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VIRULENCE OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM AND INNATE ANTIBACTERIAL HOST RESPONSES

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Unconquerable stubbornness will wear down the thickest of walls and it is only a matter of time before you break on through to the other side

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ABSTRACT

The bacterial species *Salmonella enterica* consists of a collection of closely related enteric bacteria giving rise to diverse diseases in a wide range of hosts. In the murine infection model *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes an invasive disease which in many aspects resembles human typhoid fever. The ability of this pathogen to survive and replicate within macrophages of the liver and spleen is a crucial virulence determinant which largely depends on the type III secretion system coded for by the *Salmonella* pathogenicity islands 2 (SPI-2). Innate immune recognition of bacterial pattern molecules such as the lipopolysaccharide (LPS) induces immediate defence responses such as production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), synthesized by macrophages via the action of the NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS) respectively. The newly characterized phagocyte receptor TRAPC has been shown to trigger nitric oxide (NO) production in macrophages and dendritic cells upon receptor cross-linking, suggesting a possible role for TRAPC during bacterial infection. Surprisingly we could show that in combination with bacterial infection or LPS stimulation, cross-linking of TRAPC reduces macrophage NO production. The suppression of NO associated with a slight reduction of iNOS expression possibly mediated by a dampening the TLR-4 response. For *S.* Typhimurium and many other enteric pathogens, the ability to express complete LPS molecules has conventionally been regarded as a requirement for bacterial virulence, e.g. lack of the O-antigen (the outermost part of the LPS molecule) has been shown to reduce the virulence of *S.* Typhimurium in murine infection models. However, *S.* Typhimurium has also been shown to down-regulate genes for LPS-synthesis and to reduce the chain length of the O-antigen once it resides inside macrophage-like cells. This raises the question whether *S.* Typhimurium may benefit from expression of O-antigen-deficient LPS during intracellular stages of infection. To settle this issue the fitness of defined mutants devoid of O-antigen in macrophage-like cells was studied. O-antigen-deficient mutants inhibited iNOS activity in macrophage-like cells in an apparently SPI-2 dependent manner. Consequently the mutants displayed increased growth yields within these cells compared to wild type bacteria. Production of ROI as well as RNI is critical for control of disease proliferation in the murine salmonellosis model. In *E.coli* the thioredoxin and glutathione/glutaredoxin systems have been shown to mediate protection against oxidative stress. When studying the role of these systems in *Salmonella* we could show that whereas thioredoxin 1 (TrxA) is dispensable for resistance to oxidative or NO stress *in vitro*, it is essential for bacterial growth in both epithelial and macrophage-like cells as well as for virulence *in vivo* in the murine infection model. Whereas the level of replication within macrophage-like cells correlates directly to the redox potential of TrxA, *in vivo* virulence depends on both redox dependent and independent activities of TrxA. Moreover, TrxA was shown to be required for proper function of SPI-2 and for the ability of O-antigen deficient *S.* Typhimurium to inhibit iNOS activity.

LIST OF PUBLICATIONS

I. **Eva Bjur**, Sofia Eriksson-Ygberg and Mikael Rhen
The O-antigen affects replication of Salmonella enterica serovar Typhimurium in murine macrophage-like J774-A.1 cells through modulation of host cell nitric oxide production
Microbes and Infection (2006) 8:1826-1838

II. Rutger van der Holst*, **Eva Bjur***, Mikael Rhen and Jonas Sundbäck
Fine-tuning of macrophage anti-bacterial responses by TRAPC
Manuscript

III. **Eva Bjur**, Sofia Eriksson-Ygberg, Fredrik Åslund and Mikael Rhen
Thioredoxin 1 promotes intracellular replication and virulence of Salmonella enterica serovar Typhimurium
Infection and Immunity (2006) 74:5140-5151

IV. **Eva Bjur**, Aurel Negrea, Sofia Eriksson-Ygberg, Fredrik Åslund and Mikael Rhen
Thioredoxin 1 contributes to bacterial virulence through redox-dependent and redox-independent mechanisms
Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

CAMPs	Cationic antimicrobial peptides
DC	Dendritic cells
EEA1	Early endosomal antigen 1
GFP	Green fluorescent protein
HP	Hydrogen peroxidase
NO	Nitric oxide
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
L-Ara4N	4-amino-4-deoxy-L-arabinose
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
Mal	MyD88 adaptor-like
MyD88	Myleoid differentiation factor 88
NK	Natural killer
PAMP	Pathogen associated molecular pattern
pEtN	Phosphoethanolamine
phox	NADPH phagocyte oxidase
PP	Peyer's patches
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
SARM	Sterile alpha HEAT-Armadillo motifs
SIF	*Salmonella* induced filament
SPI	*Salmonella* pathogenicity island
SCV	*Salmonella* containing vacuole
TGN	Trans Golgi network
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRAPC	Triggering receptor expressed on antigen presenting cells
TREMs	Triggering receptor expressed on myeloid cells
TRIF	TIR-related adaptor protein inducing interferon
T3SS	Type III secretion system
VAP	Vacuole associated actin polymerization

1 INTRODUCTION

1.1 SALMONELLOSIS

Salmonellae are Gram-negative bacteria with an ability to persist and cause disease in a wide range of different hosts (202). The genus consist of two different species, *Salmonella bongori* and *Salmonella enterica*, which are further divided into eight different subspecies designated subspecies I, II, IIIa, IIIb, IV, V, VI and VII (27). *S. bongori* constitutes subspecies V whereas all others belong to *S. enterica*. The different subspecies are further subdivided into approximately 2500 serovariants most of which belong to *S. enterica* subspecies I (100). *S. bongori* is associated with infection of cold blooded animals (100) and lies beyond the scope of this thesis. Serovariants capable of infecting mammals mainly belong to subspecies I and cause a variety of diseases ranging from mild gastroenteritis to severe systemic infections, including typhoid fever (100, 180). In humans typhoid fever is caused by the human specific serovar Typhi. The disease is characterised by fever, diarrhoea (30-50% of reported cases) headache, muscle pain, loss of appetite, abdominal pain and enlarged liver and spleen due to bacterial replication in these organs (180, 203). Typhoid fever poses a serious public health problem in developing countries. World wide *S. enterica* serovar Typhi is reported to cause around 22 million cases of typhoid fever leading to approximately 220 000 deaths annually. The number of cases/deaths are most likely highly underestimated due to insufficient reporting of cases in these countries (19, 180). Infection of mice with *S. enterica* serovar Typhimurium (*S.* Typhimurium), which causes gastroenteritis in humans, gives rise to a systemic disease in mice which in many aspects resembles human typhoid fever (150, 180). Thus *S.* Typhimurium infection of mice and mouse-derived cells constitute a model system for human typhoid fever. Furthermore, due to its relatively short generation time, the ease by which *Salmonella* can be genetically manipulated, and the availability of complete genome sequences make *S.* Typhimurium suitable as a model organism for intracellular bacterial infections in general. The scenarios described below concerns the mouse infection model for *S.* Typhimurium.

1.2 PATHOGENESIS AND IMMUNE RESPONSES TO INFECTION

1.2.1 Infection and systemic dissemination

In both mice and men *Salmonella* spreads via the faecal-oral route. A proportion of the bacteria survives the acidic pH of the stomach and, in mice, reaches the distal ileum and caecum where they invade M-cells and endothelial cells of the intestinal epithelium. After breaching the epithelial barrier bacteria colonize the underlying Peyer's patches (PP) where they encounter resident phagocytic cells such as macrophages and dendritic cells (DCs) (232). Additionally, the intestinal epithelium responds to infection by producing various pro-inflammatory cytokines, notably IL-8, which leads to recruitment of neutrophils and macrophages (53). Having survived this first encounter with host immune responses, bacteria enter the mesenteric lymph nodes from where they disseminate to the liver and spleen (232). To what extent bacteria exist free in blood and/or lymph, or reside shielded within phagocytic cells while

disseminating is not well established. If free in the blood *Salmonella* would be exposed to the antibacterial actions of the complement system. Thus, as *Salmonella* is able to survive and replicate within macrophages, it would be beneficial if these cells could be exploited for transport to systemic sites. *Salmonella* has been shown to, to a small extent, be taken up directly from the intestinal lumen by $CD18^+$ phagocytes and it has been suggested that *Salmonella* may be transported to systemic sites shielded in these cells (191, 233). During infection of mice with an invasion-deficient mutant tagged with green fluorescent protein (GFP), GFP-expressing bacteria exclusively resided within $CD18^+$ cells when present in the blood (233). As this study only used invasion-deficient bacteria it is not possible to conclude whether the majority of wild type *Salmonella*, if the bloodstream is the main route of systemic dissemination, exists free in the blood or reside in phagocytic cells. In addition to shielded transport in the $CD18^+$ phagocytes taking up bacteria directly from the intestinal lumen, it is possible that invading bacteria are taken up by phagocytes in the underlying tissue and are transported to systemic sites within these cells. Thus it would be interesting to study the location of tagged wild type *S.* Typhimurium in the blood stream.

1.2.2 Innate immune responses controlling the infection

Both neutrophils and macrophages are recruited to infected organs and several studies have shown that both cell types are important for control of the infection and survival of the host (45, 46, 143, 176, 205, 230). However, macrophages in the liver and spleen seem to be the main host cells exploited for bacterial residence and replication (194, 199). Thus, although macrophages play a crucial role in controlling the infection they also represent an important site for bacterial replication and mutants of *S*. Typhimurium that are unable to replicate inside macrophages are avirulent (62).

The infection triggers production of pro-inflammatory cytokines. TNF-α (mainly produced by neutrophils and macrophages) and IFN-γ, both of which activate and thus enhance the antibacterial activities of macrophages, are crucial for control of the early stages of infection (172). Macrophage activation by TNF-α and IFN-γ trigger expression of the inducible nitric oxide synthase (iNOS) responsible for production of nitric oxide (NO) (66, 204). IFN-γ additionally triggers expression of the NADPH phagocyte oxidase (phox) and MHC-II thereby promoting induction of the oxidative burst and antigen presentation to T-cells respectively (204). Thereby IFN-γ together with TNF-α trigger of the two most important defence mechanisms to *Salmonella* infection (151, 209, 234). IL-12 and IL-18 secreted by activated macrophages and DCs have also been shown to be important presumably by triggering and enhancing the production of IFN-γ (53, 217). NK cells, NKT cells and T-cells have all been reported to produce IFN-γ during *Salmonella* infection but the relative importance of each cell type during the earlier stages of infection is not clear (120). At later stages of infection however, IL-12 mediated maintenance of IFN-γ production from CD4 T-cells is crucial for clearance of the infection (113). In addition to NK cells, NKT cells and T-cells, macrophages and neutrophils have been shown to produce IFN-γ in response to *Salmonella* infection *in vivo*. However the importance of these phagocyte sources for control of infection is not clear (120).

The complement system consists of a collection of proteins present in the blood, which may be activated through a cascade of enzymatic events during infection. Activation, which can be antibody dependent (classical pathway) or antibody independent (alternative and lectin pathways), leads to activation of factor C3. Activated C3 opsonizes bacteria and may thereby facilitate phagocytosis. Additionally, other components of the complement system have, in their active form, a chemoattractive effect on neutrophils, and complement activation via the classical pathway or via the lectin pathway may cause bacterial lysis by assembly of complement factors into the membrane attack complex.

The level of C3 activation by *Salmonella* has been implicated to associate with bacterial virulence. However, this suggestion was initially based on indirect evidence where C3 activation *in vitro* was compared to virulence *in vivo* (137, 138). C3 activation has also been shown to affect bacterial clearance from the bloodstream and to participate in the control of the infection (139, 174). However, existing *in vivo* data regarding the role of C3 during infection with *S.* Typhimurium have been obtained from studies on mice challenged by the intravenous route of infection (139, 174), a process that does not represent the normal infection route. Thereby the relevance of C3 activation during a normal per oral infection is unclear. Complement activation by *Salmonella* is known to occur via the alternative pathway (137, 138). In a study by Warren *et al.* however, mice deficient in C1q, a component required for activation of the classical pathway, showed increased susceptibility to infection with *S.* Typhimurium. Interestingly the increased susceptibility seemed to be unrelated to C1q dependent activation of the complement cascade and it was hypothesized that C1q may affect macrophage bacterial killing mechanisms (229). Taken together existing data do not provide a clear picture regarding the role of the complement system during *Salmonella* infection and more studies are needed to settle this issue.

1.2.3 Adaptive immunity and clearance of the infection

A small percentage of splenic DCs contain bacteria during *Salmonella* infection. As material from *Salmonella*-induced apoptotic macrophages are phagocytosed by both bystander DCs and bystander macrophages but presented to T-cells only by bystander DCs, it has been suggested that DCs rather than macrophages are the antigen presenting cells responsible for triggering of the adaptive response necessary for clearance of the infection (239). The requirement of an efficient T-cell response for clearance of *S.* Typhimurium and survival of the host is well established (149, 163, 190). Depletion experiments and transfer of specific subsets of T-cells from vaccinated animals have shown that CD8 T-cells play a role but CD4 T-cells are more important (152, 171, 185). Regarding B-cells and the antibody response the picture is less clear. *Salmonella* infection induces a strong antibody response (28) yet the importance of this response seems to vary depending on the susceptibility of the mouse strain used, the virulence of the bacterium and the route of infection (163). However, from experiments using B-cell deficient mice, it seems clear that during infection with virulent wild type *Salmonella* of a susceptible host, B-cells play an important role in controlling the infection (164).

1.3 VIRULENCE FACTORS

Salmonella uses a number of different virulence factors to establish infection and cause disease. Many of these are encoded within *salmonella* pathogenicity islands (SPIs). Pathogenicity islands are genomic elements, present in pathogenic bacteria and absent in non-pathogenic bacteria, coding for genes required for virulence. They are believed to have been acquired by horizontal transfer indicated by their altered GC content compared to the rest of the bacterial chromosome and that they often are flanked by tRNA genes, which are known hot-spot sites for insertions (147). To date more than 15 different SPIs have been identified in *S. enterica* differing in their distribution between different serovars (97, 206, 237). SPI-1 and 2, which are required for invasion of the intestinal epithelium and survival/replication within host cells respectively, are best characterized and encode two different type III secretion systems (T3SSs) and their corresponding effector proteins (68, 98, 162, 179, 208). These systems mediate transfer of proteins from the bacterial cytoplasm into the host cell cytosol, enabling *Salmonella* to manipulate the host cell thus favouring infection and bacterial survival/replication (90). SPI-3 and SPI-4 have been suggested to participate in survival within macrophages (13, 22, 244). SPI-5 encodes effector proteins for both SPI-1 and SPI-2 and thus contributes to both invasion of the host and intracellular survival/replication of *Salmonella* (121). Some virulence factors are encoded by genes on prophage elements integrated into the bacterial chromosome. The *gipA* gene encoded by the prophage Gifsy-1 was shown to be required for bacterial replication in PP. GipA is however dispensable for epithelial invasion and systemic disease during intraperitoneal infection of mice (214). The prophage Gifsy-2 on the other hand is of major importance for virulence (64), apparently mediated by *sodC1*, encoding a superoxide dismutase which is involved in protection against oxidative stress (50, 61), and *gtgE* whose function is unknown (101). Additional virulence factors worth mentioning are the lipopolysaccharide (LPS), which will be discussed in more detail below, and the *spv* genes. The *spvRABCD* genes are expressed on the virulence plasmid of *S.* Typhimurium, and have been shown to be important for bacterial virulence, most likely by affecting the ability to replicate within phagocytic cells in the liver and spleen (95). To date a function has been ascribed only for SpvR, which regulates *spv* transcription (34, 218), and SpvB which is a mono-ADP-ribosyl transferase causing actin depolymerization (135, 182, 223). Both SpvR and SpvB are important for virulence in mice whereas SpvA is dispensible (195). The mechanism by which the SpvB-mediated actin depolymerization contributes to virulence is not known. The role of SpvC and SpvD is unclear but they may partially contribute to virulence (195).

