Evasion of LPS-TLR4 Signaling as a Virulence Determinate for *Yersinia pestis*

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*University of Massachusetts Medical School Worcester*

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Evasion of LPS-TLR4 Signaling as a Virulence Determinate for Yersinia Pestis

A Dissertation Presented

By

Sara Montminy Paquette

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of:

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Program in Immunology and Virology
A Dissertation

By

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I have learned to use the word 'impossible' with the greatest caution.

- Wernher von Braun

_Tada gan iarracht_
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ABSTRACT

*Yersinia pestis*, the gram-negative causative agent of plague, is a master of immune evasion. The bacterium possesses a type three secretion system which translocates Yop effector proteins into host immune cells to inhibit a number of immune and cell signaling cascades. Interestingly, this apparatus is not expressed at low temperatures such as those found within the flea vector and is therefore neither in place nor functional when the bacteria are first transmitted into a mammalian host. However, the bacterium is still able to avoid activating the immune system, even very early during infection.

When grown at 37°C (human body temperature) *Y. pestis* produces a tetra-acyl lipid A molecule, which is antagonistic to the human Toll like receptor 4/MD2, the major lipopolysaccharide recognition receptor. Although tetra-acyl lipid A binds this receptor complex, it does not induce signaling, and in fact inhibits the receptors interaction with other stimulatory forms of lipid A. The work undertaken in this thesis seeks to determine if the production of tetra-acyl lipid A by *Y. pestis* is a key virulence determinant and was a critical factor in the evolution of *Y. pestis* from its ancestral parent *Yersinia pseudotuberculosis*.

By examining the enzymes involved in the lipid A biosynthesis pathway, it has been determined that *Y. pestis* lacks LpxL, a key enzyme that adds a secondary acyl chain on to the tetra acyl lipid A molecule. In the absence of this enzyme, *Y. pestis* cannot produce a TLR4 stimulating form of lipid A, whereas *Y. pseudotuberculosis* does contain
the gene for LpxL and produces a stimulatory hexa acyl lipid A. To determine if the absence of LpxL in *Y. pestis* is important for virulence, LpxL from *E. coli* and *Y. pseudotuberculosis* were introduced into *Y. pestis*. In both cases the addition of LpxL led to bacterium which produced a hexa-acylated lipid A molecule and TLR4/MD2 stimulatory LPS. To verify the LpxL phenotype, *lpxL* was deleted from *Y. pseudotuberculosis*, resulting in bacteria which produce tetra-acylated lipid A and non-stimulatory LPS. Mice challenged with LpxL expressing *Y. pestis* were found to be completely resistant to infection. This profound attenuation in virulence is TLR4 dependent, as mice deficient for this receptor rapidly succumb to disease. These altered strains of the bacterium also act as vaccines, as mice infected with *Y. pestis* expressing LpxL then challenged with wild type *Y. pestis* do not become ill. These data demonstrate that the production of tetra-acyl lipid A is a critical virulence determinant for *Y. pestis*, and that the loss of LpxL formed a major step in the evolution of *Y. pestis* from *Y. pseudotuberculosis*.

These bacterial strains were also used as tools to determine the contributions of different innate immune receptors and adaptor molecules to the host response during *Y. pestis* infection. The use of LpxL expressing *Y. pestis* allowed identification of the innate immune pathways critical for protection during *Y. pestis* infection. This model also established that CD14 recognition of rough LPS is critical for protection from *Y. pestis* expressing LpxL, and activation of the IL-1 receptor and the induction of IL-1β plays a major role in this infection as well.
The lipid A acylation profile of gram negative bacteria can have a direct and profound effect on the pathogenesis of the organism. This work illustrates a previously unknown and critical aspect of *Y. pestis* pathogenesis, which can be extended to other gram-negative pathogens. The greater detail of the contributions which different host adaptor and receptor molecules make to the overall innate immune signaling pathway will allow a better insight into how gram negative infections progress and how they are counteracted by the immune system. Alterations of the lipid A profile of *Y. pestis* have important implications for the production of vaccines to *Y. pestis* and other gram negative pathogens. Taken together, this work describes a novel mechanism for immune evasion by gram negative bacteria with consequences for understanding the immune response and the creation of more effective vaccines, both of which will decrease the danger posed by this virulent pathogen.
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<th>Description</th>
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<tr>
<td>ACP</td>
<td>acyl carrier protein</td>
</tr>
<tr>
<td>ASC</td>
<td>associated speck-like protein containing a caspase recruitment domain</td>
</tr>
<tr>
<td>CARD</td>
<td>N-terminal caspase recruitment domain</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GPAT</td>
<td>glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HtrB</td>
<td>high temperature required type B gene</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-D-manno-oct-2-ulosonic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88 adaptor like protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>IκB kinase subunit NF-κB essential modifier</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide-binding oligomerization domain like receptors</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoid acid-inducible gene I</td>
</tr>
<tr>
<td>RLR</td>
<td>retinoid acid-inducible gene I like receptors</td>
</tr>
<tr>
<td>SARM</td>
<td>sterile-α and HEAT/Armadillo motifs-containing protein</td>
</tr>
<tr>
<td>T3SS</td>
<td>type three secretion system</td>
</tr>
<tr>
<td>TAK1</td>
<td>transforming growth factor-activated protein kinase 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin 1 receptor homology</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll like receptor 4</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF related adaptor protein</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain containing adaptor inducing interferon-β</td>
</tr>
<tr>
<td>Yops-</td>
<td><em>Yersinia</em> outer proteins</td>
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CHAPTER I:
INTRODUCTION
The genus *Yersinia*

The genus *Yersinia* is a member of the family *Enterobacteriaceae* of Gram-negative bacteria. They are rod-shaped, non-spore forming organisms which can grow in both aerobic and anaerobic conditions. The majority of the *Yersinia* are peritrichously flagellated, with the exception of *Y. pestis* which is non-motile (Minnich and Rohde, 2007). This genus contains fifteen species, of which only three are known to be pathogenic to humans, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Yersinia pestis*. *Y. enterocolitica* and *Y. pseudotuberculosis* are soil and water borne pathogens that cause self limiting gastroenteritis in humans, whereas *Y. pestis* is associated with bubonic plague, a radically different and highly dangerous disease (Smego et al., 1999; Zhou and Yang, 2009). However, despite the differences in pathological presentation, these three species of *Yersinia* share a great degree of genetic homology and contain similar virulence determinants. All three species contain a 70-75kb virulence plasmid (pCD1 in *Y. pestis*, pIB1 in *Y. pseudotuberculosis*, and pYV in *Y. enterocolitica*) that contains both a type three secretion system (T3SS) and the *Yersinia* outer proteins (Yops) which strongly inhibit the host immune response (Cornelis et al., 1998). Despite their genetic similarities, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* cause diverse clinical symptoms with unique characteristics, and determining the manner in which they evolved can help elucidate how the bacterium became pathogenic and thus facilitate the discovery of preventative and curative treatments.
**Yersinia enterocolitica and Yersinia pseudotuberculosis**

*Yersinia enterocolitica* is the oldest pathogenic *Yersinia*, and is the evolutionary parent of both *Y. pseudotuberculosis* and *Y. pestis* (Achtman et al., 1999). Although there are numerous serotypes of *Y. enterocolitica*, not all are associated with disease. Those serotypes that are pathogenic lead to a zoonotic, food borne illness that is transmitted via the oral-fecal route, and produces disease in both humans and animals. This is thought to be a very common infection, especially in young children, but usually the symptoms are subclinical and are therefore rarely seen or diagnosed (Fredriksson-Ahomaa et al., 2006; Smego et al., 1999).

Despite the millions of years since their evolutionary divergence, *Y. pseudotuberculosis* remains very similar to its parent *Y. enterocolitica* in its pathogenesis. Both bacteria are associated with a food borne disease with self limiting gastroenteritis, spread by oral-fecal route. The name *pseudotuberculosis* is derived from the formation of granuloma-like lesions in mesenteric lymph nodes during infection (Smego et al., 1999; Zhou and Yang, 2009).

After ingestion, *Y. enterocolitica* and *Y. pseudotuberculosis* travel to the small intestine and invade microfold (M) cells, which are specialized epithelial cells that act as antigen sampling cells. The bacteria then move across the epithelial barrier, travel to the mesenteric lymph nodes and colonize these organs, resulting in mesenteric lymphadenitis. This migration may be aided by phagocytes, which internalize the bacteria and transport them to the lymph nodes (Pujol and Bliska, 2005), where they are able to multiply extracellularly. The presence of the bacterial T3SS and Yop proteins are
thought to allow the bacteria to colonize the lymphoid organs, despite the presence of immune cells and macrophages (Pujol and Bliska, 2005) (Fredriksson-Ahomaa et al., 2006).

*Yersinia pestis*

*Yersinia pestis* is the causative agent of plague, a disease firmly embedded in the collective memory of humanity. The bacterium can be found on every continent except Australia and Antarctica and is estimated to have caused approximately 200 million deaths worldwide in at least three separate pandemics. The first known large scale pandemic was the Justinian plague, which began around 541 AD in Egypt and recurred in 8-12 year cycles throughout the Mediterranean. The second pandemic, commonly known as the Black Death, was spread throughout Europe beginning in the 12th century and occurred in continuous 2-5 year cycles up until the 17th century. This pandemic is thought to have killed up to 30-40% of the medieval European population and due to its longevity and severity it is the most historically significant and well known of the 3 major pandemics. It is credited with having had a huge impact on western society and human civilization as a whole. Numerous economic, religious, moral, medical and societal changes that occurred during the Middle Ages can be traced back to the widespread and dramatic depopulation caused by *Yersinia pestis* and other diseases common during this time period. Many popular images and conceptions about plague emanate from this time period, and have helped the bubonic plague to remain one of the most feared pathogens (Cantor, 2002; Perry and Fetherston, 1997; Smego et al., 1999).
The third pandemic began sometime in the mid-1800’s and continues to this day. This pandemic is important both because of its current status and also because during this time the *Yersinia pestis* bacterium was isolated and identified as the causative agent of plague. The bacterium was identified by Alexandre Yersin (Yersin A, 1894) and Shibasaburo Kitasato in Hong Kong in 1894, and shortly thereafter the role of the flea as an insect vector was identified. These discoveries, along with others during this time, have led to an increased understanding of the disease. When this understanding and the resulting implementation of public health measures were combined with the discovery and use of antibiotics, considerable reduction of plague associated mortality was achieved (Perry and Fetherston, 1997). However, plague infections still occur worldwide, including in the United States. The prairie dog population, along with other rodents, in the southwestern United States provides a natural reservoir for the disease, and people in close contact with these rodents have contracted the illness. In 2006 there were 13 case of plague in this region, resulting in two fatalities. This bacterium is also a concern for bioterrorism, and is a category A select agent (Butler, 2009).

The life cycle of *Y. pestis* is very different from the other pathogenic *Yersinia*. *Y. pestis* is spread by flea vector between rodent hosts, who form the natural reservoirs for this bacterium. Because a flea only ingests between 0.03-0.5μL of blood during each feeding, there must be a high titer of bacteria in the bloodstream of the host to complete the *Y. pestis* life cycle, in order to ensure transmission of the bacteria (Hinnebusch and Schwan, 1993). *Y. pestis* then obstructs the midgut of the flea, so that the next time the flea feeds, it will regurgitate bacteria into the next host (Bacot, 1919; Eisen et al., 2006;
Eisen et al., 2007; Hinnebusch and Erickson, 2008; Perry and Fetherston, 1997; Smego et al., 1999).

Humans living in close contact with infected rodents acquire the disease when they are bitten by infected fleas. Three to seven days post infection; the classical bubonic plague develops with the associated fever, headaches, body aches and other flu like symptoms, along with the characteristic swollen lymph nodes, or buboes. Septicemic plague occurs when the bacterium enters the blood stream directly and as a consequence buboes are not typically observed. The bubonic and septicemic forms of plague are very serious infections, and without antibiotic treatment are fatal in 30-70% of cases. The third and most dangerous form of *Y. pestis* infection is pneumonic plague. This type of plague occurs when an individual inhales the bacteria, by coming into close contact with an infected animal or an infected individual in later stages of the disease. As an individual becomes seriously ill and the bacteria invade the lungs, the bacteria can aerosolize when the individual coughs and hence be transmitted to other individuals. Pneumonic plague has a much shorter incubation period of only 1-3 days, and without treatment has almost a 100% fatality (Dennis, 2008; Gage KL, 2005; Perry and Fetherston, 1997).

After infecting the host subdermally, the bacteria are taken up by neutrophils and macrophages. While the bacteria are successfully killed by neutrophils, they are able to survive and replicate inside the phagolysosomes of macrophages located in lymphoid organs such as the spleen (Marketon et al., 2005; Straley and Harmon, 1984). After several days, the bacteria break free of the macrophage and enter the bloodstream, where
they rapidly reach very high titers leading to septic disease (Lukaszewski et al., 2005). The uptake of *Y. pestis* by macrophages forms a critical step in the survival of the bacteria, by providing a protected niche where replication to large numbers can occur. This also allows the bacteria to be disseminated throughout the body (Li and Yang, 2008).

The pathogenesis of *Y. pestis* has been extensively studied and it has become clear that *Y. pestis* is a master at evading the host immune response. Perhaps the most studied aspects of *Yersinia* pathogenesis are the type three secretion system (T3SS) and Yop proteins. The T3SS is specialized molecular machinery that acts to inject effector proteins into a host cell. The apparatus resembles a needle that is assembled on the surface of the bacteria and may have its evolutionary roots in the flagellum apparatus (Cornelis, 2006). The genes for this system are encoded on a virulence plasmid (pCD1) that is present in all three pathogenic *Yersinia* species (Viboud and Bliska, 2005). *Y. pestis* also contains two other virulence plasmids, which are not found in the other *Yersinia* species. These plasmids (pPCP1 and pMT1) contain important virulence proteins that help the bacteria escape detection by the host immune system (Chromy Ba, 2005; Perry and Fetherston, 1997).

T3SSs are common to many Gram-negative bacteria where they play an important role in virulence. Two types of proteins involved in the T3SS are encoded by pCD1, structural proteins that form the injectosome and non-structural effector proteins (Perry Rd, 1998), along with many other different proteins. The injectosome is a large multiprotein needle like apparatus that is comprised of 25 Ysc proteins and assembled in the
cytoplasm near the inner membrane of the bacterial cell then inserted through bacterial membrane to protrude at the surface of the bacteria. Its purpose is to penetrate the host cell membrane on contact and provide a channel through which the effector proteins can be introduced directly into the host cytoplasm. YopB, D and LcrV are the translocator proteins located at the distal end of the needle like structure. The hydrophobic YopB and YopD proteins act to form a pore in the host cell membrane, while LcrV forms a pentameric structure at the tip of the needle which aids the penetration of the host cell (Broz et al., 2007; Cornelis et al., 1998; Viboud and Bliska, 2005). Once this needle is in place and the pore formed, the effector proteins are able to translocate through the needle and into the host cytoplasm. These effector proteins, YopH,O,J,E,M, and T all have specific roles in the suppression of the host immune response (Cornelis, 2002; Zhang et al., 2008). YopH is a protein tyrosine phosphatase that blocks several pathways, including those involved in oxidative burst, focal adhesion, and B and T cell activation (Black et al., 2000; Bliska et al., 1992; de la Puerta et al., 2009). YopE, T and O all target the RhoGTPases, subsequently disrupting a large number of cellular processes including the actin cytoskeleton, cytokine production and phagocytosis (Black and Bliska, 2000; Mejia et al., 2008; Schotte et al., 2004; Viboud and Bliska, 2001; Viboud et al., 2006). YopJ acts as an acetyltransferase that inhibits MAP2 kinases and NF-κB signaling pathways by blocking phosphorylation (Mittal et al., 2006; Mukherjee et al., 2006). YopM is a leucine-rich protein that targets and depletes host Gr1+ natural killer cells (Kerschen et al., 2004; Ye et al., 2009). Together these proteins deal a significant
blow to the host immune response, facilitating the multiplication of the bacteria within macrophages and lymphoid organs.

The pPCP1 plasmid encodes for three proteins, plasminogen activator (*pla*), pesticin (*pst*), and pesticin immunity protein (*pim*). The plasminogen protease, or Pla, is an outer membrane protease that induces fibrinolysis in the host, promotes adhesion to and degradation of host cell extracellular matrix, cleaves complement protein C3, and promotes invasion of epithelial cells at the site of infection (Lahteenmaki et al., 2001; Lahteenmaki et al., 1998; Sodeinde et al., 1992). Pla is an essential virulence factor in flea borne bubonic plague, but is not necessary for septicemic plague (Sebbane F, 2006). Strains that lack Pla trigger a massive inflammatory response at the site of infection, have a million-fold reduction in virulence by subcutaneous infection and are taken up and killed by neutrophils, whereas *Y. pestis* strains containing Pla do not trigger an immune response at the site of infection (Sodeinde et al., 1992). Pesticin is an acidic, bacteriocin protein that acts as a muramidase which can lyse other *Yersinia* strains and *E. coli*, an action which is thought to promote survival of *Y. pestis* (Elgat and Ben-Gurion, 1969; Rakin et al., 1996; Vollmer et al., 1997). The pesticin immunity protein is encoded in opposite polarity on the pPCP1 plasmid and, as its name implies, allows *Y. pestis* to be immune to its own pesticin protein (Rakin et al., 1996; Sodeinde and Goguen, 1988; Vollmer et al., 1997).

The second unique virulence plasmid pMT contains the genes for murine toxin and fraction 1 (F1) capsule protein. Murine toxin is a member of the phospholipase D superfamily and when purified is toxic to mice and rabbits, however inactivating murine
toxin in *Y. pestis* does not attenuate virulence (Hinnebusch et al., 2000; Montie, 1981). Instead, it is required for the survival of *Y. pestis* in the flea by protecting the bacteria from digestion in the flea gut (Hinnebusch et al., 2002). The F1 capsule protein is a surface polymer expressed at 37°C and may have functions to inhibit invasion and prevent uptake of *Y. pestis* by macrophages (Cowan et al., 2000; Du et al., 2002). It also seems to play a critical role in the transmission of *Y. pestis* to the host via the flea (Sebbane et al., 2009).

Evasion of the immune system by *Y. pestis* is also aided by their resistance to the activity of serum and complement, which may be mediated in two different ways. Firstly, elevated levels of N-acetylglucosamine found in the *Y. pestis* LPS molecule protects the bacteria from complement, but this alone is not sufficient for the protection seen (Anisimov et al., 2005). The second possible mechanism is the expression and surface exposure of the outer membrane protein Ail, which acts as an adhesin or invasion enzyme and blocks complement mediated lysis (Bartra et al., 2008).

**Evolution of the pathogenic *Yersinia***

The three pathogenic species of *Yersinia* are all closely related. *Y. enterocolitica* believed to be the evolutionary parent of *Y. pseudotuberculosis*, which is thought to have diverged away 41-186 million years ago (Zhou and Yang, 2009). While these two species are fairly distantly related, they occupy the same natural reservoirs and give rise to similar diseases (Wren, 2003). *Y. pestis* is a much more recently evolved pathogen. It has been suggested to have evolved from *Y. pseudotuberculosis* between 1,500-20,000
years ago, although more current work suggests that the split occurred greater than 10,000 years ago (Achtman et al., 2004; Achtman et al., 1999). Although, *Y. pseudotuberculosis* shares a high degree of homology to *Y. pestis*, with 75% of the chromosomal proteins sharing 97% identity, their route of infection and pathogenesis is remarkably different (Chain et al., 2004). Because of this paradox, a great deal of recent research has been dedicated to determining how these two very similar bacteria can cause two very different diseases.

Guinet *et al.* 2008 demonstrated that, when compared directly, both pathogens have the ability to cause inflammation at the site of interdermal infection and travel to the draining lymph node. Two days after interdermal infection, mice infected with *Y. pseudotuberculosis* display a large infiltration of polymorphonuclear cells that are able to contain and eliminate the infection. This infiltration was absent in *Y. pestis* infected mice, which had a complete lack of immune cell recognition, allowing the bacteria to invade the bloodstream leading to septicemia. This result is interesting in that it revealed that it was not necessarily the route of infection that made *Y. pestis* more virulent, but its ability to suppress the immune response allowing it to escape the draining lymph nodes and invade the rest of the body (Guinet *et al.*, 2008). While experimentally these two bacteria are able to cause infection via interdermal inoculation, *Y. pseudotuberculosis* is very toxic to fleas, resulting in death of these animals, while fleas are generally resistant to *Y. pestis* infections (Erickson *et al.*, 2006; Erickson *et al.*, 2007).

The sequence of a number of *Y. pestis* and *Y. pseudotuberculosis* strains is now available and has allowed for whole genome comparisons to establish the point of
divergence and how such a virulent pathogen could have evolved from a relatively minor one (Eppinger et al., 2007; Garcia et al., 2007; Parkhill et al., 2001; Voskressenskaya et al., 2005). It has been determined that *Y. pestis* contains only 32 unique chromosomal genes compared to *Y. pseudotuberculosis* and two additional plasmids. Conversely, 317 genes were absent from the *Y. pestis* chromosome and 208 genes inactivated, which means up to 13% of the *Y. pseudotuberculosis* genome no longer functions or is present in *Y. pestis* (Chain et al., 2004). These data indicate that, while *Y. pestis* has acquired virulence genes during its divergence from *Y. pseudotuberculosis*, the genes that were lost could also be an important factor in its evolution. Further research has demonstrated that some of the deleted or inactivated regions are essential for *Y. pseudotuberculosis* viability yet dispensable in *Y. pestis* (Pouillot et al., 2008) which may hold clues as to how *Y. pestis* evolved. These genes may be important in the oral-fecal route of transmission, but dispensable for transmission in the insect vector; or critical for survival in the blood stream and lymphoid organs as opposed to the gut (Pouillot et al., 2008).

*Y. pestis* is a young pathogen, and because of this it is hard to determine exactly when and how the strain evolved. To resolve this issue, Achtman et al. utilized a three pronged approach to determine the age and divergence of *Y. pestis*. Single nucleotide polymorphisms (SNPs) and IS100 elements were screened for and analyzed, and multi-locus VNTR (variable copy numbers of tandem repeats) analysis (MLVA) was also used. Using these methods, it was determined that *Y. pestis* arose in Asia and split from *Y. pseudotuberculosis* greater than 10,000 years ago and probably had spread substantially before the first recorded pandemic. It was also suggested that the grouping of *Y. pestis*
according to three biovars (or historically and physiologically variant groups of strains) is inaccurate, and should be grouped according to molecular signatures which indicate the existence of eight separate *Y. pestis* populations (Achtman et al., 2004).

### 1.2 Bacterial Lipopolysaccharide/Lipid A Biosynthesis

**Lipopolysaccharide**

Gram-negative bacteria have a unique and distinct membrane structure, comprised of inner and outer membranes (Figure 1.1). The outer membrane is exposed to the environment, and because of this has adapted specific molecules and proteins to protect it from osmotic pressure, toxic molecules, and other challenges presented to it. The largest component of the external portion of the outer membrane is lipopolysaccharide (LPS) comprising 75% of the surface and accounting for 3-10% of the dry weight of the bacterium (Raetz Cr, 2002; Vaara, 1999).

Lipopolysaccharide, also called endotoxin, is important not only for protecting the bacterium from environmental pressures, but also has a role in a wide range of bacterial-host interactions, particularly in regards to the host immune response. The concept of endotoxin dates back to the 18th century when scientists first began to understand the concept of a toxic substance found in diseased or decomposing flesh that could trigger fever. However, it was not until the discovery of infectious microorganisms by Louis Pasteur in the 19th century and further studies by Robert Koch and Sir Alexander Ogston that scientists discovered that it was the individual components of these microorganisms, not the infection itself, that could lead to fevers and septic disease. During the middle of
the 20th century endotoxin was directly identified by Richard Pfieffer, and soon after LPS was able to be purified and its exact impact on the host immune system was able to be studied (Westphal et al., 1952) (Galanos et al., 1969; Rietschel and Westphal, 1999).

**Lipopolysaccharide structure**

LPS is a complex heteropolysaccharide molecule with three genetically and structurally distinct regions, the hydrophilic O-antigen polysaccharide, the core oligosaccharide, and the hydrophobic lipid A (Figure 1.2). This mix of hydrophobic and hydrophilic components creates an amphiphilic molecule that forms micelles or aggregates, in aqueous solutions. The O-antigen extends away from the membrane into the environment and is connected to the Kdo/lipid A moiety by the core region. Lipid A is the hydrophobic anchor of the LPS molecule, embedding the molecule into the outer membrane of the bacteria and is the source of the endotoxin potential (Luderitz et al., 1973) (Raetz et al., 2007). LPS is an essential molecule for most Gram-negative bacteria, the exception being *N. meningitidis*, which can survive without an LPS as a component of its outer membrane (Steeghs L, 2001).

Lipopolysaccharide can be found in two different forms, smooth (S-form) LPS, which has a long O-saccharide chain of up to 15 sugar residues, or rough (R-form) LPS which contains a truncated or absent O-saccharide chain linked to a heptose containing core region (Miller et al., 2005). This core region can be varied, for example *E. coli* has five varieties. Common structural elements are found within this core region comprising
of D-Hep-(1→7)-L,D-Hep(1→3) L, D-Hep(1→5)-Kdo. The core region is then bound to lipid A by Kdo, or 3-deoxy-D-manno-oct-2-ulosonic acid (Raetz et al., 2007).

