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INNATE IMMUNE RESPONSE TO *YERSINIA PESTIS*

by

Sanghita Sarkar
Master of Science, University of Calcutta, 2009

A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota
August
2015

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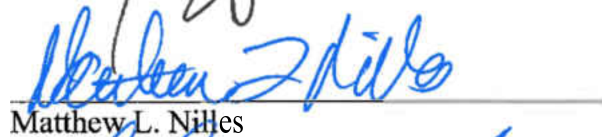
This dissertation, submitted by Sanghita Sarkar in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

David S. Bradley Chairperson



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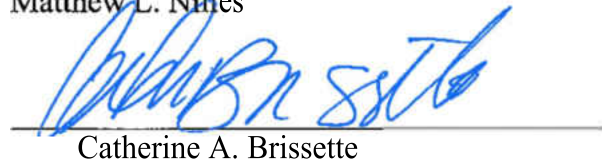

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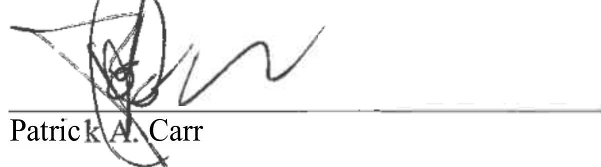

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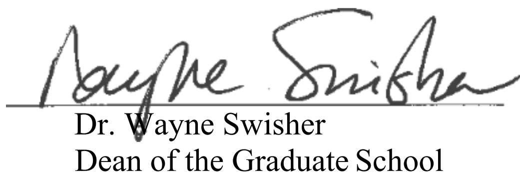

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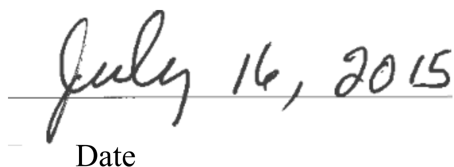

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To my dad Nimai Sarkar

ABSTRACT

This study demonstrates the various aspects of host immune response to *Yersinia pestis*. *Y. pestis,* the causative agent plague is mainly a rodent pathogen but is usually transmitted to humans via infected flea bite. After transmission to mammalian hosts, *Y. pestis* evades the host innate immune response and develops a systemic infection. Mast cells are recognized as the powerful sentinel cells responsible for controlling the early responses to a disease. We sought to determine whether mast cells are involved in the early innate immune response to *Y. pestis* and thus blocking mast cell degranulation would alter the outcome of infection. Mast cells in resistant young B10.T(6R) mice were depleted by use of anti-c-kit (ACK2) antibodies during *Y. pestis* infection. Our results demonstrated that *Y. pestis* infected anti-C kit treated mice showed lower survival rate than *Y. pestis* infected control mice suggesting mast cells are involved in early innate immune responses to *Y. pestis*. The other study is focused to understand the difference in the outcome of infection induced by endemic KIM5 and pandemic CO92 strains of *Y. pestis*. Both of these *Yersinia* strains have functional Type Three Secretion Systems (T3SS), which secretes effector molecules like Yersinia outer proteins (Yops) into the host cytosol to modulate the host immune response. KIM5 and CO92 strains express different isoforms of one of the Yop effectors, YopJ, which has been shown to inhibit NF-κB and MAPK activation in

mammalian cells. The YopJ isoform in *Y. pestis* KIM5 has two amino acid substitutions, F177L and K206E, which are not present in YopJ proteins of *Y. pestis* CO92. We show that young B10.T (6R) mice that are resistant to the KIM5 strain (LD_{50} of 14,000 CFUs) were susceptible to the CO92 strain (LD_{50} of 17 CFUs). A set of KIM5 isogenic strains expressing various YopJ isoforms allowed a demonstration that amino acid differences in YopJ were largely responsible for the increased virulence of the pandemic strain, CO92 in the B10.T (6R) mice.

CHAPTER I

INTRODUCTION

Yersinia pestis pathogenesis

The genus *Yersinia*, belonging to the *Enterobacteriaceae* family consists of eleven species, of which three species are pathogenic to humans: *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* (180). The former two bacteria are enteropathogens transmitted by contaminated food and water causing gastroenteritis. The third pathogen *Y. pestis* is the causative agent of bubonic, pneumonic and septicemic plague. Plague is transmitted via bite of an infected flea.

Plague in the Past

Plague has been one of the deadliest diseases in the world with recorded death toll of 200 million people (62) and resulting in three major pandemics. The first pandemic was the Justinian Plague (541 A.D to 544 A.D) originated in Pelusium, Egypt after spreading from Ethiopia. It quickly spread through the Middle East and the Mediterranean Basin (200). Subsequent outbreaks from 558 A.D to 640 A.D resulted in the distribution of the disease in North Africa, Europe, central and Southern Asia and Arabia. Taken together, a death toll between 15-40% for specific locales and epidemics have been ventured but it is estimated that from 541 A.D to 700 A.D there was a total population loss of 60% (200).

The start of second pandemic occurred in Sicily, Italy in 1347 probably from the steppes of central Asia via trade route. The first epidemic (1347 A.D to 1351 A.D) was referred to as Black Death and resulted in death of 17 to 28 million Europeans representing population loss of 30 to 40% (148, 260). Despite the high mortality rates, the most important effects of the Black Death epidemic resulted from the relentless epidemic cycles. Although the second pandemic was responsible for reducing majority of human population, it led to accelerate great changes in the social, economic and political development leading to implementation of policies, which brought changes in clinical research, medicine and public health regulations.

The third pandemic started in the Yünnan Province of China in the year 1855 and spread to the southern coast of China. It reached Hong Kong and Canton in 1894 and Bombay in 1898 and finally spread to Africa, Europe, Hawaii, North America and South America via steamships (34). In India alone plague was killing 12.5 million people between 1898 and 1918. Overtime, human plague cases have declined due to effective public health measures and advent of antibiotics in the 1950s.

Working independently, both Alexandre Yersin and Shibasaburo Kitasato were able to isolate the plague organism from the third plague pandemic. Initially, Kitasato was credited for the discovery, however Yersin Bacillus more accurately fits the current description of plague bacillus and Yersin used plague antiserum to cure a plague patient in 1896 (180).

Plague in the Present

Historically, plague is classified as the problem of the past. Since the plague bacillus is endemic in a variety of wildlife rodent species worldwide, it remains a current threat.

Two outbreaks of plague in Western India in 1994 followed by another outbreak in 2004 in Northern India resulted in the deaths of millions (161). Over the years there was a major shift in cases from Asia to Africa. The 3 most endemic countries are Madagascar, the Democratic Republic of Congo and Peru. Madagascar alone accounted for 30% human cases worldwide from 2004 to 2009. From 2007 to 2011, bubonic plague accounted for 86.6% of suspected cases, while pneumonic and undocumented cases accounted for 9.4% and 4% respectively (10). World Health Organization (WHO) reported 783 cases of bubonic plague cases, including 126 deaths in 2013(Figure 1).

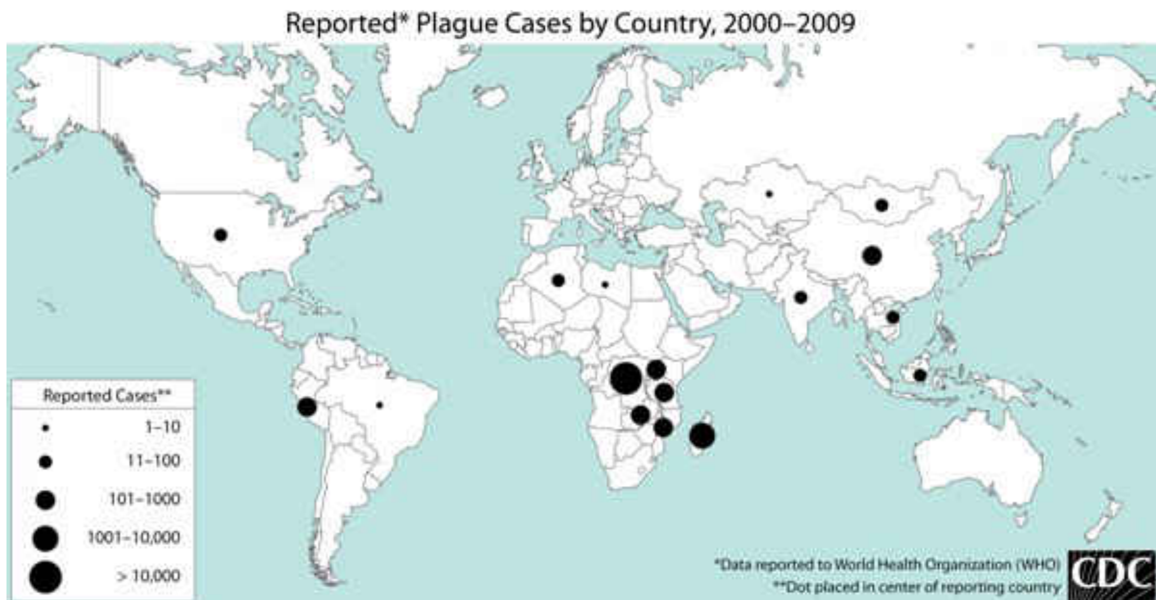


Figure 1. Reported Plague cases by country 2000-2009
http://www.cdc.gov/plague/maps/

Plague into the future

Plague is difficult to eradicate because it is widespread in wildlife rodent reservoirs. Therefore there is a critical need to understand how humans can be affected by the dynamics of these wildlife reservoirs. Plague outbreak in North America and central Asia

rodents and their risk to humans is affected by climate (71, 177) . Recent data from Kazakhstan shows that warmer springs and wetter summers increase the frequency of plague in its main host, the great gerbil (220). Such climatic conditions have shown to exist during the second and third pandemic. Although plague human cases reported are low in recent times, the threat to humanity cannot be ignored due to inherent communicability, rapid spread and high mortality state of the disease if left untreated. Also the ability of *Y. pestis* to acquire antibiotic resistance plasmid under natural conditions probably during its transit in the flea midgut is of alarm (84, 96, 102) . Recent observation of the presence of multi drug resistant plasmids similar to those acquired by *Y. pestis*, in a variety of enterobacteria isolated from retail meat products in the United States is of great concern (247). The presence of multi resistant strains of *Y. pestis* would represent a major threat to human health. Finally, the fact that plague has been weaponised throughout history from catapulting corpses over city walls to dropping infected fleas from airplanes to refined modern aerosolization of the bacteria, has led us consider *Y. pestis* as a biological weapon threat (109, 119) . Due to the high mortality rate and rapid transmission of the bacteria from one person to another *Y. pestis* is considered by Center for Disease Control (CDC) as Category A agents along with anthrax, Botulism, Small Pox, Tularemia and Viral hemorrhagic fever.

Epidemiology of *Yersinia pestis*

Alexandre Yersin was the first person to correlate between rats and plague. Masanori Ogata and Paul-Louis Simond independently discovered the role of fleas in transmission of plague during the Indian epidemic in 1897 (180). Although plague is primarily a zoonotic disease affecting rodents but humans become incidental hosts to the disease

(Figure 2). Transmission between rodents is associated with fleas. The oriental rat flea *Xenopsylla cheopis* is the classic vector for plague. Fleas obtain *Y. pestis* from feeding 0.03-0.5 µl of infected blood meal (104). A bacteremia of 10^4 CFU/ml will ensure ingestion of 300 *Y. pestis* organisms. The bacteria are cleared from some fleas but they multiply in the midgut of others. Two days post infected blood meal, the flea stomach or midgut display clusters of brown spots containing *Y. pestis*. These clusters then develop into solid dark brown masses containing the bacteria, fibrinoid like substances and hemin, which spreads throughout the stomach into the proventriculus and esophagus (13, 14). The proventriculus connects the esophagus to the stomach and also prevents regurgitation during a meal. Between 3 and 9 days post infected meal, the solid dark brown mass containing the bacteria blocks the proventriculus eventually preventing the ingested blood to reach the stomach (111). As a result when the flea next feeds, the blood sucked from the rodent is unable to reach the stomach of the fleas, instead the blood gets mixed with the bacilli present in the solid masses in the proventriculus and regurgitated into the mammalian host when the feeding attempt is terminated (111). While not all blocked fleas are responsible for transmission of the bacteria into a mammalian host, blockage is an important process ensuring transmission; unblocked fleas are rare in transmitting plague (32, 42).

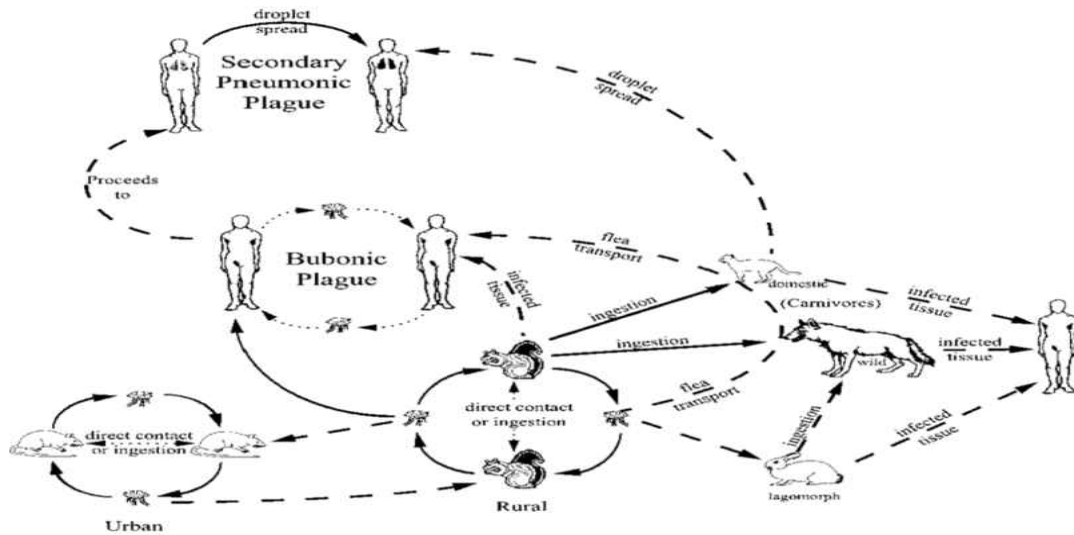


Figure 2: Plague Transmission Cycle (Used with permission) (180)**:** Human to human transmission: By droplets (pneumonic) or by fleas (bubonic). **Enzootic plague**. Resistant hosts maintain a low profile, stable rodent-flea infection cycle, e.g., voles and deer mice. **Epizootic plague**. Sensitive or moderately resistant hosts are infected by fleas or by ingestion, resulting in a highly visible die-off, e.g., rats, prairie dogs, rock squirrels. Solid lines with arrows: direct route of transmission; dashed line with arrow: occasional route of transmission; dotted line with arrows: rare route of transmission

The fate of the blocked flea is death due to starvation or dehydration. Due to lower temperature in the flea midgut (26°C), *Y. pestis* released from the fleas are unable to produce Fraction 1 capsule like antigen (F1) or any of the low calcium response stimulon (LCRS) components and are readily phagocytized by polymorphonuclear leukocytes (PMN) or macrophages. Cells phagocytized by PMNs are killed, while those phagocytized by macrophages survive intracellularly and develop resistance to phagocytosis. In a mammalian host at 37°C, by 3h, bacterial cells are resistant to phagocytosis by PMNs but not by monocytes. After 3 to 5 h, cells are resistant to uptake

by both phagocytes i.e, PMNs and macrophages (14, 43) . The expression of F1 capsular antigen and various LCRS components by the bacteria contributes resistance to phagocytosis by the host phagocytes. *Y. pestis* released into the extracellular compartments then spread systemically to spleen and liver.

The current scenario suggests that *Y. pestis* spread from the site of infected fleabite to regional lymph nodes resulting in swelling of the lymph nodes (called buboes). From the lymph nodes the organism spreads into the liver and spleen via bloodstream. For effective transmission in nature development of bacteremia of sufficient quantity and duration is essential. Infection of flea via the blood of an infected rodent completes the cycle. (180)

An important aspect of host-parasite interaction is that the parasite will develop minimum symptoms in hosts to ensure their survival and dissemination. The enzootic maintenance cycle allows *Y. pestis* to thrive endemically in nature. The domestic black rat *Rattus rattus* or the brown sewer rats *Rattus norvegicus* are the primary host for the flea vectors (35). Currently, the role of black rats in plague epidemics in medieval Europe is debated upon. Investigators challenge that black rats were rare and the cold climatic conditions across Europe would not allow the black rats to survive (107). Climatic conditions and trade routes across Europe from Asia might be responsible for spreading the epidemic in Medieval Europe (204). In North America, the primary flea vector is *Oropsylla montana*, which transfers the bacteria to the California ground squirrels (*Spermophilus beechyii*) and rock squirrels (*Spermophilus variegatus*). Chipmunks, *Tamias senex* and *T. quadrimaculatu* has shown to have high seroprevalence for *Y. pestis* (214). As Prairie dogs are the primary food source for black-footed ferrets, it is hypothesized that plague exists in both enzootic and epizootic forms within prairie dogs. There is a cyclic die-off

of prairie dogs (*Cynomys* spp.) that have an impact on black-footed ferret *(Mustela nigripes)* survival (145).

Clinical Manifestations of *Y. pestis*

Clinically Plague is present in three distinct forms in humans: bubonic, pneumonic, and septicemic. The different clinical form of plague depends on the route of infection and how bacteria disseminate throughout the body.

Bubonic Plague

The most common form of *Y. pestis* infection is bubonic plague. The usual symptoms includes fever, chills, headache and swollen lymph nodes (buboes, a characteristics sign of bubonic plague) within 2 to 6 days of contact with the organism either by flea-bite or by exposure of open wounds to infected animals. Gastrointestinal symptoms like nausea, vomiting, and diarrhea are common (108, 236) . At the initial site of an infection skin lesions may develop infrequently. Sometimes soreness in the affected lymph nodes will precede swelling. Depending on the site of the initial infection any of the lymph nodes areas can be involved: buboes are usually found in the inguinal and femoral regions but also occur in other nodes (34, 49) . Patients with bubonic plague are also characterized by bacteremia and secondary plague septicemia. Studies shows that blood culture colony ranges from <10 to 4 X 10^7/ml. Patients with colony counts greater than 100/ml have a high fatality rate.

Septicemic Plague

The second form of *Y. pestis* infection is septicemic plague. The two different forms of septicemic plague are primary and secondary. Primary septicemic plague occurs when the bacteria enter the host blood stream directly and patients with primary

septicemic plague are usually characterized by positive blood culture for the bacteria but no lymphadenopathy (206). Secondary septicemic plague develops when *Y. pestis* colonizes other areas (lymph nodes) before entering the blood stream. Symptoms developing due to primary and secondary septicemic plague include fever, chills, headache, and malaise. These symptoms are very similar to the symptoms observed during Gram-negative bacteria induced septicemia. The mortality rate is very high ranging from 30-50%. Such a high mortality rate may be because of antibiotics used to treat undifferentiated sepsis are not effective against *Y. pestis* (108).

Pneumonic Plague

The third and most rare form of *Y. pestis* infection is pneumonic plague. Pneumonic plague is the most deadly form of *Y. pestis* infection with 100% mortality rate with or without treatment. Pneumonic plague is the only form of plague that can spread from human to human. The two forms of pneumonic plague are primary and secondary.

Primary pneumonic Plague develops via respiratory droplets through close contact with the infected individual. The incubation period of primary pneumonic Plague is 1-3 days (35). The bacteria colonize the lungs and induce a flu-like illness that rapidly progresses to an overwhelming pneumonia with severe cough and bloody sputum.

Secondary pneumonic plague develops when *Y. pestis* either migrates from the lymph nodes (bubonic plague) or from blood (septicemic plague) to the lungs establishing a pneumonic infection.

Diagnosis and Treatment

To diagnose plague, sputum, blood samples and aspirates from the buboes can be used in the laboratory. A unique characteristic of *Y. pestis* is the bipolar staining and

pleomorphism. From bubo aspirates, Wright-Giemsa or Wayson stains will reveal the "safety pin" bipolar staining of *Y. pestis* (35). To isolate colonies from sputum samples *Yersinia* specific cefsulodin-irgasan-novobiocin (CIN) agar can be used (193). Recently, *Y. pestis* specific medium was developed containing combination of bile salts, irgasan and low concentrations of crystal violet to inhibit the growth of most other bacterial species (20). *Y. pestis* F1 antigen is detected by a rapid high specificity antigen detection test in clinical specimens from sputum, bubo aspirate and cerebrospinal fluid (46). Serum specimens containing seroconversions of four-fold or greater titers against *Y. pestis* F1 antigen can also be used as a diagnostic test (109).

Traditionally, streptomycin has been considered the most effective treatment for plague (35). However, streptomycin is a bacteriolytic antimicrobial agent (180). The release of endotoxic products from lysed bacterial cells could escalate the chance of systemic shock in a *Y. pestis*-infected individual. Doxycycline, tetracyclines, third-generation cephalasporins and quinolones are other alternative antimicrobials used to treat plague (186). Use of different treatment regimen depends on the different stage of disease development.

In 1995 two separate antibiotic resistant strains of *Y. pestis* were isolated (84). The first isolate, *Y. pestis* (17/95) was resistant to all recommended antimicrobial agents against the plague, except trimethoprim whereas the second isolate, *Y. pestis* (16/95) exhibited high levels of resistance to streptomycin. In both cases, resistance was carried on a conjugative plasmid capable of conjugation between *Y. pestis* strains and *E. coli*. It has been speculated that conjugation of the resistance plasmids may have taken place within a human or rat host.

Characteristics of *Yersinia pestis*

The genus *Yersinia* belonging to the *Enterobacteriaceae* family is a gram-negative, non-motile, non-spore forming coccobacillus. The organism is capable of growth between 4°C and 40°C but the optimal growth conditions for *Y. pestis* are temperatures between 28°C and 30°C (180). Growth is slow generally requiring 24 - 48 h for colony formation at 28-30°C, however the generation time of *Y. pestis* in defined media is 90 minutes. At all temperatures, *Y. pestis* requires L-methionine, L-phenylalanine, L-isoleucine, L-valine and glycine for growth. Growth restriction decreases the chances of long-term survival outside of the host. However, mammalian blood is rich in these organic nutrients and *Y. pestis* thrives within the host (26). Also the temperature shift from 26°C to 37°C in the mammalian system alters gene expression and leads to the production of virulence factors expressed by *Y. pestis*.

Yersinia pestis, like other Gram-negative bacteria, has lipopolysaccharide (LPS) in its outer membrane. The LPS of *Y. pestis* is characterized as rough because it lacks an O- group side chain due to a mutation in the O-antigen gene cluster (187). The bacteria do not have a typical capsule but do form a carbohydrate-protein envelope, termed capsular antigen or fraction 1(F1) at temperatures above 33°C.

The genome size of *Y. pestis* is ~4,380 ± 135 kb (139). The three plasmids found in wildtype isolates of *Y. pestis* are pCD1, pMT1 and pPCP1. pCD1 is a shared plasmid between pathogenic *Yersinia* species, while pMT1 and pPCP1 are unique to *Y. pestis*.

During *in vitro* growth at 37°C, in low calcium concentrations, the replication and gene expression is altered in *Y. pestis*. Growth restriction at 37°C in the absence of calcium results from shutdown of stable net RNA synthesis, inhibition of protein

synthesis and a decrease in adenylate energy charge (259). Simultaneously, virulence factors encoded on pCD1 are produced and regulated by this "low calcium response" phenomenon.

