 5-4B). Macrophages infected with $\Delta irp2$ and Δpsn however, did not show increased protection to cell death when treated with CoPP. This conflicts with previous data suggesting that HO-1 could be induced as protection against ybt-mediated damage. Perhaps HO-1 is induced by ybt-mediated damage but cannot protect against it. However, CoPP treatment mitigated the cytotoxic effects of the T3SS of *Y. pestis*.

Due to the protective effects we observed on secondary septicemic plague, we asked if CoPP was effective as a post-exposure treatment for *Y. pestis* respiratory infection that begins at 24 or 48 HPI. Mice that received CoPP treatment at 24 HPI had a significant increase in survival compared to mice in the 48 HPI-treatment group (Fig. 5-5). This shows that CoPP may be effective as a post-exposure treatment for pneumonic plague, but like antibiotics, it appears that CoPP treatment alone is not effective at promoting host survival when initiated after the onset of disease.

Discussion

Dissemination of *Y. pestis* from the lung causes secondary septicemic plague and subsequent sepsis to the host. Due to the damage induced by the bacterial infection as well as the immune cells that produce inflammatory cytokines and reactive oxygen species (ROS) that damages host cells, this

results in a hyperinflammation response in the host. Our data suggest that the primary mechanism of CoPP efficacy was through the enzymatic function of HO-1. The degradation of heme yields biliverdin and carbon monoxide, and we think the evidence supports a possible role for these products in the protection of liver tissue during septicemic plague. Carbon monoxide, the product of heme catabolism, is known to induce IL-10 which helps to establish an anti-inflammatory response to LPS [82, 83]. Perhaps not unexpectedly given the tetraacylation of lipid A, which is anti-stimulatory to TLR4, we did not find an increase in IL-10 in the serum of CoPP-treated mice. However, CO has been shown to have anti-apoptotic properties that may improve the ability of cells to resist cytokine-mediated apoptosis and liver ischemic injury [71, 93-95].

While there was no overall change in the inflammatory response, there were more CoPP-treated mice that presented with high levels of IL-6 within the serum at 72 HPI. This could be indicative of a direct effect of CoPP on IL-6 expression during infection, a result of increased bacterial growth, or sepsis. Increased bacterial growth is unlikely as there was no increase in bacterial titers in CoPP-treated livers and spleens. There is evidence of potential crosstalk between IL-6 and HO-1, and that HO-1 could be inducing IL-6 and *vice versa* [96]. This could potentially be the reason why we observed higher concentrations of IL-6 in CoPP-treated animals.

Currently, it is unclear if potential induction of IL-6 was beneficial to the host during secondary septicemic plague. There are instances in previous research that support the hypothesis that induction of IL-6 may be beneficial to

the host. CoPP-induced early IL-6 production during LPS-induced sepsis led to a faster resolution of the fever state [97]. Also, higher bacterial burdens were observed in IL-6 knockout mice orally infected with *Y. enterocolitica* [98]. They also presented with a dysregulated immune response to the *Y. enterocolitica* infection, with significantly more TNF α and MCP-1 present in IL-6 knockout sera. CoPP-induced HO-1 could be acting through IL-6 to correct cytokine response to *Yersinia* infections and lower overall bacterial burdens.

Lesions present in the livers of IL-6 knockout mice were predominantly occupied by neutrophils, whereas C57BL/6 liver lesions consisted of neutrophils and macrophages [98]. IL-6 signaling could be important at resolving *Yersinia* infections given IL-6's role in resolving acute neutrophil infiltration and promoting a shift toward recruiting monocytes and macrophages to the infection. YopM has been shown to modulate neutrophil activity to create lesions in lung tissue and prevent phagocytosis [99]. Treatment with CoPP could overcome this potential *Yersinia* neutrophil manipulation by inducing IL-6 and lowering potential lesion formations at the site of infection. Resolving inflammation from liver lesions would lower potential damage to the tissue and prevent over-infiltration of immune cells [100-102]. In addition, potential cis-signaling of IL-6 could lower pro-inflammatory cytokine expression in the liver and promote expression of anti-apoptotic factors within hepatocytes [103-106].

If we assume that the anti-apoptotic properties of HO-1 mediated protection aids in shielding macrophages from T3SS-mediated death, then this increased cellular survival phenotype could be extended to other cell types, such

as neutrophils. That could decrease potential risk of further immunopathological damage from toxic neutrophilic contents as they would be less susceptible to T3SS-mediated death.