Virulence factors of specific relevance for this thesis; SPI-1, SPI-2, the LPS and defence mechanisms against oxidative and nitrosative stress, as well as virulence gene regulation is discussed in more detail below.

1.3.1 SPI-1

The SPI-1 T3SS is required for efficient invasion of the intestine and colonization of PP (68, 144, 165). Secretion of the SPI-1 effector proteins SopE and SopE2 into the host cell cytosol leads to activation of the Rho-family GTPases Cdc42 and Rac1 (91, 215). In their active form Cdc42 and Rac1 mediate actin rearrangements and membrane ruffling, which facilitate bacterial uptake (40). The inositol phosphate phosphatase

SopB acts in concert with SopE and SopE2 to induce membrane ruffling and bacterial uptake. In addition to its phosphatase activity SopB has been suggested to activate Rac1 (251). Additionally SipA and SipC have been reported to, in a cooperative manner, be important for formation of the membrane ruffles, mediated by their ability to nucleate and bundle F-actin filaments (39, 156). The GTPase activating effector SptP inactivates Cdc42 and Rac1 thus ensuring reestablishment of an intact host cell membrane (69). When *Salmonella* reaches the PP SipB-mediated induction of caspase-1-dependent apoptosis of macrophages has been shown to be essential for colonization of PP and for the ability to disseminate to deeper tissues and cause systemic disease (165). These results were however recently challenged by Lara-Tejero *et al*. who were unable to detect any differences in PP colonization or systemic dissemination by *S.* Typhimurium in wild type or caspase-1 deficient mice (131). Additionally, in contrast to Monack *et al*. who reported increased resistance to *S.* Typhimurium infection in caspase-1 deficient mice (165), lack of caspase-1 was shown to increase the susceptibility to infection (131). The reason for these contradicting results is unclear but could possibly relate to use of different mouse strains. Evidently more studies are needed to establish the role of caspase-1 during infection with *S.* Typhimurium.

1.3.2 SPI-2

The SPI-2 T3SS secretes effector proteins across the vacuolar membrane of the *Salmonella* containing vacuole (SCV), the specialised vacuolar compartment in which *Salmonella* resides during intracellular stages of infection, into the host cell cytosol (90). SPI-2 is essential for intracellular survival and replication of *Salmonella* (42, 179, 231) evidently through interference with phagosomal maturation and macrophage oxidative and NO responses, mediated by secreted effector proteins (1, 231). The role of SPI-2 during infection will be discussed in more detail in section 1.4.

1.3.3 The lipopolysaccharide (LPS)

LPSs, complex highly conserved glycolipids, constitute the major component of the outer membrane of Gram-negative bacteria and are important for membrane stability. The LPS molecule can be divided into three different parts; the glycolipid lipid A which anchors the LPS to the outer membrane, the oligosaccharide core and the polysaccharide O-antigen (Fig. 1). Mutants that express LPS devoid of the O-antigen are designated as “rough” due to a rough colony morphology when grown on LB agar plates, whereas the wild type LPS is designated as “smooth” (241). The LPS also constitutes a conserved pattern molecule which is recognized by the host, mainly by Toll-like receptor (TLR) 4, leading to induction of innate immune responses (65). The consequences of this are discussed in section 1.4.3.1. Although LPSs are highly conserved molecules they are far from being static; the sugar composition of the core region and the O-antigen as well as the number of acyl chains on lipid A differ between different bacteria. As a consequence of this LPS from different bacteria differ in their bioactivity (225). Additionally, *Salmonella* modifies its LPS in response to environmental conditions to increase its fitness.

Salmonella Lipid A is known to be decorated with 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (pEtN), palmitate and 2-hydroxymyristate, and may

also be deacylated (57, 189). L-Ara4N, pEtN and palmitate are added to lipid A during growth in acidic low [Mg^{2+}] medium and during growth in macrophage-like cells but not when bacteria are grown in tissue culture medium or in a high [Mg^{2+}] mildly alkaline medium. The 2-hydroxymyristate decoration on the other hand is present under all these growth conditions (74) and the role of this modification in bacterial virulence is not known (225). L-Ara4N and pEtN decoration of phosphate groups on lipid A (L-Ara4N and pEtN) and the core region (pEtN) result in increased resistance to cationic antimicrobial peptides (CAMPs) (84, 133). Genes mediating the L-Ara4N modification have been shown to be required for full bacterial virulence in BALB/c mice. (85). As addition of L-Ara4N and pEtN renders the bacterial surface less anionic it has been assumed that the increased resistance to CAMPs is mediated by a decreased heterophilic attraction. In a study by Tamayo *et al.* lack of L-Ara4N dramatically decreased the resistance to CAMPs and virulence *in vivo*, whereas lack of pEtN only had a slight effect on CAMP resistance and bacterial virulence was essentially unaffected (221). As both modifications render the bacterial surface less anionic, they argued that the resistance to CAMPs is likely to be mediated by a mechanism that is not based on charge. However, Zhou *et al.* have reported that, at least under certain conditions, pEtN modifications occur to a less extent than the L-Ara4N modification (252), and Tamayo *et al.* did not provide data to show whether the L-Ara4N and pEtN modifications occurred to the same extent. In other bacteria, unable to modify lipid A with L-Ara4N however, the pEtN modification is important for resistance to CAMPs. Thus a likely explanation for the minor effect on CAMP resistance and lack of effect on virulence *in vivo*, observed for *Salmonella* in the absence of pEtN, is a compensatory increase in L-Ara4N modification (225).

The number of acyl chains on lipid A may differ and lipid A can be both acylated, by addition of palmitate, or deacylated to generate hepta- and penta-acylated lipid A respectively. Like the L-Ara4N and pEtN modifications, the addition of palmitate leads to increased CAMP resistance thought to be mediated by increased membrane density (86). Both penta- and hepta-acylated lipid A exhibit reduced ability to trigger signalling through TLR-4 and it has been suggested that these modifications occur to reduce host innate antibacterial responses (117). It has also been shown that not only the number but also the length of the acyl chains, as well as their position on lipid A, affect TLR-4 signalling (116). Disruption of the bacterial membrane was shown to induce deacylation and formation of penta-acylated lipid A and it was hypothesized that in the case of bacterial membrane damage within the host release of penta-acylated LPS may reduce TLR-4 signalling and thus benefit the bacteria (118). However, deacylation does not take place in the presence of L-Ara4N (118), which is added to lipid A in macrophage-like cells (74). Palmitoylation giving rise to hepta-acylated lipid A, which can co-exist with the L-Ara4N modification, also gives reduced TLR-4 signalling (117) and should serve the same function if released during the course of membrane damage. Possibly, penta-acylated LPS is required only under conditions other than those encountered within the host, a hypothesis that is strengthen by the finding that a mutant unable to deacylate lipid A is not attenuated in the mouse infection model (16).

Salmonella is evidently able to modify its lipid A in a multitude of ways in response to environmental stimuli. However the specific composition of lipid A under a given

condition is not fully understood. Possibly heterogeneity may be desirable from a bacterial point of view to maximise protection against antibacterial host responses.

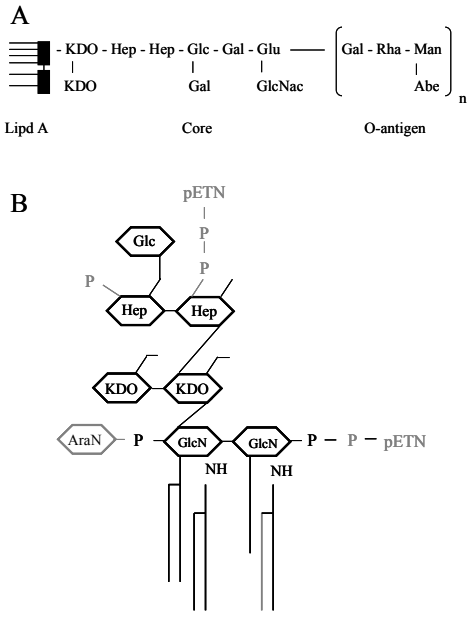

Figure 1. The full length LPS structure is shown in **A**. **B** shows lipid A and the inner core with the different modifications shaded in grey.

In addition to flagellin, Lipid A is considered as the major inducer of host responses during *Salmonella* infection (104, 225), and its modification is evidently important for increased bacterial resistance to host responses. However the core region and the O-antigen are also important for bacterial virulence and protection from host responses. Despite providing the bacterial surface with negative charge, phosphorylation of the core heptose region is required for resistance to polymyxin, a peptide antibiotic thought to act by the same mechanism as CAMPs, possibly mediated by affecting membrane stability. Phosphorylation of the core is furthermore required for virulence *in vivo* (247). The host complement system is activated at the tip of the LPS. By providing a distance between the site of complement activation and the bacterial surface the O-antigen mediates protection against the membrane attack complex (76). The composition of the polysaccharide backbone as well as the type of side chains is highly variable both between and within Gram-negative bacterial species e.g in *Salmonella* more than 50 different variants are known (134). When the abequose (Abe) side chain of *S.* Typhimurium (Fig.1) was exchanged for the tyvelose side chain found in *S. enterica* serovar Enteritidis, *S.* Typhimurium showed increased activation of complement, increased phagocytosis by macrophage-like cells and reduced virulence during intravenous infection of mice (112, 137-139). Additionally, expression of complete smooth LPS is a prerequisite for bacterial virulence, not only for *Salmonella*

but also for other enterobacteria such as *Yersinia enterocolitica*. Strains of both *Y. enterocolitica* and *S.* Typhimurium which lack the O-antigen display a reduced ability to colonize the murine host and a reduction in virulence in mouse infection models (5, 173, 211, 224). However recent studies from our laboratory (Paper I) and others (21, 130, 238) indicate that under certain conditions bacteria may benefit from expressing reduced amounts of O-antigen. This will be discussed in more detail in the Results and Discussion section for Paper I.

1.3.4 Defence mechanisms against oxidative and nitrosative stress

To be able to replicate within the host and cause disease *Salmonella* must withstand the host immune responses such as the oxidative burst and NO production of macrophages (151, 209, 234). Bacterial defence mechanisms against these host responses will be discussed in section 1.4.2.

1.3.5 Virulence regulation

During the course of infection and spread to new hosts *Salmonella* has to be able to survive/replicate outside the host e.g. in food, water and soil, in the host gastrointestinal tract, while passing through epithelial cells and finally inside macrophages. Survival/replication under such diverse conditions requires tight gene regulation ensuring that the appropriate genes are expressed in the correct environment. Use of alternative σ-factors may alter promoter preferences of the RNA polymerase and thereby also the gene expression profiles (192). The alternative σ-factor RpoS is required for survival under stress conditions such as starvation and exposure to low pH as well as virulence *in vivo* (59, 177, 242). As depriviation of nutrients and acidic pH are conditions met by the bacteria inside macrophages it was hinted between the lines that RpoS would be required for survival and replication inside these cells (59). However, it was later shown that *S.* Typhimurium LT2 strains, which carry a defect *rpoS* gene, as well as an *rpoS* null mutant survive inside J774-A.1 macrophage-like cells and bone marrow-derived murine macrophages to the same extent as an isogenic strain carrying the wild type allele (242). Survival/replication inside macrophages was later shown to largely depend on another alternative σ-factor, RpoE (106). In addition to intra-macrophage survival, RpoE has also been shown to mediate resistance to antibacterial peptides (106) and to play an important role during nutrient deprivation (119, 222), virulence *in vivo* and resistance to oxidative stress both *in vitro* and *in vivo* (222). However as the situation *in vivo* is much more complex than the situation in cell cultures, it is fully possible that RpoS is important for intracellular replication *in vivo*.

Regulation of virulence genes is governed by sensing of environmental changes. This is often mediated by two-component signal transduction systems. These systems consist of a response regulator whose activity is regulated by an integral membrane protein that senses changes in the environment. Activation of the sensor leads to autophosphorylation followed by transfer of phosphate to and thus activation of the response regulator, which in turn activates or repress expression of different subsets of target genes (15).

The PhoP/PhoQ two-component system responds to changes in [Mg^{2+}] and [Ca^{2+}]. It is activated by low concentrations, conditions which are encountered within the SCV, and inhibited by high ("extracellular") concentrations (72). PhoP/PhoQ has also been reported to respond to acidic pH within macrophages (6), and an *rpoS/phoP* double mutant was shown to be more sensitive to acidic pH than an *rpoS* single mutant (14). Garcia Véscovi *et al*. on the other hand reported that induction of PhoP/PhoQ regulated genes in response to low pH occurs independently of PhoP/PhoQ (72). Thus the results regarding induction of PhoP/PhoQ in response to low pH are inconsistent and the use of differential experimental setups in the different reports makes it difficult to definitely conclude whether or not acidic pH has a role in the activation of the PhoP/PhoQ system. Nevertheless, the PhoP/PhoQ system is important for intracellular survival and virulence, and regulates the expression of several virulence functions, e.g. it activates transcription of *sodCI*, which is involved in protection against oxidative stress (77). Via the action of PmrD, the PhoP/PhoQ system activates the PmrA/PmrB two-component system which regulates genes mediating the modifications of lipid A that render *Salmonella* more resistant to CAMPs (81, 84, 124, 133). As mentioned above PmrA and PmrA-controlled genes mediating these modifications have been shown to be required for full bacterial virulence in BALB/c mice during per oral but not intraperitoneal infection (85). It was therefore suggested that this modification would be required for defence against antibacterial host responses present within the intestine. Recently it was reported that genes required for the L-4AraN modification are expressed not only inside murine macrophage-like cells and mouse liver and spleen, but also in the intestine of BALB/c mice in a PhoP and PmrA dependent manner (158). This was a surprising finding and contradictory to the dogma that PhoP and PhoP dependent induction of PmrA are induced exclusively during intracellular stages of infection. Possibly these systems may be induced differentially under different conditions. Furthermore, considering the complex network of regulatory systems in *Salmonella*, one can envision that induction of genes controlled by these systems is more complex than what is known to date and that a combination with other regulatory systems may control which PhoP regulated genes that are induced under a given condition. Intestinal expression of the L-4AraN modification appears logical in the sense that it would mediate protection against CAMPs present in the intestine. Interestingly, the PhoP/PhoQ system was recently reported to respond to the presence of antibacterial peptides, implying that the changes in surface composition that mediate resistance to antibacterial peptides are induced directly by sensing their presence. (9). Possibly sensing of CAMPs is the inducer of PhoP regulated genes in the intestine. The PmrA/PmrB system also responds to mildly acidic pH or high iron concentrations independently of PhoP/PhoQ (213, 246). Within the host, high iron concentrations are encountered only transiently in the gastro intestinal tract but the relevance of this for PmrA/PmrB induction during infection is unclear. Furthermore the induction of PmrA in the intestine reported by Merighi *et al* was indicated to occur independently of the concentration of iron (158). However, outside the host high iron concentrations are encountered e.g. in soil and the PmrA/PmrB mediated modifications of lipid A have been shown to promote bacterial survival in this environment (175). Thus, the PmrA/PmrB system is important for adaptation to environmental conditions both inside and outside the host.

