Effects of *Francisella tularensis* infection on macrophage intracellular signaling

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## CONTENTS

**PAPERS IN THE THESIS** ........................................................................................................................................ 5  
**SAMMANFATTNING** ........................................................................................................................................ 6  
**ABSTRACT** .................................................................................................................................................... 7  
**INTRODUCTION** ............................................................................................................................................. 9  

1. *Francisella tularensis*. Taxonomy and history. ............................................................................................ 9 
   1.1. The Live Vaccine Strain ...................................................................................................................... 10  
   1.2. Tularemia. Clinical manifestations. Epidemiology ................................................................................... 10 
   1.3. Virulence factors of *F. tularensis* ........................................................................................................ 11  

2. Mammalian host defense mechanisms ............................................................................................................. 14 
   2.1. Interaction between phagocytic cells and intracellular pathogens. ....................................................... 14 
   2.2. Innate immune system in mammals. ...................................................................................................... 15  

3. Innate immune signaling in insects. ..................................................................................................................... 26 
   3.1. *Drosophila Toll* pathway .................................................................................................................... 27 
   3.2. *imd* pathway. ..................................................................................................................................... 27 

4. Laboratory models for studying the virulence of *F. tularensis* ................................................................. 29 
   4.1. Animal models. ..................................................................................................................................... 29 
   4.2. *Drosophila* as a potential model for studying *F. tularensis* .............................................................. 29 

**AIMS OF THE STUDY** ...................................................................................................................................... 31  

**RESULTS** ....................................................................................................................................................... 33 

1. Effects of *F. tularensis* infection on intracellular pathways in monocyic cells during internalization. ........................................................................................................................................... 33 
   1.1 Effects of *Francisella* infection on intracellular signaling in mouse and human cells. ........................................... 34 
   1.2 Effects of *Francisella* infection on cytokine secretion. ............................................................................. 35 

2. *F. tularensis* escapes from the phagolysosome........................................................................................... 35 

3. *F. tularensis* inhibits Toll-like receptor-mediated activation of intracellular signaling in mouse macrophages. ................................................................................................................................. 37 
   3.1 Intracellular survival and multiplication of *Francisella*. ................................................................. 37 
   3.2 *F. tularensis* inhibits secretion of proinflammatory cytokines stimulated by *E. coli* LPS or BLP. ................................................................................................................................. 37 
   3.3 *Francisella* inhibits activation of NF-κB, p38, and c-Jun signaling pathways and affects regulation of TLR2. .................................................................................................................. 38 

4. The IglB, IglC, IglD, and MglA proteins are important virulence determinants of *F. tularensis*. .................................................................................................................................................. 39
4.1 *iglB, iglC, iglD,* and *mglA* mutant strains are unable to replicate and survive inside phagocytic cells. .......................................................................................................................... 39

4.2 *iglB, iglC, iglD* and *mglA* mutant strains do not inhibit *E. coli* LPS-mediated activation of intracellular pathways. .............................................................................................................. 40

5. The role of RNS and ROS for the *F. tularensis*-induced effects on intracellular signaling. ............................................................................................................................................. 40

5.1 H$_2$O$_2$ does not affect inhibition of LPS-induced activation of intracellular signaling and secretion of TNF-$
\alpha$ by *F. tularensis* LVS. ........................................................................................................ 41

5.2 Effects of NO and peroxynitrite on the inhibition of LPS-induced activation of macrophages by *F. tularensis* LVS. ............................................................................................................. 41

5.3 SNAP reverses inhibition of intracellular pathways induced by *Francisella* in a dose-dependent manner. ....................................................................................................................... 42

5.4 Possible mechanisms of the NO-mediated effects on intracellular pathways of eukaryotic cells............................................................................................................................. 42

6 *Drosophila* .................................................................................................................................. 43

6.1 Survival and growth of *F. tularensis* in *Drosophila* haemocytes. ........................................ 43

6.2 Survival and growth of *F. tularensis* in *Drosophila*. ............................................................ 43

6.3 Effect of *Francisella* infection on intracellular signaling in *Drosophila*......................... 44

CONCLUSIONS.................................................................................................................................. 45

ACKNOWLEDGEMENTS .................................................................................................................. 46

REFERENCES................................................................................................................................... 47

PAPERS .......................................................................................................................................... 57
The thesis is based on the following papers, which will be referred to in the text by the roman numerals.

I. Telepnev, M., Golovliov, I., Sjöstedt A.


II. Lindgren, H., Golovliov, I., Baranov, V., Ernst, R.K., Telepnev, M., Sjöstedt A.


III. Telepnev, M., Golovliov, I., Grundström, T., Tärnvik, A., Sjöstedt A.


IV. Telepnev, M., Golovliov., I, Guina, T., Sjöstedt A.


V. Telepnev, M., Pettersson, M., Sjöstedt A., Stöven S.

*Drosophila melanogaster* as a model for elucidating the pathogenicity of *Francisella tularensis*. Manuscript.
SAMMANFATTNING


En sådan signalering leder till ett proinflammatoriskt immunsvar och uttryck av antimikrobiella försvarsmekanismer. Vi visar i denna avhandling att den intracellulära patogenen *Francisella tularensis* kan inducera ett proinflammatoriskt svar vid internalisering i humana och murina makrofager. Efter internalisering kan emellertid bakterien effektivt blockera MAPK och NF-κB signaleringsvägarna och därigenom sker ingen utsöndring av proinflammatoriska cytokinier ifrån de infekterade murina makrofagerna. Också i humana makrofager sker en nedreglering men någon total blockering sågs inte. Vi fann också att de infekterade cellerna inte svarade på stimuli som normalt aktiverar TLR, t.ex., LPS från *Escherichia coli* eller bakterielt lipoprotein. Denna blockering sågs endast när cellerna var infekterade med *F. tularensis* LVS men inte med avdödade bakterier eller mutanter av LVS. Dessa mutanter var defekta för uttryck av vart och ett av tre s.k. Igl proteiner samt en reglerare av dessa proteiner som benämns MglA. De senare resultaten talar för att närvaron av Igl proteiner i sig inte är tillräckliga för att effektuera de hämmande effekterna utan att det också krävs att de samtidigt kan regleras via MglA.

För att ytterligare förstå hur blockeringen av TLR-medierad cell signalering sker, studerade vi om kväveoxid, superoxid och reaktionprodukten av dessa två, peroxynitrit, påverkade förloppen. Vi observerade att tillsats peroxynitrit hade en liten påverkan medan tillsats av SNAP, som bildar kväveoxid, resulterade i ett nästan totalt återställande av den *F. tularensis*-inducerade blockeringen av TNF-α sekretion. Ytterligare analys talade för att tillsatsen av SNAP resulterade i att kväveoxid intracellulärt reagerade med superoxid och bildade peroxynitrit och att det var det senare ämnet som trots allt motverkade blockeringen medierad av *F. tularensis*.

För att ytterligare identifera en roll för Igl/Mgl proteiner kopplad till *F. tularensis* virulens så etablerade vi en modell för tularemia i *Drosophila melanogaster*. Flugan kan i många avseenden sägas efterlika dem miljö som bakterien befinner sig när den sprids av artropoder. Vi observerade att överlevnaden för flugor infekterade med Igl-mutanter var betydligt längre än för de som var infekterade med *F. tularensis* LVS.

Sammanfattningsvis talar våra resultat för att *F. tularensis* innehåller PAMP som aktiverar ett proinflammatoriskt svar så länge bakterierna befinner sig extracellulärt och vid internalisering. Däremot kan de intracellulärt lokalisera bakterierna blockera intracellulär signalering och sekretion av proinflammatoriska cytokinier helt eller delvis beroende på vilket ursprung de monocytära cellerna har. En sådan hämning kan vara betydelsefull för bakteriens förmåga att överleva intracellulärt. Proteinerna i Igl/Mgl systemet verkar ha en nyckelroll för att effektuera denna hämning. Vidare spelar Igl/Mgl systemet en central roll för *F. tularensis* förmåga att föröka sig i mammalieceller och i *D. melanogaster*. 

ABSTRACT

Microbes contain a number of structural components, also known as pathogen associated molecular patterns (PAMP), which can be recognized by the host defense system. The PAMP serve as ligands for Toll-like receptors (TLR) expressed on phagocytic cells. Binding of PAMP to TLR leads to activation of a number of intracellular transduction pathways including NF-κB and mitogen-activated protein kinase pathways (MAPK). Activation of those pathways in turn leads to activation of proinflammatory immune responses and antimicrobial defense mechanisms. In this study we show that the intracellular pathogen F. tularensis LVS can induce a proinflammatory response during internalization in both human and mouse macrophages. However, after internalization, F. tularensis LVS effectively blocks all these pathways resulting in no secretion of proinflammatory cytokines in mouse macrophages. In human macrophages, a downregulation was observed, however, there was not a complete block as in the murine cells. Furthermore, the infected cells do not become activated by stimulation with TLR agonists such as Escherichia coli LPS or bacterial lipoprotein. This phenomenon is observed only when the cells were infected with the LVS strain, but not with killed bacteria or specific mutants such as iglC, or mglA mutants, suggesting that not only their presence but also rapid regulation of the Igl proteins are needed. Thus, Igl/Mgl system plays a very important role in mediating these inhibitory effects.

To further investigate the role of the Igl/Mgl proteins in the virulence of F. tularensis, we established a novel model for tularemia, infection of Drosophila melanogaster. Using the fly model as a way to mimic the F. tularensis infection in arthropod vectors, which are important for its life cycle, we observed that bacterial mutants in each of the IglB, IglC, IglD, or MglA proteins all showed less efficient killing of the flies than did the parental LVS strain.

