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**Survival of Yersiniae in naïve and activated macrophages:**

**Role of opsonization and the plasmid-encoded type III secretion system**

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**Betty Lavonia Noel**

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Abstract of the Dissertation

**Survival of Yersiniae in naïve and activated macrophages:**

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Within the genus *Yersinia* are two closely related human pathogenic species, *Y. pseudotuberculosis* and *Y. pestis*. *Y. pseudotuberculosis* is an enteropathogen from which *Y. pestis*, the agent of plague, recently evolved. They share a virulence plasmid (pYV) that encodes a type III secretion system (T3SS) and effector proteins, termed Yops, which function to antagonize normal macrophage signaling responses. For example, YopJ action stimulates macrophages to die of apoptosis. *Y. pestis* is known to survive within macrophages, and in previous studies, *Y. pseudotuberculosis* was shown to survive within macrophages only when pYV was not expressed or had been removed, suggesting that the T3SS and/or Yops may play a negative role in intracellular survival.

There are three translocator proteins necessary to deliver Yops into the host cell: YopB, YopD, and LcrV. The LcrV protein is a known target of protective antibodies, but it is not known if anti-LcrV antibodies increase killing of *Y. pestis* in naïve or activated macrophages. We investigated how anti-LcrV antibodies and the host pro-inflammatory

cytokine IFNγ impact bacterial survival and apoptosis in cultured murine macrophages infected with *Y. pestis* KIM5. Results show that anti-LcrV antibodies reduced apoptosis at an early time point but not later in infection. Additionally, anti-LcrV antibodies were ineffective at promoting killing of KIM5 in naïve or IFNγ-activated macrophages.

While anti-LcrV antibodies are major candidates for inclusion in a vaccine, alternative antigens, such as YopB and YopD, must be investigated for their protective effect because of the variable sequences of LcrV. A complex of Yops B, D and E is being investigated for protective activity. We examined the effect of anti-YopBDE antibody opsonization on bacterial uptake as well as apoptosis of infected macrophages. Opsonization of KIM5 with anti-YopBDE antibodies did not significantly affect phagocytosis of the bacteria or decrease levels of apoptosis late in infection.

Understanding the mechanism by which *Y. pseudotuberculosis* viability decreases in a pYV-dependent manner within macrophages may help to elucidate the natural ability of *Y. pestis* to survive inside macrophages. We studied pYV- or *yop* mutants of *Y. pseudotuberculosis* to determine what T3SS-related factor caused decreased survival of *Y. pseudotuberculosis* in macrophages. We found that *Y. pseudotuberculosis* YopO, a Yop involved in phagocytosis inhibition, may be responsible for the decreased survival within macrophages.

# Table of Contents

# List of Figures

# List of Tables

# Chapter 1: Introduction

## Yersinia Pathogenesis

There are three human pathogenic *Yersinia* species: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. All three species contain an approximately 70 kb virulence plasmid (pCD1 in *Y. pestis,* pYV in *Y. pseudotuberculosis* and *Y. enterocolitica)* that encodes a type three-secretion system (T3SS) and effector proteins termed *Yersinia* outer proteins or Yops (29). *Y. pestis* is the causative agent of pneumonic and bubonic plague and the other two species cause gastroenteritis. Historically, *Y. pestis* has had a major impact on society, killing large numbers worldwide. There were three known human pandemics caused by *Y. pestis*, which had major impacts on society (145). The 5$^{th}$ through 7$^{th}$ century was marked by the Justinian plague, from the 13$^{th}$ to 15$^{th}$ century was the historically well-known Black Death, and from the 1870s until now is the modern plague (145).

Although plague still occurs today, with the development of antibiotics and increased sanitary conditions, plague is no longer a major public health concern (70). However, there are still rodent populations infected with plague, and small numbers of humans within the population are infected annually (97). It is important to further study *Y. pestis* and to create a safe and effective vaccine because there is still a natural reservoir

and because there is also the potential danger that plague may be used as an agent of bio-terrorism (67, 124).

*Y. pestis* and *Y. pseudotuberculosis*/*Y. enterocolitica* have different routes of infection (Figure 1.1 (145)). *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens and enter via the fecal-oral route (152). They infect the M-cells over the Peyer's patches of the gut-associated lymphoid tissue (102). Once penetrating M-cells, they interact with resident macrophages and migrate to the lymph nodes. Primarily, they cause a self-limiting infection that does not kill the host. *Y. pseudotuberculosis* can enter the bloodstream and causes septicemia, although this occurs rarely.

Although *Y. pestis* is closely related to *Y. pseudotuberculosis*, it has acquired two additional plasmids, pMT1 and pPCP1, that give it increased virulence (97). The pMT1 plasmid is approximately 100 kb and encodes the *Caf* operon. With prolonged growth at 37°C the expression of the *Caf* operon leads to the production of the capsular fraction 1 protein that forms an amorphous capsule (34, 113). The plasmid pPCP1 is approximately 9.6 kb and contains the plasminogen activator (Pla). Pla is an outer membrane serine protease that may be necessary for dissemination of *Y. pestis* in subcutaneous infections and for growth in the lungs during pneumonic infections (62, 64, 66).

*Y. pestis* can enter the host subcutaneously through the bite of an infected flea (55, 144). It then multiplies under the skin where it can be taken up by resident macrophages and eventually migrates to the local lymph nodes where it multiplies causing bubonic plague. Eventually, *Y. pestis* enters the bloodstream causing septicemic plague and invades many cell types and organs especially the spleen and liver where it multiplies. The host eventually succumbs to the infection and dies from septicemic shock (97). *Y.*

*pestis* can also enter the lungs through inhalation of infected aerosols causing pneumonic plague.

Several *Y. pestis* strains have been identified. Two frequently experimentally utilized strains are KIM and CO92. KIM is from the biovar Mediaevalis and was isolated in Kurdistan from a plague patient (145). CO92 is from the biovar Orientalis and was originally isolated from a plague patient in Colorado (145). The KIM genome is about 50 kb smaller than CO92 (145); however, they both contain pMT1, pCD1, and pPCP1 as well as the chromosomal high-pathogenicity island (HPI). Encoded on the HPI is the gene for a siderophore called yersiniabactin (*ybt*) that allows *Yersinia* to acquire iron from the host (13, 22, 43). The HPI is contained within the pigmentation (*pgm*) locus, a 102 kb unstable chromosomal region (21, 22, 40).

The remaining portion of the *pgm* locus is the *pgm* segment. The *pgm* segment includes the hemin storage (*hms*) locus that gives *Y. pestis* the pigmented phenotype when grown on Congo-red agar plates (22). It was shown that the *hms* locus is important for transmission between fleas and the host (22, 56)]. The *pgm* locus is important for both transmission and virulence and is known to spontaneously delete possibly through homologous recombination between its two flanking insertion elements (20, 40). Removal or loss of the *pgm* locus causes *Y. pestis* to be avirulent in humans and easily handled under laboratory conditions.

The pCD1 plasmid encodes a T3SS composed of the secretion apparatus, chaperones, Yops and the translocator proteins (YopB, YopD, and LcrV) (29). The T3SS is a multi-subunit protein complex that grants Gram-negative bacterial pathogens the ability to translocate proteins into host eukaryotic cells (146). *Yersinia* has been shown

to inject effectors preferentially into macrophages, neutrophils, and dendritic cells as opposed to B and T cells (74). It is present not only in *Yersinia* but other bacterial pathogens such as *Shigella*, *Salmonella*, and *Pseudomonas* (146). Upon contact with the host cell, the T3SS allows for the translocation of effectors into the host cytoplasm. These effectors serve to distort the normal host cell processes (131). There is a great variation among effectors between species; however, the T3SS complex is mostly structurally and functionally conserved (83, 146). There are more than 20 unique proteins that are involved in spanning the inner and outer bacterial membranes, bridging the extracellular space, and penetrating the host cell membrane (Figure 1.2 (82)), allowing translocation of multiple effectors.

Six Yops have been identified: YopH, YopO/YpkA, YopP/J, YopE, YopM, and YopT (131, 136). Each Yop helps to alter the host cell in some way. The exact function of YopM is still unclear. It is a 41 kDa protein comprising mostly leucine-rich repeats or LRRs and is known to target the host cell nucleus (59, 60). YopJ (or YopP in *Y. enterocolitica*) inhibits mitogen-activated protein kinase (MAPK) signaling cascades as well as the activation of NF-κB, a transcription factor important for the transcription of many proinflammatory genes such as tumor necrosis factor alpha (TNFα), by preventing the phosphorylation and subsequent degradation of the NF-κB inhibitor (IκB) (29, 88). TNFα is transmembrane protein that is produced mainly by monocytes and has been shown to induce other inflammatory mediators, generating an inflammatory response involving the release of chemokines and cytokines and the recruitment of immune cells such as neutrophils (114). As a result of inhibiting NF-κB, YopJ inhibits the release of TNFα, diminishing the inflammatory response. YopJ has also been shown to induce

apoptosis, which may be due to the loss of NF-κB activity (29, 87, 128). It has been recently shown that YopJ is involved in caspase-1 activation and secretion of interleukin-1β (IL-1β) (71). IL-1β is another important proinflammatory cytokine produced mainly from monocytes and macrophages and expressed due to NF-κB activation (92). Pro-IL-1β is cleaved to its active form by caspase-1, and the mature form is secreted and is known to cause fever (92).

During phagocytosis of a pathogen via a surface receptor, such as the Fc receptor, various signaling cascades are activated. The plasma membrane-bound Rho GTPase family members, (Rac-1, RhoA, and Cdc42), are activated and GTP bound (29). These GTPases regulate the polymerization of actin, driving phagocytosis. Several of the Yops (such as YopO, YopT, and YopE) have anti-phagocytic effects (39). YopO (also known as *Yersinia* protein kinase A) is a serine-threonine kinase that autophosphorylates when it binds actin and interacts with RhoA and Rac-1 (88). YopO also has GDI (Guanine Dissociation Inhibitor) activity (2, 101). YopT is a cysteine protease whose function is to cleave the membrane bound Rho family members, causing their release from the membrane (5, 29, 88). Without membrane anchoring, these proteins lose their function, halting actin polymerization. YopE is a GAP (GTP activating protein) and causes the rapid hydrolysis of GTP to GDP and as a result deactivates the Rho family members (4, 107, 110). This deactivation disrupts the actin cytoskeleton and inhibits phagocytosis (138).

The phosphotyrosine phosphatase YopH has both an antiphagocytic effect as well as an anti-inflammatory effect (38). YopH dephosphorylates proteins involved in focal adhesions such as p130Cas and FAK, thereby disrupting their interaction with the actin

cytoskeleton, causing inhibition of phagocytosis (88). YopH also is involved with suppressing the inflammatory response. It inhibits the expression of monocyte chemoattractant protein 1, a chemokine involved in macrophage recruitment. YopH has also been shown to diminish the Fc mediated oxidative burst in neutrophils and macrophages (18, 88).

The expression of the T3SS and Yop translocation is dependent on temperature, calcium levels, and host cell contact. At 37°C the T3SS is maximally induced (88), and a needle-like surface structure, the Ysc injectisome, is formed with LcrV localized at the tip (33, 82). Upon contact with a host cell, the T3SS is systematically activated. The translocators YopB and YopD are believed to form a channel in the host cell membrane, allowing the delivery of the effector Yops (49, 91). The Yops are translocated into the host cell cytoplasm, where they disrupt host cell signaling.

## LcrV and *Y. pestis* Infection

The translocator proteins YopB and YopD, as well as LcrV, are necessary to deliver the effector Yops into the host cell (98). The mechanism by which LcrV mediates translocation is not fully understood but it appears to be important for the correct assembly of the translocation channel (50, 85, 112). LcrV has been shown to localize to the tip of the injectisome (85). LcrV, also known as V antigen, has many other important roles. It has a regulatory role in Yop secretion within the bacterium (99). LcrV is also a soluble protein and is an important protective antigen (31, 85, 140).

In addition to promoting Yop translocation, LcrV has been suggested to have additional anti-host activities. Purified LcrV has been shown to inhibit chemotaxis of polymorphonuclear neutrophils into sponges (141). This effect is seen in vivo where there is an acute inflammatory response in lesions formed in response to *Y. pestis* in the liver and spleen. Following the acute inflammation, there is a decay of neutrophils and no further recruitment of these cells (48, 99). As a result, lesions with few neutrophils develop over the entire spleen and liver.

Purified LcrV has also been shown to stimulate the production of IL-10, which inhibits cytokine release through suppression of NF-κB (51). LcrV induced immunosuppression through IL-10 is thought to be important during infection (41). Researchers have studied this immunosuppressive effect in monocyte/macrophage cell lines but it is also believed that it could involve other IL-10 producing cell types as well (99). IL-10 is an anti-inflammatory cytokine that contributes to the dampening of the inflammatory response and is essential to promote healing (19). The mechanisms include the inhibition of NF-κB activation and Toll-like receptor synthesis, which prevents proinflammatory cytokine synthesis. There is controversy within the literature as to the role of IL-10 in LcrV mediated immunomodulation by *Y. pestis* and the role of IL-10 in general.

It has been shown that LcrV induces IL-10 in a Toll-like receptor 2 (TLR2) dependent manner, which is essential in the pathogenicity of *Y. enterocolitca* 0:8 (100, 117). TLR2 recognizes microbial products such as lipoprotein and peptidoglycan (1, 69, 130, 142, 147). Recombinant LcrV from *Y. enterocolitica* 0:8 leads to the inhibition of TNFα in macrophages and was found to do so in a CD14- and TLR2-dependent manner

by inducing IL-10 (117, 119). An N-terminal LcrV mutant was found to cause the loss of both TLR2 and IL-10 inducing activity in vitro. In vivo the same mutant had impaired ability to activate TLR2 and was attenuated only in wild-type C57CL/6 mice and not $IL10^{-/-}$ or $TLR2^{-/-}$ mice (117). In *Y. pestis*, LcrV mutants show varying levels of IL-10 induction in vitro (94). In mice, a LcrV-His fusion protein induces IL-10 (90). *Y. pestis* LcrV has lower IL-10 induction levels in vitro compared to *Y. enterocolitica,* and it was speculated that this difference may be due to conformational state or oligomeric state differences between the two species' proteins (106). These authors also showed that, in vitro, the interaction between LcrV and TLR2 is weak, and from that result one may assume that TLR2-dependent IL-10 induction by LcrV does not contribute to the virulence of *Y. pestis* (106). *Y. pseudotuberculosis* was shown to induce IL-10 independently of TLR2 and LcrV (9). The IL-10 induction is blocked by the T3SS, and YopJ not only inhibits expression of TNFα but also that of IL-10, which is not detectable even in vivo in mouse tissue (9).

To further compound the IL-10 controversy, the accuracy of studies using $IL10^{-/-}$ mice has come into question. $IL\text{-}10^{-/-}$ mice are resistant to both *Y. pestis* and *Y. enterocolitica* (99, 118). Recently it has been shown that $IL\text{-}10^{+/-}$ mice are also are resistant to high-dose *Y. pestis* infection (132). These researchers found that the commercial $IL\text{-}10^{-/-}$ mice contain important genomic DNA segments from the original 129 mouse strain and that two substrains of these mice are resistant to *Y. pestis* infection. They conclude that a region of DNA from the 129 mouse strain near IL-10 is what confers resistance of these commercial IL-10-deficient mice to *Y. pestis* infection (132).

## Role of Phagocytes in the Innate Immune Response

During early stages of infection, the innate immune response protects the host in a non-specific manner (81). Not only is the innate immune system important as a defensive front against pathogens, it is also important in activating the adaptive immune response and immunologic memory (59, 78). The innate immune response involves phagocytic cells as well as soluble factors such as complement proteins to mediate protection (78). The immune cells involved in this response use a variety of signaling cascades to trigger events that help prevent the establishment of infection such as phagocytosis, release of reactive oxygen species, and cytokine production (59). These phagocytic cells, including macrophages and neutrophils, are able to uptake pathogens, digest them, and create an inflammatory response (10, 59).

Once the pathogen is taken up in the phagosome, the vacuole matures and fuses with lysosomes. The phagolysosome has a lowered pH as well as many other antimicrobial substances that destroy the pathogen. These professional phagocytic cells also have the ability to recognize pathogens using Toll-like receptors (TLRs). TLRs are examples of pattern recognition receptors (PRR) that were first found in Drosophila and known to be important not only in innate immunity but in inducing adaptive immunity (79). TLRs recognize conserved microbial structures called pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS) and peptidoglycan are two examples of PAMPs that TLRs recognize. This recognition causes phagocytosis of the pathogen as well as the production of proinflammatory cytokines such as TNFα and interleukin 12 (IL-12) (57).

Production of IL-12, a heterodimeric cytokine that is essential for activation and expansion of T helper 1 cells (17), causes the activation of $CD4^+$ T helper cells and natural killer (NK) cells, stimulating the release of interferon gamma (INFγ) (15). INFγ is produced by $CD4^+$ T helper cells, NK cells, and $CD8^+$ T cytotoxic cells (75). During infection with high levels of *Y. pestis* lacking pCD1, INFγ levels peak by day 4 post-infection (19). INFγ increases the macrophage's ability to present antigens, synthesize proinflammatory cytokines, and undertake complement-mediated phagocytosis (75). Overall, INFγ activates macrophages (Figure 1.3 (78)), which increases their ability to phagocytose and upregulate the production of reactive oxygen species, and overall increases their ability to kill pathogens. Macrophages will then display the antigens from the digested pathogens in their MHC class II surface receptors, which will help to stimulate the adaptive immune response.

Phagocytosis is dependent on recognition of the pathogen. Opsonization of a pathogen by antibodies and complement proteins can cause recognition by Fc receptors, which recognize immunoglobulins, and/or complement receptors (CRs), which recognize complement proteins such as C3 (96, 137)]. The complement cascade consists of plasma proteins that react with each other to opsonize pathogens, increase opsonization of pathogens by antibodies, and induce inflammatory responses to help fight off infection (23, 78). Many of the complement proteins are proteases that circulate in their inactive forms. These precursor zymogens are distributed throughout the body and are only activated locally at sites of infection (96). Once one complement protein is activated, it then cleaves its substrate, which then cleaves its substrate, causing an amplified signaling cascade leading to a large-scale response (78).

There are three complement pathways that eventually converge and create the same effector molecules. These three pathways are: the classical pathway, the mannan-binding lectin (MBL) pathway, and the alternative pathway (78). The classical pathway is initiated by C1q binding to the pathogen and can also bind to an antibody/antigen complex during the adaptive response (80). The MBL pathway (Figure 1.4 (78)) is one of the better characterized arms of the complement cascade and is initiated by MBL, a serum protein, binding to mannose-containing carbohydrates on pathogens (57, 72, 78, 96, 133)]. The alternative pathway is initiated when a spontaneously activated complement protein binds to the surface of the pathogen (80).

The three pathways follow a sequence of reactions that ultimately produce C3 convertase, which covalently binds to the pathogen (78). C3 convertase cleaves complement protein C3 generating C3a, a peptide mediator of inflammation, and C3b (57). C3b acts as an opsonin and covalently binds pathogens targeting them for destruction by phagocytes with the C3R (96).

Fc receptor-mediated phagocytosis can increase antibacterial functions of macrophages by the production of reactive oxygen species (ROS) and cytokines, causing greater killing of pathogens (137). It has been shown that Fc receptor-mediated uptake of antibody opsonized *Y. pseudotuberculosis* is necessary to cause an oxidative burst in mouse macrophages (18). Upon ligand binding, Fc receptors initiate intracellular signaling cascades within the cell. The intracellular signals that are propagated initially during phagocytosis, in general, cause the fusion of phagosomes with lysosomes that contain digestive enzymes, lowering the pH and increasing ROS and nitric oxide production to destroy the phagocytosed pathogen (137).

Phagocytosis is an actin cytoskeleton driven process that results in pseudopod formation and the taking up of a particle (6, 96, 127). The first event involves the recognition of the particle by the surface receptor of the phagocyte. The particle is then internalized in a phagosome derived from the plasma membrane, and intracellular signaling events lead to the production of ROS and interactions between the maturing phagosome and the endocytic pathway (37, 126). This interaction causes the acidification of the phagosome. The mature phagosome contains lysosomal contents such as hydrolytic enzymes, antimicrobial peptides and iron transporters like Nramp1, which modulates iron levels within the phagosome (8, 47, 61). All of these attributes of phagosomes help to kill a wide array of bacteria and other microorganisms.

Mice express three immunoglobulin G (IgG) receptors (FcγRI, FcγRII, and FcγRIII). Fc receptors have different ligand binding alpha chains that form complexes with gamma, beta, or lambda chains (134). These chains are intracellular and act to propagate the signal from the antibody opsonized pathogen binding the alpha chain. FcγRI is a high affinity IgG2a receptor and is found on macrophages, monocytes, and dendritic cells. IFNγ stimulation causes macrophages to upregulate the FcγRI receptor on its surface. There are Fc receptors that are specific for other immunoglobulin isotypes besides IgG. FcεR recognizes the IgE isotype while FcαR interacts with IgA (111).

The most prevalent CRs on phagocytes are CR3 and CR4. Both receptors recognize C3, one of the most prevalent complement proteins. CR mediated phagocytosis involves cellular membrane ruffling that causes the formation of vacuoles that are 1-5 μm in diameter (137). The opsonized particle almost sinks into the membrane in contrast to the pseudopod formation in Fc receptor mediated phagocytosis (Figure 1.5(37)) (96,

137). It appears that uptake mediated through both CRs and Fc receptors work together to heighten phagocytosis and the resulting immune response (137).

The respiratory burst is an important tool used by phagocytes to destroy phagocytosed pathogens. The respiratory burst involves the production of superoxide anions ($O_2^-$) within the forming phagosome and the subsequent transformation of these anions into more potent products: reactive oxygen species (ROS) such as hydrogen peroxide (135). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is responsible for the production of the superoxide anion by catalyzing the reduction of molecular oxygen to superoxide (135). NADPH oxidase is vital in the immune defense as is seen in chronic granulomatous disease, where NADPH oxidase is nonfunctional and leads to increased susceptibility to bacterial and fungal infections (77, 135).

NADPH oxidase activity must be closely regulated because ROS are damaging to not only the pathogen but to the host tissues as well. NADPH oxidase is composed of many subunits. When phagocytes, such as macrophages, are not activated (naïve or in a resting state), NADPH oxidase is unassembled (115). The different subunits are found in the cytosol as well as in the membrane. The membrane components are $gp91^{phox}$ and $gp22^{phox}$. The cytosolic components of NADPH oxides are $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. Upon activation by phagocytic stimuli, the membrane subunits are brought to the developing phagosome by secretory vesicles, and the cytosolic components translocate to the phagosome along with Rac2 (115). Once all of the oxidase subunits are assembled in the phagosome, NADPH is reduced and ROS can be produced.