In a more general sense, if host cells are protected from T3SS-mediated death upon CoPP treatment, this could lead to less sIL-6R being shed from dead or dying cells. Without sIL-6R, this could promote more IL-6 cis-signaling in the liver, where hepatocytes present the classical transmembrane IL-6R. This would potentially promote a more resolutive inflammation response, by lowering IL-1 β and other pro-inflammatory cytokines [67, 68]. This would explain why CoPP-treated animals that presented as septic (>1,000 pg/mL of serum IL-6) had significantly less IL-1 β and IFN γ than the untreated controls.

Finally, we showed that HO-1 can be protective as a post-exposure treatment but could not protect mice from lethality when initiated during the disease phase (i.e. 48 HPI). Nevertheless, we were able to show tissue protection in CoPP-treated mice during the disease phase. If HO-1 induction is the main mode of action that CoPP treatment is having on the disease, then potential therapeutics that induce HO-1 along with traditional antibiotic treatment may improve patient outcomes from those suffering from pneumonic plague.

Figure 5-1. *Y. pestis* dissemination during CoPP treatment. (A-B) Groups of 5 C57BL/6 mice were treated with 5 mg/kg CoPP or equal volume of vehicle. Mice were then intranasally infected with 5,000 CFU of CO92 *Y. pestis* and at 72 HPI their livers and spleens were homogenized and plated to enumerate CFU. Data were collected from 2 independent experiments and vehicle and CoPP-treated groups were compared with Mann-Whitney test; n=10, medians are shown, † denotes animals that died prior to 72 HPI. Limit of detection was 100 bacteria. **(C)** Percent dissemination was calculated: Presence of bacteria in liver or spleen/total animals infected. The incidences of dissemination were compared statistically with Chi Square and Fisher's exact test, n=10, $p=0.11$.

Figure 5-2. Reduced severity of sepsis in animals treated with CoPP. (A-F) Groups of 5 C57BL/6 mice were treated with 5 mg/kg CoPP or equal volume of vehicle. Mice were then intranasally infected with 5,000 CFU of CO92 *Y. pestis* and at 72 HPI blood was collected by cardiac puncture and serum was analyzed by Multiplex or ELISA for presence of cytokines. Multiplex or ELISA tests were conducted by Dr. Rachel Olson. Data shown from 5 independent experiments; n=20-24. All data were analyzed using Mann-Whitney test, medians are shown * $p < 0.05$. **(A)** IL-6 was measured and septic animals were identified by having a concentration of IL-6 >1,000 pg/mL (Dotted Line). These septic animals were n=6 for vehicle, and n=12 for CoPP-treated. The cytokines of these septic animals were then compared, **(B-F)** **(B)** IFNβ **(C)** IL-10 **(D)** IL-1β **(E)** TNFα **(F)** IFNγ. CoPP-treated and vehicle septic mice proinflammatory cytokines were compared, significance was present where indicated.

Figure 5-3. CoPP treatment reduces liver pathology. At 72 HPI, CoPP-treated and vehicle-treated mice were euthanized and, blood and tissue collected. **(A-B)** Representative images from H&E-stained liver showing typical necrotic and neutrophilic inflammatory lesions (N) as well as occasional bacteria (B), and inflammatory foci (IF). Scale bar indicates 50 μ m; **(C)** Quantification of lesion severity (scored 0-4 for size and frequency of inflammatory foci, vascular inflammation, necrosis, and hemorrhage; bacterial microcolonies were noted when present). Data shown were collected in 4 independent trials, n=19-20 per group and analyzed for significance by Mann Whitney test. Severity was quantified by Dr. Deborah Anderson. **(D)** Serum levels of liver enzymes associated with injury (conducted by Comparative Clinical Pathology Laboratory): alkaline phosphatase (ALP), aspartate amino transferase (AST), and alanine aminotransferase (ALT); mean and standard deviation are indicated. Data shown were collected from three independent trials, n=20-22 per group. Data were evaluated by Student's *t*-test, **p*<0.05.

Figure 5-4. CoPP treatment protects macrophages from T3SS-mediated death.

