YERSINIA PESTIS YOPJ AND ITS

IMPACT ON INNATE IMMUNITY

A Thesis

Presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

KAYLA DANIELLE MARKS

Dr. Deborah Mae Anderson, Thesis Supervisor

MAY 2022

The undersigned, appointed by the dean of the Graduate School, have examined the Dissertation entitled

YERSINIA PESTIS YOPJ AND ITS
IMPACT ON INNATE IMMUNITY

presented by Kayla Danielle Marks,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

_____
Dr. Deborah Anderson

_____
Dr. Jerod Skyberg

_____
Dr. Solomon Odemuyiwa

_____
Dr. Carol Reinero

_____
Dr. Aaron Ericsson

DEDICATION

I am the great-granddaughter of sharecroppers, limited to a 3rd grade education.

I am the granddaughter to a home maker, who's honest living was cooking and cleaning much of her life. A widow, tasked to raise 5 black boys in 1950s rural Georgia.

The level of impoverishment both of my parents grew up with included outhouses, one of many examples of the shortcomings they were burdened with.

It is with great honor and humility that I managed to be the *second* doctor in my family... and I would be remissed to not acknowledge that. It was only through the support of my biological family, and <u>chosen</u> family, my friends, that I have been able to accomplish such a feat. At this level of rigor and scholarship, the most important attribute one can have is *perseverance*. Above all else. And I give many thanks to God to those He allowed to cross paths with mine, and change my journey for the better. Through the help of these people and my mentors, I have not only exhibited a doctoral level of scholarship, but significantly grown as a person. These people will forever have my gratitude for the strength, courage, and wisdom they poured into me during this tumultuous journey, and their support reminds me of this poem:

be softer with you,

you are a breathing thing

a memory to someone

a home to a life

And so, I will carry these sentiments with me always.

ACKNOWLEDGMENTS

I would like to acknowledge the many mentors that poured into me, and nurtured my aptitude to become a scientist.

It is the teachers that keep society afloat. The future is always in the youth. If it were not for the intervention of teachers, we would be a dozen standard deviations away from where we have progressed to now.

Their investment in students is indispensable.

Thanks to a teacher in 9th grade that saw potential in me, Mr. Jonathan Johnson, I managed to accumulate many accolades, and am now able to add Ph.D. to the list. All because someone saw potential in me.

Many thanks to my undergraduate advisors that helped me gain research experience, to my PREP advisors who pushed me, my committee who challenged me, and most of all my thesis advisor, Dr. Deborah Anderson who truly invested in me.

I can't imagine taking this journey with another Principal Investigator. I will be eternally grateful for the patience and empathy she has shown me. I am also appreciative for the times she pushed me, and more importantly, kept me motivated. The example she has modeled as a woman in science is immeasurable, and I'm so happy I chose her to go on this journey with. Career path aside, I will take on the responsibility of being a positive model to young black scientists and women scientists, and I know her teachings and example will fortify my shoulders for budding scientists to stand on.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure: Chapter 4

ABSTRACT

Yersinia pestis is one of the most virulent pathogens known to man, with the most impactful epidemics in human history. As the causative agent for the plague, *Y. pestis* has demonstrated its propensity to ravage through animal and human populations. As a zoonotic disease that can confer transmission between different species of mammals, this further illustrates the importance of plague research. Today, plague still has a foothold in several countries such as India and Madagascar. Worldwide outbreaks led to its ranking as number 5 in emerging zoonotic diseases by the Centers for Disease Control in 2019. Despite the gratuitous devastation it has displayed worldwide, *Y. pestis* has piqued interest in use for bioterrorism from multiple superpowers. Given the exhibition of its reach in the 14th century, and its conserved methods of pathogenesis in other pathogens, plague studies demonstrate a broader impact in elucidating the mechanisms of infectious diseases.

Chapter 1: Introduction

Y. pestis employs mechanisms commonly used by gram negative bacteria to reprogram host cell responses during infection. Particularly, Y. pestis constructs an injectisome when it makes contact with target cells, shuttling toxic effectors (effector proteins) into the host cell. This process triggers an inflammatory crescendo to mitigate the infection toward resolution. One of the most prominent virulence factors of Y. pestis, YopJ has a myriad of host proteins it targets for post-translational modification in an effort to dampen immune responses. This modulation at the cellular level is accompanied by major systemic impacts, including subjecting the mammalian host to sepsis. This is one of many examples of how the host immune response can become pathogenic for the host. And so, the focus of this dissertation work was to elucidate the role of YopJ in skewing the host response away from protection. The studies centered around 2 signaling pathways: type I interferon and toll-like receptor 7. We used treatment assays to block type I interferon signaling and administered type I interferon, IFNβ to observe for the impact—protective or deleterious—type I interferon has during Y. pestis infection. We also incorporated TLR7$^{-/-}$ mice in observation of its role in pathogenesis. Thus, we conclude that there is intercalation of type I interferon, toll-like receptor 7 and YopJ towards pathogenesis during Yersinia pestis infection.

Historical Impact of the Plague

  Yersinia pestis is a gram negative facultative organism and is the causative agent for the plague. *Y. pestis* is best known for being the culprit of the "Black Death" also known as the Justinian plague, which is the first of three pandemics *Y. pestis* caused. The plague began in Egypt, quickly spreading throughout the Middle East and eventually to all parts of the "known world" with most of its impact taking place in the 14th century, illustrated in Figure 1 [1].
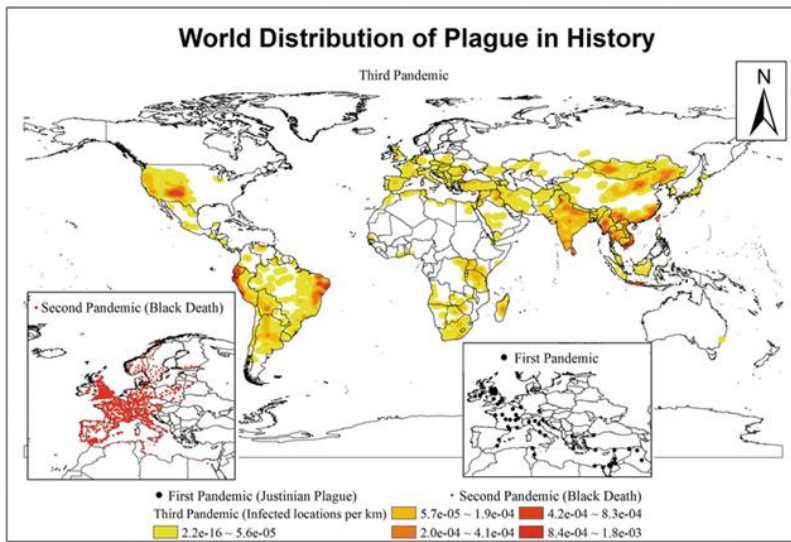
Figure 1: **World Distribution of Plague.** Illustration of the reach of transmission of Plague [2]

This pandemic killed 17-28 million Europeans (~30-40% of the population) [3, 4]. What's more, what added to the devastation of the pandemic was its occurrence in cycles. During this time period, many populations suffered from the same problem: hygiene. This is evident in the many other types of infections that were circulating at the time such as smallpox, typhus, and syphilis to name a few. And so, many developed countries such as Europe were not impervious to the

transmission and impacts of plague. An additional factor to the vicious spread of the disease is because of trade routes. As countries imported and exported goods across the world, these journeys were accompanied by pests such as rodents, which serve as natural reservoirs for the bacteria, illustrated in (Fig. 2).
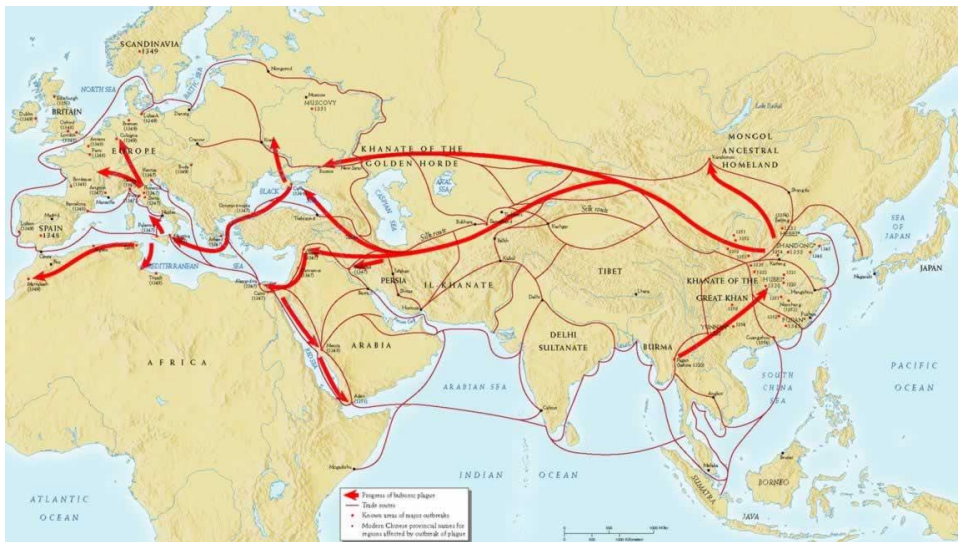
Figure 2: Spread of the bubonic plague in the 14th century. Fine lines indicate trade routes. Arrows indicate dissemination of plague [5].

In regards to infectious disease, the technological advances over the past few centuries have evolved our tools for warfare, which now includes the usage of biological weapons [6, 7]. These techniques date back to 600 B.C. in the use of poisonous herbs to lay siege in opposing countries. Additionally, during the *Y. pestis* plague of the 14th century, Russian troops catapulted plague infected human cadavers into Swedish cities [6]. During World War II it is reported that the Japanese army dropped plague infected fleas over populated areas in China and Manchuria [6, 8]. Now, many contemporary superpowers including the United States of America have explored methods for use of *Y. pestis* for

weaponry. In 1970, the propensity to use *Y. pestis* as a biological weapon progressed so much so that the World Health Organization reported the "worst case scenario of the deliberate release of 50 kg of aerosolized *Y. pestis* over a city of 5 million would result in pneumonic plague in 150,000 persons, with 36,000 casualties." Further, the bacilli would remain viable for up to 1 hour at a radius of 10 km [9]. This information taken together is a testament to the gravity of devastation that would incur upon a large populous of people. Due to globalization, this would yield unprecedented worldwide catastrophe.

The Etiology of *Yersinia pestis*

The life cycle of *Y. pestis* begins with infection of the flea, serving as the vector for transmission of the bacteria, sylvatic cycle illustrated in Figure 2 [10].
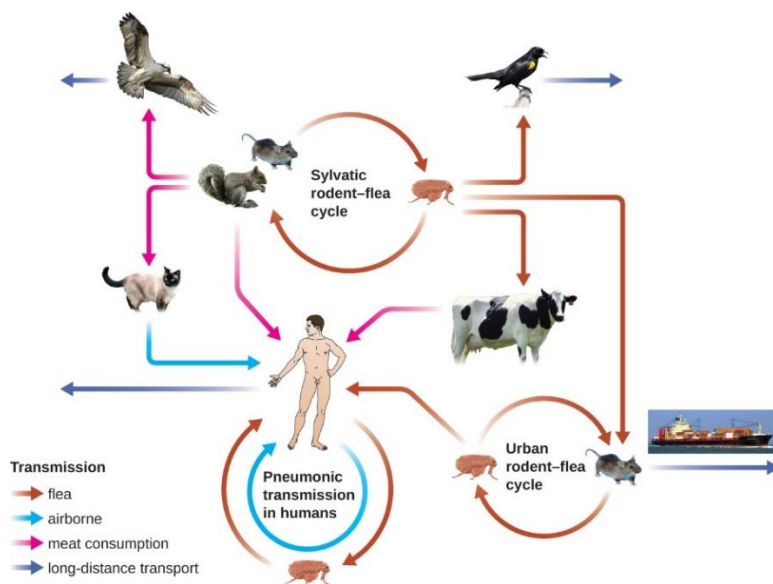
Figure 3 : Sylvatic cycle of plague. Illustration of plague transmission as a zoonoic disease. [11]

The flea becomes infected with *Y. pestis* which induces biofilm formation. The extracellular matrix in the proventriculus (midgut) will grow to physically impair the flea from taking a blood meal [12]. When the flea takes a blood meal from various mammalian hosts—cats, dogs, rodents, humans, etc.—the backwash of the flea causes parts of the biofilm to sluff off into the host, beginning the mammalian infection. The flea is an ideal vector for *Y. pestis* transmission because during feeding the flea is searching for a capillary in the host, which may require multiple bites and thus multiple exposure events of the bacteria [13]. This is also called "frustrated feeding," or failed attempts to take a blood meal due to blockage of the proventriculus. Due to the blockage, the flea continuously bites the host seeking relief from hunger. This blockage event illustrates efficient transmission of *Y. pestis* given the multiple opportunities for the bacteria to enter the bloodstream. At this point, the infection can rapidly progress within the host as the bacteria have been directly placed in the bloodstream (bacteremia/septicemia). This method of infection can also metastasize into other forms of plague such as bubonic or pneumonic. Bubonic is described as a hyperinflammatory response of lymph nodes. After bacteria are transmitted into the mammalian bloodstream, they traffic to the nearest draining lymph node. There, they exhibit their proclivity for infecting effector cells such as macrophages [14]. Due to their infectious properties as defense mechanisms against host response, they curtail many points of intervention by the host immune system. And so, they are able to form a replicative niche within these effector cells and rapidly proliferate throughout the organism without much

interference. This proliferation and subsequent cell recruitment can lead to the swelling of the lymph nodes, designated as "buboes" during infection. Pneumonic plague can develop from bubonic or septicemia but can develop from aerosolized bacteria in saliva droplets of an infected mammal. Pneumonic plague is also the most efficient form of transmission of *Y. pestis* and has the most devastating impact on populations as air borne transmission is the easiest form of transmission [15].

Molecular Mechanisms for *Yersinia pestis* Infection

Y. pestis has 2 virulence factors that determine biosafety level containment: the pCD1 plasmid and the pigmentation locus. Particularly, the pCD1 plasmid is a 70kb plasmid, with iterations found in other *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*) encodes for the type three secretion system (T3SS). The T3SS comprises of 3 types of proteins: structural proteins, chaperone proteins, and effector proteins [16]. Structural proteins make up the needle-like structure (injectosome). This structure spans from the bacterial cell wall to the target cell membrane, allowing structural and effector proteins to be shuttled from the bacterial cytosol to the target cell cytosol. Chaperone proteins accompany effector proteins through the injectisome, helping effector proteins maintain the fidelity of their structure as they are trafficked into the host cell. Lastly, the most impactful to disease outcome are effector proteins, also known as *Yersinia* outer proteins, or Yops. Yops have a wide range of enzymatic activity orchestrated towards the goal of modulating host cell responses toward a

bacterial advantage. For example, proteins like YopE and YopT interfere with

Rho GTPase activity, significantly stunting the host cell's ability to regulate actin

filament polarization [17]. This impact on cytoskeleton significantly reduces host

cell capability to phagocytize bacteria. Further, Yops also interfere with

intercellular signal transduction and proinflammatory cytokine production [18].

This yields insufficient communication to neighboring cells, rendering them

quiescent in response to the infection.

YopJ Enzymatic Activity

Of the Yops that are significant virulence factors in *Y. pestis*, YopJ is one

of the more impactful with a host of enzymatic functions. YopJ has a well

characterized history of disrupting homeostatic immune responses, driving the

outcome of infection to host disadvantage [19]. This is fortified by studies that

have used YopJ deletion mutants (ΔYopJ) or the catalytically null mutant

(YopJ$_{C172A}$) compared to wild type *Y. pestis*, significantly attenuating the strain

during infection [20]. *Y. pestis* YopJ is also a member of a larger YopJ effector

family. This family has homologs in other bacteria pathogenic to other mammals,

plants, and fish not just in *Yersinia spp.* This representation of the T3SS and

YopJ in other pathogens illustrates a broader impact on these studies.

Although the structure of *Y. pestis* YopJ has not been determined, a high

resolution molecular structure of a homolog from *Xanthomonas* has been solved

[21]. From this and numerous years characterizing the enzymatic activity of YopJ

and its family members, the acetyl transferase activity is known to be contingent

upon covalently attaching to host cell compound inositol hexaphosphate (IP_6). [22]. After this binding event, YopJ goes through a conformational change, making it suitable to exercise its functions within the host cell through allosteric activation. Additionally, contingency of the acetyltransferase activity by YopJ towards MAP kinases can be seen through the addition of acetyl-CoA in a dose dependent manner (a part of the necessary intermediate step of the binding event of IP_6 to the YopJ substrate) [23]. This further illustrates the strong affinity YopJ has for IP_6 given the positive correlation between increase in YopJ enzymatic activity and the availability of IP_6. As bacteria that produce YopJ do not produce IP_6, this limits the activity of YopJ function to within the host cell rather than in the bacterial cell.