Since the oxidative burst is crucial to the host defense against invading pathogens, some invading organisms, in order for their survival, target it. For example, *Francisella*

*tularensis*, a gram negative facultative intracellular pathogen and causative agent of tularemia, has been shown to disrupt the respiratory burst of neutrophils. *F. tularensis* is rapidly phagocytosed but was found to disrupt the assembly of NADPH oxidase in the phagosome causing bacterial survival (77).

## Mechanism of Immune Evasion of Phagocytes by *Yersinia pestis*

*Y. pestis* has evolved multiple mechanisms to evade the innate immune system. *Y. pestis* can replicate within macrophages early during infection and extracellularly during the later stages of infection. After phagocytosis, *Y. pestis* has the ability to evade degradation and to survive and replicate within phagosomes (102). *Y. pestis* can survive within both activated and naive macrophages (102, 104). At later stages, *Y. pestis* can avoid phagocytosis, using the F1 capsule or Yop translocation, and can even cause macrophages to undergo apoptosis, using YopJ for example (93).

Through the effect of certain Yops, *Yersinia* is also able to inhibit the oxidative burst, which would promote bacterial survival theoretically (30). Using *Y. pseudotuberculosis*, Bliska and Black (1995) showed that functional YopH was necessary for inhibiting the oxidative burst of macrophage-like cells (18). In order to generate an oxidative burst, Bliska and Black (1995) found that it was necessary to cross-link Fc receptors with IgG opsonized *Y. pseudotuberculosis* (18). They also found that the tyrosine phosphatase activity of YopH could globally inhibit the Fc receptor mediated signaling within macrophages. Presumably, YopH could enter the host cell and dephosphorylate the proteins involved in the Fc receptor-signaling pathway.

YadA, a surface protein expressed by *Y. enterocolitica* and not *Y. pestis*, has been shown to cause resistance to complement (35, 37). It prevents the attachment of C3b to the surface of the bacterium (26). While *Y. pestis* does not have YadA, there is some evidence that the outer membrane protein Ail found in *Y. pestis* may cause resistance to complement (11).

## Rationale

There are three main goals of this dissertation. In Chapter 2 we investigated how anti-LcrV and IFNγ impact bacterial survival and apoptosis in bone marrow derived macrophages infected with *Y. pestis*. It remains unknown if IFNγ and TNFα cooperate with anti-V antibody to increase killing of *Y. pestis* and decrease apoptosis in macrophages. We hypothesized that in order to confer protection against *Y. pestis* in vivo, both antibody and activated macrophages are important. This is based on two main pieces of evidence. The first is from Philipovskiy et al. 2005, where they depleted macrophages in mice infected with *Y. pestis* and found that there are higher bacterial loads in the liver even with anti-V antibody treatment (99). When macrophages are present combined with anti-V antibody, there are lower bacterial levels in the liver. These results showed that macrophages are important for anti-LcrV antibody mediated protection in the liver. The second piece of evidence is from Parent et al. 2006, where they passively immunized mice and showed that in mice depleted of the cytokines IFNγ or TNFα, the overall survival of the mice decreases and the bacterial loads in the organs

increase compared to the control mice (95). The results showed that IFNγ and TNFα are important during humoral defense against *Y. pestis*.

Our goal for Chapter 2 was to examine the effect of protective antibodies and activated macrophages on the intracellular survival of *Y. pestis* in vitro. We examined various in vitro readouts for protection to see if the combination of anti-V antibodies and IFNγ-stimulated macrophages was important for protection against *Y. pestis*. More specifically, we examined the effect of anti-V antibody with and without IFNγ-stimulation of macrophages on *Y. pestis* internalization, YopJ-mediated apoptosis, release of TNFα and IL-1β by *Y. pestis*-infected macrophages, intracellular survival of *Y. pestis*, and the effect on the acidification of *Y. pestis* containing phagosomes. In our studies we used the *Y. pestis* strain KIM5 because it a commonly used *Y. pestis* strain that contains all three *Y. pestis* plasmids and does not have the *pgm* locus, allowing it to be easily handled.

In Chapter 3 we continued to investigate the interaction of protective antibodies and *Y. pestis* in vitro. A complex of Yops B, D, and E (BDE) was investigated as an experimental vaccine and shown to elicit protective activity against *Y. pestis* infection in mice (58). Our goal was to determine if these newly found protective antibodies behaved similarly in our in vitro assays as anti-LcrV antibodies, since they were well studied in Chapter 2 and throughout the literature. We examined the effect of anti-BDE antibody opsonization on bacterial uptake and intracellular survival, as well as apoptosis of *Y. pestis*-infected macrophages. We also examined the effect of immune sera raised against a YopB and YopD complex (BD) or YopB alone (B) on bacterial uptake.

Finally, in Chapter 4 we wanted to look closely at the intracellular survival of *Y. pseudotuberculosis* within macrophages. In vitro, despite opsonization with protective antibodies and the presence of IFNγ-stimulated macrophages, *Y. pestis* continued to survive intracellularly. However, *Y. pseudotuberculosis* has been shown to have decreased survival within macrophages in vitro (108, 151)]. Early experiments suggested that pore formation within macrophages by *Y. pseudotuberculosis* mutants is required for the overall decreased survival of *Y. pseudotuberculosis* (108). Later experiments showed that wild-type *Y. pseudotuberculosis* has decreased survival and implicated the T3SS as being involved (151). In these experiments *Y. pseudotuberculosis* was shown to survive within macrophages only when pYV is removed or not expressed. This suggests that the T3SS and/or Yops may play a negative role in intracellular survival. We wanted to determine if the Yops caused decreased survival of *Y. pseudotuberculosis* within macrophages. In order to examine this we performed intracellular survival assays by infecting macrophages with wild-type *Y. pseudotuberculosis* and various Yop mutants and compared survival over time.

## Experimental Procedures

**Bacterial Strains**. The strains used are shown in Table 2.1. *Y. pestis pgm* mutants KIM5 and KIM5/GFP contain pCD1Ap. KIM5/GFP also contains an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible plasmid encoding green fluorescent protein (pGFP). KIM5 *yopB* mutant contains an in-frame deletion of *yopB* in pCD1Ap as

described in Lilo et al. (71). KIM5 *phoP* mutant contains pCD1Ap and an in-frame deletion of *phoP* as described in Grabenstein et al. (45). *Y. pseudotuberculosis* serogroup I strain 32777, formerly known as IP2777, contains pYV. The strains used below are shown in Table 4.1. *Y. pseudotuberculosis* serogroup I strain 32777, formerly known as IP2777, contains pYV (151). IP2666, a *Y. pseudotuberculosis* serogroup III strain also contains pYV (Table 4.1). IP2666c is cured of pYV. IP32, IP2666 *yopO*, was created using allelic exchange to delete the entire *yopO* reading frame. IP36, IP2666 *yopK*, was created using allelic exchange to insert a frame shift mutation at the Afl III restriction site of the *yopK* gene. IP37, IP2666 *yopEHOMKJ*, was created by transforming the competent IP2666c bacteria with a mutant form of pYV which contains deletions in *yopEHOMKJ*. IP2666/GFP, IP32/GFP, IP37/GFP, and IP2666c/GFP contain an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible plasmid encoding green fluorescent protein (pGFP). IP2666 pYopO was created by conjugating pYopO (encoding YopO from KIM) from S17λpir into IP2666.

**Serum and Antibodies.** Anti-LcrV serum and control serum were obtained from LcrV immunized or adjuvant only injected mice, respectively, as described in Ivanov et al (58). The monoclonal anti-LcrV antibody (mAb 7.3) has been described (54). The Hybridoma Facility at Stony Brook University generated a hybridoma producing anti-YopD mAb 248.19. Balb/c mice were immunized with YopBDE antigen (58). Following fusion of spleen cells from immunized mice, hybridoma clones producing anti-YopD mAb were identified by (ELISA) and immunoblotting. One subclone producing anti-YopD mAb was designated 248.19 and the mAb was isotyped as IgG1. The mAb was

purified by ammonium sulfate precipitation of serum free hybridoma supernatants. The precipitated protein was dissolved in PBS and dialyzed against PBS. Anti-BDE serum, anti-BD serum, anti-B serum, and control serum used in Chapter 3 were obtained as described in Ivanov et al. (58).

Pooled sera titers were determined as described in Ivanov et al. by using an antigen excess enzyme-linked immunosorbent assay (ELISA) (58). Pooled sera from mice immunized with LcrV or BDE were reacted with corresponding purified antigen, and the results of ELISA for total IgG showed the presence of specific antibodies at similar titers of $10^{-5}$, which represents the lowest dilution that gives an $OD_{450}$ value above 0.1. We decided to use 10 μl of pooled serum per infection. The rationale behind this choice was that we wanted a volume that would allow for antibody excess. We roughly calculated that if there were 100 injectisomes per bacterium, there would be roughly 500 LcrV proteins expressed on the tips (LcrV is a pentamer so there would 5 LcrV proteins per injectisome). There are about $4x10^{12}$ IgG molecules in 1 μg of purified monoclonal antibody. During infection we used $1.5x10^{6}$ bacteria so there would be approximately 750 $x10^{6}$ surface LcrV proteins. Therefore, for every 1 μg of antibody there would be over 8000 molecules of anti-LcrV for every LcrV protein, which would be an excess of antibody.

**Primary Macrophage Cell Culture.** Bone marrow derived macrophages (BMDMs) were isolated from the femurs of C57BL/6 mice (Jackson Laboratory) and prepared as previously described (102). BMDMs were seeded in 24-well cell culture plates at $1.5 \times 10^{5}$ cells per well for 24 h prior to infection in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum (HyClone), 1 mM sodium pyruvate, 2

mM glutamate, and 15% L-cell conditioned medium.

**Infection Conditions**. Bacterial cultures were grown in Heart Infusion Broth (HI) containing Ampicillin (Amp) (25 μg/ml), and Chloramphenicol (Cam) (30 μg/ml) to select for pCD1Ap or pGFP, respectively, overnight with aeration at 28°C. Overnight cultures were diluted 1:40 in HI with Amp and 2.5 mM $CaCl_2$ (and with Cam and 0.05 mM IPTG to induce expression of GFP when necessary) and incubated with aeration for 2 h at 37°C. Ten μl of KIM5 ($1.5 \times 10^6$ CFU in 1 ml) suspended in PBS were incubated with 10μl of LcrV antibody serum or control serum or 10 μl of PBS (KIM5 nonopsonized) and incubated at 37°C + 5% $CO_2$ for 10 min. Opsonization with mAb was similarly performed. The volume was then increased to 1 ml with cell culture medium, and the sample was added to the macrophages (multiplicity of infection of 10 bacteria per macrophage). The plates were centrifuged for 5 min at 95 x g to facilitate contact between macrophage and bacteria and incubated for 20 min at 37°C with 5% $CO_2$. Bacterial cultures in Chapter 4 were grown in Heart Infusion Broth (HI) containing antibiotics as needed, overnight with aeration at 28°C. Overnight cultures were diluted 1:40 in HI (with 0.05 mM IPTG to induce expression of GFP when necessary) and incubated with aeration for 2 hours at 37°C. Macrophages were then infected at an MOI of ten bacteria per macrophage.

**Phagocytosis assay.** Raw 264.7 mouse macrophage like cells (ATCC TIB-71) were grown in Dulbecco's modified Eagle's medium plus Glutamax (Gibco BRL) with 10% heat inactivated fetal bovine serum (HyClone) and 1 mM sodium pyruvate at 37°C with 5% $CO_2$. Macrophages were seeded on glass coverslips at $1.5 \times 10^5$ cells in 1 ml of medium in 24-well cell culture plates and incubated overnight. Macrophages were

infected as described above with opsonized or non-opsonized KIM5/GFP expressing GFP. The plates were centrifuged at 95 x g for 5 min to facilitate contact between macrophage and bacteria and incubated for 20 min at 37°C with 5% $CO_2$ . The wells were then washed with prewarmed PBS once and fixed with 2.5% paraformaldehyde (PFA) in PBS at room temperature for 30 min. After washing with PBS coverslips were blocked with 3% bovine serum albumin in PBS for 20 min. Extracellular bacteria were labeled with rabbit anti-*Yersinia* antiserum SB349 (16) and with goat anti-rabbit antibody conjugated to Alexa Fluor 594 (Molecular Probes). The coverslips were washed and mounted with ProLong Gold antifade reagent (Invitrogen) onto slides. Slides were examined by epifluorescence microscopy using a Ziess Axioplan2 microscope. Pictures of three fields per coverslip in at least three independent experiments were taken. The number of macrophage-associated intracellular bacteria (green only) and the number of macrophage-associated extracellular bacteria (red and green overlay) was counted and the percent internalization determined as follows: [intracellular bacteria/ (intracellular bacteria + extracellular bacteria)] x 100%.

**LDH release assay.** LDH release was determined from supernatants collected from *Y. pestis* infected BMDMs at 5 and 24 h post-infection using the CytoTox-96 nonradioactive cytotoxicity assay (Promega). Supernatants were collected and centrifuged to remove cellular debris, and LDH levels were determined in triplicate. Total LDH release was determined from supernatants from freeze-thaw lysed BMDMS. Spontaneous LDH release was determined from supernatants obtained from uninfected cells. The percentage LDH release was calculated as follows: percent LDH release=

[(infected cell LDH release – spontaneous LDH release) / (total LDH release – spontaneous LDH release)] x 100%.

**CFU assay.** At 25 min, 1.5 h, and 5 h post-infection, *Y. pestis* infected BMDMs (Chapters 2 and 4) or RAW 264.7 cells (Chapter 3) were washed with PBS and lysed with 500 μl of 0.1% Triton X-100 in PBS. The plate was then incubated at 37°C + 5% $CO_2$ for ten min. Wells were scraped and lysates collected in microcentrifuge tubes. Five hundred μl of PBS were used to wash the wells. Lysates and washes were combined and used for serial dilutions. Dilutions were plated on HI plates containing antibiotics as appropriate and incubated at 28°C for two days. The bacterial colonies were enumerated and the $Log_{10}$CFU per ml was determined from the results of three independent experiments.

**TNFα and IL-1β ELISA.** At 24 h p.i. supernatants from wells containing infected BMDMs were collected and centrifuged for 10 min at 200 x g to remove cellular debris and transferred to new tubes. Supernatants were diluted appropriately and 50 μl of each diluted sample was analyzed. The concentration of TNFα and IL-1β in the supernatant was measured using the Quantikine mouse TNFα and IL-1β immunoassay kits (R&D Systems).

**Phagosome acidification.** J774A.1 murine macrophage-like cells (ATCC TIB-67) seeded in 24-well plates on glass coverslips at 1.5 x $10^5$ cells/well were infected with KIM5 expressing GFP as described above. Determination of colocalization of phagosomes containing GFP positive *Y. pestis* with lysotracker Red DND 99 was performed by fluorescence microscopy (105). As a positive control for colocalization with Lysotracker Red DND 99, we used KIM5/GFP fixed in 2.5% PFA (105). One hour

prior to fixation, the cell culture medium was removed from the wells, and cell culture medium containing 50 nM Lysotracker Red DND 99 was added. Coverslips were fixed with 2.5% PFA and mounted onto slides. The percentage of KIM5/GFP that colocalized with Lysotracker Red was determined as described (105).

**GFP Induction Assay**. Macrophages seeded on coverslips were infected as described above with IP266/GFP and mutant strains. At 23 h p.i. media with IPTG was added to the wells to induce GFP expression in the surviving bacteria. After an additional hour incubation, the wells were then washed with prewarmed PBS once and fixed with 2.5% paraformaldehyde (PFA) in PBS at room temperature for 30 min. After washing with PBS coverslips were blocked with 3% Bovine serum albumin in PBS for 20 min. Macrophages were permeabilized with 0.1% Triton X-100 and bacteria were labeled with rabbit anti-*Yersinia* antiserum SB349 (16) and with goat anti-rabbit antibody conjugated to Alexa Fluor 594 (Molecular Probes). The coverslips were washed and mounted with ProLong Gold antifade reagent (Invitrogen) onto slides. Slides were examined by epifluorescence microscopy using a Ziess Axioplan2 microscope.

**PCR amplification of YpkA.** To confirm the presence of the yopO/ypkA genes within *Y. pestis* and *Y. pseudotuberculosis* strains, PCR was performed using the primers YpkA-F (5'-GGTACCTGGGGATGAGTAAAGCATG-3') and YpkA-R (5'-GTCGACTCACATCCATTCCCGCTC-3'). Bacterial cultures grown overnight or colonies from plates were used as sources of DNA template for the PCR. Conditions for PCR were as follows: initial 7 min melting at 95ºC followed by 30 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 2.5 min elongation at 72ºC. A final elongation at 72ºC was

done for 7 min at the end of the 30 cycles.

To amplify sequences encoding yopO/ypkA from IP2666, 32777, and CO92 to insert into the pMMB67EH expression vector, high fidelity Taq polymerase and primers YpkA-F and YpkA-R were used. Similar PCR conditions were used except: initial 7 min melting at 95ºC followed by 30 cycles of 95ºC for 20 sec, 50ºC for 20 sec, and 4.5 min extension at 72ºC. A final elongation at 72ºC was done for 7 min at the end of the 30 cycles. The resulting PCR products were processed for sequencing by the core facility at Stony Brook University. Sequences were aligned using ClastalW.

**Statistical Analysis.** Experiments were performed at least three times. The results were subjected to analysis of variance and the Tukey post test using Prism (GraphPad). Results were considered significantly different if the Probability (P) values were less than 0.05.

# Figures

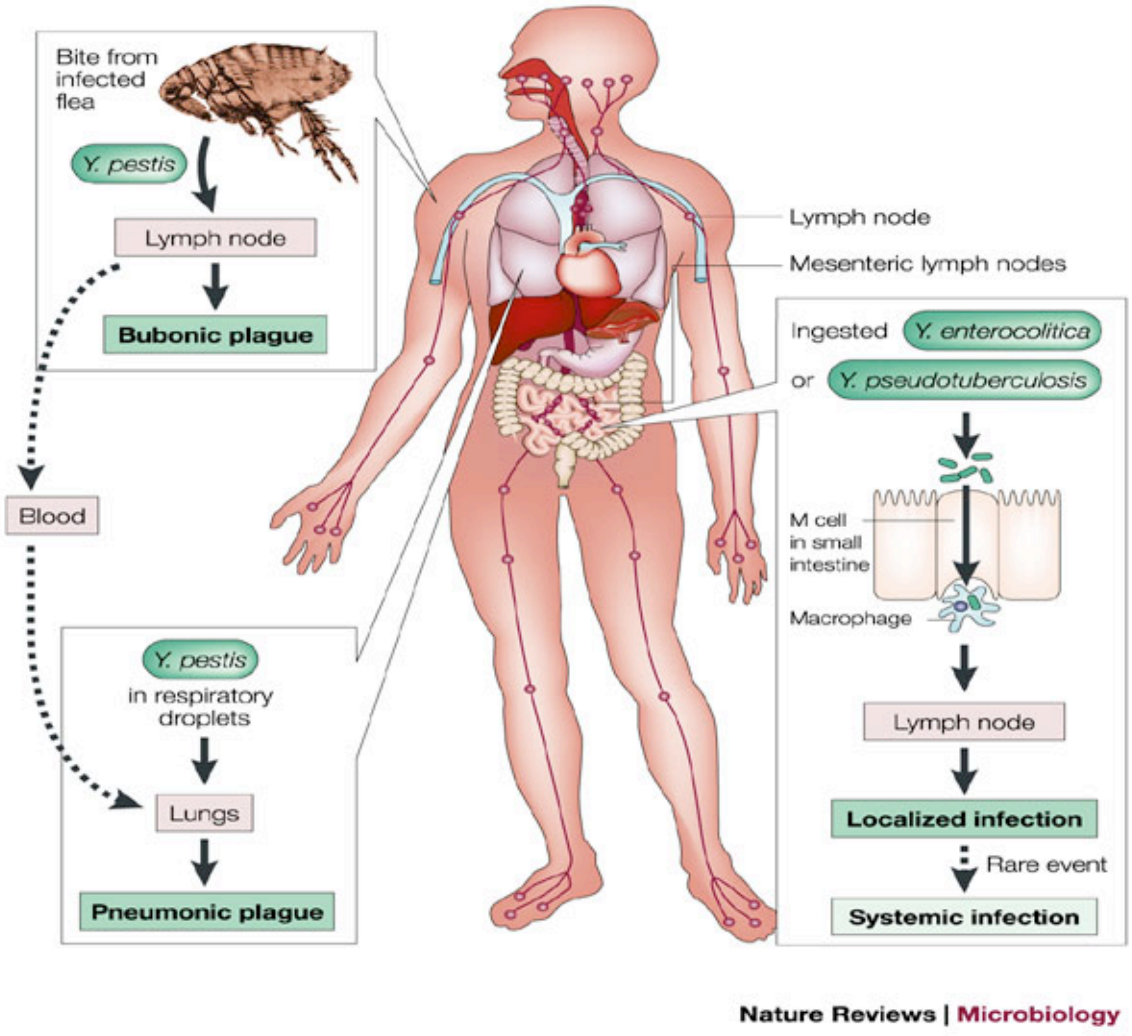
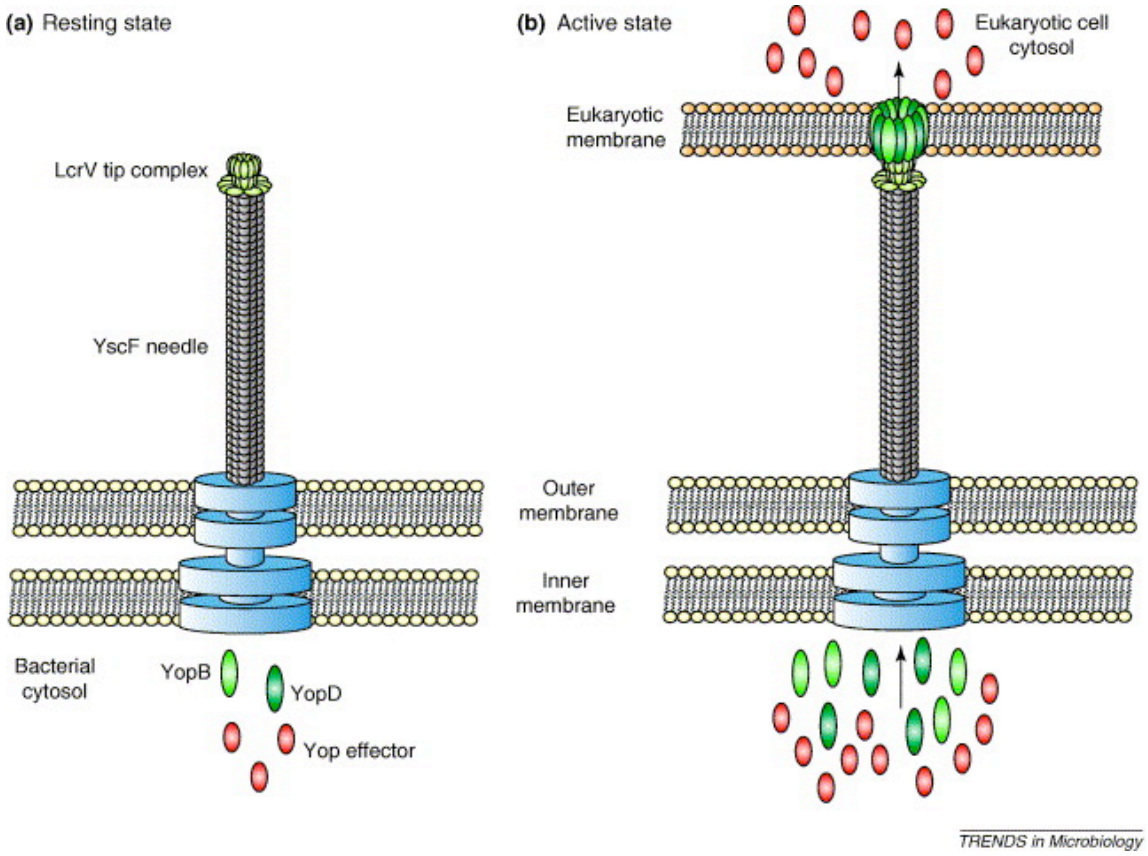



**Figure 1.1**
*Yersinia* pathogenesis. There are three human pathogenic species of *Yersinia*: *Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica*. Rodents are a reservoir for *Y. pestis* and the rodent's fleas take up the bacterium after a blood meal and transmit the bacterium to other rodents as well as humans. This results in bubonic plague in humans. Pneumonic plague is transmitted through respiratory droplets from person to person. *Y. pseudotuberculosis* and *Y. enterocolitica* are food pathogens and infect the M cells of the small intestine causing a localized infection of the lymphatic system. Permission for use of image obtained from the publisher (145).