Species Evolution

The closest evolutionary relative of *Y. pestis* is a clone derived from *Y. pseudotuberculosis* and is believed to have emerged as a separate species between 1,500 - 20,000 years ago (4). Genome sequence analysis of virulent *Y. pestis* CO92 (176) and KIM10 (Kurdistan Iran Man) (58), shows that many of the virulence factors were acquired from its parental strain, *Y. pseudotuberculosis*. Genomic-fluidity and horizontal gene transfer from a *Y. pestis*-progenitor strain diverged from *Y. pseudotuberculosis* resulted in acquisition of only two virulence plasmids and 32 additional genes (38).

Numerous pseudogenes present in *Y.* pestis are still functional in *Y. pseudotuberculosis* (45). Several of the pseudogenes encodes for factors important in adherence and invasion of eukaryotic cells in enteropathogenic species of *Yersinia.* For example adhesion and invasion molecules important in the pathology of *Y. psuedotuberculosis,* but are non-functional in *Y. pestis* are YadA and InvA (180). Other gene products identified in *Y. pestis* that are analogous to YadA and InvA in *Y. pseudotuberculosis* are YadBC (80) and YapE (127) that may aid in bacterial colonization. The loss of these genes may play a role in the unique and recent evolutionary adaption of arthropod-borne transmission in *Y. pestis* and the difference in disease pathology.

Biovars

Based on *Y. pestis* ability to ferment glycerol and reduce nitrate to nitrite, there are three biovars of *Y. pestis*: Antiqua, Medievalis, and Orientalis. Antiqua is positive for both characteristics (186), Medievalis ferments glycerol but is unable to reduce nitrates and Orientalis cannot ferment glycerol but can reduce nitrate (3). Recently, these biovars were associated to regional outbreaks with spread of disease in pandemics (186). Based on synonymous single neucleotide polymorphisms (SNPs) Achtman et al. analyzed 156 strains of *Y. pestis* and constructed a phylogenetic tree (3) that correlated with earlier biochemical and historical work. Probing for *Y. pestis* DNA and protein signatures from ancient mass graves that are historically considered to be the result of outbreaks have further confirmed the etiology of plague pandemics. *Y. pestis* was accountable for the Justinian plague and 1st pandemic (60). Samples from purported plague burial sites from the time period of the 2nd pandemic also associated *Y. pestis* with the outbreak (61). Interestingly, all of the isolates linked to the above studies appear to have originated from the biovar Orientalis. Another study using genotyping found evidence that plague spread through Europe in two distinct upsurges (98). One upsurge was caused by biovar Orientalis, while the other was caused by biovar Medievalis. A non-human pathogenic biovar Microtus was identified recently (266).

Virulence Factors

The most proficient vector for transmission of plague, *Xenopsylla cheopis,* the classic vector for transmission of plague shows poor competence in the uptake of *Y. pestis* from an infected host (138). For an infection to occur in the flea, Approximately 10^4 bacteria are required. However, the LD_{50} for most susceptible mammals can be as

low as 10 bacteria (180). For proper transmission of plague in mammalian hosts, a threshold of 10^6 CFU/ml are required. Such high bacteremia can be lethal to the mammalian host due to endotoxic shock. *Y. pestis* has adapted numerous virulence functions to ensure both proper colonization within the midgut of the flea and to allow for efficient vegetative growth within the mammalian host without causing host death.

Plasminogen activator (Pla protease): Initially, it was believed that the coagulase activity of the pPCP1-encoded plasminogen activator (Pla) was responsible for the blockage of the proventriculus and bacterial aggregation in the flea (42). The coagulase model suggests that *Y. pestis* are enveloped and actively multiply in a fibrin clot found within the bloodmeal of a flea due to the coagulase activity of Pla. However recent studies show that the coagulase activity of Pla is weak and Pla mutant are still able to form aggregates that block the *X. cheopis* flea midgut (101). Transmission electron microscopy (TEM) of the *Y. pestis* infected flea proventriculus revealed that the bacteria were tightly packed together and were surrounded by an extracellular matrix (101). *Y. pestis* was forming a biofilm within the proventriculus and this was giving rise to aggregates and a "blocked" flea. At present, the more accepted model is the Biofilm Model of Proventricular Infection (110), which suggests that the temperature shift from host (37°C) to flea (26°C) induces the expression of *Y. pestis* genes specific for biofilm formation. Pla also aids in propagation of bacteria from the subcutaneous site into circulation. *In vitro* studies show that Pla of *Y. pestis* cleaves host plasminogen to plasmin; following disruption of the extracellular matrix (ECM), and Pla can bind directly to the ECM, letting the bacteria adhere to the ECM (128). Abolition of the proteolytic activity of Pla results in decreased virulence in mice infected via peripheral

routes (215) as the bacteria are unable to spread from the site of infection to the lymph node and eventually systemically. However, Pla mutants of *Y. pestis* are still fully virulent if injected intravenously into mice (206). Pla protease is vital for bacterial colonization, but not dissemination as suggested by studies in a murine model of primary pneumonic plague (126). Also a Pla mutant of *Y. pestis* if injected intraperitoneally along with the injection of iron enhances the virulence of *Y.pestis* suggesting that rather than serving as a nutrient, iron inactivates a host factor that compensates for the mutation. Pla protease is also essential for the invasion of epithelial cells *in vitro* (55) and may play a role as a *Y. pestis* adhesin or invasin.

Iron assimilation and pigmentation: Iron is an essential nutrient that is chelated by mammalian proteins making it less available to invading pathogens. *Y. pestis* requires iron for growth and infection. The 102 kb pigmentation (*pgm)* locus in the chromosome contains a high pathogenicity island that carries 11 genes encoding for proteins involved in siderophore synthesis. The siderophore, yersiniabactin, is involved in iron acquisition during infection(18). Pigmented (Pgm^{+}) cells of *Y. pestis* do not produce a pigment but adsorb large amounts of excess hemin that causes the formation of the pigmented colonies at 26°C but not 37°C, and are virulent in mice. All clinical isolates contain the *pgm* locus (28) and strains lacking this locus (pgm^{-}) are attenuated during infection. Supplementing mice with inorganic iron during mammalian infection restores virulence, demonstrating the important role of iron-acquisition to plague virulence (205). Studies in the pulmonary challenge model restored virulence after supplementing with inorganic iron (129). Bacteria are able to colonize the lungs and thrive, but there is still a lack of pulmonary damage and inflammation.

Murine toxin: *Y. pestis* has the pMT1 plasmid, which is absent from *Y. pseudotuberculosis* and *Y. enterocolitica*. pMT1 is a 110-kb plasmid. One gene product of pMT1 is the murine toxin (MT). MT is a phospholipase D that is required for colonization of the flea gut (25). MT is also lethal to mice and rats but not other animals like chimpanzees, monkeys, pigs and rabbits (156). However, it is not essential for virulence in a mouse model (103, 199).

Fraction 1 capsule: Another virulence factor specific to *Y. pestis* that is encoded on the pMT1 plasmid is the 15.5 kDa F1 capsule-like antigen (F1). The four genes that are located within the *caf1* (capsule antigen F1) operon are *caf1, caf1A, caf1M, and caf1R.* All except *caf1R* are required for assembly of a complete Caf1 (F1) subunit (135). At 37°C but not 26°C, F1 forms a large gel-like structure or envelope around the bacterium (180). Expression of F1 antigen is correlated with resistance to phagocytosis by monocytes. A decrease in ability to phagocytize bacteria was observed when murine macrophages were infected with *Y. pestis* expressing wildtype F1 compared to macrophages infected with mutant F1 (63). Numerous animal models show that F1 is not necessary for virulence (250) and a $F1^-$ strain has been isolated from a human case of plague (254). The genetic background of mouse strains used in F1 pathogenicity studies may also contribute to the differences seen in virulence between F1 and F1 mutant *Y. pestis* (240). A decrease in virulence was observed in a F1 mutant of *Y. pestis* in BALB/c mice compared to C57BL/6 mice.

pH 6 Antigen: Another virulence factor of *Y. pestis* is the chromosomally encoded fimbrial structure PsaA (pH 6 Ag) that is anti-phagocytic in nature (106). PsaA is synthesized at 37°C in acidic media, agglutinates erythrocytes, recognized as a

fimbriae structures and released from *Y. pestis* after internalization by phagocytes (133). This may contribute to why *Y. pestis* is resistant to phagocytosis after internalization and release by macrophages (43). Also, $PsaA^{-}$ *Y. pestis* is attenuated in an intra venous mouse model of infection (134).

Lipopolysaccharide (LPS): *Y. pestis,* like other Gram-negative bacteria, has lipopolysaccharide (LPS) in its outer membrane. LPS is a ligand for Toll-like- Receptor (TLR4), one kind of pattern recognition receptor (PRR). *Y. pestis* synthesizes two forms of LPS; tetra- and hexa-acylated based on temperature (157). At 26˚C, bacteria grown in the flea gut produce a hexa-acylated LPS, which can trigger TLR4-mediated immune signaling to induce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8. But after the temperature transition from the flea gut (26˚C) to mammalian host (37˚C), *Y. pestis* synthesizes tetra-acylated LPS, which is non-stimulatory for TLR4 mediated immune signaling, as well as resistant to complement mediated lysis and phagocytosis (64, 157).

Serum resistance: *Y. pestis* is resistant to complement mediated lysis regardless if grown at 26°C or 37°C (11). Resistance to complement mediated-lysis may be responsible for the survival of the bacteria in both flea and mammals. Synthesis of Ail outer membrane protein is one of the components of resistance to complement-mediated killing. This type of serum mediated resistance is most prominent in infection models of guinea pigs, rabbits, sheep, goats, and humans, but normal mouse serum has no bactericidal properties against *Y. pestis* and a Δ*ail* strain is still fully virulent in a mouse model of infection (16). This suggests that the contribution of Ail protein to *Y. pestis* virulence is species-specific.

Type III secretion system (T3SS): One of the factors contributing to virulence is the complex type III secretion system (T3SS), which delivers multiple effector proteins to the cytoplasm of host cells in contact with the bacteria (234). The T3SS is encoded by the plasmid pCD1, (CD stands for "calcium dependence") which is a 70.5 kb plasmid. The T3SS delivers six *Yersinia* outer proteins (Yops) directly into the cytoplasm of host innate immune cells. Usually, the function of Yop translocation is to decrease phagocytic activity, cell paralysis, induce apoptosis, inhibit autophagy, and manipulate intracellular trafficking (159). Together, these functions would result in suppression of a proinflammatory response to plague. This is demonstrated in a murine model of *Y. pestis* infection (168). Mice infected with pCD1 negative *Y. pestis* have a robust inflammatory response compared to mice infected with wildtype *Y. pestis* where there is little to no inflammatory response. The following section will cover, in detail, the T3SS effector proteins of *Y. pestis* and the reported mechanisms of host immune response manipulation (Figure 3).

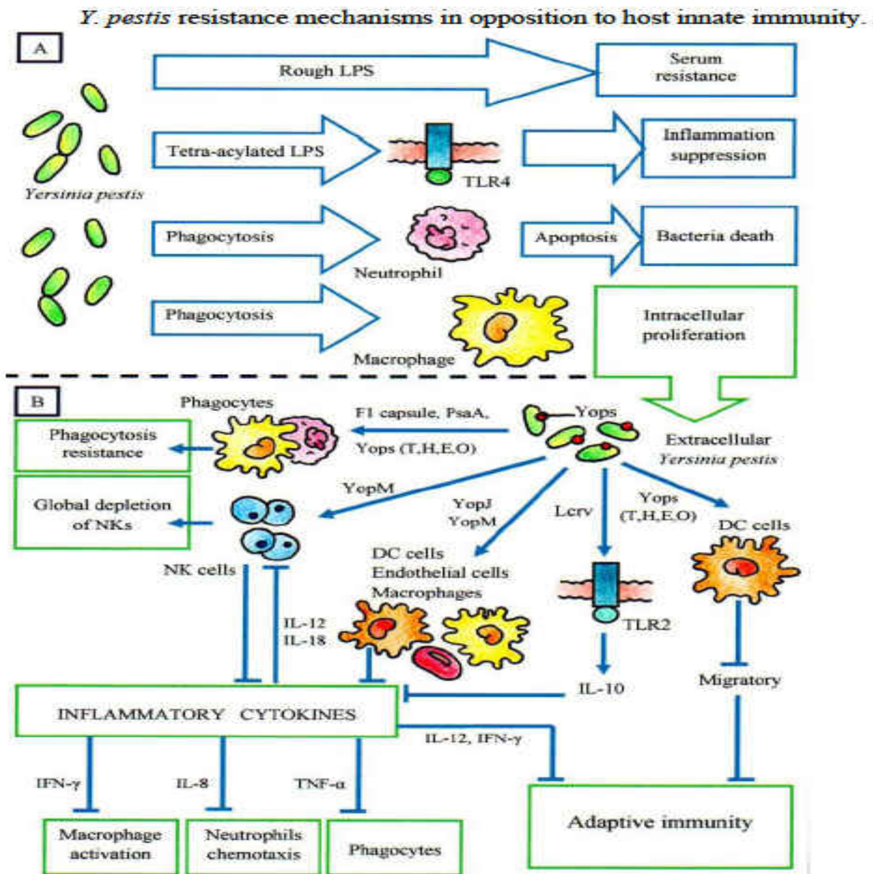


Figure 3: *Y. pestis* resistance mechanism in response to innate immunity (Used with permission) (6). (A) Resistance mechanisms at the early stage of infection. The various LPS structures of *Y. pestis* during transition between flea and host temperatures make the bacteria resistant to the complement-mediated lysis and suppress the proinflammatory response. Simultaneously, the bacteria phagocytosed by macrophages proliferate and express different virulence determinants to act on host immune response. (B) Release of *Y. pestis* into the extracellular milieu. The bacteria released from macrophages have the ability to resist phagocytosis and can inhibit the production of proinflammatory cytokines, which also attenuate the host's adaptive immunity.

The LcrV (also known as V-antigen) protein is a multifunctional protein associated with the tip of the injectisome needle complex (162). The LcrV protein interacts with the negative regulator LcrG and also associates with YopB/YopD to transport the effector molecule into the host cytoplasm (54). After being secreted into the environment, LcrV interacts with TLR2 and CD14 to stimulate the production of IL-10 (210), which is an anti-inflammatory cytokine. IL-10 then suppresses the formation of various pro-inflammatory cytokines including TNF-α and IFN-ϒ as seen in *Y. pestis* infection studies both *in vivo* and *in vitro* (170). Soluble LcrV can be found in the blood of the infected host (79) and suppresses neutrophil chemotaxis independently of T3SS Yop translocation (248). In the absence of the receptor, LcrV can directly bind to human IFN-γ (89). This binding affinity appears to be unique to *Yersinia* as studies show that an LcrV homolog, PcrV (*Pseudomonas aeruginosa*-derived), does not bind to IFN-γ (89). Thus, LcrV results in an overall down regulation of a general inflammatory response.

There are six effector proteins of the *Yersinia*-family T3SS that have been shown to modulate the host immune system: YopE, YopJ/YopP, YopM, YopH, YopT, and YpkA/YopO (234).

YopH is a protein tyrosine phosphatase. The major target of YopH is a focal adhesion molecule $p130^{Cas}$. YopH- mediated dephosphorylation of $p130^{Cas}$ leads to disruption in actin structures, including loss of focal adhesion and inhibition of phagocytosis. Other YopH targets include focal adhesion kinase (FAK), paxillin, Lck, Fyb and SKAP-HOM (50). *Y. enterocolitca*-derived YopH has also been shown to inactivate the phosphatidylinositol 3-kinase pathway (PI-3 kinase), that reduces the production of monocyte chemoattractant protein 1 (MCP1) (201). Bacterial strains

lacking YopH (CO92 Δ*yopH*) are avirulent in an intranasal and subcutaneous plague model (29) and are able to induce a host proinflammatory response (37). Vaccination with live CO92 Δ*yopH* is also protective against subsequent plague challenge (29). These data suggests that the direct ability of YopH to downregulate a proinflammatory response that contributes to plague virulence.

YopM is 46.2 kDa acidic protein that consists of leucine rich repeats (51) and is the effector protein that lacks catalytic activity. The targets of YopM were two kinases: ribosomal S6 protein kinase 1(RsK1) and protein kinase c-like 2(Prk2). YopM bind to α-thrombin and inhibits thrombin-induced platelet aggregation (130). The contribution of YopM to plague virulence has been explored by infection with YopM mutant strains of *Y. pestis*. YopM mutant strains are highly attenuated in virulence (117). Flow cytometric analysis of natural killer cells shows that there was a depletion of NK cells in the spleens and blood of *Y. pestis* KIM5 infected mice than mice infected with YopM mutant strain (117). YopM infected NK cells are defective to secrete interferon-gamma (IFN-Y) and there are undetectable levels of IL-15R mRNA. For homeostasis and maintenance of host NK cell populations, interaction of IL-15 with IL-15R is important (116). It is unclear how YopM downregulates IL-15R and whether that results in decrease in the number and function of NK cells during infection. Infection studies shows that YopM depletion of natural killer cells is organ-specific (257). Unexpectedly, antibody-mediated depletion of NK cells did not increase virulence of YopM mutant strains. Removal of neutrophils ($Gr1^+$) did increase virulence of YopM mutant strains, suggesting that neutrophils and a subset of inflammatory monocytes helps to clear Δ*yopM* *Y. pestis* (257) and neutrophils and monocytes may be a target of YopM during infection.

YopE is a GTPase activating protein targeting small RhoA-like G proteins, including RhoA, Rac1 and CDC42. YopE inhibits G proteins by inducing their GTPase activity. Cells infected by wild type Yersinia spp. undergo apoptosis, a process that does not induce any inflammatory mediators. However, in cells infected with YopE mutant strains, there is uptake of extracellular molecules and release of cytosolic proteins such as LDH, suggesting that YopE is involved in resealing pores made by the T3SS, thereby preventing leakage of cellular contents and stimulation of the host immune system. YopE also contributes to antiphagocytic activity (228).

YopT is a cysteine protease found exclusively in *Y. enterocolitica*. Studies demonstrated that YopT functions by disrupting actin microfilaments during infection. (228).

YpkA is a multidomain protein that functions as serine/threonine kinase. YpkA localizes to the cytoplasmic face of the plasma membrane in eukaryotic cells (185). It disrupts the actin cytoskeleton and contributes to the ability of *Yersinia* to resist phagocytosis by macrophages (228)

YopJ (YopP in *Y. enterocolitica*) is a 33 KDa protein that inhibits multiple MAPK signaling pathways and the NF-KB pathway (175). YopJ belongs to a family of cysteine proteases (53). Evidence suggests that YopJ functions as a deubiquitinase (224). YopJ also has acetyltransferase activity that acetylates serine and threonine residues important for activation of mitogen activated protein kinase (MAPK) kinase (MKKs) and IκB kinase complex (IκκB) pathways (152, 153, 163). The loss of cellular signaling events results in downregulation of various cytokines, chemokines and adhesion molecules. Down regulation of cytokines allows *Yersinia* to inhibit the recruitment and activation of

macrophages and neutrophils to the site of infection and thus evade the host inflammatory response (169). YopJ functions by binding to multiple members of MAPK kinase superfamily including MKKs and IkB kinase B (IKKB). In resting cells, NF-κB is bound to its inhibitor IκB and retained in the cytoplasm. IκB can be phosphorylated by IκB kinase, IKK. Following phosphorylation of IκB leads to its ubiquitination and degradation. NF-κB is then freed to enter the nucleus and affect transcription. YopJ acetylates IκB and thus prevents its phosphorylation. If phosphorylation of IκB is prevented, then IκB remains bound to NF-κB, thus blocking the NF-κB pathway (152).

YopJ can also induce LPS-stimulated apoptosis in macrophages (197). Apoptosis, or programmed cell death, is the organized disassembling of cellular structures and the lack of formation of a proinflammatory response (78). Bone marrow-derived macrophages with constitutive expression of an active inhibitor of NF-κB, inhibitor of κB kinase β (IKKß) are completely resistant to YopJ-dependent apoptosis, demonstrating that YopJ inhibits signaling upstream of IKKß (262). LPS activates apoptosis in macrophages by signaling via the adaptor protein Toll/IL-R domain-containing adaptor-inducing IFN-β (198). Together, LPS-activation can lead to anti- apoptotic signals through expression of the NF-kB-induced anti-apoptosis product, plasminogen activator inhibitor 2 (PAI-2) (95). During Gram-negative bacterial infections PAI-2 negatively regulates both apoptosis and caspase-1 induction and could act as a negative feedback loop to ensure an innate immune response without cell death. *Yersinia* species have adapted various modes to manipulate this system resulting in pro-apoptotic response without a strong induction of inflammation and thus evading the host immune response.

Pyroptosis is a form of programmed cell death that results in the proteolytic cleavage of the pro-IL-1β and -IL-18 by caspase-1 and secretion of the mature forms of these proinflammatory cytokines (78). YopJ can also inhibit caspase-1-dependent pyroptosis in activated macrophages (132). Naïve macrophages infected with *Yersinia* are targeted for apoptosis whereas activated macrophages undergo caspase-1-dependent pyroptosis (19)**.** Allelic polymorphisms seen in YopJ isolates between species, and strains may explain marked difference in the levels of apoptosis and pyroptosis observed when macrophages are infected with *Y. pestis*. *Y. pestis* KIM5 encodes for an isoform of $YopJ^{KIM}$ that stimulates higher levels of apoptosis and pyroptosis , and production of IL-1β (132). $YopJ^{KIM}$ has two amino acid substitutions (F177L, K206E), which are not present in $YopJ^{CO92}$ (132). Studies show that $YopJ^{KIM}$ has a higher binding affinity to IKKβ and increased inhibition of NF-κB, when compared to YopJ from *Y. pestis* CO92 and YopJ from *Y. pseudotuberculosis* (264). Human neutrophils are intrinsically resistant to YopJ-induced cell death (216). Activated-neutrophils can phagocytize and kill *Y. pestis* unlike macrophages wherein bacteria phagocytized survive intra cellularly (216). These findings show how complex and dynamic *Y. pestis* infection truly is and immune responses are different depending on infection routes and host.

Vaccination and Protective Immunity Against the Plague

In 1897, vaccination was used against plague (251). Heat-killed cultures of *Y. pestis* were used by Haffkine to treat plague patients. In 1970, Meyer *et al.* developed formalin-killed bacteria that were used as a plague vaccine (USP) which protected US military troops against exposure in Vietnam (251). However, whole-cell vaccines are

reactogenic (251) and might not contribute to protection against the pneumonic plague (251).

The next approach to effective plague vaccines was to target known *Y. pestis* virulence factors. Mice and rats were protected against *Y. pestis* infection when vaccinated with the F1 capsular protein (15). Moreover, passive transfer from F1-vaccinated rabbits was protective against pneumonic challenge (9). However, strains that lack F1 but cause disease were isolated, hence vaccination with just F1 would not protect against all strains of *Y. pestis.*

A subunit vaccine consisting of F1 and the virulence protein LcrV provides greater protection in different routes of infection than either subunit by itself (194). Studies show that for both bubonic and pneumonic plague, (8) passive immunization with F1/LcrV antibodies is protective in infected mice. Serum titers of F1 and LcrV antibody correlate with protection in mouse and non-human primate models (249). However, the F1/LcrV vaccine is not completely protective against aerosolized infection in African green monkeys (194). Also *Y. pestis* strains may make a variant of LcrV that will not induce a protective immune response (184). LcrV has shown to upregulate IL-10 and sequester IFN-ϒ. LcrV that lacks the immunomodulatory domain is still protective and induces lower IL-10 production *in vitro*. All these factors should be taken into consideration while assembling a putative protein in a plague vaccine. The need for an effective vaccine that is protective for all routes of infection is enhanced by the potential of plague to be aerosolized and used as a biological weapon (186).