The hydrophobic anchor lipid A molecule is the most structurally conserved element of the LPS molecule. It is generally composed of a $\beta$-(1’-6)-interlinked D-glucosaminylated hexosamine (GlcN) disaccharide backbone with phosphates bound at the 4’ position of the distal GlcN and $\alpha$-glycosidically linked to the proximal GlcN (Figure 1.3). The four primary fatty acids, or acyl groups, are bound to the disaccharide backbone by amine bonds at the 2’ and 2’ positions and by ester linkages at the 3’ and 3’ positions of the molecule. This tetra-acylated molecule is also referred to as Lipid IV$_A$ (Figure 1.3a), and has unique biological and immune stimulatory characteristics. The secondary acyl groups are attached to the Lipid IV$_A$ molecule by ester linkages to the $\beta$-hydroxyl groups on the two primary acyl groups located on the distal GlcN molecule (Figure 1.3b). This structure creates an asymmetrical fatty acid distribution on the molecule, which is a critical characteristic of the molecule. The arrangement, length, and chemical characteristics of these acyl chains strongly influence the immune stimulating potential of the molecule (Raetz et al., 2007).

**Lipid A biosynthesis in E. coli**

The biosynthesis of lipid A in *E. coli* has been examined in detail, allowing a greater understanding of the enzymatic pathway involved with the production and modification of the molecule (Figure 1.4). The enzymes involved in this pathway are encoded by single copy genes which are highly conserved across Gram-negative bacteria,
but can have some functional variability. The evolutionarily conserved enzymes are located on the intracellular side of the inner membrane, and are involved in the production of the base Kdo-lipid A molecule. The variable enzymes, including a number of modifying enzymes, are located on the outer leaflet of the inner membrane, facing the periplasmic space. These enzymes are environmentally regulated, and can be suppressed or induced by changes in temperature, nutrient availability, or growth conditions (Raetz et al., 2007).

The biosynthesis pathway of lipid A is very tightly regulated with each enzyme having tight substrate specificity. This means that the enzymes act in a sequential manner to produce the lipid A molecule. The first step in this pathway is the fatty acid acylation of the UDP-GlcNAc molecule by the LpxA enzyme (Figure 1.4), requiring the R-3-hydroxymyristoyl acyl carrier protein (ACP) to donate the C14 acyl chain to the molecule. This reaction can occur in both directions, with the acyl chain able to be removed from GlcNAc (Raetz et al., 2007; Ulaganathan et al., 2007).

The second step is the deacetylation of the newly formed UDP-3-O-(acyl)-GlcNAc by the zinc dependant enzyme LpxC (Figure 1.4). This is the first committed step of the lipid biosynthesis pathway and from this point on the pathway is unidirectional ending in the formation of a complete lipid A (Raetz et al., 2007; Young et al., 1995). LpxC is the target for a new and highly effective antimicrobial CHIR-090 which irreversibly binds to the enzyme in a two step, time dependent manner, inactivating the enzyme and shutting down the lipid A biosynthesis. As most Gram-negative bacteria cannot survive without lipid A, this compound displays a remarkable
potency at nanomolar concentrations across a broad spectrum of Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *H. pylori* and *N. meningitidis* (Barb et al., 2007a; Barb et al., 2007b; McClerren Al, 2005).

The next step in the pathway is the addition of a second C14 acyl chain at the site where LpxC removed the acetate molecule from GlcNAc. The second step is undertaken by enzymes LpxH and LpxD which mediate the formation of the lipid A disaccharide molecule. LpxH cleaves the pyrophosphate linkage of UDP-2,3-diacyl-GlcN to create 2,3-diacyl-GlcN-1-phosphate, a molecule called lipid X (Figure 1.4d). This molecule is half of the tetra-acyl lipid A molecule, which is then joined to another lipid X molecule by LpxB to create the β, 1-6'-linked disaccharide tetra-acyl lipid A precursor molecule (Figure 1.4) (Babinski et al., 2002; Buetow et al., 2007).

The next enzymes to act on this molecule are integral inner membrane proteins that contain membrane spanning regions within their N-terminal regions and active domains which are likely to face the cytoplasm. One of these enzymes, LpxK, phosphorylates the 4’ position of the disaccharide 1-phosphate molecule, creating Lipid IV$_\lambda$ (Figure 1.4) (Garrett et al., 1997). Two Kdo residues are then added to the Lipid IV$_\lambda$ molecule by KdtA (Figure 1.4) (Clementz and Raetz, 1991).

The last three enzymes in the pathway, termed the late acyltransferases, add the secondary acyl chains to the Kdo- Lipid IV$_\lambda$ molecule. In *E. coli*, LpxL is next to act on the molecule, linking a lauroyl residue to the 2’ position on the (R)-3-hydroxymyristoyl residue (Figure 1.4). The last enzyme in the pathway is LpxM, which adds a 3’ (R)-3-hydroxymyristoyl residue (Figure1.4), creating the asymmetrical acyl distribution that is
characteristic of lipid A (Vorachek-Warren Mk, 2002). *E. coli* also contains a alternative late acyltransferase, LpxP, which acts in place of LpxL at low temperatures and adds a palmitoleate to the molecule in place of a lauroyl residue (Carty et al., 1999).

When lipid A assembly is complete, the molecule is then flipped to the outer leaflet of the inner membrane by the enzyme MsbA, via an as of yet undefined mechanism (Raetz et al., 2009; Zhou Z, 1998). The O-antigen region of the LPS molecule is then attached to the lipid A-core molecule by the enzyme WaaL (Abeyrathne et al., 2005). The LPS molecule is then transported across the periplasmic space also by an unknown mechanism, and transferred to the outer leaflet of the outer membrane.

While the above biosynthesis pathway is generally conserved across Gram-negative bacteria, the lipid A molecule does display some variability. Many bacteria produce a lipid A with different numbers of acyl chains, or acyl chains are longer, shorter, or branched. These differences play a huge role in the immune activating or suppressing characteristics of the bacterium (Raetz et al., 2007). Acyl chain length is determined by the enzymes in the biosynthesis pathway. The *E. coli* acyltransferases have very tight substrate specificity. In the case of LpxA, the enzyme that adds on the first acyl chain to the glucosamine molecule, it preferentially adds a C:12 residue (Anderson and Raetz, 1987) (Bainbridge et al., 2008). However, it will add on acyl chains that have either eleven or thirteen carbon residues if that is all that is currently available in the substrate pool (Bainbridge et al., 2008). However, LpxA from other gram negative pathogens, like *N. meningitidis*, *L. interrogans*, *Bordetella* species, *P. gingivalis*, and *C. trachomatis* have either different acyl chain length requirements, or
have more relaxed specificities, and this is determined by changes in specific residues at
the catalytic site of the enzyme (Sweet et al., 2001) (Sweet et al., 2002) (Bainbridge et al.,
2008) (Odegaard et al., 1997) (Robins et al., 2009). The other enzymes in this pathway
exhibit the same sort of substrate specificity mechanisms, indicating that the enzymes are
the determinants for acyl chain length on lipid A (Bartling and Raetz, 2009) (Raetz et al.,
2007) (Bainbridge et al., 2008). *Rhizobium* species have a lipid A that contains an
especially long acyl chain and contain a unique enzyme which transfers these long acyl
chains on to the lipid A molecule (Brozek et al., 1996). Lipid A with longer or branched
acyl chains increases the thickness of the membrane, therefore altering how other
proteins can function in the membrane (Uratani et al., 1987).

**Late-acyltransferase LpxL**

The gene for LpxL was first identified in 1991 by Karow *et al.* and named *htrB*,
or high temperature requirement gene type B (Karow and Georgopoulos, 1991).
However, at this time the actual gene product remained unidentified. It was known that
this gene was necessary for bacterial growth in rich media at temperatures over 33°C, and
that mutations in this gene caused cell division arrest and profound morphology
differences in the cell wall suggesting that the gene product was involved in membrane
functions (Karow et al., 1991; Karow and Georgopoulos, 1991). At the same time as
Karrow and colleagues were investigating the *htrB* gene, Brozek *et al.* were
demonstrating the presence of late acyltransferase enzymes that added laurate and
myristate residues to Lipid IV\textsubscript{A} (Brozek and Raetz, 1990). It was later established that \textit{htrB} (also called \textit{lpxL}) encoded the lauroyl transferase (Clementz et al., 1996).

Overexpression of \textit{htrB} in \textit{E. coli} leads to an increased rate of enzymatic activity, while mutants have no detectable lauroyl transferase activity (Clementz et al., 1997). Also, when \textit{htrB} is mutated in \textit{E. coli}, the bacteria accumulate tetra-acyl lipid A in the inner membrane, due to severely reduced MsbA-mediated transported to the outer membrane. Overexpression of MsbA within the \textit{htrB} mutant bacteria promotes the export of tetra-acyl lipid A to the outer membrane, although this transfer remains highly inefficient (Zhou et al., 1998). However, at lower temperatures (below 33°C), the alternative late acyltransferase LpxP is active and can modify the tetra-acyl molecule, thus accounting for the temperature sensitivity (Figure 1.5) (Vorachek-Warren et al., 2002a). When both LpxL and LpxP are mutated the ability to grow at high temperatures is completely abolished, and primarily tetra-acyl lipid A is produced, although a small amount of pent-acyl lipid A is present due to leaky LpxM enzyme function. When all three late acyltransferases are mutated, \textit{E. coli} produces only tetra-acyl Lipid A, however this mutant can only grow on minimal media at low temperatures. All these mutant strains are highly sensitive to antibiotics, although the absence of LpxL alone appears sufficient to cause this sensitivity (Vorachek-Warren et al., 2002b).

LpxL has recently been purified and the key residues for enzyme activity have been identified. LpxL is a member of the glycerol-3-phosphate acyltransferase (GPAT) family, a diverse group of enzymes which all have a strongly conserved H(X)\textsubscript{4}D/E motif (where X is any amino acid) within their catalytic domain and utilize acyl-ACP or acyl-
CoA as their acyl donors. LpxL has four motifs which are critical for its enzymatic activity, all which show shared homology with other GPAT family members, and when mutated effect the activity of the enzyme. Mutations in the classic H(X)₄D renders the enzyme almost completely inactive, while mutations in the other 3 motifs also alter enzyme function (Six et al., 2008).

Several groups have mutated the htrB/lpxL gene in a variety of Gram-negative bacteria. Mutating htrB in S. typhimurium resulted in a significant reduction in virulence in mice, a result of the inability of the bacteria to colonize lymphatic organs and increased killing by macrophages (Jones et al., 1997). In H. influenza, mutating the htrB homologue resulted in initial temperature sensitivity, hypersensitivity to antibiotics and a loss of the secondary acyl chains from the molecule (Lee et al., 1995). Similar results were found in N. meningitidis containing mutated htrB, where the bacteria showed a decreased ability to induce TNF in human macrophage cell lines and produced tetra- and pentacylated lipid A species in contrast the hexacylated species found in the parent strain (Ellis et al., 2001; van der Ley et al., 2001). N. gonorrhoeae strains with a mutated lpxL showed a decrease in acylation over wild type, and a decrease in cytokine production in cells stimulated with LPS from the mutant strains (Ellis et al., 2001). In F. tularensis, which produces an atypical tetra-acyl lipid A, an LpxL gene has been identified and when mutated a tri-acylated lipid A species is produced. When complementing an E. coli strain which lacks lpxL with the F. tularensis lpxL, a hexa-acyl lipid A is produced, indicating that F. tularensis LpxL functions like other Gram-negative LpxL’s, despite the atypical lipid A produced by this bacteria (McLendon et al., 2007).
Yersinia lipopolysaccharide and lipid A

The pathogenic Yersinia species each have unique lipopolysaccharide characteristics, which may contribute to the growth and virulence differences observed between different serotypes and species. Y. pseudotuberculosis and Y. enterocolitica both have the S-form of LPS, with O-antigen chains connected to the core region of the molecule. These O-antigen chains display notable diversity, creating different serogroups of both Y. pseudotuberculosis and Y. enterocolitica and may account for the differences in antimicrobial and serum resistance, and other pathogenic properties seen between the different serotypes and species (Anisimov et al., 2005; Bengoechea et al., 2003; Bengoechea et al., 1998a; Bengoechea et al., 1998b; Bengoechea et al., 2004; Knirel et al., 2007). However, Y. pestis lacks this O-antigen region in its LPS, instead expressing the R-form of the molecule (Albizo and Surgalla, 1970) (Bruneteau M, 2003; Hartley Jl, 1974; Hitchen et al., 2002; Knirel et al., 2005b; Prior et al., 2001a), due to frame shift mutation in the O-antigen gene cluster in Y. pestis (Prior et al., 2001b). Generally, the lack of an O-antigen can be detrimental to the bacteria due to increased serum sensitivity; however, Y. pestis is still are able to invade the host and be complement resistant (Anisimov et al., 2005). Y. pestis expressing only lipid A are viable, and although these mutants survive, their growth is very poor and other serious defects are present (Tan and Darby, 2005). The lipid A portion of the LPS molecule also differs between the Yersinia species. Y. enterocolitica produces tetra- and hexa-acylated lipid A (Aussel et al., 2000) (Rebeil et al., 2004). Y. pseudotuberculosis produces a heterogeneous mix of lipid A containing tetra-, penta-, and hexa-acylated species. They contain a variety of secondary
acyl chains lengths, varying from C12 to C16:1, resulting in a highly complex lipid A profile. Within these two species however, no significant difference in acylation patterns are apparent when bacteria are grown at different temperatures (28°C vs. 37°C) (Rebeil et al., 2004; Therisod et al., 2002).

The lipid A in *Y. pestis* is very different from that of the other pathogenic *Yersinia*. At low temperature (26°C-27°C), *Y. pestis* produces a heterogeneous mix of tetra-, penta-, and hexa-acylated lipid A with various modifications, similar to *Y. pseudotuberculosis*. However, *Y. pestis* grown at 37°C has a notably different lipid A profile where tetra-acylated lipid A is the predominate species (Kawahara et al., 2002; Knirel et al., 2005b; Rebeil et al., 2004). These differences in acylation patterns have a considerable effect on LPS bioactivity in *Y. pestis*. When human cells, and to a lesser extent mouse cells, are cultured in the presence of either lipid A or LPS purified from *Y. pestis* grown at 37°C, it was unable to produce a strong cytokine response in comparison with LPS or lipid A from *Y. pestis* grown at lower temperatures (Kawahara et al., 2002; Matsuura et al., 2009; Rebeil et al., 2004). In addition, *Y. pestis* is also more sensitive to antibiotics when grown at 37°C verses 26°C (Rebeil et al., 2004).

Examination of the late acyltransferases of *Y. pestis* has provided insights into the mechanisms underlying the different lipid A profiles with changes in temperature (Rebeil et al., 2006). It was established that *Y. pestis* encoded *lpxM* and *lpxP* homologues, whose gene products acted as late acyltransferases, adding on a C12 and C16:1 respectively to tetra-acyl lipid A at 21°C. When *lpxM* and *lpxP* were mutated, the bacterium was unable to produce hexa-acyl lipid A at lower temperatures. However, no homologue to *lpxL* was
found (Rebeil et al., 2006). This data provides an explanation of how *Y. pestis* was able to produce hexa-acyl lipid A at low temperatures, but not at 37°C. Despite the fact that LpxM is present and functional in *Y. pestis*, due to the high substrate specificity of these enzymes, without LpxL to add on the first secondary acyl chain to the tetra-acyl molecule, LpxM does not function on the molecule, so the molecule remains with four C14 primary acyl chains.

### 1.3 Innate Immunity

#### Overview of the Innate Immune System

As science began to understand that microorganisms caused disease, an understanding of the body’s defenses also began to arise. This field of study, called immunology, looks to examine how the body defends against pathogenic organisms. Since the discovery of antibodies in 1890 by Emil von Behring and Shibasaburo Kitasato, the idea that the body is able to mount a specific response to a pathogen has become well known. This adaptive immune response utilizes antigen specific lymphocytes, such as B and T cells, to fight off specific pathogens and provide protection against further infection by that specific pathogen (Janeway Jr, 2002). However, the concept of innate immunity, how the body initially recognizes invading pathogens and starts the immune response, took longer to become established. Elie Metchnikoff first postulated the idea of cell mediated immunity and the concept that cells could phagocytose foreign bodies (Janeway Jr, 2002). However, it wasn’t until the discovery of the first Toll like receptor (TLR) in humans in 1997 (Medzhitov et al., 1997) that the picture of how the innate
immune system functioned to recognize pathogens and activate the immune response as a whole started to become clear.

The innate immune response is the first line of defense against invading pathogenic organisms. It recognizes ‘danger’ signals, which in the case of microorganisms are evolutionarily conserved molecules known as pathogen associated molecular patterns (PAMPs). These molecules include nucleic acids, bacterial cell wall components such as LPS, lipoproteins and flagellin, and viral proteins (Medzhitov and Janeway, 1998). These structures have unique properties that are conserved across a wide range of bacteria and viruses, making them ideal targets for host recognition. These molecules are recognized by pattern recognition receptors (PRR) (Akira et al., 2006). These are highly conserved germ line encoded receptor molecules that upon recognition of PAMPs initiate an intracellular signaling cascade that activates gene transcription of both pro- and anti-inflammatory cytokines, chemokines, adaptor molecules, and transcription factors. This subsequently activates other components of the innate immune response, which in turn activates and educates the adaptive immune response (Akira, 2006; Janeway Jr, 2002; Mogensen, 2009).

**Pattern recognition receptors**

Pattern recognition receptors are a large group of extracellular and intracellular receptors that recognize unique PAMPs. They fall into 3 major families, the Toll-like receptors (TLR’s), the retinoid acid-inducible gene I (RIG-I) like receptors (RLR), and the nucleotide-binding oligomerization domain (NOD) like receptors (NLR’s)
These receptors all occupy different cellular compartments, but they perform overlapping functions to ensure rapid responses to pathogens and other danger signals.

The TLR family is an evolutionarily conserved group of membrane bound receptors found in species from *C. elegans* to humans, and are probably the most extensively studied PRR to date (Akira, 2006; Mogensen, 2009). They are named after the *Drosophila* Toll, a receptor in the fly involved in development and immune responses, to which the TLRs bear homology (Medzhitov et al., 1997). Currently, 10 TLR’s have been identified in humans and 11 in mice. These receptors are found on most cell types, and although they recognize different PAMP’s, they share similar structural and cytoplasmic signaling motifs. TLR’s are formed by integral glycoproteins containing a ligand binding domain that includes a leucine rich repeat (LRR) and a cytoplasmic region that contains a Toll/Interleukin-1 receptor (TIR) domain (Slack et al., 2000). The TLR’s can be divided into two groups; TLR’s 1, 2, 4, and 6, which are found on the cell surface and recognize lipids and proteins, while TLR’s 3, 7, 8, and 9 are located in the endosomal compartment and recognize nucleic acids (Mogensen, 2009).

The second class of PRRs are the RLRs, which are a group of cytosolic nucleotide receptors. There are two receptors within this group; RIG-I, which detects virally derived dsRNA (Hornung et al., 2006; Kato et al., 2005; Yoneyama et al., 2004) and melanoma differentiation-associated gene 5 (MDA5) which recognizes polyI:C and dsRNA(Kato et al., 2006). These receptors bind cytoplasmic nucleotides via their C-terminal domains (Takahashi et al., 2009), promoting the association with adaptor proteins with their N-
terminal caspase recruitment domain (CARD). The signaling cascade triggered by this association results in type 1 interferon response which is critical for the clearance of viruses (Yoneyama et al., 2005).

The third class of PRR’s are the NLR’s, which are also cytoplasmic receptors that respond to intracellular bacteria and endogenous danger signals. This group of receptors includes NOD1 and NOD2 which recognize components of Gram-positive and Gram-negative bacterial peptidoglycan (Girardin et al., 2003a; Girardin et al., 2003b). This class of PRRs also includes the NALP proteins (the largest group of NLR’s) and IPAF, which recognize both microbial components in the cytosol, along with endogenous danger signals (misfolded proteins, crystals DNA). The recognition occurs via a C-terminal LRR and results in CARD oligomerization and the activation of inflammatory signaling (Mariathasan and Monack, 2007; Martinon et al., 2009; Martinon F, 2007; Muruve Da, 2008). These NLR proteins are a critical component of inflammasome complexes, will be described in a later section.

1.4 Toll-Like Receptor 4

Toll like Receptor 4 (TLR4) is probably the most extensively studied TLR and PRR. TLR4 was first identified as the receptor for lipopolysaccharide by examining the HeJ mutation in LPS resistant mice. Mice with a mutation in the tlr4 gene were resistant to LPS toxic shock, but highly susceptible to Gram-negative infection (Poltorak et al., 1998). From there it was determined that TLR4, in conjunction with the adapter molecule MD2, recognized the lipid A component of LPS which triggers a signaling
cascade that eventually activates NF-κB, AP1 and other proinflammatory transcription factors (Figure 1.6).

LPS is first bound by LPS binding protein (LBP), an acute phase protein found in the host bloodstream (Mathison et al., 1992). LBP then transfers LPS to CD14, a soluble glycosylphosphatidylinositol (GPI)-linked protein that is found on the membrane of mature monocytes, macrophages, and neutrophils (Arditi et al., 1993; Haziot et al., 1993). It contains a LRR domain and is considered a member of the PRR family. LPS/lipid A bound to CD14 to increase the concentration of the lipid at the cell surface and is then transferred to the MD2 protein (Tapping and Tobias, 1997; Viriyakosol et al., 2001; Wurfel and Wright, 1997; Yu et al., 1997). This increases the sensitivity of the TRL4 receptor by aiding in the transfer of LPS/lipid A to TLR4/MD2 via a currently unknown mechanism (Delude et al., 1995) (Latz et al., 2002; Meng et al., 2008).

MD2 is an essential adaptor protein associated with TLR4, and is the primary endotoxin recognition molecule (Park et al., 2009). It is a secreted soluble protein which is essential for the recognition of lipid A by TLR4 (Schromm et al., 2001), as mice deficient in MD2 are completely resistant to endotoxic shock, but hypersensitive to Gram-negative infections (Miyake et al., 2002; Nagai et al., 2002). The oligomerization of the TLR4/MD2 complex causes a conformational change in the cytoplasmic region of the proteins, which initiates the immune signaling cascade. The TIR domain of TLR4 is the essential signaling portion located in the cytoplasmic tail of the receptor. It is about 200 amino acids in length and contains three highly conserved areas, or boxes. The first box contains the domain signal sequence, while the second and third boxes play critical
roles in signaling (Brikos and O'Neill, 2008; Xu et al., 2000). Once TLR4 is dimerized, other TIR domain adaptor proteins are recruited to initiate the downstream signaling cascade (Carpenter and O'Neill, 2009). Four TIR domain adaptor proteins that promote signaling that have been identified, MyD88 (myeloid differentiation factor 88)(Medzhitov et al., 1998), Mal (MyD88 adaptor like protein) (Fitzgerald et al., 2001; Horng et al., 2001), TRIF (TIR domain containing adaptor inducing interferon-β) (Yamamoto M, 2002) and TRAM (TRIF related adaptor protein) (Yamamoto et al., 2003c). A fifth TIR domain adaptor protein SARM (sterile-α and HEAT/Armadillo motifs-containing protein) acts as a negative regulator of TRIF (Carty et al., 2006). These proteins form the distinct MyD88 dependant and TRIF dependant pathways that are activated by TLR4/MD2 signaling (Togbe et al., 2006) (Brikos and O'Neill, 2008) (Figure 1.6).

The MyD88 dependent pathway is utilized by all of the TLR’s with the exception of TLR3. Activation of the MyD88 dependant pathway results in a fast and robust activation of the IKK complex and translocation of transcription factors to the nucleus. After TLR4/MD2 activation, MyD88 and Mal are recruited to the complex and bind the TIR domain of the receptor (Nunez Miguel et al., 2007). This allows the recruitment of members of the IL-1 receptor associated kinase (IRAK) family to the Mal/Myd88 (Figure 1.6). IRAK4 followed by IRAK1 and IRAK2 are sequentially phosphorylated and associate with TRAF6 (Qian et al., 2001) (Hacker and Karin, 2006). TRAF6 acts as a ubiquitin protein ligase that adds K63- linked polyubiquitin chains on several different components of the Mal/Myd88 complex, including itself, TAK1 (transforming growth
factor-activated protein kinase 1), and NEMO (IκB kinase (IKK) subunit NF-κB essential modifier). The K63 ubiquitinated Mal/Myd88 complex facilitates the phosphorylation and activation of the IKK complex and members of the MAP kinase family. Activation of the IKK complex results in the phosphorylation and degradation of IκB, which frees NF-κB and allows it to translocate to the nucleus (Figure 1.6). NF-κB in turn activates transcription of a number of proinflammatory proteins and cytokines required for the anti-microbial response. Activation of MAP kinases leads to the stimulation of activator protein 1 (AP1), a transcription factor which in turn translocates to the nucleus and activates proinflammatory gene transcription (Carpenter and O'Neill, 2009; Mogensen, 2009).

The second TLR4/MD2 activated pathway is the MyD88 independent/TRIF dependent signaling pathway (Figure 1.6). After TLR4/MD2 activation, TRAM and TRIF associate with the TIR domain of TLR4 (Nunez Miguel et al., 2007), which in turn interacts with RIP1 and TRAF6 to activate the IKK complex. However, this activation is much slower and at a lower level than that seen in the MyD88 pathway. In addition, TRAM and TRIF can also activate the interferon (IFN) response by interacting with TRAF3 and TANK (TRAF family associated NF-κB activator) (Hacker et al., 2006). This allows the IKK related kinases TBK1 and IKKe to phosphorylate IRF3 and IRF7, which translocate to the nucleus as either hetero- or homodimers and activate the transcription of the IFN and IFN inducible genes. TRAM and TRIF also interact with NF-κB and AP-1 to induce IFN-β (Mogensen, 2009).
TLR4 expression

The type of cells on which TLR4 is expressed is crucial due to its ability to recognize pathogens and initiate a host response. In humans, TLR4 is predominantly expressed on myeloid cells, including monocytes, macrophages, neutrophils dendritic cells and granulocytes, all of which are key in the initial recognition of pathogens by the innate immune system. The promoter region of TLR4 contains features typical of a myeloid specific gene, including recognition sites for the transcription factor PU.1 and the absence of a TATA box. Many studies have shown that the levels of TLR4 mRNA can differ between these myeloid cell types and is dependent on the activation state of the cell. When alveolar macrophages were compared to autologous monocytes from healthy human subjects for example, the macrophages were found to contain lower levels of TLR4 mRNA than the monocytes, however, the surface expression levels of the receptor on both cell types was the same (Juarez et al.). Dendritic cells also differentially express TLR4, with expression determined by their developmental stage (Kadowaki et al., 2001; Visintin et al., 2001). This is illustrated by the high levels of TLR4 mRNA within monocyte precursor cells, compared with CD11b+ cells and plasmacytoid pre-dendritic cells which have no detectable TLR4 mRNA. Other studies have shown that immature dendritic cells do express low levels of TLR4 mRNA, but these levels decrease over time after activation of the cell by LPS (Kokkinopoulos et al., 2005). Langerhans cells, which are specialized dendritic cells found in the epidermis, do not express TLR4, possibly to prohibit reactions to commensal skin flora, while dermal dendritic cells, those found in
the deeper dermis layer of the skin, do express TLR4 (Takeuchi et al., 2003; van der Aar et al., 2007).