## Figure 1.2

The type III secretion apparatus. (a) The *Yersinia* secretion complex (Ysc) injectisome consists of a basal body that crosses both the inner and outer membranes. At the top of this complex is a needle-like structure composed primarily of the protein YscF. LcrV is found at the tip of the needle. In the absence of host cell contact, the Ysc injectisomes form on the bacterial surface but the translocator an effector Yops remain in the bacterial cytosol. (b) Upon host-cell contact, the injectisome switches to an active state. The translocator Yops are inserted into the host cell membrane and the effectors are translocated into the host cytoplasm, where they disturb normal cell signaling processes. Permission for use of image obtained from the publisher (82).

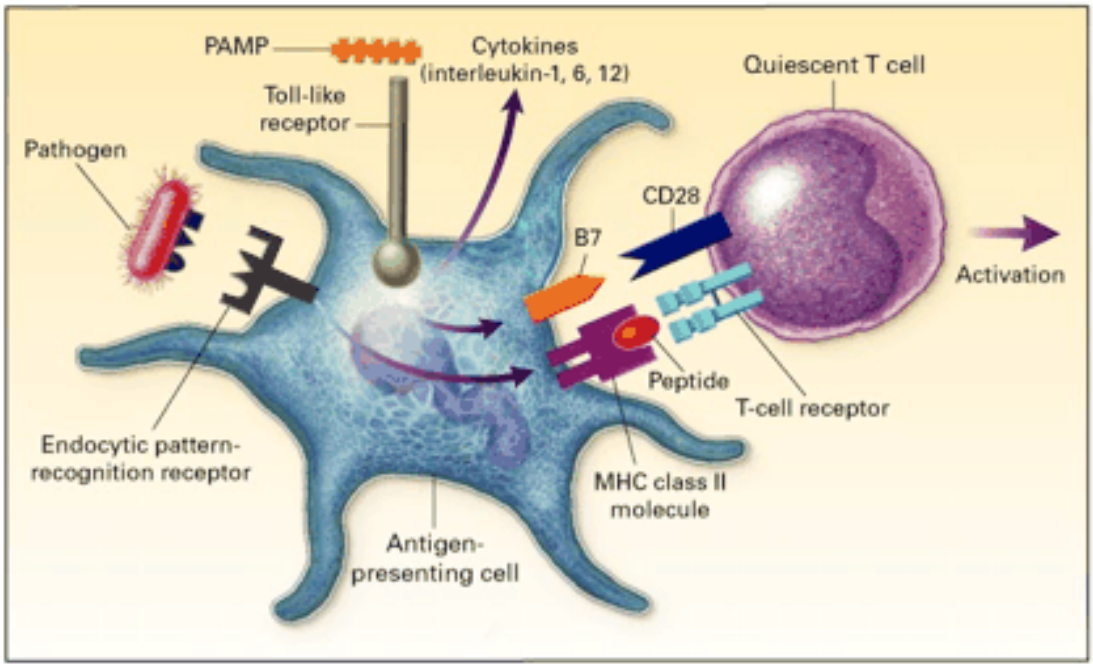



**Figure 1.3**
Activation of macrophages. When a macrophage receives stimuli from cytokines or through recognition of a pathogen-associated molecular pattern (PAMP) by a pattern-recognition receptor like the Toll-like receptor or through the phagocytosis of a pathogen, signals are generated within the macrophages that activate it. Upon receiving these stimuli the macrophage produces a number of cytokines and chemokines as well as co-stimulatory molecules to help promote an immune response. Proteins from the pathogen are processed and generate antigenic peptides that form a complex with major-histocompatibility-complex (MHC) class II molecules on the macrophage cell surface. These peptide complexes are recognized by T-cell receptors and lead to activation of the T-cell. Permission for use of image obtained from the publisher (78).

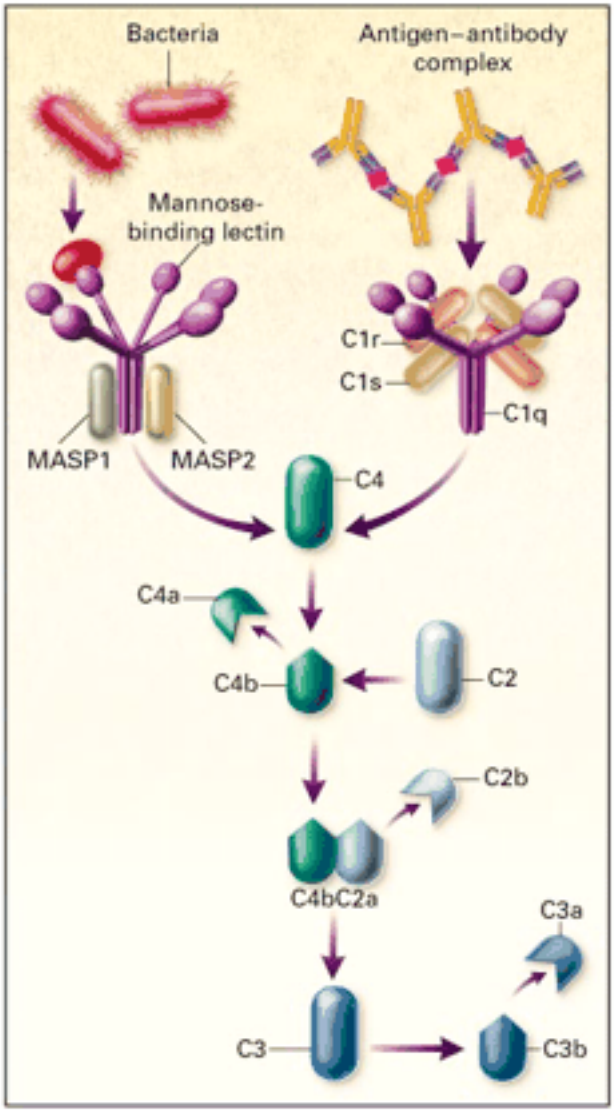



**Figure 1.4**
The mannose binding lectin pathway of the complement cascade. The mannose binding lectin (MBL) pathway is one well-characterized arm of the complement system. The MBL pathway is similar to the classical and alternative pathways in that it results in the formation of C3. The mannose binding lectin pathway is mediated by mannose-binding lectin, the pattern-recognition receptor that recognizes bacterial carbohydrates. Mannose-binding lectin is associated with mannan-binding lectin-associated proteases 1 and 2 (MASP1 and MASP2). Binding of MBL to bacterial carbohydrates activates MASP1 and MASP2 causing the cleavage off the complement proteins C2 and C4. The products of this cleavage, C2a and C4b, form C3 convertase. The C3 convertase cleaves C3 to C3a and C3b, which initiates the complement cascade. Permission for use of image obtained from the publisher (78).

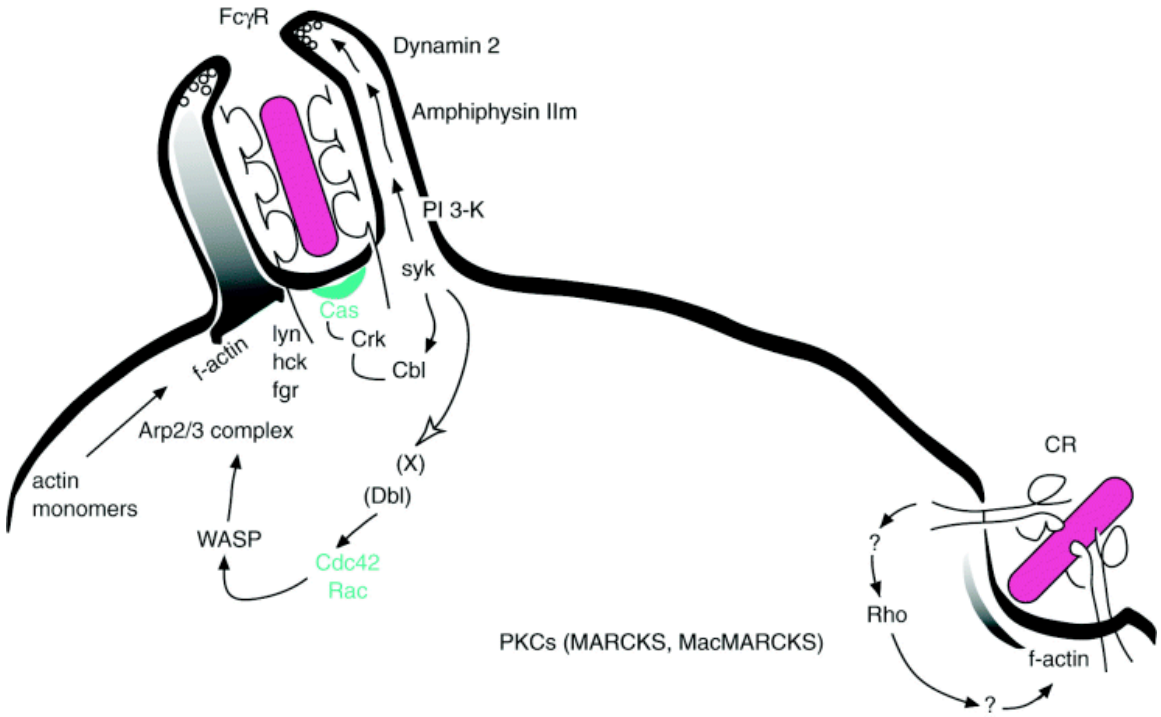



**Figure 1.5**

Phagocytosis via Fc receptors and complement receptors. Fcγ receptor (FcγR) phagocytosis involves the extension of actin filaments that surround the pathogen. The receptor immunoreceptor tyrosine activation motif (ITAM) domains propagate the phagocytosis signal by being phosphorylated by one of the tyrosine kinases such as Lyn, Fgr, and Hck. Phosphorylation of ITAM promotes recruitment of the Syk tyrosine kinases. The Syk tyrosine kinase activates Dbl family guanine nucleotide exchange factors (GEFs) leading to the activation of Rac and Cdc42. Cdc42 interacts directly with WASP and WASP interacts with the Arp2/3 complex. The Arp2/3 complex causes the polymerization of actin, which leads to the phagocytosis of the pathogen. In contrast, complement receptor (CR) mediated phagocytosis is characterized by the complement opsonized particle sinking into the membrane and there are no lamellipodia formed. This is dependent on Rho but little is known about the exact signaling processes involved. Permission for use of image obtained from the publisher (37).

# Chapter 2: *Yersinia pestis* can bypass protective antibodies to LcrV and activation with IFNγ to survive and induce apoptosis in murine macrophages


## Summary

*Yersinia pestis*, the agent of plague, uses a type III secretion injectisome to deliver Yop proteins into macrophages to counteract phagocytosis and induce apoptosis (28). Additionally, internalized *Y. pestis* can survive in phagosomes of naïve or IFNγ-activated macrophages by blocking vacuole acidification (3). The *Y. pestis* LcrV protein is a target of protective antibodies. Binding of antibodies to LcrV at the injectisome tip results in neutralization of apoptosis of *Y. pestis*-infected macrophages and is used as an in vitro correlate of protective immunity (27). The cytokines IFNγ and TNFα can cooperate with anti-LcrV antibodies (also referred to as anti-V) to promote protection against lethal *Y. pestis* infection in mice (86, 95). It is not known if these phagocyte-activating cytokines cooperate with anti-LcrV to increase killing of the pathogen and/or decrease apoptosis in macrophages.

We investigated how anti-LcrV and IFNγ impact bacterial survival and apoptosis in cultured murine macrophages infected with *Y. pestis* KIM5. *Y. pestis* KIM5 opsonized with polyclonal or monoclonal anti-LcrV were used to infect macrophages treated with or without IFNγ. Phagocytosis and survival of KIM5 and apoptosis of macrophages were measured at different time points post infection (p.i.). Results show that anti-LcrV reduced apoptosis at an early time point (5 h) but not at a later time point (24 h) in naïve

macrophages. Polyclonal anti-LcrV was unable to inhibit apoptosis at either time point in IFNγ-activated macrophages. Additionally, anti-LcrV was ineffective at promoting killing of KIM5 in naïve or activated macrophages. We conclude that *Y. pestis* can bypass protective antibodies to LcrV and macrophage activation with IFNγ to survive and induce apoptosis in murine macrophages.

## Introduction

*Y. pestis* is efficiently phagocytosed and survives within phagosomes of naïve murine macrophages when the bacteria are grown at 28°C prior to in vitro infection (46, 105, 125). *Y. pestis* can block phagosome acidification, which may be important for survival in macrophages (105). Growth of *Y. pestis* at 37°C prior to infection promotes Yop delivery during phagocytosis, and as a result the efficiency of bacterial uptake by macrophages is reduced. However, ~20-35% of 37°C-grown *Y. pestis* bacteria that associate with macrophages are internalized (32, 140). Yop-expressing *Y. pestis* that are internalized by naïve macrophages are able to survive intracellularly (71). In addition, macrophages infected with 37°C-grown *Y. pestis* die of YopJ induced apoptosis (44, 71, 140). Thus, Yop-expressing *Y. pestis* can counteract anti-bacterial functions of naïve macrophages by intracellular survival and induction of apoptosis if they are unable to avoid phagocytosis. Lukaszewski et al. showed that naïve mice infected with *Y. pestis* can harbor *Y. pestis* within $CD11b^+$ spleen macrophages for several days p.i. and that a significant percentage of these phagocytes die of apoptosis during this time period (73).

There are three translocator proteins: YopB, YopD, and LcrV. These three components are necessary to deliver the effector Yops into the host cell (85). YopB and YopD form a pore in the host membrane, and LcrV is important for the correct assembly of this translocation pore. The mechanism of how LcrV mediates translocation is not fully understood. LcrV has been shown to localize to the tip of the injectisome (85). LcrV, also known as V antigen, has many other important roles. It has a regulatory role in Yop secretion within the bacterium. It is also a soluble protein that is an important protective antigen (85, 140).

The detailed mechanism of all the effects of LcrV and their relevance during infection is still unclear. An area of debate within the literature is the mechanism of how anti-LcrV antibodies provide protection against plague. There are several proposed mechanisms. The first proposed mechanism is that anti-LcrV antibodies inhibit LcrV-elicited IL-10 production (41). Therefore, anti-LcrV antibodies may neutralize LcrV-induced immunosuppression. The second possible mechanism of protection by anti-LcrV antibodies is in preventing LcrV-mediated inhibition of PMN chemotaxis (32, 141).

The third mechanism is that LcrV antibodies decrease Yop translocation. One group found that when they used a strain of *Y. pestis* with all Yop effector genes deleted to infect mice, anti-LcrV antibodies did not exhibit a protective effect compared to the control non-protective antibody (99). This result indicates that the inhibition of Yop translocation is the most important aspect of LcrV-mediated protection. Cowan et al. (32), showed that anti-LcrV antibody-induced phagocytosis of *Y. pestis* is important and that only with phagocytosis does one observe a decrease in Yop translocation.

Mice can be protected against lethal *Y. pestis* infection by passive immunization with anti-LcrV antibodies (52-54, 63, 120, 121, 129, 143). Opsonization with anti-LcrV antibodies increases phagocytosis of *Y. pestis* by macrophages (32, 99, 140). Increased phagocytosis of *Y. pestis* mediated by anti-LcrV antibody opsonization is associated with reduced Yop translocation (32, 99) and reduced apoptosis (32, 99, 140). The ability of anti-LcrV antibodies to inhibit apoptosis in macrophages infected with *Y. pestis* is commonly used as a measure of neutralizing activity (12, 140, 143, 148).

In addition to antibodies, the cytokines IFNγ and TNFα are important for protective immune responses against *Y. pestis* infection (36, 63, 86, 95, 120, 121). *Y. pestis* can survive in macrophages activated with IFNγ (73, 103-105), but significantly reduced intracellular persistence of the bacteria is observed when macrophages are exposed to both IFNγ and TNFα (73).

Macrophages are important for anti-LcrV mediated protection against *Y. pestis* in livers of infected mice (32, 99). In addition to protecting macrophages from apoptosis, it is possible that opsonization with anti-LcrV decreases survival of *Y. pestis* in phagocytes, due to the ability of Fc receptors to activate bactericidal processes. Activation of macrophages with IFNγ is known to upregulate Fc receptor expression and function, which could further increase intracellular killing following uptake of anti-V-opsonized *Y. pestis* (63, 120, 121). It is also possible that activation per se could decrease the sensitivity of *Y. pestis*-infected macrophages to undergo apoptosis.

We investigated how opsonization with anti-LcrV antibodies, and activation with IFNγ, impacts the ability of *Y. pestis* to survive within and kill macrophages. The

findings have implications for understanding how anti-LcrV antibodies and cytokines function to protect against plague.

## Results

**Opsonization with anti-V serum increases phagocytosis of *Y. pestis* by naïve but not activated macrophages**. In order to study how anti-LcrV antibodies and IFNγ impact the ability of *Y. pestis* to survive within and kill macrophages, we utilized mouse anti-LcrV immune serum (anti-V), previously shown to be protective (58), and cultured mouse macrophages (RAW 264.7 macrophage-like cell line or BMDMs). To confirm that the anti-V used in this study contained antibodies that could increase uptake of *Y. pestis* by macrophages, a phagocytosis assay was performed. A culture of *Y. pestis* KIM5/GFP (Table 2.1) was shifted from 28°C to 37°C in the presence of IPTG to induce expression of the T3SS and GFP. Samples of the bacteria were preincubated with normal mouse control serum, anti-V or PBS (non-opsonized) for 10 min prior to infection. Naïve RAW 264.7 cells were then infected with the bacteria at a MOI of 10 for 25 min and fixed. Phagocytosis was measured by fluorescence-based microscopy. The percent internalization of non-opsonized KIM5/GFP was ~30%, which was significantly lower than the control serum condition (~45%), likely due to the absence of complement proteins in the non-opsonization condition (Figure 2.1A). KIM5/GFP preincubated with anti-V were phagocytosed to a significantly higher level (~65%) as compared to bacteria

preincubated with control serum (Figure 2.1A) showing that this serum contained anti-LcrV antibodies that could increase phagocytosis of *Y. pestis* by macrophages.

We next examined the interaction between IFNγ-stimulated macrophages and KIM5/GFP opsonized with or without anti-V. The phagocytosis assay was performed as above except that RAW 264.7 cells were activated with IFNγ (100 U/ml) for 24 h before infection. With IFNγ-activated macrophages there was an overall increase in bacterial uptake, but the increase was most dramatic for the non-opsonized and control serum-opsonized conditions (Figure 2.1B). Approximately 55-60% of non-opsonized or control opsonized KIM5/GFP were internalized. Anti-V opsonized KIM5/GFP was internalized at a slightly higher level (~75%), but this increase was not significant as compared to the control. These results showed that the activation state of the macrophage was important for the ability of anti-V to increase phagocytosis of *Y. pestis*. The fact that increased phagocytosis of *Y. pestis* was observed following exposure to IFNγ indicated that the cytokine treatment was upregulating antibacterial functions in macrophages.

**Opsonization of *Y. pestis* with anti-V serum decreases apoptosis in naïve but not in activated macrophages at an early stage of infection.** We next examined the effect of anti-V sera on YopJ-induced macrophage death using an LDH release assay. Naïve BMDMs were infected at a MOI of 10 with KIM5 (Table 2.1) grown and opsonized as above. BMDMs were also infected in parallel with the non-cytotoxic KIM5 *yopB* mutant (Table 2.1) as a control. After allowing for phagocytosis to occur for 25 min, survival of extracellular bacteria was prevented by the addition of a low concentration of gentamicin to the tissue culture medium. This infection protocol was

used because it allows for measurement of intracellular survival and apoptosis under the same infection conditions. KIM5, being a *pgm* mutant, lacks the *ripCBA* genes required for growth of *Y. pestis* in activated macrophages (103), and therefore to avoid decreases in survival due to absence of the *ripCBA* genes, the CFU-based survival assays (see Figure 2.3) were limited to a short time period (5 h). LDH release was initially determined at 5 h p.i.