Acetyltransferase activity

The YopJ effector family is well characterized to confer acetyltransferase activity [24]. Particularly, in the mammalian host, these YopJ effectors intercede in the MAP kinase pathways. *Yersinia* YopJ has several points of entry in the MAPK signaling pathway, inhibiting enzymatic function in TAK1, IKKβ, MKK2, and many others [25-27]. Congruently, YopJ homologs also target multiple kinases that participate in the MAPK pathway that they render inactive because of acetyltransferase activity. Homolog AvrA is an effector protein of *Salmonella enterica* that inhibits the JNK arm of MAPK signaling [28-30]. Comparatively, homolog VopA belongs to the pathogen *Vibrio parahaemolyticus* and is an enteric bacterium that infects salmonoids (salmon). VopA exhibits a more robust

impact on MAPK signaling, interfering with 3 different arms of MAP kinase signaling: p38, JNK, and ERK [31, 32]. Particularly, after YopJ covalently attaches to IP$_6$ and becomes enzymatically active, it transfers acetyl groups from acetyl donor acetyl-coenzyme A (Ac-CoA) and catalyzes those acetyl groups on serine, threonine, and lysine residues on target proteins [24, 33]. Acetylation at these residues prevents signal transduction. One of the integral properties of YopJ is its proclivity to inhibit MAPK pathways. The result of this intervention is especially deleterious because it prevents intracellular signaling such as NF$_\kappa$B mediated? transcription, inhibiting the host cell from killing intracellular bacteria. This also subsequently prevents extracellular signaling, inhibiting the appropriate global immune response.

MAP Kinases

YopJ has a vast catalog of deleterious impacts on MAP Kinases. Mitogen activated protein kinases or MAPKs are cell signaling proteins that have a barrage of intracellular function. Its enzymatic activity transfers phosphate groups onto threonine and tyrosine residues, contributing to proinflammatory responses, osmotic stress, apoptosis, gene expression and much more [34-37]. Regarding innate immune signaling, (most relevant to the context of YopJ function) MAP kinases play an indispensable role in curating the appropriate immune response to resolve the infection. MAP kinases also require a cascade of phosphorylation events to become active in their activation loops [36]. Phosphoryl groups need to be added to threonine and tyrosine groups to maintain an active conformation.

This is relevant because this signaling cascade leads to transcription, and subsequent translation of proinflammatory cytokines. Congruently, YopJ proactively prevents phosphorylation of MAPK proteins in the MAPK signaling cascade and consequentially skew the host cell response away from a proinflammatory response.

Innate Immunity: Toll-like Receptors

Toll-like receptors (TLRs) are the mammalian iteration of toll receptors found in drosophila [38]. TLRs are germline membrane bound receptors that recognize evolutionarily conserved microbial motifs, or PAMPs (pathogen associated molecular patterns) [39]. This is important because a built-in diverse repertoire of pathogen associated structures protects an organism from progressed encroachment of microorganisms. This is due to the early ability of the immune system to recognize and irradicate the pathogen. Particularly, intracellular TLRs (also known as endosomal TLRs) are designed to recognize nucleic acids derived from viruses and bacteria. For instance, TLR3 recognizes dsRNA, TLR9 recognizes bacterial and viral DNA rich in unmethylated CpG-DNA, and TLR7 recognizes ssRNA [40]. TLR recognition of PAMPs in turn makes TLRs pathogen recognition receptors (PRRs). As TLRs are germline receptors, this makes them an indispensable part of innate immunity. As the "first line of defense" for the immune system, innate immunity is integral to combating infections.

<u>Toll-like receptor 7: TLR7</u>

Toll-like receptor 7 (TLR7) has been characterized to have viral associated motifs (double stranded RNA, dsRNA) as its ligand [41]. TLR7 transcription begins in response to proinflammatory cytokine signaling. TLR7 Is then synthesized in the endoplasmic reticulum (ER). Much like YopJ, TLR7 is accompanied by chaperone proteins to ensure proper protein folding before exit from the ER and translocation to the Golgi apparatus [42-44]. In the Golgi, TLR7 becomes ubiquitinated and is trafficked to lysosomal organelles by adaptor proteins AP-3 and AP-4 [45]. After TLR7 activation, adaptor protein MyD88 is recruited to the C-terminal domain, triggering a flurry of other protein recruitment composing the Myddosome complex. This compound structure consists of IRAK proteins (interleukin 1 associated receptor kinases), IRF proteins (interferon regulatory factors), and TRAF6 (tumor necrosis factor receptor associated factor 6) [46]. These proteins also participate in other cell signaling pathways. The end result of TLR activation of the Myddosome is the activation of MAP kinases that regulate major inflammatory transcriptional mediators NFκB and AP-1, thus conferring proinflammatory signaling. In addition, TLR7 signaling stimulates IRF transcriptional activation, leading to the expression of type I interferon which typically is a curated response against viral infection known as the antiviral state [47]. Therefore, activation of TLR7 contributes to a pro-inflammatory cytokine signal transduction, including type I IFNs. Pathway illustrated in Figure 4.
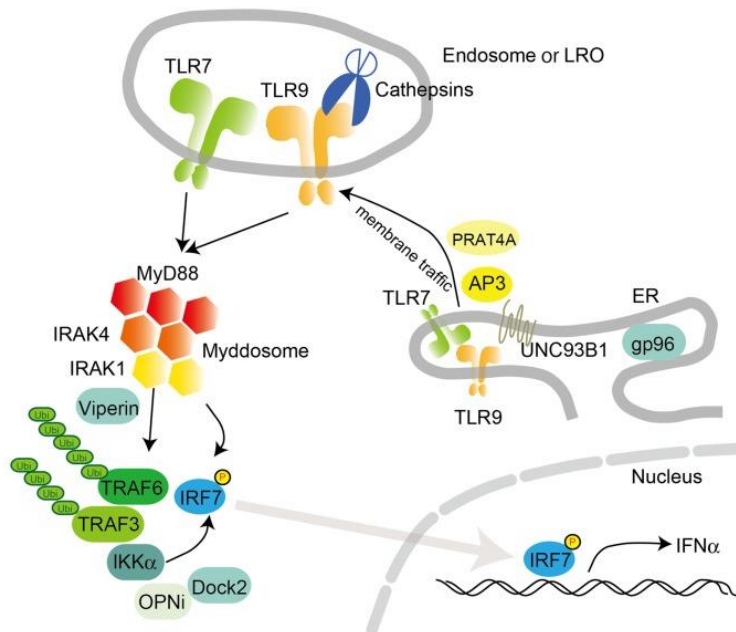
Figure 4: Intracellular signaling and trafficking of toll-like receptors. [39]

Type I interferon

Type I interferons (type I IFNs) are a part of the elaborate network of proinflammatory cytokines and a member of three different interferon families [48-50]. Initially characterized in viral models, type I IFNs were named for their ability to "interfere" with viral replication and subsequent proliferation. In the human genome, there are 13 coded interferon genes combined for all three interferon families. Type I IFN signaling is conferred after proinflammatory cytokine production. Transcription and translation of type I IFNs IFNα and IFNβ accompanies other proinflammatory cytokine production such as NF$_\kappa$B and AP-1 after immune recognition of one of many PAMPs in the membrane, cytosol, or endosome. Interferons IFNα and IFNβ serve as the ligands for IFNAR and can be produced by nearly all cell types, mouse and human [48]. Upon stimulation of

IFNAR by IFNα and IFNβ, the decoupled transmembrane receptor dimerizes and confers signal transduction. These interferons like other cytokines then go on to activate neighboring cells, particularly effector cell populations such as lymphocytes. This activation event goes on to produce interferon stimulated genes (ISGs)—a positive feedback response to a cell being activated by an interferon [51]. Activation of ISGs rapidly activates and utilizes the JAK-STAT pathway, another mediator of immunity. Stimulator of Interferon genes (STING) is also activated and induces type I IFN in cells infected with intracellular pathogens [52]. STING activation results in 1000s of gene upregulation and further contribute to inhibiting viral entry into the cell, replication, and exit from the cell [51]. Upon activation, there is an induction of type I IFNs IFNα and IFNβ. This particularly leads to autocrine responses of the infected cell, contributing to a positive feedback loop of inflammation. This positive feedback loop differentially saturates the immune response compared to other signaling pathways. Figure 5 illustrates upregulation of genes after type I IFN treatment in various cell types.
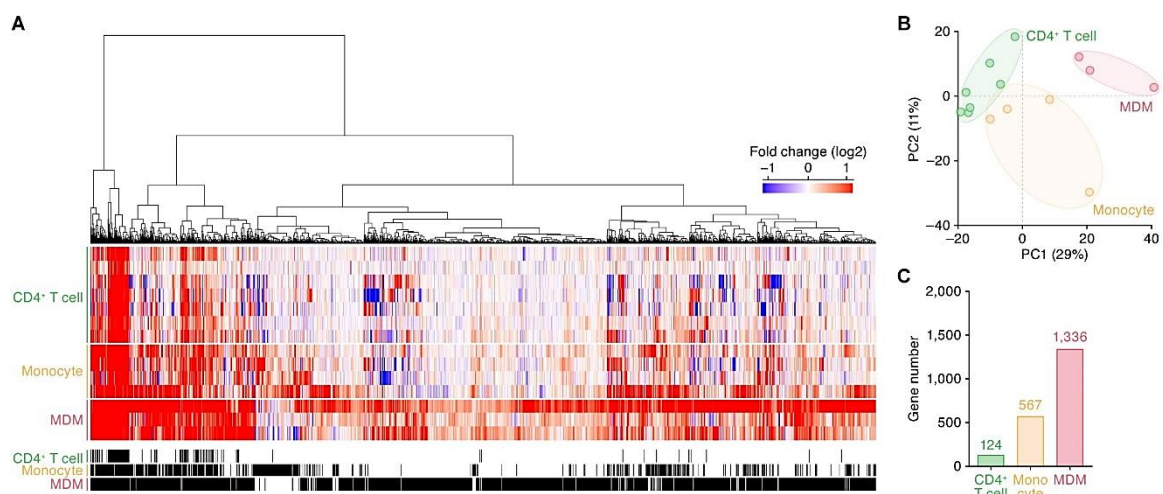
Figure 5: **Interferon gene stimulation after type I IFN treatment in monocyte derived macrophages, monocytes and CD4+ T cells.** A) heatmap of gene stimulation. B) Principal component analysis separating transcriptome data according to cell type. C) Comparison of upregulated genes amongst the cell types [53].

Given the protective nature aforementioned in type I IFNs, they can still contribute to pathogenesis. In patients with HIV, rapid progressors show stronger IFNα/β signatures than non-progressors [54]. During *Listeria monocytogenes* bacterial infection, it has also been demonstrated that IFNAR$^{-/-}$ mice survive longer and yield significantly lower bacterial load compared to wild type mice [55-57]. During infection of *Mycobacterium tuberculosis*, the bacteria upregulate type I IFNs and increase expression of negative regulators of the JAK-STAT pathway [58]. Our group has also shown a significant reduction in pathogenesis in IFNAR$^{-/-}$ mouse survival and bacterial load. And so, given the intercalated involvement of YopJ in the TLR7 and type I IFN signaling pathways, the goal of this dissertation research was to investigate the pathogenic roles of TLR7 and type I IFN during *Yersinia pestis* infection. Particularly, we wanted to elucidate the role of YopJ in modulating these normally beneficial host pathways towards pathogenesis during plague. Our long term objective is to identify new therapeutic interventions that augment the antibiotic treatment of plague in humans.

Chapter 2: *Yersinia pestis* YopJ modulation of the Type I IFN response contributes to pathogenesis.

Abstract

Yersinia pestis is a gram negative bacterium and the causative agent for the plague. *Yersinia spp.* use effector proteins to skew the host immune response toward a bacterial advantage during infection. This modulation of host immune responses particularly disrupts intracellular, and subsequently extracellular signal transduction. Effector protein YopJ has shown a disposition to effect multiple signaling pathways, including the type I IFN pathway. These data demonstrate that signaling through the type I IFN receptor (IFNAR) pathway during *Y. pestis* infection contributes to disease through survival and bacterial load. The presence of YopJ enzymatic activity, coupled with IFNAR signaling constituting this phenotype was a novel finding. Histological and pseudo-liver function tests and liver enzyme analyses corroborate the hypothesis that interaction between YopJ and the IFNAR signaling pathway exacerbate disease. Given these significant impacts on disease outcome, *Y. pestis* YopJ modulation of the type I IFN response deleteriously effects host response and the subsequent result of infection.

Introduction

Yersinia pestis is a gram-negative intracellular bacterium and the causative agent for the plague. Commonly employed by gram negative bacteria, *Y. pestis* utilizes the type III secretion system (T3SS), a virulence factor encoded

from the pCD1 plasmid [59]. This virulence factor leads to the construction of an injectosome to translocate effector proteins (*Yersinia* outer proteins, Yops) into the eukaryotic target cell. Yops modulate host cell physiology, including impaired phagocytic capability, activation of programmed cell death, and disruption of normal cytokine signaling. For example, YopH is a highly efficient tyrosine phosphatase and in its active form causes a global decrease in tyrosine phosphorylated proteins [60]. Disturbing this regulatory process has devastating effects on cell signaling because GTPase function under normal conditions triggers MAP kinase and NFκB transcription. YopK is an essential regulator of the type III secretion system and required for virulence that dictates rate and frequency of excretion of proteins [61]. Additionally interesting, yops also work together to orchestrate a specific host cell response. For example, YopE is a GTPase activating protein with rapid disruption of actin filaments throughout the cell [62]. Concurrently, YopO inhibits GTPases from converting to their active forms. YopE and YopO activity in conjunction with YopH work together towards down-regulating GTPase activity [63]. YopE and YopH also work together to compromise phagocytic capability of dendritic cells by disrupting actin filaments [64]. These mechanisms during infection aid in skewing the disease outcome towards a bacterial advantage. As a result, plague rapidly progresses as a product of the dysregulation of the host inflammatory response.

Given the mechanics *Y. pestis* uses to influence disease outcome, the exhibition of modern plague towards antibiotic resistance is concerning. Isolates from Madagascar patients in 1997 (*Yersinia pestis* 16/95 and *Yersinia pestis*

17/95) exhibited resistance towards several antibiotic treatments including streptomycin, the preferred treatment of plague [65, 66]. And so, more studies on the infectivity of *Y. pestis* is paramount to curtail potential widespread cases through natural dissemination or bioterrorism threats, devastating human populations.

Type I IFN

Congruently, during *Y. pestis* infection, there are simultaneously a multitude of immune responses to combat the infection, including the type I IFN response. Amongst the myriad of host cell responses, the type I IFN response is one of the more cogent methods of defense for innate immunity. Initially characterized in viruses, type I IFNs are cytokines that disrupt translation of proteins that viruses hijack during their life cycle that prevent viral intermediate development to proliferate in the organism [67]. This process is also known as the anti-viral state. Particularly, protein kinase RNA-activated (PKR kinase) is an interferon induced kinase that inhibits viral protein translation through phosphorylation of protein synthesis factor eIF-2α [68, 69]. And so, one of the primary methods type I IFN uses to inhibit propagation of viral progeny is the inhibition of viral polypeptides. This coupled with its initiation of proinflammatory gene transcription and translation (also referred to as interferon stimulated genes, ISGs) gives it an essential role in immunoregulation of the pro-inflammatory response, expressed in an assortment of cell types [70]. When IFNα/β (interferon alpha/beta) bind to their receptor, IFNAR (interferon α/β receptor), they induce interferon stimulated gene (ISG) production through the

JAK-STAT pathway to remedy the infection [71]. Mechanics of type I IFNs range from anti-apoptotic protein production to curtail pathogen proliferation to intercepting host pathways that pathogens hijack for replication. Hence, type I IFNs are named for their ability to "interfere" with viral replication, curated by ISG production [72]. Although initially discovered and characterized in viral models, the type I interferon response congruently plays an integral role for the host during bacterial infection [19]. However, the intricacies of this during bacterial infection still need to be explored.

Type I IFNs regulate autocrine and paracrine responses that influence disease outcome [73]. This is important because macrophages, for example play an indispensable role in mediating immune responses among effector cell populations as antigen presenters and professional phagocytic cells. Congruently, macrophages are also a target cell population of *Y. pestis* [74]. When *Y. pestis* is introduced to the mammal in the sylvatic cycle, the bacteria traffic to the nearest draining lymph node. During infection, *Y. pestis* forms a replicative niche within macrophages and other lymphocytes allowing bacteria to use macrophages as a vehicle to increase proliferation throughout the mammal, allowing for more efficient dissemination [75].

Yersinia and YopJ

In addition to being a part of the *Yersinia* family of effectors, YopJ is a member of a larger effector protein family with homologs in other pathogenic bacteria, spanning the impact of YopJ activity to multiple infectious models [26,

76]. Particularly, as a T3SS effector protein YopJ is a highly efficient modulator of cellular responses. YopJ has been primarily characterized as an acetyl transferase, preventing phosphorylation of MAP kinases and subsequent signal transduction. Of note, there are quite literally several points of entry for YopJ and its homologs to interrupt MAP kinase signaling. One relevant example is MAP3 kinase, transforming growth factor β (TGFβ) activating kinase 1 (TAK1). In *Yersinia pseudotuberculosis* YopJ targets TAK1, acetylating TAK1 on its serine and threonine residues in its activation loop [33, 77]. Inhibition of TAK1 activation by YopJ poses an obstacle for the immune system. This is because TAK1 is a MAP3 kinase that activates pathways that are germane to resolving infections such as NFκB, AP-1, and p-38 [33, 77]. As MAP kinases are necessary for an effective immune response, infections of plants and animals by bacteria expressing YopJ illustrates a larger appreciation for the barriers to host response during infection.