TNF-α and IFN-ϒ are proinflammatory cytokines essential for protective immunity against plague. Lack of both TNF-α and IFN-γ throughout the disease

progression, with a slight spike of TNF-α just before death make it difficult to detect plague in early stages of *Y. pestis* infection (168). TNF-α and IFN-γ also play an important role in F1/LcrV antibody-mediated protection against intranasal challenge with fully virulent *Y. pestis* (137) and *Y. pestis* Δ*pgm* (122). IL-17 is also important in protective immunity. After vaccination with a live-attenuated strain of *Y. pestis* there is an increase in IL-17 producing $CD4^+$ T cells. Depletion of IL-17 decreases neutrophil migration and increases bacterial burden in this model (136).

Adaptive immunity is also important in protection against plague. Adoptive transfer of $CD4^+$ and $CD8^+$ T cells from live attenuated *Y. pestis* vaccinated mice provides protection in μMT mice (184). DNA-based vaccine studies demonstrated that after LcrV vaccination, depletion of $CD8^+$ T cells decreased protective immunity after challenge (188). Mouse dendritic cells (DEC-205/$CD205^+$) targeted with a modified F1-LcrV subunit vaccine primes $CD4^+$ T cells, which induces the production of IFN-γ and is protective against subsequent challenge (59). Hence after vaccination or passive immunization, both cytokines and T cells are important in the protection against plague.

CHAPTER II

ROLE OF MAST CELLS IN *YERSINIA PESTIS* INFECTION

Introduction

Although mast cells are known for their involvement in various inflammatory disorders, they are also recognized as the powerful sentinel cells responsible for controlling the early responses to a disease. Mast cells are distributed throughout the connective tissue of the body but tend to inhabit areas of confluence between host and external environment, such as the skin and mucosal barriers. Their preferential location at the host-environment interface and their innate capacity to release preformed mediators triggers the recruitment of pathogen clearing cells from the blood to the site of infection (2). Traditionally mast cells mediators are classified into two categories: preformed mediators that include proteases (chymases and tryptases), histamine, heparin, cytokines like TNF-α (123), and chemokines CXCL1/CXCL2 (77) and those that are de novo synthesized when the cells are activated, which includes cytokines like IL-1, and eicosanoids as leukotrienes, prostaglandins and thromboxanes (85). Stimulation of mast cells leads to activation of Cl^- channels in the plasma membrane, responsible for the release of mast cells mediators (195). The release of mast cells mediators is associated with diseases such as allergy, asthma, rheumatoid arthritis and chronic inflammatory diseases (120).

In host defense mast cells utilize various mechanisms to detect pathogens. Mast cells can recognize pathogens directly through pattern recognition receptors (PRRs) such as TLRs, which are stimulated in response to conserved pathogen-associated molecular patterns (PAMPs). Type I fimbriae present on various enterobacteria and cell surface component of *Mycobacterium tuberculosis* binds to CD48 a GPI anchored protein present on mast cells (142, 164). Cross-linking of CD48 not only results in degranulation of mast cells but also results in uptake of the bacteria. Mast cell surface receptor FcRs include FcϒRII receptors and the high affinity receptor for IgE, FcεRI, both the receptors on mast cells can bind to IgG and IgE respectively and become sensitized to antigens that have been previously encountered by the host (115, 255). Subsequently mast cells can become activated resulting in degranulation following receptor cross-linking by polyvalent antigen (255). Interestingly, TLRs and FcεRI activation can have a synergistic effect on cytokine production by mast cells through the activation of mitogen activated protein kinases (MAPK) (189). Bacterial superantigens such as *S.aureus* protein A can also activate FcεRI signaling activating mast cells (90). Complement receptors C5aR can also activate mast cell degranulation (173). During infection mast cell activation is not always a result of contact with the infectious agent, their products or opsonized microbes; many stressed cells release danger signals or alarmins, which also serve as strong activators of mast cells (70, 131). Anti microbial peptides, ATP, and IL-33 released by stressed cells can activate mast cells resulting in extensive degranulation (160).

The innate immune function of mast cells located close to blood vessels include the release of histamine, TNF, proteases and vascular endothelial growth factor (VEGF)

attributes to increasing vascular permeability and edema at the site of infection (24, 94, 207, 222). Chemokines like CCL11 and CXCL8 produced by mast cells recruits eosinophils and natural killer (NK) cells respectively (31). TNF and MCP6 derived from mast cells contribute to neutrophil recruitment in bacterial peritonitis models (105) and to inflamed tissues (21, 222). Additionally mast cells enhance mucus production by epithelial cells, immobilizing the pathogen and subsequently helping to clear pathogens from surfaces of gut, nasal mucosa and bladder (22).

Mast cells not only contribute to innate immune responses but also play a major role in triggering adaptive immune response. TNF released by mast cells upregulates expression of E-selectin by local endothelium, promoting the recruitment of monocyte derived dendritic cells (DCs) (208). CCL20 produced by mast cells contributes to the influx of DCs precursors from blood to the tissues. Thus, mast cells also aid in cell trafficking to draining lymph nodes (86). Moreover, mast cells themselves can present antigen to T cells. In a recent study, antigen pulsed mast cells were shown to stimulate $CD8^+$ T cell activation, proliferation and production of T cell products such as IL-2 and granzyme B (219). Thus, mast cell and its mediators polarize the environmental milieu and form a bridge between the innate and adaptive immune response.

The contribution of mast cells to combat infection has been studied extensively. The protective role of mast cell derived TNF was first shown against *Klebsiella pneumoniae* infection (143) and against polymicrobial intra-abdominal sepsis (66). TNF released by mast cells recruit neutrophils to site of infection to clear the pathogen. A similar function of mast cells has been shown in other infection studies:

Pseudomonas aeruginosa infection of the mouse peritoneum (151), *E. coli* infection of the peritoneum and urinary tract (1), *Helicobacter felis* infection of the gastrointestinal tract (233) and *Haemophilus influenza* infection of the ear (65). Mast cells are also efficient against Gram-positive bacteria comprising *Streptococcus pyogenes, Mycoplasma pulmonis* (256) and *Listeria monocytogenes* (40). However, in the case of Salmonella infection there is suppression of mast cell degranulation. SptP (Salmonella Protein tyrosine Phosphatase) injected by the T3SS into the mast cell cytosol inhibited mast cell degranulation even after exposure to stimulants like IgE/antigen (47, 83, 113) .

Yersinia pestis, the etiological agent of plague is an obligate extracellular, gram-negative bacterium. *Y. pestis* encodes various virulence factors like F1 capsular antigen, plasminogen activator (Pla), LcrV, and the type III secretion system (T3SS). The T3SS secretes various effector molecules into the cytosol of host cells to modulate the host immune response. A previous study with LcrV has shown that LcrV induce IL-10 production, which suppresses the production of pro-inflammatory cytokines TNF-α and IFN-ϒ (248). For the spread of *Y. pestis* during infection, an anti-inflammatory environment is necessary. The inflammatory action of mast cells may counteract the immunosuppressive effect demonstrated by LcrV (210). Additionally, the inflammatory mediators released by mast cells also aid in recruiting neutrophils to the site of infection resulting in clearing of bacteria and contributing to host defense during *Y. pestis* infection. Due to highly susceptible plague models, immunological mechanisms involved in the *Y. pestis* host immune response are difficult to study.

Cromolyn sodium has a short half-life and inhibits the release of inflammatory mediators from mast cells. Cromolyn blocks chloride channels, stabilizing mast cells membranes and inhibiting mast cell mediator release (165, 195) . Our lab has shown that blocking mast cell degranulation by use of cromolyn abolished resistance to infection in DQ8αβ transgenic mice in response to *Y. pestis*. We hypothesize that mast cells are involved in the early innate immune response to *Y. pestis* and thus blocking mast cell degranulation would alter the outcome of infection. To assay mast cell contributions in response to *Y. pestis* it would be ideal to use mast cell knockout mice. But, the most reliable knockout mouse is derived from the highly plague susceptible C57BL/6 strain. So developing a mast cell knock-down model during *Y. pestis* infection will be an alternative way to study the role of mast cells in plague. To determine the role of mast cells in *Y. pestis* infection, the mast cells in resistant young B10.T(6R) mice were depleted by use of anti-c-kit (ACK2) antibodies (12) during *Y. pestis* infection. Our results demonstrated that anti-C kit treated mice showed a lower survival rate than PBS treated mice. At day 3 there was higher load of bacteria in liver and spleen in PBS treated mice as compared to anti-c kit treated mice.

Material and Methods

Mice

Young (6 to 8 weeks of age) female B10.T (6R) mice (original breeding stock a gift from Dr. Chella David, Mayo Clinic and College of Medicine, Rochester, MN) were bred and maintained in a clean laminar flow containment area within the Center of Biological Research (CBR) at University of North Dakota. Young female B10.T (6R) mice were used for mortality studies. The University of North Dakota IACUC approved all animal studies.

Yersinia pestis Infection

Young B10.T (6R) mice were infected intravenously with *Y. pestis* KIM5. All strains were grown at 26˚C with shaking overnight in Heart Infusion broth (HIB; BD Difco) followed by subculture to 0.1 A_{620}, and incubated at 26˚C with shaking to an A_{620} of 1.0. Bacteria were centrifuged at 3,220 X *g* for 5 min, washed twice in sterile phosphate buffered saline (PBS) and resuspended in PBS. For mortality studies, mice were monitored twice daily for survival for 21 days. The serial dilutions used for infection were plated on TBA (BD Difco, Sparks, MD) plates and colonies were counted after 2 d of incubation at 26˚C.

Purification of Anti C Kit 2 antibody

Anti-c kit antibody (ACK2) secreting cells were obtained from EB Brandt (Division of Allergy and Immunology and Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA). ACK2 cells were maintained and the supernatant was collected. Antibodies were precipitated with saturated ammonium sulfate (SAS) solution. The precipitate was dissolved in PBS and dialyzed against PBS buffer.

The sample was then passed in a size-exclusion Sephacryl-S-200 column and the ACK2 antibody was obtained. BCA assay or IgG ELISA quantified the monoclonal antibody obtained and western blotting was done to detect antibody against C-kit was present in the purified sample preparation.

Depletion of mast cells

For a single dose experiment, B10.T (6R) mice were injected intra-peritoneally (i.p.) with 1.5 mg of anti-c-Kit antibody before infection. For multiple dose experiment, B10.T (6R) mice were injected intravenously with 1 mg of anti-c-Kit antibody before infection followed by 4 doses of 1 mg anti-c-Kit antibody every alternate day. Over 90% of mast cells were depleted with this procedure. Toluidine blue staining of ear tissue sections and flow cytometry of ear tissue samples were used to demonstrate depletion of mast cells in ACK2 treated mice.

Identification of mast cells and neutrophils

Ears were harvested from ACK2 treated mice and prepared for flow cytometry with slight changes (121). For flow cytometry, samples were centrifuged at 200 X *g* at room temperature. Cells were resuspended in PBS containing 0.1% BSA to a density of 1 X 10^6 cells/ml. Cells were incubated for 1 h at 4°C in the dark with the following antibodies: PE-conjugated anti-FcεRI, FITC-conjugated anti-c-kit, PB-conjugated anti-CD11b, and APC-conjugated anti Gr-1 (eBioscience). The excess antibody was washed by adding 1 ml of PBS containing 0.1% BSA and centrifuged for 5 min at 200 X *g* at 4°C. The cell pellet was resuspended in 100 µl of PBS containing 0.1% BSA buffer. Mast cells were sorted and identified as c-Kit+FcERI+ cells. Several combinations of markers

were used to identify neutrophils (Gr1+ CD11b+). Flow cytometric analysis was performed on an LSR II flow cytometer (BD). The histogram overlays were constructed using FlowJo (V.7.6.4). Ears were harvested and stained by toluidine blue to detect mast cells.

Measurement of Bacterial Burden

Livers and spleens were harvested at 3 d and 6 d from young infected B10.T(6R) mice and incubated in PBS containing 0.1% collagenase D for 1 h at 37°C. The challenge inoculum for *Y. pestis* was 1000 CFUs. All organs were homogenized by passage through 70 µM pore-size nylon mesh cell strainers (Falcon-Corning Inc, Corning, NY). Homogenates were centrifuged at 3,200 X *g* at for 5 min. Pellets were resuspended in PBS containing 0.1% (vol/vol) Triton X-100 to lyse eukaryotic cells. Bacterial burden was determined by plating serial dilutions on TBA (BD Difco, Sparks, MD) plates.

Statistical analysis

Data in the Figures are expressed as the mean ± SEM. For mortality studies, statistical comparisons were carried out using Log-rank (Mantel-Cox) test in GraphPad Prism (version 5.0d, GraphPad Software Inc, La Jolla, CA). For two group comparisons, a two-tailed unpaired t-test was used. For multiple groups, one way analysis of variance (ANOVA) with Bonferroni Multiple Comparison test as a post-test, a p value of <0.05 was considered significant.

Results

Depletion of mast cells

Mast cells were depleted in B10.T(6R) mice by use of anti-c kit antibody (ACK2) (Table 1 and Figure 4). At 1 d post injection of ACK2 antibody, there was a decrease of 88.8% of mast cells in the ear tissue compared to the PBS treated mice. The decrease in mast cells was observed till day 3. Interestingly, there was an increase in the percentage of mast cells on day 5 compared to day 1 in ACK2 antibody treated mice (from 30 to 216). On day 5 in ACK2 antibody treated mice, mast cells are approximately similar in number to PBS treated mice. At day 7 mast cells are 96.6% suggesting that after anti-C-kit antibody treatment stops mast cells are recovered.

Mast cells are required for effective anti *Yersinia* defense

Mast cell depleted or control mice were infected intravenously (i.v) with 1.4 X 10^3 CFUs of *Y. pestis.* A decrease in survival was observed in mice receiving anti-c kit antibody compared to PBS treated infected mice (Figure 5). This suggests that mast cell depletion contributes to *Y. pestis* infection. Mice that received multiple doses of anti-c kit antibody showed a decrease in survival compared to mice that received single dose of anti-c kit antibody (Figure 6).

Differences in Bacterial Burden in anti-c kit antibody treated infected B10.T (6R) mice

Eventually, *Y. pestis* infection spreads systemically throughout the visceral organs. Bacterial burdens were assessed in the spleens and livers of B10.T (6R) mice infected with 1000 CFUs of *Y. pestis* KIM5. At 3 d post-infection, KIM5 infected anti c-kit treated

mice B10.T (6R) mice had1.5 X 10^7 ± 1.3 X 10^7(mean ± standard error of mean [SEM]) CFU per spleen compared to 2.8 X 10^7 ± 1.7 X 10^7(mean ± SEM) CFU or 1.8 X 10^7 ± 4.5 X 10^6 per spleen of isotype or PBS infected B10.T (6R) (Figure 6) Similarly, at 3 d post-infection, KIM5 infected anti c-kit treated mice B10.T (6R) mice 1.3 X 10^6 ± 1.25 X 10^6(mean ± standard error of mean [SEM]) CFU per liver compared to 3.5 X 10^6 ± 2.14 X 10^6(mean ± SEM) CFU or 5.4 X 10^6 ± 1.54 X 10^6 per liver of isotype or PBS infected B10.T(6R) mice(Figure 7). There was no significant change in the bacterial burden in both liver and spleen at day 3 between the anti-c-kit treated antibody and PBS or isotype treated mice. However, by 6 d post-infection both the spleen and livers of anti-c kit treated mice had higher bacterial burden (4.6 X 10^6 and 9.5 X10^5 respectively) than PBS treated mice (0 and 1.75 X 10^5 respectively) (Figure 7 and 8). Higher bacterial burden in the anti-c kit treated B10.T(6R) mice at day 6 suggests that mast cells in young B10.T(6R) mice were important in anti-Yersinia defense in the host and mast cells may be responsible to successfully induce early immune responses to *Y. pestis* .

Discussion

Young inbred female B10.T (6R) mice show an increased resistance to *Y. pestis* than other outbred mice such as Swiss Webster mice. This phenomenon of resistance observed in B10.T (6R) mice allows us to explore other factors in a *Y. pestis* infection such as host responses to *Y. pestis* or *Y. pestis* virulence factors and their effect. The innate immune response recognizes invading pathogens, eliciting an immune response, which prevents the spread of invading pathogens while an adaptive immune response is generated. A significant feature of mast cells is their ability to rapidly respond to pathogens by

releasing preformed and de novo synthesized mediators, which helps to initiate and maintain pro-inflammatory responses.

Traditionally mast cells are associated with IgE-mediated, Th2 responses to allergens but more recently they have been associated with host response during an infection. In vivo studies have shown that the role of mast cells in increased survival in infectious peritonitis, murine malaria, *Citrobacter rodentium* and *Listeria monocytogenes* infection (66, 82, 244). The decrease in survivability observed in these murine models is associated with the loss of mast cell function, either directly or indirectly. Various studies show that activated mast cells engulf pathogens and release pro-inflammatory mediators and chemotactic factors. One of the important pro-inflammatory cytokine released following mast cell activation is TNF-α. TNF-α is critical in initiating host defenses against pathogens, aids in generation of inflammatory reactions, activating endothelium, allowing the migration of immune cells into the site of infection and influencing dendritic cells and T cell recruitment, activation and function (166, 167, 223).

Depletion of mast cells showed increased mortality in anti-c-kit antibody treated B10.T(6R) mice indicating that mast cell plays a pivotal role in defense against plague. However, the mechanism of how mast cells play a role to enhance host responses against plague has yet to be determined. Loss of mast cell degranulation may impair the ability of local immune cells, such as dendritic cells, neutrophils and T cells to be recruited to the site of infection, as cytokines and chemokines are not released. Apart from recruitment of immune cells to the site of infection, loss of mast cell degranulation also might affect dendritic cell maturation and presentation of antigen to T cells. Mast cell depletion might

also increase *Y. pestis's* ability to evade the innate immune response by dampening the inflammatory reaction required by the host to suppress bacterial growth and spread.

Mast cells are a multifunctional immune cells that act as a bridge between the innate and adaptive immune system. One-way mast cells act as a bridge between innate and adaptive immunity is to function as an antigen-presenting cell. MHC class I and class II molecules are expressed on the surface of mast cells (192). Mecheri *et.al* demonstrated that mast cells pulsed with antigen *in vitro* and injected into BALB/c mice were able to activate T cells to induce antibody response (235). Upon infection or in the presence of TNF-α or IFN-ϒ mast cells express MHC class II molecules. Exosomes are vesicles that express MHC class II molecules and are released into the extracellular milieu (211). In vivo mast cell derived exosomes have demonstrated the ability to induce maturation of dendritic cells allowing stimulation of T cells and initiation of adaptive immune responses (212). Mast cells exert their effects on lymphocytes by direct cell-cell contact, or the release of exosomes. Whether naïve mast cells stimulate lymphocyte function *in vivo* is yet to be studied. However it has been shown that mast cells are activated in the periphery and migrate to draining lymph nodes where they interact and activate lymphocytes (86, 238). These data suggest that a role of mast cells is triggering adaptive immune responses.

Previous results demonstrated that cromolyn administration blocked mast cell degranulation, which showed the importance of mast cells in *Y. pestis* infection, indicating that the increased resistance might be partially due to mast cell. For the host to survive inflammatory immune responses is important against *Y. pestis* infection. *Y. pestis* has developed various tactics to down regulate host inflammatory responses. Our results demonstrated the importance of mast cells in a *Y. pestis* infection that by depleting mast

cells from resistant B10.T(6R) mice exacerbated the disease. Increased bacterial burden in anti-c-kit treated antibody than PBS treated mice demonstrated that mast cells play an important role in early host defense against *Y. pestis*. B10.T(6R) mice are compromised in the ability to mobilize neutrophils to the site of infection due to absence of mast cells. This result of increased bacterial growth in the spleen and liver of KIM5 infected B10.T (6R) mice during the later stages of *Y. pestis* infection. These results correlate with other studies showing the role of mast cells in infection and validate results obtained lately in mast cell reconstituted W/W^v mice (67).

In conclusion, these results suggest that mast cells play a role in early anti-*Yersinia* host responses. In a contest between the host and pathogen, survival of the host depends on the ability of the host to induce a robust attack on the pathogen. Due to the presence of mast cells in the body tissues, mast cells are likely to be the first inflammatory cells to contact invading pathogens. Also the fact that mast cells are the only cell-type that contain preformed inflammatory mediators makes mast cells as vanguards to control bacterial pathogens. Thus, any incapacitation of host defenses at the very early but critical stage of infection owing to loss of mast cell function, as demonstrated here, tips the balance in favor of *Y. pestis* and makes them the difference between life and death several days later.

Table-1: Total Number of Mast cells

	Total number of Mast cells			
Days	PBS (control)	Anti-C kit 2(ACK2)	% Decrease	% Increase
D1	267	30	88.8	-
D3	269	32	88.1	-
D5	214	216	-	0.93
D7	149	293	-	96.6

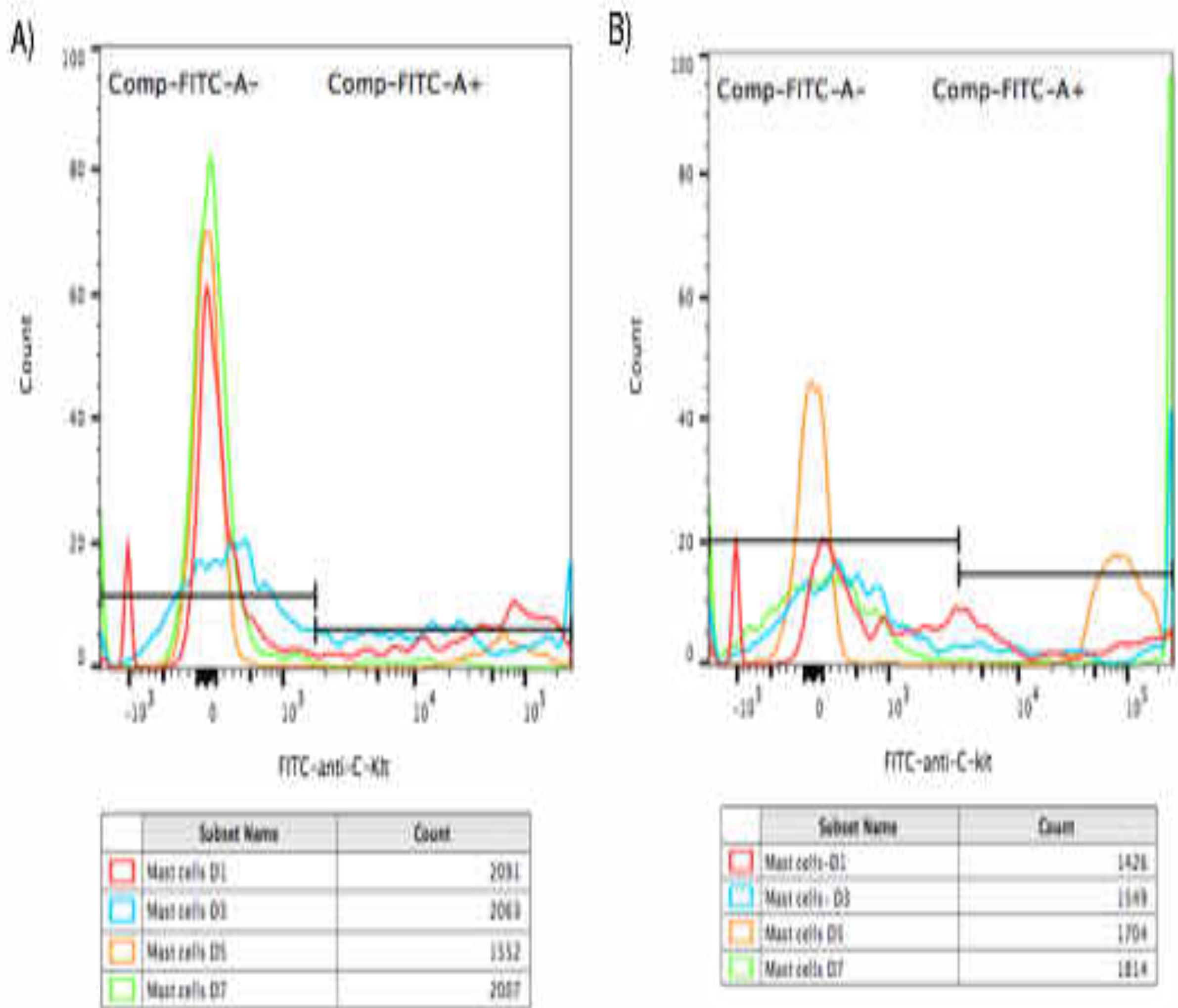


Figure 4: Mast cells were sorted and identified as c-Kit+ cells: 3 female young B10.T (6R) mice were treated with PBS (A) and Anti-C kit monoclonal antibody (B). Anti-c kit treated mice showed lower mast cell count compared to PBS treated mice. Data is representative of 2 independent experiments.