Nitric oxide, superoxide, and the reaction product of the two mediators, peroxynitrite, all are important for regulation of eukaryotic cell signaling and effectuating bactericidal mechanisms. To this end, we investigated the role of these mediators for the F. tularensis-mediated TLR-signaling inhibition. We observed that whereas peroxynitrite had little effect on the inhibition, addition of SNAP, a donor of nitric oxide, partially restored the ability of the cells to respond to LPS stimulation and secrete proinflammatory cytokine such as TNF-α. However, since FeTTPS reversed the SNAP-mediated effect, the results indicate that NO forms peroxynitrite intracellular and that the latter molecule is the effector.

Together these results indicate that F. tularensis contains PAMP that can activate proinflammatory immune response while the bacteria are located outside of phagocytic cell and during internalization, however, the intracellular bacteria have means to inhibit the activation of anti-bacterial systems of macrophages and block inflammatory responses completely in mouse cells and partially in human cells. This suppression may lead to disruption of synthesis of bactericidal effector molecules, allowing F. tularensis to survive and rapidly proliferate inside phagocytic cells. The ability to suppress the inflammatory responses is dependent on expression of the Igl/Mgl proteins. The Igl/Mgl system is also critical for the ability of F. tularensis to replicate in mammalian cells and in D. melanogaster.
INTRODUCTION

1. *Francisella tularensis*. Taxonomy and history.

*Francisella tularensis* was first described in 1911 in the publication ”A Plague-like Disease of Rodents” as a pathogen that morphologically resembled *Yersinia pestis* and caused infection and death in rodents in California. The bacterium was originally named *Bacterium tularense* but was renamed *Pasteurella tularensis* in 1957. When it was determined by DNA hybridization studies that it was not related to other *Pasteurella* species, it was given the name *Francisella* to honor Edward Francis, who dedicated his career to describe the clinical manifestation, diagnosis and histopathology of tularemia. It is the only recognized genus of the family *Francisellaceae* (Sjöstedt, 2005). The bacterium is not closely related to other human pathogens and the closest related family is *Piscirickettsiae*, which comprises intracellular bacteria pathogenic to fish. The cellular fatty acid composition of *Francisella* and the unusually high lipid content of the cell wall distinguishes the bacterium from other Gram-negative bacteria.

Before the disease was named tularemia, it was known as rabbit fever or trapper’s ailment indicating the long-recognized association of the disease with wild animals. *F. tularensis* is small, gram-negative, pleomorphic, non-motile, non-spore-forming coccoid rod, measuring 0.2 by 0.2 to 0.7 μm (Sjöstedt, 2005). *Francisella* is a strict aerobe, nonpiliated and has a thin capsule mainly composed of lipids.

There are four subspecies of *F. tularensis*, *novicida*, *tularensis*, *mediasiatica* and *holarctica*. Subspecies *tularensis* has almost only been isolated in North America and is highly virulent. In Europe, including Scandinavia, *F. tularensis* subspecies *holarctica* is the causative agent and in comparison with subspecies *tularensis* is less virulent, causing a non life-threatening disease (Ellis et al., 2002).

The other recognized *Francisella* species, *F. philomiragia*, is an infrequent pathogen but has been associated with human illness in compromised patients and in patients near drowning. The organism is extremely infectious.
1.1. The Live Vaccine Strain.

In the 1930’s, work on the development of a tularemia vaccine was started. In the United States, experiments based on the use of killed vaccines showed induction of high titers of antibodies, however, symptoms were mitigated but no protection against virulent strains was found (Tärnvik, 1989). Studies of strains attenuated by repeated passages “in vitro” in Soviet Union demonstrated that effective and safe live vaccines could be developed. The attenuated strain was actually composed of two variants of \textit{F. tularensis}. In the United States, the more virulent of the two variants was isolated and standardized for use in humans. It was named the Live Vaccine Strain (LVS) (Eigelsbach and Down, 1961). The exact cause of attenuation of the vaccine strain is still unknown and therefore, LVS is not approved by FDA for public vaccinations. Besides being used for vaccination of at-risk personal, LVS has become a very important laboratory strain for studying tularemia. Although being attenuated for humans, LVS is still highly virulent for small rodents and causes a disease similar to human tularemia (Conlan, 2004).


Tularemia is a zoonotic disease that occurs throughout most countries of the Northern Hemisphere and has a broad host distribution. While the natural reservoir of tularemia is still unknown, its association with small rodents and natural water is well established. In nature, \textit{Francisella} can survive for many weeks in soil, water or animal carcasses. It is known that it can survive and multiply in amoebae like \textit{Legionella} species. \textit{F. tularensis} is widely distributed in nature and has been isolated from many different animals, including mammalian and avian species, arthropods and domestic animals, as well as from water, soil and animal faeces. The most significant sources of human infection are wild rabbits like cottontail rabbits, other common sources of human infection are blood-sucking arthropod vectors, including ticks, mosquitoes and deerflies. There are reports of human disease transmitted by cats. Other possible routes of infection are ingestion of infected water or food and inhalation of the bacteria from contaminated soil.

Normally, the incubation period in humans lasts from 3 to 5 days, however, it may vary from 1 to 21 (Oyston et al., 2004). Various routes of infection give different clinical manifestations.
The two major types of tularemia are the ulceroglandular form, which occurs when the pathogen enters the body via the skin (insect bites or direct contact with infected animal for instance) and the respiratory form, which occurs after inhalation of, e.g., contaminated dust (Ellis et al., 2002). The latter form occurs rarely, but is characterized by a more severe disease. Less frequent is oropharyngeal tularemia, caused by intake of food or water contaminated with the pathogen. Two subspecies are of clinical importance, \textit{F. tularensis} subspecies \textit{tularensis} (clinical cases have only been reported in North America) and \textit{F. tularensis} subspecies \textit{holarctica}, which is spread over the whole Northern Hemisphere. Both are highly infectious, however only \textit{F. tularensis} subspecies \textit{tularensis} can cause a life-threatening disease with a mortality rate up to 30%, without proper antimicrobial therapy.

1.3. Virulence factors of \textit{F. tularensis}.

Little is known that explains why \textit{F. tularensis} is highly virulent (Sjöstedt, 2003). The lipopolysaccharide (LPS) of \textit{F. tularensis} has been the focus if several studies due to its unusual biological and structural properties (Dreisbach et al., 2000; Sandstrom et al., 1992; Vinogradov et al., 2002). It shows an unusual low toxicity in vitro and in vivo and it is 1,000-fold less potent that \textit{E. coli} LPS in inducing proinflammatory cytokines. The composition of the O-side chain is unique among gram-negative bacteria. The unusual functional features of \textit{F. tularensis} LPS have also been illustrated by the demonstration that the LPS and lipid A of \textit{F. tularensis} LVS failed to stimulate production of nitric oxide (NO) by rat macrophages whereas spontaneous \textit{F. tularensis} variants, expressing an antigenically distinct LPS, induced production of NO (Cowley et al., 1996). When the NO production was absent, intracellular growth occurred but it was suppressed after the antigenic shift that resulted in NO production.

Electron microscopy has revealed that fully virulent organisms of \textit{F. tularensis} are encapsulated but that this capsule is frequently lost upon hypertonic treatment or aerosolization. The capsule contains between 50 and 70\% (w/w) lipid but other components have not been identified (Sjöstedt, 2003). Stable, capsule-deficient mutants have been described. One of these was avirulent in mice and susceptible to complement-dependent lysis. When the mutant was ingested in polymorphonuclear leukocytes, it did not induce an antimicrobial response and survived in the cells. In contrast, the parent strain \textit{F. tularensis} LVS induced a respiratory burst and was killed inside the cells. Thus, the capsule appears to
be a necessary component for expression of the full virulence. It protects against serum-mediated lysis but its possession may not be advantageous intracellularly.

Mutations of the *iglB*, *iglC* and *iglD* genes located in the duplicated 33.9-kb region dramatically reduce the ability of *Francisella* to survive and multiply inside amoeba and phagocytic cells (Golovliov et al., 2003a; Lauriano et al., 2004; Nano et al., 2004). These genes are under control of the transcriptional regulator MglA (Lauriano et al., 2004). The exact function of the IglB, IglC and IglD proteins is unknown, furthermore they do not show any similarity to other characterized bacterial proteins, and the attempts to coprecipitate their possible eukaryotic or bacterial target-protein(s) have not yielded anything so far. Thus, novel virulence mechanism(s) is probably encoded inside this 33.9kb pathogenicity island.

One of the virulence factors is a 23-kDa protein, henceforth designated IglC (Intracellular Growth Locus), of *F. tularensis* LVS is upregulated during intracellular growth in murine macrophages or after exposure of extracellular bacteria to hydrogen peroxide (Golovliov et al., 1997). The deduced amino acid sequence of this 23-kDa protein showed no significant similarity to any previously characterized protein. We constructed a mutant lacking the two copies of the 23-kDa protein gene and the mutant was attenuated in vitro and in vivo (Golovliov et al., 2003b). Similarly, an IglC mutant of *F. tularensis* subspecies *novicida* also showed impaired ability to multiply intracellularly (Lauriano et al., 2004). Thus, the protein seems to play an important role for the ability of several *F. tularensis* subspecies to multiply intracellularly. A number of other attenuated protein mutants have been described in the subspecies *F. tularensis* subspecies *novicida* but the relevance of these findings for the clinically important subspecies *tularensis* and *holarctica* is not known.