Results showed that macrophages infected with the non-opsonized *yopB* mutant, deficient in Yop translocation, released very low amounts of LDH, about 2.5% of total, by 5 h of infection (Figure 2.2A). This was significantly lower LDH release as compared to BMDMs infected with control serum-opsonized KIM5 (~18%). As compared to BMDMs infected with control serum-opsonized KIM5, LDH was also significantly lower when KIM5 was opsonized with anti-V (3%) but not when the bacteria were left unopsonized (12.5%). These results were consistent with previous studies, which showed that rabbit polyclonal anti-LcrV antibodies could reduce Yop translocation and decrease apoptosis in naive J774A.1 macrophage-like cells infected with *Y. pestis* (32, 99, 140).

When the LDH assay was performed with IFNγ-activated macrophages, only the BMDMs infected with the non-opsonized *yopB* mutant released significantly lower levels of LDH as compared to the control serum condition (2.3% vs. 18%; Figure 2.2C). Thus, the ability of anti-V to inhibit apoptosis was diminished in activated macrophages.

**Opsonization with anti-V does not decrease survival of *Y. pestis* in activated macrophages.** To determine if opsonization of *Y. pestis* with anti-V would result in increased killing within BMDMs, we performed CFU assays at various times post-

infection. Naïve BMDMs were infected with opsonized or non-opsonized bacteria as above for the LDH assay. As a preliminary test to confirm that the BMDMs used were bactericidal under the conditions of the assay, a CFU assay was performed with 37°C-grown *Y. pseudotuberculosis* alongside KIM5. Internalization of non-opsonized 37°C-grown *Y. pseudotuberculosis* into BMDMs triggers a bactericidal process that requires macrophage sensing of the T3SS (108, 151). As shown in Figure 2.3A, by 5 h p.i. there was a significant decrease in CFU for *Y. pseudotuberculosis* 32777. As reported previously (71), there was no significant decrease in CFU over the same time period in BMDMs infected with non-opsonized KIM5 (Figure 2.3A). Although it is unclear why the T3SS of *Y. pestis* KIM5 did not stimulate intracellular killing in BMDMs, these results confirmed that the macrophages used were bactericidal. Furthermore, because the T3SS of KIM5 did not stimulate intracellular killing in BMDMs, we did not face the problem of multiple bactericidal processes occurring simultaneously, which could complicate analysis of the potential role of anti-V opsonization and IFNγ activation in decreasing survival of *Y. pestis* in macrophages.

Next, CFU assays were carried out using opsonized or non-opsonized KIM5. A KIM5 *phoP* mutant (Table 2.1), shown previously to be highly defective for intracellular survival (45), was included as a control. As shown in Figure 2.3B, by 5 h p.i. there was a significant (~2 log) decrease in intracellular CFU that could be recovered from the macrophages infected with non-opsonized KIM5 *phoP*. In contrast, no significant decrease in CFU was observed for the non-opsonized KIM5 *yopB* mutant, or KIM5 under any condition (non-opsonized, anti-V, or control serum; Figure 2.3B). Based on these

results, we concluded that opsonization with anti-V does not lead to intracellular killing of KIM5 by naïve BMDMs.

Seeing no evidence for intracellular killing of anti-V-opsonized KIM5 in naïve BMDMs, a CFU assay was performed with macrophages activated with IFNγ (Figure 2.3C). As with the naïve BMDMs we used KIM5 *phoP* as a control for intracellular killing. The results obtained with IFNγ-stimulated BMDMs were similar to those seen with naïve BMDMs, as only KIM5 *phoP* showed a significant decrease in CFU by 5 h post-infection (Figure 2.3C). Therefore, *Y. pestis* that was opsonized with anti-V prior to uptake was able to survive in macrophages that were activated with IFNγ.

**Opsonization of *Y. pestis* with anti-V serum does not decrease apoptosis in naïve or activated macrophages at a late stage of infection.** Under the infection conditions used in this study, in which internalized *Y. pestis* survive within macrophages (Figure 2.3) (71), the kinetics of apoptosis is extended, such that LDH release is first detected by 5 h p.i. (early stage; Figure 2.2), and continues to increase over the next 19 h (late stage) (71). To determine if opsonization with anti-V would affect apoptosis at a late stage of infection, an LDH release assay was performed on *Y. pestis*-infected naïve or activated macrophages at 24 h p.i. Interestingly, only macrophages infected with the KIM5 *yopB* mutant released significantly lower levels of LDH as compared to the control (Figure 2.2B, 2.2D). Thus, there was no significant decrease in apoptosis at 24 h in either naïve or activated macrophages when KIM5 was opsonized with anti-V prior to infection (Figure 2.2B, 2.2D).

The result with naïve macrophages was unexpected, since we had observed an ~6 fold decrease in apoptosis in BMDMs infected with anti-V opsonized KIM5 as compared to control opsonized KIM5 at 5 h p.i. (Figure 2.2A) and assumed this proportional difference would be maintained to 24 h (Figure 2.2B). Instead, the results suggested that apoptosis was accelerated in BMDMs infected with anti-V opsonized *Y. pestis* between 5 and 24 h as compared to the control infection. Since anti-V opsonized KIM5 was internalized by naïve macrophages at a higher level as compared to control opsonized KIM5 (Figure 2.1A), it was possible that larger numbers of internalized bacteria allowed for higher levels of cell death between 5 and 24 h p.i.

Macrophages treated with IFNγ appeared to be less sensitive to YopJ-dependent cell death, as the overall levels of LDH released from KIM5-infected BMDMs after 24 h were lower in activated cells as compared to the naïve phagocytes (compare Figure 2.2B and 2.2D). Activation with LPS desensitizes macrophages to YopJ/YopP-induced apoptosis during infection with *Y. pseudotuberculosis* or *Y. enterocolitica* (14, 109), and a similar phenomenon may be operating in IFNγ-activated BMDMs infected with *Y. pestis*.

**Opsonization of *Y. pestis* with anti-V serum does not alter secretion of TNFα by infected macrophages.** TNFα has been shown to cooperate with IFNγ to promote protective immunity against *Y. pestis* (121). In addition, macrophages activated with TNFα and IFNγ are more bactericidal against *Y. pestis* than macrophages treated with IFNγ alone (73). Previous studies have shown that *Y. pestis* can partially suppress secretion of TNFα from infected macrophages and that YopJ is required for this suppression (71, 149). Lilo et al. showed that after 24 h of infection ~2000 pg/ml of

TNFα was released by KIM5 infected BMDMs (71). We wanted to determine if TNFα was secreted during our infections with KIM5 and to what degree opsonization affected TNFα levels. In parallel, we determined levels of another cytokine (IL-1β) that is secreted from KIM5-infected macrophages in a YopJ-dependent manner (71).

BMDMs were infected with opsonized or non-opsonized KIM5 or non-opsonized KIM5*yopB* (Figure 2.4). Supernatants were collected at 24 h and ELISA was performed to measure TNFα and IL-1β. We found that BMDMs infected with KIM5*yopB* released ~5000 pg/ml of TNFα (Figure 2.4A). Consistent with the concept that YopJ can interfere with production of TNFα, the level of TNFα was diminished to ~2500pg/ml in KIM5-infected macrophages. When KIM5 was opsonized with control serum or anti-V, the levels of TNFα that were secreted from infected macrophages were similar to those seen with non-opsonized KIM5 (Figure 2.4A), showing that anti-V did not significantly affect secretion of TNFα. Additionally, opsonization with anti-V did not affect secretion of IL-1β from BMDMs infected with KIM5 (Figure 2.4B).

**Opsonization of *Y. pestis* with anti-LcrV mAb 7.3 does not decrease survival in macrophages or inhibit late stage apoptosis.** Polyclonal and monoclonal antibodies (mAb) that are specific for the same antigen can exhibit different activities with respect to neutralizing function (24). The anti-V used in these experiments had been shown to passively protect mice against *Y. pestis* KIM5, a conditionally virulent strain, in an intravenous infection model (58). However, it was desirable to repeat the above experiments using a well-characterized protective mAb specific for LcrV. Opsonization of *Y. pestis* with anti-LcrV IgG1 mAb 7.3 (54) increases phagocytosis and decreases

apoptosis in naïve J774A.1 cells (140). In addition, mAb 7.3 is known to provide protection against fully virulent *Y. pestis* in murine bubonic and pneumonic plague infections (52-54). We opsonized KIM5/GFP or KIM5 with mAb 7.3, and repeated the phagocytosis, intracellular survival and LDH release assays. In parallel, macrophages were infected with *Y. pestis* that was left non-opsonized or incubated with an IgG1 isotype control mAb specific for YopD (mAb 248.19). Figure 2.5 shows that mAb 7.3 significantly increased phagocytosis of KIM5/GFP by naïve (A), but not activated RAW 264.7 cells (B), as compared to the control. Opsonization with mAb 7.3 did not result in significant killing of *Y. pestis* in either naïve or activated BMDMs (Figure 2.6A and B). Opsonization of KIM5 with mAb 7.3 significantly reduced apoptosis at the early infection time point (5 h) in naïve BMDMs (Figure. 2.7A), but not at the late time point (24 h) in either naïve or activated macrophages (Figure 2.7B and D, respectively). These results were similar to those obtained with anti-V (Figures 2.1-2.3). The only difference between mAb 7.3 and anti-V was that opsonization with the former significantly reduced apoptosis in activated macrophages at the 5 h time point (Figure 2.7C).

**Anti-V opsonization does not overcome the block in acidification of *Y. pestis*-containing phagosomes.** To date, studies that have shown a block in acidification of *Y. pestis*-containing phagosomes in macrophages have used 28°C-grown non-opsonized bacteria (105). To determine if opsonization with anti-V would increase phagosome acidification, we infected J774A.1 mouse macrophage-like cells and used microscopy to measure colocalization of *Y. pestis*-containing phagosomes with the acidotropic probe Lysotracker Red DND-99 (105). Prior to infection, KIM5/GFP was grown at 28°C for 2

h or at 37°C for 2 h and then left non-opsonized or opsonized with control serum or with anti-V. Colocalization of GFP expressing KIM5 and Lysotracker Red was determined at 1.25 h p.i. As a positive control for phagosome acidification, we used KIM5/GFP fixed with PFA. Only phagosomes containing fixed KIM5/GFP (both 28°C- and 37°C-grown) showed significantly increased colocalization with Lysotracker Red (>90%) as compared to the standard condition (non-opsonized, 28°C) (Figure 2.8). We concluded that opsonization with anti-V does not overcome the block in phagosome acidification imposed by *Y. pestis* in macrophages.

## Discussion

This study focused on investigating the potential cooperative effect of anti-V opsonization and IFNγ activation in allowing macrophages to protect themselves from being colonized and killed by *Y. pestis.* We conclude that *Y. pestis* can efficiently evade the combined effects of anti-V opsonization and IFNγ activation in macrophages due to its ability to block phagosome acidification. In fact, we obtained evidence that activation with IFNγ could be counterproductive for antibody-mediated protection, as anti-V was unable to significantly reduce apoptosis at the early time point in activated macrophages infected with KIM5 (although this effect was not seen with mAb 7.3). On the other hand, activation with IFNγ increased overall levels of bacterial phagocytosis and decreased apoptosis at late stages, illustrating a protective activity of this cytokine.

We also obtained evidence that the ability of anti-V to inhibit apoptosis decreased as the time of the infection was extended. This suggests that intracellular bacteria can

contribute to apoptosis and that anti-V is not neutralizing once the bacteria are internalized. It is not clear if intracellular survival of *Y. pestis* is important for late stage apoptosis. In the case of *Y. pseudotuberculosis,* results suggest that protein synthesis is required for intracellular bacteria to induce apoptosis (151). Perhaps internalized KIM5 causes increased apoptosis by continuing to synthesize and translocate YopJ for a limited period of time within nascent phagosomes, but bacterial survival is not required per se.

In our studies we activated macrophages using IFNγ only. There is evidence that TNFα cooperates with IFNγ to kill *Y. pestis* in macrophages in absence of anti-V (73). Perhaps the addition of TNFα is required along with anti-V and IFNγ in macrophages to increase killing of *Y. pestis*. While we did not add exogenous TNFα in our CFU assays, we note that KIM5-infected macrophages secrete detectable levels of TNFα by 4 h p.i. (71), and ~2500 pg/ml TNFα was detected in supernatants by 24 h p.i. (Figure 2.4). Therefore, we can conclude that *Y. pestis* can bypass protection mediated by anti-V antibodies, pre-activation with IFNγ and the autocrine activation mediated by endogenous TNFα produced during infection with KIM5.

These results have implications for understanding the mechanism of protective immunity to plague mediated by anti-LcrV and phagocyte-activating cytokines. As our results suggest that opsonization with anti-LcrV does not dramatically increase killing of *Y. pestis* by activated macrophages, it is possible that neutrophils may be more critical for protection than macrophages (32, 99).

Consistent with this idea is the demonstration that macrophages are not essential for anti-V-mediated protection against *Y. pestis* in spleens of infected mice (32, 99). Philipovskiy et al. (99) found that the protective effect of anti-LcrV antibody in mice

involved macrophages in the liver but not in the spleen. They used fas-induced apoptosis (Mafia) transgenic mice to deplete all macrophages. They treated the mice with protective LcrV antibodies and infected with *Y. pestis*, and subsequently isolated organs and did a colony forming unit assay to enumerate the bacteria in the organs. They found that in the liver, without macrophages present, anti-LcrV was not effective and there were similar bacterial loads between mock and protective antibody-treated mice. In the spleen without macrophages, anti-LcrV treated mice had significantly less bacteria compared to mock treated mice. Overall, they concluded that macrophages are important mediators of protection in the liver and that another cell type may be important in the spleen.

Neutrophils appear to be important for anti-V-mediated protection in both spleens and livers of mice challenged with plague (32). Cowan et al. (32) showed that neutrophils are the major mediators of protection by anti-LcrV antibodies against KIM5 in mice. They used anti-Gr-1 antibody to deplete mice of their neutrophils and then treated with anti-LcrV antibodies. After this treatment they infected with *Y. pestis* strain KIM5 and found that mice depleted of neutrophils and treated with anti-LcrV were unable to reduce bacterial levels in the liver and spleen. As a result, they concluded that neutrophils are crucial to anti-LcrV mediated protection.

It is possible that TNFα cooperates with IFNγ to upregulate microbicidal processes in neutrophils. Anti-V increases phagocytosis of *Y. pestis* by murine neutrophils (32). *Y. pestis* is unable to survive in rodent (25) or human neutrophils (122). It has been shown that Yop delivery does not protect intracellular *Y. pestis* from neutrophil killing (122). However, whether anti-V opsonization accelerates killing of *Y. pestis* in neutrophils has not been examined.

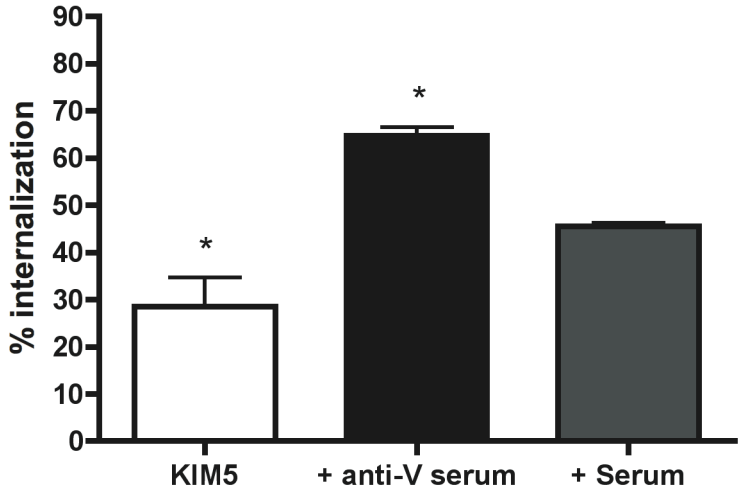
Neutralization of apoptosis in *Y. pestis*-infected macrophages by anti-V is commonly used as an in vitro correlate of protective immunity (12, 140, 143, 148). Simply put, an in vitro correlate of protective immunity is an in vitro assay whose result correlates with protection as seen in vivo. Although this assay has many positive features including ease of use, reproducibility, and a quantifiable readout, we feel the following points should be considered with respect to improving the in vitro correlate of protective immunity assay. First, our results showing that anti-V can promote uptake of *Y. pestis* into macrophages without killing the bacteria, and that anti-V may not inhibit translocation of YopJ by the intracellular population, indicates that the time point at which apoptosis levels are quantified is critically important. In addition, the activation status of the macrophage is an important variable, as we found that IFNγ-activated macrophages are less sensitive to apoptosis. Finally, *Y. pestis* strains differ in their ability to induce apoptosis (KIM has high activity (71)) and not all strains require apoptosis for virulence (68, 150). Therefore, there continues to be a need to develop an anti-LcrV neutralization assay that does not rely on macrophage apoptosis as a read out.

## Figures and Tables

**Table 2.1** *Yersinia* Strains used in Chapter 2.

| Strain | Relevant Characteristics | Reference |
|---|---|---|
| *Y. pestis* | | |
| KIM5 | pCD1Ap, pMT1$^{+}$, pPCP1$^{+}$, pgm$^{-}$, Amp$^{r}$ | (71) |
| KIM5/GFP | KIM5/pMMB207gfp3.1, Amp$^{r}$, Cam$^{r}$ | (45) |
| KIM5 *phoP* | pCD1Ap *phoP* in frame deletion | (45) |
| KIM5 *yopB* | pCD1Ap *yopB* in frame deletion of nucleotides 496-774, Amp$^{r}$ | (71) |
| *Y. pseudotuberculosis* | | |
| 32777 | pYV$^{+}$ | (116) |

A.




B.

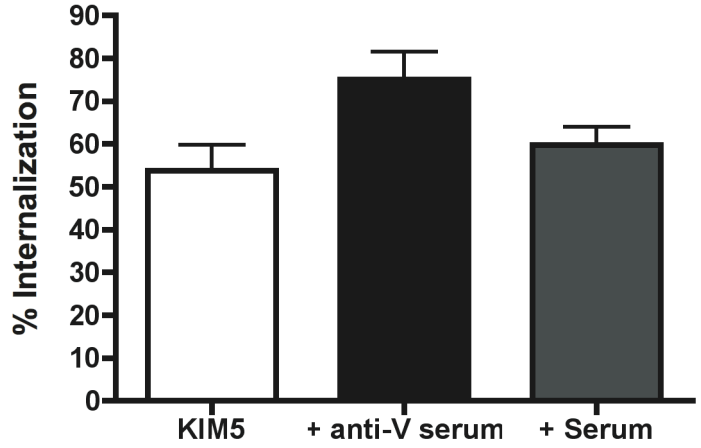
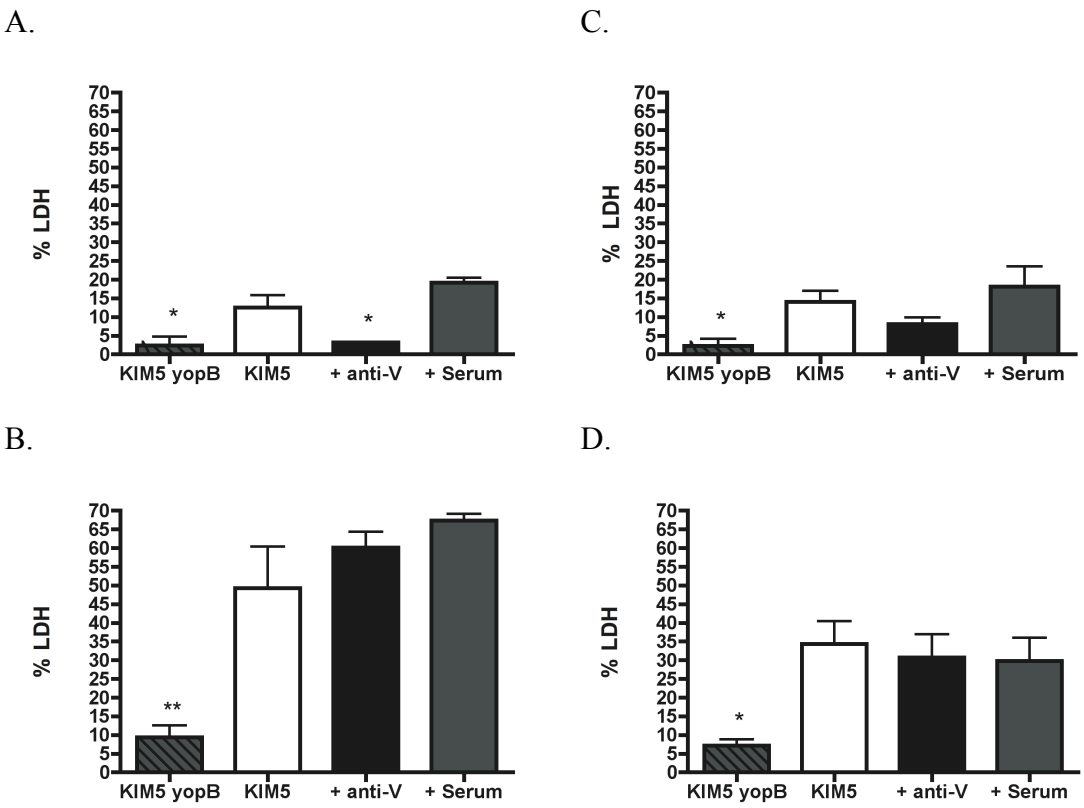



**Figure 2.1**

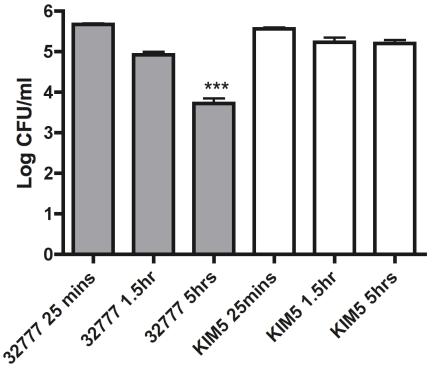
Phagocytosis assay with KIM5-infected naïve or IFNγ-stimulated Raw 264.7 macrophages on coverslips. KIM5/GFP was grown under T3SS inducing conditions and incubated with PBS (KIM5), anti-V (+ anti-V serum), or control serum (+ Serum) prior to infecting naïve macrophages (A) or IFNγ-stimulated macrophages (B). Twenty-five min post-infection the cells were fixed and extracellular bacteria immunofluorescently labeled. Extracellular and intracellular bacteria from three fields per experiment were counted by fluorescence microscopy and the % internalization determined. Results were taken from three independent experiments and averaged. The error bars are S.D. ($p<0.05$, * as compared to the control serum condition).