Expression of the SPI-1 T3SS and its effector proteins have been shown to be induced by high osmolarity, low oxygen and slightly alkaline pH, conditions which are all found in the small intestine. Additionally growth in rich medium to the late logarithmic phase of growth induces SPI-1 thus facilitating the study of SPI-1 functions (144). The PhoP/PhoQ system is known to mediate inhibition of SPI-1 by repressing expression of the main activator of SPI-1, the transcription factor HilA (11). How *Salmonella* senses the environmental conditions which induce SPI-1 is not completely understood. SirA, a member of the two-component response regulator family known to act together with the membrane sensor BarA, has been shown to participate in SPI-1 induction (7). The OmpR/EnvZ two-component system responds to changes in osmolarity as well as low pH and has been shown to be involved in the regulation of SPI-1 (145). Induction of SPI-1 expression is mainly regulated by the transcription factor HilA. SPI-1-inducing conditions activate the HilD transcription factor, which activates expression of *hilA*. HilA in turn, either directly or by inducing the transcription factor InvF, activates transcription of genes within SPI-1 (7). HilD and HilC are also reported to activate InvF independently of HilA (3). However a *hilA* mutant is essentially non-invasive (10, 21) and the role of HilA independent mechanisms is unclear.

The SsrA/SsrB two-component system responds to low [Ca^{2+}], low osmolarity and acidic pH, environmental conditions encountered within the SCV, and is essential for induction of SPI-2 (73). It is not known whether SsrA senses these signals directly or if another sensor is involved. Additionally, expression of SPI-2 partly depends on the OmpR/EnvZ two-component system and the transcriptional regulator SlyA, both of which respond to low osmolarity and low [Ca^{2+}] (73, 141). Additionally, the PhoP/PhoQ system has been reported to induce expression of SPI-2 *in vitro* (51) and in macrophage-like cells (245), indicating PhoP/PhoQ regulation of SPI-2. Another study reported that *ssrA/ssrB* is induced independently of PhoP/PhoQ in macrophage-like cells (132, 160). Beuzon *et al.* later reported that PhoP/PhoQ and SPI-2 contribute independently to virulence *in vivo* (18). However the data were not conclusive and a partial role for PhoP/PhoQ cannot be excluded based on these data. Moreover, PhoP was recently shown to regulate transcription of *ssrB* and to bind to the *ssrB* promoter region both *in vitro* and in macrophages (20). Thus existing data do not provide a clear cut picture regarding the role of PhoP/PhoQ in SPI-2 regulation. Taken together it seems like PhoP/PhoQ may have a partial effect on SPI-2 induction. If so, that would explain the apparently conflicting data obtained from studies on macrophage-like cells as different SPI-2 genes were studied in the different reports (132, 160, 245).

Evidently virulence gene regulation in *S. enterica* serovar Typhimurium is highly complex with a multifaceted network of regulators responding to different stimuli. The details on how and which of the different regulators that interact to ensure expression of the appropriate set of genes in a given environment is far from clear.

1.4 SALMONELLA AND THE MACROPHAGE

1.4.1 Macrophage responses to infection

Upon infection or tissue damage monocytes circulating in the bloodstream migrate into the infected/damaged tissue where they differentiate into macrophages. Macrophages

are professional phagocytic cells serving both a cleaning function, ingesting and degrading e.g. apoptotic and necrotic host cells, as well as pro-inflammatory antimicrobial functions (79). Upon phagocytosis the ingested material are taken up into a phagosome which matures, through ordered sequential vesicle fusion/fission events with early endosomes, late endosomes and finally with lysosomes, into a highly microbicidal phagolysosome. The maturation is accompanied by a progressive decrease in phagosomal pH and acquisition of hydrolytic enzymes, such as cathepsin D. Additionally within the phagosome/phagolysosome microbes are deprived of nutrients and exposed to reactive oxygen and nitrogen intermediates (ROI and RNI) (240). The regulation of the fusion/fission events leading to formation of the phagolysosome is largely unknown. It is thought to be partly controlled by members of the Rab family of small GTPases. Rab5 is present on early phagosomes and is believed to mediate fusion events with later endosomal compartments through its downstream effector early endosomal antigen 1 (EEA1) (184). Phagosomal maturation occurs along microtubules, which together with actin filaments constitute the cytoskeleton of eukaryotic cells (240). Microtubules are polarized tracks which guide organellar traffic within the cell. Movement along microtubules in the + (towards the cell surface) or - (towards the centre of the cell) direction is driven by the motor proteins kinesin and dynein respectively (240). Rab7 present on late endosomes has been shown to be recruited to intermediate phagosomes and to mediate interaction between the phagosome and microtubule, through its effector Rab interacting lysosomal protein (RILP), thereby promoting phagosomal movement and formation of phagolysosomes (92).

ROI, produced during the oxidative burst, and production of NO and other RNI are the most important macrophage defence mechanisms during infection with *S.* Typhimurium of macrophage-like cells, primary macrophages as well as of mice *in vivo* (55, 151, 209, 234). ROI and RNI have been shown to mainly act sequentially during the infection with the bactericidal ROI being important initially and the bacteriostatic RNI at later time points (151, 234). For production of ROI the macrophage relies on phox, which is composed of seven different subunits which assemble in the phagosomal membrane upon macrophage activation to produce superoxide (108). Superoxide passes through the outer membrane of Gram-negative bacteria and may damage periplasmic proteins but is unable to penetrate the cytoplasmic membrane. However, in the periplasm superoxide undergo both spontaneous and enzyme-mediated dismutation to generate the more reactive hydrogen peroxide which is able to enter the bacterial cytoplasm. Hydrogen peroxide may react further to generate the even more toxic hydroxyl radical and, in combination with NO, peroxynitrite (108). In phagocytic cells, NO is produced by iNOS from L-arginine and oxygen in a NADPH dependent process, and may undergo further reactions to generate an array of RNI (58, 250).

NO is highly diffusible and toxic not only to microbes but also to the host, and its production therefore needs to be tightly regulated (250). NF-κB is the main transcription factor involved in induction of iNOS transcription. Whereas direct interaction between NF-κB and the IFN-γ inducible transcription factor IRF-1 has a synergistic effect on iNOS expression, the transcription factor STAT3 interacts with NF-κB to inhibit transcription of iNOS (201, 249). Protein dimerization is necessary for iNOS activity and depends on binding of L-arginine, heme and tetrahydrobiopterin

(BH_4) as well as the formation of a disulphide bridge that covalently links the two monomers. In addition to its requirement for iNOS dimerization, L-arginine has been shown to control iNOS translation. Reduced levels of L-arginine inhibits translation without affecting the level of iNOS mRNA (54). The activity of iNOS is also controlled by protein-protein interactions. Kalirin and NAP110, expressed in neuronal cells and macrophages respectively, interact directly with iNOS to inhibit its activity (250). On the other hand Rac2, a member of the Rho family of small GTPases which is also the driving force for phox, has been shown to interact with iNOS to increase its enzymatic activity and was indicated to affect iNOS distribution within macrophage-like cells (129).

The distribution/localisation of iNOS has been suggested to constitute yet another possible level of control of iNOS activity as it was shown that iNOS partly localises to a vesicular compartment in macrophages (236, 243). That is, vesicular localisation would enable recruitment of iNOS-containing vesicles to phagosomes upon infection, thereby focusing the response onto the bacterial localisation. While Webb *et al*. observed iNOS to associate with the cortical submembranous actin as well as being localised to cytoplasmic vesicles and free in the cytoplasm, they could not detect any relocalisation or recruitment to phagosomes of iNOS upon particle ingestion or infection with *S.* Typhimurium (236). Later iNOS has been shown to associate with latex bead-containing phagosomes whereas both *S.* Typimurium and *Mycobacterium tuberculosis* have been reported to dislocate iNOS from the SCV/phagosomal membrane (38, 161). A possible explanation for the contradicting results regarding iNOS localization and its recruitment to phagosomes may be that iNOS distribution and tendency to associate with phagosomes depends on the activation state of the cells, a possibility that has been suggested previously by Webb *et al*. (236). Additionally iNOS was recently shown to form aggresomes in both human and murine macrophage cell lines (123). Aggresomes are discrete cytoplasmic inclusion bodies whose formation is triggered by the synthesis of misfolded proteins. However, this did not appear to be the rationale for formation of iNOS-aggresomes. Aggregation of iNOS was shown to limit iNOS mobility within the cell and it was suggested that iNOS-aggresome formation occurs to control iNOS activity (123). However no data showing the enzymatic activity of aggregated iNOS was presented and the biological relevance of iNOS aggresome formation remains to be determined. Taken together, existing data on iNOS localisation and its possible redistribution upon phagocytosis do not provide a clear picture and more studies are evidently needed to settle this issue.

1.4.2 Bacterial defence mechanisms and manipulation of host responses

Protection against host antibacterial mechanisms occurs at two levels, *i)* induction of stress responses which detoxify ROI and RNI or repair the damage imposed by these substances and *ii)* manipulation of host responses in order to create a less hostile environment which promotes bacterial survival and replication.

Salmonella possesses several different systems which mediate detoxification of harmful substances, and DNA damage-repair mechanisms. The superoxide dismutase SodCI, shown to be an important virulence factor, converts superoxide into hydrogen peroxide

(50, 125). Although this ROI is more toxic than superoxide, its formation is a necessary step in the superoxide-detoxification process (108). Exposure to hydrogen peroxide activates the transcription factor OxyR, which induce expression of genes involved in protection to oxidative stress, e.g. *katG* encoding hydrogen peroxidase (HP) I, a catalase which reduces hydrogen peroxide to generate water and oxygen (80). However a mutant lacking HPI and HPII, the only other catalase known to be expressed by *Salmonella*, is unaffected for survival in macrophages and virulence in mice (31). It is thus unclear what mechanisms *Salmonella* uses to resist hydrogen peroxide in macrophages *in vitro* or *in vivo*. Glucose-6-phosphate deyhdrogenase has been shown to be important for protection against hydrogen peroxide and an NO donor compound *in vitro* as well as virulence in mice, presumably through its participation in the production of NADPH which serves as a source of reducing equivalents (146). In the closely related bacteria *Escherichia coli* the glucose-6-phosphate deyhdrogenase is regulated by the SoxR/SoxS system. However, this system has been shown to be dispensable for survival of *Salmonella* in phagocytic cells and virulence in mice (60). Hence expression of glucose-6-phosphate deyhdrogenase is most likely induced by another regulator in *Salmonella*. DNA damage activates the RecA protein which induces expression of genes mediating DNA repair. This is known as the SOS response and ensures bacterial survival under DNA-damaging conditions (168, 200, 212, 227). RecA as well as the DNA-repairing enzyme RecBCD have been shown to be important for resistance to the macrophage oxidative burst and virulence *in vivo* (32, 35).

1.4.2.1 Manipulation of macrophage responses

As mentioned above, phagocytosis of a microbe is followed by phagosomal maturation into a highly bactericidal phagolysosome. *Salmonella* manipulates this development in order to generate a specialised vacuolar compartment, the SCV, which allows bacterial survival and replication. The biogenesis and details regarding the composition of the SCV is largely unclear. This is partly due to use of different cell types and bacterial strain backgrounds in existing studies. Knowledge regarding SCV maturation has largely been acquired from epithelial cells which are fundamentally different from macrophages. Certain aspects of SCV maturation may however be easier to study in epithelial cells whereby these cells may serve as a tool when deciphering the SCV maturation process. However, findings derived from epithelial cells need to be verified in macrophages, and, to avoid the risk of essential features of SCV maturation being missed, it is important to study this process also directly in macrophages. It seems clear that the SCV fuses with early endosomes, as evidenced by the transient acquisition of the early endosomal markers EEA1 and the transferrin receptor, in a Rab5 dependent manner in both epithelial cells and macrophages (1). These early markers are rapidly replaced by late endosomal markers such as Rab7, lysosomal glycoproteins e.g. LAMP-1, and the vacuolar ATPase which is responsible for phagosome acidification. SCV acidification is a requirement for secretion of SPI-2 effectors and thereby for bacterial replication (1, 103).

The function of the SPI-1 T3SS in *Salmonella* as well as T3SSs in other bacterial species, have been shown to depend on host cell cholesterol (94). At this point it is not clear whether the SPI-2 T3SS also requires cholesterol for proper function. However, bacterial replication, in epithelial cells as well as in macrophages, has been shown to

depend on the accumulation of host cell cholesterol around the SCV (36, 37). Another characteristic of the SCV, in both epithelial cells and macrophages, is the SifA dependent formation of membrane extensions from the vacuole known as *Salmonella* induced filaments (SIFs) (71, 122). SIFs are formed and extend towards the periphery of the cell along microtubules, and are required for maintenance of SCV membrane integrity (17, 29, 71). In addition to SifA, the SPI-2 effector SseJ has been shown to contribute to SCV membrane dynamics (196) and was recently shown to affect virulence *in vivo* (181).

The vesicles of the endosomal pathway as well as maturing phagosomes, are normally transported along microtubules in the cell to reach their intended target. *S.* Typhimurium has been shown to target the SCV to the Trans Golgi network (TGN) in epithelial cells (198). Retention of SCVs in this juxtanuclear position is required for bacterial replication in epithelial cells, and depend on the SPI-2-effector proteins SseF and SseG and their ability to interact with the microtubule network (127, 198). Furthermore, an *sseG* mutant exhibits reduced fitness in macrophage-like cells, primary peritoneal macrophages as well as in mice *in vivo*, thereby providing indirect evidence for the importance of *S.* Typhimurium-Golgi interactions in macrophages (99, 198). Microtubules were shown to accumulate around SCVs during infection of both epithelial cells and macrophages, a bacteria-driven process as indicated by the large dependency on bacterial protein synthesis. In epithelial cells, the accumulation of microtubules was accompanied by accumulation of both kinesin and dynein, a process shown to promote bacterial replication as well as being important for SCV membrane dynamics, including SIF-formation (82). However *Salmonella* has also been reported to inhibit the recruitment of kinesin to the SCV membrane (26). This may seem contradictory however excessive recruitment of kinesin to the SCV leads to SCV-displacement towards the periphery of the host cell and disruption of the SCV membrane presumably due to increased kinesin-mediated pulling force on the SCV (26). Hence, a balanced interaction of the SCV with both dynein and kinesin seems to be required for retention of the SCV in a juxtanuclear position, SCV membrane integrity and bacterial replication. Guignot *et al*. reported that the recruitment of dynein to the SCV depends on Rab7, possibly mediated by its downstream effector RILP (82). Rab7 is also required for SIF formation, SCV membrane integrity and bacterial replication (30, 82). Recruitment of Rab7 to the SCV membrane would thereby benefit the bacteria. The Rab7/RILP dependent recruitment of dynein to the SCV was confirmed by Marsman *et al.*, however these authors showed that this leads to fusion of SCVs with lysosomes and inhibition of bacterial replication (148). The latter study is based on experiments where RILP is overexpressed. Considering the balanced interaction of the SCV with both dynein and kinesin that seems to be required for proper maintenance of the SCV, and that RILP mediates interaction with dynein it is possible that the results by Marsman *et al.* are caused by a tilting of this balance due to overexpression of RILP. Additionally, *S.* Typhimurium was recently shown to interfere with the transport of vesicles from Golgi to the cell surface as evidenced by the accumulation of exocytic vesicles in the vicinity of SCVs. This process was shown to depend on the SPI-2 effector proteins SifA, SseF and SseG. It was suggested that the recruitment of exocytic vesicles may serve as a source for nutrients and membrane material (126).