Few studies have focused on the intracellular survival of *F. tularensis*. The pathogen seems to be well suited for survival and multiplication within macrophages. For example, we have demonstrated that very few proteins are induced during intracellular multiplication. A recent publication from our research group has added further information on how *F. tularensis* survives in monocytic cells. We demonstrated that the vaccine strain, *F. tularensis* LVS, is capable to escape the phagosome of macrophages via a mechanism that may involve degradation of the phagosomal membrane (Golovliov et al., 2003a). This was true for mouse peritoneal cells, the mouse macrophage-like cell line J774A.1, and the human macrophage-like cell line THP-1. Confocal microscopy of infected J774A.1 cells indicated that during the first hour of infection, bacteria colocalized with the late-endosomal/lysosomal glycoprotein LAMP-1, but within 3 h, this colocalization significantly decreased from approximately 60%
to 30%. Transmission electron microscopy indicated that >90% of bacteria were not enclosed by a phagosomal membrane after 2 h of infection and some were in vacuoles that were only partially surrounded by a limiting membrane (Golovliov et al., 2003a). This previously unknown virulence mechanism may explain why *F. tularensis* is so well adapted to the intracellular habitat and shows few signs of stress intracellularly. Escape from the phagolysosome has been described as a survival mechanism of a number of intracellular bacterial pathogens, such as *Shigella* spp., *Listeria monocytogenes* and *Bacillus anthracis*. In these cases, specific mechanisms have been identified that lead to the disruption of the phagosomal membrane, thus allowing bacterial entry into the cytoplasm. For example, escape of *Shigella* requires expression of a type III secretion system and of *L. monocytogenes* and *B. anthracis*, pore-forming hemolysins and phospholipases (Prior et al., 2001, Goebel, 2000 #233). Neither of those mechanisms exists in *F. tularensis*, though (Larsson et al., 2005). An example of a strategy resembling that of *F. tularensis* has been demonstrated for the fungus *Cryptococcus neoformans*, which after phagocytosis is able to disrupt the phagosomal membrane by release of polysaccharide-containing vesicles.
2. Mammalian host defense mechanisms.

2.1. Interaction between phagocytic cells and intracellular pathogens.

Phagocytosis is a receptor-mediated process that uses an actin-based mechanism to internalize particles such as bacteria, parasites, and yeasts into phagocytic cells. Professional phagocytes, including neutrophils and macrophages, are characterized by their ability to express a set of phagocytic receptors that recognize, bind to and trigger internalization of foreign objects, e.g., pathogens, debris and apoptotic cells. After internalization by phagocytic cells, microbes are located inside phagosomes. If the microbes do not possess intracellular survival mechanism, the phagosome containing the microbe fuses with the lysosome and the killing occurs within 15-30 min (Hamrick et al., 2000; Polsinelli et al., 1994; Prada-Delgado et al., 2001). Activated phagocytes produce nitric oxide and reactive oxygen species, which are converted into chloramines, hydroxyl radicals and hydrogen peroxide. These chemicals very effectively kill microbes inside phagosomes. The phagosomes are very rapidly acidified to a pH less than 5; such an environment can kill pathogens per se (Prada-Delgado et al., 2001). The lysosomes contain various hydrolytic enzymes and anti-microbial peptides.

Phagocytosis requires a variety of receptors and associated signaling pathways, the diversity of which is only beginning to be appreciated. To counteract these major clearance mechanisms, some bacterial pathogens have evolved strategies to avoid phagocytosis as an alternative to the intracellular survival mechanisms used by invasive pathogens. Other bacteria, the virulence of which involves direct interaction with host cells, exhibit contact-dependent antiphagocytic activities. Bacteria capable of interrupting phagocytic signals seem to harbor antiphagocytic properties aimed at paralyzing multiple phagocytic functions. The intracellular bacteria have developed different strategies to survive and replicate inside the phagocytic cells. They can block phagocytosis (Yersinia), escape from the phagosome by lyzing the vacuolar membrane (Listeria, Shigella), block the fusion of phagosomes with lysosomes (Legionella) or resist the antimicrobial activity of phagosomes (Salmonella).
2.2. Innate immune system in mammals.

Almost 100 years ago, Elias Metchnicov in his book “L’immunite dans les Maladies Infectieuses” described what he called natural – now termed innate immunity. Innate immunity is the first line of defense against infectious microorganisms. The innate immune system relies on pathogen-associated molecular patterns (PAMPs) for recognition of pathogens (Underhill et al., 1999). PAMPs are not always cell-associated. For instance, the mammalian lipopolysaccharide (LPS)-binding protein, synthesized in the liver, is a serum protein that binds LPS and the complex activates a conserved receptor at the surface of macrophages, causing quick detection of Gram-negative bacteria. Activation of pattern-recognition receptors leads to the expression of large numbers of antimicrobial molecules, which attack the pathogens by different mechanisms.

In mammals, the innate immune system can respond to different microbial-derived substances, including components of the cell wall such as LPS, peptidoglycans and lipoproteins (Guha et al., 2001). Bacterial DNA and double stranded RNA can also activate it, which is common for many viruses. The central role in recognition of the immunostimulatory agents and pathogens is played by Toll-like Receptors, see chapter 2.2.1 (TLRs) (Hatada et al., 2000; Zhang and Ghosh, 2000). In the response to triggering PAMPs, TLRs activate signaling pathways, which lead to expression of antimicrobial molecules, cytokines and co-stimulatory molecules (Karin and Lin, 2002). This activation of the innate immunosystem rapidly slows down infection and leads to activation other components of immune system, such as T-cells, the adaptive immunity (Schnare et al., 2001). Moreover, inflammation itself is believed to block dissemination of infection. The innate immune system is much older than the adaptive immunity and the elements of the former system can be found in almost any multicellular organism, including vertebrates, invertebrates and plants. The innate immune system of macrophages and polymorphonuclear leucocytes mediates inflammation and early response to the infectious agents that can cross one or the other barrier to cause an infection.
2.2.1. Toll-like receptors (TLRs).

A hallmark of innate immunity is recognition of microbial components that are essential for the survival of the microbe. By virtue of their essentiality, it has been possible for mammals to develop recognition receptors for so-called ‘Pathogen Associated Molecular Patterns’ (PAMPs). TLR ligands are generally excellent examples of this principle since they recognize a broad range of microbial products.

TLRs have been named due to their observed homology and functional similarity to *Drosophila*’s Toll receptors. At least 11 TLRs have been found in the mammalian genomes (Aravind et al., 2003). Each TLR contains a transmembrane receptor with an extracellular domain and an intracellular signaling domain that is homologous to the cytoplasmic tail of the IL-1 receptor. In all these receptors, the intracellular signaling domain activates intracellular signaling pathways. TLRs play very important role in regulating mammalian immune response. Recognizing a particular array of signaling molecules TLRs can direct innate immune system to combat extra- or intracellular pathogens. Specificity for broad categories of PAMPs is provided by a relatively limited diversity of TLRs. In some cases, combinations of TLRs are required for recognition of a PAMP. Depending on the microbial agent and its PAMP, recognition by TLRs may occur at the plasma membrane (e.g., TLR4) or in a phagosome (e.g., TLR2). The localization of the response also may depend on the specific cell-type. For example, intestinal epithelial cells can ingest LPS that associates with TLR4 in the Golgi complex. Thus, specific TLRs distinguish between diverse components of microbes and participate in the building of an inflammatory response appropriate for defense against a specific pathogen.

PAMPs of gram-positive organisms, such as peptidoglycan and lipopeptides, are recognized by TLR2. TLR2 in combination with TLR6 or TLR1, mediates responses to multiple microbial pathogens including mycobacteria, spirochetes, mycoplasma, and yeasts. For example, TLR2 and TLR4 have both been demonstrated to be important for diminishing the spread of tuberculosis from the lung and controlling the chronic phase infection. Although TLR4 mediates LPS signaling from enteric gram-negative bacteria, LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* activates macrophages through TLR2. Flagellin is principal component of bacterial flagella and a virulence factor and the monomeric form is
recognized by TLR5. Although many non-pathogenic commensal organisms express flagellin, only pathogenic organisms release monomeric flagellin capable of activating TLR5.

In addition to bacterial pathogens, TLRs are important for the recognition of PAMPs associated with viruses. Double-stranded RNA (dsRNA) is a molecule associated with viral infection, because it is produced by most viruses at some point during replication. Studies have shown that mammalian TLR3 recognizes dsRNA, and that activation of the receptor induces NF-κB and the production of type I IFNs. The innate immune response to fusion protein F of respiratory syncytial virus (RSV) is mediated by TLR4 and CD14. Data demonstrate that cytomegalovirus and the measles virus are recognized by TLR2. Thus, a variety of TLRs are capable of initiating an inflammatory response against viruses.

The signaling pathways triggered by TLR2 and TLR4 lead to activation of NF-κB and involve a number of different adaptor proteins and cytokines with the ultimate aim to degrade IkB thereby allowing free NF-κB to enter the nucleus and activate transcription from its target genes (Zhang and Ghosh, 2000). However, it has been demonstrated that TLR2 and TLR4 signaling results in activation of overlapping but not identical genes. For instance, TLR2 activation causes expression of IL-1β, but not IFN-γ, while activation of TLR4 leads to expression of both IL-1β and IFN-γ (Hirschfeld et al., 2001). Possible explanations are that different TLRs may activate different species of NF-κB, or that variations are caused by other pathways, activated in conjunction with NF-κB.

2.2.2. The Nuclear Factor kappa B (NF-κB) family.

A very important feature common to the innate immune response of mammals is the NF-κB/Rel family of transcription activators (Hatada et al., 2000; Kamata et al., 2002). Nuclear factor-kB is a ubiquitous heterodimeric transcription factor mainly composed of p50 and p65 subunits. In nonstimulated cells, NF-κB is present as hetero- or homodimeric proteins, sequestered by association with IkB family of inhibitory proteins.

