# Reactive oxygen and nitrogen species in host defense against *Francisella tularensis*

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# ABSTRACT

*Francisella tularensis*, the causative agent of tularemia, is a potent human and animal pathogen. Initially upon infection of the host, intramacrophage proliferation of *F. tularensis* occurs but after activation of the acquired host immunity, the phagocytes become activated to kill the bacterium. In my thesis, I focused on mechanisms utilized by *F. tularensis* to survive intracellularly and on host mechanisms responsible for macrophage-mediated killing and control of infection.

The *F. tularensis*-specific protein IglC has been previously shown to be essential to the intramacrophage proliferation and virulence of the bacterium in mice. By electron microscopy of macrophages infected with either the live vaccine strain of *F. tularensis* or an isogenic mutant, denoted $\Delta iglC$, expression of IglC was found to be necessary for the bacterium to escape from the phagosome. IFN-γ-activated macrophages significantly inhibited the escape of the live vaccine strain of *F. tularensis* from the phagosome.

iNOS and phox generate NO and $O_2^-$, respectively. These molecules and their reaction products possess both bactericidal and immunoregulatory properties. We investigated the capability of IFN-γ-activated peritoneal exudate cells from gene deficient $iNOS^{-/-}$ or $p47^{phox-/-}$ mice to control an intracellular *F. tularensis* LVS infection. iNOS was found to contribute significantly to the IFN-γ induced killing, while phox contributed only to a minor extent. Unexpectedly, bacteria were eradicated even in the absence of both a functional phox and an active iNOS. The eradication was found to depend on $ONOO^-$, the reaction product of NO and $O_2^-$, because addition of a decomposition catalyst of $ONOO^-$ completely inhibited the killing.

Studies on $iNOS^{-/-}$ or $p47^{phox-/-}$ mice infected with *F. tularensis* LVS showed phox to be important during the first days of infection, a stage when iNOS seemed dispensable. Eventually, $iNOS^{-/-}$ mice died of the infection, suggesting a role of iNOS later in the course of infection. $iNOS^{-/-}$ mice exhibited elevated IFN-γ serum levels and severe liver damage suggesting that the outcome of infection was at least in part related to an uncontrolled immune response.

Several pathogenic bacteria express Cu,Zn-SOD, which in combination with other enzymes detoxifies reactive oxygen species produced by the host. A deletion mutant of *F. tularensis* LVS lacking the gene encoding Cu,Zn-SOD was attenuated at least 100-fold compared to LVS in mice. In peritoneal exudate cells from mice, Cu,Zn-SOD was found to be required for effective intramacrophage proliferation and, in mice, important for bacterial replication at the very early phase of infection.

In summary, the most conspicuous findings were a capability of IFN-γ activated macrophages to retain *F. tularensis* LVS in the phagosome, an essential role of $ONOO^-$ in intracellular killing of *F. tularensis*, and an importance of Cu,Zn-SOD to the virulence of *F. tularensis* LVS.

# PAPERS IN THE THESIS

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

I.  Lindgren H, Golovliov I, Baranov V, Ernst RK, Telepnev M, Sjöstedt A.

Factors affecting the escape of *Francisella tularensis* from the phagolysosome. J. Med. Microbiol. 2004; 53: 953-8

II. Lindgren H, Stenman L, Tärnvik A, Sjöstedt A.

The contribution of reactive nitrogen and oxygen species for the killing of *Francisella tularensis* LVS by murine macrophages. Microbes Infect. In press.

III. Lindgren H, Stenmark S, Chen W, Tärnvik A, Sjöstedt A.

Distinct roles of reactive nitrogen and oxygen species to control infection with the facultative intracellular bacterium *Francisella tularensis*. Infect. Immun. 2004; 72: 7172-82.

IV. Lindgren H, Golovliov I, Sjöstedt A.

Cu,Zn-superoxide dismutase of *Francisella tularensis* LVS is a virulence factor. Manuscript

# SAMMANFATTNING

Bakterien *Francisella tularensis* är en potent patogen som orsakar sjukdomen tularemi (harpest) hos människa och djur. *F. tularensis* har egenskapen att tillväxa i professionella fagocyter såsom makrofager (celler specialiserade på att avdöda mikrober) och detta är centralt för bakteriens sjukdomsframkallande förmåga. Dock, när immunförsvaret utvecklas och utsöndrar cytokiner, såsom TNF-$\alpha$ och IFN-$\gamma$, aktiveras mekanismer i makrofagen som avdödar *F. tularensis* och detta är centralt för en utläkning av tularemi. Denna avhandling identifierade mekanismer hos *F. tularensis* vilka är viktiga för dess tillväxt i makrofager och i djur men även mekanismer viktiga för immunförsvarets förmåga att avdöda bakterien i makrofager och in vivo.

Efter upptaget i makrofager degraderar *F. tularensis* det fagosomala membranet och kan därefter tillväxa i cellens cytoplasma. IglC är ett *F. tularensis* protein som krävs för tillväxt i makrofager och för att orsaka sjukdom i möss. Makrofager infekterade med *F. tularensis* LVS eller en stam av *F. tularensis* LVS som saknade IglC ($\Delta iglC$) studerades med elektronmikroskopi. Resultaten visade att iglC krävdes för att bakterien skulle kunna degradera det fagosomala membranet. $\Delta iglC$ innesluten i fagosomer avdödades mycket snabbt när makrofagerna aktiverades med IFN-$\gamma$. Aktiveringen av makrofagerna förhindrade delvis *F. tularensis* LVS förmåga att degradera det fagosomala membranet och *F. tularensis* LVS avdödades effektivt, dock inte lika snabbt som $\Delta iglC$.

iNOS och phox producerar NO respektive $O_2^-$. Dessa molekyler och derivat av dessa kan både avdöda bakterier och reglera immunförsvaret. Vi undersökte om iNOS och phox bidrog till avdödningen av *F. tularensis* LVS i IFN-$\gamma$ aktiverade makrofager. Makrofager från möss som saknade en funktionellt iNOS (iNOS$^{-/-}$) eller phox (p47$^{phox-/-}$) eller från kontrollmöss (iNOS$^{+/+}$ och p47$^{phox+/+}$) infekterades. NO bidrog signifikant till den IFN-$\gamma$ inducerade avdödningen medan avdödningen i princip var oberoende av $O_2^-$ producerat från phox. ONOO$^-$, produkten av NO och $O_2^-$, förmedlade den IFN-$\gamma$ inducerade effekten eftersom tillsats av FeTPPS, vilken katalyserar nedbrytningen av ONOO$^-$, inhiberade avdödningen av *F. tularensis* LVS.

Studier av iNOS$^{-/-}$ och p47$^{phox-/-}$ möss infekterade med *F. tularensis* LVS visade att både iNOS och phox var viktiga för att kontrollera infektionen. Phox-beroende mekanismer reducerade tillväxten av *F. tularensis* LVS under de första dagarna av infektionen medan iNOS krävdes i ett senare skede för att läka ut infektionen. iNOS$^{-/-}$ möss utvecklade en allvarlig leverskada och hade förhöjda nivåer av IFN-$\gamma$ i serum vilket indikerar att dessa möss hade ett okontrollerat immunsvar. Detta i kombination med att makrofager i frånvaro av NO har en reducerad förmåga att avdöda *F. tularensis* LVS (se ovan) kan förklara den fatala utgången av infektionen i iNOS$^{-/-}$ möss.

Många bakterier utrycker Cu,Zn-SOD, vilken dismuterar $O_2^-$ och därmed bidrar till att skydda mot reaktiva syreradikaler som produceras av värden. Det krävdes minst 100 gånger fler bakterier av en stam av *F. tularensis* LVS som saknade genen som kodar för Cu,Zn-SOD ($\Delta sodC$) för att orsaka fatal infektion i möss jämfört med *F. tularensis* LVS. Specifikt så krävdes Ft-Cu,Zn-SOD för effektiv intracellulär tillväxt av *F. tularensis* LVS i makrofager och för att under de första dagarna av infektionen kolonisera lever och mjälte i möss. Resultaten visade att Cu,Zn-SOD skyddade mot reaktiva syremetaboliter när koncentrationen av NO var låg vilket återigen indikerar att ONOO$^-$ är viktig för att avdöda *F. tularensis* LVS.

# ABBREVIATIONS

| | |
|---|---|
| LVS | Live vaccine strain |
| CFU | Colony forming units |
| iNOS | Inducible nitric oxide synthase |
| phox | Phagocyte oxidase |
| XO | Xanthine oxidase |
| NO | Nitric oxide |
| $O_2^-$ | Superoxide |
| $ONOO^-$ | Peroxynitrite |
| ROS | Reactive Oxygen Species |
| RNS | Reactive Nitrogen Species |
| $LD_{50}$ | Lethal dose 50% |

# INTRODUCTION

## A. *Francisella*

### A.1 Classification and phenotype

The genus *Francisella* was named after Edward Francis, an American microbiologist who extensively studied the bacterium *F. tularensis* and the pathogenesis of tularemia. Within the family *Francisellaceae*, *Francisella* is the only recognized genus. Two recognized species, *F. tularensis* and *F. philomiragia* (reviewed in reference (Sjöstedt, 2005)), show a mutual 16S rRNA gene similarity of >98.3 %. *F. tularensis* comprises four subspecies; *F. tularensis* subsp. *tularensis, F. tularensis* subsp. *novicida, F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica.*

*F. tularensis* is a gram-negative, short rod-shaped or coccoid, aerobic organism (Sjöstedt, 2005). On enriched nutrient media such as chocolate agar colonies are distinct, convex, pale white, and reach maximum size in 3-4 days. *Francisella* exhibits a three-laminar membrane structure, similar to other gram-negative bacteria. The outermost layer, the capsule, is lipid rich. The LPS of the membrane is of the smooth type with repeating O-side chains.

***F. tularensis* live vaccine strain.** In the former Soviet Union, in the 1940's, a strain of *F. tularensis* subsp. *holarctica* was repeatedly passaged on agar and attenuated variants of the bacterium were isolated. (Conlan, 2004). In 1956 two of these attenuated isolates were transferred from Gamaleya Institute in Moscow to researchers at the US Army Research Institute of Infectious Diseases in US. Upon plating on peptone cysteine agar, two colony types were distinguished. A gray variant was avirulent in mice, whereas a blue variant was attenuated yet virulent in mice. Vaccination with the blue variant induced protection in mice from systemic challenge with virulent type A *F. tularensis.* The blue variant was designated the live vaccine strain of *F. tularensis* (*F. tularensis* LVS) and was used as a human vaccine against tularemia until the early 1990's. However, since the attenuation of *F. tularensis* LVS is not defined and the vaccine has elicited tularemia like symptoms in humans, it is no longer licensed as a human vaccine in most countries. In Sweden, though, it has been used for

immunization of laboratory personnel at risk. *F. tularensis* LVS is still virulent in mice and elicits a disease, which resembles human tularemia (Elkins et al., 2003).

## A.2. Tularemia

*F. tularensis* is the causative agent of the disease tularemia but only *F. tularensis* subsp. *tularensis* (also called type A) and *F. tularensis* subsp. *holarctica* (type B) are of clinical relevance in humans (Tärnvik and Berglund, 2003). An inoculum as low as 10 or fewer type A or type B bacilli can initiate severe infection following intradermal inoculation or inhalation, demonstrating the extreme infectivity of these subspecies. Of the two, subspecies *tularensis* is by far the more virulent of these two subspecies. If untreated, tularemia caused by subspecies *tularensis* may be associated with a mortality of 10% or more whereas death in tularemia caused by subspecies *holarctica* is rare.

The highly virulent subspecies *F. tularensis* subsp. *tularensis* is found only in North America and the less virulent subspecies *F. tularensis* subsp. *holarctica* is found in Asia, Europe and North America (Tärnvik and Berglund, 2003). Tularemia is a zoonotic disease, infecting both animals and humans. It occurs epidemically and endemically over a large part of the Northern Hemisphere. The most extensive epidemics occurred during the Second World War in Russia and comprised up to 100,000 cases per year. More recently, outbreaks of tularemia in the Northern Hemisphere have comprised 100 - 1,000 cases. The endemic centers of Sweden are located in Närke and Hälsingland. Cases also occur outside the endemic centers. The reservoir(s) of *F. tularensis* remains unknown. Ticks, lagomorphs and rodents are believed to be the most important source for spread of tularemia to human. Since these animals either die from the infection or do not develop protective immunity, they are not believed to be the reservoir. The bacterium has been demonstrated to multiply inside intracellular vacuoles of *Acanthamoeba castellanii* (Abd et al., 2003). Thus, ubiquitous protozoa might be an important environmental reservoir for *F. tularensis*.