Congruently, YopJ enzymatic activity also modulates several other intracellular pathways, highlighting its promiscuity in function. For instance, through expression of YopJ *in vitro*, ubiquitination of host protein TRAF6 was severely ablated compared to the catalytically null YopJ mutant, which suggests an element of deubiquitinase activity [78]. Another well-defined evasive maneuver of YopJ from immune recognition is it's induction of multiple forms of programmed cell death. [79]. YopJ induces caspase 8 and 3, all products of inflammasome activation that leads to cell death [80]. In conjunction with YopM, after caspase 8 activation YopJ targets IL-18 and IL-1β maturation through

inflammasomes, suppressing the immune response [81]. The anti-inflammatory nature of programmed cell death activation is further evidence that YopJ enzymatic activity contributes to a concerted effort to evade immune responses. Additionally, in macrophages YopJ is required for their induction of apoptotic cell death [79]. The implications of forming a replicative niche in macrophages (essential in galvanizing effector cell populations) and directing them to cell death has a wide breadth of consequences and contributes to mortality.

YopJ and Type I IFN

Like other cellular processes, dysregulation of type I IFN can confer severe pathogenesis, resulting in the host succumbing to the infection. Concurrently, YopJ has shown a propensity to disrupt type I interferon expression, significantly decreasing IFNβ expression [19]. This quiescent change in cellular response can contribute to the multitude of anti-inflammatory mechanisms YopJ initiates during infection. Ultimately, injection of YopJ leads to activation of programmed cell death and suppression of proinflammatory responses that could appropriately eradicate the infection. It is for this reason we sought to investigate the relationship between YopJ, a bacterial effector protein and the type I interferon response, largely referenced in the context of viral pathogenesis.

Results

To investigate the impact of YopJ on the type I interferon response, we looked at the pathway through the type I IFN receptor, IFNAR, observing for survival and bacterial load. This would give us insight into the systemic impacts of the influence YopJ has on the type I IFN response. Mice were intraperitoneally injected with MAR-1 (a monoclonal blocking antibody for IFNAR) or IgG (an isotype control) 1 day before infection and observed survival 14 DPI (days post-infection). MAR-1 blocks IFNAR signaling and its downstream activation of IFN

stimulated genes. Our findings indicate that inhibition of IFNAR confers

protection for survival of the host during KIM5- infection (Fig. 1a). When YopJ

enzymatic activity is present with functional IFNAR (the wild type/wild type

scenario), there is a significant decrease in survival compared to MAR-1 treated

mice. Next, we wanted to know the impact on bacterial load in the context of

IFNAR and YopJ activity (Fig. 1b). Infected mice treated with MAR-1 or IgG

antibody were harvested for CFU on 5 DPI. The entry point for infection, the lung,

showed no differences in bacterial load in the absence of IFNAR. Of filtering

tissues spleen and liver, CFU was significantly impacted in the spleen in the

absence of IFNAR. The bacterial load in the spleens of KIM5- infected mice

shows mice with non-functional IFNAR have a significantly less bacterial load

compared to IgG treated mice, underscoring that inhibition of IFNAR skews

towards protection (Fig. 1b). Last, we performed a cytokine analysis observing

for an anti- or proinflammatory environment and to look at downstream signaling

of IFNAR. Observations were made in the spleen, liver, and the serum as a

representation of septicemic plague. As a metric of the general proinflammatory

environment we included IL-6 in the panel, which showed no significant

differences in either tissue (Fig. 1f). However, notably, IgG treated mice sustain a

marked increase in IL-6 in the serum compared to MAR-1 treated mice. As a

representative of the general anti-inflammatory response, we included IL-10 in

the panel which also illustrates no significant or notable differences among the

tissues or serum (Fig. 1e). Similar results were observed for TNFα, included as

another marker for proinflammatory inflammation and an indicator for potential

effector cell proliferation (Fig. 1d). RANTES is downstream of type I IFN signaling and is a potent chemoattractant for monocytes and DCs, and also resulted in no notable or significant differences. The surprising results of the cytokines displaying lack of differences suggests that this general cytokine profile is not significantly impacted by IFNAR signaling during peak *Y. pestis* infection. Additionally, cytokine expression is not correlative to the protection IFNAR inhibition confers.
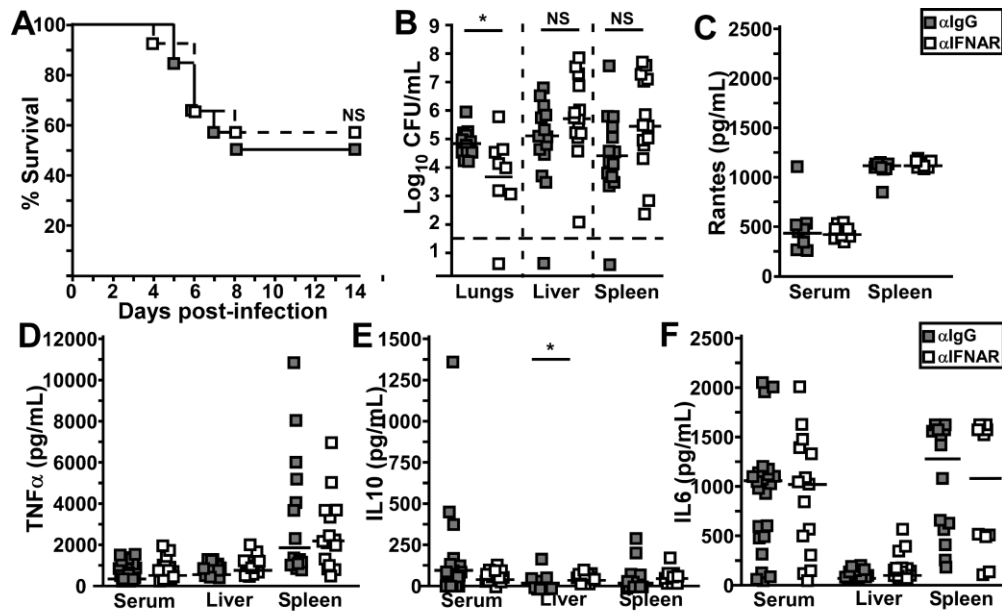
Figure 2: **Antibody inhibition of IFNAR is negligible towards disease outcome of *Y. pestis* YopJ$_{C172A}$**

infection. Mice were treated with MAR-1 (open symbols) vs IgG (closed symbols) antibodies 1 day before

YopJ$_{C172A}$ infection. Data collected in 3 independent trials. A) Survival curves of MAR-1 vs IgG treated mice

infection. There are 12 mice in each control group. B) CFU of antibody treated mice in the lung, liver, and

spleen 5 days after infection. Limit of detection is 50 CFU, bars indicate median. Cytokine expression of C)

RANTES, D) TNFα, E) IL-10, and F) IL-6. Statistics were a log-rank t-test A) or Mann-Whitney t-test B) - F)

with a p-value <0.05, NS: not significant.

Following the same experimental design as Figure 1, we did an analysis on

antibody treated mice infected with the catalytically null YopJ mutant, YopJ$_{C172A}$.

As IFNAR inhibition showed to be protective for survival of the host during KIM5-

infection, MAR-1 treatment was inconsequential to host survival during YopJ$_{C172A}$

infection (Fig. 2a). The enzymatically null YopJ mutant not depicting differences

in survival between MAR-1 and IgG treated mice as in Figure 1 further suggests

that YopJ activity modulates the type I IFN response towards mortality.

Additionally, there were no relevant differences in bacterial load for spleen or

liver tissues (Fig. 2b). Point of entry for infection denotes IgG treated mice experience significantly higher bacterial load than MAR-1 treated mice. Lastly, we did a cytokine analysis observing for expression of RANTES, TNFα, IL-10, and IL-6 in Figs. 2c-f, respectively. Observations were made in the serum, spleen, and liver. The data illustrate no significant or relevant differences within any of the tissues among the cytokine profile aside from the liver in IL-10. Particularly, survival and CFU further support the idea that YopJ is a *Yersinia* component that significantly influences the type I IFN response towards pathogenesis through mortality. These data insinuate the pairing of YopJ activity with IFNAR signaling is what exhibits a phenotype that skews antagonistic towards the host.
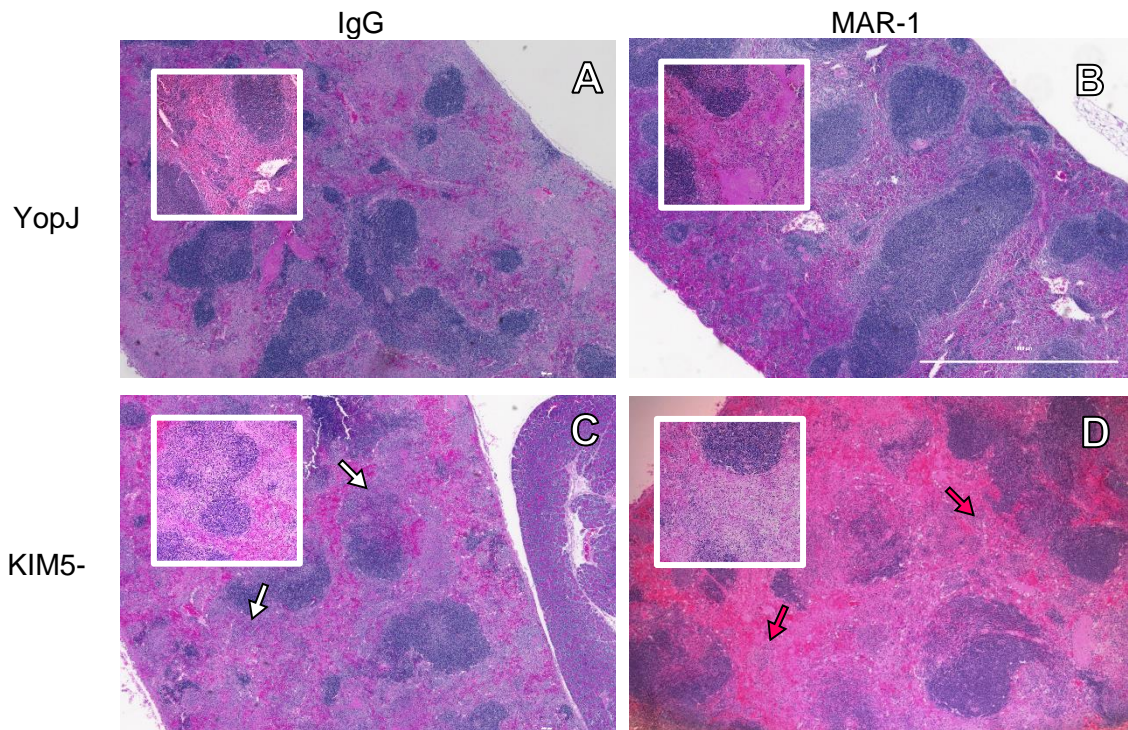
Next, we wanted to analyze histopathology of the spleen at 5DPI. We looked for how well spleens maintained their structural integrity, particularly in the red pulp and white pulp. The red pulp of the spleen consists of large amounts of connective tissue designed to filter antigens, microbes, and other foreign substances circulating from the blood, giving it a rich red color. The white pulp of the spleen, also called islets is a kind of immune epicenter of this lymphatic tissue. This is where B and T cells reside and exchange information circulating around the organism with effector cells such as macrophages and dendritic cells.

Particularly, this information exchange includes microbial motifs. From our

observations, the red pulp of the MAR-1:KIM5- mice sustained an overwhelming

amount of devastation to the red pulp (indicated by red arrows) (Fig. 3d). Tissue

morphology of the red pulp is virtually nonexistent, with the rich red color of a

healthy animal reduced to a bright pink color. Congruently, the white pulp is also

suffering from a large amount of deterioration. The histopathology juxtaposed to

IgG:KIM5- mice is considerable. Illustrated for IgG:KIM5-, much of the damage

was sustained in the islets compared to the red pulp (Fig. 3c). In regards to

YopJ$_{C172A}$ infected mice with IgG or MAR-1 treatment, neither groups bolstered

relevant histopathology of the spleen.
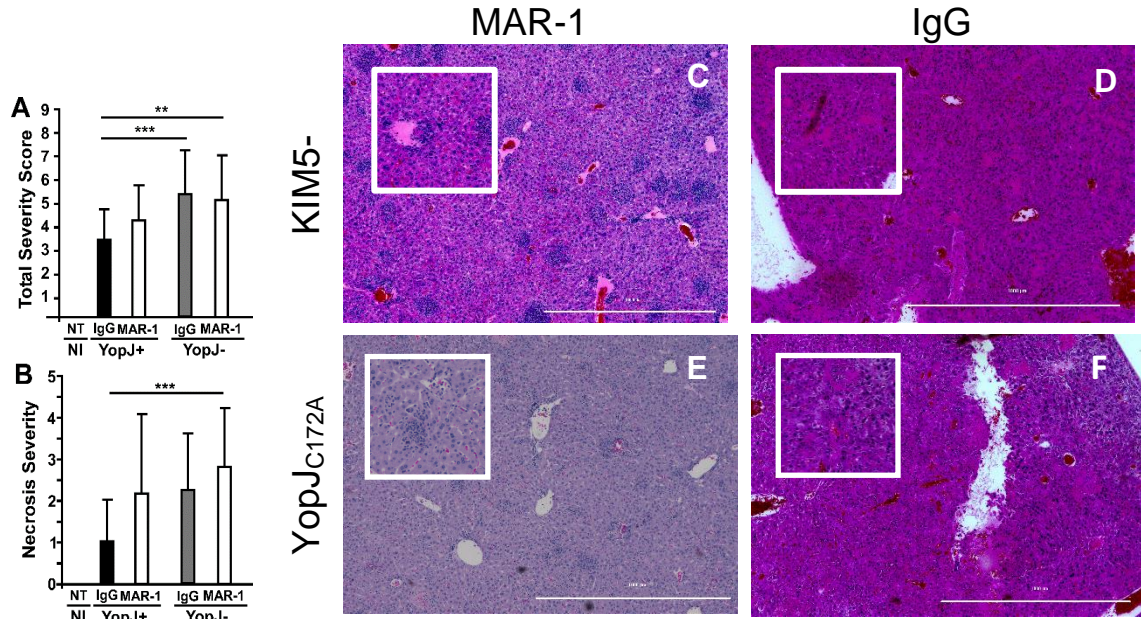
Figure 4: Inhibition of YopJ and Type I IFN exacerbate liver histopathology. Mice treated with MAR-1 or IgG antibody after infection of KIM5- or YopJ$_{C172A}$ had liver analyses on 5DPI. A) Total severity score. B) Necrosis severity score. Representative liver histology images for C) IFNAR antibody treated, KIM5- infected mice, D) Isotype antibody treated, KIM5- infected mice, E) Isotype antibody treated, YopJ$_{C172A}$ infected mice F) IFNAR antibody treated, YopJ$_{C172A}$ infected; scale bar indicates 1000 μm. Statistics were One-way ANOVA, p-value < 0.05.

Previous works have demonstrated a potential phenotype between YopJ activity and inflammatory foci formation in the liver, so we performed a histological analysis on livers in the context of YopJ and IFNAR on day 5 post-infection (Fig. 4a) [19]. The variables factored into the analysis are hemorrhaging, tissue necrosis, and inflammatory foci (as an average of size of foci and number of foci in a field). These elements make up the Total Severity Score, accounting for the loss of tissue integrity and architecture. The data show that IgG: YopJ$_{C172A}$ (Fig. 4 f) sustained significantly more severity of histology than IgG: KIM5- (Fig. 4d), and at least one and a half order of magnitude more than MAR-1: KIM5- (Fig. 4c).

28

Congruently, MAR-1: KIM5- also has markedly more severity of histopathology than IgG: KIM5-. These results are counter to the survival and CFU data and suggest that lesions in the liver are not contributing to the survival phenotype. This is interesting because despite inhibition of IFNAR and YopJ activity being protective in survival and bacterial load, they still induce more liver histopathology in size and frequency of legion formation coupled with voids of necrotic tissue than the wild type/wild type control. Further, we looked at scores for necrotic tissue (Fig. 4b). For necrotic tissue, the data is showing the same trend as in the total severity score: IgG:Yop$_{JC172A}$ ≥ MAR-1:KIM5- > IgG:KIM5-. Representative images illustrated in Figure 4c-f for each control group further suggests that despite the protection the lack of IFNAR and YopJ activity offers the host, loss of architecture of the liver are inconsequential for the host.