SPI-2 has been shown to mediate formation of an F-actin meshwork around the SCV known as vacuole associated actin polymerization (VAP). VAP formation occurs in both epithelial and macrophage-like cells and was indicated to be important for intracellular replication of *S.* Typhimurium as well as for maintenance of the SCV membrane (157). However, after their initial appearance VAP formation in epithelial cells was shown to be reduced with time post infection (159). As mentioned in section 1.3, SpvB is a bacterial mono-ADP-ribosyl transferase which inhibits host cell actin polymerization by ADP-ribosylation of G-actin (135, 223). SpvB is important for virulence *in vivo* and replication within the mesenteric lymph nodes and the spleen (95). Although no defect for intracellular replication has been demonstrated for *spvB* mutant bacteria, *spvB* expression is induced during bacterial growth in macrophage-like cells *in vitro* (63, 96, 193) and the study by Heffernan *et al*. provides indirect evidence that SpvB enables growth within macrophages *in vivo* (95). This raises the question whether the virulence function of SpvB is related to VAP. However conflicting data exist regarding this (159, 207). Furthermore as the importance of VAP has been established only by the use of actin depolymerising substances which may have other adverse effects, it has been argued that these data should be interpreted with caution until the role of VAP can be confirmed by genetic evidence (83). Thus more studies are required to verify the importance of VAP and to determine how the actin depolymerising activity of SpvB contributes to virulence.

S. Typhimurium is also capable of excluding both phox and iNOS from the SCV in a SPI-2 dependent manner, thereby minimising the encounter with the highly antibacterial ROI and RNI produced by these enzymes (38, 235).

1.4.3 Receptors mediating recognition and/or uptake

Macrophages express a wide variety of receptors on their surface mediating recognition and/or phagocytosis. These include Fc receptors, complement receptors, scavenger receptors, C-type lectin receptors, TLRs and triggering receptors expressed on myeloid cells (TREMs) (2, 43, 78). Some of these recognise both host and pathogen derived ligands and are able to mediate either inhibitory or activating signals depending on the ligand. This can be exemplified by scavenger receptors which mediate inhibitory signals e.g. during phagocytosis of apoptotic host cells and activating signals while mediating phagocytosis of bacteria (2, 78). During an infection a pathogen is likely to be recognized simultaneously by multiple receptors. Additionally, certain infections e.g. with *Salmonella*, are associated with host cell apoptosis which may induce inhibitory signals upon phagocytosis of bacteria-containing apoptotic bodies (248). Thus a given macrophage may receive both inhibitory and activating signals in a given situation and the outcome, i.e. the activation state of the macrophage is likely to be the sum of all input signals. Of the receptors expressed on macrophages TLR-4 and TREMs are of particular importance for this thesis and will be described in more detail below.

1.4.3.1 TLR-4

TLRs belong to a large group of receptors known as pattern recognition receptors due to their ability to recognise pathogen associated molecular patterns (PAMPs). The mammalian TLR family (so far) consist of ten human (TLR-1 to 10) and twelve murine (TLR-1 to 9 and TLR-11 to 13) TLRs (115). TLRs were initially considered as being expressed exclusively on the cell surface. Later however it has been shown that whereas e.g. TLR-1, 2 and 4 are expressed on the cell surface and are recruited to phagosomes upon activation, TLR-3, 7 and 9 are expressed intracellularly (4). TLRs are expressed by both immune and non-immune cells and recognize a wide variety of PAMPs from a broad range of pathogens. Ligand recognition is mediated by a leucine-rich repeat domain and triggers homo- or hetero-dimerisation depending on the specific TLR (4, 183). Receptor dimerisation triggers signalling via the cytoplasmic Toll-interleukin-1 receptor (TIR) domain through which they interact with TIR containing adaptor proteins to induce intracellular signalling. To date five TLR-associated adaptor proteins have been identified; myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal), TIR-related adaptor protein inducing interferon (TRIF), TRIF-related adaptor molecule (TRAM) and sterile alpha HEAT-Armadillo motifs (SARM) (178). The response of the activated cell differs depending on which TLR that triggers the activation. The molecular basis for how different TLRs induce diverse responses is poorly understood but differential adapter usage is believed to be part of the mechanism by which the different TLRs discriminate between different pathogens (178).

TLR-4 is mainly known as an LPS receptor but has also been shown to recognise a variety of PAMPs in addition to LPS (4, 183). With its ability to respond to LPS, TLR-4 is the main inducer of host responses to Gram-negative bacteria (105, 186, 188). The details regarding LPS-TLR-4 interactions are poorly understood. LPS-mediated signalling through TLR-4 has been shown to depend on LPS binding protein (LBP), CD14 and MD2. According to the current model LBP binds to LPS and the LPS/LBP complex in turn binds soluble or membrane bound CD14 which presents the complex to TLR-4. Additionally TLR-4 needs to be bound to the secreted protein MD2 to be able to signal (65). TLR-4 uses (at least) four different adaptors which are thought to act in pairs, MyD88/Mal and TRIF/TRAM, with Mal and TRAM acting as bridging adapters which recruit MyD88 and TRIF respectively upon receptor activation and dimerization (4, 178). MyD88 recruits IL-1 receptor associated kinase (IRAK) 4 and IRAK-1. Phosphorylation of IRAK-1 by IRAK-4 enables release of IRAK-1 from its inhibitor Toll-interacting protein (Tollip) and MyD88 where after IRAK-1 associates with TNF receptor associated factor 6 (TRAF-6). Signalling downstream of TRAF-6 leads to activation of the transcription factor AP-1 and early activation of NF-κB which trigger the production of pro-inflammatory cytokines such as TNF-α and IL-1β (65, 115, 178). Signalling mediated through TRIF/TRAM, known as the MyD88 independent pathway, appears to be more complex and is still incompletely understood. It seems clear that MyD88 independent signalling leads to activation of the interferon regulatory factor (IRF) 3 transcription factor, late activation of NF-κB and production of IFN-β and iNOS (102, 169, 178). Although TNF-α was initially shown to be induced solely by the MyD88 dependent pathway a recent study suggests that TNF-α is produced also by the MyD88 independent pathway in an IRF-3 dependent manner. MyD88 independent production of TNF-α was shown to be required for the late

activation of NF-κB in this pathway (47). It has also been suggested that two different pathways exist downstream of TRIF. One where TRIF, either directly or via RIP-1, interacts with TRAF-6 to activate NF-κB, and one where IRF-3 and possibly also IRF-7 induces e.g. IFN-β expression (169). Adding to the complexity, the transcription factor IRF-5 has recently been shown to be required for triggering of the pro-inflammatory cytokines IL-6, IL-12 and TNF-α via the MyD88 dependent pathway (219).

Activation of TLR-4 triggers both the MyD88 dependent and independent pathways (65). The rationale for two separate signalling pathways downstream of TLR-4 is not known. However recent data may provide a clue. Jiang *et al*. have shown that macrophages from mice with non-functional CD14 respond differentially to smooth and rough LPS isolated from *Salmonella*. CD14 deficient macrophages were unable to trigger the MyD88 independent pathway and the MyD88 dependent pathway was triggered only in response to rough LPS (111). Another study has compared TLR-4 responses to LPS from different Gram-negative bacteria. This showed that the relative contribution of MyD88 dependent and independent signalling differed in the response to the different LPS (253). Although the biological relevance of this is not yet clear it opens up the possibility that TLR-4 may discriminate between LPS from different bacteria and it is intriguing to speculate that this may be used by the host to optimise the response to a specific Gram-negative bacterium.

Excessive triggering of pro-inflammatory cytokines and iNOS may cause extensive tissue damage by induction of apoptosis and/or necrosis, or induce septicaemia, and would thereby be detrimental for the host (142, 228). It is therefore not surprising that a number of negative regulators for TLR-signalling have been identified, many of which have been shown to be up-regulated in response to LPS and /or other TLR-ligands (140). Identification of a soluble variant of TLR-4 in mice lead to the hypothesis that decoy TLRs may exist to balance the response. The soluble variant of TLR-4 was shown to inhibit LPS-induced production of NF-κB and TNF-α by an unknown mechanism (107). A short variant of MyD88, MyD88s, is recruited to the receptor but does not allow NF-κB signalling and may thus dampen the response by competing with MyD88 (33, 109). The inhibition appears to be specific for NF-κB as AP-1 dependent gene expression is intact in the presence of MyD88s (110). IRAK-M and alternative splice variants of IRAK-1 and IRAK-2 are believed to inhibit IRAK activity (140). The function of the non-inhibitory IRAK-2 splice variant is not known. Other intracellular proteins shown to mediate negative regulation include the PI3K, SOCS1, A20 and Tollip (140). TLR responses may also be controlled by transmembrane proteins e.g. ST2 and TNF-related apoptosis-inducing ligand receptor (TRAILR). ST2 belongs to the TIR superfamily and exists in a membrane bound (ST2L) and a soluble form (sST2). ST2L is believed to mediate inhibition by sequestration of MyD88 and Mal through its TIR domain. sST2 has been shown to inhibit LPS-induced production of pro-inflammatory cytokines in macrophages by an unknown mechanism (140). TRAILR is believed to suppress TLR responses by inhibiting the NF-κB pathway (52).

1.4.3.2 TREMs

The recently identified TREMs belong to the Ig-superfamily of receptors. They lack a cytoplasmic signalling domain and rely on association with membrane-anchored adaptor proteins for surface expression and signalling. To date two human and three murine TREMs have been characterised, all of which associate with the adaptor DAP12 (43). The cytoplasmic domain of DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) which is phosphorylated upon ligand binding to the receptor. Phosphorylation of the ITAM motif, mediated by a Src family kinase, allows recruitment of the tyrosine kinases Syk and/or ZAP70. This triggers several signalling pathways through a cascade of phosphorylation events. Eventually this leads to nuclear translocation of transcription factors which activate gene expression (12, 88). Human TREM-1 is up-regulated on neutrophils and monocytes in response to Gram-negative and Gram-positive bacterial as well as fungal stimuli but not during non-microbial inflammatory conditions. Activation of TREM-1 by cross-linking of the receptor triggers an inflammatory response in monocytes and neutrophils, a response that is augmented by LPS stimulation of the cells, indicating that TREM-1 synergizes with TLR-4 signalling (24, 25). Murine TREM-2 has been shown to be expressed on macrophage cell lines, immature DCs, microglial cells and osteoclasts (43, 48). Cross-linking of murine TREM-2 triggered production of NO in transfected macrophages indicating that TREM-2 mediates activating signals (48). Of the up till now characterised TREMs, murine TREM-2 is the only one for which possible ligands have been identified. TREM-2 has been shown to bind both Gram-negative and Gram-positive bacteria. The binding was inhibited by anionic bacterial products such as LPS and lipotechoic acid but also by other anionic carbohydrates, indicating that the binding is based on charge (49). Murine TREM-3 is expressed on macrophage cell lines and has, like TREM-1, been shown to be up-regulated in response to LPS stimulation and was indicated to mediate activating signals (41).

TREM-1 also exists as an alternative soluble splice variant found in the blood of patients with sepsis (75). The function of this soluble variant is not known but it has been suggested that it may act as a decoy receptor to dampen the response, a hypothesis that is strengthened by studies on murine soluble TREM-1. Experimental LPS-induced septic shock in mice caused an up-regulation of murine TREM-1. Interestingly mice could be rescued by treatment with a soluble TREM-1 fusion protein (25). As mentioned above the only signalling adaptor known to associate with TREMs is DAP12 which typically mediates activating signals. However recent data suggest that DAP12 also may mediate inhibitory signals by an unknown mechanism. Absence of DAP12 was shown to cause enhanced macrophage-production of pro-inflammatory cytokines in response to various known TLR ligands. DAP12 deficient mice accordingly showed an increased susceptibility to septic shock as well as increased resistance to bacterial infection with *Listeria monocytogenes* (89). Furthermore, although TREM-2 was initially believed to trigger macrophage activation, it has recently been shown that TREM-2 inhibits macrophage activation by certain but not all TLRs. Stimulation of macrophages from TREM-2-/- mice with LPS, zymosan or CpG provoked increased levels of TNF-α compared to wild type macrophages, indicating that TREM-2 signalling dampens the response triggered by TLR-4, TLR-2/6 and TLR-9. The absence of TREM-2 did not have any effect when cells were stimulated with

TLR-3 or TLR-7/8 ligands (226). Thereby TREM-2-/- macrophages responded in a similar way as DAP12 deficient macrophages to stimulation with TLR-4, TLR-2/6 and TLR-9 ligands. Additionally, TREM-2 has recently been shown to be able to mediate the TLR-inhibitory signals through DAP12 (87). DAP12-mediated inhibition has been observed for other receptors than TREMs. Cross-linking of the receptor NKp44 or Siglec-H on plasmacytoid DCs (pDC) inhibited production of IFN-α/β in response to CpG (23, 67). It has also been shown that pDCs unable to signal through DAP12 produce increased cytokine amounts in response to infection with murine cytomegalovirus compared to cells from wild type mice (210). Thus DAP12 seems to be able to mediate inhibitory as well as activating signals, however, the molecular basis for this dual function of DAP12 is not known. As mentioned above, in a given situation an immune cell may receive signals through multiple receptors and the level of the response is likely to be the balanced outcome of all input signals. Signalling by ITAM associated receptors are often counterbalanced by receptors mediating their signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM). Following phosphorylation of tyrosine residues ITIMs recruits the protein tyrosine phosphatases SHP-1 or SHP-2 or the inositol lipid phosphatase SHIP which dephosphorylate signalling proteins and thereby dampens the response. However in the case of DAP12 the inhibitory signal seems to be mediated through an ITAM motif which normally induces activating signals (87).

Although little is known about the molecular mechanism underlying DAP12 mediated inhibition, it has been reported to require phosphorylation of the same tyrosine residues that are involved in mediating activation, and possibly also the activity of Syk (87). Several possible mechanisms have been suggested on how DAP12 mediates the inhibition on TLR signalling, including recruitment of a phosphatase followed by dephosphorylation and thereby inactivation of signalling molecules, sequestration of signalling molecules or activation of a specific negative TLR regulator (87). However, none of these models provide an explanation for how an ITAM bearing receptor may mediate an inhibitory signal which depends on the same tyrosine motifs as the activation, and future studies are thus needed to shed light on this enigma.

2 AIMS

This thesis focuses on *S.* Typhimurium host-pathogen interactions, in particular the interplay between *S.* Typhimurium and its macrophage host cell.

The specific aims were to:

- Establish what role the O-antigen has for intracellular fitness in macrophages
- Study the role of the thioredoxin and glutathione/glutaredoxin systems in resistance to oxidative and NO stress, intracellular fitness and virulence *in vivo*
- Study the function of the newly characterised receptor TRAPC during bacterial infection

3 RESULTS AND DISCUSSION

3.1 PAPER I

According to the dogma, many enterobacterial pathogens, including *Salmonella*, rely on expression of smooth LPS for full virulence. This is exemplified by the reduced ability of O-antigen deficient mutants of *Yersinia enterocolotica* to colonize Peyer's patches and their inability to colonize the liver or spleen. Concomitantly these mutants also display a severe reduction in virulence in the murine infection model. Likewise mutants of *S.* Typhimurium devoid of the O-antigen display a decreased capability to colonize the gut, and a reduced virulence in the murine infection model.

In addition to its important role for bacterial virulence the LPS is also a major inducer of innate host responses mainly through recognition by TLR-4, thereby triggering production of pro-inflammatory cytokines, ROI and RNI (65, Hoebe, 2003 #424, 178, 204). Recognition by TLR-4 has been attributed to lipid A and *Salmonella* is known to modify its lipid A in a multifaceted way to increase resistance to CAMPs or reduce recognition by TLR-4 (84, 86, 117, 133). However recent data indicating that CD14 is essential for the recognition of smooth but not rough LPS (111), brings to mind the possibility that the O-antigen in addition to lipid A may influence TLR-4 recognition of Gram-negative bacteria. Additionally *Shigella* was recently shown to reduce the length of its LPS by glycosylation-mediated conformational changes of the O-antigen thereby achieving more efficient type III secretion of virulence proteins into host cells (238). Furthermore, *S.* Typhimurium has been shown to down-regulate genes for LPS synthesis (56) and to reduce the chain length of the O-antigen once the bacteria reside and replicate inside macrophage-like cells (130). Combined, these findings imply that it may be beneficial to modify also the O-antigen under certain stages of infection. This possibility intrigued us to study whether *S.* Typhimurium benefits from expressing LPS devoid of O-antigen once bacteria reside and replicate inside macrophages.