NF-κB regulates expression of many molecules involved in host defense mechanisms, including cytokines, immunoreceptors, cell adhesion molecules and inducible nitric oxide synthase (Hatada et al., 2000). NF-κB is responsible for activating the transcription of the TNF-α gene and initiating its secretion, which in turn can trigger activation of NF-κB in
neighboring or distant cells. Another known target gene for NF-κB is IL-1β (Guha and Mackman, 2001; Zhang and Ghosh, 2000). It has been shown that NF-κB is also involved in inducing the respiratory burst.

NF-κB is involved in multiple events of host defense against infectious agents. Data from several studies have shown protective role of RelA subunits of NF-κB against pro-apoptotic stimuli (Collier-Hyams et al., 2002). At the same time there is evidence that c-Rel can induce apoptosis upon certain stimulation (Duckett, 2002). One explanation can be that pro- and anti-apoptotic genes selectively bind the complex in response to a specific signal.

An important property that characterizes the NF-κB protein is a 300 amino-acid sequence in its N-terminus that is homologous between NF-κB family members. This sequence is termed the “Rel homology domain” (RHD). Through this domain, NF-κB proteins bind to DNA and form homodimers with each other or heterodimers with other NF-κB proteins. This is also the region to which IkB proteins bind. Close to the C terminus of the RHD, a conserved nuclear localization sequence β (NLS) is found that is necessary for nuclear translocation of NF-κB proteins. Sequences outside of the RHD domain show very little homology between family members.

Dimerisation is needed for NF-κB binding to DNA. All members of NF-κB family, except RelB, have been shown to form homo- or heterodimers with other NF-κB proteins. The NF-κB dimer binds to a specific DNA sequence. Different NF-κB dimers show different affinity to variants of this sequence, meaning that the transcriptional response is determined by which NF-κB protein is expressed and which is activated under certain circumstances. Rel and p105/p50 are found in almost all cell types, the other members of NF-κB family show more restricted pattern but are expressed in a variety of different cell types.

2.2.3. The NF-κB activation pathway and IKK.

Many transcription factors are not expressed until they are needed for the cells. But in some situations the cells need to make an instant response to changing conditions and this can be only achieved if the transcription factor needed is already present inside the cell. NF-κB is one such factor. Inside the cytoplasm, NF-κB is held in a complex with its specific inhibitory
protein IκB. There are over 150 known signals that can result in NF-κB activation (Pahl, 1999). No matter what the signal is, the pathway leads to activation of the IκB kinase, or IKK, which is a key component of the NF-κB signaling pathway. The most important step in the NF-κB-activating pathway is phosphorylation of IκB. The phosphorylation occurs in two conserved serine residues in the N-terminus of IκB. Three IKK subunits have been cloned and studied; they are IKKα or IKK1, IKKβ or IKK2 and IKKγ or NEMO.

IKKα and IKKβ are kinase components of IKK. They are highly homologous to each other, showing about 50% identity. They contain a leucine zipper followed by a helix-loop-helix domain. The leucine zipper is required for dimerisation and assembly of IKKα and IKKβ both of which are needed for their activity. It is most likely that different stimuli employ different sets of proteins to signal to IKK, which is not surprising considering the number of agents that can induce NF-κB. Only some of the pathways that lead to NF-κB activation have been studied in detail (Elewaut et al., 1999).

Once IκB has been phosphorylated, it is degraded by a number of proteins. The first set of proteins links a small protein, ubiquitin, to IκB. The ubiquitin system is constantly active in cells, however, it does not affect native IκB but only the phosphorylated form (Gao and Kwaik, 2000; Neish et al., 2000). IκB tagged with a chain of ubiquitin molecules becomes rapidly degraded via a special protein complex called the 26S proteasome. The 26S proteasome is a huge complex with a molecular weight of about 2 MDa. Experiments with proteasome inhibitors resulted in inhibition of activation NF-κB, despite its phosphorylation and ubiquitination, IκB remains in the complex with NF-κB, proving that all necessary components must be in place and functional.

2.2.4. Nuclear translocation of NF-κB.

As soon as the NF-κB-IκB complex falls apart, the NLS domain of NF-κB is uncovered and recognized by a number of cytoplasmic proteins that transfer NF-κB into the nucleus. The two proteins that transfer NF-κB into the nucleus are importin α and importin β. The importin α only transfers NF-κB to importin β, which in turn carries the whole complex to the nuclear pore. During passage through the nuclear pore, the complex NF-κB-importin α-importin β
disintegrates and free NF-κB gets ready to execute its transcription activating functions (Neish et al., 2000).

Once inside the nucleus NF-κB starts to activate transcription from its target genes. One of these genes is the gene for the NF-κB inhibitor, IκB, which suggests that there is an autoregulatory mechanism, preventing overactivation of NF-κB. In fact, newly synthesized IκB can be seen in cytoplasm within 30-45 min after stimulation. Then, another feature of IκB can be seen, as it is involved in bringing nuclear NF-κB back to the cytoplasm, a series of studies has shown that even in non stimulated cells IκB constantly goes in and out of the nucleus, bringing nuclear NF-κB back to cytoplasm (Huang et al., 2000; Tam et al., 2000).

![Fig. 1. An overview of the NF-κB pathway.](image)

**2.2.5. Effects of ROS and RNS on NF-κB activation.**

The reactive oxygen (ROS) and reactive nitrogen species (RNS) are well known mediators of NF-κB activation. It has been established in different models that activated macrophages produce increased amounts of both reactive oxygen and nitrogen. ROS are produced during a process, known as respiratory burst, which involves increased oxygen consumption and the
reduction of molecular oxygen to superoxide (O$_2^-$) (Dahlgren and Karlsson, 1999). Activated macrophages also produce high levels of RNS by upregulation of inducible nitric oxide synthase (iNOS). Both ROS and RNS are capable to mediate microbial killing by macrophages. RNS and ROS not only can directly kill microbes by natural toxicity and the damage inflicted by oxidation and nitration, but also can stimulate NF-κB induction by activation of IKK through the release of iron (Xiong et al., 2003). Peroxynitrite was shown to activate NF-κB in monocytes through tyrosine nitration of IκB. Furthermore nitrated IκB becomes a target for degradation, thus the nitration-based activation of NF-κB does not require activation of IKK at all.

The cellular redox state regulates the NF-κB pathway. H$_2$O$_2$ has been used as a mediator of oxidative radical stress, and it has been demonstrated that it per se has a weak intrinsic ability to activate IKK (Kamata et al., 2002). Moreover, it shows a synergistic activating effect in combination with TNF-α or PMA. It also enhances TNF-induced NF-κB activation. These effects are mediated through phosphorylation of serine residues in the activation loops of IKK. Thus, it is possible that inhibition of the NF-κB pathway upstream of IKK may be counteracted by the addition of donors of ROS or RNS since they have direct activating effects on IKK. Oxidation confers direct activation of the NF-κB pathway.

![Diagram](image)

Fig. 2. Tentative model for regulation of TNF-α secretion in monocytic cells.
2.2.6. The NF-κB pathway as a target for pathogen diversion.

Recently reported data seems to indicate that each of several intracellular parasites may have developed its own strategy for inhibition of the NF-κB pathway. The protozoan parasite *Toxoplasma gondii* prevented nuclear translocation of NF-κB in murine macrophages, resulting in inhibition of the cytokine response (Butcher et al., 2001). *Leishmania donovani* avoided to induce phosphorylation of several MAPK pathways as well as the degradation of IκB (Prive and Descoteaux, 2000). In epithelial cells infected with non-virulent *Salmonella* isolates, the NF-κB pathway was inhibited although the IκB phosphorylation step was spared, since the subsequent degradation of IκB was inhibited by preventing its ubiquitination (Neish et al., 2000). *Y. pseudotuberculosis* and *Y. entercoliticca*, although considered extracellular bacteria, employ a similar strategy. The YopJ protein, a component of a type III secretion system, blocked phosphorylation and subsequent activation of the NF-κB regulating kinases as well as MAPK kinases by affecting ubiquitination (Orth et al., 2000; Ruckdeschel et al., 1998). Infection with *E. coli* resulted in suppression of nuclear translocation of NF-κB which was dependent on secretion of protein B (EspB). Also, enterohemorrhagic and enteropathogenic *E. coli* all interfere with NF-κB activation initiated by TNF-α. As a consequence of the NF-κB, significantly lower mRNA levels of IL-8, IL-6, and IL-1 were observed compared with bacteria lacking EspB.

2.2.7. Mitogen-activated protein kinases (MAPK)

The MAP kinases are an important group of serine/threonine signaling kinases that phosphorylate and thus activate transcription factors, linking transmembrane signaling with gene induction events inside the nucleus. In mammalian cells, three major groups of MAP kinases have been identified. They include the extracellular signal-regulated kinases (ERKs), the *c-jun* amino-terminal kinases (JNKs) and p38 MAP kinases. The MAP kinases are all activated by phosphorylation of a common threonine-X-tyrosine regulatory motif by their specific upstream MAP kinase kinases. Although distinct in their activation, there is considerable co-operation between these kinases and many substrates are shared between pathways (Cobb, 1999). This family of kinases is important in a wide spectrum of cell
functions including proliferation (Mansour et al., 1994), apoptosis (Wang et al., 2000), cytokine biosynthesis (Kovalovsky et al., 2000), and cytoskeletal reorganization (Guay et al., 1997).