The clinical manifestations of tularemia depend on the portal of entry of the bacteria. The ulceroglandular form of tularemia is the most common form and is established when *F. tularensis* enters through small wounds in skin after direct contact with an infected animal or via an insect bite (reviewed in reference (Tärnvik and Berglund, 2003). At the site of infection, an ulcer develops and the regional lymph nodes become swollen and tender. Respiratory tularemia results from inhalation of contaminated dust and oropharyngeal

tularemia is the outcome of ingestion of contaminated water or food. Irrespective of clinical form, tularemia is associated with fever and general illness. Untreated tularemia caused by the less virulent strain *F. tularensis* subsp. *holarctica* might persist for weeks, followed by a period of fatigue.

**A.3 Virulence mechanisms of *F. tularensis***

### A.3.1 F. tularensis *escapes the phagosome*

By studying tissues from infected animals and by following in vitro cell infections, it is well documented that *F. tularensis* has the capability to multiply in professional phagocytes as well as in other target cells, for example hepatocytes (Conlan and North, 1992; Fortier et al., 1995; Fortier et al., 1992). Depletion of iron or addition of agents that block endosome acidification inhibits the intracellular replication of *F. tularensis* (Fortier et al., 1995). The intracellular lifestyle appears to be pivotal to the virulence of the bacterium, since mutants that fail to multiply in professional phagocytes in vitro also fail to elicit disease in mice (Golovliov et al., 2003b; Lauriano et al., 2004).

Bacteria that multiply in professional phagocytes either interfere with the maturation process of the phagosome into a phagolysosome, or escape from the phagosome, or are able to adapt to the hostile milieu (Amer and Swanson, 2002). A phagolysosome is formed after sequential interactions of the phagosome with the endocytic pathway in the cell and specific markers characterize its development. Early phagosomes contain markers such as transferrin receptors (TfRs), early endosomal antigen 1 (EEA1), and Rab 5 and they are mildly acidic (pH 6). Within 10-30 minutes after formation, the phagosome acquires the traits of a late phagosome and within 1 hour, a phagolysosome is formed. On late phagosomes, EEA1, Rab5 and TfRs are absent; instead markers such as Rab7, mannose-6-phosphate receptor and lysosomal associate protein (LAMP) appear. The pH of a late phagosome is moderately acidic (pH around 5.5) and that of a phagolysosome extremely low, << 5. In addition, the phagolysosome contains active hydrolases, such as cathepsin D, high concentrations of ions and ROS generated by phox. Most bacteria are efficiently killed in this environment.

Earlier studies concluded that *F. tularensis* survives and multiplies in an acidic membrane-enclosed compartment (Anthony et al., 1991; Fortier et al., 1995). However, recent data suggest that *F. tularensis* LVS is able to escape from the phagosome (Clemens et al., 2004;

Golovliov et al., 2003a). Both the study by Golovliov and Clemens analyzed cells infected in vitro with strains of *F. tularensis* by transmission electron microscopy and confocal microscopy (Clemens et al., 2004; Golovliov et al., 2003a). It was found that *F. tularensis* during a period after uptake resided in a membrane enclosed compartment. This compartment transiently colocalized with the late endosomal marker LAMP (Clemens et al., 2004; Golovliov et al., 2003a) but did not colocalize with the acid hydrolase, cathepsin D, and was only mildly acidified (pH 6.7) (Clemens et al., 2004). Both the study by Golovliov and Clemens showed that, within a few hours after ingestion *F. tularensis* escaped from the phagosome and resided freely in the cytoplasm. Collectively, the two studies indicate that *F. tularensis* interferes with the maturation process of the phagosome to a phagolysosome and succeeds in escaping the compartment.

The mechanism behind the escape of *F. tularensis* from the phagosome is elusive. *Listeria monocytogenes* like *F. tularensis* escapes from the phagosome (Portnoy et al., 2002). *L. monocytogenes* expresses a pore-forming cytolysin denoted listeriolysin O, which is critically required to degrade the phagosomal membrane. In addition, *L. monocytogenes* secretes two phospholipases that contribute to the escape. A genome analysis of *F. tularensis* did not reveal the presence of any hemolysins or phospholipases and therefore the mechanism of escape used by *F. tularensis* LVS appears to be distinct from that used by *L. monocytogenes*. Results from the study by Golovliov et. al indicates that capsular components might be released from the bacteria, which may associate with the membrane integrity of the vacuole (Golovliov et al., 2003a). In paper I we investigated if IglC, a protein essential to the intracellular proliferation of *F. tularensis*, is required for the escape.

### *A.3.2 IglC*

The adaptation of intracellular bacteria to the hostile environment in professional phagocytes involves modulation of their protein synthesis. However, when *F. tularensis* LVS grows in macrophages the modulation of protein synthesis is modest. In one study, only four proteins showed enhanced expression (Golovliov et al., 1997). The escape of *F. tularensis* from the phagosome for multiplication in the cytoplasm could make it less dependent on a distinct stress response. The most prominent upregulation during intracellular growth was shown for a 23-kDa protein, denoted IglC (Golovliov et al., 1997). The expression of this protein was not

upregulated at 2 h after infection of macrophages but at 6, 24 and 48 h the expression showed almost a 4-fold increase. Moreover, IglC was recently shown to be essential to the intracellular proliferation and virulence in mice of strains of subspecies *holarctica*, *tularensis* and *novicida* (Golovliov et al., 2003b; Lai et al., 2004; Lauriano et al., 2004). Additional effects of IglC are described in section A.3.4.

The gene encoding IglC (*iglC*) is part of an operon with four open reading frames denoted *iglABCD* (Nano et al., 2004). In a data bank search, IglA and B showed 30% identity to proteins involved in protein secretion in *Rhizobium leguminosarum*, whereas IglC and D showed no similarity to any protein. MglA positively regulates the *igl* operon, (Lauriano et al., 2004) and, accordingly, expression of MglA is essential to the intramacrophage proliferation and virulence in mice. In addition, expression of IglC and MglA, was required for intraamoebae survival of *F. tularensis*. Since one possible natural reservoir of *F. tularensis* is suggested to be amoebae, the MglA-regulated proteins may consequently be essential for environmental persistence.

In a recent publication it was suggested that the *igl* operon is located in a pathogenicity island (FPI) (Nano et al., 2004). The FPI comprises approximately 30 kb and harbors three large open reading frames, named pathogenicity determinant protein A, B and C (PdpA, B, C). In addition eight relatively short ORFs are located in the region between *pdpB* and *pdpC* . On one part of the FPI, there are eight open reading frames, four of which constitute the *igl* operon. None of the other ORFs in the FPI showed any significant homology to any other known protein and their function is therefore elusive.

A pathogenicity island (PI) is defined as a specialized form of a genome island that comprises a large genomic region, 10–200 kb, present on the genomes of pathogenic strains but absent from the genomes of nonpathogenic strains of the same species. In principle, the FPI appears to be identical among different subspecies of *F. tularensis*, even in low virulent strains such *F. tularensis* LVS and *F. tularensis* subspecies *novicida* (Nano et al., 2004), thus, not strictly conforming to the definition of a PI as virulence-associated but rather a genome island. A notable exception is the partial deletion of the *pdpD* gene from the genomes of virulent strains of subsp. *holarctica* and *F. tularensis* LVS.  PdpD is essential to the intracellular proliferation and virulence in mice of strains of subspecies *novicida*. The possible impact of PdpD on the virulence in subspecies *tularensis* has not been tested.

### A.3.3 Superoxide dismutase, catalase, and other potential antioxidant systems

Superoxide dismutases (SODs) represent a family of metalloenzymes, found in organisms ranging from bacteria to humans (Zelko et al., 2002). SODs exist in several different isoforms that differ in their structure and prosthetic ion/ions but all isoforms catalyze the dismutation of $O_2^-$ to hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$). Iron-SOD (Fe-SOD) and Manganese-SOD (Mn-SOD) are located in the cytoplasm of prokaryotes whereas Cu,Zn-SOD is located in the periplasm of gram-negative bacteria, anchored to the outer membrane, or secreted (Lynch and Kuramitsu, 2000). Thus, Cu,Zn-SOD in bacteria has the potential to protect against reactive oxygen species (ROS) generated from external sources. Accordingly, it has also been shown to be a virulence mechanism of many bacteria (Battistoni, 2003). The amino acid sequences of Cu,Zn-SOD from different bacterial species show extensive variation (Battistoni, 2003) and is predicted to affect the architecture of the active site channel and subunit assembly and, hence, enzyme activity. Thus, the SOD activity may vary substantially between bacterial species.

*F. tularensis* has been described to possess SOD activity (Shimaniuk et al., 1992) and the genomes of strain SCHU S4 (type A, fully virulent) and of *F. tularensis* LVS encode Fe-SOD (*sodB*) and Cu,Zn-SOD (*sodC*) (genome analysis). The importance of SOD to the virulence of *F. tularensis* is unknown. Catalase is an enzyme that converts $H_2O_2$ to water ($H_2O$) and $O_2$. The cooperation of SOD and catalase thereby efficiently prevents the formation of other, more reactive ROS such as hydroxyl radical ($OH^-$), anion ($OH^-$), or peroxynitrite ($ONOO^-$) (formation of ROS is described in section C.4). Strains of *F. tularensis* have been reported to possess catalase activity and attenuation of the strains led to a marked reduction of the catalase activity (Rodionova, 1976). This implies that catalase is a virulence mechanism in *F. tularensis*.

The genomes of SCHU S4 and of *F. tularensis* LVS also contain additional genes that encode proteins or enzymes implicated in protection against oxidative and nitrosative damage; i) Glutathione- and thioredoxin-dependent reduction systems, which help to keep the reduced environment intact in the cell (Kroncke et al., 2001), ii) Glutathione peroxidase, which like catalase reduces $H_2O_2$ and in addition reduces organic peroxides and hydrogen peroxide. This enzyme has also been shown to reduce $ONOO^-$ (Sies et al., 1997) and recently, it was demonstrated to be a virulence mechanism in *Streptococcus pyogenes* (Brenot et al., 2004), iii) Peptide methionine sulfoxide reductase, reduces oxidized methionine (Weissbach et al., 2002). In addition to restoration of the function of oxidized proteins, the oxidation reduction

cycle of methionine may be one of the prime mechanisms that cells use to scavenge ROS and RNS before they damage other targets in the cell (Weissbach et al., 2002).

The proteins and enzymes described above could be of special importance for survival of *F. tularensis* when located inside professional phagocytes, which have the capacity to generate high levels of ROS and RNS from the phagocyte oxidase (phox) (section C.1) and inducible nitric oxide synthase (iNOS) (section D), respectively. In paper IV, the importance of Cu,Zn-SOD for the virulence of *F. tularensis* LVS was tested.

### *A.3.4 Modulation of the host immune response*

*F. tularensis* LVS is able to inhibit TNF-$\alpha$ and IL-1$\beta$ secretion in macrophages by blocking the NF-$\kappa$B activation (Telepnev et al., 2003) and this inhibition is dependent on the expression of IglC. In addition, LPS derived from *F. tularensis* is rather inert. Its ability to elicit IL-1 and TNF-$\alpha$ secretion from macrophages is at least a 1,000-fold lower as compared to *E. coli* LPS (Sandström et al., 1992). Several studies have shown TNF-$\alpha$ to be pivotal in the host defense against *F. tularensis* LVS. The ability to inhibit the induction and release of proinflammatory cytokines from macrophages may be one reason why *F. tularensis* is such a successful intracellular pathogen.

*F. tularensis* LVS-infected J774 macrophages become apoptotic 12-18 h after infection (Lai and Sjostedt, 2003). This delayed induction of apoptosis suggests that a direct binding of *F. tularensis* to the macrophages or mediators produced by extracellular bacteria is not the initiating event for apoptosis. Rather, apoptosis seems to be triggered by intracellularly proliferating bacteria, because $\Delta iglC$ (*F. tularensis* LVS lacking *iglC*) did not proliferate intracellularly and did not induce apoptosis. It remains to be determined whether the induction of apoptosis is an active mechanism of *F. tularensis* LVS relying on IglC, or simply an effect of an increasing bacterial load. Nevertheless, when infected cells become apoptotic, the bacteria will be released from a host cell replete of nutrients and the infection cycle can restart. Apoptosis, in contrast to necrosis, does not induce an inflammatory response and, in fact, when a macrophage ingests an apoptotic body it is activated to secrete macrophage-deactivating stimuli such as TGF-$\beta$ and IL-10 (Ma et al., 2003). Thus, induction of apoptosis is another mechanism that, like inhibition, of NF$\kappa$B activation might reduce the inflammatory response upon infection.

### A.3.5 Capsule

A capsule acts as a mechanical barrier, by impeding the diffusion of potentially harmful host-derived substances, for example, of degrading enzymes and bactericidal peptides (Daffe and Etienne, 1999). The capsule may also enclose enzymes and molecules, which detoxify host-derived radicals or help to supply the bacterium with nutrients. Moreover, a capsule could "hide" more immunogenic parts of the bacterium, for example the LPS, from the immune response.