TLR4 expression is also found within other cell types with roles within the immune response, such as lymphoid cells. Human hematopoietic stem cells and plasma cells express TLR4 mRNA, however B cells do not (Dorner et al., 2009; Hornung et al., 2002; Mansson et al., 2006). CD4 and CD8 T cells both express TLR4, and activated CD8 T cells secrete high levels of IFNγ, perforin and granzyme in response to LPS stimulation (Komai-Koma et al., 2009; Xu et al., 2005). TLR4 is also expressed in most other tissues and cells in humans, with the exception of the liver, which does not contain detectable levels of TLR4 mRNA transcripts. Of interest is the fact that TLR4 is expressed on both melanocytes and keratinocytes in the epidermis, which may help the body respond to subcutaneous infections (Ahn et al., 2008; Kollisch et al., 2005; Lebre et al., 2007).

A variety of mouse cells and tissues express TLR4, however, in many cases the distribution is different to that seen in humans. Mouse myeloid cells have the similar levels of TLR4 to humans, however the expression in tissues such as lung, heart, kidney, and muscle are considerably higher (Dearman et al., 2009; Liu et al., 2002). In contrast to humans, mouse B cells do express TLR4 mRNA and respond to LPS (Genestier et al., 2007), whereas mouse naïve CD8 T cells lack TLR4, although after injury both CD4 and CD8 T cells have been shown to express the receptor (Cairns et al., 2006; Komai-Koma et al., 2009). Recent work also suggests that mouse serum contains different properties then human serum, which can reduce the response of macrophages to LPS, indicating that
cellular differences are not the only factor in regulating the response of TLR4 to LPS (Warren et al.). This differential expression within the different cellular subsets and species is important to consider, both when examining the route of infection and the early responses to the pathogen, and also when utilizing mice as a model system of infection.

**TLR4/MD2 recognition of LPS/Lipid A**

In human cells, the most potent activator of TLR4/MD2 signaling is hexa-acyl lipid A which can induce a strong TNF and IFNβ response at very low (picomolar) levels. However, modifications of the lipid A molecule can affect the signaling capabilities of TLR4. These modifications include reduction in the number of acyl chains, modification or branching of these chains, and modification of the GlcNAc backbone such as altering the number of phosphates or arabinose modifications (Raetz et al., 2007). In particular, tetra-acyl lipid A is known to be a potent antagonist for TLR4 signaling. When cells are stimulated with Lipid IVₐ or synthetic tetra-acyl lipid A, there is no TLR4 activation or signaling. Lipid IVₐ can also act as an inhibitor of TLR4/MD2 signaling when tetra-acyl lipid A is added to cells alongside stimulatory hexa-acylated lipid A signaling is significantly reduced or abolished (Golenbock et al., 1991; Saitoh et al., 2004a).

Recent work resolving the crystal structure of the TLR4-MD2-lipid A complex has provided a clearer picture of the interaction between TLR4/MD2 and lipid A, and how differences in the acylation pattern of lipid A affect stimulation (Park et al., 2009). The hydrophobic pocket of MD2 binds the lipid A portion of the LPS molecule. Five of the six acyl chains of hexa-acylated lipid A fit into this hydrophobic pocket, while the
sixth chain remains outside the pocket and forms a tight hydrophobic interaction with conserved phenylalanine’s of the TLR4 protein. Two MD2 proteins complex in this manner with to two lipid A molecules, and then interact with two TLR4 receptors, creating a tight symmetric complex (Figure 1.7). The phosphate groups on the lipid A molecule interact with both the MD2 and TLR4 molecules via positively charged residues on the TLR4 protein and a hydrogen bond with MD2. This binding pattern explains why modifications to the acylation pattern of lipid A effect the stimulation of TLR4. In the case of tetra-acyl lipid A, the four acyl chains fit neatly into the hydrophobic pocket, with no acyl chains outside the pocket and the glucosamine backbone oriented in a different manner. This arrangement allows the lipid A molecule to fit deeper into the MD2 hydrophobic pocket and prevents proper interaction with TLR4, thus inhibiting immune activation (Park et al., 2009).

Species specific recognition of Lipid IVₐ

Lipid A specificity varies greatly among species. For example in contrast to human TLR4/MD2, mouse TLR4/MD2 is able to recognize tetra-acyl lipid A leading to a signaling and immune activation (Akashi et al., 2001). Early work to determine the cause of this species specificity was contradictory. Several reports indicated that species specificity was conferred by MD2 (Akashi S, 2001; Muroi M, 2006), other reports indicated that TLR4 was the deciding factor (Lien et al., 2000; Poltorak et al., 2000). In order to establish what causes these differences in species specific recognition, both MD2 and TLR4 were examined and it was determined that surface charges at the lipid
A/TLR4/MD2 interface contribute to how the receptor complex recognizes lipid A. Lipid IVₐ is also an agonist for the equine TLR4/MD2 complex. In order to determine what portions of TLR4 and/or MD2 are important in this recognition, comparative analysis between humans and horses was undertaken. It was determined that the electrostatic surface potential of specific domains on the surface of both MD2 and TLR4 were critical for recognition of different forms of lipid A. By swapping these domains between horse and human TLR4 and MD2, the receptor was able to recognize and productively signal (Walsh et al., 2008).

Computational studies have shown that the ionic structure of the mouse TLR4/MD4/lipid A interface differs from that of the human complex, resulting in differential binding of Lipid IVₐ and activation of the receptor (Meng 2009). The key residues that determine this surface charge difference have been identified as well, and mutating these residues to change their charges alters how the receptor complex functions (Meng 2009, in process). These studies are critical to the understanding of how different forms of lipid A can activate TLR4/MD2.

The effect of lipid A structure on bacterial pathogenesis and the host immune response

As described previously, Gram-negative bacteria produce a wide range of lipid A species. TLR4 recognition of these different forms of lipid A has implications for both the hosts’ immune response and for the pathogenesis of the bacteria. Gram negative pathogens like *E. coli* (Imoto et al., 1985; Raetz et al., 2007), *K. pneumonia* (Clements et
al., 2007), *E. cloacae* (White et al., 1984), *H. influenzae* (Mikhail et al., 2005), and some *Shigella* species (Rallabhandi et al., 2008) produce hexa-acylated lipid A which is able to stimulate human TLR4. These pathogens infect through the mucosal pathways such as the GI tract, and while they do have the ability to cause severe disease, this usually occurs in hosts that may not be fully immunocompetent (Munford, 2008), which is advantageous both for the host and pathogen. In this situation the host is generally able to clear the infection without severe damage or septic shock, while the bacteria are shed from the host large numbers, allowing for effective dissemination and the opportunity to infect a new host. However, when these pathogens with stimulatory LPS cross the mucosal/epithelial barrier and become systemic, they can have catastrophic effects. When introduced into the bloodstream, even non-pathogenic *E. coli* can cause severe septic shock and death of the host (Ender et al., 2009; Wray et al., 2001). This is in part due to the TLR4/MD2 mediated immune response triggered in the bloodstream as well as other factors such as the activation of complement and clotting deficiencies, all of which can lead to hemorrhage and subsequent death (Munford, 2008).

In addition to *Y. pestis*, many Gram negative bacteria produce a lipid A with a reduced ability to be recognised by TLR4, including *F. tularensis* (Schilling B, 2007), *B. abortus* (Qureshi et al., 1994), *H. pylori* (Stead et al., 2008), *L. pneumophilia* (Palusinska-Szysz and Russa, 2009), *P. aeruginosa* (Bedoux et al., 2004), *C. burnettii* (Schramek and Galanos, 1981), and *V. cholerae* (Vinogradov et al., 1995). Although not all Gram-negative bacteria that produce non-stimulatory lipid A are pathogenic, those that are usually invade the body through non-mucosal routes including abrasions, cuts or
injections into the skin or bloodstream, inhalation, and the conjunctival membranes. These bacteria are characterized by their ability to become systemic and reach very high titers in the bloodstream of infected individuals. They are also capable of causing septic shock, despite their inability to stimulate human TLR4/MD2, which is thought to occur either via toxins produced by the bacteria or due to the very high bacterial titer with in the bloodstream which can trigger other innate immune receptors (Munford, 2008).

1.5 Lipid A Modifications and Vaccine Development

With the discovery of the TLRs and innate immune system, a great deal of work has gone into trying to utilize the responses from this arm of the immune system in vaccine development. The key goal of this work has been to increase the efficacy of vaccines, decreasing any side effects or negative reactions. Therefore, finding molecules that activate both innate and adaptive immunity but have low toxicity or antigen cross-reactivity is of great interest. TLR ligands are of particular interest because they have the ability to both activate and direct the adaptive immune response. Recent work done by a number of groups has examined how modifying lipid A in Gram-negative bacteria can increase the immunological properties of vaccines. A vaccine for *Neisseria meningitidis* serogroup B has been difficult to design, and the use of endogenous LPS in these vaccines is controversial because of its acute toxicity. However, work has been done to create membrane vesicles that contain *N. meningitidis* outer membrane proteins with a variety of TLR agonists, including monophosphoryl lipid A, a TLR4 agonist that has lower toxicity than standard LPS/lipid A due to its bias for activating the TRIF/TRAM
pathway rather than MyD88 (Casella and Mitchell, 2008; Fransen et al., 2007). *N. meningitidis lpxL* mutants have also been used in vaccine development, because of reduced toxicity of the LPS in these strains due to the decreased number of acyl chains on the molecule (Stoddard et al., 2009). A similar approach has been taken in generating *B. pertussis* and *F. tularensis* vaccines. LPS from the *N. meningitidis lpxl2* mutant and monophosphoryl lipid A was injected into mice along with the standard *B. pertussis* vaccine resulting in a higher efficacy of the vaccine in both cases. With *F. tularensis*, a different strategy was utilized. In this case, a strong TLR2 agonist, PorB from *N. meningitidis*, was co-administered to mice with LPS from *F. tularensis* LVS as a vaccine, after which the mice were challenged with live *F. tularensis* LVS. Mice that were vaccinated with both PorB and LPS had a survival rate of 70% after bacterial challenge, whereas only 25% of mice injected with LPS alone survived the infection (Chiavolini et al., 2008). This research demonstrates that the selective and potent activation of the TLR response can be an important strategy for vaccine development (Geurtsen et al., 2008).

### 1.6 IL-1 pathway and inflammasome activation

The IL-1 pathway is a critical and essential immune regulatory pathway. It plays a central role in the activation of the immune response, and causes the upregulation of a large number of chemokines, cytokines, growth factors, adhesion molecules and other key regulators of the immune response (O'Neill, 2002). The IL-1R is the founding member of the IL-1/TLR superfamily that now encompasses a large number of receptors critical for the innate immune response. It utilizes MyD88 as an essential adapter protein
and activates NF-kB through similar downstream signaling cascades (Medzhitov et al., 1998; Stylianou et al., 1992). Unlike TLRs which recognize PAMPs, the ligands for IL-1R are endogenous cytokines produced upon stimulation of the TLRs. Currently, 11 ligands for IL-1R have been identified at this point; the most extensively are IL-1α, IL-1β, and IL-18 (Dinarello, 2009; Slack et al., 1993).

The inflammasomes are a group of multiprotein cytoplasmic complexes that utilize NLRs to sense intracellular microbial elements and activate IL-1β and IL-18, two potent inflammatory cytokines. The cytokines IL-1β and IL-18 are produced in response to TLR activation in the pro-form, and when activated by caspase cleavage, elicit an intense inflammatory response. Therefore, the existence of two separate regulatory mechanisms provides an important safety mechanism to maintain control of their expression and release. While this complex has only recently been discovered, it has been determined to play an important role in the detection of both endogenous and exogenous danger signals. The NLR proteins act as both sensors and the molecular scaffold on which the rest of the complex is assembled (Martinon et al., 2009).

The NALP subfamily of NLR’s are comprised of 14 genes in humans and form the largest group of NLRs. Three of these proteins, NALP1, NALP2, and NALP3 act as the central sensing and scaffold proteins for the caspase 1 inflammasome. These proteins have an LRR region that functions to sense cytoplasmic pathogens, a NACHT region which is the central nucleotide domain essential for oligomerization of the complex, and an N-terminal pyrin domain (PYD) that bridges between the receptor and adaptor/effector proteins which also contain PYDs. Although IPAF is evolutionarily distant from the
NALP subfamily, it also contains LRR and NACHT domains. However, instead of having a PYD domain, it encodes a CARD domain in its N-terminal region which acts like PYD to link the receptor to other proteins containing a CARD region (Martinon et al., 2009).

After activation, associated speck-like protein containing a caspase recruitment domain (ASC) complexes with the NLR and an inflammatory caspase, leading to the cleavage and activation of the associated caspase. This activated caspase then cleaves the pro-IL-1β and pro-IL-18 present within the cytoplasm, facilitating their release from the cells (Martinon et al., 2009; Yamamoto et al., 2004). Several NLR proteins are present within the cell allowing the production of many different types of inflammasomes.

The inflammasomes recognize a wide range of different danger signals, including various microbial products. This is necessary because some microbes grow intracellularly or produce toxins which are injected into the cytoplasm. Pathogens such as S. flexneri, S. typhimurium, P. aeruginosa and L. pneumophila all activate the IPAF-caspase 1 inflammasome through factors secreted into the cytoplasm. The NALP3 inflammasome is known to be activated by bacterial pore-forming toxins like α toxin from S. aureus and listeriolyisin O from L. monocytogenes, and NALP 1 inflammasome is activated by anthrax toxin.

A number of different aspects of Y. pestis life cycle could trigger inflammasome activation. It has been shown in Y. enterocolitica that the presence of the T3SS pore alone even in the absence of Yop expression triggers the activation of caspase 1, resulting in the cleavage and secretion of IL-1β. However, in the presence of Yop effectors,
caspase 1 activation was inhibited resulting in a lack of cleavage or secretion of IL-1β (Shin and Cornelis, 2007). Three types of Yop proteins have been implicated in this inhibition, YopE, YopT and YopJ. In Y. enterocolitica YopE and YopT inhibit RhoGTPases, leading to a significant reduction in the activation of caspase 1, and resulting in the inhibition of IL-1β release (Schotte et al., 2004). Conversely, in Y. pestis and Y. pseudotuberculosis YopJ seems to activate caspase 1 and promote apoptosis in macrophages (Bergsbaken and Cookson, 2007; Lilo et al., 2008). However, in the case of Y. pestis, this effect was only seen in isolates of KIM5 strain, so this may be particular to this strain rather than an overall function of YopJ.

1.7 Thesis Objectives

The long term objective of this thesis is to increase our understanding of how Gram-negative pathogens are able to evade the recognition of innate immune receptors by altering their LPS/lipid A profile. I specifically wanted to examine how Y. pestis utilized this and other mechanisms to evade TLR4/MD2 activation in mammalian hosts by altering its lipid A profile at mammalian body temperature, and to determine if the absence of lpxL should be considered a major virulence determinant. I also wanted to determine if the loss of the lpxL gene from Y. pseudotuberculosis formed a major evolutionary step in the development of Y. pestis into a distinct and highly virulent bacterium. The experiments and data presented here were performed to determine how LPS/lipid A effect virulence of Gram-negative bacterial infections, and to determine if altering the lipid A profile during infection could change the course and outcome of any
disease condition. The findings of this research have major and direct implications for our understanding of Gram-negative bacterial infections. A greater appreciation of how bacteria evade the innate immune responses will allow us to design improved vaccines and treatments for these infections.
Figure 1.1

Figure 1.1 Schematic representation of the Gram-negative bacterial cell wall

Representation of the inner and outer membranes of a Gram-negative cell wall.

(Raetz et al., 2007)
Figure 1.2

Figure 1.2 Chemical Structure of lipopolysaccharide

Schematic representation of the structure of LPS, including lipid A, the core region, and the O-antigen outer portion of the molecule. (Ohno and Morrison, 1989)
Figure 1.3
Figure 1.3 Chemical structure of Lipid IV\textsubscript{A} and \textit{E. coli} lipid A

a) Structure of tetra acyl lipid A, also known as Lipid IV\textsubscript{A}, the precursor to lipid A in the \textit{E. coli} biosynthesis pathway.

b) Structure of classical \textit{E. coli} lipid A
Figure 1.4

Figure 1.4 Lipid A biosynthesis pathway in *E. coli*

The biosynthesis pathway for *E. coli* K-12. This general biosynthesis pathway varies between different bacterial species, including *Y. pestis*. (Raetz et al., 2007)
Figure 1.5

Carty et al JBC 1999. 274, 9677-9685
Figure 1.5 Mechanism of LpxP

LpxL is a cold shock induced enzyme in *E. coli* that at low temperatures acts in place of LpxL to add a secondary C:16 acyl chain to the Lipid IV<sub>A</sub> base molecule. LpxM can then add the final secondary acyl chain to the molecule to produce hexa-acyl lipid A. (Carty et al., 1999)
Figure 1.6

Figure 1.6 Toll-like Receptor 4 (TLR4) signaling pathway.

A schematic of the TLR4 signaling pathway. Not shown is MD2 interacting with the bound pathogen and TLR4. Activation of TLR4/MD2 leads to the downstream activation of adaptor molecules, which in turn leads to activation of NF-κB, AP-1 and IRF3 (Mogensen, 2009).
Figure 1.7

Figure 1.7 Crystal Structure of TLR4 complexed with MD2 and lipid A.

The lipid A molecule is colored in red, with the core carbohydrates as pink. The MD2 molecules are gray, TLR4 is blue, TLR4* is green.

a) Top view of the TLR4/MD2-lipid A complex. The primary and dimerization interface is shown.

b) Side view of the TLR4/MD2-lipid A complex.

(Park et al., 2009)
Table 1.1 Y. pestis lipid A species

<table>
<thead>
<tr>
<th>Lipid A species</th>
<th>Number of Acyl chains</th>
<th>Temperature expressed</th>
<th>Activation Profile</th>
<th>Mouse TLR4</th>
<th>Human TLR4</th>
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<tr>
<td>Tetra-acyl lipid A</td>
<td>4</td>
<td>37°C/26</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Penta-acyl lipid A</td>
<td>5</td>
<td>26°C</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hexa-acyl lipid A</td>
<td>6</td>
<td>26°C</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Acylation profile of lipid A expressed by wild type Y. pestis at different temperatures, and how these species are able to activate either mouse or human TLR4.
Preface to Chapter 2

This chapter has been published in Nature Immunology

Sara W Montminy, Naseema Khan, Sara McGrath, Mitchell J Walkowicz, Fiona Sharp, Joseph E Conlon, Shoichi Kusumoto, Charles Sweet, Kensuke Miyake, Shizuo Akira, Robert J Cotter, Jon D Goguen, & Egil Lien

Virulence Factors of Yersinia Pestis are overcome by a strong lipopolysaccharide response. Nature Immunology 7 (10) 1066-1073 (2006)

Naseema Khan performed Fig 2.1c-f, Fig 2.6b
Sara McGrath and Robert Cotter performed the MALDI-TOF on purified Lipid A, Fig 2.2
Fiona Sharp performed the experiments Fig 2.1 a,b
Joseph E Conlon performed the experiments in Fig 2.6c
Egil Lien performed in vivo infection experiments, the initial experiments with Jon Goguen in Figures 2.4, 2.6a,d, 2.7, 2.8.
Charles Sweet advised on the lipid A isolation and analysis of MALDI-TOF spectra
Shoichi Kusumoto, Koichi Fukase, Kensuke Miyake and Shizuo Akira provided reagents
Mitchell J Walkowicz participated in generating the pLpxL plasmid

Sara Montminy performed all other experiments, Figure 2.1g, Fig 2.2, 2.3 a-c,h, 2.4g, 2.5

Sara Montminy, Jon Goguen and Egil Lien prepared the manuscript
CHAPTER 2

VIRULENCE FACTORS OF YERSINIA PESTIS ARE OVERCOME BY A

STRONG LIPOPOLYSACCHARIDE RESPONSE
Abstract

Many Gram-negative pathogens produce a lipopolysaccharide (LPS)/lipid A with reduced ability to activate Toll-like receptor 4 (TLR4), but the role of this phenomenon in virulence has been unclear. *Yersinia pestis*, causative agent of plague, blunts TLR4-signaling by altering its lipid A at 37°C. Here we show that the resulting evasion of immune triggering is required for virulence. When genetically modified to produce a highly stimulatory LPS at 37°C, normally lethal *Y. pestis* is rendered avirulent in wild type mice, but remains virulent in animals deficient in TLR4-signaling. Both adaptive and innate mechanisms contribute to sterilizing immunity against this modified strain, which also acts as an effective vaccine against plague. Escaping TLR4-activation by lipid A alteration may contribute to virulence of various Gram-negative bacteria.
Introduction

Early recognition of invading bacteria by the innate immune system plays a crucial role in antibacterial defenses by triggering inflammatory responses that act to prevent the spread of infection and suppress bacterial growth. For many pathogens, early innate responses slow the progress of infection, buying time for adaptive immunity to develop. Pathogens with exceptional ability to evade or suppress innate responses may cause the death of the host before adaptive responses become effective. This is illustrated by the behavior of the Gram-negative pathogen *Yersinia pestis*, the causative agent of plague. In nature, *Y. pestis* depends primarily on the bite of infected fleas for transmission between mammalian hosts, causing bubonic plague (Perry and Fetherston, 1997). This requires the bacteria to establish a systemic infection distinguished by very high bacteremia -- exceeding $10^8$ per ml of blood in some human plague patients -- following inoculation of a few bacteria in the skin via flea bite. Early foci of infection contain large numbers of bacteria and are often entirely devoid of inflammatory cells (Nakajima et al., 1995; Sodeinde et al., 1992), despite the multiple and redundant mechanisms which normally trigger innate immunity. To suppress local inflammation, the pathogen employs several parallel approaches that are each necessary for virulence. These include a complex type III secretion system (T3SS) which delivers multiple effector proteins to the cytoplasm of host cells in contact with the bacteria, and the expression of Pla, an outer membrane protease with plasminogen activator activity (Cornelis, 2002; Perry and Fetherston, 1997; Sodeinde et al., 1992). The extracellular release of the T3SS-associated
protein LcrV has also been suggested to contribute to *Yersinia*-mediated immune suppression (Nakajima et al., 1995).

Toll-like receptors (TLRs) are central to the host innate immune response against invading microbes (Akira and Takeda, 2004; Liew et al., 2005). To achieve the profound suppression of inflammation observed in vivo, *Y. pestis* must either avoid inducing pro-inflammatory responses via the TLRs or actively suppress the effects of such responses. Arguably, the combination of these two approaches has the greatest potential to be effective. Several of the T3SS effectors have activities that could serve to blunt the effects of TLR simulation, including derangement of intracellular signaling, induction of apoptosis, and interference with phagocytosis (Cornelis, 2002; Perry and Fetherston, 1997; Sodeinde et al., 1992). A key pathway inducing inflammation in response to Gram-negative bacteria is the activation of TLR4-signaling by lipopolysaccharide (LPS), a major component of the Gram-negative outer membrane (Hoshino et al., 1999; Lien et al., 2000; Poltorak et al., 1998; Raetz Cr, 2002). Many types of LPS are extraordinarily potent, stimulating immune cells at picogram per milliliter concentrations. The immune-activating moiety of LPS is lipid A, a di-glucosamine unit with covalently attached acyl chains, which interacts with the host LPS receptor complex consisting of TLR4 and MD-2 to induce cellular responses (Hoshino et al., 1999; Lien et al., 2000; Poltorak et al., 1998; Raetz Cr, 2002; Saitoh et al., 2004b; Shimazu et al., 1999). The GPI-anchored molecule CD14 binds LPS to the cell surface, transfers LPS to TLR4/MD-2 and enhances cellular activation (Wright, 1990; Miyake, 2004), whereas the TLR adapters MyD88, Mal
(Tirap), TRIF and TRAM initiate the intracellular signaling cascade following interaction with the TIR domain of TLR4 (Akira and Takeda, 2004).

Lipid A structure is conserved among Gram-negative bacteria, making it an archetypical example of a pathogen-associated molecular pattern; however, it is not invariant. For example, the number, location and composition of acyl chains varies among species, is influenced by the environment, and is often heterogeneous even within a single species (Dixon and Darveau, 2005; Raetz Cr, 2002; Zähringer et al., 1999). Based upon in vitro observations, it has been hypothesized that production of a weakly stimulatory LPS could play a role in virulence of several Gram-negative pathogens (Dixon and Darveau, 2005). Hexa-acylated lipid A structures are found in Y. pestis grown at 21°C to 27°C (flea temperatures), but are predominantly tetra-acylated at 37°C (host temperature) (Kawahara et al., 2002; Knirel et al., 2005b; Rebeil et al., 2004). While hexa-acylated LPS/lipid A is normally a strong activator of human cells, tetra-acylated lipids have lower stimulatory activity (Golenbock et al., 1991; Ingalls et al., 2000; Kawahara et al., 2002; Loppnow et al., 1989; Rebeil et al., 2004). It has been suggested that the temperature-dependent remodeling of lipid A structure could be necessary for Y. pestis to achieve the high bacterial load in mammalian blood required for efficient flea infection prior to the induction of lethal shock. It is also possible that Y. pestis may need to minimize TLR4 stimulation early in infection to prevent containment by local inflammation.