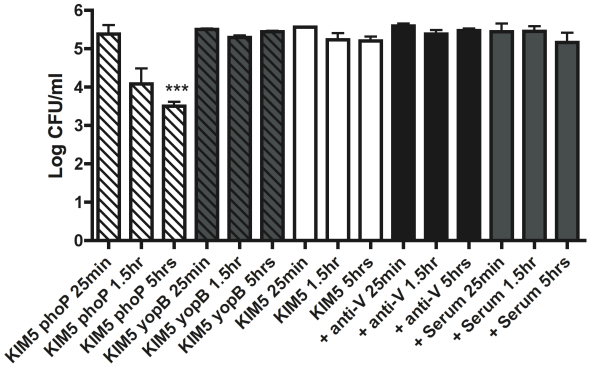

## Figure 2.2

Examination of YopJ-mediated cell death in *Y. pestis*-infected macrophages through determination of LDH release. Naïve BMDMs (A and B) and IFNγ-stimulated BMDMs (C and D) were left uninfected or infected with nonopsonized KIM5 or nonopsonized KIM5 *yopB*, or KIM5 opsonized with the indicated sera. LDH levels in supernatants of the BMDMs at 5 h (A and C) and 24 h (B and D) post-infection were measured. Results were normalized by subtracting background levels of LDH from uninfected macrophages and are shown as percent of total LDH. Results were taken from three independent experiment and averaged. Error bars are S.D. ($p<0.05$, *; $p<0.01$, ** as compared to control serum).

A.




B.




C.

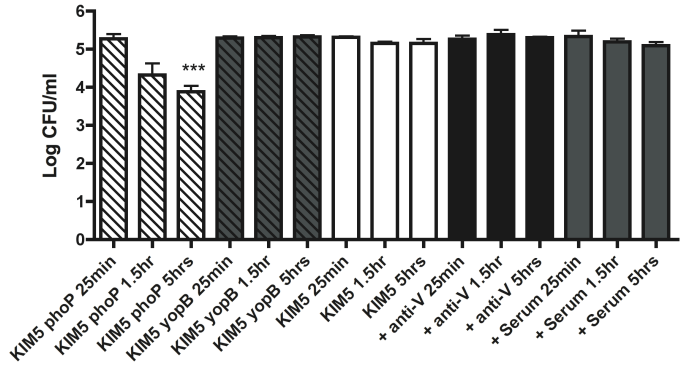
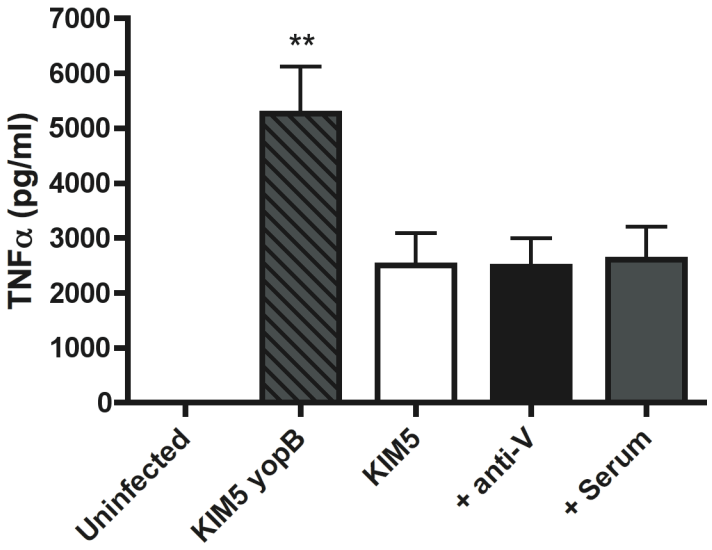



**Figure 2.3**

Comparison of the intracellular survival of *Y. pestis* and *Y. pseudotuberculosis* 32777 in BMDMs. Naïve BMDMs (A and B) and IFNγ-stimulated BMDMs (C) were infected with the indicated strains with or without opsonization with sera and intracellular bacterial survival was determined at 25 min, 1.5 h, and 5 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.001$, *** as compared to 25 min within the same strain)

A.




B.

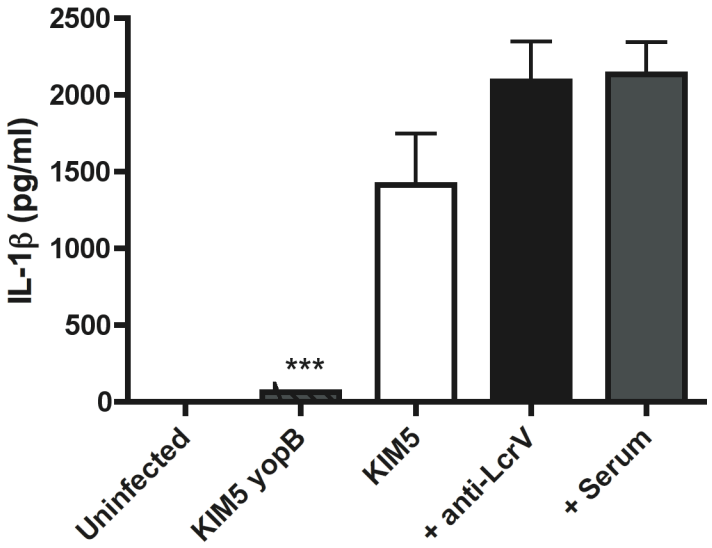
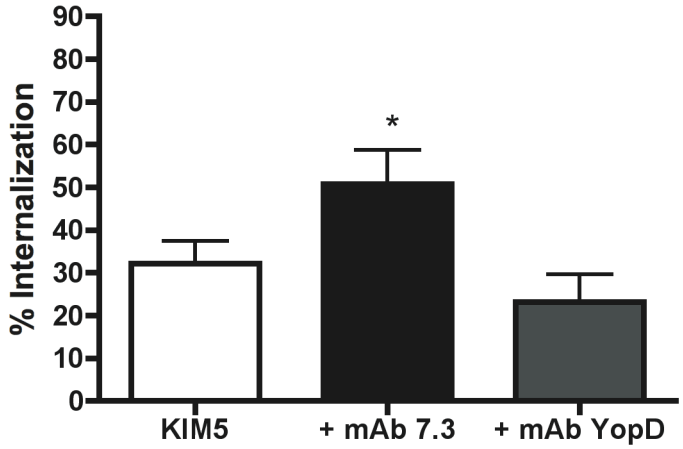



**Figure 2.4**

Monitoring TNFα and IL-1β levels in supernatants of *Y. pestis*-infected BMDMs. BMDMs were left uninfected or infected with the indicated strains. Supernatants were collected after 24 h of infection and TNFα and IL-1β levels were measured by ELISA. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.01$, **; $p<0.001$, *** as compared to serum).

A.




B.

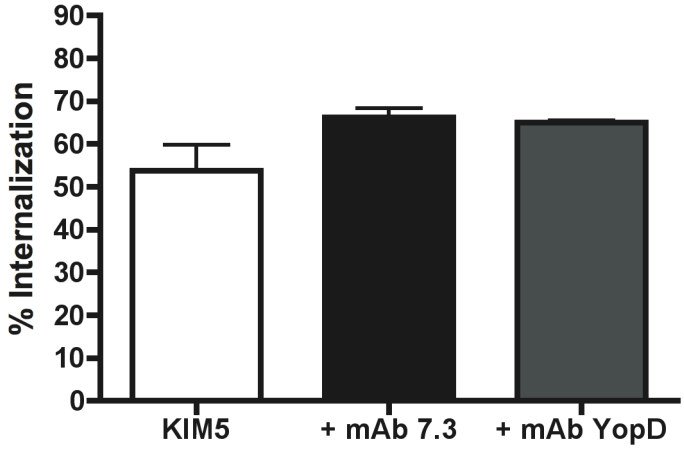
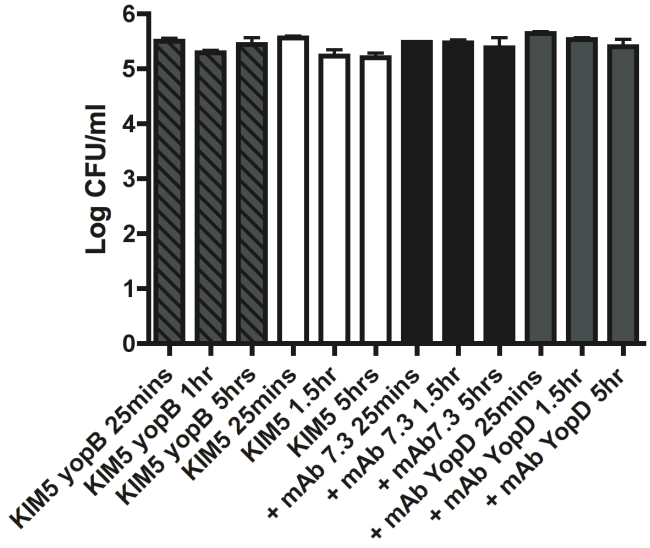



**Figure 2.5**

Phagocytosis assay with KIM5 infected naïve or IFNγ-stimulated Raw 264.7 macrophages. KIM5/GFP was left unopsonized or incubated with anti-LcrV mAb 7.3 or anti-YopD mAb prior to infecting naïve macrophages (A) or IFNγ-stimulated macrophages (B). Twenty-five min p.i. the cells were fixed and the % internalization was determined as in the legend in Fig. 2.1. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.05$, * as compared to mAb YopD).

A.




B.

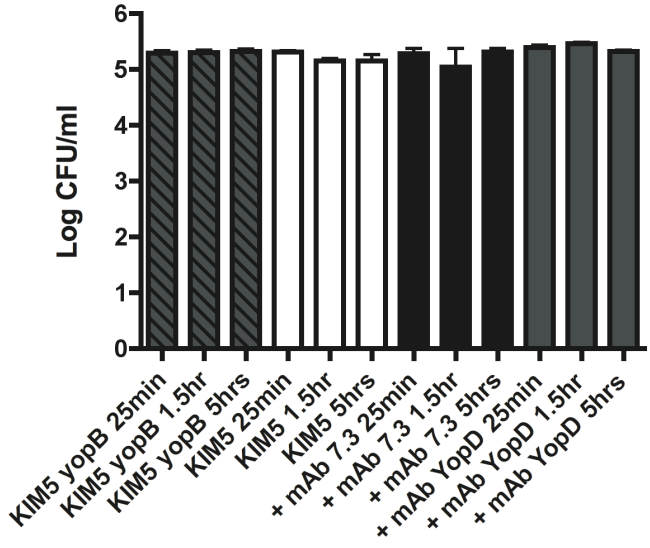



**Figure 2.6**

Effect of monoclonal antibodies on the intracellular survival of *Y. pestis* in BMDMs. Naïve BMDMs (A) and IFNγ-stimulated BMDMs (B) were infected with the indicated strains with or without preincubation with monoclonal antibodies and intracellular bacterial survival was determined at 25 min, 1.5 h, and 24 h p.i. as indicated in the legend to Fig. 2.3. Results were taken from three independent experiments and averaged. Error bars are S.D.

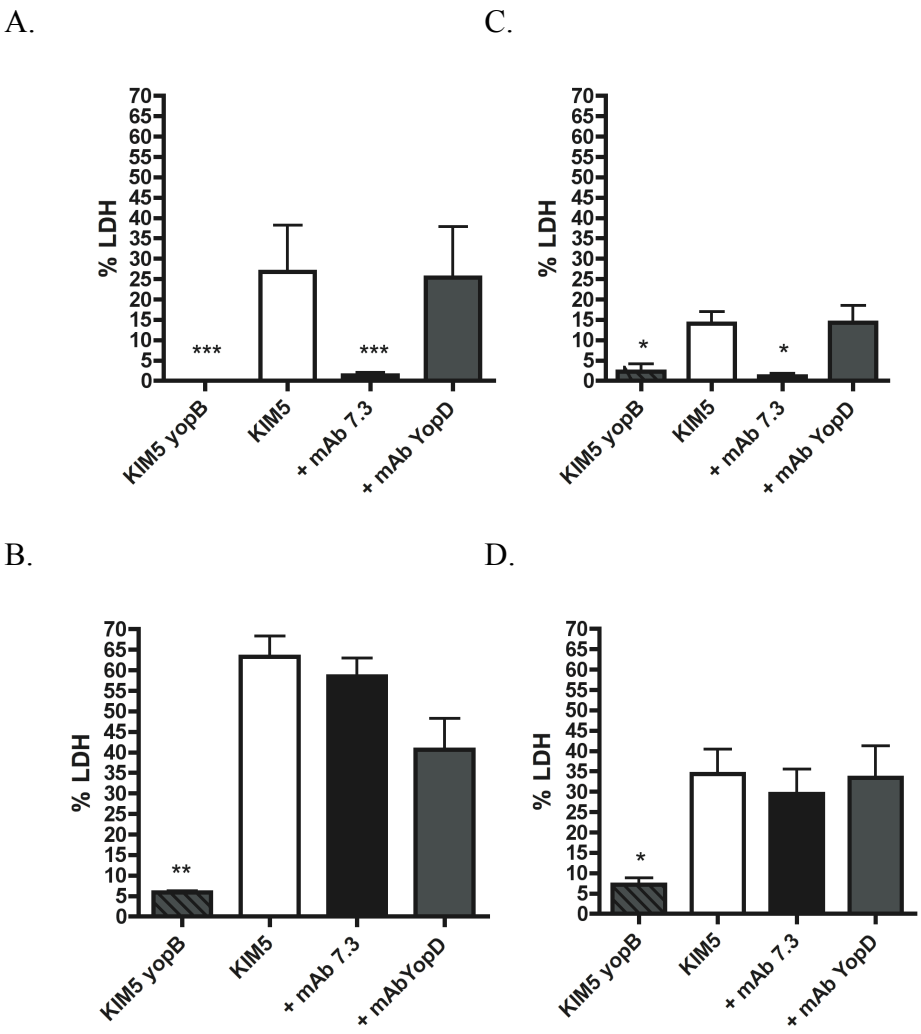
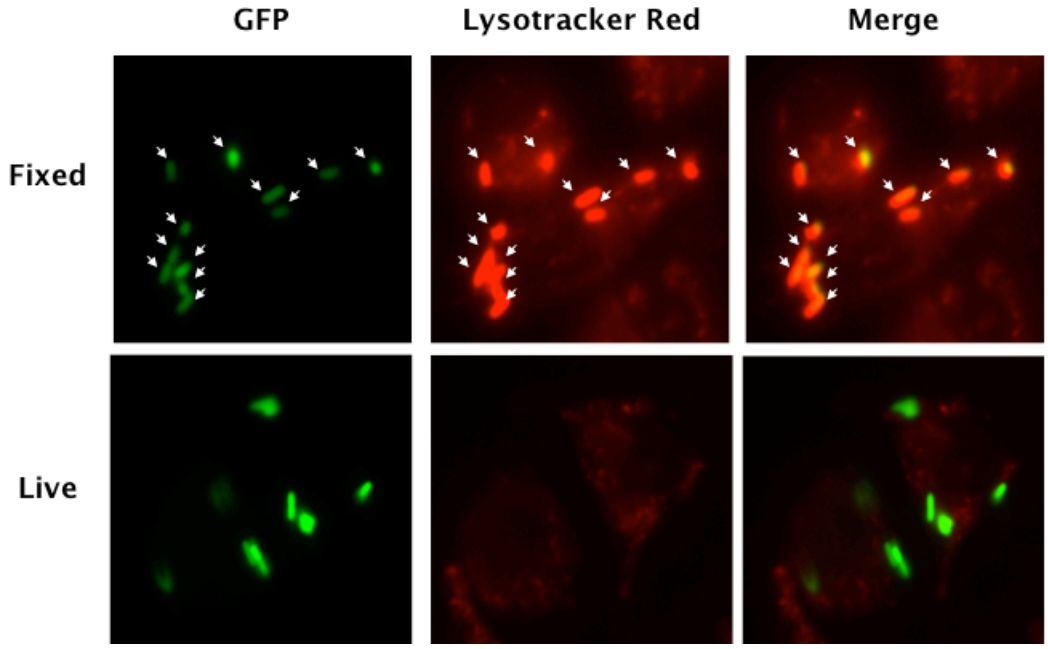



**Figure 2.7**
Effect of monoclonal antibodies on YopJ-mediated cell death through determination of LDH release. Naïve BMDMs (A and B) or IFNγ-stimulated BMDMs (C and D) were infected with the indicated strains of KIM5 with or without preincubation with monoclonal antibodies or left uninfected. LDH levels in supernatants of the BMDMs at 5 h (A and C) and 24 h (B and D) p.i. were measured as indicated in the legend to Fig. 2.2. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.05$, *; $p<0.01$, ** as compared to mAb YopD).

A.

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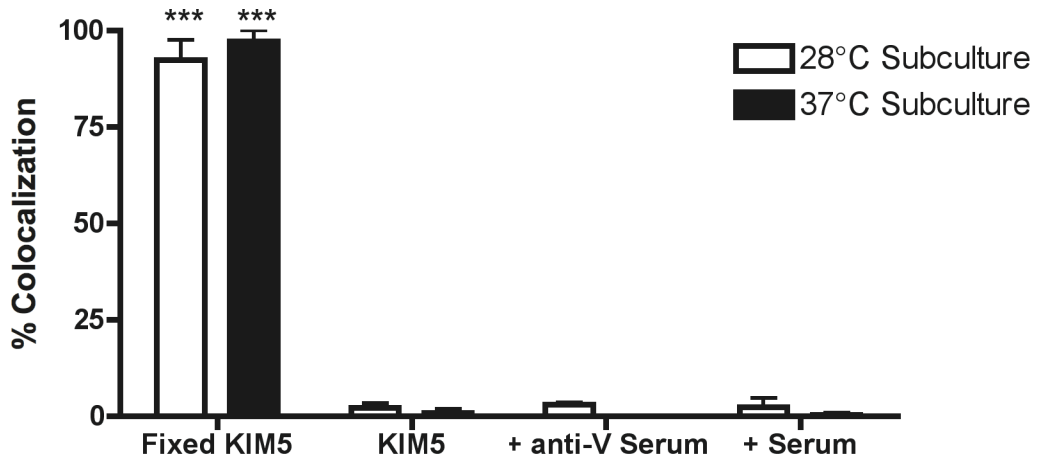



**Figure 2.8**

The effect of bacterial growth, temperature and serum opsonization on phagosome acidification within naïve J774A.1 cells. J774A.1 cells were infected with fixed or live KIM5/GFP pregrown at 28°C or 37°C and left unopsonized or opsonized as indicated. Lysotracker Red DND 99 was used to label acidic compartments and colocalization with KIM5/GFP was determined a 1.25 h p.i. by fluorescence microscopy (A). White arrow heads indicate areas of colocalization between bacteria and acidic compartments labeled with Lysotracker Red DND 99 (A). Results were taken from three independent experiments and averaged (B). Error bars are S.D. ($p<0.001$, *** as compared to KIM5 28°C).

# Chapter 3: Examination of antibodies to YopB, D and E for the ability to increase phagocytosis, survival and bactericidal functions in macrophages infected with *Y. pestis*.

## Summary

*Y. pestis* uses a type III secretion injectisome to deliver effector proteins, Yops, into macrophages. This process requires three translocator proteins YopB, YopD, and LcrV (123). LcrV is a well-known protective antigen recognized by antibodies and is a major candidate for inclusion in a subunit vaccine. One drawback for using LcrV as a vaccine is that it contains a variable region. LcrV sequences from *Y. pestis* and the other two human pathogenic *Yersiniae*, *Y. pseudotuberculosis* and *Y. enterocolitica*, are different and can be used interchangeably (84). This sequence variability may make antibody-antigen specificity a problem. Alternative antigens, such as YopB and YopD, must be investigated for their protective effect because of this (139).

A complex of Yops B, D and E (BDE) investigated as an experimental vaccine was shown to elicit protective activity against *Y. pestis* infection in mice (58). We examined the effect of anti-BDE opsonization on bacterial uptake and intracellular survival, as well as apoptosis of *Y. pestis* infected macrophages. Opsonization of KIM5 with anti-BDE did not significantly increase phagocytosis of *Y. pestis*, increase intracellular killing of the bacteria, or decrease levels of apoptosis late in infection.

We also examined the effect of immune sera raised against a YopB and D complex (BD) or YopB alone (B) on bacterial uptake. Although vaccination of mice with BD did elicit protection against *Y. pestis* in mice, opsonization of KIM5 with anti-

BD or anti-B did not significantly increase uptake of KIM5 by naïve macrophages. Anti-B antibody opsonization did significantly increase uptake of KIM5 by IFNγ-stimulated RAW 264.7 cells. These results indicate that the assays used may not be applicable for determining in vitro correlates of protection mediated by antibodies against YopB and YopD.

## Introduction

*Y. pestis* is a human pathogenic bacterium that is the causative agent of bubonic and pneumonic plague. While plague has been historically devastating, there are still natural reservoirs in the U.S. and other parts of the world (29). Although if detected early enough, plague can be easily treated with antibiotics, antibiotic resistant strains have been isolated. Because of the ease of aerosolization and the highly fatal nature of pneumonic plague, *Y. pestis* could be used as a biological weapon. This, combined with the development of antibiotic-resistant isolates, presents a need for new vaccines to aid in the prevention of plague (42).

There are no safe and effective vaccines currently available that protect against pneumonic and bubonic plague (120). Currently a subunit vaccine is being developed. This vaccine would consist of F1 and LcrV. F1 protein forms an amorphous antiphagocytic capsule on *Y. pestis* in vivo and with prolonged exposure to 37°C in vitro (34, 97). It is encoded on pMT1. Mice vaccinated with recombinant F1 have been shown to be protected against both bubonic and pneumonic plague (120). While this antigen seems promising, *Y. pestis* strains deficient for F1 have been shown to be equally

virulent as the $F1^+$ bacteria (97). Because of this, F1 alone would not make a suitable subunit vaccine.

LcrV is another protein that is being considered for inclusion in a subunit vaccine along with F1. LcrV is a multifunctional virulence protein shown to localize to the tip of the type III secretion system (T3SS) injectisome tip (85). Mice actively vaccinated with LcrV or passively immunized with anti-LcrV antibodies are protected against both bubonic and pneumonic forms of plague (129). However, *Y. pseudotuberculosis* and *Y. enterocolitica* express LcrV proteins with sequence variation that does not allow for cross protection (140). As a result, LcrV is not a perfect antigen for inclusion in a subunit vaccine. Because of these considerations, other antigens need to be investigated for potential use as vaccine candidates.

Other components of the T3SS are being studied as potential protective antigens. These include the T3SS needle component YscF as well as YopB and YopD, which are involved in effector translocation into host cells. YscF has been shown to function as a protective antigen as well as YopD (7, 76). However, YopD was only protective against F1 deficient *Y. pestis* and not $F1^+$ *Y. pestis* (7). Ivanov et al. showed that mice actively immunized with a complex of YopBDE (BDE) or a complex of YopBD (BD) were protected against $F1^-$ KIM5 and not $F1^+$ KIM5 (58).