From the genome sequence of *F. tularensis* subsp. *tularensis* strain SCHU S4, the capsule is predicted contain poly-D-glutamic acid, similar to that of *Bacillus anthracis* (Larsson et al., 2005). A capsule-deficient mutant of *F. tularensis* was attenuated in mice (Sandström et al., 1988). In vitro, the capsule conferred protection against complement-activated lysis but also triggered an oxidative burst upon phagocytosis in human neutrophils, which resulted in killing of the bacterium. The mechanism of escape of *F. tularensis* from the phagosome is not resolved but suggested to rely on the release of capsular components, which interact with the membrane of the vacuole.

## B. Host immune responses during tularemia

In order to establish infection, a microorganism has to penetrate or colonize the epithelial barrier of the body and escape killing by the innate immune mechanisms. If infection is established, specific adaptive immunity is evoked, which means expansion of specific T-cells and B-cells and subsequently production of specific antibodies. The cytokines secreted by innate immune cells, most importantly macrophages and dendritic cells, influence the developing specific immune response. IL-12 is a key cytokine in driving the development of Th1 cells that characteristically secrete IFN-γ and such a response is commonly referred to as an inflammatory immune response. This type of immune response is pivotal when a host encounters a pathogen that has developed strategies to survive in professional phagocytes since, IFN-γ in combination with other cytokines and Th1 cells activate phagocytes to kill such bacteria. Examples of IFN-γ induced events are induction of iNOS, limitation of the availability of iron, upregulation of MHC class II molecules and Fcγ-gamma receptors,

enhanced endosomal trafficking that promotes the formation of a phagolysosome and proper assembly of the phagocyte oxidase.

A major part of the information regarding immune mechanisms active in the course of tularemia has been obtained in a murine model and the current knowledge on the host immune response to *F. tularensis* has recently been reviewed (Elkins et al., 2003)

The skin is the most common route of infection of *F. tularensis* in humans. Nonetheless, the protective efficacy of the skin is striking since a thousand- to a million-fold more bacteria are required to cause a lethal infection by dermal route versus other routes of inoculation, for example, intranasal, intravenous or intraperitoneal (Fortier et al., 1991). When mice are inoculated with *F. tularensis* LVS by the dermal route, bacteria spread by the lymph to the regional lymph nodes and from there disseminate to spleen and liver. The bone marrow and lung are only colonized to a minor extent and other tissues or organs are free of bacteria.

Cells of the epidermis respond to a *F. tularensis* infection with only low levels of TNF-$\alpha$, IL-12, and IFN-$\gamma$ and not until day three after inoculation (Stenmark et al., 1999). However, systemically, IFN-$\gamma$ is present already at day 2 of infection (own unpublished observation). IFN-$\gamma$, TNF-$\alpha$, and the heterodimer IL-12p70 (composed of the subunits p40 and p35) are all produced during a primary infection with *F. tularensis* LVS. Mice deprived of either IFN-$\gamma$ or TNF-$\alpha$ do not restrict the replication of the bacteria during the early phase of the infection (Sjöstedt et al., 1996). As a consequence, the LD$_{50}$ of TNF-$\alpha$ deprived mice is several thousands times lower compared to immunocompetent mice and IFN-$\gamma$ gene-deficient mice succumb to even one single bacterium of *F. tularensis* LVS. IL-12p70- or IL12p40-deficient mice control the initial phase of the infection but are unable to eradicate the bacterium (Elkins et al., 2002). In contrast, IL12p35-deficient mice clear the infection. Thus, clearance of *F. tularensis* LVS is not dependent on IL-12 p70 but on the IL12p40 subunit.

The importance of IFN-$\gamma$ and TNF-$\alpha$  in the immune response to *F. tularensis* LVS is probably due to their critical role in activating macrophages and other phagocytes to kill the bacterium. IFN-$\gamma$ activates murine macrophages to kill LVS (Anthony et al., 1992; Fortier et al., 1992; Polsinelli et al., 1994), however, the mechanisms of killing appear to depend on the origin of the macrophages. Peritoneal cells from mice killed *F. tularensis* LVS in a NO-dependent manner and killing required endogenously secreted TNF-$\alpha$ by the macrophages (Fortier et al., 1992). In contrast, the IFN-$\gamma$ induced killing of *F. tularensis* LVS in alveolar

macrophages was found to be independent of NO and TNF-$\alpha$ (Polsinelli et al., 1994) and no effect was observed after supplementation with iron, SOD, or catalase. The two enzymes might not have access to the interior of the cell and the role of reactive oxygen metabolites in the killing therefore requires further analysis. A brief and incomplete report has suggested NO from iNOS to play a crucial role during murine tularemia (Elkins et al., 2003) whereas another report was contradictory (Chen et al., 2004). The role of iNOS and phox in macrophage killing and control of murine tularemia was studied in paper II and III in this thesis.

Neutrophils suppress the replication of *F. tularensis* LVS during the innate phase of the infection in mice and are critically required to resolve an infection with *F. tularensis* LVS (Sjöstedt et al., 1994). The presence of either CD4^{+} or CD8^{+} T cells is sufficient for eradication of infection while the absence of both subsets renders the mice unable to clear the bacteria during the final phase of the infection (Conlan et al., 1994). In addition, CD4^{+} depleted mice produce a poor IgG antibody response relative to *F. tularensis* LVS infected wild-type mice, and this indicates that antibodies are of minor importance. Nevertheless, there are several findings, which indicate that a humoral immune response is important during an infection with *F. tularensis* LVS. Transfer of *F. tularensis*-specific antibodies to naïve mice prior to challenge protects mice against an otherwise lethal dose of the *F. tularensis* LVS or a virulent strain of subsp. *holarctica* (Stenmark et al., 2003). Moreover, B-cells contribute to protection in an antibody-independent manner (Elkins et al., 1999).

Normally, mice clear a primary infection with *F. tularensis* LVS within 2-3 weeks after inoculation and then develop protective immunity to *F. tularensis* LVS. In the skin, the protective immunity is reflected by a rapid and high expression of TNF-$\alpha$ and IL-12 (Stenmark et al., 1999). These cytokines are evident within 1 day after inoculation while IFN-$\gamma$ is not evident until day 3 of infection. In parallel, the bacterium is contained in the skin and lymph nodes and eradicated within 3 days. Neutrophils contribute to the protective immunity but not to the same extent as during a primary immune response (Sjöstedt et al., 1994). CD4^{+} cells and CD8^{+} T-cells are required for protection only when the rechallenge dose of *F. tularensis* LVS exceeds a certain threshold, proving that T-cell independent mechanisms are also operative in the protective immunity to *F. tularensis* LVS (Conlan et al., 1994). Immunity acquired by the dermal route is also active against lethal doses of *F. tularensis* LVS given by other routes, for example, intravenous, intraperitoneal, and intranasal. Systemic immunity can be detected as intense proliferation of spleen cells from immune mice when exposed to killed *F. tularensis* LVS in vitro.

# C. ROS generating systems

NADPH oxidase, also referred to as phox, is expressed in professional phagocytes such as neutrophils and macrophages. It catalyzes the production of large amounts of $O_2^-$ (~10 mM inside the phagosome) upon activation. The neutrophil is particularly efficient and produces levels 2-3 times higher than those of macrophages. The important role of phox to the immune defense in exemplified by the fact that a defective enzyme results in chronic granulomatous disease (CGD) (Heyworth et. al., 2003). People with CGD have a genetic deficiency of phox components and suffers from recurrent infections. There are also other cellular sources of $O_2$, including xanthine oxidoreductase and homologues of the $gp91^{phox}$ subunit of phox. In addition, the mitochondria and certain metabolic pathways generate $O_2^-$.

## C.1 Phox

Phox is a multimeric enzyme consisting of five subunits, $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ that are constitutively expressed (Babior et al., 2002). Two of the subunits, $p22^{phox}$ and $gp91^{phox}$, constitute the membrane spanning, flavocytochrome b558 (cyt b558). When phox is inactive, the subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ are located in the cytosol. Phosphorylation of $p47^{phox}$ triggers the assembly of the subunits and the subsequent activation of phox. When active, cyt b558 serves as an electron transporter of the phox, facilitating transfer of electrons from cytosolic NADPH, across the membrane to oxygen. Thus, $O_2^-$ will be formed in certain intracellular compartments, for example, in the phagosomes and granules in neutrophils or in the extracellular milieu (Fig. 1).

Phox is activated by various receptor systems, for example, IgG receptors and CR3 (Shao et al., 2003). When macrophages phagocytose IgG-opsonized *Mycobacteria* and *Salmonella* through the Fcγ-gamma receptor, the pathogens ability to block phagosome maturation is prevented and consequently, the bacteria are degraded by mediators of the phagolysosomal pathway (Ismail et al., 2002).

IFN-γ activation of phagocytes enhances the endosomal trafficking for example by upregulation of small GTPases such as Rab5a (Alvarez-Dominguez and Stahl, 1998). Specifically, Rab5a recruits active Rac2 to *L. monocytogenes*-containing vacuoles and thereby facilitates a successful 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 affects the viability of the bacterium negatively, especially during the early phase after uptake of the bacterium (Vazquez-Torres et al., 2000).

There are several homologues of the phagocyte $gp91^{phox}$, and these homologues generate $O_2^-$ levels in the micromolar range. The $gp91^{phox}$ homologues are up-regulated in response to inflammatory mediators such as IFN-γ, LPS, and bacteria (Geiszt and Leto, 2004). Their location in epithelial cells of the salivary gland ducts and along mucosal surfaces of colon, rectum, and major airways imply a role of these enzymes in host innate defense.


## C.2 Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a molybdoflavoenzyme and is generally recognized as the terminal enzyme of purine metabolism (Harrison, 2002). In addition XOR has been reported to contribute to host resistance against invading pathogens and, accordingly, is upregulated by proinflammatory cytokines (Segal et al., 2000; Umezawa et al., 1997). The mammalian enzyme consists of interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (Harrison, 2002). Both forms of the enzyme can reduce $O_2$ to generate $O_2^-$, although XO is more powerful. Under limited oxygen tension, XOR has also been reported to simultaneously generate NO and $O_2^-$ with the subsequent formation of $ONOO^-$. In mammalian tissues, the highest levels of XOR are found in liver and intestine. In particular, the enzyme is found in the endothelia and in epithelial cells and is located in the cytoplasm or anchored to the outer surface of the cell. It is further found freely circulating in the blood in the form of XO, in breast milk and low levels are present in virtually every organ.


## C.3 Mitochondria

The electron transport chain of the mitochondria sequentially passes 2 electrons from NADH with oxygen as the final electron acceptor and water as the end product. However, the electron transport chain is leaky and side reactions with oxygen generate $O_2^-$ (Cadenas and Davies, 2000). It has been estimated that 1-2% of the total daily oxygen consumption is going to mitochondrial $O_2^-$ generation. NO enhances release of $O_2^-$ from the electron transport chain since it binds to one of the components in the chain and thereby inhibits the electron transport

(Cadenas et al., 2001). TNF-$\alpha$ activation of cells also increases the mitochondrial release of ROS.

Monoamine oxidase is an additional source of $O_2^-$ generated by the mitochondria (Cadenas and Davies, 2000). It is localized on the outer mitochondrial membrane and catalyzes the oxidative deamination of biogenic amines. In this reaction, a substantial amount of $H_2O_2$ is released that contributes to increase the steady state concentration of reactive species within both the mitochondria and cytosol.

## C.4 Chemistry of $O_2^-$

$O_2^-$ is a moderately reactive molecule capable of acting both as an oxidant or reductant in biological systems (Hampton et al., 1998). Its relative inactivity allows $O_2^-$ to diffuse considerable distances, although since being charged, it cannot easily pass biological membranes. Nevertheless, extracellularly generated $O_2^-$ can gain access to intracellular targets via cellular ion channels. At physiological pH, $O_2^-$ rapidly reduces itself (dismutation) (half-life within microseconds) to form $H_2O_2$. Dismutation of $O_2^-$ can also occur enzymatically by the action of superoxide dismutase (SOD) (section A.3.3). At low pH, protonation of $O_2^-$ occurs ($HO_2$) and this compound is membrane permeable to the same extent as $H_2O_2$.

$H_2O_2$ is a more reactive molecule compared to $O_2^-$ and because of its neutral charge, $H_2O_2$ readily passes through biological membranes. $H_2O_2$ can react with $O_2^-$ to form $OH^.$ and $OH^-$, the so-called Haber-Weiss reaction or Fenton reaction (Kehrer, 2000). This reaction requires a metal ion as a catalyst. $OH^.$ is a highly reactive molecule and diffusion-limited due to encounter with an oxidizable substrate close to its generation site. In the presence of a peroxidase, for example myeloperoxidase in neutrophils, $H_2O_2$ can react with halides to form hypohalous acid (HOX, X=Cl). $O_2^-$ can also react with NO and this results in the formation of $ONOO^-$ (Lewis et al., 1995). This reaction predominates in a system where both $O_2^-$ and NO are produced. Collectively, $O_2^-$ and $O_2^-$-derived molecules are referred to as reactive oxygen intermediates (ROS). The reactions described above are schematically summarized in Fig. 1.