Y. pestis provides an excellent model for testing the idea that LPS modification contributes to Gram-negative virulence via evasion of TLR4 signaling. In this report we
study a genetically modified strain of \textit{Y. pestis}, that expresses a potent TLR4-activating hexa-acylated LPS at 37°C. We find that this strain is avirulent in wild-type mice, despite the presence of other well established virulence determinants. Hence, both active immune suppression and stealth are critical for evading anti-bacterial host responses during plague. This emphasizes the role of TLR4-mediated innate immunity in protection against life-threatening infections, and has broad implications for understanding bacterial virulence in \textit{Y. pestis} and other organisms.

\section*{Results}

\subsection*{37°C \textit{Y. pestis} LPS inhibits TLR4 activation}

In contrast to bacteria grown at 26°C, \textit{Y. pestis} grown at 37°C poorly activated TLR4 signaling, supporting the idea that 37°C \textit{Y. pestis} LPS has low stimulatory potential (Figure 2.1 a, b) (Kawahara et al., 2002; Rebeil et al., 2004). When grown at 26°C, heat-killed \textit{Y. pestis} KIM5, an attenuated strain deficient in iron acquisition, strongly activates HEK293 huTLR4/MD-2 cells to release IL-8 and to activate luciferase reporters dependent upon transcription factors NF-κB and IRF-3 (Figure 2.1a,b). If the bacteria are grown at 37°C, this activation is not observed. Activation and nuclear translocation of these two transcription factors constitute two major branches of TLR4 signaling necessary for the induction of pro-inflammatory cytokines and type I interferons (Akira and Takeda, 2004; Liew et al., 2005). These data, together with the presence of poorly immune activating tetra-acylated \textit{Y. pestis} lipid A structures at 37°C (Kawahara et al.,
suggest that evasion of TLR4-signaling could represent a central mechanism by which \textit{Y. pestis} avoids innate immune activation.

Since tetra-acylated LPS/lipid A may antagonize activity induced by potent endotoxin (Golenbock et al., 1991; Lien et al., 2000; Loppnow et al., 1989), we also analyzed the anti-inflammatory role of 37°C \textit{Y. pestis} LPS. LPS purified from \textit{Y. pestis} KIM5 strain grown at 26°C, but not 37°C, activated human peripheral blood mononuclear cells (PBMC) (Figure 2.1c) and HEK293 huTLR4/MD-2 cells (Figure 2.1d). Furthermore, activation induced by 26°C \textit{Y. pestis} LPS and \textit{Escherichia coli} LPS was inhibited by 37°C \textit{Y. pestis} LPS and by the synthetic tetra-acylated lipid IVA in human PBMC (Figure 2.1c). Stimulation of HEK293 huTLR4/MD-2 cells as measured by the release of interleukin (IL)-8 (Figure 2.1d) and activation of NF-κB and IRF-3 dependent reporters (Figure 2.1e,f) by the 26°C LPS was also inhibited by the 37°C preparation. These observations suggest that 37°C \textit{Y. pestis} LPS has a general defect in activation of TLR4-signaling, potentially reflecting a failure to oligomerize the receptor complex (Miyake, 2004). We also observed repression of cytokine release in PBMC from cynomolgus macaques (\textit{Macaca fascicularis}) (Figure 2.1g), a model organism for studies of plague in primates. Bacteria may well contain a mixture of stimulatory and non-stimulatory LPS species, especially during transition between flea and host temperatures, with the antagonistic activity of tetra-acylated LPS blunting the induction of primate immune signaling by the active LPS species.
Y. pestis expressing lpxL synthesizes a hexa-acylated potent LPS

Mass spectroscopy studies (Kawahara et al., 2002; Knirel et al., 2005b; Rebeil et al., 2004) (Figure 2.2a) showed mainly tetra-acylated Y. pestis lipid A in Y. pestis grown at 37°C (Figure 2.3a), with hexa-acylated structures present only at lower temperatures (Figure 2.2b, 2.3b). LpxL (also called HtrB) and LpxM (MsbB) are late acyltransferases in E. coli and several other Gram-negative bacteria, adding the secondary acyl chains to the tetra-acylated precursor lipid IVA (Raetz Cr, 2002). LpxP, a third late E. coli acyltransferase, is active at low temperatures (Carty et al., 1999; Raetz Cr, 2002; Vorachek-Warren et al., 2002b). We could identify only two late acyltransferases, lpxP and lpxM, in the Y. pestis genome (Deng et al., 2002; Parkhill et al., 2001; Perry et al., 1998). This suggests that the lack of LpxL together with the low temperature specificity of LpxP is responsible for the absence of hexa-acylated lipid A at 37°C.

We cloned lpxL from E. coli K12 and expressed the gene in Y. pestis KIM5 under control of its own promoter on the vector pBR322 (pMW::lpxL or pLpxL), generating the strain Y. pestis KIM5-pLpxL. Mass spectroscopy (Figure 2.2 c,d) showed that this strain contains novel hexa-acylated structures at both 37°C and 26°C (Figure 2.2c,d, 2.3c), indicating that, as in E. coli, LpxL mediates addition of one 2’ secondary C12:0 acyl chain to lipid IV_A. Presumably, a 3’ secondary C12:0 acyl moiety is added by the endogenous LpxM at both temperatures, suggesting that LpxM enzyme function is temperature-independent in Y. pestis. In contrast to results obtained with KIM5, both 37°C and 26°C LPS from KIM5-pLpxL strongly activated HEK293 huTLR4/MD2 cells (Figure 2.3d), but not TLR2-expressing cells (Figure 2.3e). A similar observation was
made with human PBMC: only the KIM5 37°C LPS showed profoundly reduced induction of TNF, IL-6, and IL-8 release (Figure 2.3f,g). Responses of human PBMC from several healthy volunteers (n=9) followed a similar pattern (not shown), as did PBMC from Cynomolgus macaques (Figure 2.3h), indicating that primates have very low ability to respond to native 37°C *Y. pestis* LPS. Mouse cells have a higher capacity to respond to tetra-acylated lipid A than human cells, a phenomenon attributed to species differences in TLR4/MD-2 (Akashi et al., 2001; Hajjar et al., 2002; Lien et al., 2000; Poltorak et al., 2000). Indeed, mouse macrophages released limited amounts of TNF in a TLR4- and MD-2-dependent manner when exposed to the 37°C LPS from KIM5, but this response was much weaker than observed with 37°C LPS from KIM5-pLpxL or 26°C LPS from either strain (Figure 2.3i,j,k). In summary, a major deficit in response to 37°C *Y. pestis* LPS was common to cells from all species tested, and the expression of *E. coli* LpxL in *Y. pestis* resulted in the production of a novel hexa-acylated LPS at 37°C, with strong ability to activate TLR4-signaling.

**Y. pestis harboring a potent LPS is unable to produce bubonic plague**

Mammals have multiple surveillance pathways that can trigger antibacterial responses. On the other hand, *Y. pestis* has sophisticated active mechanisms for suppressing these responses, including a T3SS that intoxicates host cells in contact with the bacteria (Cornelis, 2002; Perry and Fetherston, 1997). To determine the significance of LPS stimulatory activity in this complex environment, we conducted infection experiments in mice. Many rodents are natural hosts for *Y. pestis* (Perry and Fetherston,
and mice are a well-established model for the study of plague. Fully virulent Y. *pestis* (KIM1001 strain (Sodeinde et al., 1992)) contains all known virulence factors, hence, we could evaluate the effect of a potent LPS in the context of all these other activities. KIM1001 produced 100% mortality in mice after subcutaneous (SC.) injection (a model in which the resulting disease is similar to bubonic plague) of 1,000 colony forming units (CFU) (Figure 2.4a), while infection with 100 CFU resulted in 80% mortality. In a striking contrast, the same doses of KIM1001 expressing *lpxl* (KIM1001-pLpxL) did not cause disease (Figure 2.4a). The effect of LpxL is profound: at doses of up to of $10^7$ CFU, approximately $10^6$ mean lethal doses for the KIM1001 parent strain, no mortality or apparent signs of illness were detected (Table 2.1). Limited swelling at the infection site could be observed at the higher dose of KIM1001-pLpxL bacteria. The phenotype of KIM1001-pLpxL was not confined to the C57Bl/6 mouse strain, as four out of four 129Sv mice died when infected with 1,000 CFU of KIM1001, whereas four out of four 129Sv animals survived infection with KIM1001-pLpxL.

Although the mouse TLR4/MD-2 system has a limited but significant response to tetra-acylated LPS/lipid A (Fig 2.3i) (Akashi et al., 2001; Hajjar et al., 2002; Lien et al., 2000; Poltorak et al., 2000), the resulting innate immune signaling is apparently insufficient to protect infected mice. Our data suggest that the development of systemic disease following infection at peripheral sites – as occurs in bubonic plague -- is dependent on the production of LPS with reduced TLR4/MD-2 stimulating activity.
**Y. pestis-pLpxL retains key virulence determinants**

We wanted to ensure that the presence of the pLpxL plasmid did not alter key features of the bacteria, so a number of experiments were done to examine this question. These experiments indicated that other *Y. pestis* virulence determinants were not affected by pLpxL. The presence of pLpxL had minimal effect on bacterial growth rate (Figure 2.5a), the T3SS remained fully functional, as exemplified by normal secretion of the central T3SS virulence factor YopM (Figure 2.5b), retention of Ca\(^{2+}\)-dependent growth at 37ºC, and retained T3SS – dependent cytotoxicity towards HeLa cells (data not shown).

Furthermore, resistance to polymixin B, and serum-dependent bacterial killing was preserved (Figure 2.5 c,d), the activity of the Pla protease remained normal (Figure 2.5e), and the presence of the plasmid vector by itself had no effect on virulence (Figure 2.5f). We also determined that pLpxL was stable in the absence of selection (no plasmids segregants detected among 1000 colonies after 40 generations of growth in liquid culture), and that native *Y. pestis* plasmids were retained in the presence of pLpxL.

Bacteria obtained from spleens of mice infected IV with KIM1001-pLpxL all remained ampicillin resistant following patching of 100 colonies. LpxC is the second enzyme in the lipid A biosynthesis pathway and is critical for the formation of lipid A (Raetz Cr, 2007). It has been reported that an LpxC inhibitor, CHIR-090, is a potent antibiotic, because it inhibits the formation of lipid A, which is essential for outer membrane development and survival (Barb et al., 2007a; Barb et al., 2007b; McClerren Al, 2005). CHIR-090 binds tightly to the active site of LpxC, therefore not allowing the binding of the natural substrate and stopping the formation of the base lipid A molecule. We tested
CHIR-090 against both KIM5 and KIM5-pLpxL, and found that both strains have a similar level of susceptibility to CHIR-090, and that this compound is also a potent antimicrobial agent to *Y. pestis* (Figure 2.5g)

**Protection from plague induced by *Y. pestis* pLpxL is dependent on TLR4 signaling**

A critical experiment in testing this hypothesis is verification that resistance to KIM1001-pLpxL is dependent on functional TLR4-signaling. Accordingly, we infected wild type and TLR4-/- mice with KIM1001 or KIM1001-pLpxL SC. at a dose of 1,000 CFU. Both mouse strains succumbed to infection with KIM1001, while only TLR4-/- animals died when infected with KIM1001-pLpxL (Figure 2.4b). It should be noted that the progression of disease (median time to death, Figure 2.4b), bacterial growth *in vivo*/systemic dissemination of disease as reflected by bacterial titers in the spleen (Figure 2.5h) and the development of symptoms (activity, posture, appearance of buboes) were comparable in TLR4-/- mice infected with either KIM1001 or KIM1001-pLpxL. This demonstrates that in the absence of TLR4, KIM1001-pLpxL remains fully virulent. This is consistent with other evidence suggesting that this strain retains all other virulence factors found in KIM1001 except for the ability to produce primarily tetra-acylated LPS.

In contrast to the experiments shown in Figure 2.4b, three out of three TLR4-/- animals survived SC infection with $10^5$ CFU of a *Y. pestis* KIM1001 based strain defective for the surface protease known as Pla, a strain that does not cause systemic infection by SC route (Sodeinde et al., 1992). The containment of this strain is also dependent on a substantial inflammatory reaction, although the mechanism by which Pla
suppresses this response is not known. This result suggests that TLR4 deficiency does not grossly compromise innate responses to *Y. pestis* in general, and that loss of Pla and production of stimulatory LPS, while both permitting effective innate responses, do so via distinct pathways.

MD-2, a small molecule attached in a non-covalent manner to the extracellular portion of TLR4, is a critical component of the LPS receptor complex (Shimazu, 1999; Schromm, 2001; Nagai, 2002; Miyake, 2004). The phenotype of animals deficient in MD-2 and infected with KIM1001-pLpxL should therefore resemble the one observed in TLR4-/- mice. When animals deficient in MD-2 were infected with 1,000 CFU of KIM1001 or KIM1001-pLpxL, all of the animals in both groups died (Figure 2.4c). Hence, the protection from disease induced by KIM1001-pLpxL requires both TLR4 and MD-2.

Foci of infection containing virulent *Y. pestis* are often devoid of inflammatory cells, reflecting the ability of this pathogen to evade immune responses (Nakajima et al., 1995; Sodeinde et al., 1992). Intravenous (IV) infection is a useful model for examining inflammation in tissues remote from injection site trauma. When mice are infected IV with KIM1001-pLpxL, an enhanced anti-bacterial response was revealed by extended survival time (Figure 2.4d) and a 100-fold reduction (p=0.008) in spleen bacterial titers (Figure 2.4e). Despite lower bacterial load, KIM1001-pLpxL infected animals had two-fold more TNF in spleen homogenates compared to mice infected with KIM1001 (Figure 2.4f). Livers of mice infected with wild type *Y. pestis* were characterized by masses of free bacteria, without distinct indications of local inflammation (Figure 2.4g). In contrast,
KIM1001-pLpxL provoked microabcess formation in livers from wild type mice, but not in TLR4-/- animals (Figure 2.4g). Liver tissue was normal in animals receiving 1,000 CFU of KIM1001-pLpxL SC (Figure 2.4g), and no bacteria were detected in spleens (Figure 2.4e). This indicates that SC. administration of KIM1001-pLpxL fails to establish systemic infection, presumably due to containment by effective local immunity at the infection site.

**Impact of TLR adapters and CD14 on LPS-mediated protection from plague**

Four TIR-containing adapter molecules, MyD88, Mal (also called Tirap), TRIF, and TRAM, participate in mediating cellular responses following exposure to LPS (Akira and Takeda, 2004). The adapters have different roles in inducing TLR4-dependent responses; the MyD88/Mal branch of TLR4 signaling leads to the early activation of NF-κB and the release of a number of pro-inflammatory mediators, such as TNF and IL-6 (Akira and Takeda, 2004). In the MyD88-independent pathway, TRAM/TRIF mediate activation of IRF-3 and the release of type I IFN and IFN- regulated genes such as Rantes, as well as late activation of NF-κB (Akira and Takeda, 2004). Few studies have addressed the relative contribution of the adapters in protection against Gram-negative pathogens. When we infected MyD88, Mal, TRIF and TRAM-deficient mice SC. with 1,000 CFU of KIM1001-pLpxL, MyD88-/- mice were the only animals that displayed 100% mortality Figure 2.6a). Forty percent of Mal-/- mice survived, whereas 80% of TRIF or TRAM mice survived (Figure 2.6a). TLR2-/- mice behaved as wild type mice following infection with KIM1001-pLpxL (Figure 2.6a). This result suggests that
MyD88-dependent anti-bacterial responses are essential for the TLR4-mediated protection against plague. Mal also has a significant role; whereas TRAM and TRIF mediated signaling is less important.

Most members of the family *Enterobacteriaceae* synthesize a smooth chemotype of LPS, that consist of lipid A, an oligosaccharide core, and an O-specific polysaccharide containing 20-40 repeats of a defined unit (Raetz Cr, 2002). In contrast, *Y. pestis* generates a rough LPS (Skurnik and Bengoechea, 2003), with a core comprising of 5-8 saccharides but lacking an O-polysaccharide (Knirel et al., 2005a; Vinogradov et al., 2002). CD14, which exists as both a GPI-anchored and soluble protein, has been shown to enhance responses to LPS, but is not absolutely necessary for LPS signaling via TLR4/MD-2. Recent studies have questioned the role of CD14 in responses leading to immune activation by rough chemotypes of LPS (Huber M, 2006; Jiang Z, 2005). In particular, CD14 was suggested to be dispensable for TNF release and MyD88-dependent rough LPS responses (Jiang Z, 2005). However, these conclusions were based upon experiments that utilized a deep rough Re mutant form of purified LPS (only containing lipid A plus two KDO saccharide moieties), which is not naturally found in pathogenic bacteria.

Similar to its parent strain, *Y. pestis* -pLpxL produce a rough LPS (Figure 2.6b). We were interested in determining if CD14 has any effect on the protection from lethal infection provided by the LpxL strain. Macrophages from CD14-/- animals were defective in their ability to respond to rough *Y. pestis*-pLpxL 37°C LPS, measured by the release of both TNF and Rantes (Figure 2.6c). Subsequently, we inoculated CD14-/- mice
SC. with 1,000 CFU of KIM1001-pLpxL to determine the role of CD14 in the protection against infection mediated by rough LPS. Six of nine CD14-/- animals infected with KIM1001-pLpxL died following infection; indicating a shift in LD50 of more than 4 orders of magnitude compared to wild type mice (Figure 2.6d). Our results support the idea that CD14 has a function in transferring many types of rough LPS to TLR4/MD-2 to amplify release of TNF and Rantes and anti-bacterial responses.

**Bacteria with increased TLR-activating ability as vaccine strains**

Activation of TLR signaling leads to strong induction of adaptive immune responses, mediated in part by a strong upregulation of co-stimulatory molecules on antigen-presenting cells (Akira and Takeda, 2004). In fact, many TLR ligands are promising constituents of vaccines, serving as adjuvants (van Duin et al., 2006). Bacterial strains containing increased TLR stimulation capabilities could, through their increased content of adjuvant activity, provide elevated activation of adaptive immunity and have useful properties as vaccine strains. A problem in the development of new vaccines against *Y. pestis* has been to provide protection against both pneumonic and bubonic disease. Effective humoral and cellular immunity are both thought to be necessary for optimal protection (Parent et al., 2005). We hypothesized that the attenuated *Y. pestis* strain producing potent LPS might be effective as a vaccine against plague. Mice were vaccinated with a single SC. dose (1,000 or 100,000 CFU) of KIM1001-pLpxL. Thirty to forty days later, both vaccinated and naïve mice were challenged with virulent KIM1001 at SC doses between 1,000 and 1,000,000 CFU (the latter corresponding to
approximately $10^5 \times$ LD50), or at intranasal (IN) administration, mimicking pneumonic disease, of 5,000 or 50,000 CFU (the latter approximately 150 x LD50). All mice vaccinated with KIM1001-pLpxL survived while all naïve mice died (Figure 2.7a,b, Table 2.2, Table 2.3), demonstrating that *Y. pestis* producing potent LPS is an effective vaccine against both bubonic and pneumonic plague. Thus, our experiments provide a proof of principle for the generation of attenuated strains of microorganisms with enhanced TLR-activating potential as a strategy for vaccine production.

**Contributions by adaptive immunity to LPS-mediated protection against plague**

We have demonstrated that strong innate immune activation is essential to survive acute infection with *Y. pestis* synthesizing a potent LPS. However, this does not imply that innate responses are sufficient to produce sterilizing immunity. To determine if adaptive responses were also required for protection, we infected Rag1-/- animals, lacking B and T cells and thus adaptive immunity, with KIM1001-pLpxL. While infected TLR4-/- animals all died of acute disease within six days, the Rag1-/- animals remained healthy until day 9, when the first animal appeared sick. All Rag1-/- animals died within the next few days (Figure 2.8). This implies that innate immunity contains the infection until adaptive responses sufficient for sterilization develop. We conclude that even in the face of a potent TLR4-mediated innate immunity, adaptive responses are required to fully clear infection by the *Y. pestis* -pLpxL strain, and are crucial for the long-term survival of the host.
Discussion

Our results emphasize the double-edged nature of the sensitive TLR4-dependent LPS detection system. Injection of high amounts of purified LPS into humans and research animals induces a well established syndrome called endotoxic shock. As a result, potent endotoxin is often thought of as harmful during Gram-negative infections. Our report propose that the expression of a potent LPS by some pathogens may in fact be beneficial for the host, providing early recognition of infection and effective onset of immune signaling. Subversion of TLR4-mediated immune responses by lipid A modification may be a central strategy for various Gram-negative pathogens to evade anti-bacterial mechanisms.

The general picture that emerges from our current understanding of *Y. pestis* virulence is that successful systemic infection is dependent on efficient and simultaneous suppression and/or evasion of multiple pathways ordinarily capable of eliciting protective local inflammatory responses. Our results establish that evading stimulation of TLR4 is an essential part of this strategy. This implies that the strong capacity to induce TLR4-signaling of *Y. pestis*-pLpxL is dominant over the anti-host bacterial defenses provided by T3SS and other bacterial activities. Our findings also highlight the remarkable efficiency of TLR4-mediated responses in the control of Gram-negative infections. Despite retention of the active means used by *Y. pestis* to suppress host responses, a strain producing hexa-acylated LPS and thus quickly detectable via TLR4/MD-2 fails to cause disease even when the subcutaneous inoculum exceeds $10^6$ mean lethal doses. This implies strong selection against TLR4 recognition, and suggests that adaptations to
prevent this stimulation should be common among pathogens. Several other Gram-
negative pathogens have been shown to synthesize lipid A with weak stimulatory
activity, associated with differences in number and composition of acyl chains (Dixon
and Darveau, 2005; Zähringer et al., 1999). To our knowledge, this is the first report to
establish the significance of the synthesis of such modified lipid A forms for
pathogenesis. It is also notable that Y. pestis LPS has reduced stimulatory activity for
both murine and primate systems, reflecting the broad host range of this pathogen.
Although we did not detect measurable stimulation in the primate system, murine cells
showed a limited but significant response. However, these responses were clearly not
sufficient to protect mice during infection.

The production of a weakly stimulatory LPS is not the only mechanism needed by
Y. pestis to escape destruction or containment by local inflammation. For example, key
proteins injected into host cells via T3SS and which act to block proinflammatory
signaling, along with, in most Y. pestis strains, Pla protease activity, are also required
(Cornelis, 2002; Perry and Fetherston, 1997; Sodeinde et al., 1992). Another potentially
important activity is the antagonism of TLR4 stimulation by native Y. pestis LPS
demonstrated above (Figure 2.1). Membranes may contain a mixture of stimulatory and
non-stimulatory LPS species, especially immediately following the flea bite. In primates,
and potentially other species, this antagonistic activity could ensure that membranes
containing multiple LPS species become inert with respect to TLR4 stimulation as soon
as the tetra-acylated LPS species achieves sufficient concentration in the host.
Our findings also have potential implications for the development of therapeutics and vaccines. The strong TLR4-mediated protection against *Y. pestis* producing potent LPS suggests that the development of anti-infectives based on stimulation of innate immunity, now in its infancy, has significant promise (Agrawal and Kandimalla, 2003; Ulevitch, 2004). However, incorporation of adjuvant activity into pathogens by LPS modification or other means may also be effectively applied as a novel principle for development of vaccines against some infections. The protection we observe is due to the incorporation of adjuvant activity into the bacteria, ensuring that its activity is focused precisely where needed. Such modifications may strongly attenuate virulence and stimulate immune responses, both desirable characteristics for vaccine strains.
Materials and methods:

Bacterial strains and growth conditions:

The lpxL gene from E. coli K12, including 435 bp upstream and 140 bp downstream of coding region, was cloned using Pfu Ultra polymerase (Stratagene) and ligated into the BamHI and SalI sites of pBR322, creating pMW::lpxL (or pLpxL). The control plasmid pBR322Δtet was constructed by digesting empty pBR322 with NaeI and EcoRV and then ligating the plasmid to remove the major part of the tetracycline resistant gene. The resulting plasmids were electroporated into Yersinia pestis strain KIM5 (Goguen et al., 1984) or KIM1001 (Sodeinde et al., 1992) and selected by growth on tryptose-beef extract (TB) agar supplemented with 0.25 mM CaCl₂ and 0.6 mg/mL glucose in the presence of 100 µg/ml ampicillin. All strains containing either pBR322Δtet or pMW::lpxL remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, pMT1+) is highly virulent (Sodeinde et al., 1992), while the KIM derivative KIM5 bears a chromosomal deletion designated Δpgm, which strongly attenuates virulence. KIM5 was used to limit risk of infection in in vitro studies where a virulent strain was not required, and the pgm locus contains no genes thought to impact LPS biosynthesis.

For growth analyses, the overnight cultures were diluted into 25 ml of TB broth with CaCl₂ to an OD600 of ~0.15. Growth of the cultures was monitored every 2 hours at OD600. For the generation of heat killed bacteria, cultures were resuspended in PBS and incubated at 60°C for 1 hr.
Antimicrobial assays

Polymixin B was obtained from Sigma-Aldrich. CHIR-090 was a generous gift from Dr CRH Raetz (Duke University). Overnight cultures of KIM5 and KIM5-pLpxL were swabbed on to TB agar plate as described. Antimicrobial agents were then spotted on 7mm filter paper disks (Blotting paper 703 VWR International), and growth was monitored and the diameter of diffusion was measured after 2 days. All experiments were done in triplicate.