In this chapter we investigated antibodies against BDE, BD, or YopB (B) generated in Ivanov et al. (58). The established in vitro correlates for anti-V mediated protection include an increase in phagocytosis of *Y. pestis* by macrophages as well as decreased YopJ-mediated apoptosis early in infection. We wanted to see if protective

antibodies against the translocator proteins would have similar functional activities as anti-V.

## Results

**Characterization of anti-BDE serum for the ability to increase phagocytosis of *Y. pestis* by naïve or activated macrophages**. In order to determine if antibodies raised against BDE could increase uptake of *Y. pestis* by macrophages, a phagocytosis assay was performed. An overnight culture of *Y. pestis* KIM5/GFP was shifted from 28°C to 37°C in the presence of IPTG to induce expression of the T3SS and GFP respectively. Bacteria were preincubated with normal mouse serum, anti-BDE, anti-V (used as a positive control for increased uptake), or PBS (non-opsonized KIM5) for 10 min prior to infection. Naïve RAW 264.7 cells were then infected with the bacteria at an MOI of 10 for 25 min and then fixed. Phagocytosis was measured by fluorescence-based microscopy and the percent internalization was determined. The percent internalization for non-opsonized KIM5/GFP was ~32%, which was significantly lower than the control serum (45%) (Figure 3.1A). This difference may be accounted for by the absence of complement proteins in the non-opsonized KIM5/GFP infection condition. As expected, KIM5/GFP preincubated with anti-V serum were phagocytosed to a significantly higher level (~63%) than bacteria preincubated with control serum (Figure 3.1A). The percent internalization of anti-BDE preincubated KIM5/GFP was ~ 55%, which was not significantly different from the control serum condition. This shows that this serum did

not contain anti-BDE antibodies that could significantly increase phagocytosis of *Y. pestis* by macrophages.

Next, we examined the interaction between IFNγ-stimulated macrophages and KIM5/GFP opsonized with or without anti-BDE. The phagocytosis assay was performed as described above except that the RAW 264.7 macrophages were activated with 100 U/ml of IFNγ for 24h prior to infection. With pre-activated macrophages there was an overall increase in bacterial internalization (Figure 3.1B). Non-opsonized KIM5 had a dramatic increase in uptake (~50%) by IFNγ-stimulated macrophages (Figure 3.1B). However, when compared to the control serum opsonization condition, the presence of anti-V antibodies or anti-BDE antibodies did not significantly increase internalization by IFNγ-stimulated Raw 264.7 cells. The presence of IFNγ alone was enough to increase internalization, indicating that this treatment upregulated antibacterial effects in macrophages; however the addition of anti-BDE antibodies did not increase this affect.

**Characterization of anti-BDE for the ability to decrease survival of *Y. pestis* in macrophages**. To determine if opsonization of *Y. pestis* with anti-BDE would result in increased killing within RAW 264.7 cells, we performed CFU assays at various times post-infection. Naïve Raw 264.7 cells were infected with opsonized or non-opsonized KIM5. No significant decrease in CFU was observed for non-opsonized KIM5 or KIM5 with anti-V, anti-BDE, or control serum (3.2A). These results indicate that opsonization with anti-BDE does not lead to intracellular killing of KIM5 by naïve RAW 264.7 cells.

Since there was no intracellular killing of KIM5 under any condition (non-opsonized, anti-BDE, anti-V, or control serum) by naïve RAW 264.7 cells, CFU assays

were performed with IFNγ-stimulated RAW 264.7 cells (Figure 3.2B). Since IFNγ increases antibacterial effects, we wanted to see if that combined with protective antibodies increases intracellular killing. There was no significant decrease in intracellular survival of KIM5 among any conditions, which is similar to the results seen with naïve RAW 264.7 cells. Therefore KIM5 pre-opsonized with anti-BDE was able to survive in IFNγ-stimulated RAW 264.7 cells.

**Opsonization of *Y. pestis* with anti-BDE serum and examination of apoptosis in naïve or activated macrophages at a late stage of infection.** To determine if opsonization with anti-BDE would affect apoptosis of BMDMs at a late stage of infection, a LDH release assay was performed on *Y. pestis*-infected naïve macrophages at 24 h p.i. Macrophages infected with the KIM5 *yopB* mutant released significantly lower levels of LDH as compared to control serum opsonized KIM5 (Figure 3.3). KIM5 opsonized with anti-BDE had high levels of LDH release by 24 h p.i. This result was not unexpected because phagocytosis was not significantly affected by addition of anti-BDE (Figure 3.1). Anti-LcrV was unable to decrease apoptosis at this stage of infection as well (Figure 3.3).

**Opsonization of *Y. pestis* with anti-BDE serum and determination of levels of TNFα and IL-1β secretion by infected macrophages.** We wanted to determine to what degree opsonization with anti-BDE affected levels of TNFα secretion from *Y. pestis*-infected macrophages. YopJ is required for *Y. pestis* to suppress secretion of

TNFα from infected macrophages (71, 148). We also determined the levels of IL-1β, which was shown to be secreted in a YopJ dependent manner from KIM5-infected macrophages by Lilo et al. (71). BMDMs were infected with opsonized or non-opsonized KIM5 or non-opsonized KIM5 *yopB* (Figure 3.4). Supernatants were collected at 24 h, and TNFα and IL-1β levels were measured by ELISA. We found that BMDMs infected with KIM5 *yopB* released ~5000 pg/ml TNFα (Figure 3.4A). With KIM5 without opsonization the level of TNFα secretion was diminished to ~2500 pg/ml. When KIM5 was opsonized with control serum or anti-BDE, the levels of TNFα that were released from the infected macrophages were similar to each other and to non-opsonized KIM5 (Figure 3.4A). This indicates that anti-BDE did not significantly affect the secretion of TNFα. Examining IL-1β levels showed that opsonization with anti-BDE did not affect the secretion of the cytokine from KIM5 infected BMDMs (Figure 3.4B).

**Opsonization with anti-BD or anti-B serum and measurement of phagocytosis of *Y. pestis* by naïve macrophages or activated macrophages**. We next carried out macrophage infections with *Y. pestis* KIM5 opsonized with anti-BD or anti-B immune sera generated for in vivo studies (58). To examine if the anti-BD and anti-B sera contained antibodies that could increase uptake of *Y. pestis* by macrophages, a phagocytosis assay was performed as described above. The percent internalization of non-opsonized KIM5 was ~30%, which is significantly lower than uptake with KIM5/GFP opsonized with control serum (55%) (Figure 3.5A). We included KIM5 with anti-BDE sera for comparison. The percent internalization of KIM5/GFP opsonized with anti-BDE serum was ~65%. This value was not significantly different compared to the

control serum condition. The percent internalization with anti-BD and anti-B opsonization was ~55% and ~70% respectively. However, these values are not significantly different from opsonization with control serum. This indicates that the anti-BD and anti-B sera do not contain antibodies that significantly increase phagocytosis of *Y. pestis* by naïve RAW 264.7 macrophages.

We next examined the interaction between IFNγ-stimulated RAW 264.7 cells and KIM5/GFP with or without opsonization. With IFNγ treatment there was an overall increase in uptake by RAW 264.7 cells. Over 50% of the control serum opsonized KIM5 were internalized; this was significantly more than without serum present (Figure 3.5B). Although uptake of KIM5 with anti-BDE, anti-BD, or anti-B increased with IFNγ treatment of RAW 264.7 cells, only KIM5 opsonized with anti-B was internalized significantly more compared to the control serum condition.

## Discussion

This study focused on comparing, with respect to *Y. pestis*-macrophage interactions, the functional consequences of antibodies directed against different components of the T3SS translocon. We examined uptake of anti-BDE, anti-BD, or anti-B opsonized KIM5 by naïve and IFNγ stimulated macrophages. We found that these sera did not contain antibodies that promoted increased phagocytosis as compared to control serum opsonized KIM5, except for the case of IFNγ activated macrophages, which phagocytosed significantly more anti-B opsonized KIM5 compared to control bacteria.

It was suggested that any antibody that increases phagocytosis of *Y. pestis* by macrophages would be protective (32). While the anti-BDE serum was shown to be protective, it was only shown to be so with the $F1^-$ KIM5 strain (58). When mice were challenged with $F1^+$ KIM5, the protective effect seen after $F1^-$ KIM5 challenge was no longer seen. Perhaps if the phagocytosis studies described above were performed with $F1^-$ KIM5, then a significant increase in uptake may have been seen. Or perhaps the mechanism of protection for anti-BDE does not involve increased phagocytosis as a consequence.

We also examined the effect of anti-BDE on Yop mediated apoptosis through an LDH release assay at 24 h p.i. We found that anti-BDE did not significantly affect the release of LDH and that these levels were similar to those found when infecting with KIM5 or KIM5 with control serum. This result may not be too surprising, because anti-V serum was shown to not have an effect on LDH release at 24 h p.i., even though it is protective (Chapter 2). Lastly, we examined the effect of anti-BDE opsonization of KIM5 on release of the cytokines TNFα and IL-1β from infected BMDMs. Again, we found that anti-BDE had no affect on the cytokine levels detected in supernatants collected at 24 h p.i.

An in vitro correlate for protection with anti-BDE still remains to be developed. Perhaps our experiments, primarily the phagocytosis assays with naïve or IFNγ activated macrophages, would have yielded different results if we used an $F1^-$ strain of KIM5 since that was the strain where anti-BDE and anti-BD were found to be protective (58). On the other hand, the conditions used to grow KIM5 for these assays are not expected to result in high levels of F1 expression. It was suggested that the F1 capsule could be masking

the antigens so these epitopes cannot be bound by antibodies. However, since F1 is not necessary for *Y. pestis* virulence, the YopBDE complex vaccine would be worth studying further.

# Figures

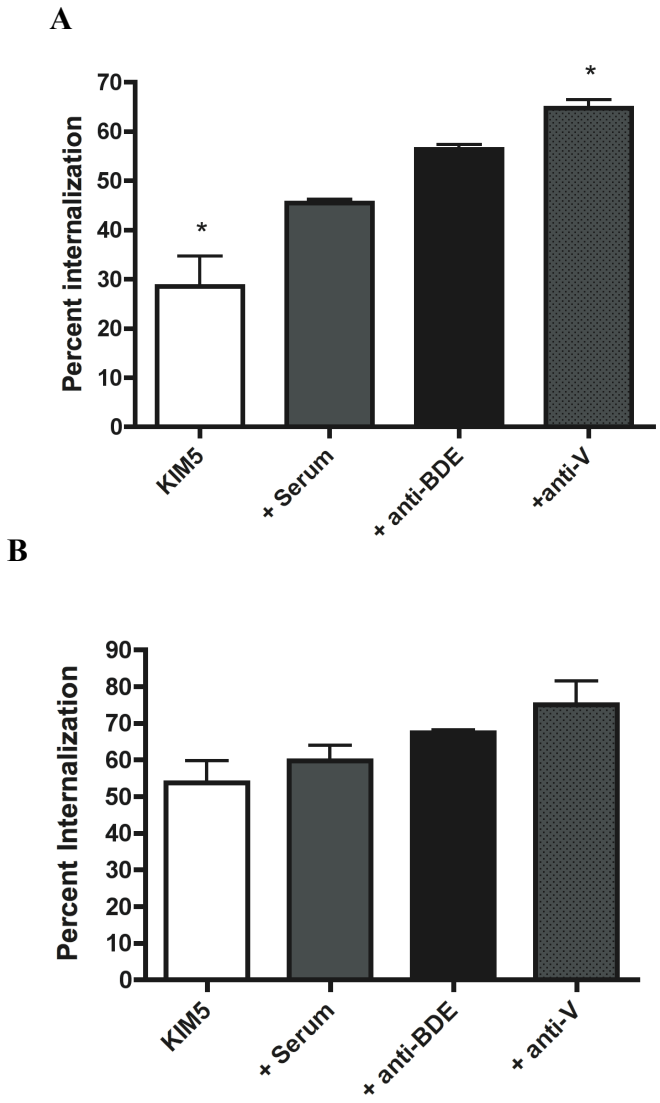



**Figure 3.1**

Phagocytosis assay with or without opsonization in KIM5-infected naïve or IFNγ-stimulated macrophages. KIM5/GFP was grown under T3SS inducing conditions and incubated with PBS (KIM5), anti-YopBDE (+ anti-BDE serum), anti-LcrV (+ anti-V), or control serum (+ Serum) prior to infecting naïve RAW 264.7 macrophages (A) or IFNγ-stimulated macrophages (B). Twenty-five min p.i. the cells were fixed and extracellular bacteria immunofluorescently labeled. Extracellular and intracellular bacteria from three fields per experiment were counted by fluorescence microscopy and the % internalization determined. Results were taken from three independent experiments and averaged. The error bars are S.D. ($p<0.05$, * as compared to the control serum condition).

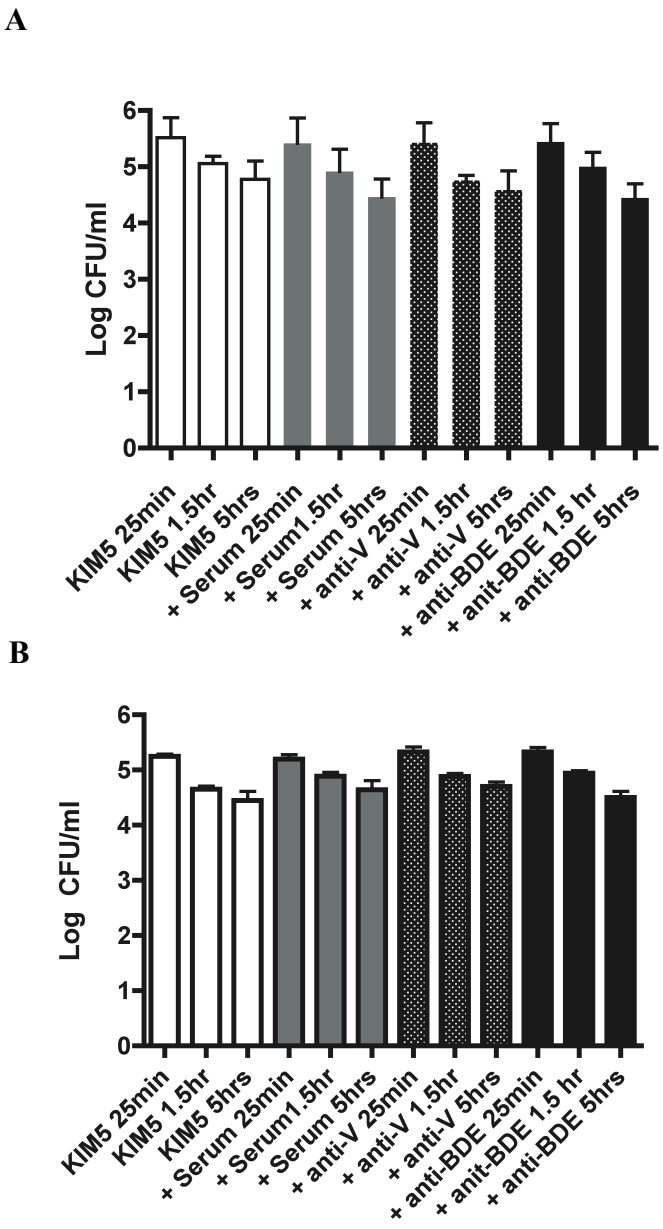



**Figure 3.2**

Comparison of the intracellular survival of opsonized and non-opsonized *Y. pestis* in macrophages. Naïve BMDMs (A) and IFNγ-stimulated BMDMs (B) were infected with the indicated strains with or without opsonization with sera and intracellular bacterial survival was determined at 25 min, 1.5 h, and 24 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D.

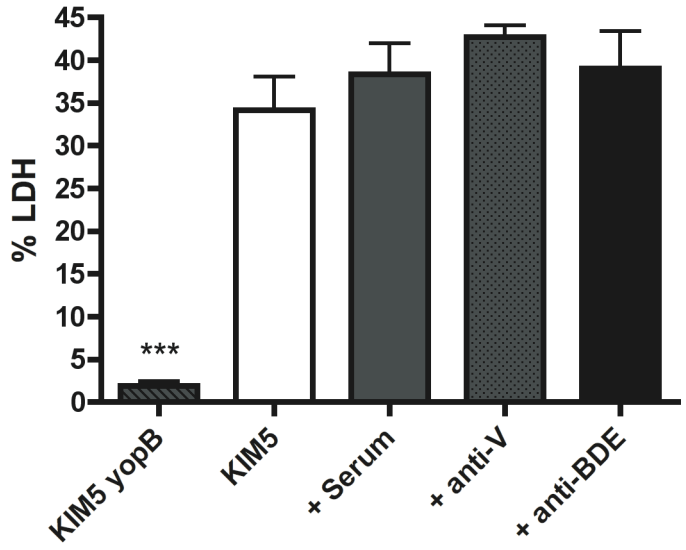



**Figure 3.3**

Examination of YopJ-mediated cell death in macrophages infected with *Y. pestis* with or without opsonization through determination of LDH release. Naïve BMDMs were left uninfected or infected with nonopsonized KIM5 or KIM5 *yopB*, or KIM5 opsonized with the indicated sera. LDH levels in supernatants of the BMDMs 24 h p.i. were measured. Results were normalized by subtracting background levels of LDH from uninfected macrophages and are shown as percent of total LDH. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.001$, *** as compared to control serum).

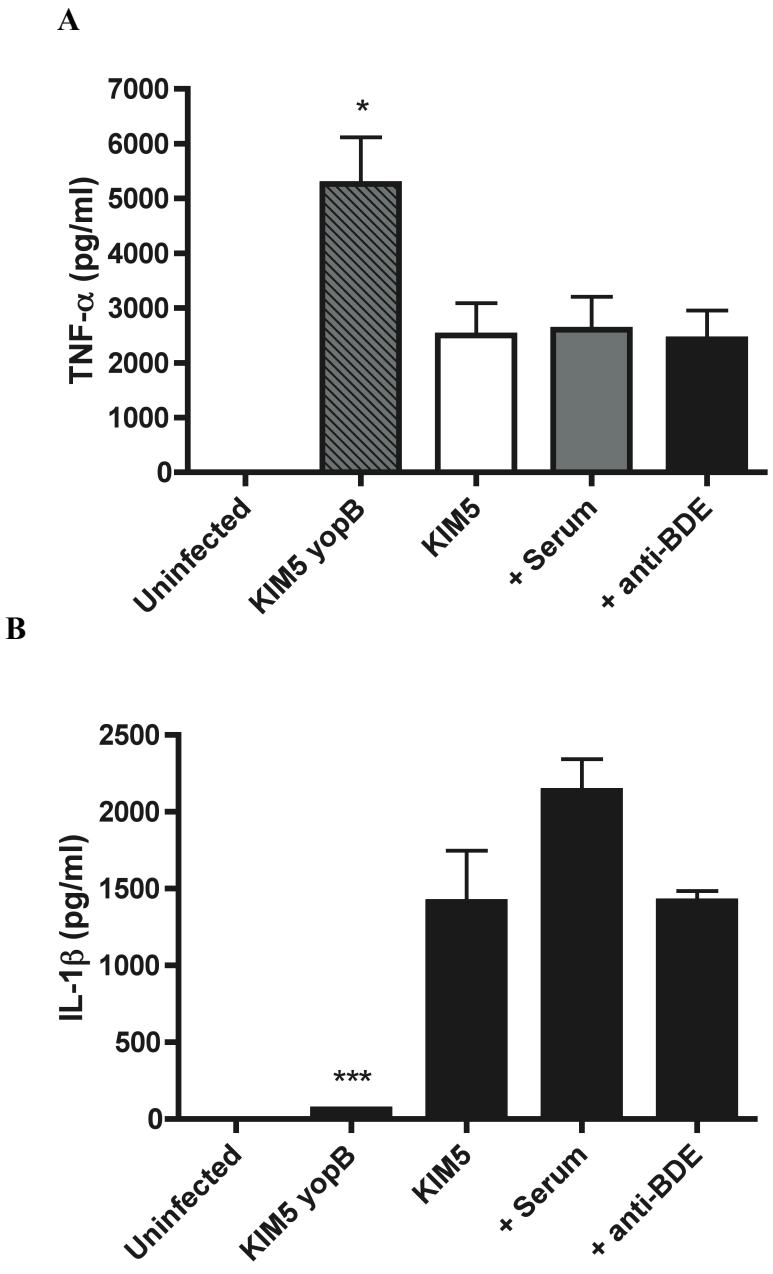
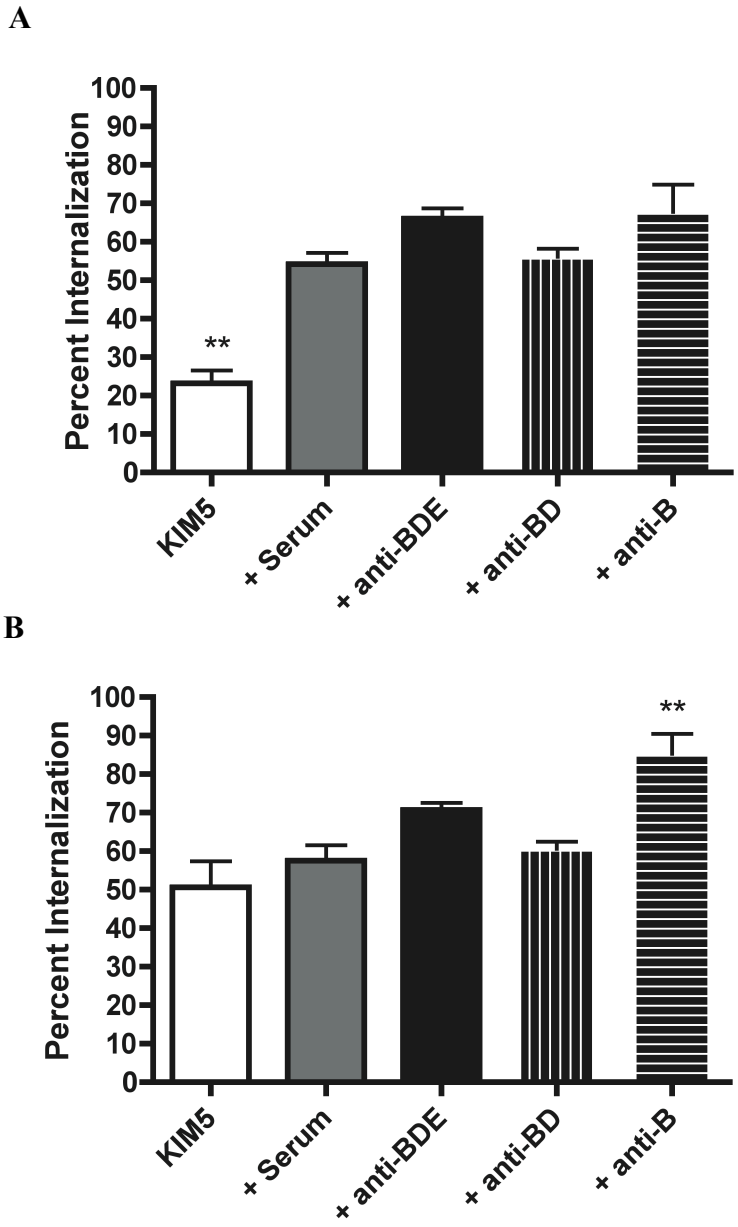



**Figure 3.4**

Monitoring TNFα and IL-1β levels in supernatants of macrophages infected with *Y. pestis* in the presence or absence of opsonization. BMDMs were left uninfected or infected with the indicated strains. Where indicated KIM5 was opsonized with anti-BDE or control serum. Supernatants were collected after 24 h of infection and TNFα and IL-1β levels were measured by ELISA. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.05$, *; $p<0.001$, *** as compared to KIM5).