## C.5 Effects of oxidative stress

In the host defense, professional phagocytes produce ROS for the destruction of the invading pathogens. Persons with a defective phox are prone to repeated infections, thus demonstrating

the importance of ROS derived from phox to the immune defense. ROS may exert its cidal effect via several routes. They oxidize DNA, react with unsaturated fatty acids in membranes thereby affecting ionic channels, inactivate membrane transport proteins or enzymes, and destabilize the lipid bilayer (Kehrer, 2000).

Besides being bactericidal, ROS also alter the signaling pathways in a cell by oxidizing sulfhydryl groups in proteins resulting in disulfide linkage between proteins or by disrupting protein-protein interaction by altering the oxidative state of the binding site. Changes in signaling pathways may modify the activity of growth factors, protein kinases, protein phosphatases and transcription factors (Kehrer, 2000). Examples of signaling pathways activated by $H_2O_2$ are mitogen activated protein kinases (MAP-kinases), and Janus kinase/signal transducers, and activators of transcription (JAK/STAT) (Hancock et al., 2001).

## D. Nitric oxide synthase

Nitric oxide synthase (NOS) is one of the largest enzymes described, 300 kDa (Bogdan, 2001; Nathan and Xie, 1994) and produces NO, one of the smallest and simplest biosynthetic products. There are three different isoforms of NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The killing of many infectious agents including bacteria, protozoa, viruses, and helminths are reported to depend on iNOS-derived NO (Bogdan et al., 2000). NOSs carry out a five-electron oxidation of the non-aromatic amino acid, L-arginine. Citrulline and NO are the end products of the reaction (Fig. 1). The three isoforms differ with respect to their regulation, the amplitude and duration of the NO production, and their cellular distribution.
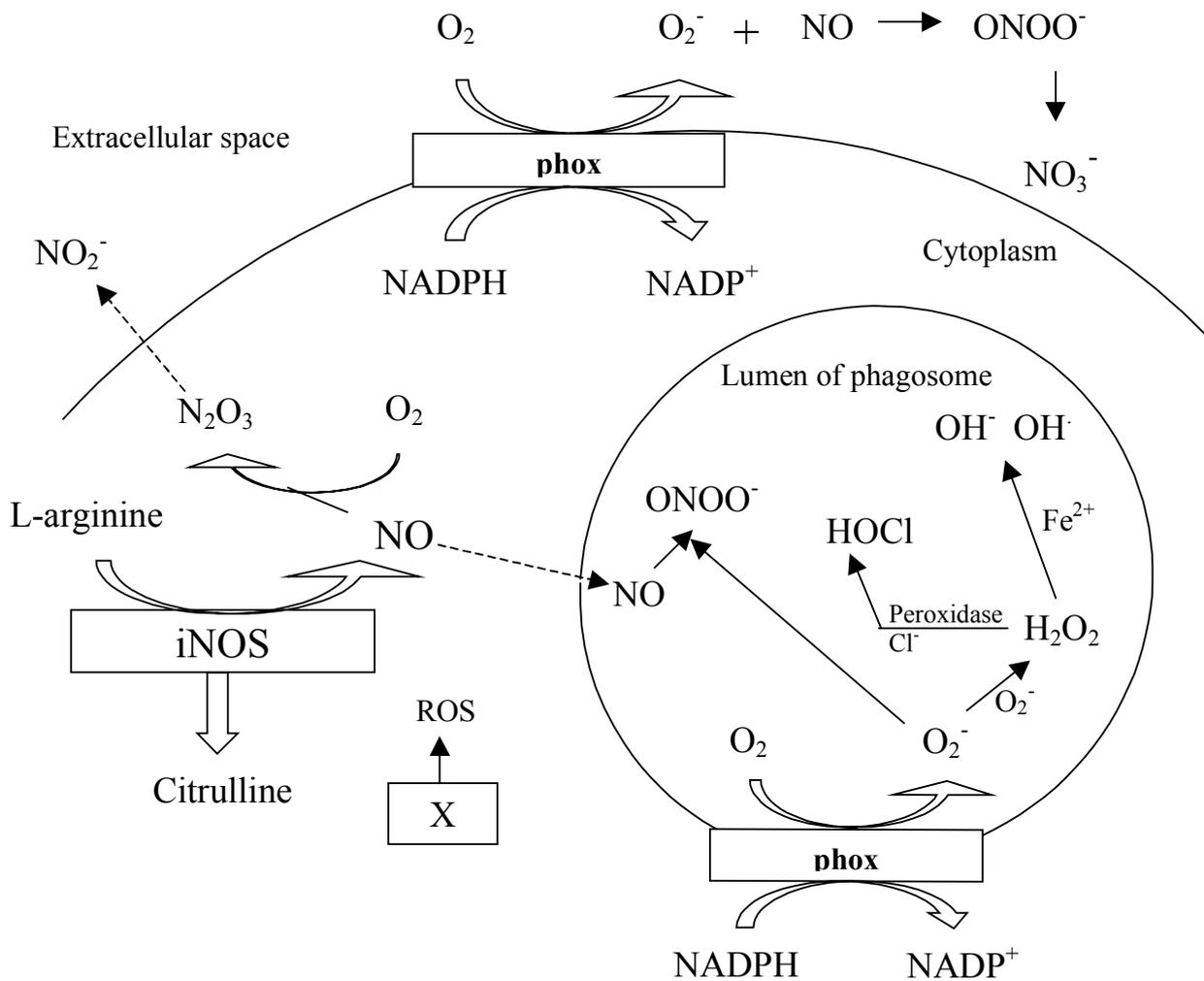
Fig. 1. Schematic illustration of ROS and RNS generation from phox and iNOS, respectively

## D.1 Regulation

eNOS and nNOS are constitutively expressed in cells but the magnitude of expression can be regulated at the transcriptional level (Forstermann et al., 1998). iNOS is tightly regulated (Bogdan, 2001; Nathan and Xie, 1994). Cytokines, immune complexes, and microbial or viral products regulate the expression of iNOS. The activity of iNOS can be modified at the transcriptional, mRNA, or protein levels. Murine macrophages are the best-studied model of iNOS gene regulation. NF-κB and Stat-1-α are critical transcription factors regulating the iNOS gene. Upon IFN-γ activation, Stat-1-α is phosphorylated and thereby translocated to the nucleus and enhances transcription of the iNOS gene. A regulatory circuit is seen between

NF-κB and NO. Early in macrophage activation, low levels of NO (0.1-10 μM) activate NF-kB and, in turn, up-regulate iNOS transcription. High levels of NO (0.2-0.5 mM) inhibit NF-kB and thereby prevent transcription of the iNOS gene and overproduction of NO.

Transforming growth factor-β (TGF-β) and cytokines produced by the Th2 cell subset negatively regulate iNOS activity (Bogdan, 2001). TGF-β inhibits the transcription of the iNOS gene, destabilizes iNOS mRNA, and induces increased iNOS protein degradation by the proteasome pathway. It is important to stress, though, that there is species-specific regulation of iNOS. One example is the human iNOS promoter region, which significantly differs from the murine promoter region (Kroncke et al., 1998; Pance et al., 2002). A nucleotide exchange of the human LPS/IFN-γ enhancer region of the iNOS gene makes it hyposensitive to LPS/IFN-γ. Also, NF-κB binds to the human iNOS promoter more weakly than to the murine iNOS promoter.

Availability of L-arginine is another limiting factor for the high output of NO by iNOS, and depends on an extracellular source of the amino acid (Mori and Gotoh, 2000). LPS stimulation of macrophages increases the L-arginine uptake. Extracellular concentration of L-arginine is strongly modulated by arginase. This enzyme degrades L-arginine to urea and ornithine. The Th2 cytokine TGF-β is known to strongly increase arginase activity. The aforementioned regulatory mechanisms of iNOS activity are only some of many described mechanisms. The tight regulation of iNOS reflects the need of a high output of NO only during certain periods of the immune response.


**D. 2 Chemical properties of NO**

NO is a simple diatomic molecule with physico-chemical and biological properties determined by its small size, absence of charge and single unpaired electron (Stamler et al., 1992). It is readily diffusible in body fluids and tissues and freely crosses cell membranes (Wink and Mitchell, 1998). The chemical half-life of NO is irreversibly proportional to its concentration. At 1 mM, its half-life is less than one second, whereas at 1 μM, its half-life is 10-15 min. Hence, the half-life of NO is extended as its concentration declines. Therefore the fate and effects of NO is strongly dependent on its initial concentration but also on the environment of production. Conditions that influence the fate and effect of NO will be described below and are schematically summarized in Fig. 2.

**D.3 Effects of NO**

At low concentrations (picomolar-nanomolar), NO reacts directly with metallo-proteins and other radicals. Examples of direct effects are activation of guanylate cyclase to produce cGMP, which in turn influences a variety of cellular responses. At low concentration NO is also thought to be anti-apoptotic, terminate lipid peroxidation induced by ROS and to act as a pro-inflammatory molecule (Bogdan, 2001). These direct effects of NO are often associated with eNOS and nNOS since these enzymes produce low levels of NO. iNOS produces high levels of NO but since NO is highly diffusible in biological systems, it will reach lower concentrations further from its source. Here it can exert direct effects as seen with NO produced from eNOS and nNOS.

At high concentrations as when produced from iNOS, NO is rapidly oxidized to reactive nitrogen species (RNS) and the effects of NO are thereby indirect (Wink and Mitchell, 1998). $N_2O_3$ is the major reaction product of $NO/O_2$ in an environment with low concentrations of reactive oxygen species. NO and $O_2$ are concentrated in biological membranes due to their increased solubility in lipids. Therefore, even in a cell that does not produce high concentrations of NO, the $NO/O_2$ reaction might proceed within membranes. The reaction between NO and $O_2^-$ occurs at near diffusion-controlled rates with a rate constant of $\sim 10^{10}\,M^{-1}\,S^{-1}$ (Wink and Mitchell, 1998). Thus, in a biological system, such as an activated macrophage, where high concentration of $O_2^-$ and NO are generated simultaneously, $ONOO^-$ will be the major RNS produced. $N_2O_3$ preferentially nitrosylates amines and thiol groups in proteins whereas $ONOO^-$ and its derivatives are powerful oxidants and nitrants. The reactions described above are schematically summarized in Fig. 1.
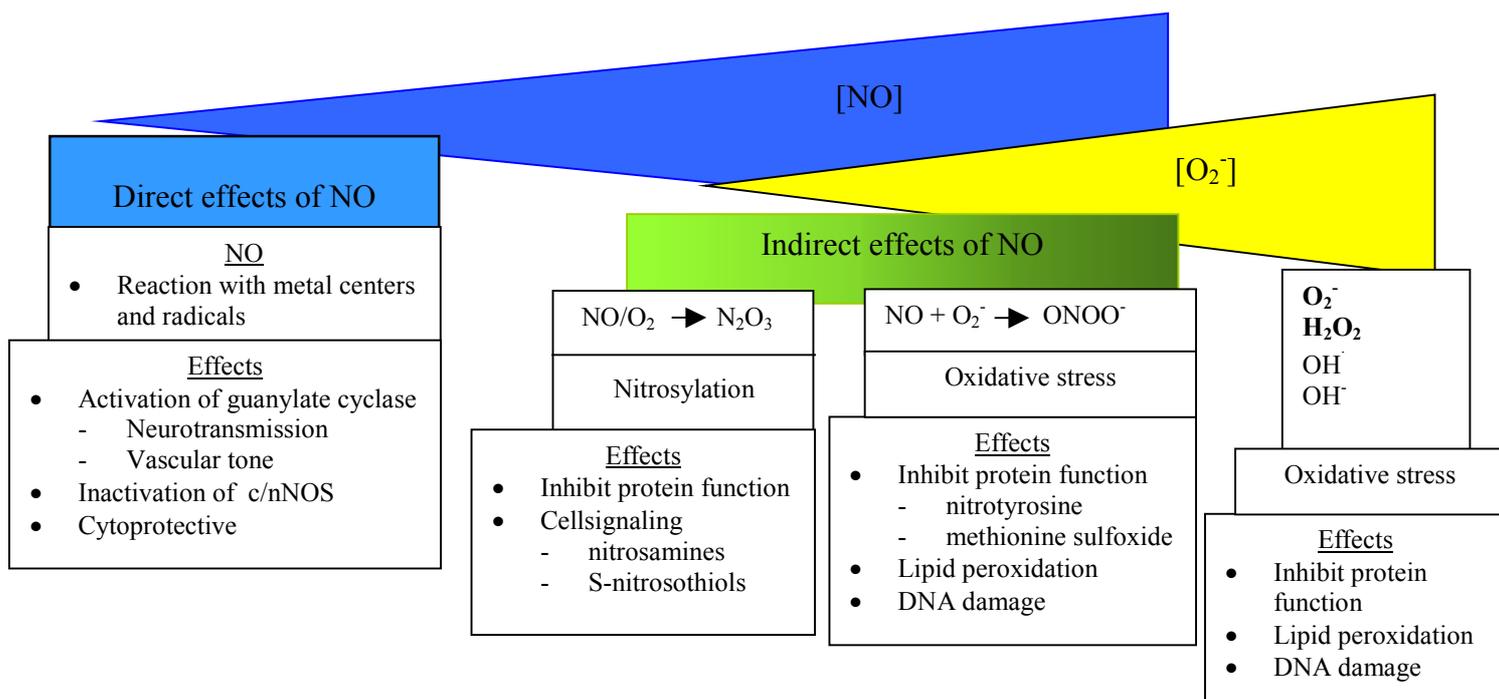
**[NO]**    **[O$_2^-$]**