Serum sensitivity assay

*Y. pestis* KIM5 and KIM5-pLpxL was diluted to a concentration of $2 \times 10^4$ CFU/mL. Of this dilution, 150 μL of bacterial culture was added to 150 μL of human serum, to make the final concentration of serum 50%. The bacteria and serum were incubated at 37°C for 1 hour, with 25 μL taken in duplicate at time 0, 30 minutes and 60 minutes and plated on TB agar. The plates were incubated for 48 hours at 37°C and CFU was determined by counting colonies per plate.

Lipid preparations:

Pyrogen-free reagents and supplies were used to the greatest extent possible in the lipid preparations. Bacteria were harvested by centrifugation at 6,000 x g. LPS was purified from bacteria by hot water-phenol extraction (Westphal et al., 1952), followed by two phenol re-extractions to remove contaminating lipoproteins and TLR2-activity (Hirschfeld et al., 2000). The chemical synthesis of the tetra-acylated precursor lipid IVA (also called 406, LA-14-PP or precursor Ia) has been reported (Liu et al., 1999).
LPS from *E. coli* O111:B4 (Sigma) was phenol re-extracted as indicated above. The absence of activation of TLR2-expressing cells (Fig. 2e), in spite of potent IL-8 release by bacterial Pam3CysSK4 lipopeptide (not shown), indicates the absence of contaminating lipoproteins in our highly purified LPS preparations.

*Lipid A preparation and mass spectroscopy*

Pyrogen-free reagents and supplies were used to the greatest extent possible in the lipid preparations. Bacteria were harvested by centrifugation at 6,000 x g. Lipid A was directly isolated from whole bacterial cells using the Bligh-Dyer two-phase chloroform-methanol-water organic extraction (Vorachek-Warren et al., 2002b). Samples were subsequently analyzed by MALDI-TOF MS using a Kratos (Manchester, UK) AXIMA CFR high performance mass spectrometer operated in both positive and negative ion modes.

*Cell Stimulation Assays:*

Human PBMC were obtained from healthy volunteers with informed consent, and isolated by centrifugation on Lymphoprep density media (Axis-Shield/Nycomed, Oslo, Norway). The UMass Institutional Review Board approved the human subject protocol. HEK293 cells stably expressing human TLR4-YFP and retroviral MD-2, or human TLR2-YFP, or empty vector pcDNA3 were as published (Latz et al., 2002). NF-κB B-luciferase (provided by K. Fitzgerald) and IRF-3-dependent 561-luciferase (a gift from G. Sen (Bandyopadhyay et al., 1995)) reporters were transfected into 293-huTLR4/MD-2 cells using Genejuice (Novagen). PBMC were cultured in X-vivo 15 medium (Cambrex)
containing ciprofloxacin and 1% FCS or 1% human serum, whereas 293 cells were stimulated in DMEM/ciprofloxacin plus 10% FCS. Wild type C57Bl/6 or Rag1-/ mice (backcrossed 10 generations into C57Bl/6) were from Jackson Laboratories (Bar Harbor, ME). TLR4/-, MyD88/-, Mal-/-, TRIF/-, TRAM/- and TLR2/- animals were generated as described (Adachi et al., 1998; Hoshino et al., 1999; Takeuchi et al., 1999; Yamamoto et al., 2003a; Yamamoto et al., 2002; Yamamoto et al., 2003b), and backbred 11 (TLR4, TLR2, MyD88), 4 (Mal) or 5 (TRIF, TRAM) generations into C57Bl/6. MD-2/- animals (Nagai et al., 2002) (source: Dr. Miyake and Japan Science and Technology Corporation) and CD14/- animals (Moore et al., 2000), a gift from K. Moore and M. Freeman, were backcrossed 7 and 10 generations into C57Bl/6, respectively. Mouse peritoneal macrophages were harvested from wild type C57Bl/6 or TLR4/- mice 3 days after injection of 2 ml thioglycollate (3%) and cultured in RPMI 1640 medium containing 10% FCS. Cells were plated at a density of 2x10⁴ cells/well (293 cells) 5x10⁴ cells/well (mouse macrophages) or 1x10⁵ cells/well (PBMC) in 96-well dishes, and stimulated 16-18 hrs before harvesting supernatant (for cytokine analysis) or cells for lysis (transfections and reporter assays, using reagents from Promega). Cytokines were measured using ELISA kits from BD Pharmingen (moTNFα, moRantes) or R&D systems (huTNFα, IL-6, IL-8). Cell lysates from luciferase reporter assays were analyzed by the addition of luciferase substrate followed by luminometry. Results are shown as one representative out of three to eight experiments, as mean of triplicates (transfection assays) or triplicates/duplicates (cytokine assays) +/- standard deviation (s.d.).
Stimulation of non-human primate cells

Cynomolgous macaque PBMC, purified by centrifugation of whole blood in CPT/sodium citrate tubes (Becton Dickinson), were obtained from Bioreclamation, Inc (Hicksville, NY). The PBMC were cultured in RPMI 1640 medium containing 10% FCS. Cells were plated at a density of $1 \times 10^5$ cells/well in 96-well dishes, and stimulated 16-18 hrs before harvesting supernatant. Cell stimulation was analyzed by the release of IL-8, using an ELISA kit from R&D systems (huIL-8 Duoset). Results are shown as mean of triplicates +/- standard deviation (s.d.).

In vivo infections:

Mice were infected with *Y. pestis* KIM1001, KIM1001-pPpxL or KIM1001-pBR322Δtet, either by SC. injection of 50 μl on the nape of the neck, IN infection with 50 μl dropped in the nostrils under ketamine/xylasine anesthesia, or IV injection of 500 μl in the tail vein. Inocula contained the indicated CFU suspended in PBS. Mice were euthanized by IP pentobarbital overdose when moribund. Survival was monitored up to 21 or 28 days, every 12 hrs during acute infection. For collection of organs, mice were sacrificed 48 hrs following IV infection or 72 hrs following SC. infection by pentobarbital overdose, spleens were homogenized in PBS to obtain bacterial titers and for cytokine analysis. Separate infection experiments with detection limits of $10^3$ or $10^1$ CFU per spleen showed similar results, with the absence of detectable bacteria in spleens following SC. infection with KIM1001-pPpxL. Livers were fixed in neutral buffered 4% formalin, followed by standard hematoxylin/eosin (H&E) staining and microscopy (100x
magnification). Inserts (KIM1001 and KIM1001-pLpxL IV) are at 1,000x magnification. A Nikon Eclipse E400 instrument and 10x or 100x-oil objectives were used. Images were captured with a Spot camera and corresponding software (Diagnostic instruments). All animal studies were approved by the UMass Institutional Animal Care and Use Committee, and the experiments followed their guidelines and regulations.

**YopM secretion analysis.**

KIM5 and KIM5-pLpxL were grown overnight at 26°C in TB medium without CaCl₂. The cultures were then diluted 1:50 in BHI media (Difco) and grown at 26°C to an OD600 of 0.2. The cultures were then shifted to 37°C and allowed to grow for another 2 hours both in the presence and absence of CaCl₂. The bacteria were spun down at 12,000 rpm and the supernatant was collected. YopM in the supernatant was precipitated with 20% trichloroacetic acid and resuspended in SDS-PAGE buffer. The samples were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk, then incubated with a YopM antibody (a gift from Susan Straley) in PBS/1% Tween 20 for 2 hrs. The membrane was then washed in PBS/tween and incubated with anti-mouse IgG peroxidase-conjugated secondary antibody (GE Healthcare) for 2 hours, washed in PBS/1% Tween 20, and the protein was detected using West Pico Chemiluminescent substrate (Pierce).

**Plasminogen activation assay**

KIM1001, KIM1001-pLpxL and a KIM1001 mutant in Pla(Sodeinde et al., 1992) were grown at 37°C on TB agar plates supplemented with 0.25mM CaCl₂ and 100mg/ml
ampicillin if needed for selection. Conversion of plasminogen to plasmin was performed as described (Sodeinde et al., 1992). Briefly, bacteria were scraped off the plates and resuspended in 0.1M Tris/0.1%Tween. Each reaction was performed in a duplicate, and contained 5 μL of bacterial solution, 10 μL of the chromogenic plasmin substrate S2251 (Val-Leu-Lys-p-nitroanalide) at a concentration of 4mM, 10 μL of 160nM human plasminogen and 80 μL Tris/tween buffer. The reaction was monitored for 1 hour with readings taken at OD405.

Statistical analysis

Differences in spleen CFU and TNF concentrations were analyzed by Mann-Whitney U-test. Statistical differences in survival were studied by Kaplan-Meyer survival analysis and the log rank test.
Figure 2.1

(a) IL-8 (ng/ml) levels in 293-huTLR4/MD2 and 293-pcDNA3 cells treated with media alone, heat killed Y. pestis 26°C, and heat killed Y. pestis 37°C.

(b) Luciferase (fold induction) levels for NF-κB and IRF-3 in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells.

(c) TNF (ng/ml) levels in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells.

(d) IL-8 (ng/ml) levels in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells.

(e) NF-κB luciferase (fold induction) in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells.

(f) IRF3-561 luciferase (fold induction) in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells.

(g) IL-8 (ng/ml) levels in 26°C Y. pestis LPS and 37°C Y. pestis LPS treated cells with Lipid IVα.

lipids (ng/ml)
Figure 2.1 *Y. pestis* 37°C LPS contains inhibitory activity.

*Y. pestis* grown at 37 °C is a poor stimulator of TLR4 signaling

**a)** HEK293 cells stably expressing TLR4/MD2 or empty vector (pcDNA3) were exposed to media alone (black bars), or heat killed *Y. pestis* KIM5 grown at 26 °C (vector temperature, light gray bars) or 37 °C (host temperature, dark grey bars) at a density of 10 bacteria per ml. Supernatants were analyzed for IL-8.

**b)** HEK293 TLR4/MD-2 cells were transiently transfected with NF-κB- or IRF-3 dependent 561-luciferase reporter constructs and then stimulated with heat-killed *Y. pestis* grown at 37 °C or 26 °C. Results are given as fold reporter induction above cells exposed to media alone.

**c)** Human PBMC were treated with 10 or 100 ng/mL of *Y. pestis* 37°C LPS or synthetic tetra-acyl lipid IV<sub>A</sub>, followed by the addition of *Y. pestis* 26°C LPS or *E. coli* LPS (10 ng/ml) and incubated for 16 hrs.

**d)** HEK 293 cells expressing human TLR4/MD2 were exposed to *Y. pestis* 37°C LPS (100 and 10 ng/ml), followed by addition of 26°C *Y. pestis* LPS (10 ng/ml). Cells were incubated for 16 hrs. To study influence on transcription factor activation, HEK293 huTLR4/MD-2 cells were transiently transfected with **e)** NF-κB- or **f)** IRF-3 dependent 561 promoter-luciferase reporters, treated with *Y. pestis* 37°C and/or 26°C LPS, and incubated for 18 hrs. Lipid concentrations are given in ng/ml.

**g)** *Y. pestis* KIM5 37 °C LPS or synthetic tetra-acylated lipid IV was added to PBMC from Cynomolgus macaque, followed by addition of KIM5 26 °C LPS. Cells were incubated for 18 hours and supernatants were analyzed by human IL-8 ELISA. Results indicate that tetra-acylated lipid A/LPS species do not activate non-human primate cells, and can inhibit non-human
primate cellular activation by more potent lipid A species in a similar fashion as human
cells. The LD50 for this primate is approximately 400 CFU by inhalation (Dr. J.
Adamovicz, USAMRIID, personal communication, used with permission).
Figure 2.2

a:

b:
Figure 2.2 Mass spectrometry analysis of lipid A from \textit{Y. pestis}

Wild type \textit{Y. pestis} KIM5 was grown at 26 °C and 37 °C and the lipid A was purified from the whole bacteria by Bligh-Dyer two-phase organic extraction. The lipid was then purified over a DE52 column and analyzed by MALDI-TOF mass spectrometry. The negative ion spectra shown here are representative of multiple extractions. Analysis of the positive ion spectra (not shown) was also done to determine the location of the arabinose at the reducing end of the molecule. 

\textbf{a)} When the bacteria is grown at 37 °C, they produce predominantly tetra-acyl lipid A. Structural analysis was also carried out for the lipid A from \textit{Y. pestis} KIM5-pLpxL expressing the acyltransferase LpxL from \textit{E. coli}.

\textbf{b)} At 26 °C, the bacteria express at least three different species of lipid A: tetra-acyl lipid A with four C14:0 acyl groups (m/z 1405), a penta-acyl lipid A that has an additional secondary C12:0 acyl chain (m/z 1587), and a hexa-acyl lipid A with additional C12:0 and C16:1 secondary acyl chains (m/z 1824). An additional species (m/z 1179) corresponds to a tri-acyl lipid A that may also be a fragment ion resulting from the loss of an acyl-linked C14:0 group from the tetra-acyl lipid A. In addition, peaks for all four of these species with the addition of an 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety are also observed (m/z 1310, 1537, 1720 and 1955). 

\textbf{c)} At 37 °C the lipid A revealed the presence of a novel hexa-acyl species (m/z 1769), which has a 12:0 acyl chain at the 2’ secondary position rather than a 16:1 acyl chain. The addition of L-Ara4N was also observed (m/z 1900). 

\textbf{d)} At 26 °C the mass spectra were similar, predominantly the hexa-acyl species (m/z 1769) and some penta-acyl lipid A (m/z 1587) and a small amount of tetra-acyl lipid A (m/z 1405). The peaks at m/z
1349, 1363 and 1377 (observed more weakly in the other spectra) may not represent tetra-acyl lipid A heterogeneity, since the mature hexa-acyl species appear as single peaks. Hence, they may not be related to lipid A species, and may be other lipids (e.g. cardiolipins) recovered from the extraction.
Figure 2.3

(a) [Chemical structure diagram]
(b) [Chemical structure diagram]
(c) [Chemical structure diagram]
(d) [Graph showing IL-8 (ng/ml) vs. lipids (ng/ml) for different conditions]
(e) [Graph showing IL-8 (ng/ml) vs. lipids (ng/ml) for different conditions]
(f) [Graph showing TNF (ng/ml) vs. lipids (ng/ml) for different conditions]
(g) [Bar graph showing IL-6 and IL-8 levels for different conditions]
(h) [Graph showing IL-8 (ng/ml) vs. lipids (ng/ml) for different conditions]
(i) [Graph showing TNF (ng/ml) vs. lipids (ng/ml) for different conditions]
(j) [Graph showing TNF (ng/ml) vs. lipids (ng/ml) for different conditions]
(k) [Graph showing TNF (ng/ml) vs. lipids (ng/ml) for different conditions]
Figure 2.3 *Y. pestis*-pLpxL synthesizes a potent LPS. a-c) Lipid A was isolated from bacteria grown in liquid culture at 37°C or 26°C. The major forms of lipid A were determined by MALDI-TOF mass spectrometry (figure 2.2). Shown are the significant lipid A structures found in *Y. pestis* KIM5 at 37°C a) or 26°C b), or in KIM5-pLpxL at both temperatures c). HEK293 cells expressing d) human TLR4/MD2 or e) human TLR2 were stimulated with LPS isolated from *Y. pestis* KIM5 grown at 26°C (♦) or 37°C (■), *Y. pestis* KIM5-pLpxL grown at 26°C (◊) or 37°C (□), or synthetic lipid IVA (●) for 18 hrs. Supernatants were analyzed by IL-8 ELISA. Human PBMC isolated from a healthy donor were exposed to LPS as described above for 16 hrs before supernatants were examined for f) TNF or g) IL-6 and IL-8 by ELISA. Similar results were obtained with cells from nine individuals. h) PBMCs isolated from Cynomolgus macaques were stimulated with increasing doses of lipid IV or LPS isolated from wild type *Y. pestis* KIM5 or KIM5-pLpxL grow at 26 °C or 37 °C for 18 hours. The supernatant was analyzed for human IL-8 by ELISA. The cells did not respond to KIM5 37 °C LPS, but strongly responded to the other forms of LPS in a similar fashion as the response observed in human PBMC. Peritoneal macrophages were isolated from i) wild type, j) TLR4-/- and k) MD-2-/-mice, and stimulated for 16 hrs with different forms of *Y. pestis* LPS and lipid IV_A as described above. Supernatants were analyzed for TNF contents by ELISA.
Figure 2.4

a

b

c

d

e

f

KIM1001

KIM1001-

-pLpxL

SC

WT

IV

WT

IV

KO

WT

KO

IV

WT

KO

IV

WT

KO
Figure 2.4 Y. pestis -pLpxL is avirulent in wild type mice. 

a) Wild type C57Bl/6 mice (n=10 per infection group) were infected subcutaneously (SC.) with either parental Y. pestis KIM1001 at 1,000 CFU (♦) or 100 CFU (■), or with Y. pestis KIM1001-pLpxL at 1,000 cfu (◊) or 100 cfu (□). Survival was monitored every 12 hrs. [(p<0.0001 in comparisons between 1000 CFU, KIM1001 vs KIM1001-pLpxL].

b) Wild type C57Bl/6 and TLR4-/- mice (n= 10 per infection group) were infected SC. with Y. pestis KIM1001 (▲, ■) or KIM1001-pLpxL (□, ◊) at a dose of 1,000 CFU (p<0.0001 for KIM1001-pLpxL, wild type vs TLR4-/-).

c) Wild type C57Bl/6 and MD-2-/- mice (n= 8 per infection group) were infected SC. with Y. pestis KIM1001 (▲, ■) or KIM1001-pLpxL (□, ◊) at a dose of 1,000 CFU (p<0.0001 for KIM1001-pLpxL in MD-2-/- mice vs wild type mice).

d) Y. pestis KIM1001-pLpxL IV infection is associated with increased survival times. Wild type C57Bl/6 or TLR4-KO mice (n=5 per group) were infected with 1000 cfu of KIM1001 or KIM1001-pLpxL iv in the tail vein. Survival was monitored every 12 hours.

e) Colony forming units (CFU) were determined in spleen homogenates from wild type mice infected intravenously (IV) or SC. with KIM1001 or KIM1001(pLpxL).

f) TNF concentration in spleen homogenates from IV infected mice from D) were measured by ELISA (p=0.008).

g) Liver sections from wild type or TLR4-/- mice infected SC. or IV with 1,000 CFU of KIM1001 or KIM1001-pLpxL were H&E stained. Arrows indicate bacteria-containing lesions, and the star indicates a microabcess containing infiltrating inflammatory cells [100x, inserts: 1,000x magnification of a bacterial mass (KIM1001 infection) or the microabcess, KIM1001-pLpxL].
Figure 2.5

(a) 

(b) 

(c) 

(d) 

(e) 

(f) 

(g) 

(h)
Figure 2.5 *Y. pestis* containing pLpxL retains key features.

a) KIM5, KIM5-pLpxL, and KIM5-pBR322Δtet were seeded in liquid cultures at an OD600 of approximately 0.15. The strains grew at similar rates at both 26 °C and 37 °C in TB broth containing calcium. Hence, the presence of pLpxL and pBR322Δtet did not significantly affect growth in vitro. b) KIM5 and KIM5-pLpxL were grown in the presence and absence of calcium at 37°C for 1 hour, and the supernatants were collected and analyzed for the presence of YopM by western analysis. Secretion of YopM in KIM5-pLpxL was comparable to KIM5, as detected by anti-YopM antibody in the absence of calcium, while no secretion is seen in the presence of calcium. c) *Y. pestis* and *Y. pestis*-pLpxL were diluted to an OD of 0.1, and the culture was then swabbed onto TB agar plates containing calcium and ampicillin for the modified strain. Whatman 7mm disks were then placed on the swabbed plates and inoculated with polymyxin B. The plates were then incubated at 37°C for 24 hours, and the zone of inhibition was measured. d) KIM5 and KIM5-pLpxL were incubated in 50% serum for one hour and then plated for CFU. Plates were incubated for 48 hours at 37°C and colonies were counted. Three experiments were averaged together and standard deviation determined from the mean averages. e) Pla activity is conserved in both KIM1001 and KIM1001-pLpxL, as indicated by the ability of the bacteria to induce cleavage human plasminogen to plasmin. Subsequently, active plasmin is measured by the ability of plasmin to cleave the chromogenic Val-Leu-Lys-p-nitroanalide (S2251) plasmin substrate, and OD405 is measured as a function of time. A *Y. pestis* KIM1001 mutant that lacks Pla activity is unable to cleave the substrate, whereas KIM1001 and KIM1001-pLpxL both contain high
Pla activity.  

f) The presence of plasmid vector does not affect KIM1001 virulence. Wild type C57Bl/6 (n=5 per group) were infected with 1000 CFU of KIM1001 or KIM1001-pBR322Δtet SC in the nape of the neck. Bacteria were resuspended in 0.05 mL of PBS. Survival was monitored every 12 hours.  

g) *Y. pestis* and *Y. pestis*-pLpxL were diluted to an OD of 0.1, and the culture was then swabbed onto TB agar plates containing calcium and ampicillin for the modified strain. Whatman 7mm disks were then placed on the swabbed plates and inoculated with CHIR-090, an LpxC inhibitor. The plates were then incubated at 37°C for 24 hours, and the zone of inhibition was measured.  

h) Three TLR4-/- mice were infected IV with 1,000 CFU of either KIM1001 or KIM1001-pLpxL. Sixty hours post-infection, spleen homogenates were prepared and plated on TB plates (KIM1001) or TB Amp plates (KIM1001-pLpxL).
Figure 2.6

a) Survival (%) over time (d) for different genotypes: TLR2-KO, TRAM-KO, TRIF-KO, MAL-KO, and MyD88-KO.

b) Western blot analysis of KIMS5 and KIMS5(pLpxL) at 37°C and 26°C, and E. coli O111:B4.

c) TNF (ng/ml) and RANTES (ng/ml) levels in WT and CD14-KO under LPS (+) and (-) conditions.

d) Survival (%) over time (d) for CD14-KO, KIM1001, CD14-KO,KIM1001-pLpxL, WT, KIM1001, and WT, KIM1001-pLpxL.
Figure 2.6 MyD88, Mal and CD14 contribute to LPS-mediated survival for KIM1001-pLpxL.

a) MyD88, Mal, TRAM, TRIF or TLR2-deficient mice (ten mice per group) were infected SC. with 1,000 CFU of KIM1001-pLpxL. Survival was monitored up to 28 days. (MyD88 vs Mal: p<0.0001. Mal vs TLR2; p=0.003).

b) Purified LPS from *Y. pestis* KIM5 and KIM5-pLpxL grown at 37°C or 26°C, and LPS from *E. coli* 0111:B4 were run on a 12% Bis-Tris gel. The LPS was visualized by silver stain. The *Y. pestis* LPS is rough (lacking an o-polysaccharide), whereas the *E. coli* LPS is smooth.

c) Peritoneal macrophages from wt or CD14-/- mice were stimulated with *Y. pestis* KIM5-pLpxL 37°C LPS (10 ng/ml) for 18 hrs. TNF and Rantes release was measured by ELISA.

d) Wild type or CD14-/- animals were infected with KIM1001 (10 wt or 10 CD14-/- animals) or KIM1001-pLpxL (10 wt or 9 CD14-/- animals) SC. at a dose of 1000 CFU Survival was monitored up to 21 days. p=0.002 (CD14-/- vs. wt mice, KIM1001-pLpxL).
Figure 2.7
Figure 2.7 *Y. pestis* harboring potent TLR4-activating ability is an effective vaccine against plague

a) Wild type mice (ten mice per group) were challenged SC with 1000 CFU of KIM1001-pLpxL or left untreated. Thirty-five days later, mice were infected SC with 1,000 CFU of virulent KIM1001 and survival was monitored.  
b) Wild type C57Bl/6 mice were vaccinated SC with 1,000 or 100,000 CFU of KIM1001-pLpxL or left untreated. Thirty to forty days later, the animals were challenged with 1,000 up to 1,000,000 CFU of virulent KIM1001. Survival was monitored up to 28 days. *Results from two experiments are pooled.

c) Wild type mice were vaccinated SC with 100,000 CFU of KIM1001-pLpxL or left untreated. Thirty days later, the animals were infected intranasally (IN) with 5000 or 50,000 CFU of KIM1001. Survival was monitored up to 28 days. *Results from two experiments are pooled.
Figure 2.8
Figure 2.8 Adaptive immunity is critical for protection against KIM1001-pLpxL.

Rag1/-/-, TLR4/-/- or wild type C57Bl/6 mice (5 mice per group) were infected SC with 1,000 CFU of KIM1001-pLpxL. Survival was monitored up to 28 days.
Table 2.1 Survival of wild type mice with high doses of KIM1001-pLpxL

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>KIM1001</th>
<th>KIM1001-pLpxL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10² CFU</td>
<td>4 of 20</td>
<td>20 of 20</td>
</tr>
<tr>
<td>1x10³ CFU</td>
<td>0 of 30</td>
<td>29 of 29</td>
</tr>
<tr>
<td>1x10⁴ CFU</td>
<td>-</td>
<td>14 of 14</td>
</tr>
<tr>
<td>1x10⁵ CFU</td>
<td>-</td>
<td>5 of 5</td>
</tr>
</tbody>
</table>

Survival of wild type mice after subcutaneous infection with increasing doses of *Y. pestis* KIM1001 or KIM1001-pLpxL, presented as surviving mice of total mice.