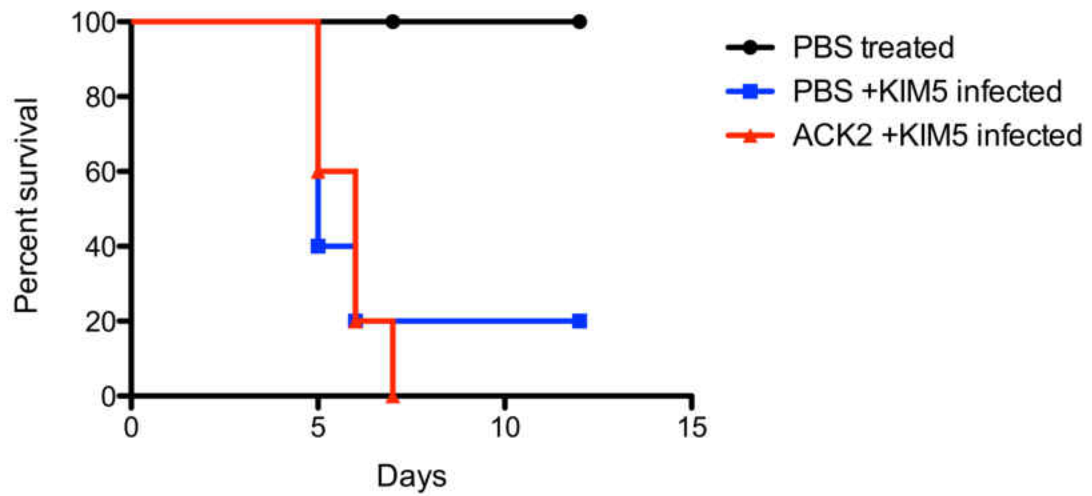


Figure 5: Survival curve of Anti-C Kit and PBS treated B10.T(6R) mice: 7 female young B10.T(6R) mice were infected with 1,000 CFUs of *Y. pestis* KIM5. Anti-C kit treated mice succumb to infection. Data is representative of 2 independent experiments.

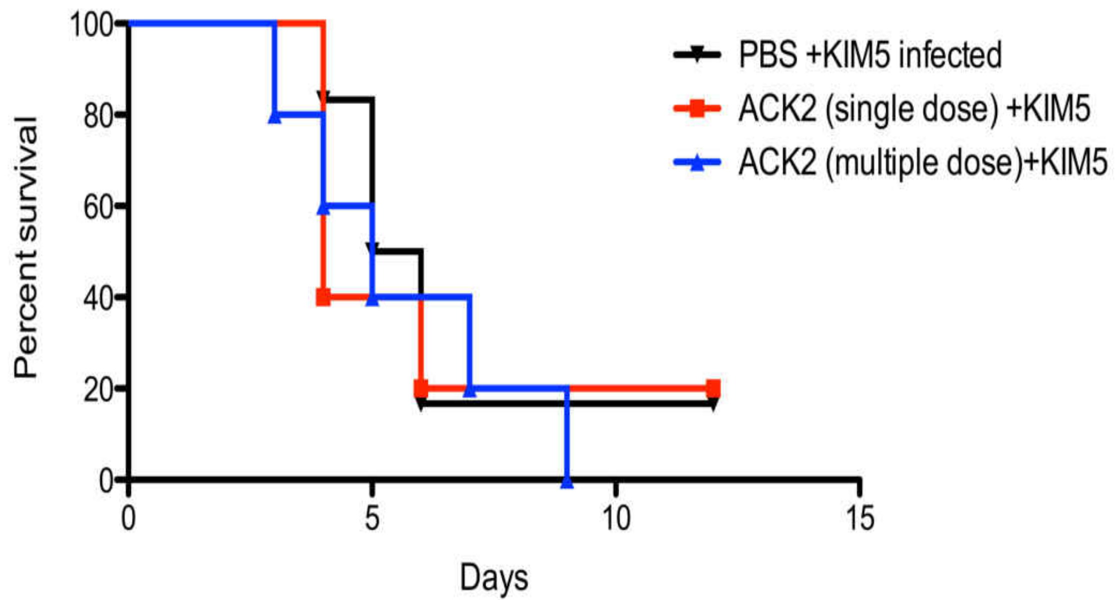


Figure 6: Survival of Anti-C Kit and PBS treated B10.T(6R) mice that received multiple doses of ACK2: 7 female young B10.T(6R) mice were infected with 1,000 CFUs of *Y. pestis* KIM5. Mice that received multiple dose of Anti-C kit antibody succumb to infection earlier than mice that received single dose of anti-c kit antibody. Data is representative of 2 independent experiments.

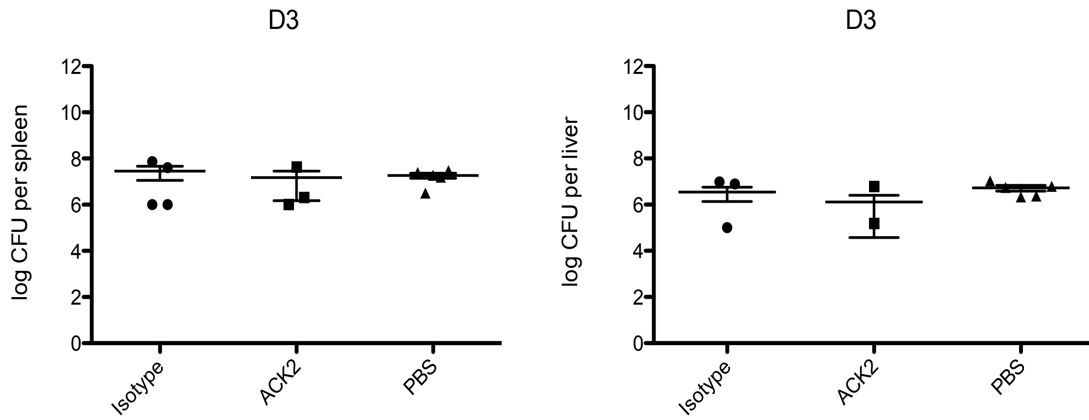


Figure 7: Bacterial burdens in the spleens and livers of infected mice at day 3 (A & B). Spleens and livers of B10.T(6R) mice challenged with 1,000 CFUs of *Y. pestis* KIM5(n=5/dose/experiment). Data is representative of 2 independent experiments; $p<0.05$ by Bonferroni's Multiple Comparisons test.

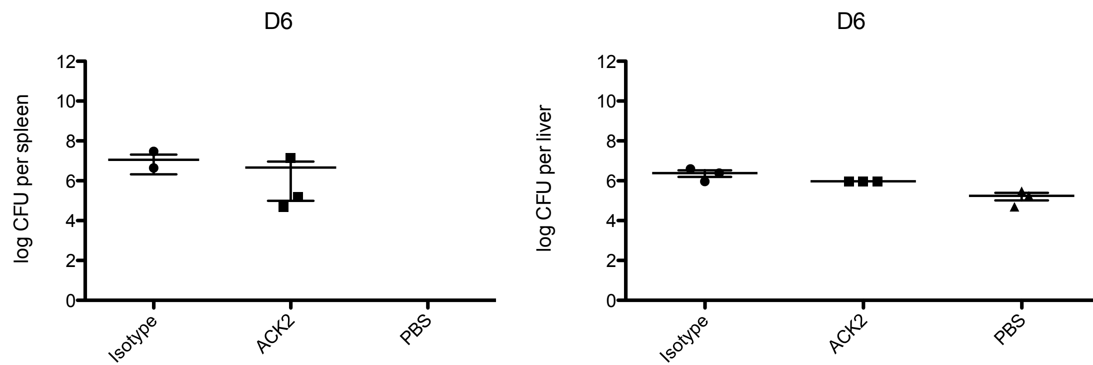


Figure 8: Bacterial burdens in the spleens and livers of infected mice at day 6 (A & B). Spleens and livers of B10.T(6R) mice challenged with 1,000 CFUs of *Y. pestis* KIM5(n=5/dose/experiment). Data is representative of 2 independent experiments; $p<0.05$ by Bonferroni's Multiple Comparisons test.

CHAPTER III

SUSCEPTIBILITY TO *YERSINIA PESTIS* IN B10.T (6R) MICE IS DEPENDENT ON YOPJ INDUCED VIRULENCE

Introduction

Three of the 11 species found in the genus *Yersinia* are pathogenic to humans: *Yersinia pseudotuberculosis, Yersinia enterocolitica* and *Yersinia pestis* (26). *Y. pseudotuberculosis* and *Y. enterocolitica* cause gastrointestinal disorders (169) whereas *Y. pestis* is the causative agent of bubonic, pneumonic and septicemic plague (180). Plague is transmitted to mammalian hosts via infected flea bites. After transmission to mammalian host, the bacteria evade the host innate immune response and colonize the proximal lymph nodes (240). The organism will proliferate in the lymph nodes, leading to enlarged and inflamed lymph nodes called buboes, a manifestation of bubonic plague. From the lymph nodes, the organism spreads systemically to spleen, liver and lungs. Colonization of bacteria in lungs can lead to secondary pneumonia and may result in person-to-person transmission causing primary pneumonic plague (180).

Y.pestis has been classified into 4 biovars depending on their ability to reduce nitrate and ferment glycerol: *Antiqua* (positive for both metabolic functions), *Mediaevalis* (cannot reduce nitrate but can ferment glycerol) and *Orientalis* (can reduce nitrate but cannot ferment glycerol) and non-human pathogenic *Microtus*. *Y. pestis* CO92 strain (Orientalis) is an American isolate from the third plague pandemic (97), while KIM5 strain

(Mediavalis) is an isolate from endemic plague foci (176). Both of these strains share 95% of sequence similarity between the 2 genomes (58), (176). The CO92 genome is ~ 50 kilobases (kb) larger than the KIM genome due to the presence of an 11 kb and several small insertions in CO92 relative to the KIM5 genome. About 27 kb of the differences is due to insertion sequence (IS) elements present in CO92 (176). CO92 has 1 less rRNA operon than KIM. Both of these strains of *Y. pestis* harbor a 70 kb highly conserved plasmid pCD1 (calcium dependence) containing ~ 50 virulence genes encoding the type III secretion system (T3SS). The T3SS delivers several effector molecules (YpkA, YopH, YopE, YopJ, YopT, YopK and YopM) into the cytosol of host cells (169). YopJ, an effector molecule secreted by the T3SS, inhibits the MAPK and the nuclear factor-kappa B (NF-κB) pathways. Delivery of YopJ to the host cells prevents the formation of anti-apoptotic factors, thereby *Yersinia* infected macrophages undergo apoptosis that requires TLR4-dependent activation of caspases (155, 198, 261, 263). YopJ is homologous to CE cysteine proteases (174) and also functions as deubiquitinase (175, 224, 267). More recent studies identify YopJ as an acetyl-transferase, activated by host-specific factor inositol hexakisphosphate (153). YopJ acetylates Ser and Thr residues important for activation of MKKs and IKKβ pathways (153, 163). YopJ mediated acetylation of MAPKKs and IKKβ directly competes with phosphorylation of these residues, thus blocking the signal transduction required for activation of MAPK and NF-Kβ transcription factors (163). Consequently, transcription of pro-inflammatory cytokines and cell survival gene expression is inhibited by YopJ mediated activity. The combined effect of YopJ mediated inhibition of cell survival genes and TLR4 dependent activation of apoptotic signaling pathways results in *Yersinia* infected macrophage cell

death. Together, lack of the various pro-inflammatory products inhibits the activation and recruitment of macrophages and neutrophils to the site of infection and thus *Y. pestis* is able to evade the host inflammatory response (169)

Y. pestis infected hosts have a lack of early inflammatory responses with immune cell depletion at sites of bacterial replication and dampening of pro-inflammatory cytokine responses. Although *Y. pestis* causes deadly infections in hosts, there exists a percentage of hosts that are intrinsically resistant to plague (48, 230). Multiple laboratory strains of inbred mice are resistant to plague but the mechanisms of resistance and susceptibility are poorly understood. Susceptible C57BL/6J mice infected with the KIM5 strain of *Y. pestis* have an LD_{50} of 20-50 CFUs (48) whereas KIM5 infected 129S2/SV.Hsd have an LD_{50} of 2 X 10^6 CFUs (180). BALB/C strains of mice are susceptible to plague infection, however, the BALB/cJ substrain is resistant to plague infection (229). The resistance was mapped to a region that coincided with the major histocompatibility complex on chromosome 17 (230). Inbred B10.T(6R) mice are significantly more resistant to *Y. pestis* KIM5 than C57BL/6 and other “susceptible” mouse strains, and this resistance decreases with age (124)

Here, the susceptibility of young and middle aged B10.T (6R) mice to *Y. pestis* CO92 Δ*pgm* was examined. Interestingly, young B10.T (6R) mice that are resistant to the KIM5 strain (LD_{50} of 14,000 CFUs) were susceptible to the CO92 Δ*pgm* strain (LD_{50} of 9-17 CFUs). To our knowledge, this is the first known report to document such a difference in the virulence of plague strains. A set of KIM5 isogenic strains expressing various YopJ isoforms allowed a demonstration that amino acid differences in YopJ were largely responsible for the increased virulence of the pandemic strain, CO92 Δ*pgm* in the

B10.T(6R) mice. Comparison of serum cytokine profiles of sera taken from young B10.T(6R) mice infected with either *Y. pestis* CO92 Δ*pgm* or KIM5 demonstrated that pro-inflammatory cytokines (e.g. IL-6, IL-1β) and chemokines (KC, RANTES, and MCP1) levels were higher in mice infected with the KIM5 strain compared to the CO92 Δ*pgm* strain. These results suggest that $YopJ^{CO92}$ is associated with lower cytokine production, and that the cytokine changes correlate with the differences seen in virulence between the CO92 Δ*pgm* and KIM5 strains of *Y. pestis.*

MATERIALS AND METHODS

Mice

6 to 8 weeks of age of female B10.T (6R) mice (original breeding stock a gift from Dr. Chella David, Mayo Clinic and College of Medicine, Rochester, MN) were bred and maintained in a clean laminar flow containment area within the Center of Biological Research (CBR) at University of North Dakota. Young female B10.T (6R) mice were used for mortality studies and to determine LD_{50} for various strains of *Y. pestis*. The University of North Dakota IACUC approved all animal studies

Table 2: Bacterial Strains Utilized

Bacterial Strains	Characteristics	Source or Reference
Y. pestis		
KIM5	Biovar Mediavalis, pgm^-, pCD1, pMT1, pPCP1	(230)
CO92 Δpgm	Biovar Orientalis, pgm^-, pCD1, pMT1, pPCP1	J.Bliska
KIM5-$YopJ^{CO92}$	KIM5 pCD1-Ap encodes YopJ L177F E206K, Ap^r	J.Bliska
KIM5-$YopJ^{YPTB}$	KIM5 pCD1-Ap encodes YopJL177F, Ap^r	J.Bliska
KIM5-$YopJ^{E206K}$	KIM5 pCD1-Ap encodes YopJE206K, Ap^r	J.Bliska
CO92-$YopJ^{KIM5}$	CO92pCD1-Tm encodes YopJF177L K206E, Tm^r	This study
CO92-$YopJ^{YPTB}$	CO92 pCD1-Ap encodes YopJL177F, Ap^r	This study
CO92-$YopJ^{E206K}$	CO92 pCD1-Ap encodes YopJE206K, Ap^r	This study

Bacterial Strains, culture conditions and plasmids

Y. pestis strains as listed in Table 1 lack the pigmentation locus and are exempt from select agent guidelines. All strains were stored at -80˚C in 25% glycerol (vol/vol). CO92 Δpgm, KIM5-YopJ^{CO92}, KIM5-YopJ^{YPTB}, and KIM5-YopJ^{E206K} were a kind gift from James B. Bliska.

Preparation of YopJ mutants in CO92Δpgm: CO92Δpgm cells were grown on Tryptose Blood Agar Base (TBA; BD Difco, Sparks, MD) plates at 26°C for 48 hrs. The CO92 Δpgm cells grown at 26°C were plated on TBA plates with 2.5mM concentration of $CaCl_2$ at 37°C for several generations to cure the pCD1 plasmid. Growth curve were performed as described by Straley et. al. Briefly, bacteria were grown in TMH medium (99) at 26°C overnight, subcultured into fresh medium at an A_{620} 0.1(plus 2.5mM $CaCl_2$) and grown until OD reaches 0.2 at 620nm. The temperature was shifted to 37°C and incubated for 7 h while taking A_{620} readings at 1 h intervals. 1 ml of sample was aliquoted into 1.5 ml tubes, centrifuged at 20000 X *g* for 5 min at 4°C and pellets separated from the supernatant, which contains secreted proteins. The pellet and supernatents were precipitated with 10%(w/v) trichloro acetic acid (TCA) followed by SDS-PAGE and silver staining to ensure that pCD1 was cured from the CO92 Δpgm cells. This CO92 ΔpCD1 was used to prepare electrocompetent cells.

Preparation of CO92 ΔpCD1 electrocompetent cells: CO92 ΔpCD1 cells were grown overnight in HIB broth, subcultured into fresh medium to an A_{620} of 0.1 and grown until OD reaches 0.5-0.8.The culture was taken in a 50 ml conical tube, centrifuged at 3220 X *g* for 20 min at 4°C. The cell pellet was washed once with cold distilled water at

3220 X *g* for 20 min. The cell pellet was dissolved in 1 ml of cold 10% PEG 8000, centrifuged at 20000xg at 3 min at 4°C. The cell pellet was dissolved in 25µl PEG/OD_{620}.

The pCD1 plasmid DNA was isolated from D27 YadA:Tm^r strain by midiprep isolation kit (Qiagen Sciences, Maryland 20874,USA) and used for transformation of CO92 Δ*pgm* $pCD1^-$.

Bacterial Challenge

Young B10.T (6R) mice were infected intravenously with various strains of *Y. pestis* as listed in Table 1. All strains were grown at 26˚C with shaking overnight in Heart Infusion broth (HIB; BD Difco) followed by subculture to 0.1 A_{620}, and incubated at 26˚C with shaking to an A_{620} of 1.0. Bacteria were centrifuged at 3,220 X *g* for 5 min, washed twice in sterile phosphate buffered saline (PBS) and resuspended in PBS. Mice were monitored twice daily for survival for 21 days. For LD_{50} studies, challenge inoculums were decimally increasing doses of 10^1 to 10^7 CFU for B10.T(6R) mice. The serial dilutions used for infection were plated on TBA (BD Difco, Sparks, MD) plates and colonies were counted after 2 days of incubation at 26˚C.

LD_{50} calculation

Mice were monitored daily from inoculation on day 1, until day 21. The experimental dose that resulted in death of 50% for the mice infected was determined using log dose probit analysis (SPSS version 21.0; IBM Corporation, Somers, NY)

Measurement of Bacterial Burden

Livers and spleens were harvested from young infected B10.T (6R) mice and incubated in PBS containing 0.1% collagenase D for 1 h at 37°C. The challenge inoculum for *Y. pestis* was the LD_{50} dose obtained from the mortality study. All organs

were homogenized by passage through 70 μM pore-size nylon mesh cell strainers (Falcon-Corning Inc, Corning, NY). Homogenates were centrifuged at 3,200 x *g* at for 5 min. Pellets were resuspended in PBS containing 0.1% (vol/vol) Triton X-100 to lyse eukaryotic cells. Bacterial burden was determined by plating serial dilutions on TBA (BD Difco, Sparks, MD) plates.

Cytokine and Chemokine Analysis

For cytokine and chemokine analysis, mice were bled retro-orbitally at day 1 and day 3 post-infection. Blood was pooled, collected in serum separation tubes, centrifuged at 8,000 X *g* for 10 min and stored at -80˚C until assayed. Spleens and livers from infected mice were harvested at day 1 and 3 post infection, homogenized by passage through a BD falcon 70 μm nylon mesh strainer and the cell pellet contents were washed in 1 ml of PBS. Sera, Splenic and liver cytokine and chemokine levels were measured using Bio-Plex Pro Mouse Cytokine/Chemokine 23-Plex Panel assay (Bio-Rad, Hercules, CA). IL-1β levels were measured by ELISA (Biorad, Hercules,CA).

Statistical Analysis

Data were analyzed using GraphPad Prism (version 5.0d, GraphPad Software Inc, La Jolla, CA) to calculate statistical significance. For two group comparisons, a two-tailed unpaired t-test was used. For multiple groups, one way analysis of variance (ANOVA) with Bonferroni Multiple Comparison test as a post-test, a p value of <0.05 was considered significant.

RESULTS

Resistant young B10.T(6R) mice are susceptible to *Y.pestis* C092

Young female B10.T(6R) mice were infected i.v (retro-orbitally) with increasing doses of *Y. pestis* KIM5 or *Y. pestis* CO92 Δ*pgm*. The LD_{50} of *Y .pestis* KIM5 Δ*pgm* in young B10.T(6R) mice was 14,000 CFUs (124) whereas the LD_{50} of *Y. pestis* CO92 Δ*pgm* in young B10.T(6R) mice was 17 CFUs (Figure 9A). Interestingly, the young B10.T(6R) mice that were resistant to KIM5 (124) were susceptible to the CO92 Δ*pgm*. (Figure 9A). The mean times to death were similar for all bacterial strains (4-5 days, data not shown). B10.T(6R) mice have decreased resistance to plague with increasing age. To determine whether susceptibility to CO92 Δ*pgm* changes with age, groups of middle aged B10.T(6R) mice were infected with increasing doses of KIM5 and CO92 Δ*pgm* strains of *Y.pestis*. The LD_{50} of middle-aged susceptible B10.T(6R) mice infected with *Y. pestis* CO92 Δ*pgm* was 23 CFUs comparable to the LD_{50} levels observed in middle aged B10.T (6R) mice infected with *Y. pestis* KIM5 where the LD_{50} was 64 CFUs (Fig 9B) similar to a previous report in this plague mouse model where resistance waned with age in female B10.T(6R) mice (124). Taken together, these results suggested that the difference in virulence between the KIM5 and CO92 Δ*pgm* strains in young B10.T(6R) mice is not due to age related factors of the mouse strain but due to differences between the bacterial strains.