**Direct effects of NO**

NO
- Reaction with metal centers and radicals

Effects
- Activation of guanylate cyclase
  - Neurotransmission
  - Vascular tone
- Inactivation of c/nNOS
- Cytoprotective

**Indirect effects of NO**

$NO/O_2 \rightarrow N_2O_3$

Nitrosylation

Effects
- Inhibit protein function
- Cellsignaling
  - nitrosamines
  - S-nitrosothiols

$NO + O_2^- \rightarrow ONOO^-$

Oxidative stress

Effects
- Inhibit protein function
  - nitrotyrosine
  - methionine sulfoxide
- Lipid peroxidation
- DNA damage

**O$_2^-$**
**H$_2$O$_2$**
OH$^.$
OH$^-$

Oxidative stress

Effects
- Inhibit protein function
- Lipid peroxidation
- DNA damage

Fig. 2. Chemistry and effects in environments with different concentrations of O$_2^-$ and NO. At low concentrations of NO, the direct effects will dominate. At high concentrations of NO, but low concentrations of O$_2^-$, nitrosative chemistry will dominate. When NO and O$_2^-$ are produced simultaneously, nitration and oxidative stress will dominate. At high levels of ROS, low levels of NO are cytoprotective by terminating the ROS-induced oxidative damage for example of lipid peroxidation.

### D.4.1 Nitrosylation, nitration and oxidation in bacterial killing

Nitrosylation of proteins in a bacterium involved in metabolism, respiration or with antioxidant properties might inhibit their function and thereby affect the viability of the bacteria. However, nitrosylation of proteins is reversible since a nitrosylated protein is easily denitrosylated by cellular reductants, shifts in pH and pO$_2$, transition metals and thiols (Mannick and Schonhoff, 2002). Hence, it is not until the reducing capacity in a bacterium is exhausted that nitrosylation affects its viability. Furthermore, nitrosylation might benefit the bacterium by activating transcription factors that induce transcription of genes encoding proteins that metabolize RNS and ROS. For example, the OxyR transcription factor of *E. coli* is activated by both H$_2$O$_2$ and by nitrosylating agents and genes under control of OxyR encodes proteins that metabolize nitrosants (Marshall et al., 2000).

ONOO$^-$, and derivatives thereof, nitrosate tyrosine and tryptophan residues and oxidize methionine in proteins, and similar to nitrosylation of proteins, this might affect the viability

of the bacterium negatively (Radi, 2004). Nitration is an irreversible modification and is therefore a more toxic event compared to nitrosylation (Mannick and Schonhoff, 2002). Oxidized methionine, however, might be reduced by methionine sulfoxide reductase. Lipid peroxidation is another effect induced by ONOO$^-$ that disrupts the membrane integrity of the cell and thereby enhances the access of toxic radicals to the interior of the bacterial cell (Bogdan, 2001). Further, ONOO$^-$ and derivatives thereof damage DNA and, if at the same time DNA repair proteins are modified and thereby inhibited, this is deleterious to the invading pathogen.

The resistance/sensitivity of a given bacterium to different RNS is dependent on its ability to prevent or reverse the oxidative, nitration and nitrosylation event. In section A.3.3., examples of systems utilized for this purpose are described.

### D.4.2 *NO modulates the immune response*

At an early stage of infection, NO acts as a pro-inflammatory molecule, inducing vasodilation and the recruitment of neutrophils (Bogdan, 2001). NK-cell development and cytotoxicity have also been reported to partly rely on NO (Cifone et al., 2001). Moreover, NO influences the type of immune response elicited. Low levels of NO in combination with IL-12 during the differentiation phase of Th0 cells, will favor the development Th1-cells and thus an inflammatory immune response (Niedbala et al., 1999). RNS also acts as a suppressor of the immune response. For example, RNS inhibit T-cell proliferation and induces apoptosis of T-cells since lymphocytes have a minimal capacity to replenish their GSH content (Kroncke et al., 2001). High concentrations of NO down-regulate the expression of members of different adhesion molecule families and thereby inhibit the recruitment of inflammatory cells to the infection site (Bogdan, 2001).

# AIMS OF THE THESIS

- To investigate the mechanisms behind the IFN-γ-induced killing of *F. tularensis* LVS by macrophages. Specifically we asked:

    - is the escape of *F. tularensis* LVS from the phagosome inhibited in IFN-γ activated macrophages?
    - are phox-dependent mechanisms involved in the killing of *F. tularensis* LVS?
    - are iNOS-dependent mechanisms involved in the killing of *F. tularensis* LVS?

- To establish if similar killing mechanisms are active against virulent strains of *F. tularensis*.

- To investigate the roles of iNOS- and phox-derived radicals to the control of murine tularemia

- To investigate if Cu,Zn-SOD expressed by *F. tularensis* LVS contributes to the virulence of the bacterium.

- To study if the expression of IglC is required for *F. tularensis* to escape from the phagosome.

## RESULTS AND DISCUSSION

### E. Factors affecting the escape of *F. tularensis* LVS from the phagosome (paper I)

*F. tularensis* degrades the phagosomal membrane and proliferates in the cytoplasm of macrophages (Golovliov et al., 2003a) but is efficiently killed when macrophages are activated with IFN-γ (Fortier et al., 1992). The intracellular growth of *F. tularensis* LVS requires the expression of a 23-kDa protein denoted IglC (Golovliov et al., 2003a). The mechanism behind the escape of *F. tularensis* LVS from the phagosome is unknown and in this work we asked if IglC is involved in this process. We also investigated if the IFN-γ induced killing of *F. tularensis* LVS was related to containment of the bacterium in the phagosome.

Adherent thioglycolate-elicited peritoneal macrophages (PEC) from C57BL/6 mice were infected in vitro with either *F. tularensis* LVS or Δ*iglC* (a deletion mutant of *F. tularensis* LVS lacking expression of IglC). Electron microscopy of infected macrophages revealed that > 99% of Δ*iglC* bacteria were located in phagosomes with intact membranes. In contrast, 97% of *F. tularensis* LVS were localized free in the cytoplasm. Similarly to a previous study (Golovliov et. al), Δ*iglC* was unable to multiply intracellularly, and 85% of the bacteria were killed by 18 h. In contrast, the number of *F. tularensis* LVS increased 1.6 $\log_{10}$ CFU within 18 h. Thus, expression of IglC is necessary for *F. tularensis* LVS to escape from the phagosome. We suggest that escape from the phagosome is a necessary step that *F. tularensis* LVS has to complete before being able to proliferate intracellularly.

In IFN-γ activated cells, the number of *F. tularensis* LVS bacteria that was observed within phagosomes with intact membranes was significantly higher than in cells without IFN-γ treatment, 20% versus 3% respectively ($p < 0.0001$). A great majority, >99%, of Δ*iglC* bacteria were found in phagosomes with intact membranes irrespective of IFN-γ treatment. IFN-γ activated macrophages killed both bacteria; at 5 h 92% of the Δ*iglC* bacteria and 50% of *F. tularensis* LVS bacteria were killed. By 18 h, 93% of *F. tularensis* LVS bacteria were killed and Δ*iglC* were eradicated. In summary, by IFN-γ activation of the macrophages, escape of *F. tularensis* LVS from the phagosome was partly prevented and the bacteria killed. However, virtually all Δ*iglC* bacteria were retained in the phagosome and they were significantly more susceptible to the IFN-γ induced killing than *F. tularensis* LVS.

The general view is that professional phagocytes when activated by cytokines, such as IFN-γ, counteract the survival strategies of intracellular pathogens and direct them into to the phago-lysosomal pathway for destruction. The high susceptibility of Δ*iglC* to the IFN-γ-induced killing indicates that the membrane-enclosed compartment is bactericidal for *F. tularensis*. It is possible that *F. tularensis* LVS during the initial phase of the infection in IFN-γ activated macrophages resides in such compartments and becomes irreversibly damaged although managing to escape into the cytoplasm. It is also possible that *F. tularensis* LVS is exposed to bactericidal molecules in the cytoplasm. Moreover, it is likely that the retention of *F. tularensis* LVS in the phagosome is more efficient in vivo when the macrophages are activated not only by IFN-γ but also by other cytokines.

It is unlikely that IglC directly protects the bacterium against killing, because our unpublished findings indicate that the phenotypes of mutants lacking expression of either the IglB or IglD proteins are similar in susceptibility to macrophage defense to that of Δ*iglC*. Moreover, a deletion mutant lacking expression of a regulator of the *igl* operon, MglA, has a similar phenotype (Lauriano et al., 2004). Altogether, our data indicate that, most likely, the coordinated regulation and joint expression of all proteins of the *igl* operon are necessary for the escape of *F. tularensis* LVS from the phagosome and its efficient adaptation to the intracellular habitat.

### F. The contribution of reactive nitrogen and oxygen species to the killing of *F. tularensis* LVS by murine macrophages. (paper II)

In paper II we further investigated the mechanisms behind the IFN-γ-induced killing of *F. tularensis* LVS by macrophages. IFN-γ induces the expression of iNOS in macrophages, which catalyzes the production of high levels of NO and primes cells for efficient phox-dependent $O_2^-$ production (Bogdan et al., 2000; Prada-Delgado et al., 2001). NO and $O_2^-$ are precursors of other RNS and ROS, which are highly reactive and known to contribute to the killing of many intracellular bacterial pathogens. Adherent inflammatory peritoneal macrophages from iNOS$^{-/-}$ mice and p47$^{phox-/-}$ mice and control littermate mice were infected with *F. tularensis* LVS to delineate the roles of RNS and ROS in the killing of the bacterium.

## F.1 Role of iNOS-derived RNS

Within 6 h of infection, IFN-γ-activated macrophages from iNOS$^{+/+}$ mice killed 97% of internalized bacteria, as compared to 28% by macrophages from iNOS$^{-/-}$ mice. At 24 h, macrophages from iNOS$^{-/-}$ mice had eradicated most of the bacteria. This delayed killing of *F. tularensis* LVS in macrophages from iNOS$^{-/-}$ mice demonstrated that: i) iNOS-derived NO is a major mediator of the IFN-γ induced killing; ii) killing mechanisms independent of iNOS contribute to the killing. Addition of NMMLA, a competitive inhibitor of iNOS, to macrophage cultures from iNOS$^{+/+}$ mice reduced the killing capacity to that observed in macrophage cultures from iNOS$^{-/-}$ mice.

## F.2 Role of phox-derived ROS

Within 24 h of infection, IFN-γ-activated macrophages from p47$^{phox-/-}$ mice eradicated *F. tularensis* LVS as efficiently as did macrophages from p47$^{phox+/+}$ mice. Also macrophages from p47$^{phox-/-}$ mice treated with NMMLA still expressed a bactericidal effect, thus demonstrating the presence of killing mechanisms independent of both iNOS and phox. However, the residual killing in macrophages without both a functional phox and iNOS was even less potent than in macrophages without a functional iNOS since eradication was not achieved at 48 h. Hence, phox-dependent mechanisms are partly responsible for the retained bactericidal activity in macrophages that do not produce NO from iNOS.

In summary, the killing of *F. tularensis* LVS by IFN-γ-activated macrophages was highly dependent on NO from iNOS. In the absence of iNOS, the killing partly relied on phox but in its presence, phox was dispensable. In addition, IFN-γ-activated macrophages possessed mechanisms independent of both iNOS and phox, which were weakly bactericidal against *F. tularensis* LVS.

### *F.2.1 Role of ONOO$^-$*

In a cell-free assay we generated NO and ONOO$^-$, a reaction product of NO and O$_2^-$, and determined the effects of these components on *F. tularensis* LVS. NO generated from SNAP did not kill *F. tularensis* LVS during a 6 h incubation period. In contrast, ONOO$^-$, when generated from SIN-1, efficiently killed *F. tularensis* LVS.

To find out whether NO mediated the IFN-γ-induced killing in the form of ONOO$^-$ in macrophages, FeTPPS, a decomposition catalyst of ONOO$^-$, was supplied to the IFN-γ activated macrophages. The effect of FeTPPS was striking; the bactericidal effect elicited by IFN-γ was completely blocked and macrophages, irrespective of mouse origin or treatment, supported growth of *F. tularensis* LVS to a similar extent as non-activated cells. The effect of FeTPPS strongly suggests that ONOO$^-$ mediated the IFN-γ-induced killing in macrophages from iNOS$^{+/+}$, iNOS$^{-/-}$, p47$^{phox+/+}$, and p47$^{phox-/-}$ mice and also when cells were treated with NMMLA.