In general, the ERK 1/2 pathway is activated by growth factors, mitogenic stimuli and tumor promoters (Cobb, 1999), whereas environmental stress and inflammatory cytokines stimulate the p38 and SAP/JNK pathways (Bellmann et al., 2000; Kovalovsky et al., 2000). The activated MAPK are responsible for phosphorylating and activating numerous transcription factors which function to stimulate the synthesis of various inflammatory proteins including the cytokines TNF-α, IL-1β, and IL-6. The MAPK are also involved in the transcriptional regulation of iNOS and cyclooxygenase-2 (COX2) (Chen et al., 1999). Information is rather limited regarding MAPK signaling in macrophages, particularly in primary macrophages. The most studied MAPK signaling pathway in macrophages is initiated through LPS which signals by means of CD14 and the TLR4 (Guha and Mackman, 2001; Rao, 2001). The LPS mediated activation of p38, ERK 1/2 and JNK is initiated by a surprising number of pathways. In all cases, however, p38, ERK 1/2 and JNK are activated by phosphorylation mediated through MAPK kinases, which in turn are activated by MAPK kinase kinases.

![Fig. 3. An overview of the MAPK p38 pathway.](image-url)
2.2.8. MAPK as targets for pathogen diversion.

Interestingly, a number of studies have implicated the MAP kinases as important cellular targets for infectious organisms. *Yersinia enterocolitica* has been shown to suppress TNF-α production by inhibiting ERK 1/2, p38 and JNK kinase activities, with this suppression being dependent upon expression of the bacterial protein YopJ (Palmer et al., 1999). It has been suggested that the broad effects depend on the cleavage of highly conserved ubiquitin-like molecules, disrupting posttranslational changes of numerous regulatory proteins. Infection of the RAW 264.7 macrophage cell line with *Leishmania donovani* resulted in a limited response by these macrophages to PMA and IFN-γ. These results correlated with a decreased activation of ERK 1/2 and increased phosphatase activity within infected cells (Nandan et al., 1999). Infection of macrophages with pathogenic *Candida albicans* results in activation of MKP-1 (MAPK phosphatase-1) and reduced levels of MEK 1/2 and ERK 1/2 (Ibata-Ombetta et al., 2001). Infection of mouse macrophages with *T. gondii* results in initial activation of MAPK and JNK pathways followed by rapid inhibition (Butcher et al., 2001). ERK 1/2 and p38 MAPK were activated at the early stage of *Ehrlichia chaffeensis* infection of human monocytes; however, the activations of ERK 1/2 and p38 MAPK by LPS treatment were subsequently reduced in *E. chaffeensis*-infected monocytes compared with those in uninfected monocytes (Lin and Rikihisa, 2004).

MAPK appear to be targets of pathogenic importance during mycobacterial infections. For example, it was demonstrated that treatment of the human monocytic cell line THP-1 with Man-LAM results in diminished reactivity to PMA, LPS and IFN-γ stimulation. This reduction in the macrophage response was associated with decreased ERK activation in treated cells, suggesting that Man-LAM may limit macrophage activation by reducing ERK 1/2 phosphorylation (Knutson et al., 1998). Thus, it appears as if mycobacteria in general will activate MAPK but more virulent mycobacteria appear able to limit the level of MAPK activation. This diminished MAPK activation in macrophages infected with pathogenic mycobacteria may account, at least in part, for the decreased production of inflammatory mediators in macrophages following infection with pathogenic compared to non-pathogenic mycobacteria.

A recent study on the mechanisms of apoptosis induced by *F. tularensis* identified that concomitant with the development of apoptosis, phosphorylation of p42/p44 MAPK (Erk1/2)
occurred and it was demonstrated that levels of apoptosis were markedly diminished if cells were treated with an inhibitor of MEK1/2 and that this inhibitor also abrogated phosphorylation of p42/p44 MAPK (Hrstka et al., 2005). They also noted that a significant inhibition of p38 MAPK activity occurred in infected cells and, on the basis of results with an inhibitor of p38 MAPK, SB203580, proposed that the latter inhibition is an important component in the pro-apoptotic activity of *F. tularensis*.

Together these data strongly support the ability of pathogens to modulate MAPK activation in macrophages or other host cells and thus provide one mechanism by which these organisms can limit a host immune response.

### 2.2.9. IFN-γ-mediated effects on cellular signaling and control of intracellular infections.

IFN-γ profoundly affects intracellular signaling in monocytic cells. After binding to its receptor, cytoplasmic Janus kinases (Jak1 and Jak2) become activated and phosphorylate specific tyrosine residues on the signal transducers and activators of transcription (STAT) molecules. Activated and phosphorylated STAT factors form homodimers, translocates to the nucleus, and bind specific STAT-binding DNA sequences. IFN-γ modulates the expression and activation of more than 200 proteins (Der et al., 1998). However, only a few of all IFN-γ-induced proteins have been shown to exert a direct role in pathogen elimination (Bach et al., 1997) but still, IFN-γ is the most important factor to endow macrophages with a cidal activity. It enhances the endosomal trafficking by upregulation of small GTPases such as Rab5a (Alvarez-Dominguez and Stahl, 1998). This has been shown to be critical for conferring anti-*Listeria* activity since Rab5a recruits active Rac2 to *L. monocytogenes*-containing vacuoles. This results assembly of the phox (Prada-Delgado et al., 2001) and a subsequent ROS-dependent killing of the bacterium. Similarly, the phox is assembled and becomes active in IFN-γ-activated macrophages infected with *S. typhimurium* which restricts the pathogen multiplication, especially during the early phase of infection (Vazquez-Torres et al., 2000). Another IFN-γ-mediated effect is upregulation of IGTP, a GTP-binding protein, which is involved in clearance of *Toxoplasma* (Taylor et al., 2000) and Nramp1, a macrophage-restricted lysosomal protein mediating transport of ions over the lysosomal membrane that is necessary for clearance of *Leishmania, Salmonella*, and *Mycobacterium* spp (Prada-Delgado et al., 2001; Searle et al., 1998) but not *F. tularensis*. In addition, IFN-γ induces the
production of reactive nitrogen species (RNS) with microbicidal activity (Anthony et al., 1992; Fortier et al., 1992; Polsinelli et al., 1994). IFN-γ has been shown to play a crucial role during both the innate and acquired immune response to F. tularensis infection. Mice genetically deficient in IFN-γ and mice treated with monoclonal antibodies neutralizing IFN-γ succumb to a few LVS organisms; the LD₅₀ being a million-fold lower than in immunocompetent mice. Ex vivo, the capability of F. tularensis to proliferate in mononuclear phagocytes is dramatically affected when the phagocytes are exposed to IFN-γ (Fortier et al., 1992; Lindgren et al., 2004; Lindgren et al., 2005).

Besides conferring an important anti-microbial effect, pretreatment with IFN-γ has been shown to render monocytes and macrophages more responsive and sensitive to LPS (Boehm et al., 1997). Primary human monocytes increased TNF-α transcription and formed more stable TNF-α mRNA when they were primed with IFN-γ for several hours prior to LPS stimulation (Hayes et al., 1995). The mechanism underlying the priming effect appears to be complex. In part, IFN-γ upregulates the receptor for LPS, CD14. It also appears to alter proteins, which target mRNA turnover (Suk and Erickson, 1996). In addition to this well-known priming effect, there have been some studies suggesting that IFN-γ may have a direct effect on transcription and induce TNF-α gene and protein expression (Beutler et al., 1986).

3. Innate immune signaling in insects.

Drosophila has independent immune signaling pathways, which lead to activation of the NF-κB transcription factor. The Toll pathway primarily responds to fungal and gram-positive bacterial infection. Induction of Toll/antifungal signaling pathway leads to activation of NF-κB homologs called Dorsal and Dif, resulting in a production of antimicrobial peptides such as drosomycin. Dorsal is also required for early embryogenesis. By contrast, LPS induces immune deficiency (imd) signaling pathway, leading to activation the third Drosophila NF-κB homolog, Relish, which activates expression of antibacterial peptides such as Diptericin (Hedengren et al., 1999; Rutschmann et al., 2000). The two pathways are homologous to the mammalian Toll-like receptor (TLR) and tumor necrosis factor receptor (TNFR) signaling pathways accordingly. Relish protein is synthesized as a precursor protein, which is cleaved
and activated upon stimulation in a same manner as p105, p100 NF-κB precursor proteins in mammals (Dushay et al., 1996). At the same time some antimicrobial peptides, such as cecropin, can be activated by either pathway and may have both antibacterial and antifungal activities (Ekengren and Hultmark, 1999; Lemaitre et al., 1996).

In Drosophila, infection also leads to activation of JNK and JAK/STAT pathways (Lagueux et al., 2000; Sluss et al., 1996). The function of the JNK pathway in immunity has not been fully established, while JAK/STAT pathway is necessary for the induction of some complement-like proteins, which have been shown to participate in bacterial opsonization, promoting phagocytosis (Lagueux et al., 2000; Levashina et al., 2001).

### 3.1. Drosophila Toll pathway.

Toll is transmembrane protein initially identified as an important component in embryogeneses, followed studies have shown its involvement in innate immunity (Gobert et al., 2003; Wu and Anderson, 1997). Stimulation of the Toll pathway by Gram-positive bacteria is mediated through extracellular peptidoglycan recognition protein (PRGP) (Michel et al., 2001). Stimulation of Toll pathway by fungi, instead, depends on a serine protease, Persefone, and a protease inhibitor (Ligoxygakis et al., 2002). Two transcription factors activated by Toll pathway are Dif and Dorsal, they induce expression of several antimicrobial peptides such as drosomycin, defensin and metchnicov. Dif and Dorsal are homologous to mammalian NF-κB protein. Another downstream member of Toll signaling pathway – Cactus protein is a homologous to mammalian I-κB protein.