YopJ isoforms were responsible for the difference in the outcome of infection in young B10.T (6R) mice

One of the differences between *Y. pestis* KIM5 and *Y. pestis* CO92 is a variance in YopJ. The YopJ isoform in *Y. pestis* KIM5 has amino acid differences at two sites,

F177L and K206E, compared to YopJ of *Y. pestis* C092 (264). To understand whether the different isoforms of YopJ were responsible for the difference in the outcome of infection, young female B10.T(6R) mice were infected with mutants expressing different isoforms of YopJ. Mutations that allowed production of the CO92 YopJ in the KIM5 (KIM5-YopJ^{CO92}) strain increased the virulence of *Y. pestis* KIM5 to approximately the same value as the *Y. pestis* CO92 Δ*pgm* strain (Figure 9A). The LD_{50} of the KIM5-YopJ^{CO92} strain was found to be 10 CFUs, significantly reduced from the KIM5 strain (14,000 CFUs) (Figure 9A) but similar to the virulence to that of CO92 Δ*pgm* strain (Figure 9A). On the contrary, the CO92-YopJ^{KIM5} infected B10.T (6R) mice were found to have an LD_{50} of 243 CFUs (Figure 10A), which lowered the virulence level of the CO92 Δ*pgm* strain. The combined data suggested that although CO92 YopJ expression in a less virulent strain (KIM5) leads to increased virulence but KIM5 YopJ expression in CO92 did not show a reverse effect suggesting that there might be other potential virulence factors in the CO92 strain of *Y. pestis* that might be responsible for the increased virulence seen with the CO92 strain in this study. To determine which specific amino acid polymorphisms of YopJ were responsible for the increased virulence in CO92 strain, young female B10.T (6R) mice were infected with *Y. pestis* strains expressing either the F177L (YopJ^{YPTB}) or E206K mutations on either the KIM5 or CO92 background. The LD_{50} of KIM5-YopJ^{YPTB} and KIM5-YopJ^{E206K} were found to be 3.6 X 10^4 and 2.6 CFUs respectively (Figure 10B). Similarly the LD_{50} of CO92-YopJ^{YPTB} and CO92-YopJ^{E206K} in CO92 background were found to be 2,600 and 62,000 CFUs respectively (Figure 10B). Taken together these data suggest that not a specific amino

acid polymorphism but both of the amino acid polymorphisms were responsible for the difference in virulence of the two strains.

Differences in Bacterial Burden in CO92 infected B10.T (6R) mice

Eventually, *Y. pestis* infection spreads systemically throughout the visceral organs (26). Bacterial burdens were assessed in the spleens and livers of B10.T (6R) mice infected with 14000 CFUs of *Y. pestis* KIM5, 17 CFUs of *Y. pestis* CO92 Δ*pgm,* 10 CFUs of KIM5-$YopJ^{CO92}$ and 250 CFUs of CO92-$YopJ^{KIM}$. At 3 d post-infection, KIM5 infected young B10.T (6R) mice had $9.3 \times 10^7 \pm 3.3 \times 10^7$(mean ± standard error of mean [SEM]) CFU per spleen compared to $1.9 \times 10^7 \pm 1.1 \times 10^7$(mean ± SEM) CFU per spleen of CO92 Δ*pgm* infected B10.T (6R) (Figure 10A). Similarly, at 3 d post-infection, the livers of KIM5 infected B10.T (6R) mice had an average bacterial count of $4.9 \times 10^7 \pm 6.3 \times 10^6$ (mean ± SEM) compared to $1.1 \times 10^7 \pm 7.7 \times 10^6$ (mean± SEM) that was comparatively lower than the bacterial count observed in the spleens of KIM5 or CO92 Δ*pgm* infected B10.T (6R) mice (Figure 11C and 11A). However, by 6 d post-infection both the spleen and livers of CO92 Δ*pgm* infected B10.T (6R) mice contained fewer bacteria (9.8×10^4 and 1.86×10^5 respectively) than KIM5 infected mice (2.5×10^7 and 2.83×10^6 respectively) (Figure 11B and 11D). However, the *Y. pestis* strains expressing different isoforms of YopJ (KIM5-$YopJ^{CO92}$ or CO92-$YopJ^{KIM}$) did not show a significant change in bacterial burden. These data suggest that CO92 Δ*pgm* infected young B10.T (6R) mice were able to successfully induce immune responses to *Y. pestis* and were able to clear the bacteria from visceral organs.

Analysis of cytokines and chemokines

To understand the mechanism of resistance/susceptibility, cytokine and chemokine levels were analyzed in the sera of B10.T (6R) mice infected with *Y. pestis* KIM5, *Y. pestis* CO92 Δ*pgm,* KIM5-YopJ^{CO92} and CO92-YopJ^{KIM}. Susceptible B6 mice showed higher levels of IL-6 in serum post infection with different doses of KIM5 than resistant BALB/cJ mice (229). IL-6 and RANTES levels were higher in CO92 Δ*pgm*, compared to KIM5 infected B10.T (6R) mice independent of dose (Figure 12A,B, C). KC and MCP-1 levels were elevated at only the lower dose, 100 CFUs of CO92 Δ*pgm* compared to KIM5-infected mice (Figure 12B &C). Interestingly, at 100 CFUs 1 d post infection, there was no difference in serum IL-1β levels between KIM5 and CO92 Δ*pgm* but there was a significant difference in the IL-1β levels at 3 d post infection. With the mutant strains (KIM5-YopJ^{CO92} and CO92-YopJ^{KIM}) either at 100 or 1,000 CFUs there was a statistical significant difference in the sera IL-1β levels at 1 d post infection (Figure 13). Conversely, at 1,000 CFUs there was a statistical significant difference in sera IL-1β levels between the KIM5 and C092 Δ*pgm* strains and the mutant strains (KIM5-YopJ^{CO92} and CO92-YopJ^{KIM}) at 1 d post infection. However, there was no significant difference observed in the sera IL-1β levels at 3 d post infection with the mutant strains (KIM5-YopJ^{CO92} and CO92-YopJ^{KIM}). Since *Y. pestis* infections spread systemically throughout the visceral organs (229), cytokine levels were followed to examine if a trend similar to that seen in serum occurred in spleens or livers. B10.T(6R) mice were infected with 100 or 1,000 CFUs of *Y. pestis* KIM5, *Y. pestis* CO92 Δ*pgm,* KIM5-YopJ^{CO92} and CO92-YopJ^{KIM}. At 1 d post infection, IL-1β levels were lower in the spleen of mice when infected with 100 CFUs of KIM5 strain than CO92 compared to 1000 CFUs infected

mice. There was no significant difference observed in the IL-1β levels in the liver at day 1 post infection when mice were infected with 1000 CFUs of *Y.pestis* KIM5 or CO92 but a statistically significant difference was observed in the livers between the mutant strains (Yp-YopJ^{CO92} and Yp-YopJ^{KIM}). At 100 or 1000 CFUs IL-1β levels were significantly higher in KIM5 infected mice in the spleen or liver at days 3 post infection than CO92 infected mice suggesting an immunosuppressive role of YopJ^{CO92}. This was further demonstrated when the mutant strain show a similar trend in suppressing the IL-1β levels in the spleen or liver when Yp-YopJ^{CO92} expression in the KIM5 strain lowered IL-1β production compared to the expression of Yp-YopJ^{KIM} in CO92 when B10.T (6R) mice were infected with 1000 CFUs of bacteria.

IL-1 receptor antagonist (IL-1Ra) levels in *Y. pestis* infected B10.T (6R) mice

IL-1 receptor antagonist proteins are expresses by macrophages and neutrophils to dampen the pro-inflammatory response. We sought to determine whether production of IL-1Ra by YopJ^{CO92} is able to dampen the pro-inflammatory immune response (Figure 14). As expected at day 3 there was a higher amount of IL-1Ra in the sera of CO92 infected B10.T (6R) mice than KIM5 infected B10.T (6R) mice irrespective of the dose used (100 or 1000 CFUs). At day 3 both the CO92 infected spleen and liver showed higher amount of IL-1Ra than KIM5 infected B10.T (6R) mice suggesting that YopJ of CO92 might be responsible for suppressing the immune response (lower IL-1β production).

Discussion

Y.pestis has developed innumerable ways to manipulate and evade host immune response. *Y.pestis* like other Gram-negative bacteria has lipopolysaccharide (LPS) in its

outer membrane. LPS is a ligand for Toll-like-Receptor (TLR4). Based on temperature, *Y.pestis* synthesizes two forms of LPS: tetra and hexa-acylated LPS (157). At 26ºC, bacteria grown in flea gut produce a hexa-acylated LPS, which elicits TLR4-mediated immune signaling to induce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (157). After infection of the mammalian host, the temperature shift from 26ºC to 37ºC lets *Y.pestis* synthesize tetra-acylated form of LPS that is inhibitory for TLR4 mediated immune signaling resulting in resistance to complement-mediated lysis and repression of pro-inflammatory cytokines (64, 157). During early stages of infection, the bacteria are phagocytosed by macrophages and neutrophils at the site of infection. Flow cytometry data and histological evidence suggests that *Y. pestis* are killed by neutrophils and that neutrophils can control *Y.pestis* growth up to two days post infection (140). However, in the phagolysosome of the macrophages *Y.pestis* can survive and proliferate (221). This intracellular growth is important for *Y.pestis* pathogenesis to express various virulence determinants like F1 antigen and V antigen and various effector molecules secreted by the type III secretion system (T3SS) (188).

YopJ one of the effector molecules of T3SS, is an acetyl transferase and inhibits NF-Kβ and MAPK signaling pathways. Additionally, $YopJ^{KIM}$ has higher affinity for IKKβ, triggers apoptosis, caspase-1 activation and IL-1β secretion than other isoforms of YopJ (265). The amino acid substitution present in YopJ in *Y. pestis* KIM5 at two sites, F177L and E206K, compared to the YopJ present in *Y. pestis* CO92 accounts for the various phenotypes seen in bone marrow derived macrophages (265). How these polymorphsims in YopJ affects virulence of *Y.pestis* and or host response is not known.

Previous reports demonstrated that young B10.T(6R) female mice are highly resistant to infection with *Y.pestis* KIM5 and this resistance is abolished with age (124). Studies show that B10.T(6R) mice are resistant to *Trichuris muris* infection (69). H-2^q haplotype of B10.T(6R) mice is associated with resistance to *Trichuris muris* infection(69). B10.T(6R) mice is a substrain of C57BL/10SnSg mice that differ at H-2 locus . Other inbred mice that are resistant to *Y.pestis* infection are 129 substrains or BALB/cJ mouse strain, which is also linked to H-2^b or H-2^{bc} and H-2^d respectively (230). However, whether this resistance to *Y.pestis* is related to H-2q haplotype is not known.

Using this model of resistant B10.T (6R) mice, we sought to determine the LD_{50} of B10.T(6R) mice when infected with *Y.pestis* CO92 strain. As shown in this study the LD_{50} observed in young female B10.T (6R) mice was 17 CFUs when infected with CO92 strain of *Y.pestis* while the LD_{50} observed with KIM5 strain of *Y.pestis* was 14000 CFUs. Interestingly, the young B10.T(6R) mice that were resistant to KIM5 strain of *Y. pestis* were susceptible to the CO92 strain of *Y. pestis*. A similar experiment was done with female middle-aged B10.T(6R) mice infected with KIM5 or CO92 strain of *Y. pestis*. The LD_{50} observed for CO92 was 23 CFUs comparable to 64 CFUs when female middle-aged B10.T(6R) mice were infected with *Y. pestis* KIM5. These results suggested that the difference in virulence observed between the two strains is not due to age related factors but due to a difference in the bacterial strain. We hypothesize that in our model, different isoforms of YopJ present in KIM5 and CO92 strain of *Y.pestis* is responsible for the difference in the outcome of infection. As shown in Figure 2, production of $YopJ^{CO92}$ in KIM5 strain increased virulence level of KIM5 to same value as *Y. pestis* CO92 suggesting that YopJ is responsible for the difference in virulence. However, when

YopJ^{KIM5} was expressed in CO92 strain the LD_{50} levels did not increase to same value as KIM5 strain but lowered the virulence level of CO92 strain indicating that there are other possible virulence factors in CO92 that is responsible for the increased virulence seen in *Y. pestis* CO92 strain.

The kinetics of our infection model suggests that bacterial clearance begins at spleen followed by liver at three days post infection. There was higher bacterial burden in the spleens and or liver of KIM5 infected B10.T (6R) mice compared to CO92 infected mice. The higher bacterial burden observed in KIM5 infected mice might be due to the higher amount of bacteria (LD_{50} dose of 14000 CFUs) inoculated into the mice compared to the LD_{50} doses (10,20, 250 CFUs) used for the other strains (CO92, Yp-YopJ^{CO92} and Yp-YopJ^{KIM}).

The immunological response observed in young B10.T(6R) mice when infected with KIM5 or CO92 strain of *Y.pestis* were compared by analyzing various cytokine and chemokine level. IL-6, and RANTES levels were higher in young female B10.T(6R) mice when infected with *Y. pestis* CO92 strain compared to infection by KIM5 strain irrespective of the CFUs doses used. On the contrary, IL-1β levels were higher in the sera of B10.T (6R) mice infected either with 100 or 1000 CFUs of *Y. pestis* KIM5 compared to *Y. pestis* CO92 both at days 1 and 3 post infection. IL-6 down regulate the proinflammatory response (112, 202) , strongly supports the importance of the cytokine in both neutrophil recruitment and regulation of the immune response. KIM5 infected B10.T (6R) mice exhibited lower levels of IL-6, and this in return may influence levels of proinflammatory mediators IL-1β. In humans, there is a positive correlation between serum IL-6 levels and disease outcome in septic patients (36, 41, 56). In experimental

sepsis models, blockage of IL-6 increases survival in a polymicrobial sepsis model (218). Since the host is thought to succumb to sepsis during the late stages of *Y. pestis* infection (27) and IL-6 is elevated in both clinical and experimental models, the elevated levels of IL-6 in CO92 infected B10.T(6R) mice in our model may be a result of YopJ-induced sepsis. The lower IL-6 levels observed in KIM5 infected B10.T(6R) mice may be more reflective of a controlled inflammatory response, with the anti-inflammatory role of IL-6 downregulating other inflammatory mediators like IL-1β.

Splenic and liver IL-1β levels were also significantly elevated in KIM5 infected B10.T(6R) mice than CO92 infected B10.T(6R) mice. This is in accordance to the findings observed in *in vitro* studies where $YopJ^{KIM}$ triggers caspase-1 activation and which in turn elevates the IL-1β levels whereas $YopJ^{CO92}$ suppresses IL-1β activation in bone marrow derived macrophages (264). Mutants expressing different isoforms of YopJ showed that $YopJ^{CO92}$ when expressed in KIM5 strain suppresses IL-1β levels whereas expression of $YopJ^{KIM}$ in CO92 strain elevated IL-1β levels. These findings suggest that YopJ of *Y. pestis* CO92 is able to suppress the production of these cytokines in B10.T(6R) mice. Although the IL-1β levels were higher in the later stages of infection yet there was no significant difference in the IL-1β levels at day 6 post infection (data not shown) between the KIM5 or CO92 strain. This observation can be explained that at later stages of *Y. pestis* infection, normally, it is too late for the host to clear the bacteria and the host succumbs rapidly to sepsis due to the systemic burden of the growing bacteria and overwhelming cytokine production by the host (56).

These results suggest a model whereby the recognized YopJ allele of KIM5 strain has higher apoptotic or caspase-1 activating potential (264) which in turn increases the IL-1β

levels. The phenotype observed by $YopJ^{KIM}$ might be due to F177L mutation. On the other hand, $YopJ^{CO92}$ allele evolved from *Y. pseudotuberculosis (132)* has lower cytotoxic and caspase-1 activating potential and hence produce lower IL-1β levels. This observation might be due to the E206K codon substitution present in the YopJ of CO92 strain.

Taken together we demonstrated that the YopJ of *Y. pestis* KIM5 is an important virulence factor and contributes to the susceptibility observed in our previous plague resistant B10.T (6R) mice model. Additionally, our data indicated that the YopJ of *Y. pestis* CO92 had the ability to suppress cytokine production *in vivo* and thus modulating host immune responses. Our future studies are targeted at better understanding the role of *Y.pestis* induced sepsis using this model of B10.T (6R) mice.

Figures

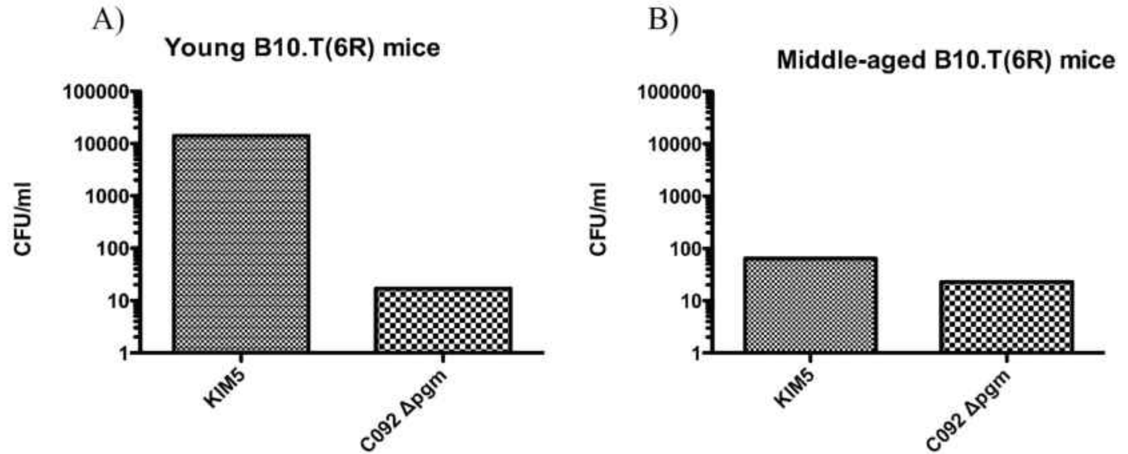


Figure 9: Difference in LD_{50} between the KIM5 and CO92 Δ*pgm* strain of *Y.pestis*.(A) Young (6-8wks) female B10.T(6R) mice were infected i.v via the retro-orbital sinus with increasing doses of *Y.pestis* KIM5 & CO92 strains . After 21 days of monitoring LD_{50} was calculated using log-dose probit analysis (at a 95% confidence level). (B) LD_{50} of middle aged female B10.T(6R) mice. Data are representative of 2 independent experiments(n=7/dose group/experiment)

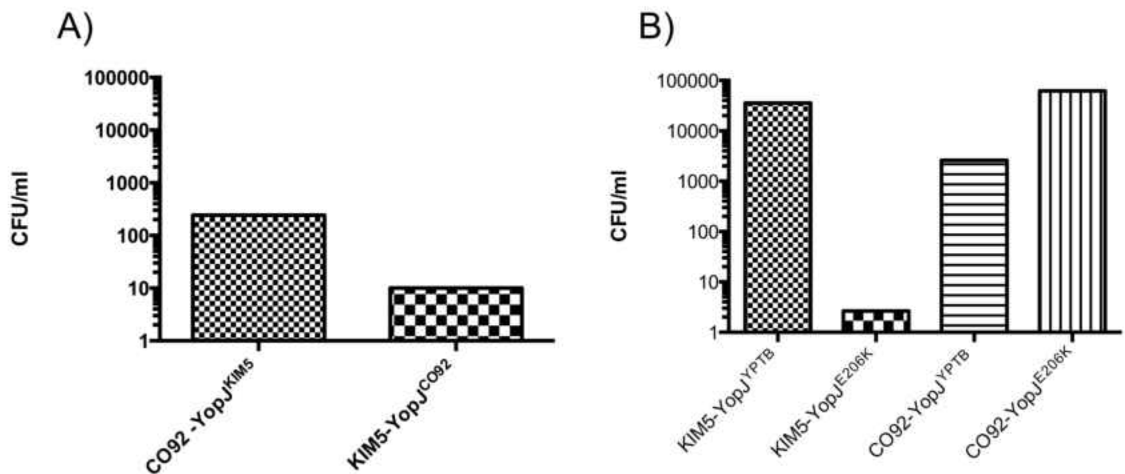


Figure. 10: Different isoforms of YopJ are responsible for the difference in the outcome of infected mice. A) Young (6-8wks) female B10.T(6R) mice were infected i.v via the retro-orbital sinus with increasing doses of *Y.pestis* KIM5-$YopJ^{CO92}$ & CO92-$YopJ^{KIM5}$. After 21 days of monitoring LD_{50} was calculated using log-dose probit analysis (at a 95% confidence level). B) Young female B10.T(6R) mice were infected i.v via the retro-orbital sinus with increasing doses of *Y.pestis* strains expressing either F177L (YPTB) or E206K mutations on either KIM5 or CO92 background (KIM5-$YopJ^{YPTB}$, KIM5-$YopJ^{E206K}$, CO92-$YopJ^{YPTB}$ and CO92-$YopJ^{E206K}$). Data are representative of 2 independent experiments(n=7/dose group/experiment)

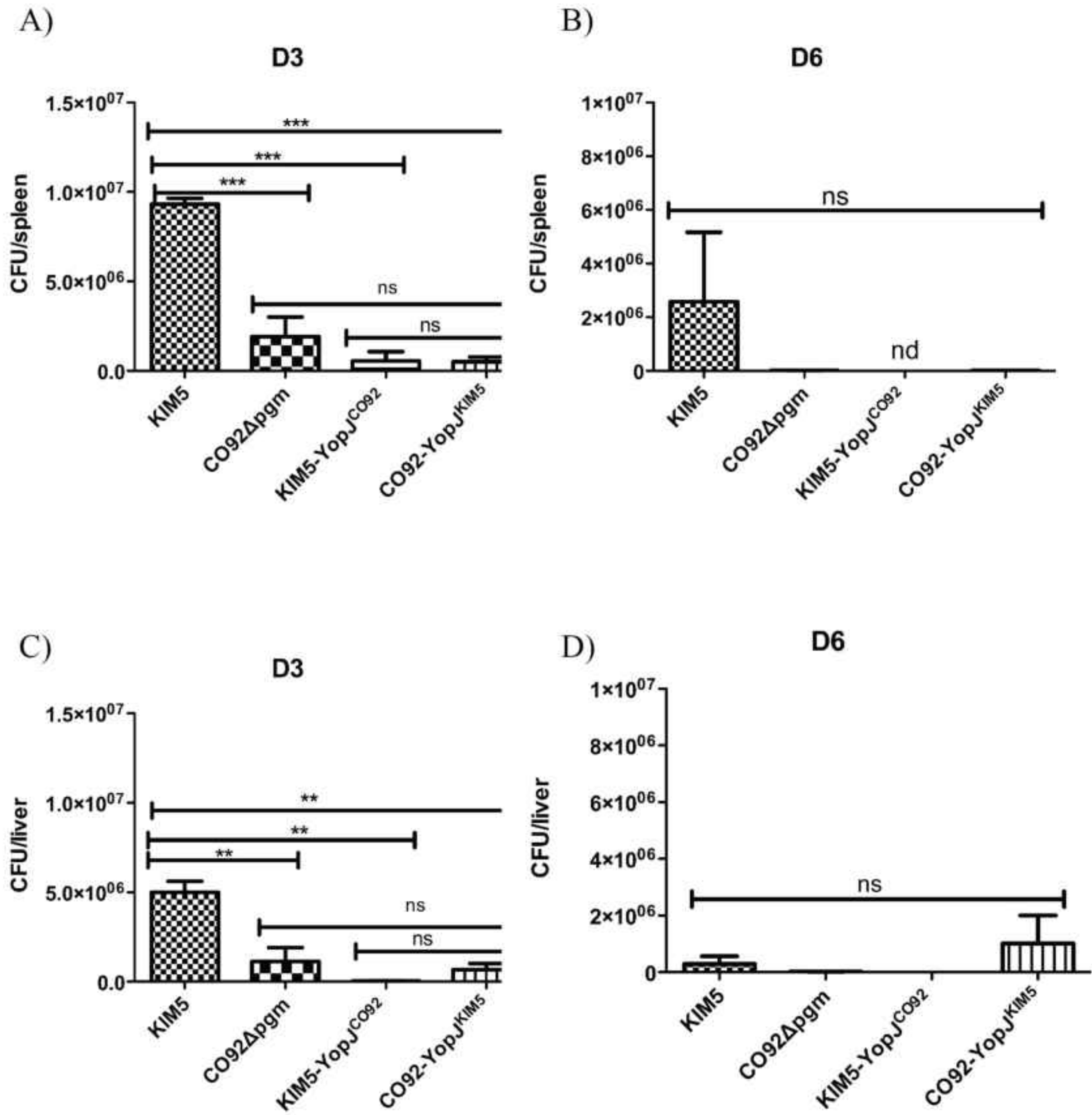


Figure 11: Bacterial burdens in the spleens and livers of infected mice B10.T(6R) mice were infected with various strains of *Y.pestis(* KIM5, CO92 Δ*pgm,* KIM5-$YopJ^{CO92}$ and CO92-$YopJ^{KIM}$) with LD_{50} dose :14000 CFUs, 17 CFUs, 250 CFUs and 10 CFUs respectively. Spleen (A&B) and Liver (C&D) were collected at d3 and d6 post infection (n=3/dose/experiment). Data is representative of 2 independent experiments; $p<0.05$ by Bonferroni's Multiple Comparisons test.