	αIgG: KIM5-			αMAR-1: KIM5-		
	average	range	σ	average	range	σ
Glucose (mg/dL)	101.89	58-300	75.19	148.14	63-322	93.08
Urea Nitrogen (mg/dL)	25.44	16-34	6.88	20.29	17-25	4.19
Albumin (g/dL)	2.87	2.4-3	0.36	2.88	2.5-3.3	0.23
AST (U/L)	639.58↑	116-1121	289.84	596.09	93-1239	378.34
Total Bilirubin (mg/dL)	0.45	0.1-1.3	0.28	0.28	0.2-0.4	0.09
ALT (U/L)	144.00	22-317	77.15	156.67	48-270	95.62
ALP (U/L)	24.00	16-34	6.98	42.71	16-131	43.32

	αIgG: YopJ$_{C172A}$			αMAR-1: YopJ$_{C172A}$			Uninfected		
	average	range	σ	average	range	σ	average	range	σ
Glucose (mg/dL)	117.00	62-210	53.60	150.40	73-250	68.92	354.10	285-476	73.00
Urea Nitrogen (mg/dL)	21.50	13-35	6.82	27.60	20-57	16.44	30.40	25-36.6	4.40
Albumin (g/dL)	2.74	2.5-3.2	0.24	2.76	2.3-3	0.23	3.18	2.88-3.42	0.18
AST (U/L)	368.17	80-1123	290.87	316.11	142-629	161.20	126.00	83-211	46.53
Total Bilirubin (mg/dL)	0.34	0.1-0.9	0.23	0.18	0.1-0.3	0.10	0.35	0.2-0.6	0.14
ALT (U/L)	138.36	45-305	78.37	110.22	44-278	87.56	33.00	19-52	10.93
ALP (U/L)	58.73	16-340	93.89	44.00	25-64	13.91	101.00	73-126	22.03

Figure 5: Pseudo-liver function tests and liver enzyme activity analysis. After antibody treatment and infection of KIM5- or YopJ$_{C172A}$, at 5DPI mice were harvested for serum and liver analytes were measured. Data represents 2 trials. Statistics were student's *t-test*, p-value < 0.05. * = significance between groups. ↑ = higher value in significance.

Given the significant change in tissue morphology we observed in the liver, we performed a pseudo-liver function test and liver enzyme analysis looking for differences in analytes that could contribute to disease (Fig. 5). The analytes in this liver panel taken together give insight to liver function during infection. Serum samples of infected mice under specified treatment conditions were collected at 5DPI. Glucose measures gluconeogenesis, the ability of the liver to produce glucose. Urea nitrogen, also known as BUN is a biproduct of protein breakdown after meal consumption. Albumin can be described a carrier protein, shuttling various hormones, vitamins, and enzymes throughout the organism. Albumin also helps fluid in the blood from leaking into tissues. Low levels of albumin are found with hepatic biosynthetic failure. Total protein measures 2 classes of protein in the blood, albumin and globulin. ALT (alanine

aminotransferase) and AST (aspartate aminotransferase) are liver specific and non-specific proteins, respectively. Additionally, if the AST/ALT ratio is greater than 2:1 this is also a sign of liver disease. Total Bilirubin is bound to liver protein albumin and is made during the process of breaking down blood and excess accumulation in the blood and can be indicative of liver dysfunction. Lastly, ALP is a liver enzyme also important for breaking down proteins and if accumulated in excess can be a sign of liver damage.

For 7 of the 8 analytes in the panel, at least 3 of the 4 infected control groups are significantly different compared to uninfected. However, of those 7 analytes, 6 of the infected control groups are not significantly different from each other. Glucose, Total protein, Urea Nitrogen, ALT, Albumin, AST and ALP represent this phenotype. AST is the only analyte that shows significance between the infected groups, IgG: KIM5- and IgG: YopJ$_{C172A}$. This significant increase, influenced by the liver specific enzyme ALT would suggest that AST is increased due to a non-hepatic mechanism. And so, in regards to liver function these data together suggest *Y. pestis* infection results in significantly worsened liver function independent of YopJ and IFNAR.

Methods

Mouse Challenges and Animal work

- **Ethics Statement:** All animal procedures were performed in compliance with guidelines of 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 [82].

- C57BL/6J mice were the inbred strain background of TLR7$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME). Wild-type C57BL/6J and mutant strains were bred in-house at the University of Missouri. All infected mice were monitored by daily assignment of health scores, which involved assessments of their appearance and activity. Animals that survived to the end of the 14-day observation period or were identified as moribund (defined by pronounced neurologic signs, inactivity, and severe weakness) were euthanized by CO_2 asphyxiation followed by bilateral pneumothorax or cervical dislocation, according to the American Veterinary Medical Association Guidelines on Euthanasia. Mice were a minimum of 6 weeks old day of challenge. Mice were dropwise intranasally infected with *Yersinia pestis* at an inoculum of 2.5×10^7.

Histology processing and scoring

- After intranasal infection of *Yersinia pestis*, mice were euthanized at designated time points. Necropsies were performed pre- and post-mortem, harvesting the liver for histological analysis. Tissues were fixed in

zinc formalin for a minimum of 24 hours, after which further processing onto microscope slides was outsourced through IDEXX BioAnalytics© in Columbia, MO. Histology slides were blinded, and three individual field images were taken per liver to account for the landscape of the entire liver section and counted towards their respective control group. Basis of scoring: hemorrhaging, necrotic tissue and inflammatory foci (an average of the number and size of foci in a given field image).

Analyses

- Cytokines: Tissues were homogenized in 1mL of PBS in whirl pack bags and then processed via ELISA kit per manufacturer instructions.

- Pseudo-liver function analysis: At 5DPI, cardiac stick procedure was performed on mice to harvest blood. Samples were deposited in heparin tubes and centrifuged for 2 minutes at 14,000 rpm to separate serum from the blood. Serum was collected from the top layer of samples and submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory in Columbia, MO for the pseudo-liver function test and liver enzyme analysis. Until the performance of the analysis, samples were stored in 1.5 mL Eppendorf tubes at $-80°C$.

Bacterial Stains and Load Quantification

- *Yersinia pestis* strains KIM5- and YopJ$_{C172A}$ were grown fresh from a frozen stock by streaking for isolation onto heart infusion agar (HIA) plates. Subsequent colonies were grown in 40 mL of heart-infusion broth (HIB) with 2.5M of $CaCl_2$ for 24 hours with aeration at 125 rpm. Multiple

frozen stock of bacterial culture was frozen in -80°C at 0.2 OD_{600} to use for
experiments. For each experiment, a frozen stock was thawed and grown
in the HIB liquid broth for 24 hours and then used for infection. All work
was conducted at biosafety level 2 (BSL2).

- To quantify CFU, tissues were homogenized in 1mL of sterile PBS and
  serially diluted in PBS, in duplicate, up to 5 dilutions in duplicate on
  heart infusion (HIA) agar. After ~48 hours, colony forming units were
  counted.

Antibody Application

- IFNAR and Isotype antibodies from Bio X Cell © in Lebanon, NH were
  intraperitoneally injected into mice at a dose of 2.5mg 1 day before
  mice were infected.

Discussion

The goal of this study was to understand the molecular mechanisms that contributed to the phenotype we saw as a result of the YopJ and type I IFN interaction in previous studies. These data demonstrate that type I IFN in the presence of YopJ enzymatic activity confers a detrimental impact on the host, illustrated in the survival curves. YopJ and type I IFN signaling must both be present to have a significant reduction in survival of mice. This is complemented by bacterial load of the spleen, where MAR-1 treated mice have a significantly lower bacterial load. These data together demonstrate the overall impact the YopJ and type I IFN interaction has on disease outcome. Surprisingly, the cytokine analysis didn't display major differences between the treatments within the serum, spleen, or liver. We anticipated that cytokine expression would show differences because during wild type *Y. pestis* infection the immune response does progress to septicemia, curating an overactive immune response and cytokine storm. Our group has also shown that IFNAR^{-/-} mice are less sensitive to *Y. pestis* infection [83]. And so, we hypothesized the impact of YopJ on type I IFN is what is contributing to a hyper immune response. Our group has observed the phenotype previously with another innate immune receptor, TLR7. Knock out of TLR7 compared to wild type mice during peak *Y. pestis* infection resulted in less type I IFN, IFNβ expression in the serum compared to wild type mice [19]. This gives credence to the idea that of the myriad of ways YopJ interferes with intracellular signal transduction, there's a direct correlation to germline immune receptors. Congruently, there were differences in survival of TLR7^{-/-} mice compared to wild type mice during wild type *Y. pestis* infection. And so, the

35

protection of MAR-1 treatment does not translate to cytokine expression in a meaningful way to use cytokine expression as a metric for disease progression.

Observing for spleen histology, MAR-1 treatment contributes to disease progression, competing with the conferred protection seen in survival and CFU. This means that despite the advanced disease progression illustrated in the spleen histology of MAR-1:KIM5- mice, they still sustain significantly less bacterial load in the spleen than IgG:KIM5- mice. This, with the lack of differences observed in YopJ$_{C172A}$ infected and treated mice suggests that IFNAR enhances bacterial growth of the spleen independent of YopJ. This is further supported by the lack of difference in bacterial load of YopJ$_{C172A}$ infected, treated mice. And so, there are more bacteria present to inject YopJ and kill the tissue and macrophages, a targeted cell type during *Y. pestis* infection in lymph nodes. As prior mentioned, what is interesting is despite IFNAR contributing to bacterial load, this does not translate to disease progression in spleen histology. During infection, there is a feedback loop that presents itself between bacterial presence and the host immune response. Bacteria will trigger a response, and while that response is subsequently building on itself, it can cause substantial damage to host tissues in an effort to eradicate the infection through the release of toxic effectors such as reactive oxygen species. This in turn also creates an advantageous opportunity for bacterial proliferation because of this growing nutrient-rich environment, caused by tissue damage. And so, even though IFNAR contributes to bacterial load of the spleen, it is very surprising to see substantially worsened tissue histology in MAR-1 treated mice compared to IgG.

This study also accounted for 2 different analyses of the liver. Plague is characterized to have the host succumb to infection through a few different ways, including liver failure. This coupled with our findings in a previous study with differences in lesion formation in TLR7$^{-/-}$ mice and IFNAR$^{-/-}$ mice compared to wild type mice made observing for histopathology relevant [19]. Thus, we first accounted for liver histology to see if there were any notable differences in architecture, as we have previously seen in TLR7$^{-/-}$ mice compared to wild type mice [19]. After scoring, we saw significant differences between the IgG:YopJ$_{C172A}$, MAR-1:KIM5-, and IgG:KIM5- controls. Interestingly, controls missing one of the target proteins (YopJ or IFNAR) conferred significantly more disease severity in liver morphology through inflammatory lesion formation and hepatocyte death than the wild type/wild type control. Like what we observed in the spleen, this was surprising because despite the absence of YopJ and IFNAR being protective for survival, they yielded more severe liver pathology. And so IFNAR contribution to pathogenesis is surprisingly independent of liver and spleen histopathology.

To further understand the pathology in the liver phenotype, we conducted a pseudo-liver function analysis to see if there is a correlation between the compromised liver morphology and liver function. Particularly, Glucose and BUN levels of infected groups were significantly lower than uninfected mice. This is interesting because studies have shown a positive correlation between *Y. pestis* infection with starvation and weight loss [84]. Having said that, as prior mentioned there are no significant differences between the infected controls. And so, starvation as a result of *Y. pestis* infection likely is unrelated to IFNAR signaling and YopJ enzymatic activity. Accounting for all the analytes in the liver

panel, there's also no correlation between liver damage and YopJ or IFNAR activity as there are no significant differences between the control groups. This means that liver histopathology is independent of liver function.

Due to the coordination of YopJ and IFNAR controlling for bacterial growth in the spleen, this could mean YopJ-injected cells are altered in how they signal through IFNAR or what genes are regulated by IFNAR signaling. And so, the next step in the study could elucidate what happens to IFNAR signaling when YopJ is injected into the same cell. Observations can be made for what proteins are modified and clarify what impacts bacterial load. Particularly, we can examine for macrophages and neutrophils as they are targets of elimination by the T3SS [14, 85]. These cells control bacterial growth, so perhaps IFNAR signaling somehow enhances the YopJ-dependent killing of macrophages and neutrophils or their activity.

Chapter 3: TLR7 and Type I IFN contribute to mortality during *Yersinia pestis* infection.

Abstract

Yersinia pestis is a gram negative bacterium and the causative agent for the plague. *Y. pestis* utilizes the type III secretion system (T3SS) to translocate effector proteins into the target cell that reprogram intracellular immune responses. Here, we test how the modulation of host response by effector proteins impacts innate immune receptor toll-like receptor 7 (TLR7). The data demonstrate that TLR7 contributes to mortality but has no effect on bacterial load. Additionally, the cytokine profile showing an increase in wild type mice compared to TLR7$^{-/-}$ mice in IL-10 and TNFα alludes to a demonstration of sepsis. Lastly, we explored the role of type I interferon, IFNβ, during infection. TLR7 activates upon recognition of viral motifs with subsequent signal transduction leading to type I IFN. Previous studies have shown a significant increase in IFNβ of wild type mice serum at peak infection compared to TLR7$^{-/-}$ mice. And so, we did an IFNβ treatment assay to observe for recapitulation of the wild type phenotype in TLR7$^{-/-}$ mice, which demonstrates that type I interferon IFNβ contributes to pathogenesis through TLR7. These data together illustrate the capacity of *Y. pestis* infection to skew host response to pathogenic.

Introduction

The innate immune system utilizes a myriad of germline encoded pathogen recognition receptors (PRRs), including toll-like receptors (TLRs)

toward combating invasive microbes [39]. TLRs are one of the many arbiters of

the innate immune system, serving as transmembrane bound receptors that play

a key role in the initial immune response to pathogens. Their wide range of

evolutionarily conserved antigens serving as ligands is evidence that TLRs are

indispensable during infection. This repertoire covers a breadth of pathogen

associated structures such as dsRNA (TLR3) and unmethylated CpG DNA,

commonly found in microbes and rarely found in vertebrate genomes (TLR9) [86,

87]. Particularly, TLR7 is designated as an endosomal PRR and has been

characterized to recognize viral associated configurations (ssRNA) as ligands

[88, 89]. Like other members of its family, once activated, a proinflammatory

signal cascade is triggered to neighboring leukocytes [89]. The mounted

response disseminates systemically in the host, increasing host capability to

eradicate the infection. Differentially expressed in various cell types, human and

murine TLR7 is predominant in plasmacytoid dendritic cells (pDCs) and B cells

with inducible expression in macrophages during infection [90-92]. During viral

infections, TLR7 activation aids the host response in efficiently mediating

infections such as influenza A virus [93]. Additionally, administration of TLR7

agonists during influenza infection of rats confers protection [94]. However, the

role of TLR7 during bacterial infections is not yet entirely clear. For instance,

TLR7 activation has shown to be more pernicious during polymicrobial infectious

studies of the gut, contributing to kidney failure and mortality compared to TLR7$^{-/-}$

mice [95]. Congruently, other studies have also shown a direct correlation

between type I IFN induction of IFNβ after TLR7 dependent recognition of phagosomal bacteria in dendritic cells and slightly in macrophages [96].

The causative agent for the plague, *Yersinia pestis* is a well-documented example of a pathogen with a wide range of evasive maneuvers for immune system recognition. *Y. pestis* utilizes the type III secretion system, an injectosome that many bacteria employ to translocate effector proteins (*Yersinia* outer proteins, yops) to the host/target cell [60]. Yops modulate host cell physiology, impairing normal cell responses and giving the bacteria an advantage during infection. Particularly, a lot of yops work in tandem to evade innate immune responses. For instance, during natural infection when *Y. pestis* transitions from the flea vector to the mammalian host, its LPS conformation changes from hexaacylated (26°C) to tetraacylated (37°C). This is important because TLR4 recognizes the hexaacylated conformation, thus, TLR4 is unable to recognize *Y. pestis* in the mammalian host.

YopJ is a significant virulence factor and one of the more enzymatically versatile effector proteins including acetyltransferase activity. An example of an impacted pathway is the MAP kinase pathway. Perturbation of MAP kinase function through post-translational modifications can be detrimental as MAPK function is indispensable to combat infection [24, 97]. MAP kinases reside in the cytosol and participate in many PRR pathways, including TLR7 [89]. By nature, MAP kinases go through a multi-level phosphorylation event to confer enzymatic activity after the terminal domains of PRRs have been phosphorylated through receptor activation [98]. After the MAP kinase signaling cascade, the result of all

PRR activation leads to proinflammatory signal transduction ranging from NFκB

to interferon production. This cell signaling event activates autocrine and

paracrine responses of neighboring cells, exponentially propagating cytokines to

fight the infection. This signal cascade also includes the type I interferon (type I

IFN) response. Type I IFNs—IFNα and IFNβ—physically '"interfere" with viral

replication by intercepting host pathways that viral machinery hijack during their

life cycle towards proliferation [89]. The type I IFN response is characterized as a

curated response to viral infections. And so, the impact of effector protein YopJ

on intracellular signaling and subsequent proinflammatory responses comprises

much to appreciate. This information taken together illustrate the capacity of

YopJ to interfere with normal host cell processing, including TLR7 mediated type

I IFN responses, justifying the investigation of the role of TLR7 during *Y. pestis*

infection.