To analyse this, isogenic *waaL* and *waaK* deletion mutants, lacking the O-antigen ligase and the N-acetylglucoseamine transferase respectively, as well as a Δ*waaKL* double mutant were generated. When assayed for fitness and ability to induce host cell NO production in macrophage-like J774-A.1 cells, the Δ*waaL*, Δ*waaK* and Δ*waaKL* mutants triggered lower production of NO and consequently displayed higher intracellular growth yields compared to wild type bacteria. If the reduction in macrophage NO production was caused by differential recognition by TLR-4, the reduced NO levels should be accompanied by reduced iNOS expression. Interestingly, immunoblotting experiments showed equal induction of iNOS expression in cells infected with wild type, Δ*waaL* or Δ*waaK* mutant bacteria. As this experiment indicated that these mutants interfere with the enzymatic activity of iNOS we continued by analysing the requirement for bacterial protein synthesis for inhibition of iNOS. When bacterial protein synthesis was inhibited during infection of J774-A.1 cells the Δ*waaL* mutant was unable to interfere with the activity of iNOS. Furthermore we could show that the iNOS inhibition was unrelated to SPI-1 but seemed to require the SPI-2 T3SS. Interestingly, while the Δ*waaK* mutant which still expressed slight amounts of O-antigen inhibited the activity of iNOS, the partial restoration of O-antigen expression

in the Δ*waaKL* mutant, obtained with complementation with *waaK* and *waaL in trans*, restored macrophage NO levels and bacterial growth yield to wild type levels. Thus a delicate balance in O-antigen expression seems to regulate the ability to inhibit iNOS activity.

Our results clearly show that *S.* Typhimurium benefits from expressing LPS devoid of the O-antigen during growth in macrophage-like cells, and provide a possible explanation for the observation by Lähteenmäki *et al.* that *S.* Typhimurium down-regulates the chain length of the O-antigen during growth in macrophage-like cells (130). That is, *S.* Typhimurium may modify the O-antigen to enable interference with iNOS activity and possibly also other antibacterial macrophage-responses. In this scenario our mutants already express the required LPS structure whereas the wild type needs time to modify the LPS rendering the mutants able to inhibit iNOS activity more efficiently. In this context it is interesting to note that *S.* Typhimurium has been shown to shed its LPS during infection of epithelial cells. Furthermore, LPS shedding was only observed with wild type bacteria expressing smooth LPS (70). Possibly the rationale for this shedding is to rapidly change the LPS structure into one containing less O-antigen to enable efficient iNOS inhibition.

In recent experiments J774-A.1 cells were primed by a 2h treatment with IFN-γ (1000 U/ml) prior to infection. This did not affect the background levels of NO in uninfected cells, but deprived the Δ*waaL* mutant of its ability to suppress macrophage NO production compared to wild type bacteria (Fig. 2 unpublished data). Hence, the level of *Salmonella*-mediated iNOS inhibition seems to correlate with the activation state of the cells. One explanation for this could be that the level of iNOS inhibition dependent on the dose of the inhibitor as well as on the kinetics of iNOS expression. Possibly, priming with IFN-γ enables the J774-A.1 cells to trigger iNOS expression, and thereby NO production, more rapidly in response to infection compared with non-primed cells. Bearing in mind that the suppression of iNOS activity is indicated to depend on SPI-2 and that macrophage NO production is reported to inhibit SPI-2 expression (154), one can envision that a more rapid induction of NO production may inhibit SPI-2 to the extent that it cancels out the benefit of lacking the O-antigen.

Whatever the identity of the bacterial protein/s (factor X) mediating the inhibition of iNOS activity, the inhibition may occur at one or several different levels. Production of NO depends on the presence of L-arginine, and iNOS activity is partly controlled by arginase-mediated degradation of L-arginine. Furthermore, the intracellular storage of L-arginine is not enough to support the NO production and iNOS activity also rely on transport of L-arginine into the cell by arginine transporters (167). Thus factor X may control iNOS activity by inducing expression of host cell arginases or inhibiting arginine transport. Treatment of cells with an arginase inhibitor did not affect the ability of the Δ*waaL* mutant to suppress iNOS activity (unpublished data) but the possible effect on L-arginine transport remains to be studied. Additionally, iNOS is active only as a dimer and requires a number of co-factors for dimerization, hence factor X may also inhibit iNOS dimerization by inducing degradation, inhibit expression or sequester cofactors required for activity. Factor X may also inhibit iNOS activity by inducing expression of inhibitory proteins, or by reducing the disulfide bridge necessary for dimerization.

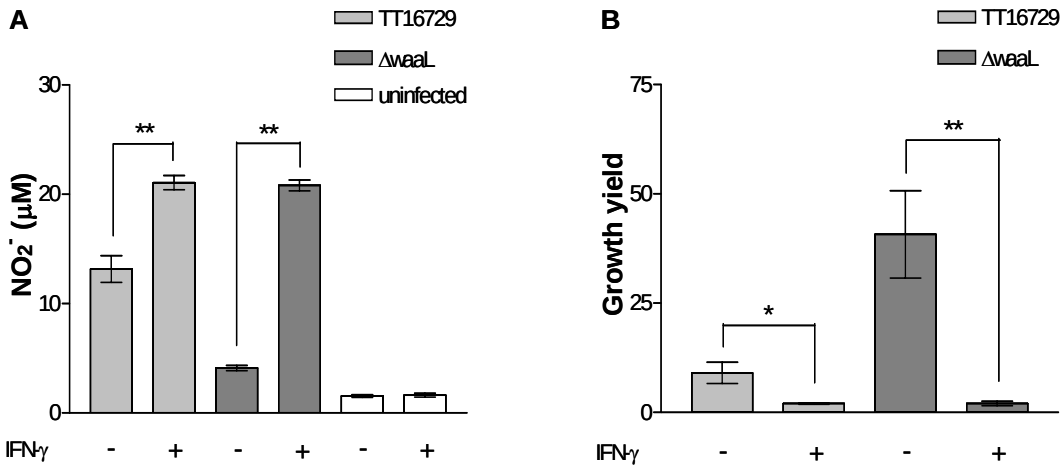

Figure 2. J774-A.1 cells were pretreated with IFN-γ for 2h prior to infection with wild type or Δ*waaL* mutant bacteria. NO production (**A**) and bacterial growth yields (cfu at 16h/cfu at 2h) (**B**) were measured 16h post infection.

3.2 PAPER II

The recently characterized novel member of the mouse TREM receptor family triggering receptor expressed by antigen presenting cells (TRAPC) is, like TREM-1, transcriptionally up-regulated by LPS on macrophage-like cells, and cross-linking of TRAPC induces production of NO in macrophage-like cells and DCs (manuscript under revision in Eur. J. Immunol.). The production of NO by murine phagocytic cells is important for the control of intracellular bacterial replication not only of *Salmonella* but also other important pathogens, including *Listeria monocytogenes* and *Mycobacterium tuberculosis*. Thereby the ability of TRAPC to induce NO expression strongly suggests a role for TRAPC in the defence against bacteria and intrigued us to study the effect of TRAPC-cross-linking during bacterial infection of macrophage-like cells.

When combined with infection with the Gram-negative bacteria *E. coli* TG1 or *S.* Typhimurium, or stimulation with LPS, cross-linking of TRAPC on J774-A.1 cells, in contrast to what would be expected considering the priming effect of TRAPC-cross-linking on macrophages, inhibited host cell NO production. For *S. enterica* serovar

Typhimurium the reduction in NO levels correlated with increased intracellular bacterial replication.

Like all other up till now identified members of the TREM receptor family, TRAPC contains an ITAM motif in its cytoplasmic domain through which it associates with the ITAM-containing membrane-anchored adaptor protein DAP12. ITAM mediated signalling typically generates activating signals (12, 88), however, an increasing number of reports show that DAP12 also may mediate inhibitory signals (23, 67, 87, 89, 210). TREM-2 was recently shown to have an inhibitory effect on TLR signalling and to be able to mediate this effect through DAP12 (87, 226). In one set of samples, the suppression of macrophage NO production, obtained when TRAPC cross-linking was combined with infection or LPS stimulation, was accompanied by slightly reduced iNOS levels indicating that the suppression of NO production occurs on the level of signalling rather than interference with iNOS activity. The transcription factor NF-κB has a crucial role for induction of iNOS expression, and is activated during TLR-4 signalling through degradation of Ikβ (115, 201). Immunoblotting experiments with cross-linked non-infected macrophage-like cells show that cross-linking of TRAPC triggers degradation of Ikβ. Ongoing experiments will reveal whether the inhibition of macrophage NO production, obtained with TRAPC cross-linking combined with Gram-negative infection or LPS stimulation, is a consequence of inhibition of the NF-κB pathway.

It may seem puzzling that while IFN-γ has a synergistic effect on host cell NO production triggered by cross-linking of TRAPC (manuscript under revision in Eur. J. Immunol.), bacterial infection or LPS stimulation acts inhibitory. One can however envision at least two explanations for this apparent contradiction. *i*) TREM-2 has been shown to bind specifically to Gram-negative and Gram-positive bacteria as well as to yeast, and this binding can be inhibited by bacterial products such as LPS, lipoteichoic acid, and peptidoglycan but also anionic carbohydrate molecules, including dextran sulfate, suggesting that ligand recognition is based partly on charge. It is tempting to speculate that bacteria have evolved to use certain DAP12 associated receptors to their advantage. In this scenario, by inducing DAP12 mediated inhibition of antibacterial macrophage responses, bacteria would promote their own survival. It remains to be investigated if bacteria are able to bind these DAP12 associated receptors, including TRAPC, directly, and what mechanism(s) underlies the TRAPC-mediated regulation of innate immune effector functions. *ii*) A full scale pro-inflammatory signalling will inevitably cause a certain degree of tissue damage to the host, and is therefore undesirable unless absolutely necessary. It is possible that TRAPC participates in the decision whether full scale signalling should be triggered or not and that the signal mediated by TRAPC (activating or inhibitory) is determined by the activation state of the cell. That is, triggering of TRAPC in conjunction with TLR-4 signalling in activated cells or in combination with other pro-inflammatory stimuli, e.g. IFN-γ may be a signal of high level of danger and that full scale signalling is required. Triggering of TRAPC in combination with TLR-4 signalling on naive cells in the absence of additional pro-inflammatory stimuli on the other hand may lead to TRAPC mediated dampening of the TLR-4 mediated response to avoid unnecessary damage to the host. This scenario may however be too simplistic. DAP12 -/- mice are more susceptible to septic shock due to excessive induction of inflammatory responses (89) i.e. a certain

degree of inhibition seems to be required to avoid detrimental damage to the host. Most likely in a given situation a given macrophage receives signals from a multitude of receptors, and there may also be cross-talk between some of these receptors. Several inhibitory and activating signals may be required to fine tune the response to ensure induction of the appropriate one.

3.3 PAPER III AND IV

Host cell production of ROI is, together with production of RNI, the most important host defence mechanism towards infection with *S.* Typhimurium in the murine infection model. Thus it is not surprising that a number of defence mechanisms, which protect *Salmonella* from these host responses, have been identified. However, there are still genes potentially important for protection against ROI/RNI but whose importance has not been evaluated in *Salmonella*.

ROI may affect the redox status of cytosolic proteins and oxidize cysteine sulfhydryl side chains essential for protein folding, enzymatic activity or for the chelating of essential metal cofactors. In *E. coli*, the thioredoxin and glutathione/glutaredoxin systems encode enzymes which shuttle redox potential from NADPH to cytosolic substrates, thereby providing a repair system for oxidized sulfhydryl groups in cytosolic proteins (Fig. 3). Through thioredoxin 1 (TrxA) and 2 (TrxC) the thioredoxin system transfers reducing equivalents to DsbD, located in the cytoplasmic membrane, for transfer to the periplasmic space thus providing the periplasmic oxidoreductase DsbC with electrons. DsbC in turn, by reducing wrongly paired disulfides, plays an important role in assuring correct protein folding of periplasmic proteins. Both thioredoxin 1 and 2 are in turn continuously reduced, mainly by thioredoxin reductase (TrxB) which derives its reducing equivalents from NADPH.

The glutathione/glutaredoxin system, which relies on glutathione as a redox-shuttle from NADPH to oxidized targets, consists of the glutathione-synthesizing enzymes γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB), the glutathione oxido-reductase (Gor) and glutaredoxin 1 (GrxA), 2 (GrxB) and 3 (GrxC) (8). For *E. coli* it has been shown that null mutants for single components of the thioredoxin or glutathione/glutaredoxin system do not have any profound effects on viability during standard *in vitro* growth conditions (187, 197). However, when *trxA*, *trxC* and *grxA* are deleted simultaneously the combination is lethal (216). Hence, the thioredoxin and glutathione/glutaredoxin pathways could be envisioned to formally constitute parallel mechanisms for transfer of electrons. Deficiencies in the thioredoxin or glutathione/glutaredoxin pathways are indeed reported to sensitize bacteria such as *E. coli, Helicobacter pylori* and *Rhodobacter sphareoides* to hydrogen peroxide *in vitro* (44, 136, 220), whereas inactivation of a homologue for glutathione peroxidase (*gpxA*) in *Neisseria* is reported to cause increased sensitivity to paraquat (166). Additionally, in *E. coli* glutaredoxin has been suggested to act as a "S-nitrosyl sink" in the protection against nitric oxide stress (93).

The annotated *S. enterica* serovar Typhimurium LT2 genome sequence contains close homologues to the components of the *E. coli* thioredoxin and glutathione/glutaredoxin

systems, however, apart from the role of DsbA and its paralogue SrgA for SPI-2 secretion and virulence, the importance of these systems for *Salmonella* virulence have not previously been studied. Thus deletion mutants of the *trxA*, *trxB*, *trxC*, *gshA*, *grxA* and *dsbC* genes were generated to establish the importance of the thioredoxin and glutathione/glutaredoxin systems for protection against ROI and RNI, intracellular replication and *in vivo* virulence of *S.* Typhimurium. These mutants were subsequently probed for sensitivity to oxidative and NO donor substances *in vitro*, intracellular fitness in epithelial and macrophage-like cells as well as for virulence *in vivo*. Surprisingly, except the *gshA* mutant, which was more sensitive to oxidative substances when grown under SCV-like conditions, none of the mutants displayed increased sensitivity to the substances tested. Furthermore, the *trxA* mutant was the only mutant that showed a reduced growth yield in both epithelial and macrophage-like cells as well as a severe reduction in virulence *in vivo*. In addition to its role as a reductase *E. coli* TrxA has been shown to harbour a chaperone function and to be able to interact with a large number of different proteins some of which are involved in oxidative stress response (128). By complementing the Δ*trxA* mutant with a collection of *trxA* constructs differing in their redox potential we could show a straight correlation between TrxA redox-activity and fitness in macrophage-like cells. Additionally, competition experiments *in vivo* between *trxA* mutants complemented *in trans* either with wild type *trxA* or redox-inactive *trxA* clearly showed that *Salmonella* virulence depends on redox active TrxA.