### F.2.2 Data supporting the formation of ONOO$^-$

The rate of formation of ONOO$^-$ from O$_2^-$ and NO is rapid ($\sim 10^{10}$ M$^{-1}$ S$^{-1}$) but highly dependent on the concentration of and distance between the two reactants (Wink and Mitchell, 1998). In our experimental system (paper II), IFN-γ–activated macrophages from iNOS$^{+/+}$ and p47$^{phox+/+}$ produced NO, measured indirectly as NO$_3^-$ and NO$_2^-$ accumulation in the culture supernatants, and exhibited an enhanced capacity to produce O$_2^-$ from phox as determined by luminol-dependent chemoluminescence. Thus, the chemical environment in these cells favors the formation of ONOO$^-$. In addition, the ratio of NO$_3^-$ and NO$_2^-$ was above 1.0 and since ONOO$^-$ decomposes mostly to NO$_3^-$ (Lewis et al., 1995; Pfeiffer et al., 1997), the high ratio further supports the conclusion that ONOO$^-$ is formed in cells from iNOS$^{+/+}$ and p47$^{phox+/+}$ mice. As a comparison, the ratio of NO$_3^-$ and NO$_2^-$ is 0.3 when NO is autooxidated (NO + O$_2$) and not consumed in the reaction with O$_2^-$ to generate ONOO$^-$ (Lewis et al., 1995; Pfeiffer et al., 1997). These results support the assumption that ONOO$^-$ was formed in the macrophage cultures and thereby strengthen the conclusion that the effect of FeTPPS was due to removal of ONOO$^-$.

### F.2.3 Mechanisms behind ONOO$^-$ formation

The results of paper II showed that macrophages genetically deficient in phox and deprived of NO formation still killed *F. tularensis* LVS, whereas macrophages deprived of ONOO$^-$ completely lost their ability to contain the bacteria. Thereby it can be postulated that a source of ONOO$^-$ that is independent of phox and iNOS exists. The mitochondria constitute such an alternative source of O$_2^-$ and the production may result from NO-mediated inhibition of the mitochondrial respiration (Wink and Mitchell, 1998). Production of O$_2^-$ by xanthine oxidase

is another alternative source (Harrison, 2002). Possibly, macrophages from p47$^{phox-/-}$ mice produced ONOO$^-$ via one or several of such alternative pathways. Alternative sources of NO are eNOS, nNOS or mtNOS, which could lead to formation of ONOO$^-$ in the absence of iNOS. There are also alternative chemical reactions that may yield ONOO$^-$ as an end product, for example, from the reaction between NO$^-$ and O$_2^-$. NO$^-$ is produced in a number of biological processes and readily available (Kirsch and de Groot, 2002). In addition, both xanthine oxidase and xanthine dehydrogenase have been reported to produce NO and O$_2^-$, resulting in formation of ONOO$^-$ (Harrison, 2002). Thus, ONOO$^-$ formation may occur even in the absence of phox, iNOS, or both of these enzymes. Most likely, the pathways described above are mainly compensatory as our results indicated that in the presence of iNOS, NO is a main source for ONOO$^-$ formation.

In essence, based on several lines of direct and indirect evidence, the results presented in paper II suggest that ONOO$^-$ has a major role in the killing of *F. tularensis* LVS by IFN-γ-activated murine macrophages. iNOS-dependent NO production contributes significantly to the formation of ONOO$^-$ whereas O$_2^-$ generated from phox plays a less important role.

### G. Importance of Cu,Zn-SOD to the macrophage virulence of *F. tularensis* (paper IV)

Cu,Zn-SOD belongs to a family of enzymes that efficiently catalyze the dismutation of O$_2^-$ to H$_2$O$_2$ (Battistoni, 2003). Cu,Zn-SOD is often located in the periplasm of gram-negative bacteria although there are examples of bacteria where the enzyme is anchored to the membrane or secreted. Because of its location, Cu,Zn-SOD has the potential to protect against ROS generated from external sources. Accordingly, it has also been shown to be a virulence mechanism in many bacteria. In paper IV, we asked if Cu,Zn-SOD contributes to the virulence of *F. tularensis*. To this end, a Cu,Zn-SOD mutant, Δ*sodC*, was constructed in *F. tularensis* LVS by allelic replacement. In a first set of experiments the mutant phenotype was analyzed with regard to proliferation and survival in inflammatory adherent peritoneal macrophages from mice.

As opposed to the wild type of *F. tularensis* LVS, Δ*sodC* failed to proliferate in macrophages from p47$^{phox+/+}$ and p47$^{phox-/-}$ mice. IFN-γ activated macrophages efficiently killed both *F. tularensis* LVS and Δ*sodC*, eradication being achieved within 24 h. When NMMLA, a competitive inhibitor of iNOS, was supplied, the killing of LVS was partly inhibited whereas

killing of $\Delta sodC$ was as efficient as in its absence. Thus, killing of $\Delta sodC$ by IFN-γ-activated macrophages was fully efficient independent of NO.

In conclusion, Cu,Zn-SOD allows *F. tularensis* LVS to proliferate intracellularly and protects the bacterium against killing by IFN-γ-activated macrophages that do not produce NO from iNOS. In addition, the results provide evidence that macrophages produce ROS in the absence of phox since $\Delta sodC$ was as attenuated in macrophages from p47$^{phox-/-}$ mice as in macrophages from p47$^{phox+/+}$ mice.

The failure of Ft-Cu,Zn-SOD to protect *F. tularensis* LVS against killing in macrophages with an intact NO production may be due to the rapid reaction of NO with $O_2^-$ ($k = \sim 10^{10}\,M^{-1}\,s^{-1}$). Thus, at high levels of NO, Ft-Cu,Zn-SOD may no longer have access to its substrate, since $O_2^-$ is consumed in the reaction with NO. As a result, ONOO$^-$ will be formed and the bacteria killed. In the absence or at low levels of NO, ONOO$^-$ may still be formed in macrophages and contribute to the killing of *F. tularensis* LVS, as suggested in paper II. But under these conditions, the levels of ROS likely increase since the ROS precursor $O_2^-$ is not consumed at the same extent to form ONOO$^-$. Thus, at low concentrations of NO, Ft-Cu,Zn-SOD dismutates $O_2^-$ and may together with other ROS-detoxifying enzymes, such as catalase, effectively protect the bacterium against damage and death.

SodCI, which is the best characterized of three Cu,Zn-SODs expressed by *S. typhimurium* (St-Cu,Zn-SOD) has the highest catalytic activity ever reported for a naturally occurring enzyme ($1.3 \times 10^{10} M^{-1}\,s^{-1}$) (Pesce et al., 2000). A Cu,Zn-SOD deletion mutant of *S. typhimurium* (St-$\Delta sodC1$) is attenuated with regard to survival in IFN-γ-activated phagocytes and virulence in mice (De Groote et al., 1997). However, abrogation of either the host expression of iNOS or phox restores the virulence of St-$\Delta sodC1$. Based on these results the authors suggested that the major role of St-Cu,Zn-SOD was to dismutate $O_2^-$ and thereby prevent the formation of toxic reactants, presumably ONOO$^-$. Thus, St-Cu,Zn-SOD has the potential to compete for its substrate $O_2^-$ even in the presence of high levels of NO, probably due to its high catalytic activity. These results further imply that, relative to St-Cu,Zn-SOD, the catalytic activity of Ft-Cu,Zn-SOD is weak, since it did not protect *F. tularensis* LVS against killing in the presence of high levels of NO.

**H. Growth and survival of fully virulent strains of *F. tularensis* in murine macrophages (Additional data)**

The reason for the attenuation of *F. tularensis* LVS has not been identified. The comparative data that exist show that fully virulent strains of subspecies *holarctica* and *tularensis* grow unrestrictedly in mice (Chen et al., 2004) and induce cytopathogenicity in murine macrophages to a greater extent than does LVS (Mack, 1994). These observations might be explained by faster replication of virulent strains in professional phagocytes and this was investigated. Furthermore, we tested if fully virulent strains are killed by macrophages in an iNOS-dependent manner as demonstrated for *F. tularensis* LVS (paper II). To this end, inflammatory adherent peritoneal macrophages from mice were infected with *F. tularensis* subsp. *holarctica* strain 200 (type B), *F. tularensis* subsp. *tularensis* strain SCHU S4 (type A), or *F. tularensis* LVS and analyzed with regard to their ability to control the number of intracellular bacteria.

In untreated macrophages, the number of type A and type B bacteria increased almost 1.0 $\log_{10}$ already during the first 6 h while the number of LVS did not increase (Fig 3A). By 20 h, all three strains had increased $>1\log_{10}$ but counts of type A and B bacteria were $>1 \log_{10}$ higher ($p < 0.05$ versus LVS for both strains), than *F. tularensis* LVS (Fig. 3B). In summary, the initiation of proliferation of the fully virulent strains was faster compared to LVS but thereafter the generation time of the three strains appeared to be similar.

As shown before, killing of *F. tularensis* LVS in macrophages activated with IFN-γ was highly effective (paper II). At 6 h, 89% of internalized *F. tularensis* LVS bacteria were killed. In contrast, no significant killing of type A and type B bacteria occurred (Fig. 3). Thereafter, the viability of all three strains decreased and at 20 h, the number of type A and type B were significantly higher than of LVS (Fig. 3). Thus, the fully virulent strains of *F. tularensis* were more resistant to the IFN-γ induced killing by macrophages than LVS.
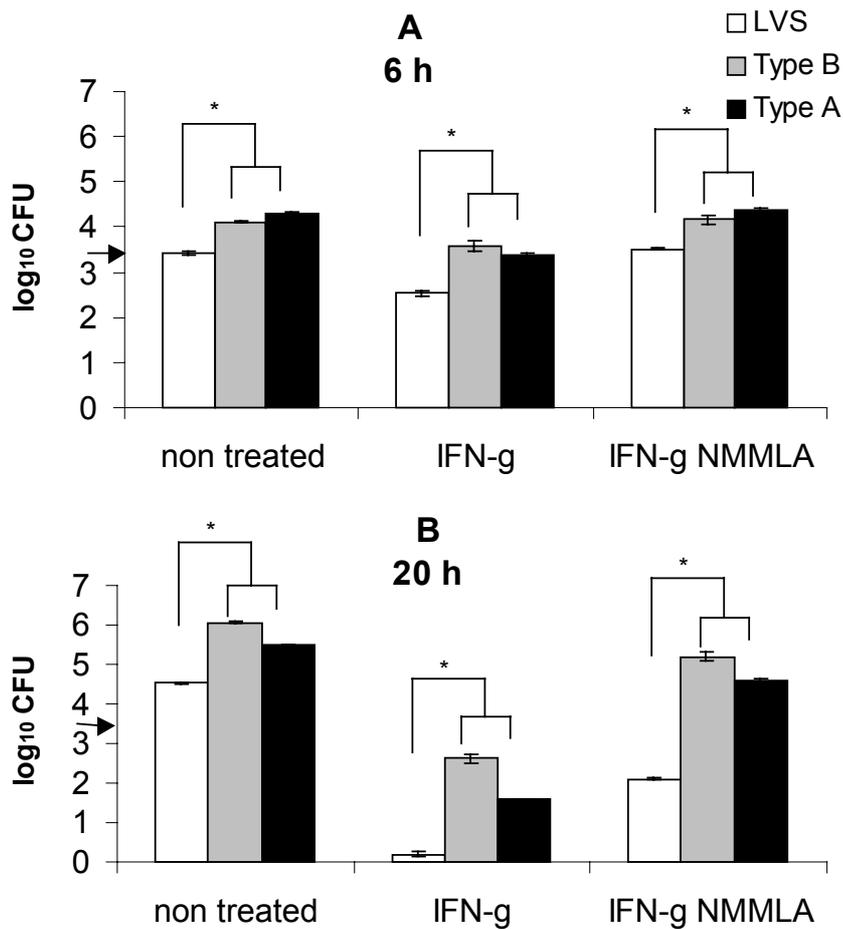
Fig. 3. Number of intracellular *F. tularensis* LVS (LVS), type B and type A bacteria in infected murine macrophages. Open bars represent unstimulated cells and gray bars cell cultures activated with IFN-γ. Black bars represent IFN-γ-activated cell cultures supplemented with NMMLA. Arrows indicate the inoculum at the start of infection. The lower limit of detection was 1.0 $\log_{10}$ CFU and if the average bacterial numbers were below this level, they are indicated as 0.2 $\log_{10}$ in the graphs. Error bars represent standard error of mean of triplicate samples. * $p < 0.05$ according to the Wilcoxon rank sum test.