RAW 264.7 macrophages were seeded overnight with 50 μ M of CoPP or vehicle. Cells were infected with 50 MOI of *Y. pestis* CO92 for 2 hours **(A)** Gentamicin protection assay was performed on RAW 264.7 cells infected at a MOI of 50, analyzed for statistical significance by Mann-Whitney test, data shown were combined from 2 independent trials, $n=12$, $*p<0.05$. **(B)** Supernatant LDH was also measured and compared to total cell lysis control. The data were analyzed for statistical significance by Two-way ANOVA and Sidak's Multiple Comparisons test, data shown were combined from 2 independent trials with the mean and SEM indicated, $n=12$, $*p<0.05$.

Figure 5-5. Post-exposure treatment with CoPP protects mice from pneumonic plague. Groups of 5 age-matched male and female C57BL/6 mice were challenged by intranasal infection with *Y. pestis* CO92. Intraperitoneal CoPP treatment was initiated at 24 (dashed line) or 48 (solid line) HPI; additional treatments were given every 48 hours over the 7-day observation period. Data shown were collected in 3 independent trials. Data were evaluated by Gehan-Breslow-Wilcoxon log rank test, n=15 per group; * $p<0.05$.

Supplemental Figure 5-1. CoPP treatment induces HO-1 expression in the liver.

(A) Comparison between daily and every other day dosing was assessed by measuring HO-1 in the liver homogenates at the indicated time points. Data were compared using One-Way ANOVA and Tukey's post-test, * $p < 0.05$, $n = 10-11$, ns denotes not significant.

(B) Dosing regimen for the studies shown in Figures 1-3. Pre-treatment with CoPP (5 mg/kg) or vehicle was provided 24 hours prior to challenge. Additional treatments were provided every other day for the duration of the observation period.

Supplemental Figure 5-2. Correlation between bacterial growth in the lungs and serum IL-6 in *Y. pestis*-infected mice. Groups of 8 age-matched male and female C57BL/6 mice were challenged by intranasal infection with *Y. pestis* CO92. Data shown collected from 2 independent trials. CFU and serum IL-6 data were correlated using a Pearson Correlation Coefficient, $r= 0.9508$, $n=16$, $p< 0.0001$.

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Chapter 6. Discussion

Pneumonic plague is an aggressive disease characterized by bacterial proliferation in the lungs, leading to bacterial dissemination throughout the body [1-4]. This dissemination leads to a secondary septicemia causing organ damage and septic shock [2, 5, 6]. Due to this two-front assault that *Y. pestis* carries out on the mammalian host during the pneumonic infection, there is little wonder as to why the fatality rate is so high if left untreated. While the incidences of *Y. pestis* infections are low in the United States, the treatment model that is defined in this body of work allows for the testing of potential therapeutics for bacterial agents that can cause pneumonia and potentially lead to sepsis [7, 8].

Streptococcus pneumoniae and *Klebsiella pneumoniae* which maybe both extremely drug resistant and prevalent nosocomial pneumonic infections present a health concern that this particular treatment model can address [9-14]. Improved treatments are needed that can aid immune clearance of the infection without applying selective pressures on the bacteria that could generate antibiotic resistance [15, 16]. Since *Y. pestis* is such an aggressive pathogen, if cobalt protoporphyrin (CoPP) treatment is successful against such a pathogen, then it is likely to aid the host in other bacterial infections.

Our research indicates that yersiniabactin aids in the establishment of a permissive environment that is favorable to bacterial colonization of the lung and ybt increases lung damage in the host. The evidence put forth in this work leads us to believe that the permissive state established by yersiniabactin is due to lung damage, however, it is not currently known if the damage is due to iron

deprivation. We also observed that the presence of *ybt* and the *pgm* locus induced transcription of heme oxygenase-1. We initially thought that *ybt* could be inducing HO-1 and its anti-inflammatory activity to avoid clearance in the lungs. After inhibiting HO-1 activity by zinc protoporphyrin (ZnPP), there was no significant change in survival during pneumonic plague. Zinc protoporphyrin leads to degradation of BACH1 and upregulates *Hmox1* expression, similar in action to CoPP [17, 18]. However, ZnPP is a competitive inhibitor of HO-1, but does not inhibit other BACH1-repressed genes that are involved with redox regulation or cell survival [19]. This means that the main host factor that is being manipulated throughout these experiments is HO-1. However, it became apparent that HO-1 was being induced in response to the damage inflicted by the *pgm* locus. By using CoPP to induce the host factor HO-1 prior to and during the infection we noticed an increase in survival in the mice intranasally infected with either *pgm*⁻ or *pgm*⁺ wild-type *Y. pestis*.