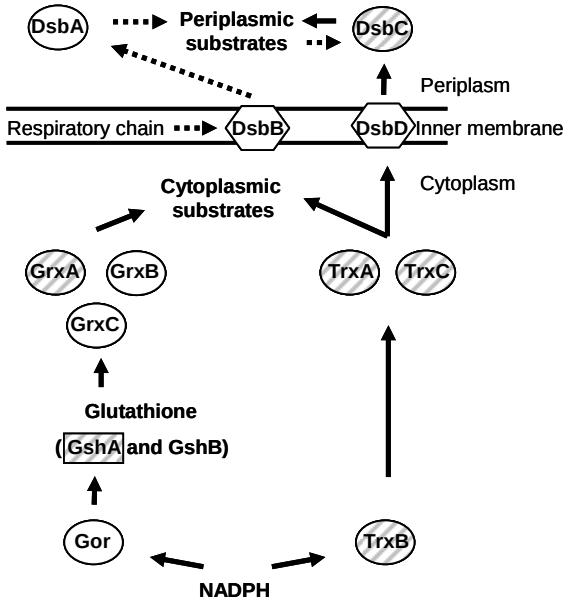

Figure 3. A schematical overview of the thioredoxin and glutathione/glutaredoxin systems in *Escherichia coli* is shown. Reduction and oxidation are indicated by solid and broken arrows respectively. Double arrows indicate disulfide bond isomerization. Members of either of these systems covered in this study are shaded in grey.

The phenotype of the *trxA* mutant, with its pronounced decrease in intracellular fitness and *in vivo* virulence, strongly resembles the phenotype of an *ssaV* mutant, which is unable to secrete SPI-2 effector proteins. In paper IV we therefore continued by studying if the phenotype of the *trxA* mutant was connected to SPI-2. Initial experiments in which macrophage-like cells were infected with wild type, Δ*trxA*, Δ*ssaV* or Δ*trxA*Δ*ssaV* mutant bacteria indicated a possible mechanistic connection between TrxA and SPI-2. SPI-2 is required for growth within the SCV, and we could furthermore show that catalytically active TrxA is required for bacterial adaptation to a sudden shift from rich medium to growth under SCV-like conditions as well as for SPI-2 functionality under these conditions. In paper I we showed that a strong reduction of the O-antigen levels or complete absence of O-antigen enables *S.* Typhimurium to inhibit the iNOS activity of macrophage-like cells. The inhibition of iNOS was furthermore indicated to depend on SPI-2. Accordingly, a compound recently shown to inhibit T3SS in *Yersinia* (114), *Chlamydia trachomatis* (170) and *S.* Typhimurium (Negrea et al. submitted manuscript) deprived the Δ*waaL* mutant of its ability to inhibit macrophage NO production (unpublished data). As we in paper I show that the inhibition of iNOS is independent of SPI-1, these results provide further evidence for the involvement of SPI-2 in the inhibition of iNOS activity. As SPI-2 depends on TrxA for proper function (Paper IV), and the inhibition of iNOS activity by O-antigen deficient *S.* Typhimurium seemed to depend on SPI-2, the next question was whether TrxA is required for iNOS inhibition.

When analyzing the importance of TrxA redox activity for iNOS inhibition, in contrast to the Δ*trxA* single mutant (Paper III), no linear correlation could be established between TrxA redox potential and intracellular fitness in the Δ*waaL* background. However, a correlation was observed in the sense that complementation with the more reducing variants of TrxA restored the intracellular fitness, whereas the more oxidizing variants of TrxA, in spite of inducing NO levels similar to the Δ*waaL* single mutant, only caused a partial restoration of the growth yield that did not differ significantly from that of Δ*trxA*Δ*waaL* complemented with the empty cloning vector pBAD33. Surprisingly, while complementation with a catalytically inactive variant of t*rxA* did not reduce the NO levels of infected J774-A.1 cells, it partially restored the intracellular growth yield of the Δ*waaL*Δ*trxA* double mutant. In the very stringent statistical analysis used in this study the partial complementation mediated by catalytically inactive t*rxA*, was not significant but in an ordinary two-sided T-test it gave a p-value < 0.05. In contrast, the catalytically inactive *trxA* did not mediate any complementation of the Δ*trxA* single mutant. Combined these observations imply that under certain conditions TrxA may contribute to intracellular fitness of *S. enterica* serovar Typhimurium through a redox-independent mechanism. Furthermore, competition experiments *in vivo*, where a *trxA* mutant complemented with the empty pBAD33 cloning vector was competed against *trxA* mutant bacteria complemented with either wild type *trxA* or catalytically inactive *trxA*, clearly showed that TrxA contributes to virulence, mainly through a redox- dependent but also through a redox-independent mechanism.

Considering the major importance of ROI and RNI in the defence against *Salmonella*, both during infection of macrophages *in vitro* and *in vivo* (151, 234), and the role of TrxA as a reductase, it may seem surprising that the *trxA* mutant does not show

increased sensitivity to oxidative or NO stress *in vitro*. However this does not in any way rule out an important role for TrxA in the protection against ROI and/or RNI. If TrxA is required for inhibition of production of ROI/RNI rather than repair of the damage or ROI/RNI detoxification, absence of TrxA will not affect the sensitivity to these compounds.

4 CONCLUDING REMARKS

The ability to adapt to host environment is essential for bacterial virulence. In the murine model system for typhoid fever adaptation of *S.* Typhimurium to the intra-macrophage environment is crucial for virulence, and largely depends on SPI-2. This thesis defines important, SPI-2 related, roles for the O-antigen of the LPS and TrxA, a component of the thioredoxin system, for bacterial adaptation to macrophage and host conditions.

Expression of a full length O-antigen has conventionally been regarded as a requirement for enterobacterial virulence. The importance of smooth LPS for colonization of the host is well established for *Salmonella* as well as for other enteric bacteria. However, the role of the O-antigen during intracellular stages of infection has not previously been studied with clearly defined LPS mutants. O-antigen depletion in *S.* Typhimurium was accompanied by a higher bacterial fitness in non-activated murine macrophage-like cells. This macrophage-adapted phenotype associated with suppression of iNOS activity and thereby the production of NO, and strongly depended on SPI-2 and TrxA. Interestingly, *S.* Typhimurium was recently shown to down-regulate the O-antigen chain length during growth in macrophage-like cells (130), providing our data with a possible biological relevance. TrxA was in turn shown to affect SPI-2 activity under SCV-like conditions. By its requirement for proper function of SPI-2 it is possible that TrxA contributes to macrophage-adaptation and virulence through SPI-2. The following question is whether TrxA mediates suppression of iNOS activity by a direct interaction with iNOS i.e. is TrxA a SPI-2 effector protein? However, TrxA does not have the translocation motif found in many, but not all, SPI-2 effector proteins (153, 231). Furthermore, considering its effect on SPI-2 functionality it seems more likely that the requirement for TrxA is secondary and that TrxA is required for the secretion and/or function of one or more SPI-2 effector proteins.

At this stage we cannot conclude whether the SPI-2-dependency for iNOS inhibition originates from the ability of SPI-2 to promote bacterial intracellular replication through its effect on vesicular trafficking, or whether there is a specific iNOS-targeted SPI-2 effector protein. It is also unclear how O-antigen deficiency relates to the SPI-2 dependent inhibition of iNOS. In *Shigella*, a reduction in LPS length, mediated by a conformational change of the O-antigen, potentates the T3SS, thereby enabling more efficient secretion of effector proteins into host cells (238). Possibly, the efficiency of the SPI-2 T3SS correlates with the length of the LPS also in S. Typhimurium, with T3SS activity being enhanced in an O-antigen deficient background. Regarding the SPI-2 dependency, a simple explanation could be that SPI-2-assisted bacterial intracellular replication associates with consumption of host L-arginine. If one further assumes that SPI-2 activity is enhanced in an O-antigen deficient background, and that this translates into increased intracellular replication, host cell resources of L-arginine would be depleted, and iNOS would be inactivated in lack of the key substrate. However this would require up-regulated transport of host L-arginine across both the vacuolar membrane of the SCV and the bacterial membranes. Thereby it seems more likely that the inhibition of iNOS is a direct consequence of SPI-2 activity. Here it is

interesting to note that TrxA recently was shown to act as a chaperone for a *Helicobacter pylori* encoded arginase. Furthermore, the *H. pylori* TrxA, in conjunction with the arginase, protected the bacterium against ROI and RNI (155). Possibly *S.* Typhimurium affects iNOS by encoding a SPI-2-secreted and iNOS-targeting effector, e.g. an arginase that requires TrxA either for its function or translocation.

Subsequent studies focusing on host cell immune response regulators opened yet another potential bacterial strategy for coping with host cell NO expression. Cross-linking of the novel TREM-family member protein TRAPC previously found to induce a moderate NO production in uninfected macrophage-like cells and DCs. However, in contrast to what would be expected, when combined with infection with wild type *S.* Typhimurium, cross-linking of TRAPC on macrophage-like cells suppressed host cell NO production. This pathway of NO inhibition appeared unrelated to the one dependent on SPI-2, in that a similar suppression was achieved upon infection with a laboratory *E. coli* strain devoid of SPI-2 as well as with LPS stimulation. It remains to be determined whether bacteria are able to exploit TRAPC to their own benefit or whether the inhibitory function of TRAPC is a mechanism used by the host to balance the response to an invading pathogen to avoid unnecessary damage to the host inflicted by an excessive pro-inflammatory response.

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6 REFERENCES

1. **Abrahams, G. L., and M. Hensel**. 2006. Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells. Cell Microbiol **8**:728-37.
2. **Aderem, A., and D. M. Underhill**. 1999. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol **17**:593-623.
3. **Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee**. 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in Salmonella typhimurium. Mol Microbiol **47**:715-28.
4. **Akira, S., and K. Takeda**. 2004. Toll-like receptor signalling. Nat Rev Immunol **4**:499-511.
5. **al-Hendy, A., P. Toivanen, and M. Skurnik**. 1992. Lipopolysaccharide O side chain of Yersinia enterocolitica O:3 is an essential virulence factor in an orally infected murine model. Infect Immun **60**:870-5.
6. **Alpuche Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller**. 1992. Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. Proc Natl Acad Sci U S A **89**:10079-83.
7. **Altier, C.** 2005. Genetic and environmental control of salmonella invasion. J Microbiol **43 Spec No**:85-92.
8. **Aslund, F., and J. Beckwith**. 1999. The thioredoxin superfamily: redundancy, specificity, and gray-area genomics. J Bacteriol **181**:1375-9.
9. **Bader, M. W., S. Sanowar, M. E. Daley, A. R. Schneider, U. Cho, W. Xu, R. E. Klevit, H. Le Moual, and S. I. Miller**. 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell **122**:461-72.
10. **Bajaj, V., C. Hwang, and C. A. Lee**. 1995. hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. Mol Microbiol **18**:715-27.
11. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee**. 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol Microbiol **22**:703-14.
12. **Barrow, A. D., and J. Trowsdale**. 2006. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. Eur J Immunol **36**:1646-53.
13. **Baumler, A. J., J. G. Kusters, I. Stojiljkovic, and F. Heffron**. 1994. Salmonella typhimurium loci involved in survival within macrophages. Infect Immun **62**:1623-30.
14. **Bearson, B. L., L. Wilson, and J. W. Foster**. 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. J Bacteriol **180**:2409-17.
15. **Beier, D., and R. Gross**. 2006. Regulation of bacterial virulence by two-component systems. Curr Opin Microbiol **9**:143-52.
16. **Belden, W. J., and S. I. Miller**. 1994. Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. Infect Immun **62**:5095-101.
17. **Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden**. 2000. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. Embo J **19**:3235-49.
18. **Beuzon, C. R., K. E. Unsworth, and D. W. Holden**. 2001. In vivo genetic analysis indicates that PhoP-PhoQ and the Salmonella pathogenicity island 2 type III secretion system contribute independently to Salmonella enterica serovar Typhimurium virulence. Infect Immun **69**:7254-61.
19. **Bhan, M. K., R. Bahl, and S. Bhatnagar**. 2005. Typhoid and paratyphoid fever. Lancet **366**:749-62.

20. **Bijlsma, J. J., and E. A. Groisman**. 2005. The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. Mol Microbiol **57**:85-96.
21. **Bjur, E., S. Eriksson-Ygberg, and M. Rhen**. 2006. The O-antigen affects replication of Salmonella enterica serovar Typhimurium in murine macrophage-like J774-A.1 cells through modulation of host cell nitric oxide production. Microbes Infect **8**:1826-38.
22. **Blanc-Potard, A. B., F. Solomon, J. Kayser, and E. A. Groisman**. 1999. The SPI-3 pathogenicity island of Salmonella enterica. J Bacteriol **181**:998-1004.
23. **Blasius, A. L., M. Cella, J. Maldonado, T. Takai, and M. Colonna**. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. Blood **107**:2474-6.
24. **Bouchon, A., J. Dietrich, and M. Colonna**. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol **164**:4991-5.
25. **Bouchon, A., F. Facchetti, M. A. Weigand, and M. Colonna**. 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. Nature **410**:1103-7.
26. **Boucrot, E., T. Henry, J. P. Borg, J. P. Gorvel, and S. Meresse**. 2005. The intracellular fate of Salmonella depends on the recruitment of kinesin. Science **308**:1174-8.
27. **Boyd, E. F., F. S. Wang, T. S. Whittam, and R. K. Selander**. 1996. Molecular genetic relationships of the salmonellae. Appl Environ Microbiol **62**:804-8.
28. **Brown, A., and C. E. Hormaeche**. 1989. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. Microb Pathog **6**:445-54.
29. **Brumell, J. H., D. L. Goosney, and B. B. Finlay**. 2002. SifA, a type III secreted effector of Salmonella typhimurium, directs Salmonella-induced filament (Sif) formation along microtubules. Traffic **3**:407-15.
30. **Brumell, J. H., P. Tang, S. D. Mills, and B. B. Finlay**. 2001. Characterization of Salmonella-induced filaments (Sifs) reveals a delayed interaction between Salmonella-containing vacuoles and late endocytic compartments. Traffic **2**:643-53.
31. **Buchmeier, N. A., S. J. Libby, Y. Xu, P. C. Loewen, J. Switala, D. G. Guiney, and F. C. Fang**. 1995. DNA repair is more important than catalase for Salmonella virulence in mice. J Clin Invest **95**:1047-53.
32. **Buchmeier, N. A., C. J. Lipps, M. Y. So, and F. Heffron**. 1993. Recombination-deficient mutants of Salmonella typhimurium are avirulent and sensitive to the oxidative burst of macrophages. Mol Microbiol **7**:933-6.
33. **Burns, K., S. Janssens, B. Brissoni, N. Olivos, R. Beyaert, and J. Tschopp**. 2003. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med **197**:263-8.
34. **Caldwell, A. L., and P. A. Gulig**. 1991. The Salmonella typhimurium virulence plasmid encodes a positive regulator of a plasmid-encoded virulence gene. J Bacteriol **173**:7176-85.
35. **Cano, D. A., M. G. Pucciarelli, F. Garcia-del Portillo, and J. Casadesus**. 2002. Role of the RecBCD recombination pathway in Salmonella virulence. J Bacteriol **184**:592-5.
36. **Catron, D. M., Y. Lange, J. Borensztajn, M. D. Sylvester, B. D. Jones, and K. Haldar**. 2004. Salmonella enterica serovar Typhimurium requires nonsterol precursors of the cholesterol biosynthetic pathway for intracellular proliferation. Infect Immun **72**:1036-42.
37. **Catron, D. M., M. D. Sylvester, Y. Lange, M. Kadekoppala, B. D. Jones, D. M. Monack, S. Falkow, and K. Haldar**. 2002. The Salmonella-containing vacuole is a major site of intracellular cholesterol accumulation and recruits the GPI-anchored protein CD55. Cell Microbiol **4**:315-28.