Results
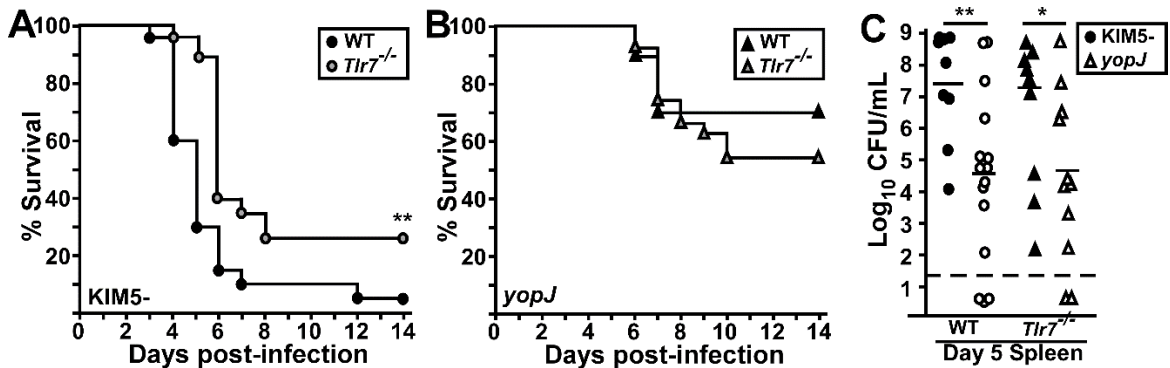
Figure 1: **TLR7 contribution to mortality is independent of bacterial load in the spleen and YopJ.** Wild type and TLR7^{-/-} mice were infected with either *Y. pestis* KIM5- (wild type) or YopJc172A. A) Survival curve of KIM5- infection in wild type or TLR7^{-/-} mice, *n* =25 (WT) and *n* = 20 (TLR7^{-/-}). B) Survival curve of wild type and TLR7^{-/-} mice during YopJc172A infection. C) At 5DPI, mice were harvested for spleens for measurement of bacterial load. Statistics are Mann-Whitney t-test, p-value ≥ 0.05.

Previous studies from our lab have only shown infections in the *Y. pestis* D27 strain. For this study, we used *Y. pestis* KIM5- as the wild type strain and so we repeated relevant assays. Additionally, we increased the dose to observe a more defined phenotype between the control groups. And so, to begin exploring the relationship between the impact on TLR7 by YopJ, we looked at how TLR7 effects mouse survival during wild type *Y. pestis* infection. Wild type and TLR7^{-/-} mice were intranasally infected with *Y. pestis* KIM5- and observed for the development of lethal disease for 14 days (Fig. 1a). The survival curve illustrates that during wild type *Y. pestis* infection—which also includes YopJ enzymatic activity—TLR7 significantly contributes to mortality. Next, we wanted to observe if the survival phenotype in TLR7^{-/-} mice happens in a YopJ dependent manner. And so, we conducted another survival study with YopJc172A (Fig. 1b). The data

demonstrate that YopJ enzymatic activity is inconsequential to survival of TLR7$^{-/-}$

mice during infection through lack of significance. Congruently, to better

understand the relationship between TLR7 and YopJ, we observed their impact

on bacterial load in the spleen as an indicator of disease progression. The spleen

is a lymphatic tissue and filters the blood. The progression of *Y. pestis* infection

can lead to bacteremia and sepsis. This makes the spleen a good candidate to

observe for disease progression [100, 101]. Wild type and TLR7$^{-/-}$ mice were

intranasally infected with either *Y. pestis* KIM5- or YopJ$_{C172A}$ and at 5DPI, spleen

homogenate was used to measure bacterial load (Fig. 1c). The results

demonstrate that when YopJ enzymatic activity is absent, bacterial growth of *Y.*

pestis is significantly attenuated in the host, independent of TLR7. Thus, even

though TLR7 contributes to mortality, the TLR7 phenotype is independent of

YopJ activity. This is because there are no differences in survival and bacterial

load of the spleen for TLR7$^{-/-}$ mice in the absence and presence of YopJ

enzymatic activity during infection. And so, moving forward we disregarded YopJ

as a relevant contributor to the TLR7 phenotype during *Y. pestis* infection.
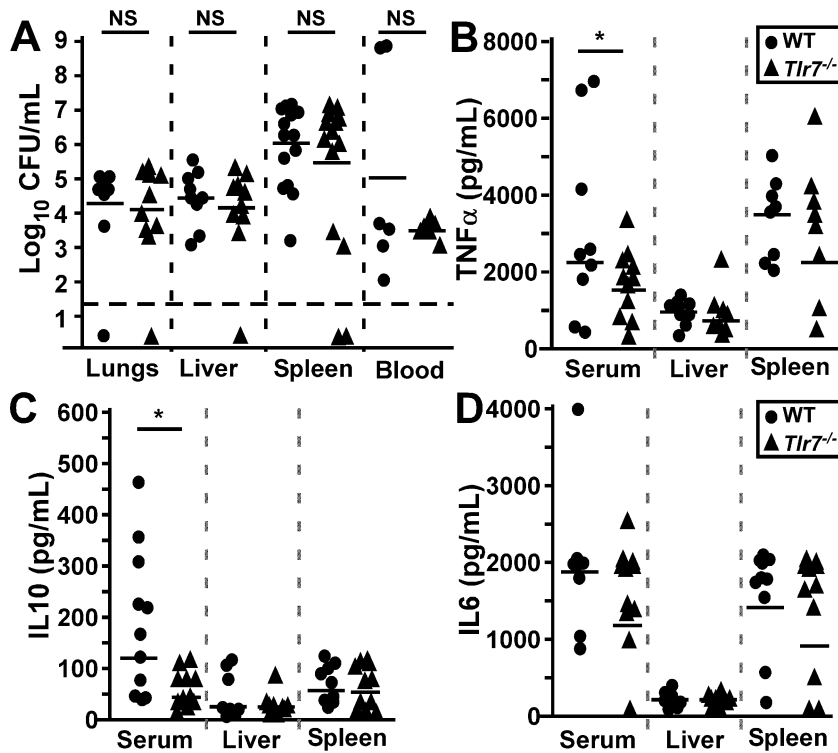
Figure 2: The systemic inflammatory environment and not bacterial load is impacted by TLR7^{-/-}. Wild type and TLR7^{-/-} mice were intranasally infected with *Y. pestis* KIM5-. A) CFU of lung, liver, spleen, and blood of wild type and TLR7^{-/-} mice at 5 DPI. Limit of detection indicated by dotted line at 50 CFU. B) At 5DPI, liver, spleen, and blood were collected to measure cytokines B) TNFα, C) IL-10 and D) IL-6. Statistics were Mann-Whitney t-test with a p-value > 0.05. Data represent 3 trials.

In this experiment we wanted to further understand the role of TLR7 during infection, and so we expanded the bacterial load study to filtering tissues lung and liver to observe for differences in the absence and presence of TLR7. Additionally, we added CFU of the blood in the panel to see if there are TLR7 dependent differences in septicemia (Fig. 2a). Unlike what we observed in the spleen, the data demonstrate no significant or noteworthy differences in the lung, liver, or blood. Next, we measured cytokine expression in wild type and TLR7^{-/-} mice to understand host immune responses during infection. As prior mentioned,

the spleen is a lymphatic tissue, an information exchange hub for professional antigen presenting lymphocytes and leukocytes such as dendritic cells and macrophages [102]. Congruently, during natural infection *Y. pestis* traffics to the nearest draining lymph node and forms a replicative niche within effector cells such as macrophages [20, 79]. And so, the spleen as an environment for direct host-pathogen interaction makes it a viable candidate for observation. Serum was collected as it depicts the impacts of inflammation circulating throughout the organism. As stated earlier the disease progresses to bacteremia and that process can elicit a myriad of host cell responses, also making it an ideal candidate for observation. TNFα is a proinflammatory cytokine that stimulates leukocytes such as macrophages and upregulates nitrogen oxygen species during bacterial infection [103]. Given that, illustration of wild type mice retaining significantly more TNFα in the serum compared to TLR7$^{-/-}$ mice suggest differences in cell recruitment (Fig. 2a). IL-6 was used as a general proinflammatory marker, and the data shows no noteworthy differences between wild type and TLR7$^{-/-}$ mice (Fig. 2b). There is also a significant increase in anti-inflammatory signaling (IL-10) in the serum of wild type mice compared to TLR7$^{-/-}$ mice (Fig. 2b). Overall, these data suggest that TLR7 modulates the systemic inflammatory response, and this may correlate with increased mortality.
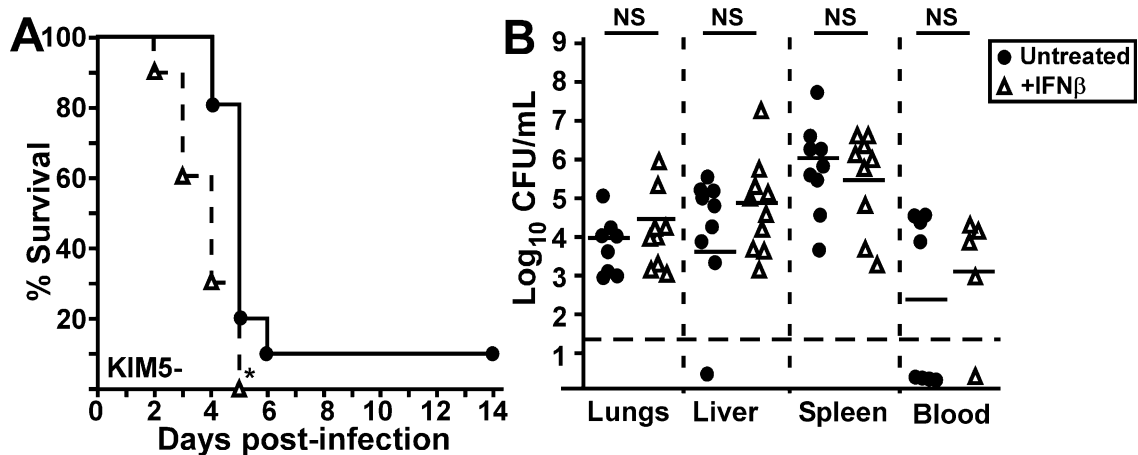
Figure 3: Excess type I IFN, IFNβ, worsens disease in TLR7$^{-/-}$ mice. TLR7$^{-/-}$ mice are intraperitoneally injected with an IFNβ antibody one day before infection, and days 1 and 3 after infection of *Y. pestis* KIM5-. A) Survival curve of IFNβ treated and untreated TLR7$^{-/-}$ mice, 10 mice/group. B) CFU of lung, liver, spleen, and blood of treated and untreated mice collected at 3 DPI. Statistics were a A) log-rank t-test and B) Mann-Whitney t-test with a p-value > 0.05. Data represent 3 trials.

Our lab has previously shown there is significantly more IFNβ during wild type *Y. pestis* infection in the serum of wild type mice compared to TLR7$^{-/-}$ mice. We also showed that IFNβ signaling causes a YopJ-dependent increase in susceptibility of mice to *Y. pestis* KIM5- infection (chapter 2). To determine if loss of IFNβ contributed to the increased resistance of TLR7$^{-/-}$ mice, we provided IFNβ treatment to mice beginning 24 hrs before infection and studied the impact on mortality. This difference in cytokine expression led us to investigate if we could recapitulate this wild type/wild type scenario. Particularly, if administration of IFNβ to TLR7$^{-/-}$ mice would replicate the worsened disease phenotype seen in wild type mice. To do this, we treated TLR7$^{-/-}$ mice with 3 doses of IFNβ and observed for survival for 14 days (Fig. 3a) [99]. The results indicate IFNβ treatment to TLR7$^{-/-}$ mice significantly decreased survival, mirroring the phenotype seen in wild type mice during wild type infection.

Congruently, IFNβ treatment worsens bacterial load in the lung, liver, blood, and spleen (Fig. 3b). We looked at bacterial load in IFNβ treated vs untreated mice. Due to the high mortality rate that occurs early in the infection, 3DPI was designated as peak infection of TLR7$^{-/-}$ mice with IFNβ treatment. The results show in the lung, liver, blood and spleen a small, but not significant increase in median bacterial load in treated mice compared to untreated.

Methods

Mouse Challenges and Animal Work

- **Ethics Statement:** All animal procedures were performed in compliance
  with guidelines of 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 [82].

- C57BL/6J mice were the inbred strain background of TLR7$^{-/-}$ mice
  (Jackson Laboratories, Bar Harbor, ME). Wild-type C57BL/6J and mutant
  strains were bred in-house at the University of Missouri. All infected mice
  were monitored by daily assignment of health scores, which involved
  assessments of their appearance and activity. Animals that survived to the
  end of the 14-day observation period or were identified as moribund
  (defined by pronounced neurologic signs, inactivity, and severe weakness)
  were euthanized by CO_2 asphyxiation followed by bilateral pneumothorax
  or cervical dislocation, according to the American Veterinary Medical
  Association Guidelines on Euthanasia. Mice were a minimum of 6 weeks
  old day of challenge. Mice were dropwise intranasally infected with
  Yersinia pestis at an inoculum of 2.5×10^7.

Histology processing and scoring

- After intranasal infection of *Yersinia pestis*, mice were euthanized at
  designated time points. Necropsies were performed pre- and post-
  mortem, harvesting the liver for histological analysis. Tissues were fixed in

zinc formalin for a minimum of 24 hours, after which further processing onto microscope slides was outsourced through IDEXX BioAnalytics© in Columbia, MO. Histology slides were blinded and three individual field images were taken per liver to account for the landscape of the entire liver section and counted towards their respective control group. Basis of scoring: hemorrhaging, necrotic tissue and inflammatory foci (an average of the number and size of foci in a given field image).

Analyses

- Cytokines: Tissues were homogenized in 1mL of PBS in whirl pack bags and then processed via ELISA kit per manufacturer instructions.

- Pseudo-liver function analysis: At 5DPI, cardiac stick procedure was performed on mice to harvest blood. Samples were deposited in heparin tubes and centrifuged for 2 minutes at 14,000 rpm to separate serum from the blood. Serum was collected from the top layer of samples and submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory in Columbia, MO for the liver panel analysis. Until the performance of the analysis, samples were stored in 1.5 mL Eppendorf tubes at -80°C.

Bacterial Strains and Load Quantification

- *Yersinia pestis* strains were grown fresh from a frozen stock by streaking for isolation onto heart infusion agar (HIA) plates. Subsequent colonies were grown in 40 mL of heart-infusion broth (HIB) with 2.5M of $CaCl_2$ for

24 hours with aeration at 125 rpm. Multiple frozen stock of bacterial culture was frozen in -80°C at 0.2 OD_{600} to use for experiments. For each experiment, a frozen stock was thawed and grown in the HIB liquid broth for 24 hours and then used for infection. All work was conducted at biosafety level 2 (BSL2).

- CFU quantification: tissues were homogenized in 1mL of sterile PBS and serially diluted in PBS, in duplicate, up to 5 dilutions in duplicate on heart infusion (HIA) agar. After ~48 hours, colony forming units were counted.

<u>Antibody Application</u>

- IFNβ for mouse treatment was procured from PBL Assay Science © in Piscataway, NJ. Using syringes, TLR7$^{-/-}$ mice were intraperitoneally injected at a dose of 2.5 U suspended in 200 mL of 1% bovine serum albumin in sterile PBS (saline). Treatments were 1 day before infection and days 1 and 3 after infection. Protocol amended from established treatments [99].

Discussion

This study was to understand the role of TLR7 in the pathogenesis of plague. Particularly, we wanted to explore the impact of *Yersinia* effector protein YopJ on TLR7 and overall host response. *Y. pestis* infection has been well characterized to progress the host response to septicemia and a cytokine storm. This overwhelmed immune response in turn contributes to mortality due to the perturbed immune homeostatic environment. The data illustrated in this paper such as the survival curve comparing wild type to TLR7$^{-/-}$ mice displays a marked difference in host response during *Yersinia* infection. Here, TLR7 interestingly shows that it contributes to mortality during wild type infection, which includes YopJ enzymatic activity. However, survival of TLR7$^{-/-}$ mice is independent of YopJ enzymatic activity. Comparatively, bacterial load of the spleen shows also that TLR7 has no significant impact on bacterial load. This means that the mechanism TLR7 transduction modulates the circumstances of infection does not impact bacterial load nor is contingent upon YopJ enzymatic activity.

We next wanted to observe the cytokine profiles within various tissues to give insight to the immune environment during infection. The data shows a significant increase in IL-10 in wild type mice compared to TLR7$^{-/-}$ mice. This is interesting because our wild type/wild type control depicts markedly more of a general anti-inflammatory environment in the serum compared to its counterpart that is missing an innate immune receptor in myeloid cells. Comparatively, TLR7$^{-/-}$ mice also showed significantly less TNFα expression. This suggests

TLR7 isn't directly involved in bacteria-host interactions, but rather involved in the systemic inflammatory response. This contradictory inflammatory environment could be explained by a difference in cellular recruitment or sepsis. As a part of its proinflammatory nature, TNFα expression also contributes to effector cell proliferation [104, 105]. The notion of having more effector cells in circulation can create a problem for the host immune response because of the secondary damage those cells impose on host tissues in an effort to eradicate the infection. And so, the cytokine profile could mean effector cell proliferation has more of an impact on infectious outcome than the cultivated anti-inflammatory environment. To address this, exploring the differences in cellular recruitment and infiltration would be a viable next step in this experiment. Comparatively, studies have shown TNF as a significant mediator in the manifestation of sepsis [106]. The involvement of TNF in septicemia was so impactful that monoclonal antibodies for TNF was recommended for treatment. Additionally, during sepsis there is also a positive correlation between an increase in TNF and a dampened anti-inflammatory response [106]. And so, the cytokine profile could also suggest a role for TLR7 toward sepsis during infection.