## Figure 3.5

Phagocytosis assay with or without opsonization in KIM5-infected naïve or IFNγ-stimulated macrophages. KIM5/GFP was left unopsonized or incubated with anti-BDE, anti-BD, or anti-B prior to infecting naïve RAW 264.7 macrophages (A) or IFNγ-stimulated macrophages (B). Twenty-five min post-infection the cells were fixed and the % internalization was determined as in the legend to Figure 3.1. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.01$, ** as compared to Serum).

# Chapter 4: Decreased survival of *Y. pseudotuberculosis* in macrophages is associated with expression of YopO

## Summary

*Yersinia pseudotuberculosis* and *Yersinia pestis* are two closely related human pathogenic bacterial species. *Y. pseudotuberculosis* is an enteropathogen from which *Y. pestis* recently evolved into the highly infectious and lethal causative agent of bubonic and pneumonic plague (145). They share a virulence plasmid (termed pYV in *Y. pseudotuberculosis* and pCD1 in *Y. pestis*) that codes for a type III secretion system (T3SS) and effector proteins, *Yersinia* outer proteins (Yops), which function to antagonize normal macrophage signaling responses(29). *Y. pestis* is known to survive within macrophages, and in recent studies, *Y. pseudotuberculosis* was shown to survive within macrophages only when pYV had been removed or was not expressed, suggesting that the T3SS and/or Yops may play a negative role in intracellular survival (151). Understanding the mechanism of the decreased survival of *Y. pseudotuberculosis* within macrophages may help to understand the remarkable ability of *Y. pestis* to survive inside phagocytes.

Murine bone marrow derived macrophages were infected with *Y. pseudotuberculosis* strains IP2666 wild-type (wt), IP2666 pYV$^-$ (IP2666c), IP2666 *yopO* (IP32), IP2666 *yopK* (IP36), or IP2666 *yopEHOMKJ* (IP37). CFU assays were performed at 25 min, 1.5 h, and 5 h p.i. Survival was also examined using a GFP

induction assay at 24 h p.i. Our results suggest that YopO may be responsible for the decreased survival of IP2666 within macrophages.

To assess if YopO was the pYV-encoded factor that caused intracellular killing of IP2666, we complemented IP32 with *yopO* from *Y. pestis* KIM5 encoded on an IPTG inducible expression vector. Survival assays were performed, and we found that *yopO* from KIM5 was unable to complement IP32. The predicted sequences of YopO from *Y. pseudotuberculosis* and *Y. pestis* strains KIM5 and CO92 were compared and amino acid differences were found, which could possibly explain the inability of *yopO* from KIM5 to complement as well as the difference in intracellular survival between *Y. pseudotuberculosis* and *Y. pestis*.

## Introduction

*Y. pseudotuberculosis* is a human enteropathogen closely related to *Y. pestis*. *Y. pseudotuberculosis* enters the host via the fecal-oral route and infects the gut associated lymphoid tissue by penetrating the M-cells over the Peyer's patches (102). After penetrating the M-cells, the bacteria interact with resident macrophages, and they can subsequently migrate to lymph nodes. Primarily, this agent causes a self-limiting infection that rarely is fatal to the host.

*Y. pseudotuberculosis* and *Y. pestis* both contain an approximately 70 kb virulence plasmid termed pYV or pCD1, respectively (29). The virulence plasmid encodes a T3SS composed of a secretion apparatus, chaperones, effector proteins (Yops), and translocator proteins (YopB, YopD, and LcrV). There are six effectors that have a variety of

functions that serve to distort the host immune response (88). For example, YopJ has been shown to inhibit the release of TNFα, which diminishes the inflammatory response, as well as induce apoptosis (29). YopO, also known as *Yersinia* protein kinase A (YpkA), is one of several Yops that has been shown to have an antiphagocytic effect.

YopO/ YpkA contains two distinct domains, a kinase domain and a GDI (guanine nucleotide dissociation inhibitor) domain, as well as an actin binding domain. YopO/YpkA is known to disrupt the actin cytoskeleton. The kinase domain and the GDI domain have been shown to additively contribute to the disruption of the actin cytoskeleton (65). The kinase activity of YopO/YpkA has been shown to be dependent on actin association in vitro. The kinase domain has also been shown to phosphorylate and thereby inhibit Gαq, which is a subunit of a heterotrimeric G-protein involved in many pathways such as RhoA activation, that leads to the inhibition of stress fiber formation and any pathway dependent on Gαq activation (89). The GDI domain binds to and inhibits nucleotide exchange in Rac and RhoA, causing inhibition of stress fiber formation (65).

While *Y. pseudotuberculosis* and *Y. pestis* have many similarities there are also several differences. *Y. pestis* can survive exceptionally well within macrophages. However, *Y. pseudotuberculosis* 32777 has decreased intracellular survival within macrophages over time (151). The T3SS was shown to be a major determinant for decreasing intracellular survival of *Y. pseudotuberculosis* 32777. When macrophages were infected with *Y. pseudotuberculosis* lacking the virulence plasmid, the bacteria were able to survive well within macrophages (151).

We investigated whether the survival defect of *Y. pseudotuberculosis* 32777 in macrophages was also found in another strain, IP2666. We also tried to elucidate what virulence plasmid associated factor(s) cause *Y. pseudotuberculosis* to be unable to survive intracellularly. We determined that *Y. pseudotuberculosis* lacking only YopO was able to survive intracellularly, indicating that YopO may be the factor that when present causes decreased intracellular survival of *Y. pseudotuberculosis*.

## Results

***Y. pseudotuberculosis* wild-type strains have decreased survival in macrophages.** Zhang et al. showed that *Y. pseudotuberculosis* strain 32777 that had been grown for maximal expression of the T3SS exhibited a significant survival defect within macrophages (151). To determine if this survival defect was specific to 32777, a serogroup I strain, we performed CFU assays at various times post-infection using 32777 and IP2666, a serogroup III strain. BMDMs were infected with 32777 or IP2666 at a MOI of 10 and intracellular survival was monitored by CFU assay.

As expected, 32777 exhibited decreased CFUs by 5 h p.i. (Figure 4.1). IP2666 had similar bacterial levels as compared to 32777 over time. The Log CFU/ml decreased significantly by 5 h p.i. compared to 25 min p.i. (Figure 4.1). This indicates that *Y. pseudotuberculosis*, from both serotype I and III, grown under T3SS permissive conditions, displays a significant survival defect within macrophages. We decided to

continue our studies with IP2666 since we had a panel of *yop* mutants already derived from this strain.

***Y. pseudotuberculosis* wild-type strain has decreased survival in macrophages compared to a pYV$^-$ or *yopEHOMKJ* mutants.** To examine the intracellular survival of *Y. pseudotuberculosis* strains lacking pYV, or all Yop effectors, CFU assays were performed using IP2666 as a control for decreased intracellular survival, and IP2666c, a pYV cured strain, as well as IP37, a multi-effector mutant (*yopEHOMKJ*). It was previously shown that 32777c could survive well intracellularly (151) so we predicted that IP2666c would be able to survive better than IP2666. In fact, IP2666c did show increased survival over time in BMDMs as compared to IP2666 (Figure 4.2), confirming that *Y. pseudotuberculosis* strains behave similarly within macrophages regardless of serotype and that removal of pYV results in increased intracellular survival. IP37 was also able to survive better in BMDMs through 5 h p.i. as compared to IP2666 (Figure 4.2), indicating that the pYV associated factor responsible for increased intracellular killing was one of the effectors lacking in this strain (YopE, YopH, YopO, YopM, YopK, or YopJ).

Next we set out to identify the Yop responsible for the survival defect seen in wild-type *Y. pseudotuberculosis*. Zhang et al. showed that a 32777 *yopJTEH* strain and a 32777 *yopJ* strain were both unable to survive inside macrophages through 24 h p.i. as compared to 32777c (151). Since 32777 and IP2666 had behaved similarly in our assays, we deduced that Yops J, T, E and H were not responsible for the intracellular survival

defect. Since IP37 was defective for these Yops as well as Yops M, O and K, we decided to look at the intracellular survival phenotypes of *yopO* and *yopK* single mutants.

BMDMs were infected with 37°C-grown IP2666, IP2666c and IP37, as controls for intracellular survival phenotypes, as well as IP32 (IP2666 *yopO*) or IP36 (IP2666 *yopK*). IP36 showed a significant survival defect at 5 h p.i. compared to the 25 min time point (Figure 4.3), indicating that YopK is not responsible for the survival defect. In contrast, IP32 did not show a significant survival defect over time within BMDMs (Figure 4.3), indicating that YopO could be the effector responsible for the decrease in intracellular survival within macrophages seen in infections with wild-type *Y. pseudotuberculosis*.

**IP2666 *yopO* shows increased survival within macrophages by GFP induction assay.** To examine the intracellular survival phenotype of the *yopO* mutant further, we used a GFP induction assay. BMDMs grown on coverslips were infected with 37°C grown IP2666/GFP, IP32/GFP, IP37/GFP, or IP2666c/GFP. GFP was induced at 23 h p.i., and coverslips were collected at 24 h p.i. and then fixed. We then examined intracellular survival by fluorescence-based microscopy. Only bacteria that are alive within the macrophages will be able to express GFP.

We found that very few IP2666 were GFP positive by 24 h p.i. IP37 and IP2666c had the most GFP-positive bacteria present (Figure 4.4). While IP32 had many GFP positive bacteria as compared to IP2666, there were not as many as with IP37 or IP2666c. Overall, these results were similar to the CFU assay.

**IP32 complemented with *yopO/ypkA* from KIM5 does not show a survival defect within macrophages.** To confirm that the absence of YopO caused the ability of IP32 to survive within BMDMs, IP32 was complemented with *yopO* from *Y. pestis* KIM5. The strain IP32 pYopO was used to infect BMDMs, and the CFU assay was performed at various time points p.i. We found that IP32 pYopO did not show a survival defect through 24 h p.i. (Figure 4.5). This result indicates that pYopO from *Y. pestis* is unable to complement the intracellular survival defect phenotype seen with IP2666.

Since we were unable to complement IP32 with *yopO* taken from *Y. pestis* KIM5 we decided to examine the predicted sequences of YopO from *Y. pestis* and *Y. pseudotuberculosis*. The alignment revealed minor sequence differences (Figure 4.6). Specifically, between *Y. pestis* and *Y. pseudotuberculosis* there were three amino acid changes: H566N, T598A, and Q647R. These changes were all found in the GDI domain of the protein (Figure 4.6). These differences may explain the inability of *Y. pestis yopO* to complement a *Y. pseudotuberculosis yopO* mutant.

## Discussion

We determined that 37°C grown pYV$^+$ *Y. pseudotuberculosis* strains from two serogroups, I and III, show decreased survival in BMDMs. These results suggest that this phenotype is conserved in *Y. pseudotuberculosis* strains, but absent from at least one *Y. pestis* strain (KIM5). From performing infection assays with various *Y. pseudotuberculosis* pYV$^-$ or *yop* mutants and determining the CFU at various times p.i.,

we were able to determine that a deletion in *yopO* caused increased intracellular survival of *Y. pseudotuberculosis*. We hypothesize that YopO, which is known as an antiphagocytic Yop, may be somehow alerting the macrophage to the intracellular presence of bacteria. This then stimulates the killing of the bacteria.

To further confirm the validity of our CFU assays, we complemented IP32 with *yopO* from KIM5. We expected that complementation would result in the increased intracellular killing of IP32. However, we observed no survival defect of the complemented strain. Examination of the predicted sequence of *Y. pestis* YopO revealed sequence differences as compared to *Y. pseudotuberculosis* YopO. These differences involve three amino acid changes located at positions 626, 658, and 647 (Figure 4.6). All three amino acid changes are in the GDI domain (shown in purple in Figure 4.6). Perhaps these differences contribute to the ability of *Y. pestis* to be able to survive intracellularly, although it is not readily clear why these specific changes within the GDI domain would yield this effect. These studies could have implications for *Y. pestis* infection. Perhaps *Y. pestis* YopO is not functional within *Y. pseudotuberculosis* or the unique sequence of *Y. pseudotuberculosis* YopO somehow alerts the macrophages and causes intracellular killing.

It is paradoxical that the T3SS, which is necessary for virulence, may also be responsible for intracellular killing of the bacteria. However, *Y. pseudotuberculosis* is still virulent, so despite the intracellular killing the bacteria are still able to counteract the host immune response. Perhaps that intracellular survival defect could explain why *Y. pseudotuberculosis* produces self-limiting infections. It would be valuable to continue the complementation studies and to complement IP32 with *yopO* from *Y.*

*pseudotuberculosis* to confirm that YopO is the cause of the decreased intracellular survival.

## Figures and Tables

**Table 4.1** *Yersinia* Strains used in Chapter 4

| Strain | Characteristics | Reference |
|---|---|---|
| *Y. pseudotuberculosis* | | |
| 32777 | $pYV^+$ | (116) |
| IP2666 | $pYV^+$ | (4) |
| IP2666/GFP | IP2666/pMMB207gfp3.1, $Amp^r$ | This Study |
| IP2666c | $pYV^-$ | (43) |
| IP2666c/GFP | IP2666c/pMMB207gfp3.1, $Amp^r$ | This Study |
| IP37 | $pYV^+$ *yopEHOMKJ* | (25) |
| IP37/GFP | IP37/pMMB207gfp3.1, $Amp^r$ | This Study |
| IP36 | $pYV^+$ *yopK* | This Study |
| IP32 | $pYV^+$ *yopO* | This Study |
| IP32/GFP | IP32/pMMB207gfp3.1, $Amp^r$ | This Study |

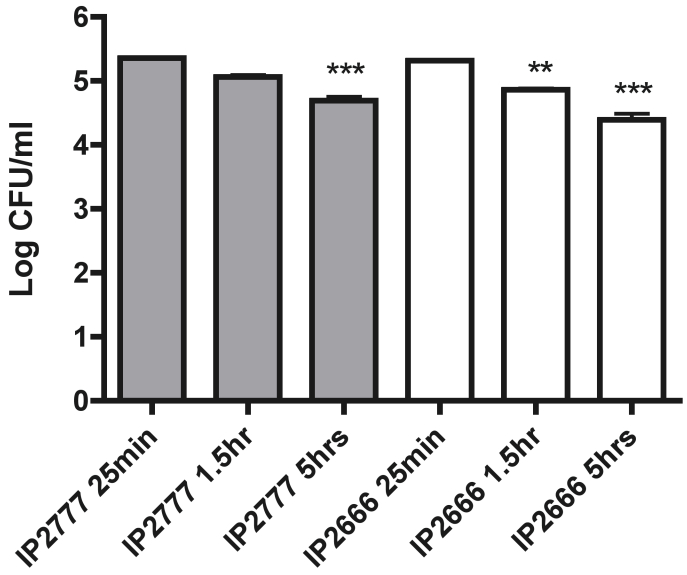



**Figure 4.1**

Comparison of the intracellular survival of *Y. pseudotuberculosis* 32777 and *Y. pseudotuberculosis* IP2666 in BMDMs. Naïve BMDMs were infected with the indicated bacterial strains that were grown for 2 h at 37°C and intracellular bacterial survival was determined at 25 min, 1.5 h, and 5 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.01$,**; $p<0.001$, *** as compared to 25 min).

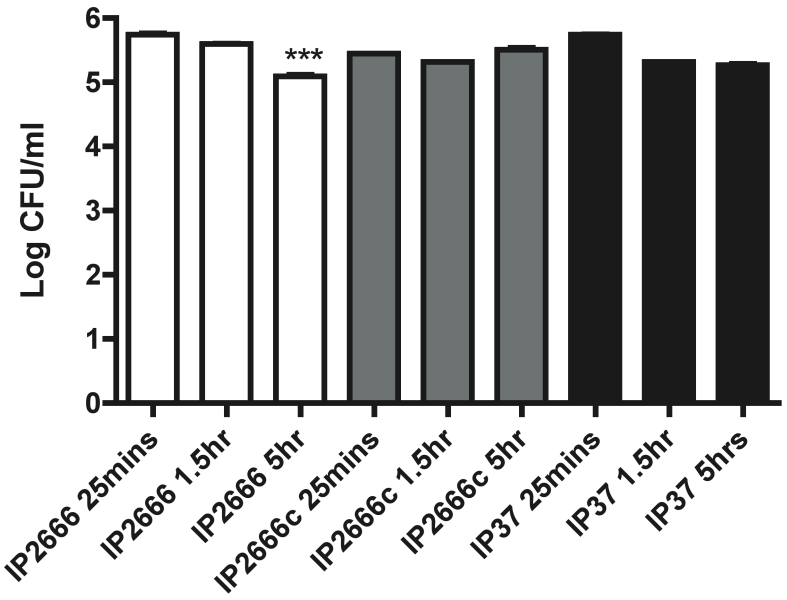



**Figure 4.2**

Comparison of the intracellular survival of *Y. pseudotuberculosis* IP2666, *Y. pseudotuberculosis* IP2666c, and *Y. pseudotuberculosis* IP37 strains in BMDMs. Naïve BMDMs were infected with the indicated strains and intracellular bacterial survival was determined at 25 min, 1.5 h, and 5 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.001$, *** as compared to 25 min).

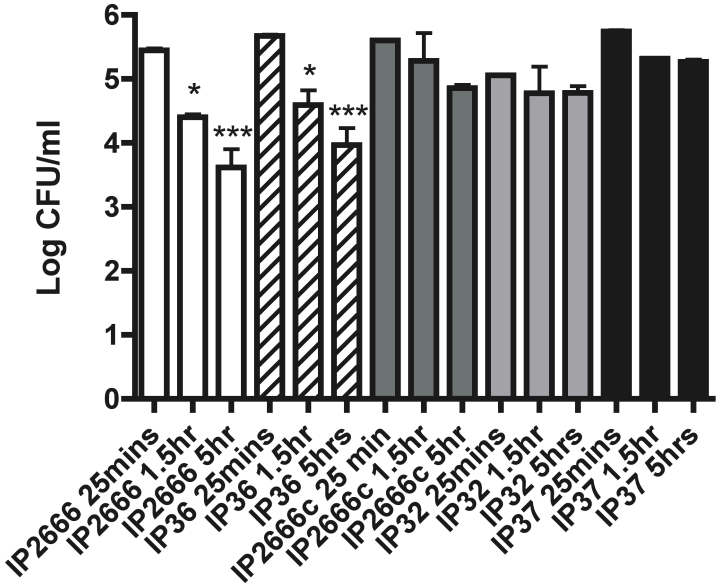



**Figure 4.3**
Comparison of the intracellular survival of wild-type *Y. pseudotuberculosis* with strains lacking pYV or Yops in BMDMs. BMDMs were infected with the indicated strains and intracellular bacterial survival was determined at 25 min, 1.5 h, and 5 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.05$, *; $p<0.001$, *** as compared to 25 min).

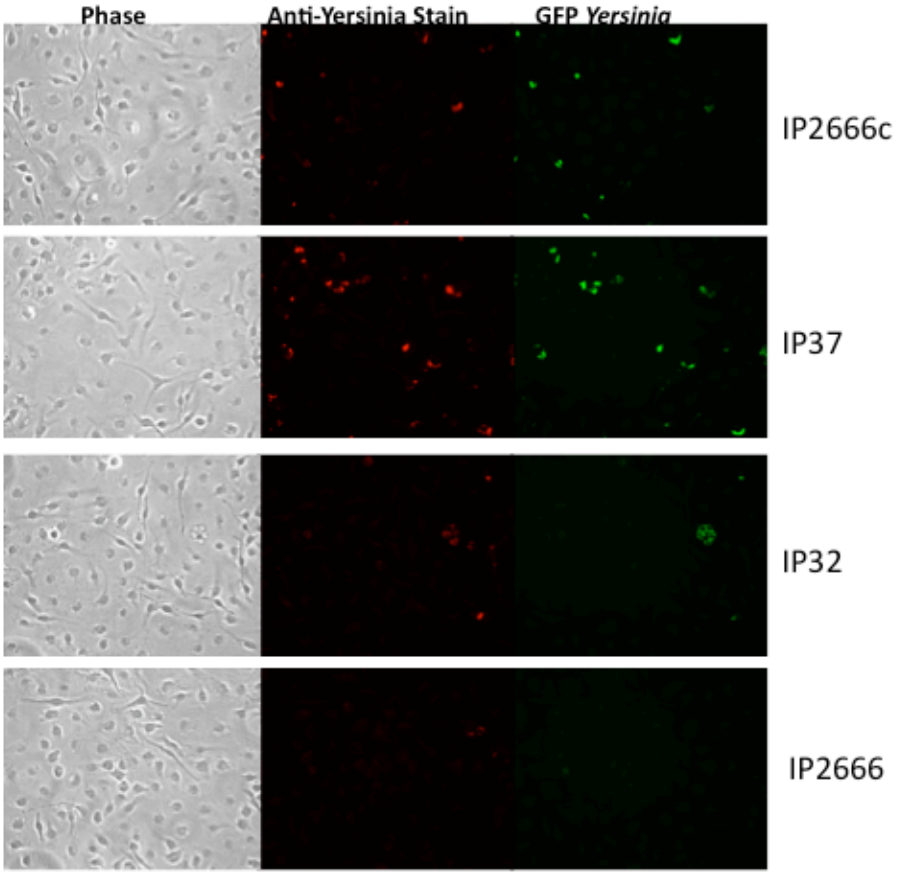



**Figure 4.4**
GFP induction assay with *Y. pseudotuberculosis* pYV mutants in BMDMs. BMDMs were infected with the indicated strains and intracellular bacterial survival was determined at 24 h p.i. by GFP induction assay.