38. **Chakravortty, D., I. Hansen-Wester, and M. Hensel**. 2002. Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. J Exp Med **195**:1155-66.
39. **Chang, J., J. Chen, and D. Zhou**. 2005. Delineation and characterization of the actin nucleation and effector translocation activities of Salmonella SipC. Mol Microbiol **55**:1379-89.
40. **Chen, L. M., S. Hobbie, and J. E. Galan**. 1996. Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses. Science **274**:2115-8.
41. **Chung, D. H., W. E. Seaman, and M. R. Daws**. 2002. Characterization of TREM-3, an activating receptor on mouse macrophages: definition of a family of single Ig domain receptors on mouse chromosome 17. Eur J Immunol **32**:59-66.
42. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow**. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol Microbiol **30**:175-88.
43. **Colonna, M.** 2003. TREMs in the immune system and beyond. Nat Rev Immunol **3**:445-53.
44. **Comtois, S. L., M. D. Gidley, and D. J. Kelly**. 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in Helicobacter pylori. Microbiology **149**:121-9.
45. **Conlan, J. W.** 1996. Neutrophils prevent extracellular colonization of the liver microvasculature by Salmonella typhimurium. Infect Immun **64**:1043-7.
46. **Conlan, J. W.** 1997. Critical roles of neutrophils in host defense against experimental systemic infections of mice by Listeria monocytogenes, Salmonella typhimurium, and Yersinia enterocolitica. Infect Immun **65**:630-5.
47. **Covert, M. W., T. H. Leung, J. E. Gaston, and D. Baltimore**. 2005. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science **309**:1854-7.
48. **Daws, M. R., L. L. Lanier, W. E. Seaman, and J. C. Ryan**. 2001. Cloning and characterization of a novel mouse myeloid DAP12-associated receptor family. Eur J Immunol **31**:783-91.
49. **Daws, M. R., P. M. Sullam, E. C. Niemi, T. T. Chen, N. K. Tchao, and W. E. Seaman**. 2003. Pattern recognition by TREM-2: binding of anionic ligands. J Immunol **171**:594-9.
50. **De Groote, M. A., U. A. Ochsner, M. U. Shiloh, C. Nathan, J. M. McCord, M. C. Dinauer, S. J. Libby, A. Vazquez-Torres, Y. Xu, and F. C. Fang**. 1997. Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. Proc Natl Acad Sci U S A **94**:13997-4001.
51. **Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel**. 1999. Environmental regulation of Salmonella pathogenicity island 2 gene expression. Mol Microbiol **31**:1759-73.
52. **Diehl, G. E., H. H. Yue, K. Hsieh, A. A. Kuang, M. Ho, L. A. Morici, L. L. Lenz, D. Cado, L. W. Riley, and A. Winoto**. 2004. TRAIL-R as a negative regulator of innate immune cell responses. Immunity **21**:877-89.
53. **Eckmann, L., and M. F. Kagnoff**. 2001. Cytokines in host defense against Salmonella. Microbes Infect **3**:1191-200.
54. **El-Gayar, S., H. Thuring-Nahler, J. Pfeilschifter, M. Rollinghoff, and C. Bogdan**. 2003. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J Immunol **171**:4561-8.
55. **Eriksson, S., J. Bjorkman, S. Borg, A. Syk, S. Pettersson, D. I. Andersson, and M. Rhen**. 2000. Salmonella typhimurium mutants that downregulate phagocyte nitric oxide production. Cell Microbiol **2**:239-50.
56. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton**. 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. Mol Microbiol **47**:103-18.
57. **Ernst, R. K., T. Guina, and S. I. Miller**. 2001. Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect **3**:1327-34.

58. **Fang, F. C.** 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest **99**:2818-25.
59. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney**. 1992. The alternative sigma factor katF (rpoS) regulates Salmonella virulence. Proc Natl Acad Sci U S A **89**:11978-82.
60. **Fang, F. C., A. Vazquez-Torres, and Y. Xu**. 1997. The transcriptional regulator SoxS is required for resistance of Salmonella typhimurium to paraquat but not for virulence in mice. Infect Immun **65**:5371-5.
61. **Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan, and J. S. Kroll**. 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol Microbiol **25**:785-96.
62. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron**. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A **83**:5189-93.
63. **Fierer, J., L. Eckmann, F. Fang, C. Pfeifer, B. B. Finlay, and D. Guiney**. 1993. Expression of the Salmonella virulence plasmid gene spvB in cultured macrophages and nonphagocytic cells. Infect Immun **61**:5231-6.
64. **Figueroa-Bossi, N., and L. Bossi**. 1999. Inducible prophages contribute to Salmonella virulence in mice. Mol Microbiol **33**:167-76.
65. **Fitzgerald, K. A., D. C. Rowe, and D. T. Golenbock**. 2004. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. Microbes Infect **6**:1361-7.
66. **Forstermann, U., I. Gath, P. Schwarz, E. I. Closs, and H. Kleinert**. 1995. Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control. Biochem Pharmacol **50**:1321-32.
67. **Fuchs, A., M. Cella, T. Kondo, and M. Colonna**. 2005. Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. Blood **106**:2076-82.
68. **Galan, J. E., and R. Curtiss, 3rd**. 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci U S A **86**:6383-7.
69. **Galan, J. E., and Y. Fu**. 2000. Modulation of actin cytoskeleton by Salmonella GTPase activating protein SptP. Methods Enzymol **325**:496-504.
70. **Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay**. 1997. Release of lipopolysaccharide from intracellular compartments containing Salmonella typhimurium to vesicles of the host epithelial cell. Infect Immun **65**:24-34.
71. **Garcia-del Portillo, F., M. B. Zwick, K. Y. Leung, and B. B. Finlay**. 1993. Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. Proc Natl Acad Sci U S A **90**:10544-8.
72. **Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman**. 1996. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. Cell **84**:165-74.
73. **Garmendia, J., C. R. Beuzon, J. Ruiz-Albert, and D. W. Holden**. 2003. The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the Salmonella typhimurium SPI-2 type III secretion system. Microbiology **149**:2385-96.
74. **Gibbons, H. S., S. R. Kalb, R. J. Cotter, and C. R. Raetz**. 2005. Role of Mg2+ and pH in the modification of Salmonella lipid A after endocytosis by macrophage tumour cells. Mol Microbiol **55**:425-40.
75. **Gibot, S., and A. Cravoisy**. 2004. Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. Clin Med Res **2**:181-7.
76. **Goldman, R. C., K. Joiner, and L. Leive**. 1984. Serum-resistant mutants of Escherichia coli O111 contain increased lipopolysaccharide, lack an O antigen-containing capsule, and cover more of their lipid A core with O antigen. J Bacteriol **159**:877-82.

77. **Golubeva, Y. A., and J. M. Slauch**. 2006. Salmonella enterica Serovar Typhimurium Periplasmic Superoxide Dismutase SodCI Is a Member of the PhoPQ Regulon and Is Induced in Macrophages. J Bacteriol **188**:7853-61.
78. **Gordon, S.** 2002. Pattern recognition receptors: doubling up for the innate immune response. Cell **111**:927-30.
79. **Gordon, S.** 2003. Alternative activation of macrophages. Nat Rev Immunol **3**:23-35.
80. **Green, J., and M. S. Paget**. 2004. Bacterial redox sensors. Nat Rev Microbiol **2**:954-66.
81. **Groisman, E. A., J. Kayser, and F. C. Soncini**. 1997. Regulation of polymyxin resistance and adaptation to low-Mg2+ environments. J Bacteriol **179**:7040-5.
82. **Guignot, J., E. Caron, C. Beuzon, C. Bucci, J. Kagan, C. Roy, and D. W. Holden**. 2004. Microtubule motors control membrane dynamics of Salmonella-containing vacuoles. J Cell Sci **117**:1033-45.
83. **Guiney, D. G., and M. Lesnick**. 2005. Targeting of the actin cytoskeleton during infection by Salmonella strains. Clin Immunol **114**:248-55.
84. **Gunn, J. S., K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller**. 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol **27**:1171-82.
85. **Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller**. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of Salmonella enterica serovar typhimurium. Infect Immun **68**:6139-46.
86. **Guo, L., K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett, and S. I. Miller**. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell **95**:189-98.
87. **Hamerman, J. A., J. R. Jarjoura, M. B. Humphrey, M. C. Nakamura, W. E. Seaman, and L. L. Lanier**. 2006. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. J Immunol **177**:2051-5.
88. **Hamerman, J. A., and L. L. Lanier**. 2006. Inhibition of immune responses by ITAM-bearing receptors. Sci STKE **2006**:re1.
89. **Hamerman, J. A., N. K. Tchao, C. A. Lowell, and L. L. Lanier**. 2005. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. Nat Immunol **6**:579-86.
90. **Hansen-Wester, I., and M. Hensel**. 2001. Salmonella pathogenicity islands encoding type III secretion systems. Microbes Infect **3**:549-59.
91. **Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan**. 1998. S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell **93**:815-26.
92. **Harrison, R. E., C. Bucci, O. V. Vieira, T. A. Schroer, and S. Grinstein**. 2003. Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Mol Cell Biol **23**:6494-506.
93. **Hausladen, A., C. T. Privalle, T. Keng, J. DeAngelo, and J. S. Stamler**. 1996. Nitrosative stress: activation of the transcription factor OxyR. Cell **86**:719-29.
94. **Hayward, R. D., R. J. Cain, E. J. McGhie, N. Phillips, M. J. Garner, and V. Koronakis**. 2005. Cholesterol binding by the bacterial type III translocon is essential for virulence effector delivery into mammalian cells. Mol Microbiol **56**:590-603.
95. **Heffernan, E. J., J. Fierer, G. Chikami, and D. Guiney**. 1987. Natural history of oral Salmonella dublin infection in BALB/c mice: effect of an 80-kilobase-pair plasmid on virulence. J Infect Dis **155**:1254-9.
96. **Heithoff, D. M., C. P. Conner, U. Hentschel, F. Govantes, P. C. Hanna, and M. J. Mahan**. 1999. Coordinate intracellular expression of Salmonella genes induced during infection. J Bacteriol **181**:799-807.

97. **Hensel, M.** 2004. Evolution of pathogenicity islands of Salmonella enterica. Int J Med Microbiol **294**:95-102.
98. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden**. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269**:400-3.
99. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden**. 1998. Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol Microbiol **30**:163-74.
100. **Herrera-Leon, S., M. Saco, A. Martinez Silvestre, L. Silveira, A. Echeita, and M. A. Usera**. 2005. Molecular characterization of a new serovar of Salmonella bongori 13,22:z39:- isolated from a lizard. Res Microbiol **156**:597-602.
101. **Ho, T. D., N. Figueroa-Bossi, M. Wang, S. Uzzau, L. Bossi, and J. M. Slauch**. 2002. Identification of GtgE, a novel virulence factor encoded on the Gifsy-2 bacteriophage of Salmonella enterica serovar Typhimurium. J Bacteriol **184**:5234-9.
102. **Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, K. Crozat, S. Sovath, J. Han, and B. Beutler**. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature **424**:743-8.
103. **Holden, D. W.** 2002. Trafficking of the Salmonella vacuole in macrophages. Traffic **3**:161-9.
104. **Honko, A. N., and S. B. Mizel**. 2005. Effects of flagellin on innate and adaptive immunity. Immunol Res **33**:83-101.
105. **Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira**. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol **162**:3749-52.
106. **Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts**. 1999. The alternative sigma factor, sigmaE, is critically important for the virulence of Salmonella typhimurium. Infect Immun **67**:1560-8.
107. **Iwami, K. I., T. Matsuguchi, A. Masuda, T. Kikuchi, T. Musikacharoen, and Y. Yoshikai**. 2000. Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. J Immunol **165**:6682-6.
108. **Janssen, R., T. van der Straaten, A. van Diepen, and J. T. van Dissel**. 2003. Responses to reactive oxygen intermediates and virulence of Salmonella typhimurium. Microbes Infect **5**:527-34.
109. **Janssens, S., K. Burns, J. Tschopp, and R. Beyaert**. 2002. Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol **12**:467-71.
110. **Janssens, S., K. Burns, E. Vercammen, J. Tschopp, and R. Beyaert**. 2003. MyD88S, a splice variant of MyD88, differentially modulates NF-kappaB- and AP-1-dependent gene expression. FEBS Lett **548**:103-7.
111. **Jiang, Z., P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos, M. Freudenberg, and B. Beutler**. 2005. CD14 is required for MyD88-independent LPS signaling. Nat Immunol **6**:565-70.
112. **Jimenez-Lucho, V. E., K. A. Joiner, J. Foulds, M. M. Frank, and L. Leive**. 1987. C3b generation is affected by the structure of the O-antigen polysaccharide in lipopolysaccharide from salmonellae. J Immunol **139**:1253-9.
113. **John, B., D. Rajagopal, A. Pashine, S. Rath, A. George, and V. Bal**. 2002. Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to Salmonella typhimurium. J Immunol **169**:2545-52.
114. **Kauppi, A. M., R. Nordfelth, U. Hagglund, H. Wolf-Watz, and M. Elofsson**. 2003. Salicylanilides are potent inhibitors of type III secretion in Yersinia. Adv Exp Med Biol **529**:97-100.
115. **Kawai, T., and S. Akira**. 2006. TLR signaling. Cell Death Differ **13**:816-25.

116. **Kawasaki, K., R. K. Ernst, and S. I. Miller**. 2004. 3-O-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of Salmonella typhimurium, modulates signaling through Toll-like receptor 4. J Biol Chem **279**:20044-8.
117. **Kawasaki, K., R. K. Ernst, and S. I. Miller**. 2004. Deacylation and palmitoylation of lipid A by Salmonellae outer membrane enzymes modulate host signaling through Toll-like receptor 4. J Endotoxin Res **10**:439-44.
118. **Kawasaki, K., R. K. Ernst, and S. I. Miller**. 2005. Inhibition of Salmonella enterica serovar Typhimurium lipopolysaccharide deacylation by aminoarabinose membrane modification. J Bacteriol **187**:2448-57.
119. **Kenyon, W. J., D. G. Sayers, S. Humphreys, M. Roberts, and M. P. Spector**. 2002. The starvation-stress response of Salmonella enterica serovar Typhimurium requires sigma(E)-, but not CpxR-regulated extracytoplasmic functions. Microbiology **148**:113-22.
120. **Kirby, A. C., U. Yrlid, and M. J. Wick**. 2002. The innate immune response differs in primary and secondary Salmonella infection. J Immunol **169**:4450-9.
121. **Knodler, L. A., J. Celli, W. D. Hardt, B. A. Vallance, C. Yip, and B. B. Finlay**. 2002. Salmonella effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. Mol Microbiol **43**:1089-103.
122. **Knodler, L. A., B. A. Vallance, M. Hensel, D. Jackel, B. B. Finlay, and O. Steele-Mortimer**. 2003. Salmonella type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. Mol Microbiol **49**:685-704.
123. **Kolodziejska, K. E., A. R. Burns, R. H. Moore, D. L. Stenoien, and N. T. Eissa**. 2005. Regulation of inducible nitric oxide synthase by aggresome formation. Proc Natl Acad Sci U S A **102**:4854-9.
124. **Kox, L. F., M. M. Wosten, and E. A. Groisman**. 2000. A small protein that mediates the activation of a two-component system by another two-component system. Embo J **19**:1861-72.
125. **Krishnakumar, R., M. Craig, J. A. Imlay, and J. M. Slauch**. 2004. Differences in enzymatic properties allow SodCI but not SodCII to contribute to virulence in Salmonella enterica serovar Typhimurium strain 14028. J Bacteriol **186**:5230-8.
126. **Kuhle, V., G. L. Abrahams, and M. Hensel**. 2006. Intracellular Salmonella enterica redirect exocytic transport processes in a Salmonella pathogenicity island 2-dependent manner. Traffic **7**:716-30.
127. **Kuhle, V., D. Jackel, and M. Hensel**. 2004. Effector proteins encoded by Salmonella pathogenicity island 2 interfere with the microtubule cytoskeleton after translocation into host cells. Traffic **5**:356-70.
128. **Kumar, J. K., S. Tabor, and C. C. Richardson**. 2004. Proteomic analysis of thioredoxin-targeted proteins in Escherichia coli. Proc Natl Acad Sci U S A **101**:3759-64.
129. **Kuncewicz, T., P. Balakrishnan, M. B. Snuggs, and B. C. Kone**. 2001. Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. Am J Physiol Renal Physiol **281**:F326-36.
130. **Lahteenmaki, K., P. Kyllonen, L. Partanen, and T. K. Korhonen**. 2005. Antiprotease inactivation by Salmonella enterica released from infected macrophages. Cell Microbiol **7**:529-38.
131. **Lara-Tejero, M., F. S. Sutterwala, Y. Ogura, E. P. Grant, J. Bertin, A. J. Coyle, R. A. Flavell, and J. E. Galan**. 2006. Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. J Exp Med **203**:1407-12.
132. **Lee, A. K., C. S. Detweiler, and S. Falkow**. 2000. OmpR regulates the two-component system SsrA-ssrB in Salmonella pathogenicity island 2. J Bacteriol **182**:771-81.
133. **Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman**. 2004. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in Salmonella enterica. J Bacteriol **186**:4124-33.