The last experiment addresses part of the catalyst of this study: the impact of IFNβ as a result of the relationship between TLR7 and YopJ. In that previous work we found a significant increase in IFNβ expression in serum of wild type compared to TLR7$^{-/-}$ mice [19]. The last experiment observed if the phenotype of the wild type/TLR7$^{-/-}$ survival could be reproduced through IFNβ treatment to TLR7$^{-/-}$ mice. Given IFNβ treatment resulted in a significant decrease in survival

compared to untreated, this suggests that IFNβ is a contributing factor to the deleterious mechanism of TLR7 signaling to the host during infection. We also analyzed bacterial load to get further insight into another mode of pathogenesis. Also, interestingly, the CFU in this experiment mirrors the CFU of the wild type and TLR7$^{-/-}$ comparison in that there are not significant differences between IFNβ treated and untreated mice. And so IFNβ treatment of TLR7$^{-/-}$ mice recapitulates disease observed in the wild type/wild type scenario. This suggests that type I IFN, through excess IFNβ has a role in TLR7 mediated pathogenesis during *Y. pestis* infection.

The study concludes that TLR7 contributes to mortality, however given the methods commonly used in infectious diseases to determine disease progression—survival, CFU, cytokine profile, and histology—we have been unable to determine what that molecular mechanism is responsible for this phenotype. Given the cytokine profile we could target further exploration toward the role of TLR7 and its impact on sepsis. This would add resolution to the molecular methods TLR7 contributes toward pathogenesis.

Chapter 4: Transcriptomics of human cells during *Yersinia pestis* infection largely impacts global host response.

Abstract

Transcriptomics is a form of analysis that observes for the cellular changes in RNA transcripts and is particularly useful for observation of these changes under different conditions. In Yersinia studies, it is understood that because of the tools Yersinia employs for infection, there is an immense amount of intracellular modulation of normal host cell responses. Particularly, in regards to the type III secretion system, Yop-dependent anti- and proinflammatory responses is evidentiary of the fact that elucidation and characterization of intracellular signal transduction during infection is pertinent to mitigating the infection. And so, these studies were conducted towards the long term goal of identifying interferon stimulated genes and genes associated with TLR7 signaling activated by Y. pestis. Here, we used an in vitro infection of HEK293 cells (human cells) infected with Y. pestis KIM5- (pgm-, T3SS) to further explore the impression Yop-mediated modulations has on the host cell. This study particularly contributes insight into the human cell

host response. The data indicate vast differences in cellular processes during infection compared to the uninfected control seen in RNA, ribosomal, and metabolism regulation.

Introduction

Yersinia pestis is a gram negative bacterium that employs infectious techniques conserved in many other pathogens. Specifically, it uses the type III secretion system to translocate effector proteins (Yersinia outer proteins, yops) into the target cell to modulate intracellular transduction. Enzymatic function of these yops aim to reprogram the host inflammatory response from protective to contributing to pathogenesis. This is illustrated in YopE stifling cytoskeleton rearrangement, subsequently dampening host cell capability to phagocytize cells [17]. YopJ also contributes to modulation of intracellular signaling through its acetyltransferase activity, directly impacting transcription of NF$_{\kappa}$B, IL-1β, IL-18, and caspase induced cell death [81, 97]. Yop-dependent modulation of signaling pathways also reprograms inflammatory signaling from protective to pathogenic such as the type I interferon response [83]. This leads to the downregulation of staples in inflammatory responses such as NF$_{\kappa}$B [107, 108]. Given the multiple places in cellular responses and regulation effected by Yop-dependent activity, a global approach to understanding impacted pathways during infection is the next logical step.

Transcriptomics provides the complete set of RNA transcripts produced by the genome of a cell [109]. Under different conditions—such as different types of

infections with mutants to identify what virulence factors effect what cellular

processes—makes this is a useful tool for understanding gene regulation. This

approach also allows for categorization of genes, such as for cancer and

inflammatory responses, giving insight into pathways that are affected by the

conditions imposed upon the cell.

Results
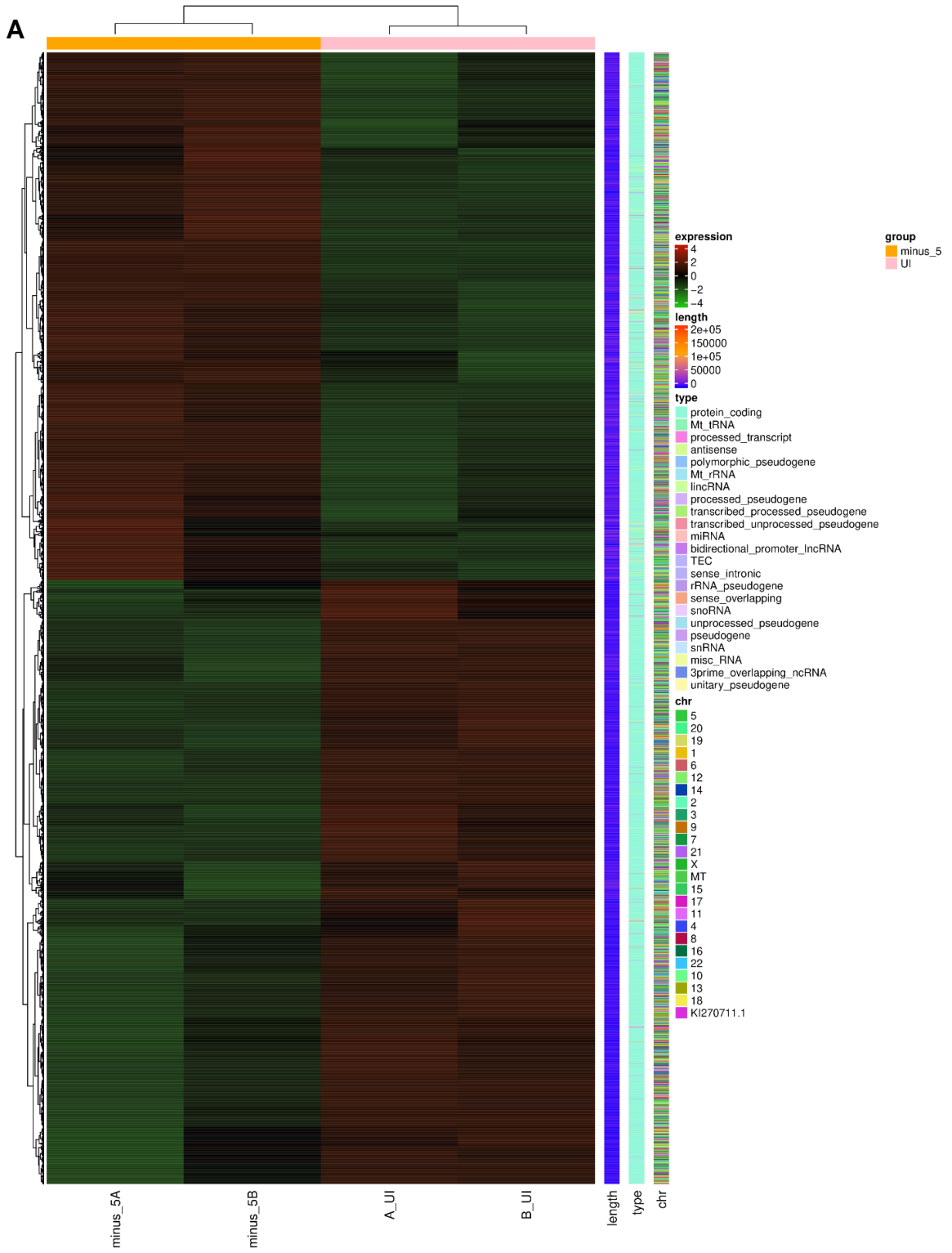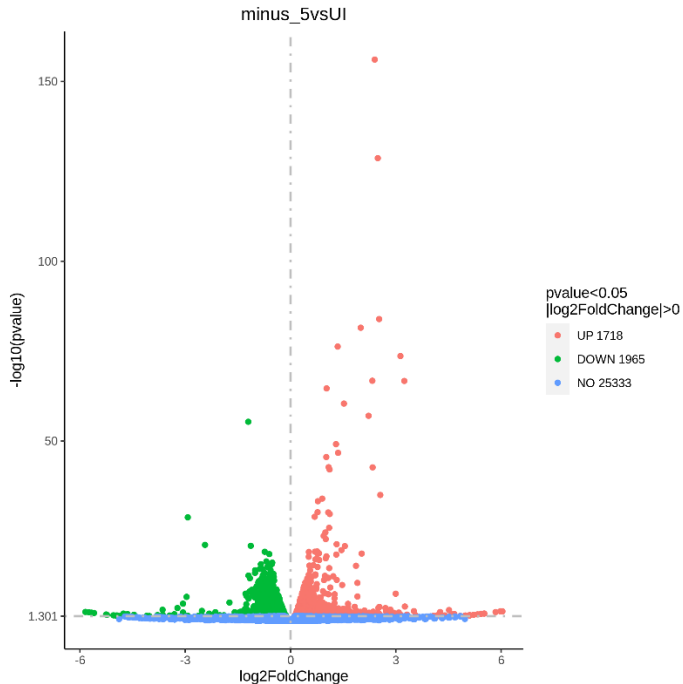
Figure 1: *Y. pestis* **infection elicits firm differences in gene expression.** HEK293 cells were infected for 4 hours *in vitro,* and RNA was collected for analysis. A) Heatmap of gene regulation. B) Volcano plot of gene regulation.

We wanted to observe for differences in gene expression. Using HEK293 cells, we did an *in vitro* infection with *Y. pestis* KIM5- (pgm-, T3SS+) for 4 hours and collected the supernatant to observed for changes in the transcriptome. Duplicate samples of infected and uninfected HEK293 cells (human kidney cells) were studies in the analysis. We had a heatmap constructed to illustrate differences in gene regulation between infected and uninfected controls (Fig. 1a). The data demonstrate stark visual differences between the infected and uninfected controls, suggesting large changes in gene regulation during infection. Duplicate samples also showed some variation in intensity but consistently contrasted with the opposing control group. This is corroborated by the different type of RNAs that were measured in the analysis (illustrated in the description of Fig. 1a). The

quantification of the changes in gene expression is shown in Figure 2b. The

volcano plot shows fold changes in genes that are up and downregulated during

infection. There is a relevant difference in up and down regulated genes given

the distance the dot clusters of upregulated genes (red, left) and downregulated

genes (green, right) are away from each other from the center point, 0 (indicated

by the dotted line). Collectively, there is a difference of more than 3,500 genes

regulated differently in the experiment. And so, *Y. pestis* infection has a large

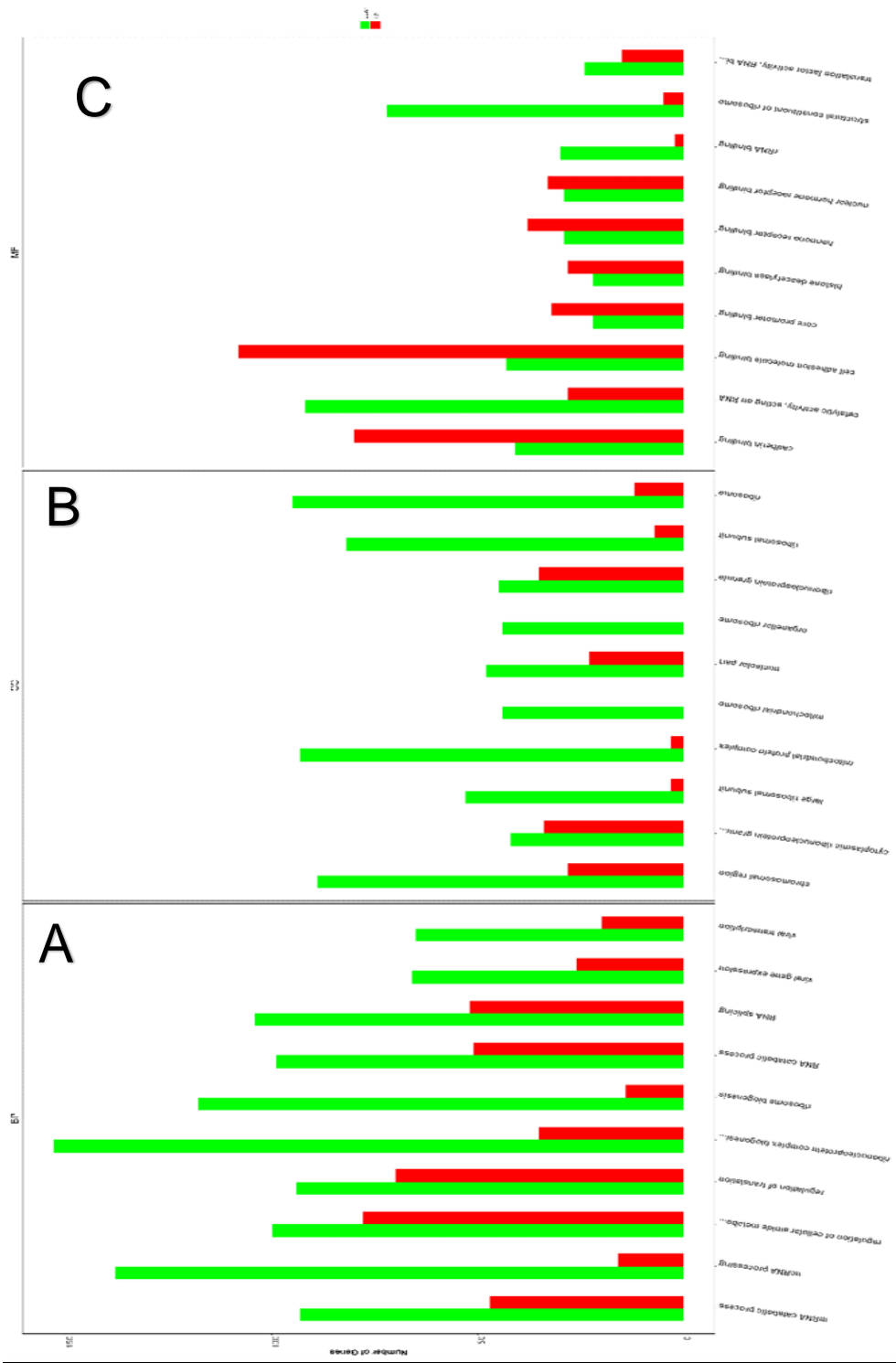impact on intracellular responses during infection.

Figure 2: Gene ontology analysis. Green is down regulated genes; red is upregulated genes. Data shown as gene ratio. A) Biological processing B) Cellular Components C)

Next, we had a gene ontology analysis constructed. Its general premise is if genes associated with a given biological process are differentially expressed in the given disease, that biological process is implicated in that disease. And so, this assay highlights those biological processes, cellular components, and molecular functions of those genes. Overall, for biological processing (Fig. 2a), there are substantially more genes that are downregulated compared to upregulation in all of the analytes in the gene ratio. Notably, there is an increase in downregulation of translation, ribosome biogenesis, RNA catabolic process, and viral gene expression and transcription. Downregulation in translation and ribosome biogenesis are interesting because they all directly reflect protein expression. A downregulation in translation of proteins has a global impact on the cell, affecting not only inflammatory responses but cell maintenance and respiration. Ribosome biogenesis is the process of making ribosomes, and this process is indispensable for protein synthesis. Congruently, downregulation here could also play a role in the reduction in translation. Lastly, the RNA catabolic process is ultimately an output for the breakdown of RNA, which would also lead to the subsequent decline in the translation and thus activity of proteins. The cellular components (Fig. 2b) mirrors the trend in biological processing with each analyte depicting more downregulation than upregulation. As mitochondria have their own genetic information, mitochondrial ribosomes also function to translate mitochondrial mRNA to mtDNA. The decline in ribosomal activity can also be specifically seen in other cell organelles (organellar ribosome). These downregulated genes suggest a large reduction in ATP production and a large

impact on organelle function. Lastly, the analysis of molecular function shows

more of an increase in the upregulation of analytes, but the analytes that show

an increase in downregulation are additionally interesting (Fig. 2c). Core

promoter binding is where RNA polymerase binds to begin transcription and is

downregulated. Translation activity of RNA binding also shows downregulation.

Lastly, the catalytic activity of RNA show a threefold increase in upregulation.

And so, despite the other places in RNA activity showing downregulation, this

means that whatever RNA transcripts are present are being modified. These data

together suggest that *Y. pestis* infection with Yop activity significantly curtail

normal cell function and processes globally. This highlights specifically where

during infection the cell is stunted, outside of the well characterized places of

inflammation.

Methods

Sample Quality Control

Please refer to QC report for methods of sample quality control.

Library Construction, Quality Control and Sequencing

Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis using either dUTP for directional library or dTTP for non-directional library.

For the non-directional library, it was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification (**Figure A**).