### 3.2. imd pathway.

Imd is an adapter protein homologous to TNFR-interacting protein receptor interacting protein (RIP) (Georgel et al., 2001). The imd pathway activates expression of many antimicrobial peptide genes in response to Gram-negative bacterial infection (Hultmark, 2003). Although in mammals LPS binds to the TLR4 receptor complex, LPS stimulation of the imd pathway in Drosophila does not require Toll.
Like p105 and p100, Relish is a NF-κB precursor protein with NF-κB-like N-terminal domain and a C-terminal I-κB-like ankyrin repeat domain (Dushay et al., 1996). In non-stimulated cells, Relish C-terminal I-κB-like domain prevents nuclear translocation of the N-terminal NF-κB domain. Upon stimulation of the antibacterial signaling pathway, Relish is proteolytically cleaved and the NF-κB N-terminus is translocated into the nucleus, while the stable C-terminus stays in the cytoplasm. This makes Relish unique among NF-κB precursor proteins (Silverman and Maniatis, 2001). Relish protein activates expression of the antimicrobial agents diptericin, cecropin, and attacin as well as defensin and mechnicov.

Another branch of the imd pathway leads to activation of JNK signaling. JNK is not required for the activation of antimicrobial peptides genes, but is involved in regulation of immediate response to injury. Data show that JNK might be involved in wound healing and melanization as part of insect innate immunity (Park et al., 2004; Silverman et al., 2000).

Fig. 4. Schematic model of the signaling via Toll and imd pathways in D. melanogaster.
4. Laboratory models for studying the virulence of *F. tularensis*.

4.1. Animal models.

Experiments on live animals such as laboratory mice provide important clues for understanding host-pathogen interactions. Experimental tularemia in mice resembles very closely infection in humans and is considered to be an appropriate model. Moreover, rodents are naturally infected with *F. tularensis*. Additionally, animals can be used for obtaining macrophages for "ex vivo" experiments. However there are drawbacks, since animal experiments require special permissions, facilities and are relatively costly. An alternative way to study interaction between intracellular pathogens and phagocytic cells is to use cell lines. The cell lines available represent a broad range of mammalian species, are easy to cultivate and the results can provide significant amount of information for the researcher. Cell lines can be used for studies of effects of intracellular pathogens on intracellular pathways, as well as for evaluating abilities of newly produced mutant strains to survive and replicate inside phagocytic cells.

4.2. *Drosophila* as a potential model for studying *F. tularensis*.

Although *Drosophila* is an insect and under normal condition does not suffer from tularemia it might become a niche among different laboratory models for studying *F. tularensis*. The advantages of using *Drosophila* for studying human pathogens are that the flies are easy to cultivate in large numbers and the intracellular signaling pathways are well described. In fact, the fruit fly has been used for studying of virulence mechanisms of the human pathogens such as *Pseudomonas, Listeria, and Mycobacterium* (Dionne et al., 2003; Mansfield et al., 2003; Schneider and Shahabuddin, 2000). Infection experiments using *Drosophila* can provide additional and supportive information to those performed on mammalian cell lines. The flies can also be used as a tool to determine virulence of newly generated bacterial mutants. Since flies are relatively transparent GFP-labeled bacteria can be used to establish its tropism inside the body.
Another very intriguing approach is to use *Drosophila* as a model for insect-vectors. Fruit-flies are not blood-feeding insects, however, it is interesting to find out whether *F. tularensis* can pass from infected larvae through all stages of its development to adult fly and their immune defense mechanisms may resemble those of the arthropods that naturally carry *F. tularensis*. There is a hypothesis that mosquitoes become infected as early as at larval stage, by feeding on amoeba infected with *Francisella*. Feeding experiments on *Drosophila* larvae can be a way to test this hypothesis.
AIMS OF THE STUDY

- To characterize the inflammatory response in mouse and human monocytic cells to *F. tularensis*.
- To characterize the early phase of the *F. tularensis* infection in monocytic cells.
- To characterize the role of the Mgl/Igl system for the interaction between *F. tularensis* and monocytic cells.
- To establish a model of tularemia in *D. melanogaster* as a way to study the interaction between *F. tularensis* and arthropod vectors and to study the effects of the infection on intracellular signaling pathways.
RESULTS

1. Effects of *F. tularensis* infection on intracellular pathways in monocytic cells during internalization.

*Francisella tularensis* LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocytic and human peripheral blood mononuclear cells (paper I).

Generally, a bacterial infection of monocytic cells results in the activation of a pro-inflammatory response mediated via activation of TLRs by conserved bacterial components such as lipopolysaccharide (LPS) or bacterial lipoproteins (BLP). The regulation of the proinflammatory response depends on intracellular signaling pathways. Central roles in these intracellular pathways have been assigned to the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Disruption of these pathways causes impaired secretion of antimicrobial effectors such as TNF-α, IL-1, and IL-8.

It is known that *F. tularensis* expresses an atypical and unique LPS molecule with very limited endotoxic ability and, thus, it is possible that the bacterium stimulates only a weak or no proinflammatory response. To this end, we investigated the monocytic response to the *F. tularensis* infection by using an in vitro model where bacteria were added to cell cultures and performed an analysis of the cellular response with regard to intracellular signaling and secretion of cytokines. We also assessed whether there were any differences between human and mouse cells.

Four different types of the cells were used in this study; the J774A.1 and THP-1 cells were received from American Type Culture Collection, VA, peritoneal exudate cells (PEC) were obtained from Balb/cJ mice 3 days after intraperitoneal injection of thioglycolate and the peripheral blood mononuclear cells were prepared from human donor’s blood.

One day prior to the start of the experiment, the adherent cells were dispersed on the surface of cell-culture dishes at appropriate densities and incubated overnight. Next day, the cells were infected with *F. tularensis* LVS, ΔiglC, or heat-killed bacteria (HK) at an MOI of 500. The ΔiglC strain lacks expression of a 23-kDa protein that has been shown to be up-regulated
during intracellular growth of *Francisella* and, thus, may be important for intracellular survival and virulence of the pathogen. At 30, 60, 120 and 300 min after infection, supernatant were collected for future cytokines assays and the cell-lysates were prepared for immunoblot assays.

### 1.1 Effects of *Francisella* infection on intracellular signaling in mouse and human cells.

To study the effects of *Francisella* infection on intracellular signaling pathways, immunoblot assays were conducted and the blots were probed with antibodies against the following proteins: phospho-\(\text{I}\beta\alpha\), \(\text{I}\beta\alpha\), phospho-cJun, phospho-p38, and p38.

Addition of either live *F. tularensis* LVS, \(\Delta\text{iglC}\), or killed bacteria to all cell types resulted in increased phosphorylation of \(\text{I}\beta\alpha\), cJun and p38 proteins within 30 to 60 min, except for p38 in PBMC. However, this initial activation of \(\text{I}\beta\alpha\), cJun and p38 was soon followed by subsequent down-regulation when the cells were infected with live *F. tularensis* LVS, but not with \(\Delta\text{iglC}\) or HK bacteria. The only difference was observed in THP-1 cells that showed continuous phosphorylation of \(\text{I}\beta\alpha\) and to some extent cJun even 300 min after infection. In contrast, infection of all cell-types with either \(\Delta\text{iglC}\) or HK caused persistent phosphorylation of \(\text{I}\beta\alpha\), cJun, and p38 at all time points, demonstrating a similar pattern to that resulting after addition of *E. coli* LPS. At the same time, irrespective of cell type, *F. tularensis* LPS did not cause any changes in phosphorylation of any of the three studied proteins.

To determine whether the lack of phosphorylation of those proteins was due to potential cell death we checked the number of intracellular bacteria and the viability of eukaryotic cells. Previously it has been demonstrated that there is no dramatic increase in intracellular bacteria number during first 6 hour of infection. However in this experimental model we found that, regardless of cell type, numbers of intracellular bacteria increased up to 2.0 log\(_{10}\) within 6 h. A possible explanation is that the bacterial numbers increased due to continuous uptake rather than proliferation of intracellular bacteria. The viability of the eukaryotic cells was determined by measuring levels of lactate dehydrogenase (LDH) in supernatants. No significant increase of LDH was observed in any cell type during 6 h period, thus we consider the effects on the intracellular signaling to be an effect related to the *F. tularensis* infection and not to indirect cytopathogenic effects.
1.2 Effects of *Francisella* infection on cytokine secretion.

To determine the amounts of secreted TNF-α in the supernatants of the cell-cultures, media were collected and an ELISA assay was used for analyses. Addition of *E. coli* LPS resulted in induction of high levels of TNF-α in all cell types. In contrast, *F. tularensis* LPS did not induce detectable levels of TNF-α. Infection of all cell types with live *F. tularensis* LVS or Δ*iglC* initially stimulated secretion of high levels of TNF-α, but while the TNF-α concentrations continuously increased in cells infected with the mutant strain, there was a significant decrease in TNF-α concentration in all cell types except in THP-1 infected with *F. tularensis* LVS. Killed *Francisella* also induced high levels of TNF-α in the same manner as *E. coli* LPS or Δ*iglC* in all cell types except in J774 cells.

These findings show the importance of the IglC protein for *Francisella* virulence. An interesting discovery is that there is a bimodal effect on the intracellular signaling of the *F. tularensis* LVS infection; an initial activation followed by a down-regulation. The differences between the response of the mouse and human cells to the *Francisella* infection observed in this study may be one explanation why LVS is attenuated for humans but still highly virulent for small rodents.

2. *F. tularensis* escapes from the phagolysosome.