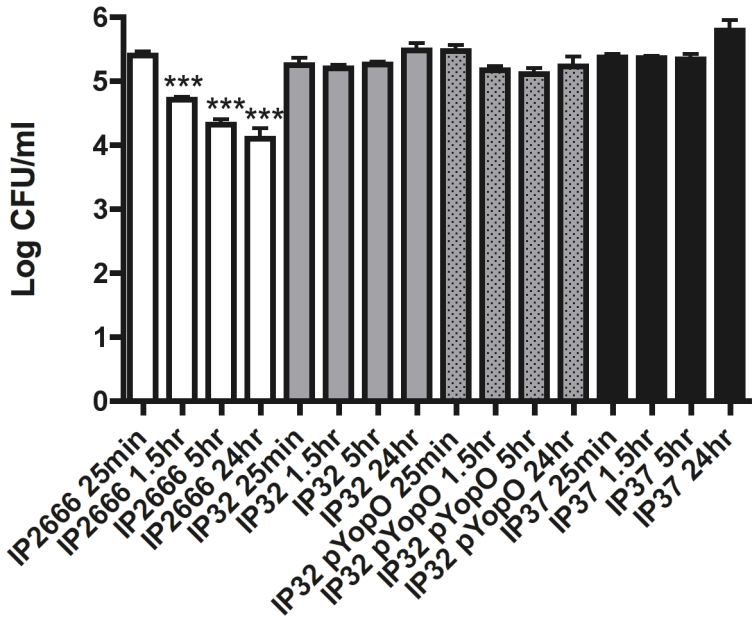



**Figure 4.5**
Comparison of the intracellular survival of *Y. pseudotuberculosis* IP32 and IP32 pYopO in BMDMs. BMDMs were infected with the indicated strains and intracellular bacterial survival was determined at 25 min, 1.5 h, 5 h, and 24 h p.i. by CFU assay. Results were taken from three independent experiments and averaged. Error bars are S.D. ($p<0.001$, *** as compared to 25 min).


## Figure 4.6

Sequence alignment of YopO from *Y. pestis* and *Y. pseudotuberculosis*. *Y. pestis* sequence is in blue and *Y. pseudotuberculosis* sequence is in green. The kinase domain is in red, the GDI domain in purple, and the Actin binding domain is in gray. The variable amino acids are in bold (between 541 and 660).

# Chapter 5: Conclusions

## Summary

*Yersinia pestis*, the agent of plague, uses a type III secretion injectisome to deliver into macrophages Yop proteins that have many diverse functions such as opposing phagocytosis and inducing apoptosis. Additionally, internalized *Y. pestis* can survive in phagosomes of naïve or IFNγ-activated macrophages by blocking vacuole acidification. While plague has had devastating affects historically, it is less common today, although there are still cases of plague worldwide. This decline is due in part to increased sanitary conditions as well as effective antibiotic treatment. However, because of the high fatality rate of pneumonic plague, the ease of aerosolization of *Y. pestis*, and the isolation of multi-drug resistant strains, there is concern that plague could be used as a bioweapon. Because of this, it is important to not only develop an effective and safe vaccine but to also continue to study *Y. pestis* and its interaction with the cells of the immune system, and its ability to survive within these cells and derange the normal immune response.

In this work, we sought to examine the intracellular survival of *Y. pestis* in both naïve and IFNγ activated macrophages. The first two parts sought to examine in vitro the interactions between *Y. pestis,* opsonized with antibodies found to be protective in vivo, and cultured macrophages. We used anti-LcrV immune sera and anti-BDE immune sera. With anti-V we first asked how opsonization with these antibodies affects the ability of *Y. pestis* to survive within and kill macrophages.

We used both the Raw264.7 murine macrophage-like cell line and murine BMDMs as our sources of macrophages. The benefit of using a cell line is that the

population of cells remains relatively consistent between experiments. We also wanted to use primary cells to better mimic the cells encountered in vivo. While there were many choices on types of macrophages we could utilize, we decided to use BMDMs for many reasons. One reason is that they are readily isolated from the femurs of mice. Another reason is that one can harvest and derive large numbers of cells. The drawback is that these cells are derived and are in a sense activated by factors in the medium. We feel that this is acceptable because we are comparing these "naïve" cells to cells from the same source that have been additionally treated with IFNγ.

It is known that the cytokines IFNγ and TNFα can cooperate with anti-LcrV to promote protection against lethal *Y. pestis* infection in mice. It remains unknown if these phagocyte-activating cytokines cooperate with anti-LcrV to increase killing of the pathogen and decrease apoptosis in macrophages. This led us to ask how opsonization with anti-V affects the ability of *Y. pestis* to survive within and kill IFNγ-activated macrophages.

We also looked at the percentage of anti-V opsonized bacteria that were internalized by both naïve and IFNγ activated macrophages. The phagocytosis assays with naïve macrophages were confirmatory, to determine whether our serum contained anti-V antibodies that increase phagocytosis as reported previously in the literature. We then wanted to expand that knowledge and investigate the impact of IFNγ activation and the presence of anti-V on internalization of opsonized KIM5. We found that with IFNγ activation there was an overall increase in bacterial uptake. Approximately 55-66% of non-opsonized or control serum-opsonized KIM5 were internalized. Anti-V-opsonized

KIM5 was internalized at ~ 75%, but this increase was not significant as compared to the control.

We extended the anti-V serum studies to include looking at how anti-V mAbs and IFNγ impact bacterial survival and apoptosis in cultured murine macrophages infected with *Y. pestis* KIM5. Overall, our results show that anti-LcrV reduced apoptosis at an early time point (5 h) but not at a later time point (24 h). Polyclonal anti-LcrV serum was unable to inhibit apoptosis at either time point in IFNγ-activated macrophages. Additionally, anti-LcrV was ineffective at promoting killing of KIM5 in naïve or activated macrophages. We concluded that *Y. pestis* can bypass protective antibodies to LcrV and macrophage activation with IFNγ to survive and induce apoptosis in murine macrophages.

While LcrV is being studied for inclusion in a subunit vaccine, there are some drawbacks. One such drawback for using LcrV is that it can exhibit amino acid variability. LcrV sequences from *Y. pestis* and the other two human pathogenic *Yersiniae*, *Y. pseudotuberculosis* and *Y. enterocolitica*, are different at the amino acid sequence level and can be used interchangeably. This sequence variability may make antibody-antigen specificity a problem. Alternative antigens, such as YopB and YopD, must be investigated for their protective effect because of this.

A complex of Yops B, D and E (BDE) is being investigated for the ability to elicit protective antibodies following vaccination of mice. Anti-BDE has been shown to be protective in mice against infection by KIM5 lacking the F1 capsule. It has been suggested that any antibody that promotes phagocytosis of *Y. pestis* could be protective. To investigate whether anti-BDE contained antibodies that could promote bacterial

uptake, similar to anti-V, we examined phagocytosis of anti-BDE opsonized KIM5 by both naïve and IFNγ activated macrophages. We found that opsonization with anti-BDE did not significantly increase phagocytosis of *Y. pestis* by naïve or activated BMDMs.

We further wanted to investigate the effect of anti-YopBDE opsonization on intracellular survival of KIM5, apoptosis of infected naïve and IFNγ macrophages, and the ability of macrophages to release two cytokines: TNFα and IL-1β. Our results indicated that opsonization of KIM5 with anti-YopBDE did not significantly increase intracellular killing of the bacteria, decrease levels of apoptosis late in infection, or affect cytokine release compared to KIM5 alone or KIM5 opsonized with control sera.

We also examined the interaction of KIM5 with macrophages after opsonization of the bacteria with YopBD-generated immune sera or YopB-generated immune sera. We examined the effect of these sera on bacterial uptake. Opsonization of KIM5 with anti-BD or anti-B did not increase uptake of KIM5 by naïve macrophage. Upon IFNγ-stimulation only anti-B antibody opsonization significantly increased uptake of KIM5 by RAW 264.7 cells. Recent studies examining anti-D mAbs have shown that some individual mAbs may decrease Yop translocation, while others may actually increase Yop translocation (M. Ivanov, unpublished data). Drawing from this new data, since the BD serum is a combination of anti-B antibodies as well as anti-D antibodies against many different epitopes, perhaps this serum contains antibodies that simultaneously promote and inhibit Yop translocation. Anti-B alone may be more effective for this reason.

Since we did not see an effect on the intracellular survival of *Y. pestis*, even with opsonization with protective immune sera, the third part of this work sought to examine the basis for the decrease of intracellular survival in macrophages of the closely related

species *Y. pseudotuberculosis*. *Y. pseudotuberculosis* and *Y. pestis* are two closely related human pathogenic bacteria species. In recent studies, *Y. pseudotuberculosis* was shown to survive within macrophages only when pYV had been removed or was not expressed, suggesting that the T3SS and/or Yops may play a negative role in intra-macrophage survival. Understanding the mechanism of the killing of *Y. pseudotuberculosis* within macrophages may help to understand the ability of *Y. pestis* to survive inside macrophages.

First we wanted to determine if different serogroups of *Y. pseudotuberculosis* had decreased survival within macrophages. We used 32777 from serogroup I and IP2666 from serogroup III and infected BMDMs. CFUs were determined at various time points post-infection. Our results showed that both strains of *Y. pseudotuberculosis* had decreased survival within macrophages. Next we tried to determine what virulence plasmid-related factor contributed to this decreased intracellular survival. Macrophages were infected with *Y. pseudotuberculosis* strains IP2666 wild-type (wt), IP2666 pYV- (IP2666c), IP2666 *yopO*, (IP32) IP2666 *yopK* (IP36), and IP2666 *yopEHOMKJ* (IP37). Intracellular survival assays were performed and bacteria were enumerated at 25 min, 1.5 h, 5 h, and 24 h post-infection (p.i.). At 25 min and 1.5 h p.i., similar levels of all strains were present. Levels of IP2666 and IP36 began to decrease significantly by 5 h p.i. while IP2666c and IP37 strains continued to survive within macrophages. IP32 was shown to survive by 5 h p.i. and even through 24 h p.i. Similar results were obtained using a green fluorescent protein (GFP) induction assay, where IP2666 strains induced to express GFP at 24 h p.i. were visualized by fluorescence microscopy. These results suggest that YopO may be responsible for the decreased survival of IP2666 within macrophages.

To determine if YopO was the pYV encoded factor that caused intracellular killing of IP2666, we complemented IP32 with *yopO* from KIM5 encoded on an IPTG inducible expression vector. Survival assays were performed, and we found that *yopO* from KIM5 was unable to complement IP32. The predicted sequences of YopO from *Y. pseudotuberculosis* and *Y. pestis* strains KIM5 and CO92 were compared and amino acid differences were found, which could possibly explain the inability of *yopO* from KIM5 to complement as well as the difference in intracellular survival between *Y. pseudotuberculosis* and *Y. pestis*.

Taken together, these results confirm that *Y. pestis* is well adapted to survive within macrophages. Despite the presence of protective antibodies and the engaging of CR and Fc receptors, this finely evolved organism can successfully avoid degradation and survive in phagosomes within macrophages. Presumably this is due to the ability of Yops to diminish the oxidative burst upon engaging the Fc receptors as well as the ability of *Y. pestis* to avoid acidification of the phagosome. Interestingly, even with IFNγ, which activates the macrophages and increases antimicrobial processes, *Y. pestis* has evolved to be able to withstand these conditions. The closely related species *Y. pseudotuberculosis* has diminished survival within macrophages, which may be due to the presence of YopO somehow alerting the macrophages. More work needs to be done to understand why *Y. pseudotuberculosis* has this diminished survival, while *Y. pestis* is fully able to survive within macrophages.

## Implications

LcrV has been extensively studied. LcrV has been shown to localize to the tip of the T3SS injectisome (85). It also has a regulatory role in Yop secretion. The exact mechanism of how LcrV mediates translocation of effectors is not fully understood (99, 140). In addition to promoting Yop translocation, LcrV has other anti-host activities. Purified LcrV has been shown to stimulate IL-10, an immunosupressing cytokine. This LcrV-induced immunosupression is thought to be important during infection (41). While researchers have studied LcrV-induced immunosuppression in monocyte/macrophage cell lines, it is suggested that other IL-10 producing cells could be involved as well (99).

Another proposed anti-host function of LcrV involves neutrophils. Purified LcrV has been shown to inhibit polymorphonuclear neutrophils chemotaxis into sponges (141). This effect is also seen in vivo where there is an acute inflammatory response due to plague infection in lesions formed in the liver and spleen. Following the acute inflammation there is a decay in neutrophils and almost no further recruitment of these cells (99). As a result, neutrophil depleted lesions develop over the spleen and liver. The detailed mechanism of all the effects of LcrV and their relevance during infection remain unclear.

It is known that LcrV is a potent protective antigen. Anti-LcrV (anti-V) has been shown to be protective for both pneumonic and bubonic forms of plague. Mice can be protected against lethal *Y. pestis* infection by passive immunization with anti-V antibodies (63). However, an area of uncertainty within the literature is the mechanism of protection against plague mediated by anti-V. There are several proposed mechanisms. One mechanism is that anti-V antibodies neutralize LcrV and inhibit the

effect of LcrV elicited IL-10 production (41). Anti-V could prevent the immunosuppressive effect of IL-10 and allow a proper immune response to mount and aid in the clearance of the infection. Another purposed mechanism of protection is the prevention of LcrV-induced inhibition of neutrophil chemotaxis by anti-V antibodies (32, 141). There is evidence that anti-V increases uptake of *Y. pestis* by neutrophils (32) and that *Y. pestis* is unable to survive within neutrophils (25, 122). Perhaps anti-V antibodies neutralize LcrV, which allows an influx of neutrophils to clear the infection.

The third proposed mechanism of anti-V mediated protection involves Yop translocation. Anti-V has been shown to increase phagocytosis of *Y. pestis* by macrophages. This phagocytosis seems to be important because only when *Y. pestis* was phagocytosed in the presence of anti-V was there a decrease in Yop translocation (32). Concurrent with a decrease of Yop translocation, reduced apoptosis of macrophages is also seen (32, 140). Another group found that when mice were infected in the presence of anti-V antibodies or nonprotective control antibodies with an effectorless strain of *Y. pestis,* there was no difference in bacterial colonization levels (99). This result indicates that inhibition of Yop translocation may be one of the most important aspects of anti-LcrV antibody mediated protection.

There is also evidence that anti-V antibody may be more important in the context of *Y. pestis* interaction with one cell-type versus another. It has been shown that anti-V antibody mediated protection in mice involved macrophages in the liver but not the spleen (99). Researchers used fas-induced apoptosis (Mafia) transgenic mice to deplete all macrophages. They treated the mice with anti-V antibodies and infected with *Y. pestis*. They then isolated organs and used CFU assays to enumerate the bacteria present.

They found that without macrophages present in the liver, anti-V was not effective and there were similar bacterial loads between mock and protective antibody treated mice. However, in the macrophage-depleted spleen, anti-V treated mice had significantly less bacteria present compared to the mock treated mice (99). This indicates that macrophages are important mediators of protection in the liver and that another cell type may be important in the spleen.

There is also evidence that neutrophils are the mediators of protection by anti-LcrV antibodies against *Y. pestis* infection in mice (32). Anti-Gr-1 antibodies were used to deplete neutrophils from mice. The mice were then treated with anti-V antibodies and infected with *Y. pestis*. It was found that mice depleted of neutrophils and treated with anti-V are unable to reduce bacterial levels in the liver and spleen. As a result, this group concluded that neutrophils are crucial to anti-V mediated protection.

In addition to anti-V and the macrophage and neutrophil cell types, the cytokines IFNγ and TNFα have been shown to be important for protective immune responses against *Y. pestis* infection (95). While *Y. pestis* can survive within IFNγ-activated macrophages, it has been shown that the combination of IFNγ and TNFα activated macrophages causes significantly decreased intracellular survival (73).

The question still remains how anti-V mediates protection in vivo. In our in vitro experiments, we find that anti-V opsonization with naïve macrophages causes an increase in phagocytosis, and a decrease in macrophage apoptosis seen early in infection. We found that with IFNγ activation there is an increase in phagocytosis, although this increase is overall in each condition and not significant when compared to control serum opsonization. There is also a decrease in apoptosis seen early in infection. It is

surprising that late in infection, by twenty four hours, the anti-LcrV mediated protection (measured by inhibition of YopJ induced apoptosis) is no longer seen. This could be because anti-V increases the amount of bacteria that are internalized by the macrophages. With this phagocytosis comes a decrease of Yop translocation, which may cause an inhibition/reduction in apoptosis early on. However, because there are more intracellular bacteria, and these are presumably no longer opsonized, Yops may still be translocated intracellularly, which might cause the return of apoptosis late in infection. We also know from our CFU assays that anti-V opsonization is not causing an increase in intracellular killing so once the significantly larger amount of bacteria is internalized, they are free to replicate and presumably translocate Yops from within the host cell.

Despite others having shown that macrophages are important in anti-V mediated protection in vivo, at least in the liver, in vitro we have not seen bacterial clearance by macrophages in the presence of anti-V. This could be for many reasons. It is possible that anti-V antibodies are protective because of the combined effects of all three proposed mechanisms. This would be difficult to take into account in vitro. Cowan et al. showed that neutrophils are important for protection within the liver and spleen (32). So perhaps the combination of both macrophages and neutrophils is important. *Y. pestis* can survive within macrophages but are unable to survive in neutrophils if taken up. Since anti-V promotes uptake by neutrophils and they are known to clear the bacteria, perhaps this is important for protection. In addition, perhaps with the neutralization of LcrV-induced IL-10 production, the macrophages and neutrophils will have increased bactericidal activity since there are no immunosuppressive signals. Also, since IFNγ and TNFα have been shown to be crucial for protection against *Y. pestis*, in vivo there would be many cell

types that could produce these cytokines, while in vitro we only added IFNγ and have shown that even in the presence of anti-V, *Y. pestis* is still able to diminish levels of TNFα produced by macrophages.

It is also important to examine the interaction between *Y. pestis* and alternative protective antibodies. The question of why anti-BDE is protective and why it is only protective against $F1^-$ *Y. pestis* still remains. Perhaps the F1 capsule blocks the epitopes that anti-BDE recognizes. However, LcrV has been shown to localize to the tip of the T3SS needle and is protective against $F1^+$ *Y. pestis*. Why would the capsule not block anti-V epitopes? Or is the protective effect of anti-V due to sequestering of secreted LcrV? We were unable to promote killing of *Y. pestis* in vitro in the presence of protective antibodies and/or IFNγ and only witnessed loss of intracellular survival of *Y. pseudotuberculosis*.

We found that YopO may be responsible for loss of intracellular survival in *Y. pseudotuberculosis*. When *Y. pseudotuberculosis* expresses *yopO* from *Y. pestis* it survives over time. Perhaps this is why we are unable to show loss of intracellular survival of *Y. pestis* in vitro. In our experiments, we show that early during infection there is an initial inhibition of YopJ translocation. Late in infection, we find that this inhibition is lost. Since the Yops are not completely inhibited, perhaps YopO is free to translocate, and since it is modified from *Y. pseudotuberculosis* YopO it has lost the ability to cause intracellular killing. Perhaps *Y. pestis* expressing *yopO* from *Y. pseudotuberculosis* would lose this ability to survive.

## Future Studies

There are many further aspects of these studies that can be developed. One future study would involve repeating intracellular survival assays with IFNγ- and TNFα-activated macrophages. There is evidence that the combination of both cytokines is effective in neutralizing *Y. pestis*. Perhaps with the addition of anti-V plus the dual activation condition, loss of intracellular survival would be seen in vitro. However, since anti-V had no affect on diminishing the intracellular survival of opsonized KIM5 by naïve or IFNγ-activated BMDMs, it would be of value to study the interaction with neutrophils. There is evidence that neutrophils could play an important role in anti-V mediated protection; whether or not a difference in survival can be seen in vitro would be interesting to determine. The overall mechanism of protection for anti-V still remains to be elucidated and may involve a combination of factors, such as an increase in phagocytosis combined with neutralization of the IL-10 inducing ability of anti-V.

Work with anti-BDE serum left many questions remaining to be answered. If anti-BDE is protective in vivo, are there any in vitro correlates for this protection? It would be interesting to examine bacterial uptake, intracellular survival, and macrophage apoptosis in infection assays with $F1^-$ KIM5 opsonized with anti-BDE. Perhaps since in vivo anti-BDE was only protective with $F1^-$ KIM5, in vitro this same strain would yield positive results. It also cannot be assumed that anti-BDE will have similar in vitro protection correlates as LcrV. The mechanism of protection of anti-BDE could be further studied.

Finally, there are a few future experiments that can be done to further study the decreased survival of *Y. pseudotuberculosis* in macrophages. First, IP32 should be complemented with pYopO from IP2666. This would help to confirm that YopO is the factor that, when expressed, causes diminished survival in macrophages. If the expression of pYopO in IP32 leads to the decreased intracellular survival, then there are a few paths the project could take. One direction would be to closely examine the differences between the YopO of *Y. pestis* and *Y. pseudotuberculosis*. Several mutants can be created that change YopO of *Y. pseudotuberculosis* to resemble that of *Y. pestis*. In that way, one could determine the area of the sequence that is somehow responsible for YopO causing decreased survival.

Another direction would be to determine if pYopO from IP2666 inserted into KIM5 *yopO* could cause decreased intracellular survival within macrophages. A final direction could be to determine how YopO causes decreased survival within macrophages. Is YopO, which is known to have an antiphagocytic role, perhaps helping the bacteria escape from the macrophage? This would explain a decrease in CFU found in our studies. Perhaps when YopO is not present the bacteria are unable to escape, which would look like intracellular survival in our assays as opposed to an inability to escape.

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The images for Figures 1.1-1.5 in Chapter One of this dissertation are reprints of the images as they appear in **Wren, B. W.** 2003. The *yersiniae*--a model genus to study the rapid evolution of bacterial pathogens. Nat Rev Microbiol **1:**55-64; **Mota, L. J.** 2006. Type III secretion gets an LcrV tip. Trends Microbiol **14:**197-200; **Medzhitov, R., and C. Janeway, Jr.** 2000. Innate immunity. N Engl J Med **343:**338-44; and **Ernst, J. D.** 2000. Bacterial inhibition of phagocytosis. Cell Microbiol **2:**379-86. Permission for the use of these images has been obtained from the publisher of each journal.

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