For the **directional** library, it was ready after end repair, A-tailing, adapter ligation, size selection, **USER enzyme digestion**, amplification, and purification (**Figure B**).
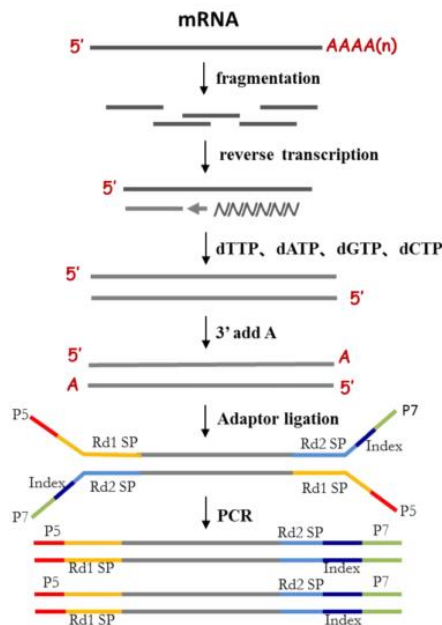
Figure A: Workflow of non-directional library construction   **Figure B:** Workflow of directional library construction
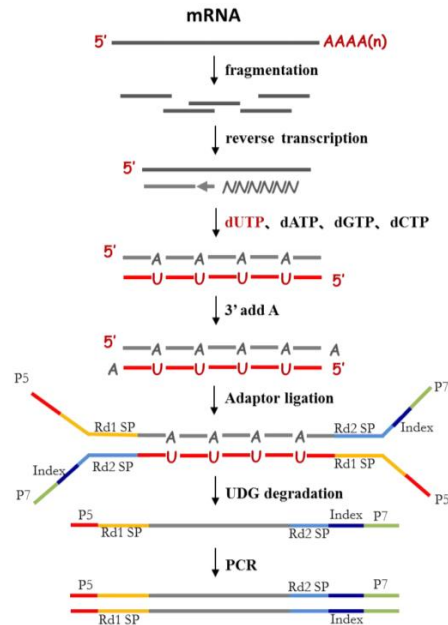
The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries will be pooled and sequenced on Illumina platforms, according to effective library concentration and data amount.

Clustering and sequencing

The clustering of the index-coded samples was performed according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Data Analysis: performed at Novogene, Beijing, China

Quality control

Raw data (raw reads) of fastq format were firstly processed through in-house perlscripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using

Hisat2 v2.0.5. We selected Hisat2 as the mapping tool for that Hisat2 can

generate a database of splice junctions based on the gene model annotation file

and thus a better mapping result than other non-splice mapping tools.

Quantification of gene expression level

featureCounts v1.5.0-p3 was used to count the reads numbers mapped to each

gene. And then FPKM of each gene was calculated based on the length of the

gene and reads count mapped to this gene. FPKM, expected number of

Fragments Per Kilobase of transcript sequence per Millions base pairs

sequenced, considers the effect of sequencing depth and gene length for the

reads count at the same time, and is currently the most commonly used method

for estimating gene expression levels.

Differential expression analysis

(*For DESeq2 with biological replicates*) Differential expression analysis of two

conditions/groups (two biological replicates per condition) was performed using

the DESeq2 R package (1.20.0). DESeq2 provide statistical routines for

determining differential expression in digital gene expression data using a model

based on the negative binomial distribution. The resulting P-values were adjusted

using the Benjamini and Hochberg's approach for controlling the false discovery

rate . Genes with an adjusted P-value <=0.05 found by DESeq2 were assigned

as differentially expressed.

(*For edgeR without biological replicates*) Prior to differential gene expression

analysis, for each sequenced library, the read counts were adjusted by edgeR

program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (3.22.5). The P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression.

Enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways. The Reactome database brings together the various reactions and biological pathways of human model species. Reactome pathways with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. The DO (Disease Ontology) database describes the function of human genes and diseases. DO pathways with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. The DisGeNET database integrates human disease-related genes. DisGeNET pathways with

corrected Pvalue less than 0.05 were considered significantly enriched by differential expressed genes. We used clusterProfiler software to test the statistical enrichment of differentially expressed genes in the Reactome pathway, the DO pathway, and the DisGeNET pathway. Gene Set Enrichment Analysis

Gene Set Enrichment Analysis

(GSEA) is a computational approach to determine if a pre-defined Gene Set can show a significant consistent difference between two biological states. The genes were ranked according to the degree of differential expression in the two samples, and then the predefined Gene Set were tested to see if they were enriched at the top or bottom of the list. Gene set enrichment analysis can include subtle expression changes. We use the local version of the GSEA analysis tool http://www.broadinstitute.org/gsea/index.jsp, GO、KEGG、Reactome、DO and DisGeNET data sets were used for GSEA independently.

SNP analysis

GATK (v4.1.1.0) software was used to perform SNP calling. Raw vcf files were filtered with GATK standard filter method and other parameters (cluster:3; WindowSize:35; QD < 2.0 ; FS > 30.0; DP < 10.

AS analysis

Alternative Splicing is an important mechanism for regulate the expression of genes and the variable of protein. rMATS(4.1.0) software was used to analysis the AS event.

PPI analysis of differentially expressed genes

PPI analysis of differentially expressed genes was based on the STRING database, which known and predicted Protein-Protein Interactions.

Fusion Analysis

Fusion gene refers to the chimeric gene formed by the fusion of all or part of the sequences of two genes, which is generally caused by chromosome translocation, deletion and other reasons. We used Starfusion software (1.9.0) to detect genes that are fused. Star-fusion is a software package uses fusion output results of STAR alignment to detect fusion transcripts, including SATR alignment, SATRfusion. predict, SATR-fusion.filter was used to correct the predicted results ofStar-fusion to ensure the accuracy of the results.

Discussion

During the infectious process of any pathogen, it is understood that a part of the process of pathogenesis is the physical encroachment the pathogen imposes on host target cells. This imposition includes the usage of toxic effectors such as effector proteins in *Y. pestis*, which serves as one of the primary virulence factors during infection. Most infectious studies are targeted towards elucidating inflammatory host responses. However, this study was an analysis of RNA transcripts produced by the genome during infection, providing a global outlook on the status of the cell. Figure 2 illustrates the drastic differences in general gene regulation between infected and uninfected controls. The results of this heatmap, coupled with the quantification of up and downregulation of ~3,500 genes shows there are many parts of cellular processing that effected by infection. We also had a gene ontology analysis constructed to gain resolution on what cellular processes are particularly impacted by infection: biological process, cellular components, and molecular function. The results are represented as a ratio of the infected and uninfected control and demonstrate 2 of the 3 categories (biological process and cellular component) show down regulation in all analytes. A downregulation in regulation for translation and ribosome biogenesis are objective examples of impairment of cellular processing. A downregulation in viral associated genes is also interesting given the several examples of IFNAR induced pathogenesis in infectious diseases.

The analysis limits interpretation to general changes in host cell function. And so, the next steps for exploration would include more specific host responses

targeted towards IFNAR and TLR7. Highlighting regulation of genes that contribute to these pathways during wild type and mutant infection would offer more insight into how these pathways are made pathogenic during infection.

These data taken together highlight the impression *Y. pestis* has on an infected cell and its global repercussions. The results of this study also provides intracellular components to target for therapeutics during infection.

Chapter 5: **Discussion**

The focus of this dissertation work was to explore the role of pathogenesis of the type I IFN response through IFNAR and toll-like receptor 7 during *Y. pestis* infection. Additionally, given the many points of intervention in these signaling pathways, we also wanted to explore the potential of YopJ modulation in these pathways toward pathogenesis. Innate immune receptors TLR7 and IFNAR were also justified to study for a couple of reasons. Activation of TLR7 can downstream trigger type I IFN production as it is designed to recognize viral motifs. Additionally, these pathways have a lot of overlap in the proteins that participate in their signaling cascades. Most importantly, many of these proteins are targets for post-translational modification by YopJ. And so, understanding of the role of these proteins during infection presents a broader impact toward the goal of curating targeted antibiotic treatment of *Y. pestis*.

In chapter 2, we delved into the role of *Yersinia pestis* YopJ mediated type I IFN responses using an IFNAR blocking antibody to elucidate that interaction. The data demonstrate a positive correlation between the type I IFN response and YopJ enzymatic activity toward pathogenesis as it relates to survival and bacterial load. This was illustrated by the performance of 2 survival curves with mice treated with either an IFNAR monoclonal blocking antibody (MAR-1) or an isotype control (IgG). Each survival curve was either infected with wild type *Y. pestis* or the catalytically null YopJ mutant, YopJ$_{C172A}$. In the KIM5- survival curve, there was significant decrease in mouse survival of IgG treated mice compared to MAR-1 treated mice. Congruently, the YopJ$_{C172A}$ survival curve

show no significant or relevant difference between the control groups. These data, coupled with the complementary CFU of the spleen aptly suggest that YopJ manipulates the IFNAR pathway towards pathogenesis. Conversely, in chapter 3, we also explored the role another innate immune receptor, TLR7 toward pathogenesis in a YopJ dependent manner. Here we conducted a survival curve with wild type and TLR7$^{-/-}$ mice infected with KIM5-. As TLR7$^{-/-}$ mice survived significantly more than wild type mice, during wild type infection, the data illustrate that TLR7 contributes to mortality. However, unlike what we saw in the IFNAR study, this phenotype did not translate in a YopJ dependent manner. This is illustrated in the second survival curve of wild type and TLR7$^{-/-}$ mice infected with YopJ$_{C172A}$ demonstrating no significant difference between the control groups. We also observed for CFU of the spleen. Interestingly, TLR7 contribution to mortality did not translate to bacterial load. And so, even though IFNAR and TLR7 present deleterious impacts on the host, only IFNAR pathogenesis showed conditional pathogenesis in a YopJ dependent manner.

Next, we analyzed cytokine profiles as another representation of the state of the immune response. We observed for IL-6, TNFα, IL-10, and RANTES. These cytokines served as markers for proinflammatory responses, cell recruitment, anti-inflammatory responses and downstream signaling of type I IFN, respectively. For the IFNAR study, surprisingly, there were not any positive correlations with protection or pathogenesis in the cytokine profile. Comparatively, in the TLR7 study, wild type mice conferred significantly more TNFα and IL-10 expression in the serum. And so, this is another point in the

infection where induced pathogenesis of innate immune receptors IFNAR and

TLR7 do not overlap. Having said that, these data compared to the findings in the

transcriptome project in chapter 4 are interesting because during wild type

infection there are many cellular processes that are downregulated. Particularly,

from the gene ontology analysis, all the analytes in the Biological Process and

Cellular Components part of the study depicted downregulation. This phenotype

seen in regulation of translation, ribosome biogenesis, organellar ribosome, and

translation activity of RNA (to name a few) illustrates the global imposition on the

cell beyond inflammatory responses. It is understood that during *Y. pestis*

infection of cells there would be a global impact on cellular processing. However,

the transcriptomics study combined with the cytokine profile illustrates in what

ways and to what degree Yop modulation of host cell physiology deleteriously

impacts the cell, subsequently contributing to pathogenesis.

In the IFNAR study, we also explored histological disease progression of

the spleen and liver. Histopathology of the spleen was surprising because MAR-1

treated mice yielded substantially more loss of integrity of splenic architecture of

the white and red pulp compared to IgG treated mice. This is interesting because

even though the MAR-1 treated mice sustained more damage, they significantly

survive better and experience significantly less bacterial proliferation.

Comparatively, liver histology demonstrated the same phenotype where the

protection MAR-1 conferred in survival and bacterial load did not translate to

histopathology of the liver. YopJ$_{C172A}$ infection—also understood to be

attenuated—had significantly more pathology in legion formation and necrotic

tissue than the IgG:KIM5- control, the pathologically worst control. And so, this suggests that histology of filtering tissues have a negative correlation with protection. Due to the significant differences in liver histopathology, we investigated further to see if histopathology was a reflection of liver function. And so, we performed a pseudo-liver analysis observing for changes in liver proteins that could give us insight into the state of the liver and how that has been potentially affected by the infection and treatment. The panel of analytes conferred no significant difference between the infected except for AST, and even that was only between the 2 IgG treated groups. This wasn't a notable contribution of data because as stated before, the attenuation YopJ$_{C172A}$ compared to wild type infection is well understood. And so, liver function is independent of IFNAR and YopJ. Given the severe disease progression illustrated in histology—large amounts of hepatocyte death in the liver and degradation of the white pulp in the spleen—this still leaves us with more to understand. Further exploration on how the host subverts this decline in tissue integrity, maintaining significantly better survival and lower bacterial load.

Lastly, in the TLR7 study we wanted to explore the role of type I IFN in a TLR7 dependent manner. Our group has previously seen a significant increase in IFNβ in wild type mice compared to TLR7$^{-/-}$ mice. This further lead to the hypothesis that there is a TLR7 dependent, pathogenic type I IFN response during *Y. pestis* infection. And so, we conducted an IFNβ treatment assay in TLR7$^{-/-}$ mice to observe if we could reproduce the phenotype seen during wild type infection in wild type mice. The data demonstrates that type I IFN, IFNβ

does significantly contribute to mortality. The protection TLR7$^{-/-}$ mice once had

against wild type infection was lost after IFNβ treatment. However, bacterial load

does not show any relevant differences between treated and nontreated groups.

Interestingly, both the survival curve between wild type and TLR7$^{-/-}$ mice and

bacterial load mirror the data in the IFNβ treatment experiment. And so, in our

effort to understand the role of TLR7 during infection, we could only find

pathologic differences in survival. Additionally, this was all demonstrated

independent of YopJ. These data compared to the findings of the IFNAR study,

demonstrating type I IFN induced pathogenesis in a YopJ dependent manner

are fascinating. Particularly because the result of the IFNβ study contributed to

disease progression, in a TLR7 dependent manner, even though TLR7

dependent pathogenesis is independent of YopJ enzymatic activity. In its totality,

these data demonstrate that the pathologic role of IFNAR and TLR7 are directly

independent of each other. Further, whatever overlap these receptors have in

worsening infection may include unidentified mediators or regulators in their

pathways.

BIBLIOGRAPHY

1. Bramanti, B., et al., *Plague: A Disease Which Changed the Path of Human Civilization*, in *Yersinia pestis: Retrospective and Perspective*, R. Yang and A. Anisimov, Editors. 2016, Springer Netherlands: Dordrecht. p. 1-26.

2. Acharya, S., et al., *Television News Channel in Transition A Response to COVID Pandemic 2020 Rathore Academic Research Publications*. 2020.

3. Gottfried, R.S., *The Black Death: Natural And Human Disaster In Medieval Europe*. 1983, New York: Collier Macmillan.

4. Ziegler, P., *The black death. .* 1991, Wolfeboro Falls, N.H.: Alan Sutton Publishing Inc.

5. *The Spread of Black Death*. Available from: http://plaza.ufl.edu/wlclapp/The%20Spread%20of%20Black%20Death.htm.

6. Riedel, S., *Biological Warfare and Bioterrorism: A Historical Review.* Baylor University Medical Center Proceedings, 2004. **17**(4): p. 400-406.

7. Wheelis, M., *Biological Warfare at the 1346 Siege of Caffa.* Emerging Infectious Diseases, 2002. **8**(9): p. 971-975.

8. EM Eizen Jr, E.T., *Medical Aspects of Chemical and Biological Warfare.* Office of the Surgeon General, Borden Institute, Walter Reed Army Medical Center, 1997: p. 415-423.

9. Consultants, W.G.o., *Health Aspects of Chemical and Biological Weapons. Geneva: World Health Organization.* 1970: p. 98-109.

10. Eskey, C.R., *Fleas as Vectors of Plague.* American journal of public health and the nation's health, 1938. **28**(11): p. 1305-1310.

11. Lin-Cereghino, G., *Geoff's Essential Microbiology 2019 edition*. 21 Plague and Lyme Disease. 2019.

12. Hinnebusch, B.J. and D.L. Erickson, *Yersinia pestis biofilm in the flea vector and its role in the transmission of plague.* Current topics in microbiology and immunology, 2008. **322**: p. 229-248.

13. Bland, D.M. and B.J. Hinnebusch, *Feeding Behavior Modulates Biofilm-Mediated Transmission of Yersinia pestis by the Cat Flea, Ctenocephalides felis.* PLOS Neglected Tropical Diseases, 2016. **10**(2): p. e0004413.

14. CAVANAUGH, D.C. and R. RANDALL, *The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague.* J Immunol, 1959. **83**: p. 348-63.

15. cdc.gov. *FAQ plague*. 2017; Available from: https://web.archive.org/web/20170314062829/https://www.cdc.gov/plague/faq/.

16. Coburn, B., I. Sekirov, and B.B. Finlay, *Type III secretion systems and disease.* Clin Microbiol Rev, 2007. **20**(4): p. 535-49.

17. Andor, A., et al., *YopE of Yersinia, a GAP for Rho GTPases, selectively modulates Rac-dependent actin structures in endothelial cells.* Cellular Microbiology, 2001. **3**(5): p. 301-310.

18.   Trosky, J.E., A.D. Liverman, and K. Orth, *Yersinia outer proteins: Yops.* Cell Microbiol, 2008. **10**(3): p. 557-65.

19.   Dhariwala, M.O., R.M. Olson, and D.M. Anderson, *Induction of Type I Interferon through a Noncanonical Toll-Like Receptor 7 Pathway during Yersinia pestis Infection.* Infect Immun, 2017. **85**(11).