Factors affecting the escape of *F. tularensis* LVS from the phagosome (paper II).

*F. tularensis* can survive and proliferate inside phagocytic cells, however, when macrophages are pre-activated with IFN-γ they can effectively kill the bacterium (Anthony et al., 1992; Fortier et al., 1992; Lindgren et al., 2005). Intracellular survival and growth of *F. tularensis* LVS in resident macrophages is characterized by escape from the phagosome and proliferation inside the cytoplasm of infected cell but the Δ*iglC* mutant did not proliferate (Golovliov et al., 2003b). The molecular mechanism by which *Francisella* escapes is so far unknown. In this study we tried to answer the question if the IglC protein is important for the escape of *Francisella* and also to elucidate the mechanisms behind the IFN-γ-induced killing of bacteria.
The results show that around 97% of *F. tularensis* LVS bacteria were localized free in cytoplasm of the infected cells, while over 99% of Δ*iglC* bacteria were inside phagosomes with intact membranes. In accordance with previous studies, *F. tularensis* LVS proliferated inside the resident cells and bacterial numbers increased 1.6 log₁₀ within 18 h of infection. In contrast, the Δ*iglC* strain was effectively killed during the same period of time and bacterial numbers decreased dramatically. These observations indicate the importance of the IglC protein for the ability of *Francisella* to escape from phagosomes and therefore to stay alive inside phagocytic cells.

Activation of the macrophages with IFN-γ resulted in a significant increase in the number of intra-phagosomal *F. tularensis* LVS bacteria, but did not have a significant effect on the localization of the Δ*iglC* bacteria. Furthermore, IFN-γ activation led to rapid killing of Δ*iglC* and eradication was achieved within 18 h. Although the killing was slower when compared with the mutant strain, also *F. tularensis* LVS bacteria were eventually eradicated in IFN-γ stimulated cells.

We also looked at the lipid composition of Δ*iglC* compared to *F. tularensis* LVS in an attempt to answer the question whether it could explain the difference in intracellular viability of these two strains and contribute to the escape from the phagosome. However, no significant changes in lipid composition were found.

The crucial role of IFN-γ for controlling and eliminating an intracellular bacterial infection has been thoroughly studied. It has been suggested that the escape from the phagosome will shield parasites from bactericidal mechanisms. However, in light of our findings, it is possible that bacteria are subject to effects of reactive nitrogen and oxygen species in IFN-γ-activated macrophages that result in irreversible damage and prevent subsequent multiplication but not escape from phagosomes. Alternatively, the *F. tularensis* bacteria may even in the cytoplasm be affected by cidal mechanisms.

Although the importance of the IglC protein for the ability of *Francisella* to escape from the phagosome is significant, it should be remarked that the Δ*iglC* mutant also lacks the ability displayed by *F. tularensis* LVS to inhibit TLR-signaling, thus, its role during the intracellular infection appears to be complex and very important for *F. tularensis*. 

*Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signaling and secretion of TNF-α and IL-1 from murine macrophages (paper III).

In this study we looked at effects of intracellularly located *F. tularensis* bacteria on NF-κB and MAPK signaling following addition of LPS, a TLR4 agonist, or BLP, a TLR2 agonist. Cells of the J774A.1 mouse macrophage-like cell line were used in the experiments. The two bacterial strains used were *F. tularensis* LVS and ΔiglC.

3.1 Intracellular survival and multiplication of *Francisella*.

*F. tularensis* LVS can survive and multiply inside murine phagocytes. J774 cells were infected with *Francisella* at an MOI 500 for 2 h, after the uptake, extracellular bacteria were removed by washing and medium containing gentamicin to prevent extracellular growth of *Francisella* was added. The uptake of the LVS strain and the mutant was very similar. In resident cells the number of intracellular *F. tularensis* LVS bacteria increased about 2.0 log_{10} in 24 h whereas ΔiglC increased only 1.0 log_{10}. Stimulation of the cells with either LPS or BLP did not have any impact on the intracellular survival and multiplication of the LVS strain but it prevented multiplication of ΔiglC.

3.2 *F. tularensis* inhibits secretion of proinflammatory cytokines stimulated by *E. coli* LPS or BLP.

Stimulation of J774 cells with LPS or BLP resulted in very strong secretion of TNF-α and within 1 h, the levels increased from <15 to >600 pg/ml with LPS and to >200 pg/ml with BLP. Only in the cells stimulated with LPS, detectable levels of IL-1β were observed after 24 h of stimulation. When cells were infected with *F. tularensis* LVS, no production of either TNF-α or IL-1β was observed. Moreover, the bacterium inhibited production of TNF-α and
IL-1β in response to LPS or BLP stimulation. By use of chloramphenicol treatment, we observed that the \textit{F. tularensis} LVS-induced inhibition required live bacteria but did not require de novo bacterial protein synthesis. In contrast, infection with \( \Delta \text{iglC} \) did not result in such an inhibition on the cells. These results were further supported by Northern blot analysis of TNF-α and IL-1β mRNA transcripts. While there was a significant increase in TNF-α and IL-1β mRNA levels in LPS-induced cells, they were completely absent in \textit{F. tularensis} LVS-infected cells.

We also have investigated whether \textit{F. tularensis} LPS may interfere with \textit{E. coli} LPS-induced signaling and thereby prevent secretion of cytokines. However, we found no evidence that the \textit{F. tularensis}-derived LPS stimulated secretion of TNF-α per se or inhibited secretion induced by \textit{E. coli} LPS.

### 3.3 \textit{Francisella} inhibits activation of NF-κB, p38, and c-Jun signaling pathways and affects regulation of TLR2.

The operons encoding TNF-α and IL-1 have motifs in their promoter regions recognized by NF-κB. Therefore, it was of interest to investigate if the intracellular bacteria affected NF-κB signaling. To study the effect, we examined regulation of the specific NF-κB inhibitor, IκB, and looked at DNA binding activity of nuclear NF-κB.

We have demonstrated previously that infection of J774 cells with \textit{F. tularensis} LVS but not \( \Delta \text{iglC} \) resulted in inhibition of LPS- or BLP-induced degradation of IκB. Furthermore, as a result, there was no nuclear NF-κB bound to DNA in the LVS infected cells. At the same time, as in previous experiments, we found that \( \Delta \text{iglC} \) did not prevent either the degradation of IκB or nuclear translocation of NF-κB.

To answer the question if other transcription factors involved in regulation of TNF-α and IL-1 are affected by the \textit{F. tularensis} infection, we have analyzed the mitogen-activated protein kinase (MAPK) pathway, known to be activated by LPS. We analyzed phosphorylation of transcription factor cJun and p38 proteins. Once again, we found that LPS- or BLP-induced phosphorylation of both cJun and p38 proteins was completely inhibited in \textit{F. tularensis} LVS but not \( \Delta \text{iglC} \) infected cells.
We also investigated the duration of the inhibition and asked whether the suppression of was long-lasting. LPS was added at 5 h after infection with *F. tularensis* LVS and activation analyzed 60 min later. In this case, EMSA revealed that in infected, LPS-stimulated cells, NF-κB activation occurred at the same level as observed in non-infected, LPS-stimulated cells whereas there was no phosphorylation of c-Jun and no secretion of TNF-α in the infected cells. In contrast, TNF-α levels were high, > 1,500 pg/ml, in cultures with non-infected cells.

Together, these results show that intracellular *Francisella* bacteria inhibit multiple intracellular signaling pathways, including those involving NF-κB, cJun and p38. Furthermore, *Francisella* is capable to inhibit activation of intracellular signaling via both TLR2 and TLR4. The inhibitory effect on the NF-κB pathway lasted < 5 h whereas the effect on c-Jun and TNF-α secretion was observed at least 5 h after the infection. The inhibition of the pathways requires the expression of the IglC protein.

4. The *IglB, IglC, IglD, and MglA* proteins are important virulence determinants of *F. tularensis*.

Mechanisms of inhibition of TLR-mediated signaling conferred by *Francisella tularensis* (paper IV).

Our previous studies demonstrated the importance of the 23-kDa protein IglC for virulence of *F. tularensis*. In this study we expanded the scope and examined the roles of other members of the *igl* (intracellular growth locus) operon as well as the regulator of the operon, MglA (macrophage growth locus).

4.1 *iglB, iglC, iglD, and mglA* mutant strains are unable to replicate and survive inside phagocytic cells.

The expression of the proteins encoded by *iglB, C, D* and *mglA* genes is upregulated during intracellular growth of *Francisella* (Golovliov et al., 1997). We have studied the abilities of the mutant strains to survive and multiply inside macrophages. The J774 cells were infected
with either *F. tularensis* LVS or one of the mutants and viable counts were determined after 0, 24 and 48 hours of infection. The results clearly demonstrate, that there is a significant difference in viability of the strains, and that all mutants show impaired proliferation inside the eukaryotic cells compared to parental LVS strain. The slight decrease of LVS bacteria after 48 hours is explained by cells lysis and killing of *Francisella* by the antibiotic in the cell medium. The differences in survival between the strains are even more pronounced in PEC, where the *iglC* mutant strain was rapidly killed within 24-36 hours (Golovliov et al., 2003a).

### 4.2 *iglB, iglC, iglD and mglA* mutant strains do not inhibit *E. coli* LPS-mediated activation of intracellular pathways.