- indicates not done. Data are representative of two to six experiments.
Table 2.2 Survival of vaccinated wild type mice after subcutaneous challenge

<table>
<thead>
<tr>
<th>Vaccination (KIM1001-pLpxL)</th>
<th>Challenge (KIM1001)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1x10^3 CFU</td>
<td>0 of 20</td>
</tr>
<tr>
<td>None</td>
<td>1x10^5 CFU</td>
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<tr>
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</tr>
<tr>
<td>1x10^5 CFU</td>
<td>1x10^6 CFU</td>
<td>5 of 5</td>
</tr>
</tbody>
</table>

Survival of wild type mice vaccinated subcutaneously with *Y. pestis* KIM1001-pLpxL and naïve mice (None) after subsequent subcutaneous challenge with increasing doses of *Y. pestis* KIM1001, presented as surviving mice of total mice. Data representative of two to five experiments.
Table 2.3 Survival of vaccinated wild type mice after intranasal challenge

<table>
<thead>
<tr>
<th>Vaccination (KIM1001-pLpxL)</th>
<th>Challenge (KIM1001)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5x10³ CFU</td>
<td>0 of 19</td>
</tr>
<tr>
<td>1x10³ CFU</td>
<td>5x10³ CFU</td>
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</tr>
<tr>
<td>1x10⁵ CFU</td>
<td>5x10³ CFU</td>
<td>5 of 5</td>
</tr>
<tr>
<td>1x10⁵ CFU</td>
<td>5x10⁴ CFU</td>
<td>5 of 5</td>
</tr>
</tbody>
</table>

Survival of wild type mice vaccinated subcutaneously with *Y. pestis* KIM1001-pLpxL and naïve mice (None) after subsequent intranasal challenge with increasing doses of *Y. pestis* KIM1001, presented as surviving mice of total mice. Data representative of two to three experiments.
Preface to Chapter III

The work in this chapter has been performed by the following:

Gregory Vladimer performed the \textit{in vitro} IP2666 infection on PBMCs Fig 3.7d

Megan Murphy and Jon Goguen constructed the IP2666\textit{Δ}lpxL strain Fig 3.3

Dan Weng performed the growth curve and Polymyxin B assays Figure 3.3 b,c,d, 3.5a-d

Ashley Beasley and Katie Stamper at John Hopkins University performed MALDI-TOF, Fig 3.2

Egil Lien and Gregory Vladimer performed \textit{in vivo} infections Fig 3.6, 3.7a,b

Sara Montminy Paquette performed all other experiments, Figures 3.1, 3.2, 3.3a, e-g, 3.5e, 3.7c,e.
CHAPTER III

PRESSURE TO EVADE LPS/TLR4 AND IL-1 SIGNALING IN THE
EVOLUTION OF A HIGHLY VIRULENT PATHOGEN
Early detection of invading microbes by the host innate immune system is critical for mounting an effective inflammatory response to prevent the spread of infection. Some pathogens have evolved the ability to evade or actively suppress the innate immune response, allowing the rapid development of a fatal infection before the adaptive immune system can respond. One such bacterium, *Yersinia pestis*, the gram-negative causative agent of plague, is a master of immune evasion. *Y. pestis* relies in part on the bite of infected fleas for transmission between mammalian hosts (Perry and Fetherston, 1997). In order to develop bubonic plague via the flea vector, *Y. pestis* must establish systemic bacteremia from the small number of bacteria delivered by the fleabite. After transmission via fleabite, the bacteria multiply producing foci of infection which contain a large number of bacteria, yet few inflammatory or immune cells (Sodeinde et al., 1992). In order to achieve this, *Y. pestis* utilizes a number of different mechanisms to avoid innate immune recognition and suppress inflammation. Our group has previously determined that the structure of the lipopolysaccharide (LPS)/Lipid A molecule is a critical factor for the virulence of *Y. pestis*. The bacterium expresses tetra-acyl lipid A in its LPS structure at 37°C, which is antagonistic to the human TLR4 (Toll-like receptor 4) and its co-receptor MD2, thus allowing *Y. pestis* to inhibit the TLR4/MD2 pathway at the initial stage of signaling, effectively halting innate immune activation. Other virulence mechanisms include a type III secretion system (T3SS) which delivers effector proteins (termed Yops) directly into the host cell cytoplasm, and Pla, an outer membrane protease with plasminogen activator activity (Cornelis, 2002; Perry and Fetherston, 1997; Sodeinde et al., 1992).
Toll-like receptors (TLRs) are vital to the recognition of pathogen associated molecular patterns (PAMPs) expressed by invading pathogens such as LPS, lipoproteins, flagellum, CpG, unmethalated DNA, and viral RNA (Kumar et al., 2009; Takeda and Akira, 2004). TLR4 is activated by the lipid A portion of lipopolysaccharide (LPS), a major component of the Gram-negative bacterial outer membrane. Initially lipid A associates with MD-2, which acts as an essential co-receptor facilitating the binding of LPS to the cytoplasmic leucine rich repeat (LRR) portion of the TLR4 receptor, leading to the dimerization of two receptors. This dimerization initiates an intracellular kinase cascade culminating in the activation of downstream transcription factors such as NF-κB and interferon regulatory factors (IRFs), which lead to the production of inflammatory cytokines such as TNFα, IL-8, pro-IL-1β, and pro-IL-18 along with type I interferons (IFNs) (Akira, 2006; Beutler, 2009; Kumar et al., 2009).

The IL-1 pathway is another immune signaling pathway that is critical for protection from bacterial pathogens. It activates a broad and potent range of pro-inflammatory molecules that help control infection. The IL-1 receptor binds IL-1α and IL-1β, which then activates a downstream pathway that utilizes many of the same adaptor molecules found in the TLR pathway and potently activates NF-κB (O'Neill, 2000). IL-1β is a very powerful pro-inflammatory molecule, and because of this its expression is under very tight control. Secretion of this molecule is dependent on two signals; first is the production of pro-IL-1β upon TLR4 mediated NF-κB activation. This pro- form of the cytokine is sequestered in the cytoplasm until a second signal stimulates the activation of caspase 1, which cleaves pro-IL-1β into its active form, after which it is then...
secreted from the host cell. Once secreted, mature IL-1β binds to the IL-1 receptor which induces a potent inflammatory response including the induction of fever, edema, and the production of leukocytes (Dinarello, 2009; Dunne and O'Neill, 2003).

*Y. pestis* expresses tetra-acyl lipid A at 37°C due of the absence of the enzyme LpxL, which plays a critical role in the lipid A biosynthesis pathway via the addition of a secondary acyl chain to the tetra-acyl lipid structure. In order to determine if this tetra-acyl lipid A structure is important for virulence of the bacteria we expressed *E. coli* LpxL in *Y. pestis* (*Y. pestis*-pLpxL), and found that the bacteria are now able to produce stimulatory hexa-acylated lipid A/LPS. *Y. pestis*-pLpxL was also avirulent in wild-type, but not TLR4-/- mice. This demonstrates that lipid A/LPS acylation patterns are a critical virulence determinant for *Y. pestis* and possibly other Gram-negative pathogens (Montminy et al., 2006).

The divergence of *Y. pestis* from its ancestral parent *Y. pseudotuberculosis* is thought to have occurred between 1,500 and 20,000 years ago (Achtman et al., 1999), with recent research indicating the date is closer to 10,000 years ago (Achtman et al., 2004). *Y. pseudotuberculosis* causes self-limiting gastroenteritis, a mild disease when compared with a potentially lethal *Y. pestis* infection (Smego et al., 1999). *Y. pseudotuberculosis* is transmitted by oral route, where they invade the ileum epithelial layer and then migrate to the Peyer’s Patches, where they replicate and further migrate to the mesenteric lymph nodes. At this point the disease is usually contained by the immune system and the patient recovers, where in *Y. pestis*, after lymph node colonization, the disease rapidly becomes systemic (Dennis, 2008). However, despite the differences in
pathology, the two bacteria share a high degree of genetic homology, and both contain a virulence plasmid that encodes genes for a type three secretion system (T3SS) and Yop effector proteins. When the whole genomes of both strains were compared, it was determined that the *Y. pseudotuberculosis* genome contains over 300 genes that have been lost or inactivated in *Y. pestis*, including *lpxL* (Chain et al., 2004; Pouillot et al., 2008). Interestingly, *Y. pseudotuberculosis* produces a number of lipid A species at 37°C, including hexa-acylated lipid A which is able to stimulate TLR4 in human cells (Rebeil et al., 2004; Skurnik et al., 2000; Therisod et al., 2002) indicating that LpxL in this bacterium may be functional. Conversely, while the acquisition of new genes such as Pla and the F1 capsule have proven important in *Y. pestis*’ divergent pathogenic profile, the loss of many genes, including *lpxL*, may also have played a critical role in the development of *Y. pestis* into a highly virulent pathogen. Here, we examine how the presence of *lpxL* affects virulence in these two Yersinia species, and what other immune pathways besides TLR4/MD2 are important for host clearance of *Y. pestis* expressing LpxL.

**Results**

**Absence of *lpxL* is a unique characteristic of *Y. pestis***

*In-silico* analysis comparing the genomes of all sequenced *Yersinia* species (including the Pestoides strains, which are thought to be the oldest strains of *Y. pestis* (Achtman et al., 2004; Bearden et al., 2009; Garcia et al., 2007)) revealed that *Y. pestis* is the only family member which does not contain a homologue for *E. coli lpxL* (data not
shown), a late acyltransferase in the lipid A biosynthetic pathway, which enables *Y. pestis* to produce hexa-acylated lipid A at 37°C. However, other *Yersinia* species all contain *lpxL* homologues that have a high sequence similarity and identity (Figure 3.1a, b). Interestingly, *Y. pseudotuberculosis*, the evolutionary parent of *Y. pestis*, has a putative *lpxL* in a region of the chromosome that was lost from *Y. pestis* (Chain et al., 2004; Pouillot et al., 2008), as does *Y. enterocolitica*, the ancestor of both strains. These bacteria produce a heterogeneous mix of lipid A species at 37°C and LPS that is stimulatory into human cells, indicating that LpxL enzyme could be functional (Rebeil R, 2006; Therisod et al., 2002). This suggests that the loss of *lpxL* may have been a critical step in the evolution of *Y. pestis* into a highly virulent pathogen.

**Activity of *Y. pseudotuberculosis* LpxL**

While it was known that *Y. pseudotuberculosis* had an *lpxL* homologue in a chromosomal region that was lost from *Y. pestis*, it had yet to be determined if that gene was functional. To address the activity of the *Y. pseudotuberculosis* LpxL enzyme, we cloned *Y. pseudotuberculosis lpxL* from the IP2666 (Simonet and Falkow, 1992) strain into pBR322 to create pYptbLpxL (pBR322::YptbLpxL), and introduced this plasmid into *Y. pestis* KIM5. When we isolated and analyzed the lipid A from KIM5-pYptbLpxL, we found that the lipid A profile bore a striking resemblance to the lipid A profile from IP2666 (Figure 3.2a, b). Previously we had cloned *E. coli* lpxL into pBR322 (pLpxL), and found that the addition of this enzyme drove the *Y. pestis* to produce almost exclusively a hexa acylated lipid A, similar to the lipid A profile seen in *E. coli*.
However, with the addition of the *Y. pseudotuberculosis* LpxL, we found that *Y. pestis* now produced a much more heterogeneous mix of tetra-, penta-, and hexa-acylated lipid A (Figure 3.2b). This profile closely resembles that seen with IP2666 (Figure 3.2a). The addition of *Y. pseudotuberculosis* LpxL to *Y. pestis* causes the bacteria to revert to its parental strain lipid A profile, rather than exclusively making hexa-acyl lipid A like in the case with KIM5-pLpxL (Montminy et al., 2006).

Many gram negative bacteria have survival or pathogenic defects when utilizing tetra acyl lipid A as the anchor for their LPS (McLendon et al., 2007; van der Ley et al., 2001; Vorachek-Warren et al., 2002b). This is due to the increased outer membrane fluidity seen in these mutants, which make the bacteria more susceptible to both environmental pressures such as osmotic changes, and to the actions of complement and antimicrobial agents. However, *Y. pestis* survives quite well with tetra-acyl lipid A, and is resistant to the action of serum and antimicrobial peptides (Knirel et al., 2007). To determine if the factors that allow *Y. pestis* to thrive while producing tetra-acyl lipid A were passed down from the parental *Y. pseudotuberculosis*, or were acquired during *Y. pestis’* evolution we generated an in-frame, non polar deletion of *lpxL* from IP2666 (Figure 3.3). As tetra-acylated lipid A is a critical virulence determinant for *Y. pestis*, this mutant was introduced into a *Y. pseudotuberculosis* pYV deficient strain of IP2666, which lacks the T3SS and Yop effector proteins in order to avoid possibly increasing virulence of the strain. When the lipid A was isolated and analyzed from the resulting strain, IP2666Δ*lpxL*, we found that tetra-acyl lipid A was the predominant species produced (1405 m/z), with some penta-acyl lipid A present as well (1587 m/z) (Figure
3.2c). The lipid A profile of IP2666ΔlpxL bore a striking resemblance to wild type Y. pseudotuberculosis, indicating that the loss of this enzyme from Y. pseudotuberculosis was sufficient for the production of tetra-acyl lipid A. The phenotype was rescued by the addition of LpxL, as the complement strain IP2666ΔlpxL-pYptbLpxL produced a lipid A profile like that of the parent IP2666 strain (Figure 3.2d). This data demonstrates that the loss of lpxL in Yersinia leads to the production of tetra-acyl lipid A.

Stimulatory Properties of Tetra- and Hexa-Acylated lipid A/LPS

Knowing that hexa-acylated lipid A was produced by LpxL expressing Yersinia strains, and that this form of lipid A is highly stimulatory to human cells, we wanted to examine the stimulatory capabilities of the LPS from KIM5-pYptbLpxL, IP2666ΔlpxL and IP2666ΔlpxL-pYptbLpxL. To that end, LPS was purified from both the KIM5 and IP2666 strains grown at 37°C, and then used to stimulate a variety of mammalian cell types. The LPS produced from the KIM5-pYptbLpxL and IP2666 strains were highly stimulatory for TNF in human PBMCs (Figure 3.4a). These LPS samples were also able to stimulate the production of IL-8 in HEK293 cells expressing TLR4/MD2 (Figure 3.4b). The LPS did not however stimulate HEK293 TLR2 cells, indicating that it is free of contaminating lipoproteins (Figure 3.4c), and no response was seen in HEK293 cells containing pcDNA3 alone (Figure 3.4d).

In contrast to the strains in which we added lpxL, IP2666ΔlpxL produced almost exclusively tetra-acyl lipid A, and was non-stimulatory in both the human PBMCs and in HEK293TLR4/MD2 cells (Figure 3.4a, b). This mimics what is seen in with KIM5 LPS,
which is also predominantly tetra-acylated. When \( lpxL \) is added back to the deletion strain, the stimulatory capability LPS was restored, indicating that the lack of LpxL, and the resulting tetra-acylated lipid A, is responsible for the non-stimulatory characteristics of these strains.

Tetra-acylated lipid A is also known to be a strong antagonist for TLR4, and we have previously shown that KIM5 LPS can suppress pro-inflammatory response in human PBMCs. To examine whether this was also the case with IP2666\( \Delta lpxL \), human PBMCs were incubated with either 10\( \text{ng/mL} \) or 100 \( \text{ng/mL} \) of IP2666\( \Delta lpxL \) LPS or KIM5 LPS and with 10 \( \text{ng/mL} \) of stimulatory LPS. Cells that were treated with either IP2666\( \Delta lpxL \) or KIM5 LPS had a marked reduction in the amount of TNF\( \alpha \) produced (Figure 3.4e). When equal amounts of stimulatory and non-stimulatory LPS were added to the cells, a 50% reduction was seen in TNF\( \alpha \) production, and when a tenfold higher concentration of non-stimulatory LPS was added, the TNF\( \alpha \) levels were near completely abolished (Figure 3.4e), indicating that tetra-acyl lipid A/LPS can act as antagonist.

Mouse TLR4 is able to respond to tetra-acyl lipid A due to differences in the receptor complex which have recently been identified. Key differences in several amino acid residues on the surfaces of both TLR4 and MD2 alter how the lipid A molecule interacts with the receptor, allowing the mouse receptor to respond to tetra-acyl lipid A, while the human receptor is antagonized (Park et al., 2009; Meng, J, 2009). However, even though tetra-acyl lipid A is recognized to some degree, it still induces much less activation than the hexa-acyl lipid A. When immortalized mouse macrophages were stimulated with KIM5 or IP2666\( \Delta lpxL \), they responded to a lesser degree than when the
cells were stimulated with LPS from the strains which contained LpxL (Figure 3.4f).
Immortalized macrophages derived from TLR4-/ mice, however, did not stimulate at all,
indicating both that this is a TLR4 mediated event, and that there were no contaminates in
the LPS preps that may be stimulating the cells through a different pathway (Figure 3.4g).

Characteristics of *Yersinia* strains with altered acylation patterns

Altering the LPS/lipid A molecule in gram-negative bacteria can drastically
change how the bacteria grow and function. In order to determine if the changes in the
acylation pattern modified any of the key characteristics of the strains, the growth and
antimicrobial sensitivity of the strains were examined. Both the *Y. pestis* and *Y.
pseudotuberculosis* mutant strains grew at the same rate as the parent strains (Figure 3.5
a, b) indicating that the alteration of the lipid A did not affect growth in these strains.
Sensitivity to polymyxin B was also determined by disk diffusion assay. Polymyxin B is
a cyclic cationic peptide that is a very effective antimicrobial against gram negative
bacteria by binding lipid A and altering the outer membrane, making it more permeable
(Tsubery et al., 2000). As *Y. pestis* grown at higher temperatures (37°C) has been shown
to be more sensitive to polymyxin B then bacteria grown at lower temperatures
(Anisimov et al., 2005), we wanted to determine if acylation pattern played any direct
role in this difference in sensitivity. We observed that none of the mutant strains were
significantly more sensitive to serum than their parent strain. KIM5-pLpxL and KIM5-
pYptbLpxL were as resistant to polymyxin B as KIM5, while IP2666ΔlpxL and
IP2666ΔlpxL-pYptbLpxL were not significantly more susceptible then IP2666 (Figure
Thus, the change in acylation pattern in this bacterium does not alter their susceptibility to polymyxin B.

We also analyzed the LPS by silver stain, and saw that the addition of *Y. pseudotuberculosis* LpxL did not drastically alter the migration pattern of the extracted LPS. KIM5-pYptbLpxL still had a rough LPS phenotype and did not contain an O-saccharide chain like *Y. pseudotuberculosis* (Figure 3.5e). IP2666ΔlpxL did show a slight shift in the band present on the silver stain gel, which may be due to the reduction in acyl chains on the lipid A. The complemented strain appeared similar to IP2666, in that the slight downward shift is gone.

**Altered *Y. pestis* Acylation patterns attenuates virulence**

We have previously shown that altering the lipid A pattern by expression of *E. coli* LpxL completely attenuates the virulence of *Y. pestis* in mice, demonstrating the expression of tetra-acyl lipid A is a critical virulence determinant. *Y. pseudotuberculosis* produces hexa-acyl lipid A at 37°C, and has a much higher LD50 in mice than *Y. pestis* (Pouillot et al., 2005). Mice are excellent models for both diseases, and are natural reservoirs for infection with *Y. pestis*, which provides an ideal system to examine the difference in acylation patterns during infection. In order to determine if expression of *Y. pseudotuberculosis* LpxL would have this same effect on virulence as expression of *E. coli* LpxL, we transformed fully virulent KIM1001 with pYptbLpxL and infected mice subcutaneously with 500 CFU of the bacteria. We found that at this dose, the bacterium was not able to produce bubonic plague, as we saw with the bacteria expressing *E. coli*
LpxL, while mice infected with the wild type strain completely succumbed to disease by day 8 (Figure 3.6a). We also found that this lethality was TLR4 dependent, and TLR4-/- mice are susceptible to infection at the same rate as mice infected with wild type \textit{Y. pestis} (Figure 3.6b).

As \textit{Y. pestis} expressing LpxL is able to elicit a TLR4/MD2 immune response and appears to be largely avirulent we next sought to determine if this strain could be used as a vaccine. We found that infection with the \textit{Y. pseudotuberculosis} LpxL expressing strain of KIM1001 provided protection from subsequent infection with the wild type KIM1001. Mice that were initially infected with 500 CFU KIM1001-pYptbLpxL and subsequently challenged 30 days post infection with KIM1001 were protected from disease when challenged with a subcutaneous dose of KIM1001 at a CFU of 1000, twice the infectious dose of the initial infection (Table 3.2). These mice were fully protected from disease.

The IL-1 pathway protects against infection with \textit{Y. pestis}-pLpxL

Strains of \textit{Y. pestis} that express hexa acyl lipid A allow us to examine the potential contribution of different components of the innate immune system during active infection. Normally, this cannot always be undertaken with \textit{Y. pestis} due to high virulence and active immune evading characteristics of the pathogen. However, these mutant strains provide sufficient attenuation of the bacteria, without altering other important virulence component, therefore facilitating closer examine the critical
pathways involved in plague progression and by extension in many other gram negative infections.

We therefore infected a number of key immune knockout mice, including TNFR I, IL-1RI, IL-12 p40, and Type 1 IFN-R with KIM1001-pLpxL. We found the most profound defect in survival was in IL-1R knockout mice (Figure 3.7a), and further infection experiments determined there was only 40% survival with the KIM1001-pLpxL in those animals compared to wild type mice (Table 3.3). We had also infected MyD88 deficient mice with 1000 CFU of wild type KIM1001, and found that they had a significantly higher rate of death than wild type mice (p=0.0004) (Figure 3.7b). MyD88 is an essential adapter for the IL-1 pathway, indicating that the IL-1 pathway may play a key role in protection during *Y. pestis* infection.

To further examine the role the IL-1 pathway plays in *Y. pestis* infection, we infected PBMCs *in vitro* with KIM5, KIM5-pLpxL, and KIM5-pYptbLpxL (Figure 3.7c). During active infection, the KIM5-pLpxL and KIM5-pYptbLpxL strains induced an IL-1β response, while a response was absent from KIM5 (Figure 3.7c). PBMCs were also infected with the *Y. pseudotuberculosis* strains at an MOI of 10 (Figure 3.7d), and in these cells the deletion of *lpxL* leads to dramatic reduction in the production of IL-1β. IP2666 was able to induce a strong IL-1β response, as did the IP2666Δ*lpxl*-pYptbLpxL strain. However, IP2666Δ*lpxl* IL-1β stimulation levels are at the same levels as unstimulated cells (Figure 3.7d).

In order to assess if TLR4 stimulation and subsequent activation of NF-κB is essential for the production of pro-IL-1β in this system, we also infected both wild type
and TLR4-/- immortalized mouse macrophages with KIM5, KIM5-pLpxL and KIM5-pYptbLpxL (Figure 3.7e). TLR4 signaling is required for the secretion of IL-1β in this system, as cells deficient for this receptor do not have any IL-1β response, whereas the wild type cells have a robust response. These data indicate that the production of tetra-acyl lipid A is unable to induce the IL-1 response during *Yersinia* infection.

**Discussion**

The deletion of *lpxL* from the *Y. pestis* chromosome appears to provide an evolutionary benefit to the bacteria. It enables the bacteria to effectively evade TLR4/MD2 signaling, providing another mechanism of innate immune evasion. Of the genus *Yersinia*, only *Y. pestis* is a highly virulent pathogen. However, all the pathogenic *Yersinia* contain the type three secretion system and Yop effector proteins, which are highly effective at suppressing the immune response in the host cytoplasm (Cornelis, 2002; Pujol C, 2005). While the acquisition of such virulence determinants such as Pla, murine toxin, and the F1 protein are critical in *Y. pestis* virulence (Perry and Fetherston, 1997), the loss of LpxL may have provided a turning point in the evolution of the bacteria. Pestoides F, a fully virulent strain of *Y. pestis* isolated from the former Soviet Union, lacks Pla and has an a number of characteristics that defines it as one of the most ancient *Y. pestis* strains isolated and sequenced (Garcia et al., 2007). This isolate also lacks the gene for LpxL, indicating the loss of this chromosomal region that contains the gene occurred very early in the evolution of *Y. pestis*. 
All sequenced strains of *Y. pseudotuberculosis* contain *lpxL* (Figure 3.1a). However, unlike *E. coli*, which produces one major form of lipid A, *Y. pseudotuberculosis* contains a heterogeneous mix of lipid A. Early analysis of *Y. pseudotuberculosis* lipid A shows that there is a high degree of variability in the strain, more so than is seen in any of the other *Yersinia* or in gram negative bacteria in general (Krasikova et al., 1978). Subsequent mass spectrometry analysis confirmed that *Y. pseudotuberculosis* expresses a considerable number of lipid A species with a large number of different modifications (Rebeil R, 2004; Therisod et al., 2002). Our analysis, however, show less heterogeneity. We see instead three major lipid A structures, with little of the acyl chain number and length variability or modifications seen in other published works. This may be due to the difference in strains used for this work. Previous work looked at either *Y. pseudotuberculosis* PB1 O:1b or ATCC 29833, whereas our work and analysis was done using IP2666. The isolation and treatment of the lipid A molecule may also play a role in the observed differences. While most other groups isolate their lipid A from previously purified LPS, we have isolated it directly from the bacteria, thus reducing the amount of harsh chemical treatments the molecule would be exposed to. Finally, differences in growth media, growth temperature, and nutrients can all affect the acylation pattern of lipid A, especially in *Y. pseudotuberculosis*. We are also repeating the mass spectrometry analysis of the lipid A from these strains with an independent collaborator to confirm our structures.

When *Y. pseudotuberculosis* *lpxL* was introduced into *Y. pestis* and the lipid A from this strain isolated, we found that *Y. pestis*-pYptbLpxL produces a lipid A that
closely mimics what we see with wild type *Y. pseudotuberculosis* strain IP2666. This is likely due to the fact that the gene is under the control of its own promoter, thus mimicking the expression levels found in *Y. pseudotuberculosis*. However, since it is expressed on a plasmid, the copy number of the gene would be higher that what is found endogenously, thus possibly driving the expression of *lpxL* to a higher level and causing a shift to a completely hexa acyl species like what is shown with IP2666Δ*p*LpxLpYptbLpxL. The reason for this discrepancy has yet to be determined and is being investigated.