20.   Monack, D.M., et al., *Yersinia-induced Apoptosis In Vivo Aids in the Establishment of a Systemic Infection of Mice.* Journal of Experimental Medicine, 1998. **188**(11): p. 2127-2137.

21.   Roden, J., et al., *Characterization of the Xanthomonas AvrXv4 Effector, a SUMO Protease Translocated into Plant Cells.* Molecular Plant-Microbe Interactions®, 2004. **17**(6): p. 633-643.

22.   Mittal, R., et al., *The acetyltransferase activity of the bacterial toxin YopJ of Yersinia is activated by eukaryotic host cell inositol hexakisphosphate.* J Biol Chem, 2010. **285**(26): p. 19927-34.

23.   Mittal, R., et al., *The Acetyltransferase Activity of the Bacterial Toxin YopJ of Yersinia Is Activated by Eukaryotic Host Cell Inositol Hexakisphosphate.* Journal of Biological Chemistry, 2010. **285**(26): p. 19927-19934.

24.   Mukherjee, S., *Yersinia YopJ Acetylates and Inhibits Kinase Activation by Blocking Phosphorylation.* Science, 2006. **312**(5777): p. 1211-1214.

25.   Mittal, R., S.-Y. Peak-Chew, and H.T. McMahon, *Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(49): p. 18574-18579.

26.   Ma, K.W. and W. Ma, *YopJ Family Effectors Promote Bacterial Infection through a Unique Acetyltransferase Activity.* Microbiol Mol Biol Rev, 2016. **80**(4): p. 1011-1027.

27.   Zhang, Z.M., et al., *Mechanism of host substrate acetylation by a YopJ family effector.* Nat Plants, 2017. **3**: p. 17115.

28.   Jones, R.M., et al., *Salmonella AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade.* Cell Host & Microbe, 2008. **3**(4): p. 233-244.

29.   Wu, S., et al., *Salmonella typhimurium infection increases p53 acetylation in intestinal epithelial cells.* Am J Physiol Gastrointest Liver Physiol, 2010. **298**(5): p. G784-94.

30.   Lu, R., et al., *Enteric bacterial protein AvrA promotes colonic tumorigenesis and activates colonic beta-catenin signaling pathway.* Oncogenesis, 2014. **3**(6): p. e105.

31.   Trosky, J.E., et al., *VopA Inhibits ATP Binding by Acetylating the Catalytic Loop of MAPK Kinases.* Journal of Biological Chemistry, 2007. **282**(47): p. 34299-34305.

32.   Trosky, J.E., et al., *Inhibition of MAPK signaling pathways by VopA from Vibrio parahaemolyticus.* J Biol Chem, 2004. **279**(50): p. 51953-7.

33.   Paquette, N., et al., *Serine/threonine acetylation of TGF -activated kinase (TAK1) by Yersinia pestis YopJ inhibits innate immune signaling.* Proceedings of the National Academy of Sciences, 2012. **109**(31): p. 12710-12715.

34.   Dhanasekaran, D.N. and E.P. Reddy, *JNK signaling in apoptosis.* Oncogene, 2008. **27**(48): p. 6245-6251.

35.   Rincón, M. and R.J. Davis, *Regulation of the immune response by stress-activated protein kinases.* Immunological Reviews, 2009. **228**(1): p. 212-224.

36.   Huang, G., L.Z. Shi, and H. Chi, *Regulation of JNK and p38 MAPK in the immune system: Signal integration, propagation and termination.* Cytokine, 2009. **48**(3): p. 161-169.

37.   Johnson, G.L. and K. Nakamura, *The c-jun kinase/stress-activated pathway: regulation, function and role in human disease.* Biochim Biophys Acta, 2007. **1773**(8): p. 1341-8.

38.   Imler, J.L. and J.A. Hoffmann, *Toll receptors in Drosophila: a family of molecules regulating development and immunity.* Curr Top Microbiol Immunol, 2002. **270**: p. 63-79.

39.   Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.

40.   Zhang, S.-Y., et al., *TLR3 Deficiency in Patients with Herpes Simplex Encephalitis.* Science, 2007. **317**(5844): p. 1522-1527.

41.   Patinote, C., et al., *Agonist and antagonist ligands of toll-like receptors 7 and 8: Ingenious tools for therapeutic purposes.* European journal of medicinal chemistry, 2020. **193**: p. 112238-112238.

42.   Liu, B., et al., *Folding of Toll-like receptors by the HSP90 paralogue gp96 requires a substrate-specific cochaperone.* Nature Communications, 2010. **1**(1): p. 79.

43.   Yang, Y., et al., *Heat Shock Protein gp96 Is a Master Chaperone for Toll-like Receptors and Is Important in the Innate Function of Macrophages.* Immunity, 2007. **26**(2): p. 215-226.

44.   Takahashi, K., et al., *A protein associated with Toll-like receptor (TLR) 4 (PRAT4A) is required for TLR-dependent immune responses.* Journal of Experimental Medicine, 2007. **204**(12): p. 2963-2976.

45.   Nakatsu, F. and H. Ohno, *Adaptor Protein Complexes as the Key Regulators of Protein Sorting in the Post-Golgi Network.* Cell Structure and Function, 2003. **28**(5): p. 419-429.

46.   Balka, K.R. and D. De Nardo, *Understanding early TLR signaling through the Myddosome.* Journal of Leukocyte Biology, 2019. **105**(2): p. 339-351.

47.   Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses.* Nature Immunology, 2004. **5**(7): p. 730-737.

48.   McNab, F., et al., *Type I interferons in infectious disease.* Nature Reviews Immunology, 2015. **15**(2): p. 87-103.

49.   Lee, A.J. and A.A. Ashkar, *The Dual Nature of Type I and Type II Interferons.* Frontiers in Immunology, 2018. **9**.

50.   Wack, A., E. Terczyńska-Dyla, and R. Hartmann, *Guarding the frontiers: the biology of type III interferons.* Nature Immunology, 2015. **16**(8): p. 802-809.

51. Schneider, W.M., M.D. Chevillotte, and C.M. Rice, *Interferon-Stimulated Genes: A Complex Web of Host Defenses.* Annual Review of Immunology, 2014. **32**(1): p. 513-545.

52. Nakhaei, P., J. Hiscott, and R. Lin, *STING-ing the Antiviral Pathway.* Journal of Molecular Cell Biology, 2010. **2**(3): p. 110-112.

53. Aso, H., et al., *Comparative Description of the Expression Profile of Interferon-Stimulated Genes in Multiple Cell Lineages Targeted by HIV-1 Infection.* Frontiers in Microbiology, 2019. **10**.

54. Rotger, M., et al., *Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque.* Journal of Clinical Investigation, 2011. **121**(6): p. 2391-2400.

55. Auerbuch, V., et al., *Mice Lacking the Type I Interferon Receptor Are Resistant to Listeria monocytogenes.* Journal of Experimental Medicine, 2004. **200**(4): p. 527-533.

56. Carrero, J.A., B. Calderon, and E.R. Unanue, *Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection.* J Exp Med, 2004. **200**(4): p. 535-40.

57. O'Connell, R.M., et al., *Type I interferon production enhances susceptibility to Listeria monocytogenes infection.* J Exp Med, 2004. **200**(4): p. 437-45.

58. Manca, C., et al., *Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway.* J Interferon Cytokine Res, 2005. **25**(11): p. 694-701.

59. Ferber, D.M. and R.R. Brubaker, *Plasmids in Yersinia pestis.* Infection and immunity, 1981. **31**(2): p. 839-841.

60. Viboud, G.I. and J.B. Bliska, *Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis.* Annu Rev Microbiol, 2005. **59**: p. 69-89.

61. Holmström, A., et al., *Virulence plasmid-encoded YopK is essential for Yersinia pseudotuberculosis to cause systemic infection in mice.* Infection and Immunity, 1995. **63**(6): p. 2269-2276.

62. Black, D.S. and J.B. Bliska, *The RhoGAP activity of the Yersinia pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence.* Molecular Microbiology, 2002. **37**(3): p. 515-527.

63. Barz, C., et al., *TheYersiniaSer/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1.* FEBS Letters, 2000. **482**(1-2): p. 139-143.

64. Bliska, J.B., *Yop effectors of Yersinia spp. and actin rearrangements.* Trends in Microbiology, 2000. **8**(5): p. 205-208.

65. Dennis, D.T. and J.M. Hughes, *Multidrug resistance in plague.* N Engl J Med, 1997. **337**(10): p. 702-4.

66. Guiyoule, A., et al., *Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of Yersinia pestis.* Emerging infectious diseases, 2001. **7**(1): p. 43-48.

67. Changotra, H., et al., *Type I and Type II Interferons Inhibit the Translation of Murine Norovirus Proteins.* Journal of Virology, 2009. **83**(11): p. 5683-5692.

68.	Gale, M., S.-L. Tan, and M.G. Katze, *Translational Control of Viral Gene Expression in Eukaryotes.* Microbiology and Molecular Biology Reviews, 2000. **64**(2): p. 239-280.

69.	Samuel, C.E., *The eIF-2 alpha protein kinases, regulators of translation in eukaryotes from yeasts to humans.* Journal of Biological Chemistry, 1993. **268**(11): p. 7603-7606.

70.	Schoggins, J.W., et al., *A diverse range of gene products are effectors of the type I interferon antiviral response.* Nature, 2011. **472**(7344): p. 481-485.

71.	Horvath, C.M., *The Jak-STAT pathway stimulated by interferon alpha or interferon beta.* Sci STKE, 2004. **2004**(260): p. tr10.

72.	George and James, *The JAK-STAT Pathway at Twenty.* Immunity, 2012. **36**(4): p. 503-514.

73.	O'Riordan, M., et al., *Innate recognition of bacteria by a macrophage cytosolic surveillance pathway.* Proceedings of the National Academy of Sciences, 2002. **99**(21): p. 13861-13866.

74.	Marketon, M.M., et al., *Plague bacteria target immune cells during infection.* Science, 2005. **309**(5741): p. 1739-41.

75.	Cavanaugh, D.C. and R. Randall, *The Role of Multiplication of Pasteurella Pestis in Mononuclear Phagocytes in the Pathogenesis of Flea-Borne Plague.* The Journal of Immunology, 1959. **83**(4): p. 348-363.

76.	Orth, K., *<Function of the Yersinia effector YopJ.pdf>.* Current Opinion in Microbiology, 2002. **5**: p. 38-43.

77.	Meinzer, U., et al., *Yersinia pseudotuberculosis Effector YopJ Subverts the Nod2/RICK/TAK1 Pathway and Activates Caspase-1 to Induce Intestinal Barrier Dysfunction.* Cell Host & Microbe, 2012. **11**(4): p. 337-351.

78.	Sweet, C.R., et al., *YopJ targets TRAF proteins to inhibit TLR-mediated NF-κB, MAPK and IRF3 signal transduction.* Cellular Microbiology, 2007. **9**(11): p. 2700-2715.

79.	Monack, D.M., et al., *Yersinia signals macrophages to undergo apoptosis and YopJ is necessary for this cell death.* Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10385-90.

80.	Bergsbaken, T. and B.T. Cookson, *Macrophage Activation Redirects Yersinia-Infected Host Cell Death from Apoptosis to Caspase-1-Dependent Pyroptosis.* PLoS Pathogens, 2007. **3**(11): p. e161.

81.	Ratner, D., et al., *Manipulation of Interleukin-1β and Interleukin-18 Production by Yersinia pestis Effectors YopJ and YopM and Redundant Impact on Virulence.* The Journal of biological chemistry, 2016. **291**(19): p. 9894-9905.

82.	Council, N.R., *Guide for the care and use of laboratory animals*. 8 ed. 2011, Washington, DC: The National Academies Press.

83.	Patel, A.A., et al., *Opposing roles for interferon regulatory factor-3 (IRF-3) and type I interferon signaling during plague.* PLoS Pathog, 2012. **8**(7): p. e1002817.

84.	Reed, D.S. and M.J. Martinez, *Respiratory immunity is an important component of protection elicited by subunit vaccination against pneumonic plague.* Vaccine, 2006. **24**(13): p. 2283-2289.

85.   Sorg, J.A., et al., *Rejection of Impassable Substrates by Yersinia Type III Secretion Machines.* Journal of Bacteriology, 2005. **187**(20): p. 7090-7102.

86.   Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-738.

87.   Notley, C.A., et al., *DNA methylation governs the dynamic regulation of inflammation by apoptotic cells during efferocytosis.* Scientific Reports, 2017. **7**(1): p. 42204.

88.   Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.* Science, 2004. **303**(5663): p. 1526-9.

89.   Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.* Science, 2004. **303**(5663): p. 1529-31.

90.   Hornung, V., et al., *Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides.* The Journal of Immunology, 2002. **168**(9): p. 4531-4537.

91.   Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8α+ DC correlates with unresponsiveness to imidazoquinolines.* European Journal of Immunology, 2003. **33**(4): p. 827-833.

92.   Barr, T.A., et al., *TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells.* European Journal of Immunology, 2007. **37**(11): p. 3040-3053.

93.   To, E.E., et al., *Intranasal and epicutaneous administration of Toll-like receptor 7 (TLR7) agonists provides protection against influenza A virus-induced morbidity in mice.* Scientific Reports, 2019. **9**(1).

94.   Hammerbeck, D.M., et al., *Administration of a dual toll-like receptor 7 and toll-like receptor 8 agonist protects against influenza in rats.* Antiviral Res, 2007. **73**(1): p. 1-11.

95.   Jian, W., et al., *Toll-like Receptor 7 Contributes to Inflammation, Organ Injury, and Mortality in Murine Sepsis.* Anesthesiology, 2019. **131**(1): p. 105-118.

96.   Mancuso, G., et al., *Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells.* Nature Immunology, 2009. **10**(6): p. 587-594.

97.   Philip, N.H., et al., *Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF-κB and MAPK signaling.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. 7385-90.

98.   Plotnikov, A., et al., *The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2011. **1813**(9): p. 1619-1633.

99.   Kumaran Satyanarayanan, S., et al., *IFN-β is a macrophage-derived effector cytokine facilitating the resolution of bacterial inflammation.* Nature Communications, 2019. **10**(1).

100. Sebbane, F., et al., *Role of the Yersinia pestis plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague.* Proceedings of the National Academy of Sciences, 2006. **103**(14): p. 5526-5530.

101.	Arnold, K., *Yersinia pestis infection in Vietnam. II. Quantitative Blood Cultures and Detection of Endotoxin in the Cerebrospinal Fluid of Patients with Menigitis.* The Journal of Infectious Diseases, 1976. **133**.

102.	*What are the organs of the immune system?* 2006, Cologne, Germany: Institute for Quality and Efficiency in Health Care (IQWiG).

103.	Matthews, N., *Tumour-necrosis factor from the rabbit. II. Production by monocytes.* British Journal of Cancer, 1978. **38**(2): p. 310-315.

104.	Fahlman, C., et al., *Tumor necrosis factor-alpha (TNF-alpha) potently enhances in vitro macrophage production from primitive murine hematopoietic progenitor cells in combination with stem cell factor and interleukin-7: novel stimulatory role of p55 TNF receptors.* Blood, 1994. **84**(5): p. 1528-1533.

105.	Guilbert, L.J., et al., *Analysis of the synergistic stimulation of mouse macrophage proliferation by macrophage colony-stimulating factor (CSF-1) and tumor necrosis factor a (TNF-α).* Journal of Leukocyte Biology, 1993. **54**(1): p. 65-72.

106.	Spooner, C.E., N.P. Markowitz, and L.D. Saravolatz, *The role of tumor necrosis factor in sepsis.* Clinical Immunology and Immunopathology, 1992. **62**(1): p. S11-S17.

107.	*<Disruption of Signaling by Yersinia Effector YopJ, a Ubiquitin-Like Protein Protease.pdf>*.

108.	Cao, Y., et al., *Yersinia YopJ negatively regulates IRF3-mediated antibacterial response through disruption of STING-mediated cytosolic DNA signaling.* Biochim Biophys Acta, 2016. **1863**(12): p. 3148-3159.

109.	Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nature Reviews Genetics, 2009. **10**(1): p. 57-63.

VITA

Kayla Marks was raised in Albany, GA, born to her loving parents Charlie Edward Marks Jr. and Arvilla Lena Troup-Marks. Her proclivity for success is evidentiary through her many achievements in high school. She won multiple national speech competitions each year of high school, was chosen to be on the student council for the state of Georgia, and was selected as a LEDA Scholar, a highly selective program housed at Princeton University designed to prepare underrepresented students to become the nation's future leaders through the most accredited institutions in the country. She did this all while managing to be the captain of her soccer team and a well-trained pianist. She pursued her post-secondary education at Rochester Institute of Technology in Rochester, NY, receiving her bachelor's degree in Biomedical Sciences. During her time there, she was very involved in her on-campus community, organizing and facilitating many events toward the betterment of women and people of color. After receiving her bachelor's, she transitioned to a post-baccalaureate program at the University of Missouri for 2 years to strengthen and fortify her research skills and competitiveness to get her Ph.D. Afterwards, she was admitted into the Molecular, Pathogenesis, and Therapeutics program under the mentorship of Dr. Deborah Anderson. During her time at the University of Missouri, she not only grew as a scientist, but as a person, and will be eternally grateful for the relationships she forged and the lessons she learned. These character building experiences will aide and follow her throughout the duration of her career.