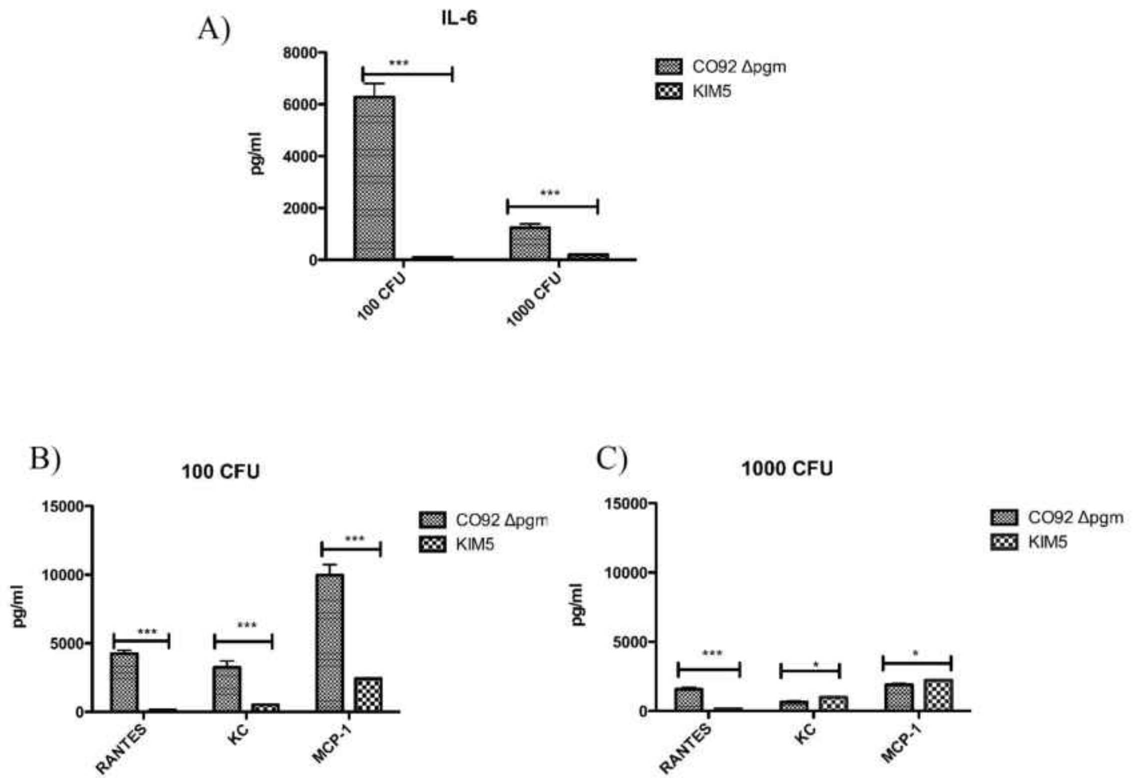


Figure 12: IL-6 and chemokine levels in KIM5 and CO92 Δ*pgm* infected B10.T(6R) mice. B10.T (6R) female mice were infected with 100 CFUs or 1000 CFUs of KIM5 and CO92 Δ*pgm* and sera was collected at day 3 post infection to measure IL-6 (A) and chemokine (B&C) respectively. Data is representative of 3 independent experiments; $p<0.05$ "*" $p<0.001$ "***" by Student's t test.

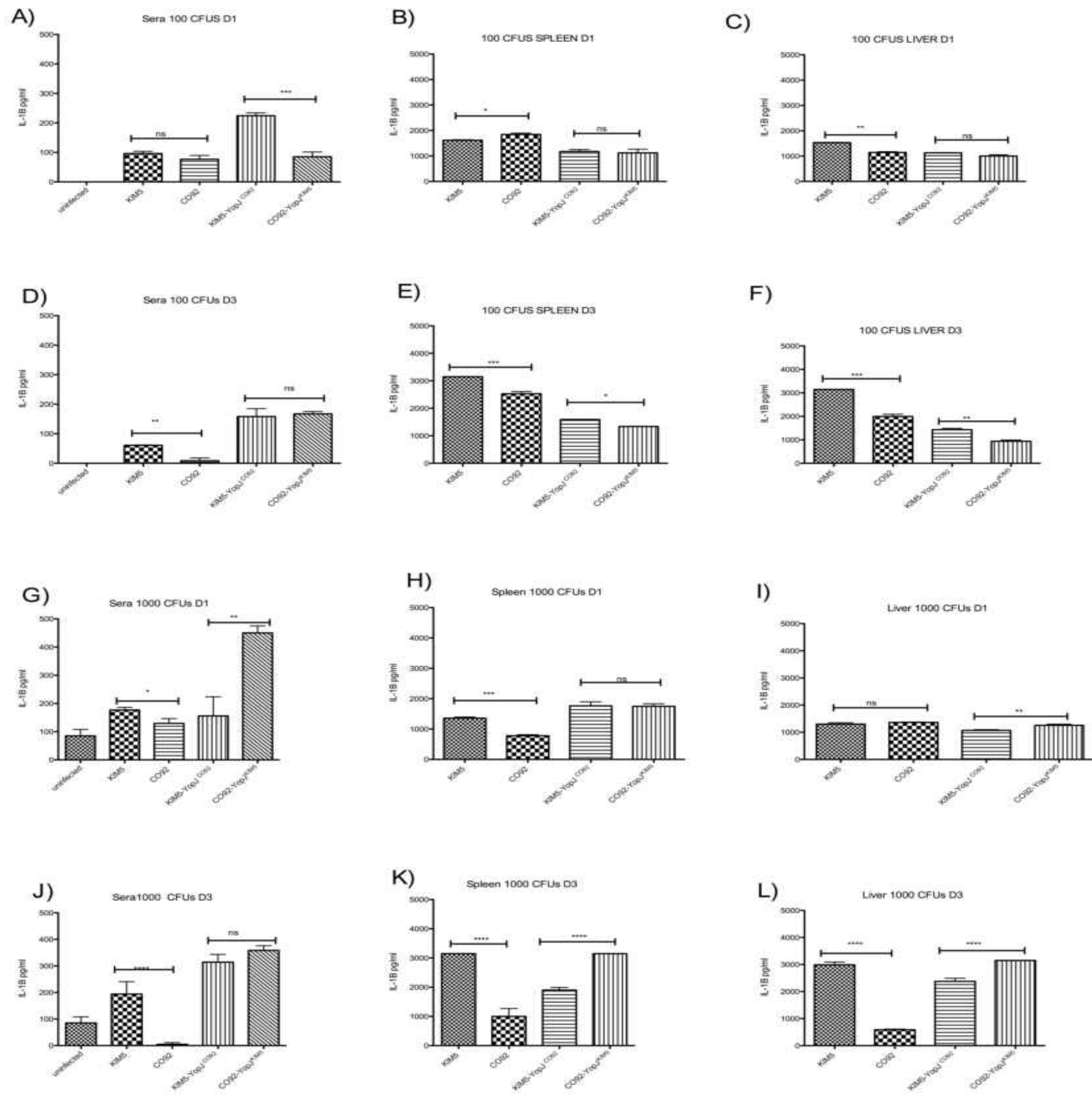


Figure 13: IL-1β levels in infected B10.T (6R) mice. B10.T(6R) mice were infected with various strains of *Y.pestis (*KIM5, CO92 Δ*pgm,* Yp-YopJ^{CO92} and Yp-YopJ^{KIM}) at 100 CFUs (D-I) and 1000 CFUs (K-P). IL-1β levels were measured in the sera, spleens and liver at days 1 and 3 post infection by ELISA. $p<0.05$ =* $p<0.001$=*** or $p<0.01$**

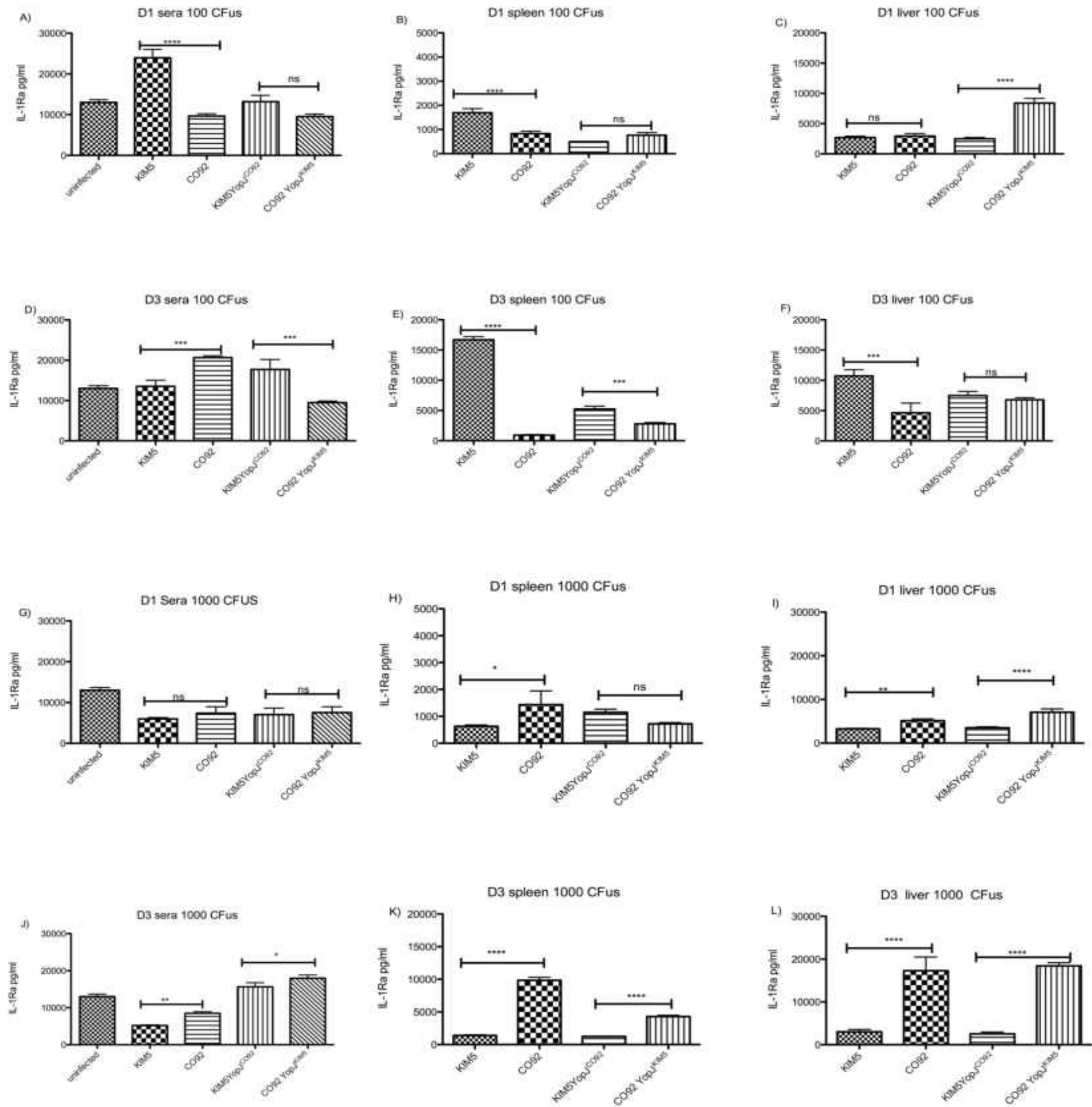


Figure 14: IL-1Ra levels in infected B10.T (6R) mice. B10.T(6R) mice were infected with various strains of *Y. pestis (*KIM5, CO92 Δ*pgm,* Yp-YopJ^{CO92} and Yp-YopJ^{KIM}) at 100 CFUs (A-F)) and 1000 CFUs (G-L). IL-1Ra levels were measured in the sera, spleens and liver at days 1 and 3 post infection by ELISA. p<0.05 =* p<0.001=*** or p<0.01**

CHAPTER IV

ROLE OF IRON IN RESPONSE TO *YERSINIA PESTIS* INFECTION

Introduction

Yersinia pestis, a Gram-negative bacterium belonging to the *Enterobacteriaceae* family is the causative agent of plague. Plague is transmitted either by bite of an infected flea or can directly be transmitted by aerosol causing bubonic and pneumonic plague respectively (180). Humans inoculated with a live attenuated *Y. pestis* strain or *Y. pestis* EV76 as a live attenuated vaccines provides protection against bubonic and pneumonic plague (91, 150) .

Classified as a category A select agent, *Y. pestis* requires special precautions because of the risk of transmission of plague. One of the major obstacles to study pathogenesis of *Y. pestis* is that many laboratories do not have access to biosafety level 3 (BSL-3). As a result, laboratories cannot work with the fully virulent strain of *Y. pestis*. Hence use of attenuated strain of *Y. pestis* that are exempt from CDC select agent list is required. Such strains lack either pCD1 or the pigmentation (*pgm*) locus (179). Upon contact with host cells, the pCD1 plasmid encodes a type III secretion system that secretes effector molecules to the cytosol of host cells that modulate the host immune response (228, 234). One of the virulence components in the 102 kb *pgm* locus is the yersiniabactin (Ybt siderophore) dependent iron transport system that is important for pathogenesis of *Y. pestis* from peripheral sites (191). Mutants that lack *pgm* or pCD1 can be used to study in

BSL-2 laboratories. However, the disease caused by the attenuated strains does not recapitulate the disease progression observed with the fully virulent strains of *Y. pestis* (158). Compared to the wild type strains, attenuated strains are unable to cause disease from natural routes of infection.

Efficient iron acquisition systems are essential for *Y. pestis* to spread systemically in the host. The Ybt system includes a series of enzymes responsible for the siderophore's biosynthesis. The iron/yersiniabactin complex is attached to the outer membrane (OM) receptor Psn and transferred into the periplasm via the TonB-dependent energy pathway. Transfer of iron into the cytoplasm is initiated when the iron/siderophore complex attaches itself to the periplasmic surface of the inner membrane-localized ATP-binding cassette (ABC) transporter YbtP/YbtQ. The YbtP/YbtO contains two permease and two ATP-binding domains. For bacterial infection to occur by subcutaneous routes, a functional Ybt transporter is required to acquire iron in early stages of the bubonic plague in mice (18, 74). Another study from a bubonic plague model suggests that the manganese- and iron-specific ABC transporter Yfe is also important for full *Y. pestis* virulence (17). Other ABC transporters for iron (Yfu and Yiu) and hemin (Hmu) are not required for virulence in the mouse model (93, 118, 225). The transporters Yfe and Feo are redundant in function in ferrous iron acquisition under microaerophilic growth conditions (182).

Various studies demonstrate the role of iron in host response against pathogens. Iron overload makes the host susceptible to pathogens as seen in infection studies with *Listeria monocytogenes* (241), *Candida albicans* (149) and in polymicrobial lung infections (172). Iron stimulates bacterial growth (178) and inhibits the antimicrobial

activity of leukocytes. (92,114). Iron overload impairs both T and phagocytic cell functions. Th1 and Th2 cell proliferation (226), expression of NO synthase (iNOS) in murine macrophage cell line, and impairment of phagocytic and neutrophil cell function (239) are also affected by iron overload. A genetic condition that is associated with iron overload is hereditary hemochromatosis. Recently, non-pigmented *Y. pestis* strain UC91309 was isolated from a researcher with fatal, septicemic plague. The patient had higher level of iron deposits in the liver and increased levels of serum ferritin, iron, and iron saturation. Sequencing of the patient's genome linked his death with the HFE C282Y mutation associated with hereditary hemochromatosis (44, 81, 190).

Y. pestis KIM5 lacks the *pgm* locus and KIM5 is attenuated when given subcutaneously to C57BL/6J mice. *Y. pestis* KIM5 , when injected intranasally into mice, there were less bacterial growth in the lungs. Mice did not develop pneumonia as they would have developed with the fully virulent strain of *Y. pestis* (129). However, strain KIM5 was able to grow in the spleen and liver of intranasally infected mice suggesting that the *pgm* locus is important for iron uptake in the lungs. Alternative studies show that with iron sulfate administration to mice followed by infection with KIM5 Δ*pgm* strain subcutaneously, mice died; these results suggest that ferrous sulfate could bypass the need for yersiniabactin, rendering KIM5 *Δpgm* fully virulent. Mice developed histological lesions in the livers and spleens resembling the lesions observed in mice when infected by the fully virulent strains of *Y. pestis* (33, 129). However, use of the same method to induce pneumonic plague by administering ferrous chloride followed by infection with KIM D27 did not show the same results as the subcutaneous model of plague (100). Moreover, a dose higher than 0.5 mg per mouse of ferrous chloride was shown to be toxic.

The toxicity of inorganic iron and the need to treat patients with iron resulted in the development of less toxic colloids comprising of carbohydrates conjugated to ferric hydroxides (57). Administration of iron dextran to mice following infection was found to be nontoxic. Previously we have shown that the LD_{50} of young B10.T (6R) mice for *Y. pestis* CO92 Δ*pgm* strain was 17 CFUs whereas the LD_{50} of young B10.T(6R) mice for *Y. pestis* KIM5 was 14,000 CFUs when infected retro-orbitally (i.v.). But when young B10.T (6R) mice were infected with 17 CFUs of *Y. pestis* CO92 *Δ pgm* strain and 14000 CFUs of KIM5 subcutaneously, all mice survived. Here, we develop a bubonic plague model in B10.T (6R) resistant mice using iron dextran following infection that will mimic the natural infection route caused by the fully virulent *Y. pestis* and also to understand the role of iron in the host immune system.

We demonstrate that resistant B10.T (6R) mice when treated with iron dextran before and after infection with various strains of *Y. pestis* subcutaneously mimics the infection route caused by the fully virulent *Y. pestis*. To our surprise, supplementing the B10.T (6R) mice with iron, followed by infection with KIM5 or CO92 s.c, lowered the mean day of death in KIM5 and CO92 infected young female resistant B10.T(6R) mice. The observed LD_{50} of B10.T(6R) mice when infected with CO92 Δ*pgm* strain and KIM5 s.c with iron is found to be 20 CFUs and <1000 CFUs respectively whereas the LD_{50} of B10.T(6R) mice when infected with CO92 Δ*pgm* and KIM5 s.c without iron is 6*10^5 CFUs and >10^5 CFUs respectively. Also, supplementing the resistant B10.T (6R) mice with iron dextran following *Y. pestis* infection, suppresses the host immune response as shown by the lower activity of neutrophils. Iron alone suppressed the ROS levels of neutrophils. KIM5 or CO92 Δ*pgm* infected neutrophils treated with iron had lower ROS levels than

KIM5 or CO92 Δ*pgm* infected neutrophils without treatment with iron. This suggests that iron might be a contributing factor in suppressing the host immune system in response to *Y. pestis*. Taken together, this mouse model provides an intriguing system to study host immune response to *Y. pestis*.

MATERIALS AND METHODS

Mice

6 to 8 weeks of age of female B10.T(6R) mice (original breeding stock a gift from Dr. Chella David, Mayo Clinic and College of Medicine, Rochester, MN) were bred and maintained in a clean laminar flow containment area within the Center of Biological Research (CBR) at University of North Dakota. Young female B10.T(6R) mice were used for mortality studies and to determine LD_{50} for various strains of *Y. pestis*. The University of North Dakota IACUC approved all animal studies.

Iron Treatment

Prior to infection with *Y. pestis*, all female young B10.T (6R) mice were injected intraperitoneally (i.p) with 4 mg of iron dextran 1 day before and every alternate day post infection.

Bacterial Challenge

Young B10.T(6R) mice were infected with various strains of *Y. pestis,* KIM5 or CO92 Δ*pgm* either subcutaneously or intravenously. All strains were grown at 26˚C with shaking overnight in Heart Infusion broth (HIB; BD Difco) followed by subculture to 0.1 A_{620}, and incubated at 26˚C with shaking to an A_{620} of 1.0. Bacteria were centrifuged at 3,220 x *g* for 5 min, washed twice in sterile phosphate buffered saline (PBS) and resuspended in PBS. Mice were monitored twice daily for survival for 21 days. The challenge inoculums were decimally increasing doses of 10^3 to 10^5 CFU for KIM5 strains and decimally increasing doses of 10^1 to 10^3 CFUs for CO92 Δ*pgm* strains. The serial dilutions used for infection were plated on TBA (BD Difco, Sparks, MD) plates and colonies were counted after 2 d of incubation at 26˚C.

Isolation Of Neutrophils

Neutrophils were collected from young B10.T(6R) mice as previously described (141). Briefly, neutrophils isolated from mouse peritoneal cavity were further separated and purified by MACS neutrophils isolation kit (Macs Miltenyi Biotec, San Dieogo, CA). For flow cytometry, cells were centrifuged for 5 min at 200 X *g* at room temperature. Cells were resuspended in PBS containing 0.1% BSA to a density of 1 X 10^6 cells/ml. The cells were fluorescently stained for 1h at 4°C in the dark with the following antibodies: FITC-conjugated anti-F4/80, PE-conjugated anti Ly-6G (eBioscience). The excess antibody was washed by adding 1 ml of PBS containing 0.1% BSA and centrifuged for 5 min at 200 X *g* at 4°C. The cell pellet was resuspended in 500 µl of PBS containing 0.1% BSA buffer. Neutrophils were sorted and identified as Ly-6G$^+$ cells whereas macrophages were identified as F4/80$^+$ cells. Flow cytometry analysis was performed on an LSR II flow cytometer (BD). The histogram overlays were constructed using FlowJo (V.7.6.4).

Measurement of Reactive Oxygen Species (ROS)

The neutrophils isolated were used to determine ROS levels. 2 X 10^5 cells /well were plated in a 96 well plate. Cells were washed three times with 1X Hank's Balanced Salt solution (HBSS) (Gibco Life technologies, Grand Island, NY) treated with 1 X 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA)/media solution and incubated for 30-60 minutes at 37°C. (Briefly, the DCFH-DA diffuses into cells and is deacetylated by cellular esterase to non fluorescent 2', 7'- Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2',7' Dichlorodihydrofluorescein (DCF) by ROS). The solution was removed and washed multiple times with 1X HBSS. The DCFH-DA loaded cells were treated with desired oxidant PMA (positive control), PBS (negative

control), iron, and bacteria (KIM5, CO92 Δ*pgm)* with and without iron in 100µl of medium. ROS levels were immediately analyzed. A kinetic analysis in increments of 10 minutes up to 2 hours was done using a fluorometric plate reader (Bio Tek) at 480nm/530 nm.