Our data with IP2666Δ*p*LpxL shows that *Y. pseudotuberculosis* can survive without *lpxL* and with tetra acyl lipid A as the anchor molecule for LPS, which is interesting since many gram negative bacteria have growth or functional defects when only tetra-acyl lipid A is present (Lee et al., 1995; Vorachek-Warren et al., 2002b). IP2666Δ*p*LpxL shows no growth or survival defect when in comparison with either the parent or the complemented strain. The compensating mechanisms present that allow the bacteria to survive with only tetra-acyl lipid A are currently unknown, however they appear conserved in *Y. pseudotuberculosis* and *Y. pestis*. Part of the answer may be in the growth rate of the bacteria. *Yersinia* has a much slower doubling time than many gram negative bacteria, such as *E. coli* or *H. influenza*, which has a doubling time of 30 minutes. Slower growth rate may allow for MsbA, a membrane enzyme that transports the lipid A molecule across the periplasmic space to the outer membrane, to move tetra-acyl lipid A across the membrane to the outer leaflet. MsbA transports tetra-acyl lipid A poorly (Zhou et al., 1998) (Reynolds and Raetz, 2009), so the slower growth rate may allow for the tetra-acyl lipid A to be transported to the outer membrane.
As we had shown previously, *Y. pestis* producing a hexa acylated lipid A is both avirulent and protects from subsequent reinfection. Wild type KIM1001 has a mean lethal dose of 10 CFU, while KIM1001-pYptbLpxL is avirulent in mice at a dose of 500 CFU. This effect is not seen in TLR4 deficient mice however, indicating that the protection seen by the LpxL producing strain is dependent on the activation of the innate immune response. Preliminary data suggests that TLR4 is critical as well for the protection of mice from *Y. pseudotuberculosis*. In a single experiment, 3 out of 4 TLR4-/- mice orally infected with $5 \times 10^8$ CFU of IP2666 succumbed to disease by day 5, whereas only 2 out of 5 wild type mice died (data not shown). While this experiment was only performed once with a low number of animals, it does indicate that TLR4 is involved in protection from disease during *Yersinia pseudotuberculosis* infection. A potentially interesting experiment to conduct would be to generate a ΔlpxL strain in IP2666 with pYV and then infect mice to see if there is an increase in virulence, however do to biosafety concerns, this has not been done. It can be speculated however, given the low potency LPS produced by this strain combined with its lack of growth defects, that this strain would prove to be more virulent then its parent. It is unlike to be as virulent as *Y. pestis* with this modification alone, however. *Y. pseudotuberculosis* containing pPla does not become more virulent, even when the O-saccharide chain is reduced to allow for enzymatic activity (Pouillot et al., 2005). Therefore, it is likely that a number of modifications need to be made to *Y. pseudotuberculosis* to render it as virulent as *Y. pestis*. However, it can be assumed that the bacteria would be less likely to activate TLR4 *in vivo*, and would be able to survive longer and reach higher bacterial number in the host then the parent strain.
It was also established that KIM1001-pYptbLpxL protects against infection from wild type KIM1001. There is a great degree of interest in developing vaccines for gram negative infections, especially *Y. pestis*. This is due to the current lack of an effective vaccine for *Y. pestis* licensed in the United States, and because of its potential as a bioterrorism agent. Therefore, developing a highly protective vaccine is of great value. We have shown that infection with KIM1001-pYptbLpxL is able to initiate a successful protective response, and to provide immunity from subsequent infection against KIM1001, the wild type *Y. pestis* strain. This makes the strain an ideal vaccine candidate, because it is both avirulent my subcutaneous infection and provides complete protection from reinfection. However, it still contains all critical virulence determinants so it cannot be used as a live-attenuated vaccine. Therefore, another strategy must be used. One such method would be to express *Y. pseudotuberculosis* LpxL in an attenuated *Y. pestis* strain and to see if this strain is also able to protect against disease. It has been shown that when *E. coli* LpxL was introduced to D27, an avirulent strain of *Y. pestis* and then used to infect mice, those mice survived infection and are also protected from disease when infected with fully virulent *Y. pestis* (Szaba et al., 2009). This further demonstrates that *Y. pestis* expressing a potent LPS may be a vaccine candidate due to its ability to stimulate and be cleared by the immune system and to provide lasting immunity.

There has also been a great deal of work dedicated to developing vaccines that have TLR stimulating capabilities, because of the TLRs ability to activate not only the innate immune system, but to activate and educate the adaptive immune response. Recent work with *N. meningitidis* has shown that modifying the LPS/lipid A structure of this
bacterium to be less potent is useful as a TLR agonist in outer membrane vesicles. When this LPS is used in conjunction with other antigens, like outer membrane proteins, in the development of outer membrane vesicle vaccines against both \textit{N. meningitidis} and \textit{B. pertussis}, a higher degree of protection is seen (Steeghs L et al., 1999; Stoddard et al., 2009; Geurtsen et al., 2008). With \textit{Y. pestis}, increasing the potency of the LPS/lipid A molecule is advantageous because it allows for the bacteria to be recognized by TLR4 and drive the immune response. Developing a \textit{Y. pestis} vaccine with an endogenous stimulatory LPS and other highly antigenic proteins, similar to work done with \textit{N. meningitidis}, could be an effective strategy for a successful vaccine. There may be potential side effects to the increased potency of the LPS, as this may increase inflammation and lead to septic shock. However, the mice infected with this strain do not have any apparent signs of illness, indicating that this may not be an issue with live bacteria. Further studies on the mice need to be done to determine what type of inflammation occurs in these animals, if any. This includes monitoring body temperature to look for disease and analysis of what cytokines are being produced during infection. This would allow us to more closely determine how robust the immune response to \textit{Y. pestis} expressing LpxL is, and if it has the potential to be detrimental to the vaccinated individual.

Changes in the acylation pattern of lipid A can drastically alter how the immune system recognizes the bacteria. Tetra-acyl lipid A effectively suppresses TLR4/MD2 signaling, and this in turn can have serious consequences on the function of other immune pathways (Zahringer et al., 2001) (Golenbock et al., 1991) (Matsuura et al.,
2009). Activation of TLR4 allows for the production of pro-IL-1β, a critical component of the IL-1 pathway, which activates a host of other downstream molecules that are critical for an effective immune response (Dunne and O'Neill, 2003) (Dinarello, 2009). Y. pestis has been suggested to shut down the IL-1 pathway by blocking activity of caspase 1, which cleaves pro-IL1β into the active form of the cytokine that can be released from the cell (Schotte et al., 2004) (Lilo et al., 2008) (Bergsbaken and Cookson, 2007; Shin and Cornelis, 2007). We show here that the production of tetra-acyl lipid A greatly inhibits the induction of pro-IL-1β, which in turn can suppress this pathway. This illustrates yet another critical mechanism by which the production of tetra-acyl lipid A suppresses the immune response against Y. pestis. When Y. pestis is modified to produce potent LPS, the IL-1 pathway can be activated, which is essential for clearance of infection. This effect is apparent in IL-1β deficient mice, as these mice have a severe defect in surviving infection with the modified strain. While much work has been done looking at Yersinia and the IL-1/caspase-1 response, this is the first time that the importance of the IL-1 pathway during Y. pestis infection in particular has been able to be examined using in vivo infection, due to the attenuated nature of the modified Y. pestis.

The data presented here are preliminary, future work includes looking at the activation of caspase-1 in terms of the different inflammasome components, and creating strains that are both deficient in the Yop proteins but express lpxL, to determine if the presence of potent LPS is able to override the caspase-1 suppressing activities.
Taken together the data presented illustrate that production of tetra-acyl lipid A, which results in dampening of the immune response, was an important evolutionary step for the development of *Y. pestis* into a highly virulent pathogen.
Materials and methods:

Yersinia lpxL Sequences

The lpxL sequences from Yersinia were located by BLAST (NCBI) search using the E. coli K12 lpxL gene (Annotation NC_000913.2). The sequence of Y. pseudotuberculosis IP2666 lpxL was determined by sequencing of the cloned region in pBR322. The gene annotation for the other genes are as follows: Y. pseudotuberculosis IP31758-CP000720.1, YPIII-CP000950.1, IP32953 BX936398.1, PB1/+-CP001048.1, Y. enterocolitica 8081 NC_008800.1, Y. enterocolitica type 0:9 AM941739. The Y. mollaretii ATCC43969 lpxL is between 9103 and 10023, Y. bercovieri ATCC43970 lpxL is between 9617 and 10537, Y. frederiksenii ATCC33641 lpxL is between 213902 and 214822, and Y. intermedia ATCC 29909 lpxL is between 30241 and 31161. These genomes have not yet been annotated.

Bacterial strains and growth conditions:

The bacterial strains used are listed in table 1. The KIM5 and KIM1001-pLpxL containing strains were made as previously described in Montminy, et. al., 2006. The lpxL gene from IP2666, including 480 basepairs upstream and 266 basepairs downstream of the coding region, was cloned by colony PCR using Pfu Ultra polymerase (Stratagene) and ligated into the BamHI and SalI sites of pBR322, creating pYtbLpxL. This inactivated the tetracycline gene in pBR322, and the mutants are tetracycline sensitive. The resulting plasmid was electroporated into Yersinia pestis strain KIM5(Goguen Jd, 1984) or KIM1001(Sodeinde et al., 1992) and selected by growth on tryptose-beef extract.
(TB) agar supplemented with 0.25 mM calcium chloride in the presence of 100 µg/ml ampicillin. KIM1001 (pPCP1+, pCD1+, pMT1+) is highly virulent while the KIM derivative KIM5 bears a chromosomal deletion designated Δpgm, which strongly attenuates virulence (Perry and Fetherston, 1997; Sodeinde et al., 1992). KIM5 was used to limit risk of infection in in vitro studies where a virulent strain was not required, and the pgm locus contains no genes thought to impact LPS biosynthesis. Y. pseudotuberculosis IP2666ΔlpxL was generated by PCR amplifying a 1052 bp upstream fragment and 569 bp downstream fragment of lpxL. A nonspecific linker sequence was inserted, and the two fragments were then joined by sewing PCR, making an 879 bp deletion in the gene (Figure 3.3). This fragment was cloned into pRE107 and transformed into β2155, an E. coli mating strain. This strain was then mated to IP2666 pYV-, and resulting colonies were screened for the deletion. The strains KIM-3001, KIM-3001ΔYopB,J,E and KIM-3001ΔYopJ,E were a gift from Dr Greg Plano (University of Miami) (Bartra et al., 2006).

Lipopolysaccharide and lipid A purification:
Pyrogen-free reagents and supplies were used to the greatest extent possible in the LPS and lipid A preparations. Bacteria were grown in 1 liter cultures overnight and then harvested by centrifugation at 3,000 x g. LPS was purified from bacteria by hot water-phenol extraction (Westphal et al., 1952), followed by two phenol re-extractions to remove contaminating lipoproteins and TLR2-activity (Hirschfeld et al., 2000). LPS from E. coli O111:B4 (Sigma) was phenol re-extracted as indicated above. The absence of
activation of TLR2-expressing cells (Figure 3.2c), in spite of potent IL-8 release by bacterial Pam3CysSK4 lipopeptide, indicates the absence of contaminating lipoproteins in our highly purified LPS preparations. LPS preparations were run on a NuPAGE® Novex 12% Bis-Tris Gels (Invitrogen) and then visualized by silver stain using BioRad Silver Stain kit (161-0443). Lipid A was directly isolated from whole bacterial cells using the Bligh-Dyer two-phase chloroform-methanol-water organic extraction (Vorachek-Warren et al., 2002a). Samples were subsequently analyzed by MALDI-TOF MS using a Kratos (Manchester, UK) AXIMA CFR high performance mass spectrometer operated in both positive and negative ion modes.

Cell Isolation and Culture:

Human PBMC were obtained from healthy volunteers with informed consent, and isolated by centrifugation on Lymphoprep density media (Axis-Shield/Nycomed). The UMass Institutional Review Board approved the human subject protocol. Red blood cells were lysed with red blood cell lysis buffer (Sigma). Immortalized macrophage cell lines were a gift from Kate Fitzgerald and generated as described (Hornung V, 2008). HEK293 cells stably expressing human TLR4-YFP and retroviral MD-2, or human TLR2-YFP, or empty vector pcDNA3 were as published (Latz et al., 2002). All cells were cultured in DMEM supplemented with 1-glutamine, 10% (vol/vol) FCS (HyClone) and with or without 100μg/mL ciprofloxacin. PBMCs and mouse macrophages were plated at a density of 1x10^5 - 2x10^6 cells per mL in DMEM and simulated with either the indicated amount of purified LPS or bacteria. Immortalized mouse macrophages were
stimulated in the presence of 1ng/mL recombinant GM-CSF (Peprotech). Bacteria used to stimulate cells were grown overnight at 37°C, expanded the next day for three hours to enter log growth phase, then washed three times with DMEM to remove bacterial growth media. The bacteria were added to cells, after three hours the cells were treated with 50μg/mL of gentamicin and incubated for another three hours; the supernatants were spun to clear the bacteria.

_Growth Analysis_

For growth analysis of both KIM5 and IP2666 strains, overnight cultures were diluted into 3 ml of TB broth with calcium chloride to an OD600 of 0.1. Growth of the cultures was monitored every 2 hours at OD600.

_Antimicrobial and serum sensitivity assays_

For antimicrobial and serum sensitivity assays of both KIM5 and IP2666 strains, overnight cultures were diluted into 3 ml of TB broth with calcium chloride to an OD600 of 0.1. These cultures were then allowed to grow for three hours to enter log phase. For the antimicrobial assay, the cultures were then swabbed on to TB agar plates of uniform concentration and volume, and then disks containing Polymyxin B (BD Sensi-Disc Cat no 230926 300units per disk) were placed on the agar. The plates were then inverted, placed at 37°C and incubated for 48 hours. The zone of inhibition was then measured. For the serum assay, the bacteria were diluted to 4 x10³ CFU/mL, and then 50% serum was added to the cultures. At the timepoints, 25 ul of the culture was plated in triplicate.
The plates were then inverted, placed at 37°C and incubated for 48 hours. The colonies were then counted and CFU/percent survival from time zero was determined.

Mice:

C57BL/6 were originally obtained from Jackson Laboratories and bred in our facilities. TNFR1 KO, IL-1R KO, IL-12p40KO, and IL-1R-deficient (Ilir1−/−) mice were from Jackson Laboratories. Mice deficient in TLR4 (Trl4−/−) were provided by S. Akira. Mice deficient in IFNAR KO were provided by J. Sprent at Scripps. All mouse strains were bred and maintained in specific pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School.

ELISA:

Cell culture supernatants were assayed for TNFα, IL-8, IL-1β with ELISA kits (R&D Systems) according to the manufactures’ instructions.

In vivo infections:

Mice were infected in a contained animal biosafety level 3 facility with fully virulent Y. pestis KIM1001, KIM1001-pEcLpxL or KIM1001-pYtbLpxL by subcutaneous (SC) injection of 50 μL in the nape of the neck. Inoculums containing the indicated CFU were suspended in PBS. Mice were euthanized by IP pentobarbital overdose or CO2 affixation when moribund. Survival was monitored up to 21 or 28 days, every 12 hrs during acute infection. All experiments involving live animals and Y. pestis were regulated by the
University of Massachusetts Medical School Department of Animal Medicine, the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee.

Statistical analysis

Statistical differences in survival were studied by Kaplan-Meyer survival analysis and the log rank test. Alignments and statistical analysis was done using DNAstar and Prism.
Figure 3.1

a

Y. pseudotuberculosis IP31758
Y. pseudotuberculosis YPIII
Y. pseudotuberculosis IP32953
Y. pseudotuberculosis PB01
Y. enterocolitica 8081
Y. enterocolitica Type 09
Y. mollaretii
Y. bercovieri
Y. frederiksenii
Y. intermedia

E. coli K12

Y. pseudotuberculosis IP31758
Y. pseudotuberculosis YPIII
Y. pseudotuberculosis IP32953
Y. pseudotuberculosis PB01
Y. enterocolitica 8081
Y. enterocolitica Type 09
Y. mollaretii
Y. bercovieri
Y. frederiksenii
Y. intermedia

E. coli K12

b

Y. pseudotuberculosis IP31758
Y. pseudotuberculosis YPIII
Y. pseudotuberculosis IP32953
Y. pseudotuberculosis PB01
Y. enterocolitica 8081
Y. enterocolitica Type 09
Y. mollaretii
Y. bercovieri
Y. frederiksenii
Y. intermedia

E. coli K12

<table>
<thead>
<tr>
<th>Percent Identity</th>
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<td>1</td>
</tr>
<tr>
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</tr>
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<td>2</td>
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<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>
Figure 3.1 \textit{lpxL} in \textit{Yersinia}

Punitive genes for \textit{lpxL} were identified by BLAST analysis using E. coli K12 \textit{lpxL} within sequenced \textit{Yersinia} strains. Punitive \textit{LpxL} genes were located in all sequenced \textit{Yersinia} with the exception of \textit{Y. pestis}.

a) Clustal W alignment of punitive \textit{lpxL} genes in sequenced \textit{Yersinia} strains. Yellow shading denotes area of sequence homology, with the consensus sequence is listed above.

b) Percent identity and divergence of the \textit{lpxL} sequences generated after clustal W alignment.
Figure 3.2

a

b
Figure 3.2 Mass spectrometry analysis of lipid A from *Y. pestis* and *Y. pseudotuberculosis*

KIM5-pYptbLpxL, IP2666, IP2666ΔlpxL, and IP2666ΔlpxL-pYptbLpxL was grown at 37 °C and the lipid A purified from whole bacteria by Bligh-Dyer two-phase organic extraction. The lipid was then further purified over a DE52 column and analyzed by MALDI-TOF mass spectrometry. The negative ion spectra shown here are representative of multiple extractions. Analysis of the positive ion spectra (data not shown) was also undertaken to determine the location of the modifications of the molecule.

a) Negative mode MALDI-TOF analysis of the IP2666 strain. Three predominant species present; tetra-acyl lipid A with four C14:0 acyl groups (m/z 1405), a penta-acyl lipid A with an additional secondary C12:0 acyl chain (m/z 1587), and a hexa-acyl lipid A with two additional C12:0 acyl chains (m/z 1769). b) KIM5-pYptbLpxL analysis shows a very similar acyl chain pattern, there also appears to be some additional tetra-acyl 1405 present, but in very low amounts. c) In contrast, IP2666ΔlpxL has a predominant tetra acyl peak (m/z 1405), with a pent-acyl peak at m/z 1587. There is no hexa acyl lipid A present in the spectra. When the strain is complemented with pYptbLpxL, the hexa acyl form becomes the predominant peak (m/z 1771).
Figure 3.3
Figure 3.3 Deletion of *lpxL* from IP2666

The IP2666 strain was generated by deleting a 879 base pair segment from the *lpxL* gene. The upstream and downstream regions of the gene were amplified by PCR and joined together with a small linker region to create a non polar deletion. This fragment was then cloned into a suicide vector, introduced into an E. coli mating strain, and then transferred to IP2666 pYV-. Colonies were tested for homologous recombination by PCR, with the mutant form exhibiting a smaller *lpxL* fragment when compared to the parent strain.
Figure 3.4
Figure 3.4 *Yersinia* strains expressing LpxL synthesis a potent LPS, while those lacking the gene produce non-stimulatory, antagonistic LPS

LPS from both *Y. pestis* and *Y. pseudotuberculosis* was isolated by hot water-phenol extraction, repurified, and used to stimulate a) human PBMCs, or HEK293 cells expressing b) TLR4/MD2, c) TLR2, or d) empty vector. Cells were stimulated for 18 hours and the supernatants were analyzed by either TNF or IL-8 ELISA. e) Human PBMCs were costimulated with LPS from either IP2666 ΔlpxL or KIM5, and various other stimulatory forms of LPS for 18 hours. Supernatants were collected and analyzed for TNF by ELISA. f) Immortalized wild type or g) TLR4/- mouse macrophages were stimulated with LPS as above, and the supernatants were analyzed for mouse TNF. LPS from *Yersinia* strains that expressed LpxL were able to stimulate a robust response from all the cell types, whereas the strains lacking *lpxL* produced non-stimulatory, antagonistic LPS.
Figure 3.5

(a) OD600 over time for KIM5 and KIM5-pYptbLpxL.

(b) OD600 over time for different strains: IP2666, IP2666ΔLpxL, IP2666ΔLpxL-pYptbLpxL.

(c) Bar graph showing zone of inhibition for KIM5, KIM5-pLpxL, and KIM5-pYptbLpxL.

(d) Bar graph showing zone of inhibition for IP2666, IP2666ΔLpxL, and IP2666ΔLpxL-pYptbLpxL.

(e) Image showing gel results with LPS from different strains: KIM5 LPS, IP2666 LPS, IP2666ΔLpxL LPS, IP2666ΔLpxL-pYptbLpxL LPS, and E. coli LPS.
Figure 3.5 Characteristics of *Y. pestis* and *Y. pseudotuberculosis* strains

a)- b) KIM5 and IP2666 strains were seeded in triplicate and incubated at 37°C in three milliliter cultures at an OD of 0.1. Cultures were then monitored every two hours at an OD600. No significant difference in growth between the parent and mutant strains was apparent.

c) - d) KIM5 and IP2666 strains were swabbed onto culture agar and then disks containing 300 units of Polymyxin B were placed on the agar in triplicate. The plates were inverted and incubated at 37°C for 48 hours, and the zone of inhibition from the antibiotic was measured. No significant difference was detected between the parent and mutant strains.

e) One microgram of LPS from the KIM5 and IP2666 strains was separated on a 12% Bis Tris gel, and the LPS visualized by silver stain. KIM5 and KIM5-pYptbLpxL exhibited a rough LPS phenotype, whereas IP2666 has more of an o-polysaccharide core region. The mutant strains again resemble their parent. The *E. coli* 0111:B4 displayed a smooth LPS, as denoted by the banding pattern of the molecule.
Figure 3.6

(a) Survival rate of different strains of bacteria:
- KIM1001
- KIM1001-pLpxL
- KIM1001-pYptbLpxL

(b) Survival rate of different strains of mice:
- C57BL/6
- TLR4-/-
Figure 3.6 Y. pestis-pYptbLpxL is avirulent in wild type mice

a) Wild type C57Bl/6 mice were infected with 500 CFU of KIM1001, KIM1001-pLpxL, or KIM1001-pYptbLpxL subcutaneously in the nape of the neck.

b) Wild type C57Bl/6 or TLR4-/- mice were infected with 500 CFU of KIM1001-pYptbLpxL subcutaneously at the nape of the neck.

Survival was monitored every 12 hours during acute infection.
Figure 3.7

(a) Kaplan-Meier survival curves for wild type and IL-1R-/- KIM1001-pLpxL.  KIM1001-pLpxL showed significantly lower survival compared to wild type.  

(b) Kaplan-Meier survival curves for MYD88-/- and C57BL/6.  MYD88-/- showed significantly lower survival compared to C57BL/6.

(c) Bar graph showing hu IL-1β (pg/mL) for different conditions: KIM5, KIM5-pLpxL, KIM5-pYK6-LpxL, unstimulated.  MOI 1 and MOI 0.1 conditions are shown.

(d) Bar graph showing hu IL-1β (pg/mL) for different conditions: unstimulated, IP69K, IP69K-pLpxL, IP69K-pYK6-pLpxL, ALUM-pYK6-LPS.  Conditions are shown.