Previously, we have demonstrated that infection of macrophages with the $\Delta iglC$ strain, unlike infection with *F. tularensis* LVS, has no affect on TLR-mediated intracellular signaling (Telepnev et al., 2003). In this study we examined effects of $\Delta iglB$, $\Delta iglD$, and $\Delta mglA$ mutants on *E. coli* LPS-induced secretion of TNF-$\alpha$. Data show that in all cell cultures infected with mutant strains and induced with *E. coli* LPS, levels of TNF-$\alpha$ were even higher than those in non-infected LPS stimulated cells, while no detectable TNF-$\alpha$ was found in cells infected with *F. tularensis* LVS. These results suggest that expression of each of the IglB, IglC, IglD and MglA proteins is necessary for *Francisella* to inhibit TLR-mediated intracellular signaling pathways.

Since multiple mutations in the *igl* operon as well as deletion of the *mglA* gene, encoding a known regulator of the *igl* operon, have a prominent impact on the intracellular survival of *Francisella*, we conclude that these effectors must work in concert for expression of the full virulence of the bacterium.

### 5. The role of RNS and ROS for the *F. tularensis*-induced effects on intracellular signaling.

Mechanisms of inhibition of TLR-mediated signaling conferred by *Francisella tularensis* (paper IV).
5.1 H$_2$O$_2$ does not affect inhibition of LPS-induced activation of intracellular signaling and secretion of TNF-α by *F. tularensis* LVS.

NF-κB signaling can be affected by the intracellular redox status (Karin and Lin, 2002). It has been demonstrated that IKK can be a direct target for H$_2$O$_2$ (Kamata et al., 2002). We examined the effect of H$_2$O$_2$ on phosphorylation of cJun and p38 proteins and secretion of TNF-α in *F. tularensis*-infected cells upon LPS stimulation. Phosphorylation of those proteins was not affected in infected cells and they were not capable to secrete measurable levels of TNF-α. Thus the addition of H$_2$O$_2$ to cell cultures did not rescue LPS induced TLR signaling, inhibited by *F. tularensis* LVS.

5.2 Effects of NO and peroxynitrite on the inhibition of LPS-induced activation of macrophages by *F. tularensis* LVS.

Although we did not find any effect of H$_2$O$_2$ on *Francisella* mediated inhibition of intracellular signaling, it was possible that addition of RNS such as NO and peroxynitrite may have an effect. We have examined the effects of SNAP, a donor of NO, SIN-1, a donor of peroxynitrite, and DPI, an inhibitor of flavoenzymes such as phagocyte oxidase and nitric oxide synthase. The results demonstrate that addition of SIN-1 has no effect on *Francisella*-induced inhibition of intracellular pathways. This might be due to very short life span of peroxynitrite due to its extremely high reactivity, and thus relatively low concentrations inside the cells, which may not be sufficient to affect the intracellular signaling. In contrast, DPI-treated *F. tularensis*-infected, LPS-induced cells showed only low levels of TNF-α secretion. The most prominent changes were observed when the cells were treated with SNAP, the donor of NO. While SNAP did not affect TNF-α secretion in non-infected cells stimulated with LPS, it almost completely reversed the *F. tularensis*-induced inhibition of the LPS stimulation.

We also examined effects of these compounds on phosphorylation of IκB, cJun and p38. The Western blot analysis confirmed that no activation of NF-κB and MAPK pathways had taken place when *F. tularensis* cells were treated with SIN-1, but addition of either DPI or SNAP partly or fully restored the ability of the infected cells to respond to LPS stimulation.
5.3 SNAP reverses inhibition of intracellular pathways induced by \textit{Francisella} in a dose-dependent manner.

We examined how different concentrations of SNAP affected inhibition of TNF-\(\alpha\) secretion induced by \textit{Francisella}. The most profound effect was when SNAP was used at a concentration of 1 mM whereas no detectable TNF-\(\alpha\) was found at concentration 0.1mM. We did not observe any effect on the number of intracellular \textit{F. tularensis} bacteria when 1 mM of SNAP was used. We also examined whether or not the combination of SNAP and LPS can cause cytopathogenic effect in the eukaryotic cells, but the release of LDH within 6 h was not significantly increased compared to non-infected cells. Thus, the effect of SNAP is dose-dependent and affects intracellular signaling of host cells or bacterial inhibitory mechanisms but not the viability of either the host or bacterial cells.

5.4 Possible mechanisms of the NO-mediated effects on intracellular pathways of eukaryotic cells.

It has been shown that ferric ions can affect intracellular signaling. One possible explanation for the effect of SNAP is that it may trigger release of intra-cellular iron that in turn can activate signaling pathways. To examine this hypothesis, we studied the effect of combining SNAP and deferoxamine (DFO), a known ferric chelator, on TNF-\(\alpha\) secretion in infected cells induced with \textit{E. coli} LPS. Indeed, the secretion of TNF-\(\alpha\) in DFO-treated cells infected with \textit{Francisella} and stimulated with SNAP and LPS was significantly reduced when compared to the cells not treated with DFO.

We also investigated the possibility that NO can undergo enzymatic transformation inside the cells and be converted into peroxynitrite, which is known to be a potent activator of NF-\(\kappa\)B. To answer this question, we used FeTPPS, a decomposition catalyst of peroxynitrite. The addition of FeTPPS to \textit{Francisella}-infected cells induced with LPS and SNAP almost completely abolished TNF-\(\alpha\) secretion and phosphorylation of I\(\kappa\)B, cJun and p38 proteins. These findings suggest that peroxynitrite is the most likely effector molecule for the activation of NF-\(\kappa\)B and MAPK signaling in infected cells. Another possibility is that peroxynitrite
directly affects the Igl/MglA expression system inside the bacterial cells and this will be the subject to further investigation.

6 Drosophila.

Drosophila melanogaster as a model for identifying Francisella tularensis virulence mechanisms (paper V).

6.1 Survival and growth of F. tularensis in Drosophila haemocytes.

Drosophila hemocyte-like malignant blood neoplasm-2 (mbn-2) cells were infected at an MOI of 500 for two hours, extracellular bacteria were washed away, and fresh medium containing 5μg/ml of gentamicin was added. Efficiency of the uptake was confirmed by fluorescent microscopy, using GFP-expressing F. tularensis LVS. The results have shown that after two to four hours of uptake almost 100% cells were infected. The ability of Francisella to survive and multiply in mbn-2 cells were studied at two different temperatures: 25°C – the normal condition and 30°C, a higher temperature that may benefit F. tularensis. In both experiments, we have found that Francisella survived and proliferated within mbn-2 cells, reaching the peak numbers after two to three days, when intracellular bacterial number increased up to 3 log₁₀.

6.2 Survival and growth of F. tularensis in Drosophila.

To investigate if the fruit fly can be used as a model for Francisella infection we performed injection experiments with adult flies. First we established that there is a dose-dependence in fly survival. Injection of 50,000 to 100,000 CFU per fly killed Drosophila within eight to twelve days, however even low dose as 10 CFU per fly was lethal, and flies died within about 3 weeks.

At the same time we studied whether or not there is a difference between survival and proliferation of the parental LVS strain and mutant strains lacking igl or mgl genes. The
results demonstrate that although mutant strains are less virulent for fruit flies they still are able to survive and to some extent even to multiply, affecting survival of the flies. This indicates that there must be a significant difference between mammalian and insect host immune responses to Francisella infection.

We also tried to establish if Francisella can be transmitted from one developmental stage to another, from larvae to adult fly. In this experiment, the Drosophila larvae were fed on F. tularensis LVS or ΔiglC bacteria. The results show that the mutant strain is less virulent for Drosophila, but at the same time, we were unable to find any viable Francisella in developed adult flies, regardless of the Francisella strain used. It seems that during metamorphosis, Drosophila clears the infection, probably due to cell death that takes place and thus exposes the parasite to the various components of the immune system.

6.3 Effect of Francisella infection on intracellular signaling in Drosophila.

It was of interest to study the insect’s intracellular signaling pathways during infection. We analyzed transcription of different antimicrobial peptides in infected flies. The results show that the flies infected with the parental F. tularensis LVS strain showed expression of diptericin during first seven days after injection, however, ΔiglC infection in flies induced expression of diptericin only during first day of infection.

Experiments involving different Drosophila strains lacking or over-expressing signaling components of the imd pathway show that it plays a very important role in controlling Francisella infection in flies. Flies carrying mutations in that pathway died much faster than LVS infected flies or those that had constitutive over-expression.

Altogether, the data show that despite significant differences in the immune system between mammals and insects, there are mechanisms used by F. tularensis that are important for infection on both types of experimental models. These findings will form a starting point for further studies.
CONCLUSIONS

- *F. tularensis* bacteria initially activates both NF-κB and MAPK intracellular pathways during uptake in mouse and human monocytic cells. This activation is subsequently down-regulated at varying degrees in human and mouse cells.

- *F. tularensis* escapes from the phagosome. IFN-γ-activated macrophages are able control the infection but the escape mechanism is still partly functional in the activated macrophages.

- Intracellularly located *F. tularensis* bacteria inhibit TLR2- and TLR4-mediated activation of NF-κB and MAPK signaling. The inhibition leads to impaired secretion of proinflammatory cytokines.

- The IglB, IglC, IglD, and MglA proteins are important virulence determinants of *F. tularensis*. Mutant strains, lacking either one of the proteins do not multiply at all or less efficiently in murine macrophages than does the parental LVS strain.

- A donor of nitric oxide can reverse the *F. tularensis*-mediated inhibition of LPS-induced NF-κB and MAPK activation and TNF-α secretion. The reversal of the nitric oxide-mediated effect by FeTPPS indicates that ONOO⁻ is an important mediator.

- By establishing a model of tularemia in *D. melanogaster*, we demonstrated that the Igl proteins are important for expression of the full virulence of *F. tularensis* in the fly.

- *F. tularensis* LVS but not the ΔiglC strain caused pronounced and long-lasting stimulation of fly’s humoral immune response.
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