Survival rates between the *pgm*⁻ and *pgm*⁺ *Y. pestis* intranasal infections when treated with CoPP give us a unique investigation into the role of sepsis and septicemia during pneumonic plague. We saw a significant increase in CoPP-treated mice survival that had been infected with *pgm*⁺ *Y. pestis*. However, there was not a full protection against the infection gained by the CoPP treatment with around 60% of the infected mice surviving. There was 100% survival of the CoPP-treated mice infected with *pgm*⁻ *Y. pestis*. The *pgm*⁺ *Y. pestis* infection leads to pneumonia and septicemia whereas the *pgm*⁻ infection only leads to septicemia [20].

Firstly, it indicates that inducing HO-1 during *Y. pestis* infection has a more positive effect on septicemia outcomes. This may be due to the fact that induction of HO-1 helped to modulate the inflammatory response during sepsis, leading to a potentially more productive immune response. Previous research suggests that induction of HO-1 during a sepsis response significantly improves survival [21-23]. Our research indicated that there were lowered amounts of pro-inflammatory cytokines in mice suffering from sepsis. Added to that was there were more mice that presented with high amounts of IL-6 in the CoPP-treated group. While that ran counter to our hypothesis of CoPP treatment potentially lowering the pro-inflammatory cytokine production during infection, a possible explanation could be that HO-1 was controlling the inflammatory response and preventing it from producing a cytokine storm-like event [24-26]. In this situation, CoPP treatment would allow for more mice to survive high levels of inflammation during sepsis [22, 27-31]. These are likely contingent on the byproducts of HO-1 activity, namely carbon monoxide and biliverdin [32-35].

Whereas in the lungs, we found that protection from lung damage was the most critical factor that was conferred by inducing HO-1. The literature stated that VEGF (vascular endothelial growth factor) would be upregulated upon HO-1 induction. However, we did not observe elevated levels of the growth factor in CoPP-treated mice [36-40]. A way that inducing HO-1 could be protecting lung barrier that does not involve promoting growth factors within the lung, is by protecting lung cells from T3SS-mediated cellular death. We have shown in both MHS and RAW macrophages that when treated with CoPP, these cells are able

to resist cellular death mediated by the T3SS of *Y. pestis*. Most of the work on T3SS seems to indicate that cells undergo apoptosis, and induction of HO-1 has been shown to induce anti-apoptotic factors and inhibition of cellular death [30, 33, 41, 42].

YopJ is one of the T3SS-effectors that causes disruption of MAPK3/7 and IKK β signaling and induces apoptosis [43-45]. However, there was no change in the induction of *Hmox1* when infected with a *Y. pestis* catalytic-null YopJ strain (data not shown). This could indicate that YopJ is not the effector that is affecting HO-1 induction. To investigate this circumstance further, there should be testing of differing Yop mutants to determine which Yop is responsible for inhibiting *Hmox1* production. Transitioning to a pneumocyte model to better resemble the lung epithelium, will be necessary to determine how Yops affect overall cellular death in the lungs, and how this is prevented by CoPP treatment.

We observed increased IL-6 levels in both the blood and the lungs of CoPP-treated animals infected with *Y. pestis*. Induction of HO-1 by CoPP either allowed for endurance of inflammatory cytokines or upregulated IL-6 production during the infection. This may be the action of HO-1 crosstalk with IL-6, or inducing fever more quickly in order to resolve sepsis, or some novel pathway that HO-1 activity interacts with IL-6 during the *Y. pestis* infection [46, 47]. A way to investigate this phenomenon would be to measure the production of IL-6 from *Hmox1* knock-out mice during a *Y. pestis* infection. Comparison of those data to wild type could elucidate the role IL-6 plays in the resolution of *Y. pestis* infections and whether the HO-1 interplay with IL-6 improves infection outcomes.