Cytokine Analysis

Iron dextran treated B10.T (6R) mice were infected with various strains of *Y. pestis* at LD_{50} dose (KIM5=14,000 CFU; CO92-YopJ^{KIM5}= 10 CFUs; CO92= 17 CFUs; KIM5-YopJ^{CO92} = 243 CFUs) Sera was collected at 1 d, 3 d, 5 d, 7 d post infection to measure IL-1β levels by ELISA.

LD_{50} calculation

Mice were monitored daily from inoculation on day 1, until day 21. The experimental dose that resulted in death of 50% for the mice infected was determined using log dose probit analysis (SPSS version 21.0; IBM Corporation, Somers, NY)

Statistical analysis

Data in the Figures are expressed as the mean ± SEM. For mortality studies, statistical comparisons were carried out using Log-rank (Mantel-Cox) test in GraphPad Prism (version 5.0d, GraphPad Software Inc, La Jolla, CA). For two group comparisons, a two-tailed unpaired t-test was used. For multiple groups, one way analysis of variance (ANOVA) with Bonferroni Multiple Comparison test as a post-test, a p value of <0.05 was considered significant.

Results

Rapid increase in infection when mice were treated with iron dextran

To determine whether the outcome of infection would be similar in mice infected via peripheral routes that mimic the natural route of plague infection, B10.T (6R) mice were infected with *Y. pestis* KIM5 or CO92 Δ*pgm*. All bacterial strains are conditionally virulent strains of *Y. pestis* due to the lack of the pigmentation locus (Δ*pgm*) (179). A major component contributing to virulence in the *pgm* locus is the yersiniabactin-dependent iron transport system (181) that is important for infection via peripheral routes. These conditionally virulent strains of *Y. pestis* are not capable of inducing disease via peripheral routes of infection as observed with pgm^+ strains of *Y. pestis* (237). However, they are fully virulent when administered intravenously. When infected intravenously (i.v.) the LD_{50} for *Y. pestis* CO92 Δ*pgm* and *Y. pestis* KIM5, in young female B10.T(6R) mice was 17 CFUs and 14,000 CFUs, respectively. We show here that when young female B10.T (6R) mice were infected subcutaneously (s.c.) with *Y. pestis* KIM5 or CO92 Δ*pgm* all mice survived. The LD_{50} of young female B10.T (6R) mice infected s.c. with CO92 Δ*pgm* or KIM5 was shown to be 6 X 10^5 CFUs and $>10^5$ CFUs, respectively. However, supplementing young female B10.T (6R) mice with iron, prior to s.c. infection, resulted in LD_{50}'s of 20 CFUs for the CO92 Δ*pgm* strain and <1000 for the KIM5 strain, and the mean day of death was lowered (3-4 days) with both KIM5 and CO92 Δ*pgm* strains (Fig 15). We then examined if iron supplementation would alter the resistance observed to KIM5 infection in young female B10.T (6R) mice when infected i.v. The LD_{50} of KIM5 in young female B10.T (6R) mice when infected i.v following iron supplementation was 140 CFUs (Fig 16), 2 fold lower than the LD_{50} observed without the

addition of iron, suggesting that iron manipulation might alter the immune response of the resistant B10.T(6R) mice making the resistant mice more prone to infection.

Analysis of cytokines

IL-1β levels were analyzed in the sera of B10.T (6R) mice infected with *Y. pestis* KIM5 or CO92 Δ*pgm* (Figure 17). At day 1, there was a statistically significant difference in the sera IL-1β levels between KIM5 and CO92 Δ*pgm* infected B10.T(6R) mice. Iron dextran treated CO92 Δ*pgm* infected B10.T(6R) mice showed a lower IL-1β level production than iron dextran treated KIM5 infected B10.T(6R) mice. Conversely, at day 3, 5 and 7 there was no statistically significant difference between iron treated KIM5 or CO92 Δ*pgm* infected B10.T(6R) mice, however there was a lower production of IL-1β levels when B10.T(6R) mice were infected with CO92 Δ*pgm* suggesting an immunosupressive role of iron.

Suppression of neutrophil ROS levels by iron

Various reports indicate the role of iron in suppression of the immune response (246). Iron has been shown to regulate nitric oxide synthase activity in neutrophils. Here we show that neutrophils isolated (Figure 18) and treated with iron alone is suppressing the ROS levels. Phorbol Myristate Acetate or PMA activates neutrophils but addition of iron to these activated neutrophils show a lower production of ROS levels. KIM5 or CO92 Δ*pgm* infected neutrophils treated with iron had lower production of reactive oxygen species (ROS) levels than KIM5 or CO92 Δ*pgm* infected neutrophils without iron treatment with iron (Figure 19). This suggests that iron might be a contributing factor in suppressing the host immune system in response to *Y. pestis.*

Discussion

A major problem to identify and characterize new immunogens is the need to undertake experiments with wild type *Y. pestis*. However, to work with the fully virulent *Y. pestis* a BSL-3 facility is required. Use of attenuated strains is more common due to the availability of BSL-2 facilities in most laboratories. Use of KIM5 or CO92 Δ*pgm* strains of *Y. pestis* that lack the pigmentation (*pgm*) locus is more usual. The *pgm* locus encodes the biosynthetic proteins for siderophore yersiniabactin that scavenges iron from the mammalian system and is important for progression of disease via peripheral routes of infection (180). Mice infected with *Y. pestis pgm* strains via peripheral routes (intradermal or subcutaneous) are unable to cause disease. Hence disease progression induced by *Y. pestis pgm* strains does not recapitulate the disease that observed with the fully virulent strain of *Y. pestis* via natural routes of infection (75), (158), (129). As shown by Galvan et.al, infection of mice was unsuccessful after administering KIM5 by the intranasal route; the bacteria proliferated poorly in the lungs and showed no significant histopathology (87). Numerous studies indicated that iron injections given to mice could suppress *pgm* attenuation in *Y. pestis* (33). Treatment of mice with ferric chloride and iron sulfate following intranasal infection with KIM5 was able to induce pneumonic plague. Proliferation of bacteria in the lungs and survival curves showed that the spread of infection was similar to that observed with wild type virulent *Y. pestis* (5),(125),(129). Interestingly, we have shown a similar trend where treating the B10.T (6R) with iron dextran following subcutaneous infection with the KIM5 or CO92 Δ*pgm* strain showed a lower survival rate than non iron-dextran treated mice (Figure 15). Subcutaneous infection with CO92 Δ*pgm* or KIM5 was shown to have an LD_{50} of 6 X

10^5 CFUs and >10^5 CFUs, respectively in B10.T (6R) mice. Prior to subcutaneous infection, supplementing young female B10.T (6R) mice with iron, resulted in LD_{50}'s of 20 CFUs for the CO92 Δ*pgm* strain and <1000 for the KIM5 strain, and the mean day of death was lowered (3-4 days) with both KIM5 and CO92 Δ*pgm* strains. We then studied if iron supplementation would alter the resistance observed to KIM5 infection in young female B10.T (6R) mice when infected intravenously (i.v). Following iron supplementation, intravenous infection of young female B10.T (6R) mice with *Y. pestis* KIM5 lowered the LD_{50} to 140 CFUs (Figure 16), 2 fold lower than the LD_{50} observed without the addition of iron, suggesting that iron manipulation might alter the immune response of the resistant B10.T (6R) mice making the resistant mice more prone to infection. A potential caveat for iron-treated mouse models is that iron can impact the host's innate immune responses, where virulent bacteria can multiply better in iron-rich phagocytes (88, 154, 242). Also, iron administration renders mice more susceptible to human pathogens, a method that has been used to develop a variety of infectious-disease models (217, 243, 258).

Bacterial pathogens prevent the early recruitment of neutrophils to infected organs during infection. This provides an obvious advantage because neutrophils with their various antimicrobial capabilities would otherwise phagocytize the pathogen and undergo apoptosis. *Y. pestis* manipulates a variety of mechanisms in the host immune response to evade and survive the early stages of infection. Evidence based on studies in various animal models of pneumonic or bubonic plague indicates that following airway or intradermal infection with *Y. pestis,* there is a delay in the early stimulation of a pro-inflammatory immune response as well as the recruitment of neutrophils to the site of

infection (5, 30, 125) .

The TTSS is one of the factors that contribute to virulence in pathogenic *Yersinia* strains. The effector proteins secreted by the TTSS are used by the pathogen to subvert early innate immune responses (52, 234). In addition to the pCD1 plasmid encoded virulence factors, the chromosomal 102-kb *pgm* locus is also important for virulence of *Y. pestis* in mammals and transmission of *Y. pestis* via blocked fleas. Efficient iron acquisition systems are required for *Y. pestis* to spread and grow in mammalian hosts. The Pgm^+ phenotype of *Y. pestis* shows various traits comprising of adsorption of large amounts of hemin at 26°C, mediated by expression of several iron regulated proteins, ability to grow in iron chelated media ta 37°C, important for virulence in mice infected by peripheral routes of injection and sensitivity to bacteriocin pesticin. Non- pigmented (Pgm^-) *Y. pestis* lack all the above-mentioned traits. As iron is important for growth of *Y. pestis* in mammals, iron overload is equally harmful for the host. Various studies shows that iron overload in certain tissues was proposed to play a role in pathogenesis of HIV and its opportunistic infection (23). Other studies show that iron overload may predispose a patient to *Candida albicans* infections (149). The hereditary condition 'hemochromatosis', which is associated with excessive build-up of iron makes people more vulnerable to the plague (190). In neutropenia, availability of decreased lactoferrin is associated with impaired iron-withholding defense system, hence increased risk of infection. Transferrin (39, 68, 72, 196), a serum protein that binds iron with high affinity, restricts free iron availability to levels insufficient for microbial growth (241). Various studies indicate a link between iron metabolism and cell-mediated immunity (245). Both iron deficiency and iron overload can exert subtle effects on the immune status by

altering the T and B cell proliferation (226, 245). Additionally, iron plays an important role in the antimicrobial activities of neutrophils and macrophages (231, 232), regulating expression of iNOS gene (246).

The results of the present study show that treatment of neutrophils with iron dextran (10 ng/ml) *in vitro* suppresses the ROS levels. When neutrophils were infected with KIM5 or CO92 Δ*pgm* there was a higher production of ROS level as compared to neutrophils that were treated with iron dextran following infection with either *Y. pestis* KIM5 or CO92 Δ*pgm*. This result suggests that suppression of ROS levels might be attributed to iron overload. Since neutrophils are the first cell type to respond to the site of infection and also aids to clear the pathogen, this function of neutrophils might be responsible why we are seeing a lower mean day death (3-4 days) and lower LD_{50} when *Y. pestis* infected mice are supplemented with iron. Supplementing the mice with iron suppresses the immune response by lowering the recruitment of neutrophils as well ROS production by neutrophils. As a result neutrophils are unable to clear the bacteria that resulted in lower LD_{50}.

In conclusion, the present study shows that disturbances of iron metabolism may profoundly affect ROS production by neutrophils, thus altering the course and outcome of infection. An alternative experiment with iron chelation might be responsible for canceling the iron induced immune suppression thus offering the potential for new strategies of immune intervention in plague.

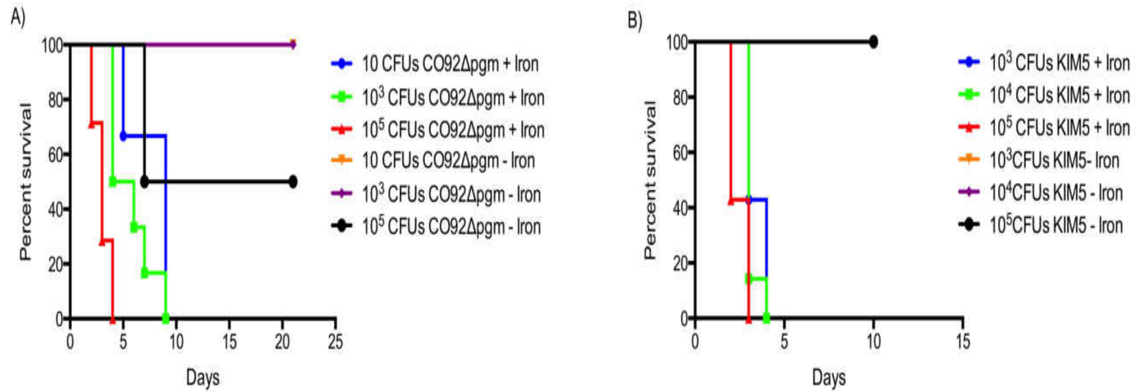


Figure 15: Survival curve of B10.T (6R) mice infected with KIM5 and CO92 Δ*pgm* strains subcutaneously (A&B). Each group of mice underwent iron supplementation by receiving iron dextran (4mg/ml) i.p before infection and every alternate days post infection. Mice were challenged with indicated doses of KIM5 and CO92 Δ*pgm* strains. and monitored twice daily for 21 days. Data is representative of two independent experiments (n=7/dose group/experiment).

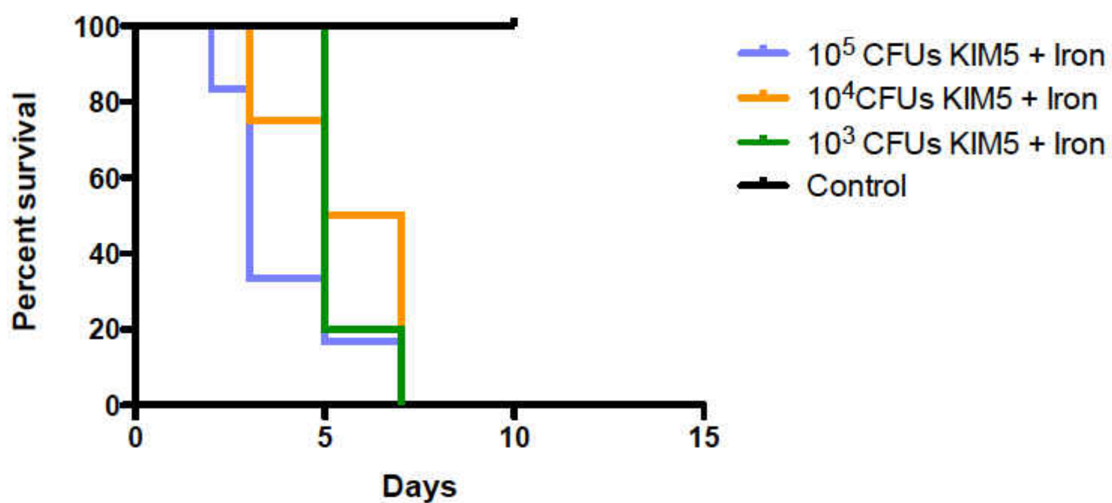


Figure 16: Survival curve of B10.T(6R) mice infected with KIM5 strain intravenously. Each group of mice underwent iron supplementation by receiving iron dextran (4mg/ml) i.p before infection and every alternate days post infection. Mice were challenged with indicated doses of KIM5 and CO92 Δ*pgm* strains. Survival curves are assessed by monitoring the mice twice daily for 15 days. Data is representative of two independent experiments (n=7/dose group/experiment).

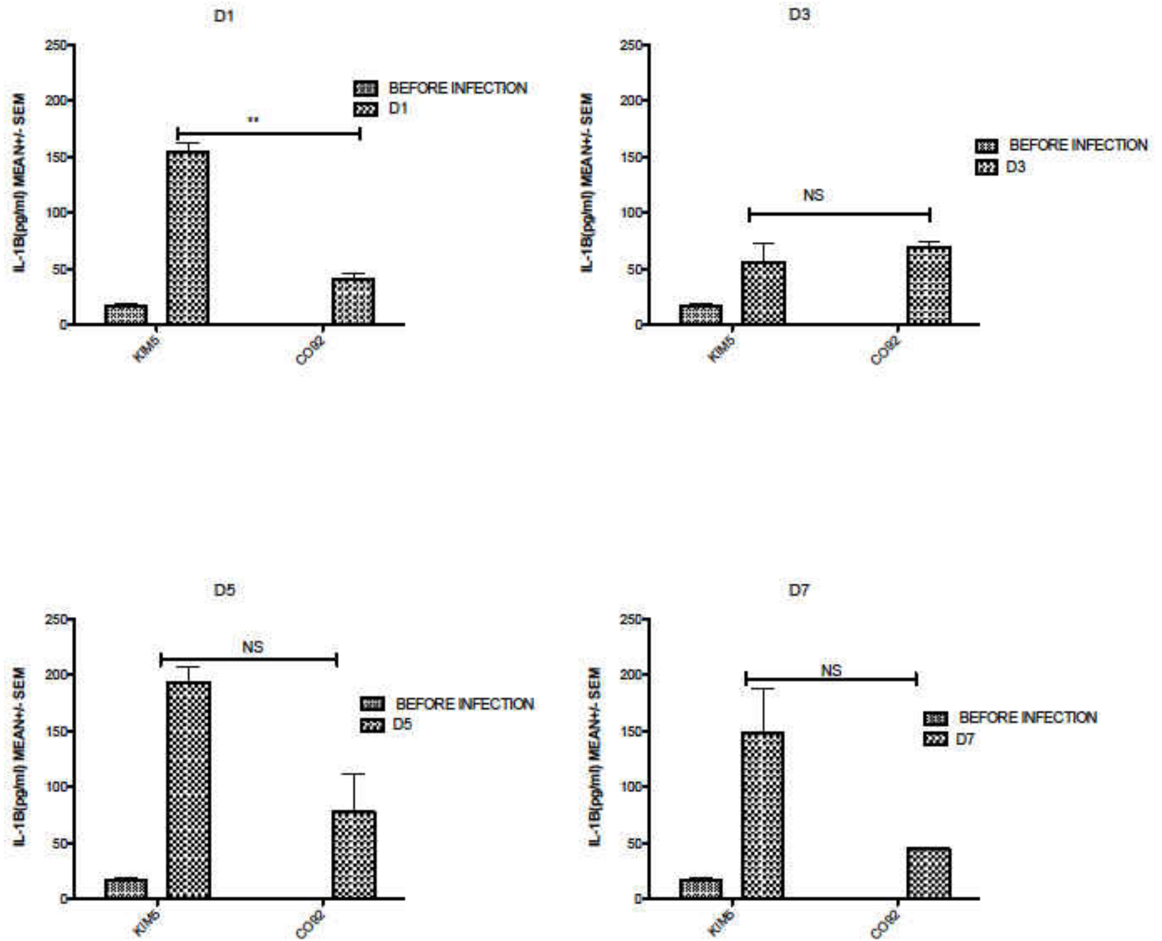
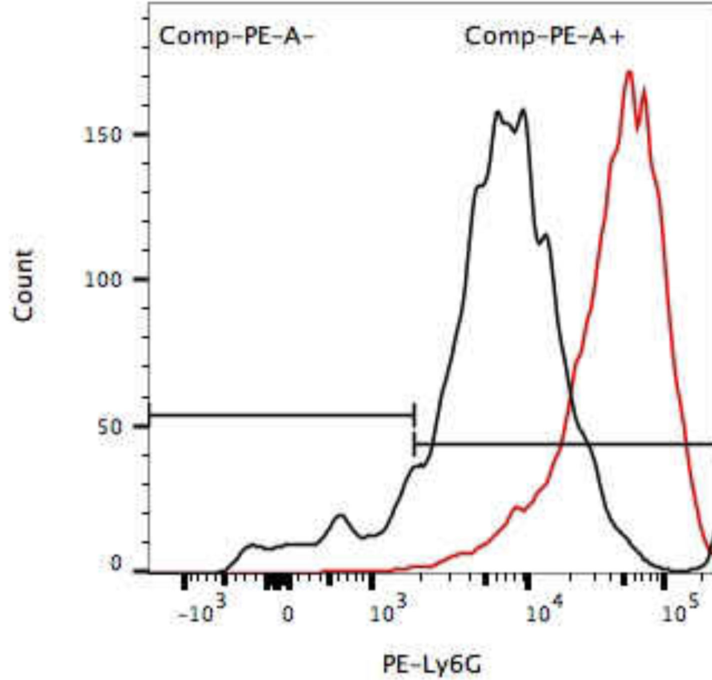


Fig 17: Iron dextran treated B10.T(6R) mice were infected with various strains of *Y. pestis* at LD_{50} dose (KIM5=14,000 CFU; CO92 = 17 CFUs). Every alternate day post infection, sera was collected to measure IL-1β by ELISA. $p<0.05$ =* $p<0.001$=*** or **



	Subset Name	Count	Median : Comp-PE-A	Comp-PE-A+ :: Freq. of Parent	Freq. of Parent
☐	Unstimulated Cells	2112	6460	n/a	51.2
☐	Stimulated Cells	7977	48725	99.0	79.8

Fig 18: Neutrophils were sorted and identified as Ly6G+ cells: Neutrophils isolated from female B10.T (6R) mouse peritoneal cavity and sorted by flow cytometry. Black open square: Unstimulated peritoneal cells from B10.T (6R) mice. Red open square: 3% thioglycollate stimulated peritoneal cells from B10.T (6R) mice. Y-axis: neutrophil cell count.

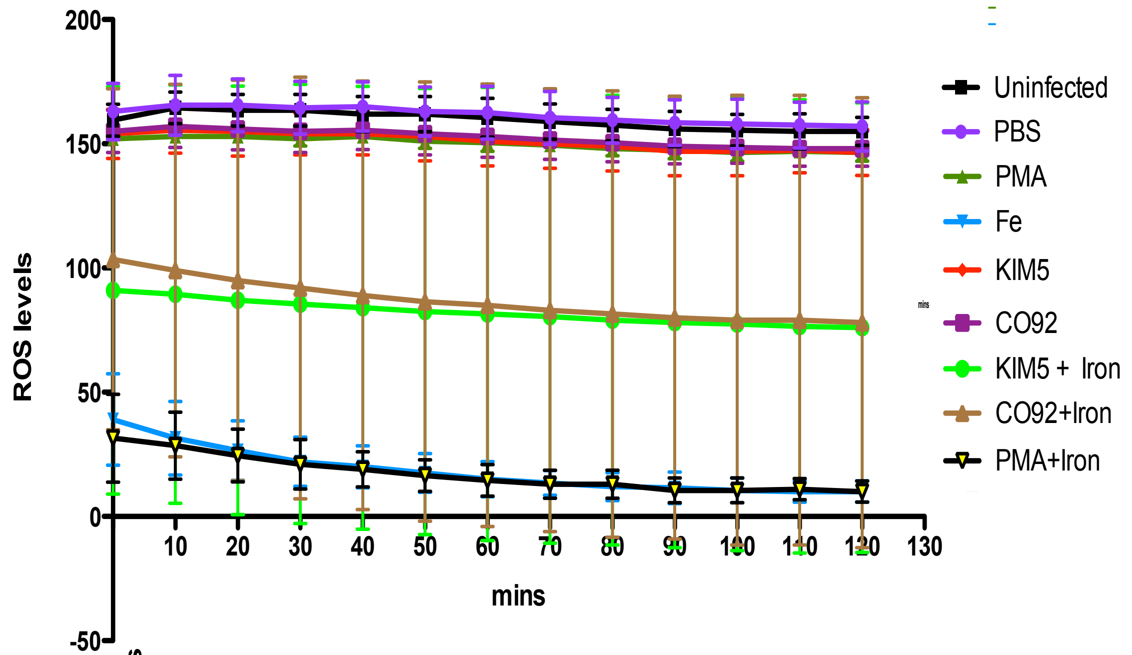


Fig 19: Iron dextran treated neutrophils isolated from B10.T (6R) mice showed lower ROS activity. Neutrophils were infected with *Y. pestis* KIM5 or CO92 at MOI of 10. ROS activity was measured using a fluorometric plate reader (Bio Tek) at 480nm/530 nm.