In macrophages treated with IFN-γ and NMMLA, the fully virulent strains type A and type B proliferated but not as efficient as in untreated macrophages (Fig. 3). In contrast, this treatment only partly inhibited the killing of *F. tularensis* LVS (Fig. 3 and paper II). Thus, the IFN-γ-induced killing of the fully virulent strains relied entirely on iNOS-derived NO, while

killing of *F. tularensis* LVS occurred in the absence of iNOS, although, not as efficiently as in its presence.

In conclusion, the fully virulent strains of *F. tularensis* started their intracellular replication more rapidly after uptake than did *F. tularensis* LVS. The fully virulent strains were susceptible to iNOS-derived RNS and, in fact, RNS was a prerequisite to kill these bacteria. If ONOO⁻ is the toxic RNS executing the killing of the virulent strains, as suggested for *F. tularensis* LVS, will be examined in future experiments by supplementation of FeTPPS to the cultures. The more successful intracellular replication of fully virulent strains revealed in the present experiments may contribute to explain their higher virulence compared to *F. tularensis* LVS.

## I. Distinct roles of reactive nitrogen and oxygen species to control infection with *F. tularensis* (paper III).

RNS and ROS formed as a result of iNOS and phox activity, respectively, can act as direct bactericidal molecules but also as immunoregulatory molecules in vivo (Bogdan, 2001). In the study described in paper III, we investigated the role of the molecules in the murine host response to *F. tularensis* LVS. To this end, iNOS (iNOS$^{-/-}$) or phox (p47$^{phox-/-}$) gene-deficient mice and control mice (iNOS$^{+/+}$ and p47$^{phox+/+}$) were inoculated intradermally with *F. tularensis* LVS. The viability of *F. tularensis* LVS in specified organs/tissues were followed at different time-points after inoculation. In addition, the resulting immune response was characterized with respect to the cytokine response and the development of *F. tularensis*-specific T-cells.

### I.1 Role of phox

Phox was critically important for the control of murine tularemia. In p47$^{phox-/-}$ mice, the LD$_{50}$ by intradermal infection was found to be 4.4 x 10$^3$ CFU of *F. tularensis* LVS and this was almost 200-fold lower than in p47$^{phox+/+}$ mice. After an intradermal inoculation of 5 x 10$^2$ log$_{10}$ CFU of *F. tularensis* LVS, the number of bacteria on day 4 was > 1.0 log$_{10}$ higher in spleen and liver of p47$^{phox-/-}$ than in p47$^{phox+/+}$ mice. This difference in the bacterial numbers was consistent throughout the infection period. Nevertheless, after day 4, both p47$^{phox-/-}$ and p47$^{phox+/+}$ mice acquired the ability to restrict infection and finally to eradicate the bacteria. In contrast, bacterial numbers in the skin of p47$^{phox-/-}$ mice did not differ from

those of p47$^{phox+/+}$ mice during the first 7 days. Thus, the increased number of *F. tularensis* LVS in spleen and liver of p47$^{phox-/-}$ mice was not related to loss of control of infection at the inoculation site. Hence, the high susceptibility of p47$^{phox-/-}$ mice was most likely related to faster replication of *F. tularensis* LVS in spleen and liver during the first days of infection.

When p47$^{phox-/-}$ mice received a higher inoculum, 5 x 10$^4$ CFU of *F. tularensis* LVS, the role of phox during the first days of infection was even more pronounced. On day 5, the number of bacteria in liver of p47$^{phox-/-}$ mice reached 7.25 log$_{10}$ CFU which was > 2.5 log higher than in p47$^{phox+/+}$ mice. In addition, bacteria disseminated to lung, blood and peritoneum of p47$^{phox-/-}$ mice but were not detected in p47$^{phox+/+}$ on day 5 of infection or thereafter. These results further demonstrate that the susceptibility of p47$^{phox-/-}$ mice is related to loss of control of the infection during the first days of infection.

Compared to previously studied IFN-γ- and TNF-α-deficient mice (Sjöstedt et al., 1996), p47$^{phox-/-}$ mice controlled an infection relatively well. In the absence of IFN-γ or TNF-α, mice succumb to an infection with *F. tularensis* LVS within one week; bacterial loads in spleen and liver exceeding 9 log$_{10}$ (Sjöstedt et al., 1996). The comparatively high resistance of p47$^{phox-/-}$ mice demonstrates that other mechanisms besides those dependent on phox are operative during the innate immune phase to control replication of *F. tularensis*. In line with this, p47$^{phox-/-}$ mice secreted IFN-γ with the same kinetics as p47$^{phox+/+}$ mice. Surprisingly, TNF-α was not detected in serum from any mouse strain infected. However, none of the mouse strains here used exhibited an exacerbation of the same magnitude as described for TNF-α-deficient mice. Therefore it seems likely that low, but still biologically significant levels of TNF-α is produced in skin, liver and spleen, and contributes to local control of *F. tularensis* in vivo.

Collectively, our data indicate an important role of phox-dependent ROS for control of the early phase of the *F. tularensis* infection, when innate immune mechanisms are operative. Nevertheless, when given a low inoculum, p47$^{phox-/-}$ mice resolved the infection, demonstrating that an adaptive immune response develops also in p47$^{phox-/-}$ mice. Thus, phox-dependent mechanisms seem to prevent *F. tularensis* LVS from reaching numbers that would overwhelm the developing T-cell-mediated immune response.

### I.1.1 Possible mechanisms behind the phox-dependent control of murine tularemia

The high susceptibility of p47$^{phox-/-}$ mice may be related to a lack of phox-dependent macrophage-mediated effector mechanisms. According to results of paper II, however, killing of *F. tularensis* LVS by IFN-γ-activated macrophages was independent of phox. There may be discrepancies between the conditions encountered in vitro and in vivo that could explain this difference. One explanation derives from experiments on mice deficient in neutrophils.

Neutrophils have been shown to play a crucial role for control of murine tularemia (Sjöstedt et al., 1994). An important early function of neutrophils is to lyse infected hepatocytes, which otherwise permit the unrestricted intracellular growth of *F. tularensis* LVS (Conlan and North, 1992). Neutrophils lyse infected host cells via a ROS-dependent mechanism (Dallegri et al., 1984). Hence, the phox-dependent control of bacterial replication during the innate phase of the infection could be mediated via host cell lysis. Alternatively, *F. tularensis* may be killed when ingested by murine neutrophils although such killing has not been demonstrated formally. In this regard, it has been demonstrated that human neutrophils effectively kill *F. tularensis* by formation of hypochloric acid (Löfgren et al., 1983). It is produced as a result of the reaction between hydrogen peroxide and chloride anions and thus is not formed in neutrophils from p47$^{phox-/-}$ mice and they may therefore lack an effective neutrophil-mediated killing of *F. tularensis*.

## I.2 Role of iNOS

iNOS was essential for the control of murine tularemia. iNOS$^{-/-}$ mice succumbed to even the lowest challenge inoculum of 20 CFU by intradermal administration and the mean time to death of iNOS$^{-/-}$ mice was $26.4 \pm 1.8$ days. When given an inoculum of $5 \times 10^2$ CFU, bacterial numbers in liver and spleen within the first six days of infection were similar in iNOS$^{-/-}$ and wildtype mice. Thereafter, bacterial numbers decreased in iNOS$^{+/+}$ mice but remained stationary in iNOS$^{-/-}$ mice until they eventually died. In skin, starting from day 4, the iNOS$^{-/-}$ mice showed higher bacterial numbers than did the wild-type mice. Thus, in contrast to ROS produced by phox, iNOS-derived RNS are dispensable to the control of *F. tularensis* LVS in spleen and liver during the first week but at the same time contribute to the control of infection in skin. Thereafter, RNS are essential to clear the infection.

Histological examinations of livers and assay of serum levels of s-ALT and s-AST, which are sensitive markers for liver damage, revealed that iNOS$^{-/-}$ mice suffered from a severe liver

damage. It developed early during infection and already on day 6, livers from iNOS$^{-/-}$ mice contained large necrotic areas with loss of normal anatomic architecture. In comparison, livers from iNOS$^{+/+}$ mice were moderately affected with clusters or small aggregates of hepatocyte necrosis. The severity of liver damage in iNOS$^{-/-}$ mice increased with time and on day 9, serum levels of s-ALT and s-AST were higher than in any other group of mice. Thus, NO prevented an early and progressive liver damage in mice infected with *F. tularensis* LVS.

In summary, iNOS$^{-/-}$ mice controlled the infection in spleen and liver during the first 6 days as efficiently as iNOS$^{+./+}$ mice but still developed a severe liver damage during this period. Thereafter, the liver damage in iNOS$^{-/-}$ mice progressed and the mice were unable to clear the infection.

### *I.2.1 Assessment of the nature of the immune response in iNOS$^{-/-}$ mice*

T-cells are essential to clear an infection with *F. tularensis* LVS. Nevertheless, mice deficient in T-cells survive for 3-4 weeks before succumbing to a primary, otherwise sublethal *F. tularensis* LVS infection (Conlan et al., 1994). This course of infection resembles that observed in iNOS$^{-/-}$ mice and it was tested whether iNOS$^{-/-}$ mice eventually succumbed to a *F. tularensis* LVS infection due to a failure of these mice to develop a *F. tularensis*-specific T-cell response. iNOS$^{-/-}$ and iNOS$^{+/+}$ mice were primed by intradermal inoculation of a low-dose of *F. tularensis* LVS, treated with moxifloxacin on days 15-22, and killed on day 35 for preparation of splenocyte cultures. Cells from primed mice of both strains displayed a strong proliferative T-cell response to heat-killed *F. tularensis*, stimulatory indices being $\geq 10.5$ whereas cells from naïve iNOS$^{-/-}$ and iNOS$^{+/+}$ mice showed a low or non-significant proliferative response (stimulatory indices $\leq 2.4$).

IFN-$\gamma$, TNF-$\alpha$ and IL12p40 are critically required to resolve murine tularemia (Elkins et al., 2003). In response to heat-killed *F. tularensis*, splenocytes from iNOS$^{-/-}$ mice primed by *F. tularensis* LVS infection secreted IFN-$\gamma$ at a level much higher than did cells from primed iNOS$^{+/+}$ mice. In response to an infection with *F. tularensis* LVS, iNOS$^{-/-}$ mice produced IFN-$\gamma$ and on day 6 and thereafter, IFN-$\gamma$ serum levels were significantly higher than those in iNOS$^{+/+}$ mice. In addition, IL-12p40 and IL-6 but not TNF-$\alpha$ and IL-4 was detected in the serum of infected mice. Thus, even though iNOS$^{-/-}$ mice produce an excess of IFN-$\gamma$ and normal levels of IL12p40 and probably TNF-$\alpha$ locally (regarding TNF-$\alpha$ se section I.1), this is insufficient to control the infection. The increased IFN-$\gamma$ serum levels in iNOS$^{-/-}$ mice might

be due to the high bacterial burden. However, the increased IFN-γ levels could also reflect an overactive immune response due to the absence of the immunosuppressive effects of NO (Bogdan, 2001; Bogdan et al., 2000; Coleman, 2001; van der Veen, 2001).

The strong recall proliferative and IFN-γ response of T-cells demonstrated that a *F. tularensis*-specific T-cell mediated immunity developed in both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice during the course of infection. The production of IFN-γ, and not IL-4, showed that the ensuing response is of the Th1-type. Thus, the high susceptibility of iNOS$^{-/-}$ mice was not related to a failure of these mice to develop a Th1 T-cell response.

### *I.2.2 Possible mechanisms behind the iNOS-dependent control of murine tularemia*

The failure of iNOS$^{-/-}$ mice to clear the infection even in the presence of a Th1-type response strongly suggests RNS to be important to the eradication of *F. tularensis* LVS in mice. This is in line with the results of paper II, i.e., that NO via formation of ONOO$^-$ mediates the killing of *F. tularensis* LVS by IFN-γ activated macrophages.

Despite their control of *F. tularensis* LVS, an early liver damage developed in iNOS$^{-/-}$ mice. This suggests that NO may have a direct cytoprotective role in liver during murine tularemia by preventing tissue damage elicited by the immune response. Analogously, it was concluded that NO was cytoprotective in liver of mice infected with *S. typhimurium* (Alam et al., 2002). In addition, the immune response in iNOS$^{-/-}$ mice, as disclosed by high serum levels of IFN-γ might be overactive during the later stage of the infection leading to progressive liver damage. This is in line with extensive evidence on the important immunoregulatory role of NO (Bogdan, 2001; Bogdan et al., 2000; Coleman, 2001; van der Veen, 2001).

Altogether, the results suggest that iNOS-derived RNS serve bactericidal, cytoprotective, and immunosuppressive roles in murine tularemia.

### I.3 Role of iNOS and phox during infection with other intracellular pathogens

In paper III, it was shown that the roles of the host-derived RNS and ROS to the control of murine tularemia were distinct depending on the stage of infection and the organ localization of *F. tularensis* LVS (Fig. 4). Such a stage- and organ-specific role of ROS and RNS has also been described in other infection models.