Y. pestis has many ways to avoid ROS-mediated killing [48-52].

Yersiniabactin has been shown to reduce ROS-generation capabilities of neutrophils [53, 54]. Yersiniabactin can also bind to Cu^{2+} to form a superoxide dismutase mimic to protect bacteria from reactive oxygen species [55]. However, while neutrophils are producing and secreting ROS into the tissue environment in an effort to kill the bacteria, for which *Y. pestis* is protected against, they are also damaging the lung [56, 57]. This hypothesis of ROS damage contributing to pneumonia needs further testing. Direct measurement on the effect that CoPP treatment has on ROS generation and lipid peroxidation of lung epithelial tissue needs to be conducted. If there is a reduction of ROS-mediated lung damage upon CoPP treatment, then this treatment could be an effective co-treatment to use to prevent other bacteria from disseminating from the lungs, such as *Klebsiella* and *Streptococcus*. Combinatorial treatment with drugs that induce HO-1 and antibiotics would be extremely helpful due to the cytoprotective role HO-1 plays in tissue integrity, thereby preventing dissemination. If dissemination did occur, then induction of HO-1 would prevent sepsis by downregulating TLR4 signaling to lessen the effects of potential endotoxic shock from bacterial lysis.

In the literature there is evidence that there is a reduction of neutrophil recruitment when HO-1 activity is induced by CoPP treatment [58-60]. We did not see a significant change in the numbers of neutrophils upon CoPP treatment but there were elevated numbers present in the vehicle-infected group. CoPP treatment could be decreasing the number of neutrophils present in the lungs or enabling them to resist cellular death, thus lowering toxic content release and

preventing lung damage. Perhaps later time points must be measured in order to establish if neutrophil numbers are decreasing in a meaningful way as the disease progresses toward more immunopathology.

In the post-infection treatment study, we observed a similar survival phenotype in mice that received CoPP treatment 24 HPI as those that were pre-treated. However, this protection against respiratory *Y. pestis* infection was not seen when CoPP treatment began at 48 HPI. Since the fever response in *Y. pestis* is most often used as a marker to begin treatment, using telemetry to monitor the body temperature of the animal could be used to see if HO-1 administration is important pre or post-fever [61, 62]. Constant monitoring of the fever response during CoPP treatment could also show if the induction of HO-1 helps to ameliorate the pathophysiological response to sepsis.

CoPP is not FDA-approved, but has been proposed for clinical use as a HO-1 inducer [18, 63]. However, this may be unlikely as CoPP has been shown in rats and hamsters to lower and deplete hepatic cytochrome P-450 [64, 65]. This enzyme is important for the enzymatic breakdown of drugs and dysfunction of cytochrome P-450 has been linked to liver disease, hepatitis, and dysregulation of copper metabolism [66-68]. The limited ability to metabolize drugs such as acetaminophen while undergoing CoPP treatment, makes CoPP a less optimal candidate for an inducer of HO-1 during the course of a bacterial infection [64]. This dysfunction of cytochrome P-450 may be a potential reason as to why CoPP treatment did not improve overall liver function during *Y. pestis* infection. Other pharmaceutical inducers of HO-1 need to be studied such as CP-

312 or curcumin, to gauge their effectiveness during an infection and to see if the side effects of CoPP treatment can be avoided [69-72].

Overall this treatment method, in which we have augmented the capability of the host to resolve a fatal disease without the use of antibiotics, is a novel and necessary way to approach the goal of antibacterial and therapeutic research. Combinatorial therapy with immune modulators could extend the usefulness of antibiotics by preventing antibiotic resistance and improving patient health during the infection. With more bacterial strains becoming resistant to antibiotics, the data presented in this dissertation may change the way that infections are treated such that the correction of the natural immune response by inducing HO-1 is used to prevent mortality from pneumonic plague and other bacterial pneumonia.

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Vita

Joshua Willix was born May 2, 1984 in Brevard, NC. He is the son of Marie (Lance) Owen and Terry Willix, and the brother of Matthew Willix. He grew up in Brevard, NC and graduated from Brevard High School in 2002. He did his undergraduate work at University of North Carolina at Charlotte (UNCC), in Charlotte, NC. He graduated in 2007 with a Bachelor of Science in Biology and with Biology Honors. While at UNCC, he worked on studying the viable but non-culturable state of recurrent uropathogenic *E. coli* infections under the mentorship of Dr. Todd Steck. After spending a few years in industry as a laboratory technologist at LabCorp in Burlington, NC, he began work towards a Doctorate in Philosophy in 2011 at the University of Missouri. At Mizzou, he began working for Deborah Anderson, studying bacterial pathogenesis using a murine pneumonic plague model and developing novel treatments from those studies. In the future, he intends to devote himself to improving treatment options for patients suffering from antibiotic resistant bacterial infections. In his spare time he enjoys biking, hiking, and writing Dungeons and Dragons campaigns.