CHAPTER V

HUMORAL IMMUNE RESPONSE TO *YERSINIA PESTIS*

Introduction

Yersinia pestis, the etiological agent of plague possess virulence factors encoded on the chromosome and three plasmids; pCD1, pMT1 and pPCP1. The pMT1 and pPCP1 plasmid are unique to *Y. pestis* whereas the pCD1 plasmid is present in all three pathogenic strains of *Yersinia.* The pCD1 plasmid encodes V antigen (LcrV) and a group of conserved *Yersinia* outer proteins (Yops), which is essential for virulence in *Y. pestis.* Yops and V antigen are expressed maximally by *Y. pestis* at 37°C either *in vitro* conditions under low calcium conditions or when in contact with mammalian cells. The effector molecules Yops secreted by the T3SS are secreted into the cytosol of eukaryotic host cells, which then subverts the innate immune response. YopH, a tyrosine phosphatase, disrupts the link between focal adhesions and actin cytoskeleton; YopT, a cysteine protease; YopM, inhibits cysteine protease caspase-1 and helps in depletion of natural killer (NK) cells; YopE indirectly depolymerizes actin microfilaments; and YopB, YopD, YopK and YopN helps in controlling translocation of Yops to the cytosol of eukaryotic host cell. LcrV is involved in resistance to phagocytosis. The pMT1 is a 100 kb plasmid that encodes the murine toxin and the F1 capsular antigen. The pPCP1 is a 10 kb plasmid that encodes the bacteriocin, pesticin, and a plasminogen activator protease

(Pla) necessary for virulence from subcutaneous route of infection. Other virulence factors are the chromosomally encoded pH 6 antigen and lipopolysaccharide (LPS).

Diagnosis of human plague infections mostly involves immune responses to F1 antigen. Animal infection studies have identified several antigens: F1 antigen and LcrV protein. Animals actively or passively immunized with F1 or LcrV antigen has shown to be protective against pneumonic plague (7). Whole cell vaccines such as Cutter USP and EV76 were used to vaccinate humans. Due to safety concerns, both of these vaccinations have been discontinued (227). After live attenuated and whole cell vaccines were found to have significant limitations, a combinatorial subunit vaccine was developed that includes F1 and V antigens of *Y. pestis* (227). The immunogenic properties conferred by the F1/V subunit vaccine and production of IgG1 antibodies was demonstrated by human phase I trials. These studies correlate with resistance against *Y. pestis* infection (252, 253). There are potential problems associated with F1/V subunit vaccine due to the occurrence of naturally occurring $F1^-$ strains, which are fully virulent in human infection. The F1 protein portion of the vaccine would not be able to provide any defense against an $F1^-$ strain of *Y. pestis*. Another problem of the subunit vaccine is the immunosuppressive property of the V antigen, as V antigen induces IL-10 production, which suppresses the inflammatory immune response to *Y. pestis* (59). The needle structure of T3SS YscF provide partial protection against *Y. pestis* infection and activates a robust IgG1 suggesting the inclusion of YscF into F1/V subunit vaccine.

Previously in our lab we have shown that young B10.T (6R) mice were resistant to *Y. pestis* KIM5 and this resistance decreases with age (124). The LD_{50} observed in young female B10.T (6R) mice was 14,000 CFUs whereas the LD_{50} observed in susceptible

female C57BL/6J mice were 20-50 CFUs. Here we evaluate whether the presence of *Yersinia* antibodies that are present in resistant B10.T (6R) mice but not susceptible C57BL/6J mice accounts for the survivability of the resistant mice post reinfection. Young B10.T(6R) mice and C57BL/6J mice were infected with the LD_{50} dose of *Y. pestis* KIM5 (14,000 CFUs for B10.T(6R) and 40 CFUs for C57BL/6J). Subsequently, after 6 months, mice that survived the LD_{50} dose of *Y. pestis* were reinfected and sera was collected and used to evaluate the humoral immune response. We observed that antibodies to YopH, YopM, YopB and F1 were found in the resistant mice compared to the susceptible mice.

Materials And Methods

Mice

All mice were housed and bred in Center for Biological Research (CBR), at the University of North Dakota. Inbred B10.T (6R) mice were a gift from Dr. Chella David, Mayo Clinic and College of Medicine, Rochester, MN. Female B10.T (6R) Female C57BL/6 mice were bred at CBR at University of North Dakota. Mice were between 6-8 weeks of age when challenged with *Y. pestis* for the studies discussed here.

Bacterial strains utilized

All of the bacterial strains (Table 3) were grown in TMH overnight at 26°C, subcultured into fresh TMH medium to an A_{620} of 0.1 and grown until the A_{620} reached 0.2. The temperature was shifted to 37°C and incubated for 6 h while taking A_{620} reading at 1 h intervals. 1 ml of sample was taken into 1.5 ml micro-centrifuge tubes, centrifuged at 20,000 X *g* for 5 min at 4°C, pellets contained the secreted proteins. The pellet was precipitated with 10% (w/v) trichloro acetic acid (TCA) and proteins were separated followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

Table 3: *Y. pestis* strains Utilized

Bacterial Strains	Source
Y. pestis	
KIM8, pCD1$^-$, pla$^-$	Lab stock
KIM8-3002.1, pCD1 Δ*yopB* [8-388](Lcr$^+$) pMT1 pPCP1$^-$	(213)
KIM8-3002.8, pCD1 Δ*lcrGV2* [LcrGΔ6-95] [LcrVΔ1-268]	(76)
KIM8-3002 Δ*caf1*	Lab stock
KIM8-3002 *yopK*::res	Lab stock
KIM8-3002*yopH*::res	Lab stock
KIM8-3002*yopE*::res	Lab stock
KIM8-3002*yopJ*::res	Lab stock
KIM8-3002*yopT*::res	Lab stock
KIM8-3002*yopM*:: res	Lab stock
KIM8-3002 *ypkA*:: res::kan::res	Lab stock

Bacterial Challenge

Young B10.T (6R) and C57BL/6 mice were infected with *Y. pestis* KIM5 intravenously. All strains were grown at 26˚C with shaking overnight in Heart Infusion broth (HIB; BD Difco) followed by subculture to 0.1 A_{620}, and incubated at 26˚C with shaking to an A_{620} of 1.0. Bacteria were centrifuged at 3,220 X *g* for 5 min, washed twice in sterile phosphate buffered saline (PBS) and resuspended in PBS. Mice were monitored twice daily for survival for 21 days. The challenge inoculums were LD_{50} doses: 14000 CFUs for B10.T(6R) mice and 40 CFUs for C57BL/6 mice. The serial dilutions used for infection were plated on TBA (BD Difco, Sparks, MD) plates and colonies were counted after 2 d of incubation at 26˚C.

Mice that survived the first dose of infection were re-challenged after 6 months with lethal doses of *Y. pestis* KIM5 14000 CFUs for B10.T(6R) mice and 2,000 (100 LD_{50} for C57BL/6J) CFUs for C57BL/6 mice. Sera were collected at d1, d3, d5, and d7 for anti-Yersinia antibodies.

Western Blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 12.5% (wt/vol) polyacrylamide gels. Samples were boiled for 10 min prior to loading the gels. The proteins were transferred to Immobolin-P membrane (Millipore Corp, Bellercia,MA) utilizing carbonate buffer (pH-9.9). Primary antibody (sera from resistant and susceptible mice) was added to a dilution of 1:1000. The membranes were washed and secondary antibody alkaline phosphatase conjugated anti mouse IgG was used to observe proteins by development with nitroblue tetrazolium and 5- bromo-4-chloro-3-indolyphosphate (NBT-BCIP, Fisher Scientific, Fair Lawn, NJ)

Results

To identify some *Y. pestis* virulence determinants, that are expressed and immunogenic during bubonic plague in resistant mice, young B10.T(6R) or C57BL/6J mice were infected with *Y. pestis* KIM5 at a dose of 14,000 CFUs or 40 CFUs respectively. Mice that survived the LD_{50} dose of *Y. pestis* were re-infected with lethal doses of *Y. pestis* KIM5. Sera were collected at day 1 and day 5 post reinfection. Convalescent sera from B10.T(6R) mice showed higher production of antibodies to YopB, YopH, YopJ, YopM, and F1 compared to convalescent sera from C57BL/6J mice. The naïve B10.T(6R) or C57BL/6J mice did not show any change in difference in the intensity of bands following infection with *Y. pestis* KIM5. This result suggests that the convalescent sera of resistant B10.T (6R) mice contain anti-Yersinia antibodies, which might be important for the survival of resistant B10.T (6R) mice following reinfection as they retain the antibodies.

Discussion

Previously it was shown that convalescent plague serum recognizes Yops. After plague infection with cell extracts from either *Y. pseudotuberculosis* or *Y. enterocolitica* (146) as the antigen source, human and mouse serum contained antibodies to Lcr plasmid-specific proteins of ~36 and ~24 kDa. Another study showed that using *Y. pseudotuberculosis* extracts as the antigen source, *Y. pestis* infected guinea pigs had an immune response to F1 and to plasmid-specific antigens with molecular masses of 76, 41, and 34 kDa, (180). Moreover, human convalescent serum from plague patients contains antibodies to YopM and V antigen (171). Most of the human cases sera were obtained from human cases of bubonic plague resulting from flea-bite, while our studies used mice that recovered from intravenously (lethal doses) challenged *Y. pestis* infection. *Y. enterocolitica* infected rats

make antibodies to V antigen, YopM, and YopH, and also to YopD and YopE, while antibody to V antigen and YopH was observed in *Y. enterocolitica* infected rabbits (73). Since most of the antigens analyzed are required for full virulence of the organism by the subcutaneous or systemic route of inoculation, we think the results obtained here will be similar to those seen after infection from natural routes.

In our study, the greatest magnitude in antibody responses were seen in resistant B10.T (6R) mice compared to C57BL/6J mice. In addition to F1, the antigens that induced significant antibody responses are V antigen, YpkA, YopB, YopJ, and YopM, all encoded on the Lcr plasmid, which is in accordance with various studies (73, 147, 171). V antigen is a protective antigen as shown in both active and passive immunization studies. Suppression of pro inflammatory cytokines TNF-α and IFN-ϒ by V antigen also supports the idea that V antigen is secreted in vivo (27). YpkA, YopJ and YopM are critical for full virulence of *Yersinia pestis.* (54, 117, 171, 183). These effector proteins are translocated to the host target cell cytoplasm in *in vitro* models. However, YopM also functions extra-cellularly. Therefore, it is noteworthy that an immune response was generated to YopM. Also antibodies to YopB were generated, and it is known that YopB along with YopD, YopN, and YopK, is involved in the translocation of other Yops in *Yersinia* species (53, 228) to the host cytosol. These studies suggest that YopB is expressed in vivo, which induces an immune response generating antibodies in vivo. A lower magnitude in antibody response to YopH observed in our study (both in the sera of resistant B10.T(6R) and susceptible C57BL/6J mice), compared to the findings in rabbits (73), may be due to the differences in the route of infection, as we used an intravenous challenge model whereas studies performed with rabbits used an orogastric route of

infection and with a different species of *Yersinia* (73). Another reason may be that some antigens are differentially expressed (146).

The antigens that elicited minimal or no antibody responses post re-challenge may be due to a number of reasons. Firstly, proteins might be expressed in vivo but at sub immunogenic amount thus not inducing an immune response. Another possibility might be antigen-presenting cells are not available to the proteins, either because the proteins are located intra-cellularly or they are translocated directly by intimate cell contact from the bacteria to the host cytoplasm, hence no humoral immune response is generated. Lastly, the observed lack of antibody response to some antigens may be due to the assay method. An immune response may have been generated but it was below the limit of detection, hence could not be identified by immunoblot analysis. Additionally, the antigenic preparations used in this study were not in their native conformation hence we would not detect antibodies to conformational epitopes of the native protein.

Our results also suggest that resistant B10.T (6R) mice had a higher magnitude antibody response than C57BL/6J susceptible mice, which might attribute to the survival of B10.T (6R) mice post reinfection. This study suggests that humoral immune response to plague is an integral factor contributing to the resistance mechanism observed in B10.T (6R) mice. Analyzing IgG antibody response to the antigens used in this study both in acute and convalescent sera from plague infected mice and comparing that with *Yersinia* infected human cell lines would give an insight into understanding the mechanism of humoral immune responses to plague.

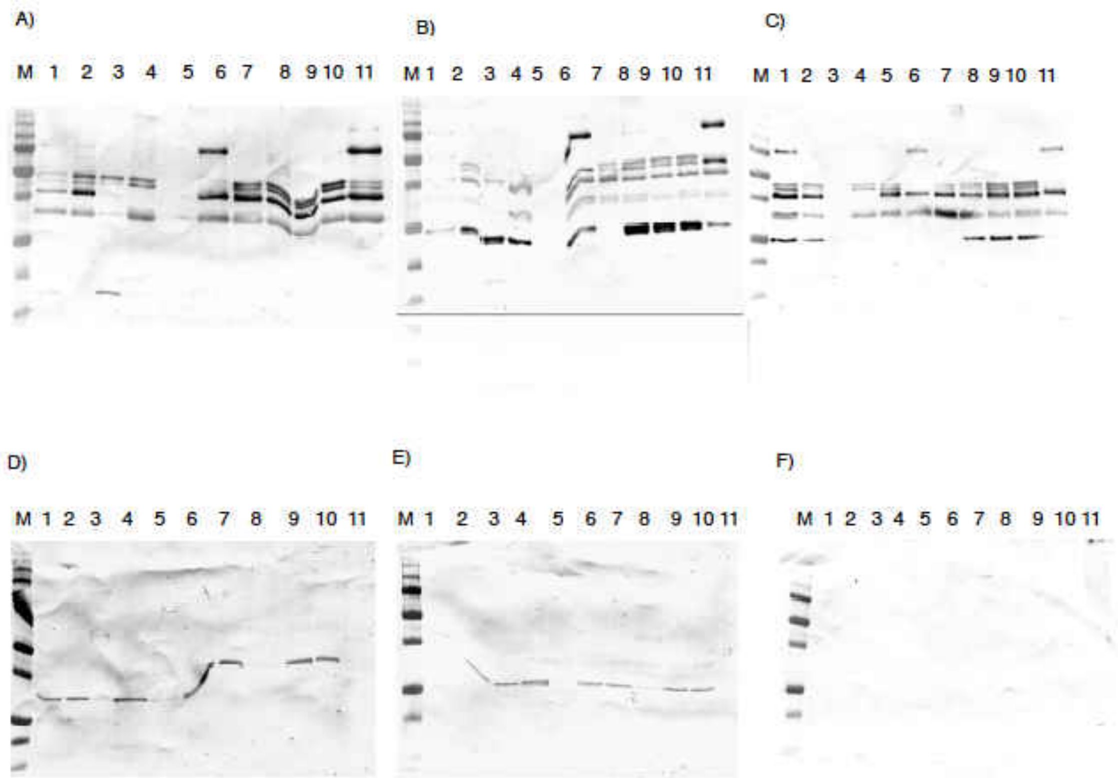


Figure 20: Convalescent sera of KIM5 infected B10.T (6R) mice (A-C) and C57BL/6 mice (D-F). Lanes; 1: D27x, 2: F1, 3:LcrVG, 4: YopB, 5:YopK, 6:YopH, 7:YopE, 8:YopJ, 9:YopT, 10: YopM, 11:YpkA. B10.T (6R) mice showed increased antibody response than C57BL/6 mice post reinfection with *Y. pestis* KIM5. (A) and (D) Convalescent sera, (B) and (E) day 1 post reinfection, (C) and (F) day 5 post reinfection

CHAPTER VI

DISCUSSION

Throughout evolution pathogens have developed numerous mechanisms to evade the host immune response. For a pathogen to infect and cause disease in a host, the pathogen must not elicit a pro-inflammatory response within the host. The manipulation of the host immune response allows the bacteria to grow and multiply while allowing the host cells to undergo apoptosis. Therefore, it is necessary to understand the strategy used by the bacteria to evade host response and use this knowledge to develop new treatments to fight the bacteria by giving the host immune system the advantage. One of the many advantages of using the mouse model is their striking resemblance to humans in anatomy, physiology and genetics. Over 95% of the mouse genome is similar to human genome, hence making mouse models effective tools to study progression of diseases. Inbred mouse strains are well characterized, as they are genetically uniform enabling reproducible studies. Because each inbred strain possesses unique combinations of phenotypes and alleles, some strains are susceptible to specific diseases whereas others are resistant. Sub strains of inbred 129 mice are highly resistant to infections with the KIM5 strain of *Y. pestis* (48, 230) whereas BALB/c or C57BL/6J strains of mice are susceptible to plague infection. The number of bacteria in the organs of 129 mice was 10- to 100- fold less than susceptible B6 mice. Resistance to 129 mice

is located on the DNA segment near the IL-10 gene (230).This resistance was not due to the presence of a functional Nramp1 (Slc11a1) protein. Nramp1 has been associated with resistance to a number of intracellular pathogens and functions as a macrophage and neutrophil cationic transporter. Susceptibility to infection has been linked to mutations in *nramp1* (144). Whether B10.T (6R) mice possess a functional Nramp1 protein and is associated with resistance is not clear. However, resistance was not increased to plague due to the presence of Nramp1 on a C57BL/6J mice (230). Moreover our model also shows that young B10.T (6R) mice are resistant to *Y. pestis* KIM5 but susceptible to *Y. pestis* CO92 Δpgm. Therefore, it is unlikely that Nramp1 protein influences plague resistance in our model.

The difference in resistance and susceptibility observed in young B10.T(6R) mice is not due to the genetic background of the mouse but due to the difference in *Y. pestis* strains. KIM5 is a Mediavalis strain whereas CO92 is an Orientalis strain. One of the other differences between the strains is in YopJ effector proteins secreted by the T3SS. The YopJ isoform in *Y. pestis* KIM5 has two amino-acid substitutions, F177L and K206E, which are not present in YopJ proteins of *Y.pestis* CO92 (264). We have shown that YopJ was responsible for the difference in outcome of infection in B10.T (6R) mice. A set of mutants expressing different isoforms of YopJ confirmed that the difference in amino acid was largely responsible for increased virulence of the pandemic strain, CO92 in the B10.T (6R) mice.

YopJ is a T3SS effector protein with acetyl-transferase activity that binds with MAPK kinases (MKKs) and inhibitor of kappa B kinase beta (IKKβ) (163). The YopJ from *Y. pestis* KIM5 is a distinct isoform with an increased ability to bind IKKβ (264).

This leads to higher levels of IL-1β release and increased caspase-1 activation, compared to other YopJ isoforms.

In vivo and comparative analysis provided insight into the differences in virulence observed. The most important finding was elevated levels of IL-1β in the sera of KIM5 infected B10.T (6R) mice compared to CO92 infected B10.T (6R) mice. A lower level of IL-1β production seen in the sera, spleen and liver of CO92 infected B10.T (6R) mice suggesting that YopJ expressed in CO92 is immunosuppressive to the host. Also higher production of IL-1Ra in the sera of CO92 infected mice suggests that YopJ of CO92 dampens the immune response. This is in accordance to the findings observed in *in vitro* studies where $YopJ^{KIM}$ triggers caspase-1 activation and which in turn elevates the IL-1β levels whereas $YopJ^{CO92}$ suppresses IL-1β activation in bone marrow derived macrophages (264). Mutants expressing different isoforms of YopJ showed that $YopJ^{CO92}$ when expressed in KIM5 strain suppresses IL-1β levels whereas expression of $YopJ^{KIM}$ in CO92 strain elevated IL-1β levels. IL-6, and RANTES levels were higher in young female B10.T (6R) mice when infected with *Y. pestis* CO92 strain compared to infection by KIM5 strain irrespective of the CFUs used. IL-6 down regulate the pro-inflammatory response (112, 202) , strongly supporting the importance of the cytokine in both recruitment of neutrophil and modulation of the immune response. KIM5 infected B10.T (6R) mice exhibited lower levels of IL-6, and this in turn may impact levels of pro-inflammatory mediators IL-1β. Since the host is thought to succumb to sepsis during the late stages of *Y. pestis* infection (27) and IL-6 is elevated in both clinical and experimental models, the elevated levels of IL-6 in CO92 infected B10.T (6R) mice in our model may be a result of YopJ-induced sepsis.

C57BL/6 mice are resistant to *Y. enterocolitica* via the intravenous route of infection (194). Interestingly, the 129 strains of mice are only moderately resistant to oral infection with the attenuated *Y. enterocolitica (203)*. The route of infection is important for the progression of the disease. Most of the *Y. pestis* strains used in the laboratory are attenuated. Hence, infection of mice via subcutaneous route with these attenuated strains of *Y. pestis* would not cause any disease. So to mimic the natural route of infection, mice can be supplemented with iron and attenuated strains can be used to infect mice subcutaneously that mimic the natural route of infection. We observed a lower mean day to death (3-4 days) and lower LD_{50} when *Y. pestis* infected mice are supplemented with iron. Additionally, when neutrophils were infected with KIM5 or CO92Δpgm, there was a higher production of ROS level as compared to neutrophils that were treated with iron dextran following infection with either the KIM5 or CO92Δpgm strain of *Y. pestis*. This result suggests that suppression of ROS levels might be attributed to iron overload. Supplementing the mice with iron suppresses the immune response by lowering the recruitment of neutrophils as well ROS production by neutrophils. As a result neutrophils are unable to clear the bacteria, resulting in lower LD_{50}.

Young female B10.T (6R) mice can also produce a strong humoral immune response to plague (29). We have shown that the convalescent sera of resistant B10.T (6R) mice showed a higher magnitude in antibody response than susceptible C57BL/6 mice. Analysis of IgG antibody response to the antigens used in this study both in acute and convalescent sera from plague infected mice and comparing that with *Yersinia* infected human cell lines would give an insight into understanding the mechanism of humoral immune responses to plague.

Conventionally, mast cells (MCs) are regarded as playing a vital role in IgE-mediated, Th2 response to allergens. Mast cells are distributed in the host-environment interface making these cells accessible to various pathogens. Mast cells also contain pre-formed mediators, which contributes to the early defense of the innate immune response. A unique property of MCs is the ability of long-term proliferation after activation. MC-deficient mice (C57BL/6 (Kit^{W-v}) and WBB6F1 (Kit^{W}/Kit^{W-v}) have shown to be more susceptible to skin infections caused by *Pseudomonas aeruginoa (209)*. MC deficient mice showed skin sections >2-fold larger than those of wildtype mice. Also recruitment of neutrophil was decreased, along with their ability to clear the bacterial burden from the site of infection. The impaired ability of MCs is also observed in *Lysteria monocytogenes* infected MCs depleted mice. In our study we show that depletion of mast cells by anti-c kit (ACK2) monoclonal antibody showed increased mortality in anti-c-kit antibody treated B10.T (6R) mice indicating that mast cells might play a pivotal role in defense against plague. Increased bacterial burden in anti-c-kit treated antibody than PBS treated mice demonstrated that mast cell plays an important role in early host defense against *Y. pestis*. The ability to recruit neutrophils to the site of infection by mast cell mediators is impaired in ACK2 treated B10.T (6R) mice due to the absence of mast cells. These results correlates with other studies showing the role of mast cells in infection and validate results obtained lately in mast cell reconstituted W/W^{v} mice (67).

The combined studies performed in our laboratory have allowed us to derive a working model of resistance to plague in young B10.T (6R) mice. The different findings in these studies related to host immune responses against *Y. pestis* infections provide a background for further studies in plague infection.

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