(e) Bar graph showing mIL-1β (pg/mL) for different conditions: KIM5, KIM5-pLpxL, KIM5-pYK6-LpxL, ALUM-pYK6-LPS, unstimulated.  Comparison between C57BL/6 and TLR4-/- conditions is shown.
**Figure 3.7 IL-1 response is critical for protection from *Y. pestis* infection**

a) Wild type C57Bl/6 or IL-1R-/- mice were infected with 1000 CFU of KIM1001 or KIM1001-pLpxL subcutaneously at the nape of the neck. Survival was monitored every 12 hours during acute infection.

b) Wild type C57Bl/6 or MyD88-/- mice were infected with 1000 CFU of KIM1001 or subcutaneously at the nape of the neck. Survival was monitored every 12 hours during acute infection.

c) Human PBMCs were infected with KIM5, KIM5-pLpxL or KIM5-pYptbLpxL or d) IP2666, IP2666ΔlpxL, or IP2666ΔlpxL-pYptbLpxL for three hours, treated with gentamicin and then incubated for three hours longer. The supernatants were collected, treated with cipro to kill any remaining bacteria, and then analyzed for IL-1β by ELISA. Bacteria expressing LpxL induce IL-1β, whereas KIM5 does not.

e) Immortalized wild type C57Bl/6 or TLR4-/- mouse macrophages were infected with KIM5, KIM5-pLpxL or KIM5-pYptbLpxL for three hours, treated with gentamicin and then incubated for three hours longer. The supernatants were collected and then analyzed for IL-1β by ELISA. Cells deficient for TLR4 did not secrete IL-1β in response to infection with any of the KIM5 strains, where as the wild type cells were able to mount a robust response.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Plasmid</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM5</td>
<td>Δpgm</td>
<td>(Goguen et al., 1984)</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM5-pLpxL</td>
<td>Contains <em>E. coli</em> K12 <em>lpxL</em></td>
<td>(Goguen et al., 1984)</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM5-pYptbLpxL</td>
<td>Δpgm, Amp resistant, tet sensitive</td>
<td>This work</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM1001</td>
<td>Fully virulent</td>
<td>(Sodeinde et al., 1992)</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM1001-pLpxL</td>
<td>Contains <em>E. coli</em> K12 <em>lpxL</em></td>
<td>(Sodeinde et al., 1992)</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>KIM1001-pYptbLpxL</td>
<td>Amp resistant, tet sensitive</td>
<td>This work</td>
</tr>
<tr>
<td><em>Y. pseudotuberculosis</em></td>
<td>IP2666</td>
<td></td>
<td>(Simonet and Falkow, 1992)</td>
</tr>
<tr>
<td><em>Y. pseudotuberculosis</em></td>
<td>IP2666 pYV-</td>
<td>Lacks T3SS and Yops</td>
<td>(Simonet and Falkow, 1992)</td>
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<td><em>Y. pseudotuberculosis</em></td>
<td>IP2666ΔlpxL</td>
<td>In frame, non polar deletion of <em>lpxL</em></td>
<td>This work</td>
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<td>IP2666ΔlpxL + pYptbLpxL</td>
<td>Complement strain</td>
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Table 3.2 Vaccination with KIM1001-pYptbLpxL

<table>
<thead>
<tr>
<th>Vaccination (KIM1001-pYptbLpxL)</th>
<th>Challenge (KIM1001)</th>
<th>Survival</th>
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<tr>
<td>None</td>
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</tr>
<tr>
<td>500 CFU</td>
<td>1000 CFU</td>
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</tr>
</tbody>
</table>

Survival of wild type mice vaccinated subcutaneously with *Y. pestis* KIM1001-pYptbLpxL and naïve mice (None) after subsequent challenge with *Y. pestis* KIM1001, presented as surviving mice of total mice. Data representative of two to three experiments.
### Table 3.3 Survival of Receptor knockout mice after challenge with KIM1001-pLpxL

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Number surviving of total</th>
<th>Percent Survival</th>
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<tbody>
<tr>
<td>C57Bl/6</td>
<td>5 of 5</td>
<td>100%</td>
</tr>
<tr>
<td>IFNαR KO</td>
<td>8 of 10</td>
<td>80%</td>
</tr>
<tr>
<td>TNFR I KO</td>
<td>5 of 8</td>
<td>63%</td>
</tr>
<tr>
<td>IL-12 p40 KO</td>
<td>6 of 11</td>
<td>55%</td>
</tr>
<tr>
<td>IL-1RI KO</td>
<td>4 of 10</td>
<td>40%</td>
</tr>
<tr>
<td>TLR4 KO</td>
<td>0 of 5</td>
<td>0%</td>
</tr>
</tbody>
</table>

Survival of wild type and knock out mice after subcutaneous infection with 1000 CFU *Y. pestis* KIM1001-pLpxL, presented as percent and surviving mice of total mice.
CHAPTER IV

DISCUSSION
Overview

The focus of the research presented in this thesis has been to determine if the presence of tetra-acyl lipid A in *Yersinia pestis* LPS acts as a critical virulence and evolutionary determinant. Specifically, I set out to determine if evasion of LPS-mediated TLR4/MD2 signaling and subsequent dampening of innate immunity are essential for the extreme pathogenicity of the bacteria. I demonstrate that by producing tetra-acyl lipid A at 37°C (mammalian body temperature) *Y. pestis* is not only able to dampen TLR4 activation, but also inhibit the IL-1 pathway, a critical component of the host response to gram negative bacteria. I also illustrate that the production of tetra-acyl lipid A is a consequence of the loss of LpxL, a key enzyme in the lipid A biosynthesis pathway, and furthermore that expression of *lpxL* in *Y. pestis* severely attenuates virulence. To that end, when *lpxL* was introduced to *Y. pestis*, the bacteria produced hexa-acyl lipid A which was able to activate the innate immune system. Thus, the loss of this critical enzyme from *Y. pestis* formed a major evolutionary step in the development from its parent *Y. pseudotuberculosis*. Unlike other *Yersinia* family members which produce mild disease upon infection, the loss of LpxL from *Y. pestis* has enabled the bacteria to evolve into a highly virulent pathogen able to completely evade the immune response leading to devastating disease. This work describes the novel finding that the production of tetra-acyl lipid A is an essential virulence determinant for *Y. pestis*, and a major factor in the evolution of a highly virulent pathogen.
Yersinia lipid A acylation patterns

It had been previously established that when grown at 37°C *Y. pestis* produced tetra-acyl lipid A, and that LPS from these bacteria was non-stimulatory (Kawahara et al., 2002; Rebeil et al., 2004; Rebeil et al., 2006). However, when grown at lower temperatures (21°C to 27°C), the bacteria produced stimulatory hexa-acylated lipid A/LPS. It remained unclear why the bacterium produced the tetra-acyl lipid A at higher temperatures and what affect this had on virulence. Generally, gram-negative bacteria produce a large array of lipid A species, which can be further altered by environmental cues such as temperature and can differentially activate TLR4/MD2 (Miller et al., 2005). However, producing tetra-acyl lipid A exclusively is unusual, due to the increase in membrane fluidity which can leave the bacterium vulnerable to osmotic and environmental stress. Also, the parent of *Y. pestis*, *Y. pseudotuberculosis*, contains a heterogeneous mix tetra, penta, and hexa lipid A, indicating that alteration of lipid A biosynthesis may have been acquired during the evolution of *Y. pestis* from its parent (Rebeil et al., 2004; Therisod et al., 2002). The first question posed in this work was how *Y. pestis* is able to produce tetra-acyl lipid A at 37°C. By examining the genes involved in the *E. coli* lipid A biosynthesis pathway, and comparing them to homologues found in *Y. pestis*, it was discovered that *Y. pestis* lacked the gene *lpxL* responsible for the addition of a secondary acyl chain to tetra-acyl lipid A late in the biosynthesis pathway. *Y. pestis* does however contain a gene for *lpxP*, another late acyltransferase, which enables the bacterium to produce hexa-acylated lipid A only at lower temperatures. During these studies *E. coli lpxL* was cloned into pBR322 under the control of its own promoter.
(pLpxL), and introduced to *Y. pestis*. The plasmid pBR322 was used because it is tolerated well by *Y. pestis*, which has three endogenous plasmids and typically does not tolerate exogenous plasmids. The lipid A from this new strain was isolated and analyzed by MALDI-TOF mass spectrometry. This revealed that *Y. pestis* was able to produce novel hexa-acyl lipid A with a mass of 1769 m/z at both 26°C and 37°C when expressing LpxL (Figure 2.2c, d). This hexa-acyl lipid A included two C:12 secondary acyl chains on the tetra-acyl lipid, indicating that both *E. coli* LpxL and the endogenous *Y. pestis* LpxM (the last late acyltransferase in the pathway) were capable of adding the C:12 acyl chains, which was novel finding. In *E. coli*, LpxM adds a C:14 chain and LpxP attaches a C:16 chain. While LpxP may function in the same manner in *Y. pestis* and *E. coli*, *Y. pestis* LpxM has an unexpected novel function of adding a C:12 chain to the lipid A, which is a new function for the LpxM enzyme.

Analysis of lipid A from *Y. pseudotuberculosis* proved to be more challenging than *Y. pestis*. The lipid A profile for *Y. pseudotuberculosis* has not been extensively studied, although two groups have undertaken mass spectrometry on the lipid A molecule directly (Rebeil et al., 2004; Therisod et al., 2002). This data showed a high degree of heterogeneity in the spectra, much greater than that seen with *Y. pestis* at 26°C or with *Y. enterocolitica*, the distant ancestral parent of *Y. pseudotuberculosis*. These spectra show tetra-acyl lipid A with a mass of 1405m/z, as well as several different forms of penta- and hexa-acyl lipid A with a C:16 modification. While it is important to note that MALDI-TOF is not quantitative, none of the previously published spectra identified one form of lipid A that seemed to dominate the spectra. However, analysis of the IP2666 strain
revealed a notably different profile. While a mix of lipids was still present, C:16 modifications were absent and the 1769 hexa-acyl peak dominated the spectra (Figure 3.2a). This hexa-acyl species was unique for *Y. pseudotuberculosis* and has not currently been reported in literature. There are several reasons this spectra may differ from published accounts. First, the LPS/Lipid A from IP2666 has not previously been examined, and it may have different characteristics given the lipid A from this bacteria are so variable. Secondly, the isolation method used in this work is different that published accounts. While previous groups have isolated lipid A secondarily from extracted LPS, I used the Bligh-Dyer lipid A isolation technique (Vorachek-Warren et al., 2002b). Isolating lipid A from previously purified LPS subjects the sample to two hot acid extractions, the first during the repeated hot phenol extractions to purify the LPS, the second, a boil in an acidic sodium acetate/SDS solution. These two harsh chemical treatments can strip the lipid A molecule of secondary modifications and possibly remove acyl chains, resulting in the appearance of heterogeneity of the molecule. The Bligh-Dyer lipid A isolation technique extracts the lipid A directly from the bacteria and results in less fragmentation and degradation on the lipid A. In this case, the lipid A is only exposed to a sodium acetate/SDS boil, and then an organic extraction that is much gentler to the molecule and results in less possible chemical modifications. The directly isolated lipid A is then purified over an anion exchange column, dried, and then frozen at -80°C immediately to ensure against further degradation of the molecule. This method is superior to isolating from LPS because, while more time consuming and lower yields, it
results in less degradation of the lipid A molecule, which is why it was used in these studies.

To address this issue and confirm the lipid A analysis, I analyzed the IP2666 strain several times to confirm the spectra, although due to external technical difficulties the results were variable further analysis is underway. MALDI-TOF performed on directly LPS confirmed the previously obtained lipid A profile shown in Figure 3.2a. I also attempted to analyze the lipids on a Finnigan LTQ electrospray mass spectrometer, however the sensitivity of this machine was less than with a MALDI-TOF. Therefore, I was only able to detect residual SDS molecules remaining from the isolation procedure. This lab has now begun a new collaboration, and future experiments planned include a fresh isolation of IP2666 lipid A and a reanalysis to confirm the lipid A profile.

Analysis of the lipid A from IP2666ΔlpXL and IP2666ΔlpXL-pYptbLPXL is much less complex (Figure 3.2c,d). IP2666ΔlpXL displays a distinct primary tetra-acyl lipid A molecule which very closely mimics that seen with KIM5 lipid A (Figure 3.2c). IP2666ΔlpXL-pYptbLPXL by contrast has a major hexa-acylated peak at 1769 m/z, mimicking KIM5-pLPXL (Figure 3.2d). The shift in the acylation patterns of Y. pestis between the wild type and LpxL expressing strains is pronounced, as is the shift between IP2666 and IP2666ΔlpXL-pYptbLPXL. lpXL is under the control of its native promoter and therefore mimicking natural expression as much as possible. However, the exogenously expressed gene is at higher copy number than normally found in E. coli or Y. pseudotuberculosis, and is subject to different levels of control. This increase in copy number may strongly drive the system more than would normally occur with a single
copy of the gene. Further experiments to be conducted involve placing a single copy of lpxL gene either directly into the chromosome or a Y. pestis specific vector, thus mimicking endogenous expression in order to determine if the acylation pattern remains the same. If LpxL was expressed at a more endogenous level, it is possible that the acylation pattern seen in these bacteria would shift to a heterogeneous mix of lipids, similarly to that seen in Y. pseudotuberculosis

**Attenuation of Virulence in Y. pestis strains expressing LpxL**

The LPS purified from Y. pestis strains expressing LpxL is stimulatory via TLR4/MD2, and unlike wild-type Y. pestis is able to activate human primary cells. However, apart from the changes in lipid A/LPS, Y. pestis contains a multitude of active mechanisms to suppress the immune response. Together these mechanisms can override even the most robust innate immune response. To determine if changes in acylation patterns had any significant effect on the virulence of Y. pestis, pLpxL and pYptbLpxL were introduced into fully virulent KIM1001. The mice were subcutaneously infected with this strain in order to mimic natural infection from a flea bite. Remarkably, all the mice infected with the LpxL expressing strains of Y. pestis survived the infection, and did not display any outward signs of sickness. Mice were infected with up to 1x10⁶ CFU of KIM1001-pLpxL and never appeared to suffer any ill effects (Table 2.1). This extreme attenuation in virulence was remarkable given that all other virulence determinants were in place. Further experimentation showed that when delivered subcutaneously, infection with KIM1001-pLpxL did not become systemic, but instead remained as a localized
infection. Mice that were infected intravenously did eventually succumb to disease, but at a notably reduced rate, with evidence of a robust and widespread immune response when compared to animals that received intravenous KIM1001 (Figure 2.4d-g). The effect seen by this modification is profound, indicating that the acylation state of the LPS greatly affects the virulence of the bacteria. Previous work had shown that bacteria grown at 26°C had a higher LD50 in mice than bacteria grown at 37°C, however in both cases the bacteria were still extremely virulent (Perry and Fetherston, 1997). These data show that by permanently altering the acylation state of *Y. pestis* to hexa acyl at 37°C, the bacteria can be rendered completely avirulent, unlike what is seen when the bacteria is merely grown at a lower temperature before inoculation. Wild type *Y. pestis* grown at 26°C expressing hexa-acyl lipid A, while stimulatory, may not be cleared by the mouse before it changes its acylation pattern due to both the time it takes for the bacteria to be fully cleared at the site of inoculation, and due to the T3SS that also comes into effect upon temperature shift. The time between the flea bite inoculation with low temperature grown *Y. pestis* expressing immunostimulatory LPS and when the bacteria shift their genetic profile in response to the increased temperature may not be sufficient for complete clearance of the bacteria before both the lipid A profile changes and the T3SS becomes active. Also, the resident dendritic cells in the subcutaneous layer of the skin, the Langerhans cells, do not express TLR4 (van der Aar et al., 2007) (Takeuchi et al., 2003), so they may not be able to recognize the bacteria. Melanocytes and keratinocytes do express TLR4 (Ahn et al., 2008) (Kollisch et al., 2005) (Lebre et al., 2007), and keratinocytes release proinflammatory cytokines in response to LPS stimulation (Lebre et
al., 2007), so these cells may be able to recognize and alert the immune system, but how
effective they are at inducing the immune response has yet to be determined. The
combination of low bacterial inoculation during the flea bite and the lack of cells
available in the subcutaneous layer of the skin to recognize the hexa acyl lipid A and
mount a rapid and effective immune response may explain why the bacteria are not
cleared from the host before they can change their genetic profile and lipid A structure
and become a systemic infection.

This attenuation was TLR4-mediated, as mice deficient for the receptor quickly
fall ill and succumb to disease (Figure 2.4b, Figure 3.6b). However, the robust innate
immune response initiated by TLR4/MD2 signaling is not sufficient to clear the infection,
as when infected with KIM1001-pLpxL RAG-/- mice eventually die from the disease,
although the time to death is delayed (Figure 2.8). The innate immune response may not
be able to clear the infection entirely due to LPS tolerance that may be induced after
prolonged stimulation. Defining the cellular subsets responsible for the robust immune
response seen at both the site of infection and systemically is of great interest, and is a
future direction of the project. This includes an examination of the cellular subsets that
are recruited to the site of infection, as well as deciphering the adaptive immune response
to the infection. Further characterization of KIM1001-pYptbLpxL will also take place,
with preliminary experiments shown in Figure 3.6 and Table 3.2. These results indicate
that this strain functions in a similar manner to KIM1001-pLpxL, and also appears to
have no growth or survival defects. It would be of interest to determine how different
adaptor and receptor mutant mice respond to infection with this strain, since the acylation pattern is somewhat different from that of *Y. pestis* expressing *E. coli* LpxL.

**Use of LpxL expressing strains as a vaccine**

The idea of modifying the LPS/Lipid A molecule of a bacteria to activate TLR4 during infection is a novel concept in vaccine development. As discussed in the introductory Chapter 1, the idea of using TLR agonists to act as potent adjuvants in vaccines has been gaining recognition (Lahiri et al., 2008; Zhu et al., 2008). As our understanding of the link between the innate and adaptive immunity grows, the concept of using specific TLR agonists to activate these host defense systems is becoming central to vaccine development. This work proposes a new model for *Y. pestis* vaccine development, and vaccine development as a whole. Wild type mice infected with the LpxL expressing strains are protected from subsequent infection with wild type *Y. pestis*, demonstrating that these strains are important potential vaccine candidates (Tables 2.2, 2.3, 3.2). The production of an attenuated *Y. pestis* strain as a vaccine candidate has long been the focus of research from various groups. The majority of this work has been focused on generating deletional mutants of various *Y. pestis* virulence factors or isolating different bacterial components with high antigenicity (F1, LcrV) (Feodorova and Corbel, 2009). However, these vaccines have failed to show complete protection from infection in non-human primates, and while tolerated well by humans in trials the degree of protection they impart in humans is controversial. The work presented here illustrates potent protection with a live attenuated strain of *Y. pestis* in immunocompetent
mice. Nevertheless, using this strain as a live attenuated vaccine in a human population is not feasible due to its pathogenicity in immunocompromised mice or by intravenous infection. Therefore, modifications would have to be made to the strain to further attenuate virulence while maintaining the high level of protection. Work by Szaba et al has shown that adding pLpxL to the pgm deficient attenuated strain of *Y. pestis* D27 attenuates the virulence of the strain during intranasal infection, and protects against subsequent reinfection with fully virulent *Y. pestis* (Szaba et al., 2009). They also reported a robust T cell response is elicited by this vaccination, and that protection from reinfection is dependent on the memory T cell response not on antibody production. This is critical as most *Y. pestis* vaccine research has been devoted to creating a vaccine that produces high vaccine antibody titers. This report demonstrates that creating a vaccine that will elicit both antibody production and a potent memory T cell response is essential for creating an effective vaccine. Further work to be undertaken includes continuing to modify *Y. pestis* to selectively attenuate virulence while expressing a potent LPS/lipid A, to create a live attenuated vaccine that produces a robust antibody and T cell response while making it safe for human use. Another possible strategy would be to create outer membrane vesicles containing both antigenic *Y. pestis* proteins and stimulatory LPS, in a similar manner to the strategy employed with *N. meningitidis* (Casella and Mitchell, 2008; Fransen et al., 2007; Stoddard et al., 2009).
Examining the roles of different innate immune pathways during *Y. pestis* infection

The data presented in this thesis demonstrates that the production of immune stimulatory LPS is able to fully attenuate the virulence of a highly dangerous pathogen. In addition, these strains of *Y. pestis* expressing LpxL, allow us to further study in detail the role of individual adapters within the TLR pathway that are critical for protection during active infection, and also which other innate immune pathways may be essential for protection from disease.

In chapter 2, different components of the TLR4 downstream signaling cascade were identified as being important for protection from *Y. pestis* infection. MyD88 is essential not only because of its role as a central adapter for TLR4, but also possibly due to its roles in other immune pathways which were further explored in Chapter 3. The adaptor Mal was also crucial, in that over half the Mal deficient mice infected with KIM1001-pLpxL succumbed to disease, demonstrating the most pronounced phenotype of any of the adaptor knockouts tested with the exception of MyD88 (Figure 2.6a). Interestingly, TRIF and TRAM deficient mice were not particularly susceptible to the disease, indicating that the alternative TLR4 signaling pathway does not play a critical role in protection against *Y. pestis* (Figure 2.6a). We also explored the contribution of the TLR4 signaling protein CD14, which had previously been reported to not be involved in the recognition of deep rough LPS from a mutant strain of *E. coli* (Huber M, 2006; Jiang Z, 2005). Mice deficient in CD14 were found to be highly susceptible to *Y. pestis* infection and produced significantly lower chemokines in response to *Y. pestis*-pLpxL LPS, indicating that CD14 does recognize rough LPS and is essential for protection from
disease (Figure 2.6c,d). These data illustrate not only the components of the LPS- 
TLR4/MD2 pathway that are being utilized during infection, but also the unique infection 
model that these strains present. The virulent strains of Y. pestis-pLpxL contain all the 
remaining powerful virulence factors, allowing us to explore which TLR4 adaptor or 
accessory proteins are involved in the TLR4 signaling cascade during active and 
fulminant infection, not just those required during LPS stimulation or some other phase 
of infection. These virulent strains also allow us to further explore which other 
components of the innate immune system interact with or are mediated by TLR4.

One such example is the IL-1 pathway, which activates a number of very potent 
cytokines such as IL-1\(\alpha\), IL-1\(\beta\), and IL-18. IL-1\(\beta\) in particular has been the subject of 
intense study recently due to its ability to strongly suppress infection, and its role in 
autoimmune disease when disregulated (Dinarello, 2009). In chapter 3, we illustrated 
that when IL-1R mice were infected with KIM1001-pLpxL, they displayed a 
substantially decreased survival, more than was apparent with other receptor knockouts 
that were thought to be involved in the response to Y. pestis (Table 3.3, Figure 3.7b). The 
MyD88 knockout mice also had a significantly higher rate of mortality than wild type and 
TLR4 knockout mice (Figure 3.7a). This indicates MyD88 is important, not just for 
protection mediated by TLR4 or potentially other TLRs, but also for the involvement of 
the IL-1 pathway. This illustrates the complex nature of the innate immune signaling 
pathway, and the high degree of redundancy and crosstalk between the pathways. MyD88 
is a central adapter to TLR4 and IL-1, which explains why mice deficient in this protein 
succumb to disease so quickly. Without this adapter, two major protective arms of the
innate immune system are deactivated, which leads to rapid onset of disease and death (Figure 4.1). Many of the other downstream adapters in the pathway, such as TRIF and TRAM, have much less of an impact on the outcome of \( Y.\ pestis \)-pLpxL infection (Figure 2.6a). This is due to the fact that this arm of the TLR4 pathway plays more of a role in activating IFN and IFN-inducible genes, which is not essential for protection in this model (Table 3.3) (Fitzgerald Ka, 2003; Sato et al., 2003; Yamamoto et al., 2003a).

Therefore, to address the importance of the IL-1 pathway, experiments were undertaken to examine the levels of IL-1\( \beta \) release in cells infected with \( Y.\ pestis \) then stimulated with LPS. It was determined from these experiments that cells infected with \( Y.\ pestis \) secreted small amounts of IL-1\( \beta \), whereas cells infected with the LpxL expressing strains secreted a much higher level of the cytokine (Figure 3.4). This increase in IL-1\( \beta \) release is TLR4 mediated, as cells deficient for this receptor did not secrete substantial amounts of the cytokine (Figure 3.4). This indicates that a strong TLR4 mediated signal is necessary for the production of pro-IL-1\( \beta \), which during active infection is cleaved by caspase-1 and released from the cell. Without this strong TLR4 signal, only low levels of the cytokine are released (Figure 3.4g). Future experiments will include the examination of the levels of pro and active IL-1\( \beta \) in the cell by western analysis, in order to assess the amount of pro-IL-1\( \beta \) production verses the level cleaved into activated IL-1\( \beta \). While it is highly likely that the increase of IL-1\( \beta \) secretion during infection with LpxL expressing strains is due to TLR4 activation and subsequent production of pro-IL-1\( \beta \), a second possibility is that the increase cleavage by caspase-1 is due to additional inflammasome activation (Figure 4.1).
IL-1β is activated by caspase-1 cleavage, which is in turn activated by the assembly of an inflammasome complex that cleaves pro-caspase-1 into its active form (Mogensen, 2009). The signals that initiate the assembly of an inflammasome complex remain under intensive study, although it does appear to involve at least two danger signals. The first signal is the activation of a PRR, while the second signal may involve some sort of cellular damage (Mogensen, 2009). In the case of *Y. pestis*, there may be multiple mechanisms to dampen or eliminate either of these signals to prevent activation of caspase-1. The production of tetra-acyl lipid A blocks activation of TLR4, which may effectively block the production of pro-IL-1β, however, the presence of the T3SS of *Y. pestis* may act as a secondary signal of cellular damage. Previous reports have shown that the T3SS needle apparatus alone can induce the activation of caspase-1 (Shin and Cornelis, 2007), and that several of the Yop proteins work to suppress this activation (Schotte et al., 2004). However, other research on the activity of YopJ have contradict this finding (Bergsbaken and Cookson, 2007; Lilo et al., 2008). Therefore, the exact role the inflammasome plays during *Y. pestis* infection is still remains unclear. The modified strains of *Y. pestis* that express LpxL and a potent LPS provide an excellent tool to help distinguish how the inflammasome is being activated by *Y. pestis*, what inflammasome components are being assembled, and which Yop proteins may be working to thwart this activation. The first step in answering these questions is to determine which NLR proteins, such as NALP3; Ipaf; ASC; and caspase-1, are involved in *Y. pestis* induced inflammasome activation. This can be undertaken by both *in vitro* methods involving cell lines deficient in these proteins, such as immortalized mouse macrophages or bone
marrow derived macrophages from knockout mice, and *in vivo* utilizing inflammasome deficient mice. The NALP3 inflammasome is perhaps the best candidate for activation by *Y. pestis*, as it has previously been shown to be involved in activation of caspase-1 (Sutterwala et al., 2006), and is upregulated by NF-κB activation (Bauernfeind et al., 2009). However, it is a possibility that other sensors may also be involved, therefore all inflammasome components will be investigated in future experiments.
Figure 4.1

TLR4 bound with MD2 and lipid A

CD14

TRIF

TRAM

MAL

MyD88

IRAK4

IRAK1/2

TRAF6

TAB2/3

TAK1

NF-κB activation

IL-1R

IL-1β

IL-1β

IL-1β

Pro-IL-1β

Caspase 1

Pro-Caspase 1

Activated Inflammasome Complex

Pro-IL-1β

Induction of Pro-IL-1β

NF-κB

Proinflammatory cytokines
Figure 4.1 Interaction between TLR4 and IL-1 pathways during stimulation with *Yersinia* expressing LpxL

Model of the interaction between TLR4/MD2 and hexa-acyl lipid A produced by *Yersinia* expressing LpxL and the activation of the MyD88 pathway which leads to the upregulation of proinflammatory cytokines like pro-IL-1β. Pro-IL-1β is then cleaved by activated caspase-1, secreted from the cell and binds to the IL-1R. The IL-1R pathway also utilizes MyD88 and potently activates NF-κB, which in turn further upregulates the pro-inflammatory response.
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