Phox-derived ROS, as in murine tularemia, critically restricted the growth of *S. typhimurium* during the first days of infection and mice with a defective phox died within one week of infection, even to a modest inoculum (Mastroeni et al., 2000). This result was mirrored in a macrophage-killing assay where the initial killing was executed by phox-dependent mechanisms (Vazquez-Torres et al., 2000). Thus, in murine salmonellosis the role of phox at an early stage of the infection was even more striking than in tularemia and might relate to a phox-dependent killing by macrophages. In contrast to a *S. typhimurium* infection, phox was of minor importance to control an infection with *M. tuberculosis* (Cooper et al., 2000) and similar to infection with *F. tularensis* LVS, the role of phox was transient and only required until the development of antigen specific T-cell response. Common to all three pathogens is that control of bacterial growth is independent of RNS at an early stage of infection but thereafter they are required to eradicate these bacteria (Mastroeni et al., 2000; Scanga et al., 2001).
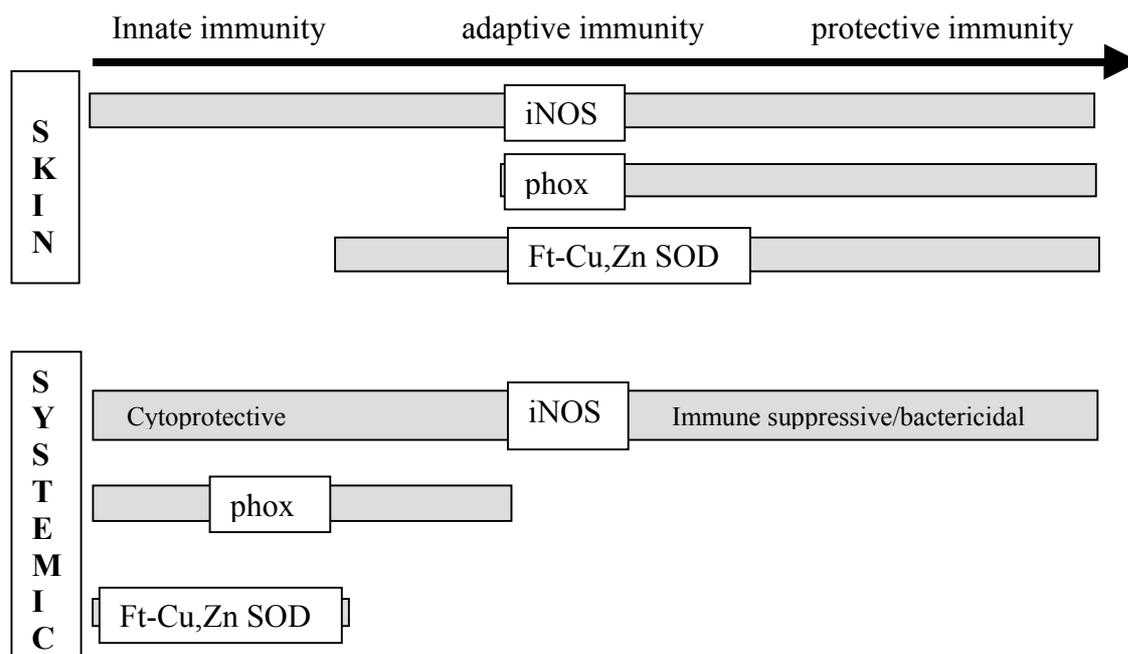
Fig. 4. The roles of iNOS and phox-derived radicals have time- and tissue-specific roles in the host immune response during murine tularemia. Similarly, the role of Ft-Cu,Zn-SOD to the proliferation and survival of *F. tularensis* LVS varies with the time of infection and tissue distribution of the bacteria.

An RNS- and ROS-dependent organ specific control was described for cutaneous leishmaniasis (Blos et al., 2003). RNS were needed for the killing of the parasite in the skin and draining lymph nodes, whereas the activity of phox was dispensable for the resolution of the acute skin infection but essential for the clearance of the parasite in the spleen.

Together these studies exemplify that the host immune response is not only pathogen-specific but also varies with the organ localization of a specific pathogen.

### J. Importance of Ft-Cu,Zn-SOD to the virulence of *F. tularensis* LVS in mice (paper IV)

The ability to proliferate in professional phagocytes is essential for the virulence of *F. tularensis*. As described in paper IV, the Ft-Cu,Zn-SOD-deficient strain, $\Delta sodC$ failed to proliferate in macrophages and it was demonstrated that its $LD_{50}$ in mice was more than 100 times higher than for the wild-type LVS strain.

The nature of the attenuation of $\Delta sodC$ was further investigated by following the bacterial numbers in infected mice. According to the results, the importance of Ft-Cu,Zn-SOD to the bacterial survival seemed to vary with the localization of bacteria. On day 2 after intradermal inoculation, $\Delta sodC$ was barely detected in spleen and liver whereas *F. tularensis* LVS reached 3-4 $\log_{10}$ CFU. Using intravenous administration of bacteria, the number of $\Delta sodC$ bacteria decreased already during the first hours of infection. After the first two days of infection, however, the role of Ft-Cu,Zn-SOD for the replication of the bacterium was apparently dispensable, at least in spleen and liver, since in these organs the increase and subsequent decrease of the number of $\Delta sodC$ and LVS bacteria were similar.

In the skin, $\Delta sodC$ bacteria proliferated to the same extent as *F. tularensis* LVS during the first days of infection. Thus, the lower number of $\Delta sodC$ in spleen and liver compared to *F. tularensis* LVS on day 2 was not related to a local control of infection after intradermal administration. However, at a later stage of the infection Ft-Cu,Zn-SOD protected the bacterium since $\Delta sodC$ was eradicated from skin already by day 7 whereas the number of *F. tularensis* LVS was about 3.5 $\log_{10}$.

Altogether these results show that Ft-Cu,Zn-SOD contributes to the virulence of *F. tularensis* LVS by facilitating efficient colonization of and proliferation in liver and spleen during the very early stage of infection. In addition, it protects *F. tularensis* LVS against killing in the

skin at a later stage of the infection. The time- and tissue-specific roles of Ft-Cu,Zn-SOD are schematically summarized in Fig. 4.

**J.1 Role of iNOS to the killing of Δ*sodC* in vivo**

As described in paper IV, iNOS-derived NO was dispensable for killing of Δ*sodC* by IFN-γ activated macrophages while killing of *F. tularensis* LVS was dependent on NO. It was also tested whether iNOS-derived NO was dispensable to the killing of Δ*sodC* in vivo. The results showed that Δ*sodC* was cleared from spleen and liver of iNOS$^{-/-}$ mice within 14 days after intradermal administration of bacteria. In contrast, as shown in paper III and IV, *F. tularensis* LVS was not eradicated and the mice eventually died. Thus, the persistence of *F. tularensis* LVS in spleen and liver of iNOS$^{-/-}$ mice was due to the protection afforded by Cu,Zn-SOD and apparently iNOS-derived NO counteracted this protective effect since iNOS$^{+/+}$ mice cleared the infection. Based on the results of infection of iNOS$^{-/-}$ mice and macrophages from iNOS$^{-/-}$ mice, we propose that Ft-Cu,Zn-SOD protects *F. tularensis* LVS against ROS-induced killing in environments with a low concentration or an absence of NO.

Neither Δ*sodC* nor *F. tularensis* LVS bacteria were cleared from skin of iNOS$^{-/-}$ mice. Thus, in skin, in contrast to in spleen and liver, iNOS-derived NO is necessary for the eradication of both strains. Obviously, Ft-Cu,Zn-SOD is active and protective when the bacterium is located in the skin since Δ*sodC* was eradicated more rapidly than *F. tularensis* LVS from skin of p47$^{phox+/+}$ mice (se above). An explanation to this paradox might be that the generation of ROS in skin is less efficient than in spleen and liver and not sufficient to kill Δ*sodC* despite deficient expression of Ft-Cu,Zn-SOD.

**J.2 Role of phox to the killing of Δ*sodC* in vivo**

Results in paper IV showed that Δ*sodC* did not regain its virulence in p47$^{phox-/-}$ mice. This indicates that, besides the phox-dependent ROS control of *F. tularensis* LVS during the first days of infection (paper III), there must be additional sources of ROS. In blood, circulating leukocytes express phox and in addition platelets (Weyrich and Zimmerman, 2004) and xanthine oxidase (Harrison, 2002) are potential sources of ROS. To reach spleen and liver *F. tularensis* LVS has to transverse endothelial barriers, which express homologues of gp91$^{phox}$ and xanthine oxidase (Geiszt and Leto, 2004; Harrison, 2002). Possibly, Ft-Cu,Zn-SOD protects *F. tularensis* LVS against such sources of ROS described above during its dissemination to spleen and liver. Such a scenario is in line with the rapid decrease in viability of Δ*sodC* after intravenous administration.

In general, $O_2^-$ generated from homologues of gp91$^{phox}$ is considered to participate in cell signaling. But there are reports that support a direct bactericidal role of ROS produced from these sources and, furthermore, that Cu,Zn-SOD expressed in bacteria can protect against this attack. An invasive *E. coli* strain engineered to overexpress Cu,Zn-SOD survived significantly better in HeLa cells compared to its control (*E. coli* without Cu,Zn-SOD-expressing plasmid) (Battistoni et al., 2000). Moreover, if the cells were treated with NADPH oxidase inhibitors both strains survived equally well. In another study, the viability of *Salmonella choleraesuis* in Caco-2 epithelial cells decreased significantly if the gene encoding Cu,Zn-SOD (*sodCI* or *sodCII*) was inactivated (Battistoni, 2003). Xanthine oxidase is induced in response to inflammatory cytokines and has been shown to contribute to the host immune response against *S. typhimurium* (Umezawa et al., 1997) and *Burkholderia cepacia* (Segal et al., 2000).

### J.4.1 Why is Ft-Cu,Zn-SOD dispensable for the bacterial survival in spleen and liver after day 2 of infection?

The acquired ability of Δ*sodC* to proliferate between day 2 and 4 could either reflect that over time, other, more powerful protection systems are induced in the bacterium or that it resides in an environment where it is not accessible to ROS-induced killing. *F. tularensis* LVS infects and proliferates in both professional phagocytes and non-phagocytic cells for example, hepatocytes. It is therefore likely that Δ*sodC* after day 2 of infection resides in non-phagocytic cells where it possibly can proliferate due to lack of ROS.

In the course of the immune response, *F. tularensis*-specific, cytokine secreting T-cells develop, which efficiently lyse infected cells. The released bacteria will be phagocytosed and killed by highly activated professional phagocytes, which presumably express high levels of iNOS and accordingly Ft-Cu,Zn-SOD no longer protects the bacterium. In macrophages, ONOO$^-$ mediated the IFN-$\gamma$-induced killing of *F. tularensis* LVS and its formation was highly dependent on NO production from iNOS. Possibly, the final eradication of *F. tularensis* LVS by phagocytes in the mice also relies on this mechanism.

In summary, it is likely that *F. tularensis* LVS during its dissemination through the bloodstream and during colonization of spleen and liver encounters ROS and our results indicate that the expression of Ft-Cu,Zn-SOD is critically required for its survival during these phases. Several pieces of evidence indicated that Ft-Cu,Zn-SOD protects the bacterium against ROS-induced killing in environment where NO is not produced. The lack of

contribution of Ft-Cu,Zn-SOD to the later phases of infection could therefore be related to an increased production of NO over time, or that the interaction between RNS and ROS, leading to ONOO⁻ formation, varies depending on the anatomical location.

# CONCLUSIONS

- IFN-γ–activated macrophages significantly inhibit the escape of *F. tularensis* LVS from the phagosomes.

- ONOO⁻ has a major role in the IFN-γ-induced killing of *F. tularensis* LVS by murine macrophages. NO production from iNOS contributes significantly to the formation of ONOO⁻ whereas $O_2^-$ generated from phox plays a less important role.

- The virulent strains, SCHU S4 and FSC200 initially proliferate faster in macrophages than does *F. tularensis* LVS but thereafter the generation times of the three strains appear to be similar.

- The virulent strains, SCHU S4 and FSC200, are killed by murine macrophages and this is strictly dependent on iNOS-derived NO.

- Phox-dependent mechanisms prevent *F. tularensis* LVS to reach numbers that would overwhelm the developing T-cell-mediated immune response in mice.

- iNOS-derived RNS serve bactericidal, cytoprotective and immunosuppressive roles in mice, which is essential to clear the infection.

- Ft-Cu,Zn-SOD contributes to the virulence of *F. tularensis* LVS by

  - facilitating the intracellular proliferation of *F. tularensis* LVS in macrophages from mice.

  - protecting *F. tularensis* LVS from killing in IFN-γ activated macrophages from mice that do not produce iNOS-derived NO.

  - facilitating efficient colonization of and proliferation in liver and spleen during the very early stage of infection in mice

- Expression of IglC is necessary for *F. tularensis* LVS to escape from the phagosome and the subsequent proliferation in the cytoplasm.

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