134. **Lerouge, I., and J. Vanderleyden**. 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev **26**:17-47.
135. **Lesnick, M. L., N. E. Reiner, J. Fierer, and D. G. Guiney**. 2001. The Salmonella spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. Mol Microbiol **39**:1464-70.
136. **Li, K., C. Pasternak, and G. Klug**. 2003. Expression of the trxA gene for thioredoxin 1 in Rhodobacter sphaeroides during oxidative stress. Arch Microbiol **180**:484-9.
137. **Liang-Takasaki, C. J., N. Grossman, and L. Leive**. 1983. Salmonellae activate complement differentially via the alternative pathway depending on the structure of their lipopolysaccharide O-antigen. J Immunol **130**:1867-70.
138. **Liang-Takasaki, C. J., P. H. Makela, and L. Leive**. 1982. Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. J Immunol **128**:1229-35.
139. **Liang-Takasaki, C. J., H. Saxen, P. H. Makela, and L. Leive**. 1983. Complement activation by polysaccharide of lipopolysaccharide: an important virulence determinant of salmonellae. Infect Immun **41**:563-9.
140. **Liew, F. Y., D. Xu, E. K. Brint, and L. A. O'Neill**. 2005. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol **5**:446-58.
141. **Linehan, S. A., A. Rytkonen, X. J. Yu, M. Liu, and D. W. Holden**. 2005. SlyA regulates function of Salmonella pathogenicity island 2 (SPI-2) and expression of SPI-2-associated genes. Infect Immun **73**:4354-62.
142. **Lirk, P., G. Hoffmann, and J. Rieder**. 2002. Inducible nitric oxide synthase--time for reappraisal. Curr Drug Targets Inflamm Allergy **1**:89-108.
143. **Lissner, C. R., R. N. Swanson, and A. D. O'Brien**. 1983. Genetic control of the innate resistance of mice to Salmonella typhimurium: expression of the Ity gene in peritoneal and splenic macrophages isolated in vitro. J Immunol **131**:3006-13.
144. **Lostroh, C. P., and C. A. Lee**. 2001. The Salmonella pathogenicity island-1 type III secretion system. Microbes Infect **3**:1281-91.
145. **Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee**. 2000. Multiple factors independently regulate hilA and invasion gene expression in Salmonella enterica serovar typhimurium. J Bacteriol **182**:1872-82.
146. **Lundberg, B. E., R. E. Wolf, Jr., M. C. Dinauer, Y. Xu, and F. C. Fang**. 1999. Glucose 6-phosphate dehydrogenase is required for Salmonella typhimurium virulence and resistance to reactive oxygen and nitrogen intermediates. Infect Immun **67**:436-8.
147. **Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay**. 2000. Salmonella pathogenicity islands: big virulence in small packages. Microbes Infect **2**:145-56.
148. **Marsman, M., I. Jordens, C. Kuijl, L. Janssen, and J. Neefjes**. 2004. Dynein-mediated vesicle transport controls intracellular Salmonella replication. Mol Biol Cell **15**:2954-64.
149. **Mastroeni, P.** 2002. Immunity to systemic Salmonella infections. Curr Mol Med **2**:393-406.
150. **Mastroeni, P., and M. Sheppard**. 2004. Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. Microbes Infect **6**:398-405.
151. **Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan**. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. J Exp Med **192**:237-48.
152. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche**. 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- Salmonella vaccines. Microb Pathog **13**:477-91.

153. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson**. 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature **413**:852-6.
154. **McCollister, B. D., T. J. Bourret, R. Gill, J. Jones-Carson, and A. Vazquez-Torres**. 2005. Repression of SPI2 transcription by nitric oxide-producing, IFNgamma-activated macrophages promotes maturation of Salmonella phagosomes. J Exp Med **202**:625-35.
155. **McGee, D. J., S. Kumar, R. J. Viator, J. R. Bolland, J. Ruiz, D. Spadafora, T. L. Testerman, D. J. Kelly, L. K. Pannell, and H. J. Windle**. 2006. Helicobacter pylori thioredoxin is an arginase chaperone and guardian against oxidative and nitrosative stresses. J Biol Chem **281**:3290-6.
156. **McGhie, E. J., R. D. Hayward, and V. Koronakis**. 2001. Cooperation between actin-binding proteins of invasive Salmonella: SipA potentiates SipC nucleation and bundling of actin. Embo J **20**:2131-9.
157. **Meresse, S., K. E. Unsworth, A. Habermann, G. Griffiths, F. Fang, M. J. Martinez-Lorenzo, S. R. Waterman, J. P. Gorvel, and D. W. Holden**. 2001. Remodelling of the actin cytoskeleton is essential for replication of intravacuolar Salmonella. Cell Microbiol **3**:567-77.
158. **Merighi, M., C. D. Ellermeier, J. M. Slauch, and J. S. Gunn**. 2005. Resolvase-in vivo expression technology analysis of the Salmonella enterica serovar Typhimurium PhoP and PmrA regulons in BALB/c mice. J Bacteriol **187**:7407-16.
159. **Miao, E. A., M. Brittnacher, A. Haraga, R. L. Jeng, M. D. Welch, and S. I. Miller**. 2003. Salmonella effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. Mol Microbiol **48**:401-15.
160. **Miao, E. A., J. A. Freeman, and S. I. Miller**. 2002. Transcription of the SsrAB regulon is repressed by alkaline pH and is independent of PhoPQ and magnesium concentration. J Bacteriol **184**:1493-7.
161. **Miller, B. H., R. A. Fratti, J. F. Poschet, G. S. Timmins, S. S. Master, M. Burgos, M. A. Marletta, and V. Deretic**. 2004. Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. Infect Immun **72**:2872-8.
162. **Mills, D. M., V. Bajaj, and C. A. Lee**. 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol Microbiol **15**:749-59.
163. **Mittrucker, H. W., and S. H. Kaufmann**. 2000. Immune response to infection with Salmonella typhimurium in mice. J Leukoc Biol **67**:457-63.
164. **Mittrucker, H. W., B. Raupach, A. Kohler, and S. H. Kaufmann**. 2000. Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. J Immunol **164**:1648-52.
165. **Monack, D. M., D. Hersh, N. Ghori, D. Bouley, A. Zychlinsky, and S. Falkow**. 2000. Salmonella exploits caspase-1 to colonize Peyer's patches in a murine typhoid model. J Exp Med **192**:249-58.
166. **Moore, T. D., and P. F. Sparling**. 1996. Interruption of the gpxA gene increases the sensitivity of Neisseria meningitidis to paraquat. J Bacteriol **178**:4301-5.
167. **Mori, M., and T. Gotoh**. 2004. Arginine metabolic enzymes, nitric oxide and infection. J Nutr **134**:2820S-2825S; discussion 2853S.
168. **Motohashi, N., and Y. Saito**. 2002. Induction of SOS response in Salmonella typhimurium TA4107/pSK1002 by peroxynitrite-generating agent, N-morpholino sydnonimine. Mutat Res **502**:11-8.
169. **Moynagh, P. N.** 2005. TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. Trends Immunol **26**:469-76.
170. **Muschiol, S., L. Bailey, A. Gylfe, C. Sundin, K. Hultenby, S. Bergstrom, M. Elofsson, H. Wolf-Watz, S. Normark, and B. Henriques-Normark**. 2006. A small-molecule inhibitor of type III secretion inhibits different stages of the

infectious cycle of Chlamydia trachomatis. Proc Natl Acad Sci U S A **103**:14566-71.
171. **Nauciel, C.** 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection. J Immunol **145**:1265-9.
172. **Nauciel, C., and F. Espinasse-Maes**. 1992. Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection. Infect Immun **60**:450-4.
173. **Nevola, J. J., B. A. Stocker, D. C. Laux, and P. S. Cohen**. 1985. Colonization of the mouse intestine by an avirulent Salmonella typhimurium strain and its lipopolysaccharide-defective mutants. Infect Immun **50**:152-9.
174. **Nishikawa, F., S. Yoshikawa, H. Harada, M. Kita, and E. Kita**. 1998. The full expression of the ity phenotype in ityr mice requires C3 activation by Salmonella lipopolysaccharide. Immunology **95**:640-7.
175. **Nishino, K., F. F. Hsu, J. Turk, M. J. Cromie, M. M. Wosten, and E. A. Groisman**. 2006. Identification of the lipopolysaccharide modifications controlled by the Salmonella PmrA/PmrB system mediating resistance to Fe(III) and Al(III). Mol Microbiol **61**:645-54.
176. **O'Brien, A. D., I. Scher, and S. B. Formal**. 1979. Effect of silica on the innate resistance of inbred mice to Salmonella typhimurium infection. Infect Immun **25**:513-20.
177. **O'Neal, C. R., W. M. Gabriel, A. K. Turk, S. J. Libby, F. C. Fang, and M. P. Spector**. 1994. RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in Salmonella typhimurium. J Bacteriol **176**:4610-6.
178. **O'Neill, L. A.** 2006. How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol **18**:3-9.
179. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman**. 1996. Identification of a pathogenicity island required for Salmonella survival in host cells. Proc Natl Acad Sci U S A **93**:7800-4.
180. **Ohl, M. E., and S. I. Miller**. 2001. Salmonella: a model for bacterial pathogenesis. Annu Rev Med **52**:259-74.
181. **Ohlson, M. B., K. Fluhr, C. L. Birmingham, J. H. Brumell, and S. I. Miller**. 2005. SseJ deacylase activity by Salmonella enterica serovar Typhimurium promotes virulence in mice. Infect Immun **73**:6249-59.
182. **Otto, H., D. Tezcan-Merdol, R. Girisch, F. Haag, M. Rhen, and F. Koch-Nolte**. 2000. The spvB gene-product of the Salmonella enterica virulence plasmid is a mono(ADP-ribosyl)transferase. Mol Microbiol **37**:1106-15.
183. **Pandey, S., and D. K. Agrawal**. 2006. Immunobiology of Toll-like receptors: emerging trends. Immunol Cell Biol **84**:333-41.
184. **Pfeffer, S. R.** 2005. Structural clues to Rab GTPase functional diversity. J Biol Chem **280**:15485-8.
185. **Pie, S., P. Truffa-Bachi, M. Pla, and C. Nauciel**. 1997. Th1 response in Salmonella typhimurium-infected mice with a high or low rate of bacterial clearance. Infect Immun **65**:4509-14.
186. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler**. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282**:2085-8.
187. **Prinz, W. A., F. Aslund, A. Holmgren, and J. Beckwith**. 1997. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. J Biol Chem **272**:15661-7.
188. **Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo**. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med **189**:615-25.
189. **Raetz, C. R., and C. Whitfield**. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem **71**:635-700.
190. **Ravindran, R., and S. J. McSorley**. 2005. Tracking the dynamics of T-cell activation in response to Salmonella infection. Immunology **114**:450-8.

191. **Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli**. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol **2**:361-7.
192. **Rhen, M., and C. J. Dorman**. 2005. Hierarchical gene regulators adapt Salmonella enterica to its host milieus. Int J Med Microbiol **294**:487-502.
193. **Rhen, M., P. Riikonen, and S. Taira**. 1993. Expression of Salmonella typhimurium virulence plasmid genes in cultured macrophages. Infect Agents Dis **2**:285-7.
194. **Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay**. 1997. Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J Exp Med **186**:569-80.
195. **Roudier, C., J. Fierer, and D. G. Guiney**. 1992. Characterization of translation termination mutations in the spv operon of the Salmonella virulence plasmid pSDL2. J Bacteriol **174**:6418-23.
196. **Ruiz-Albert, J., X. J. Yu, C. R. Beuzon, A. N. Blakey, E. E. Galyov, and D. W. Holden**. 2002. Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane. Mol Microbiol **44**:645-61.
197. **Russel, M., and A. Holmgren**. 1988. Construction and characterization of glutaredoxin-negative mutants of Escherichia coli. Proc Natl Acad Sci U S A **85**:990-4.
198. **Salcedo, S. P., and D. W. Holden**. 2003. SseG, a virulence protein that targets Salmonella to the Golgi network. Embo J **22**:5003-14.
199. **Salcedo, S. P., M. Noursadeghi, J. Cohen, and D. W. Holden**. 2001. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. Cell Microbiol **3**:587-97.
200. **Sassanfar, M., and J. W. Roberts**. 1990. Nature of the SOS-inducing signal in Escherichia coli. The involvement of DNA replication. J Mol Biol **212**:79-96.
201. **Saura, M., C. Zaragoza, C. Bao, A. McMillan, and C. J. Lowenstein**. 1999. Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription. J Mol Biol **289**:459-71.
202. **Saylers, A., and D. Whitt**. 2002. Bacterial Patogenesis - a molecular approach, 2nd ed. ASM Press, New York, USA.
203. **Schaechter, M., N. Engelberg, B. Eisenstein, and G. Medoff**. 1998. Mechanisms of Microbial Disease, 3rd ed. Wiliams and Wilkins, Baltimore, USA.
204. **Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume**. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol **75**:163-89.
205. **Seiler, P., P. Aichele, B. Raupach, B. Odermatt, U. Steinhoff, and S. H. Kaufmann**. 2000. Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating Mycobacterium tuberculosis. J Infect Dis **181**:671-80.
206. **Shah, D. H., M. J. Lee, J. H. Park, J. H. Lee, S. K. Eo, J. T. Kwon, and J. S. Chae**. 2005. Identification of Salmonella gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. Microbiology **151**:3957-68.
207. **Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden**. 1999. Influence of the Salmonella typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect Immun **67**:213-9.
208. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden**. 1996. Identification of a virulence locus encoding a second type III secretion system in Salmonella Typhimurium. Proc Natl Acad Sci U S A **93**:2593-2597.
209. **Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan**. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity **10**:29-38.

210. **Sjolin, H., S. H. Robbins, G. Bessou, A. Hidmark, E. Tomasello, M. Johansson, H. Hall, F. Charifi, G. B. Karlsson Hedestam, C. A. Biron, K. Karre, P. Hoglund, E. Vivier, and M. Dalod**. 2006. DAP12 signaling regulates plasmacytoid dendritic cell homeostasis and down-modulates their function during viral infection. J Immunol **177**:2908-16.
211. **Skurnik, M., R. Venho, J. A. Bengoechea, and I. Moriyon**. 1999. The lipopolysaccharide outer core of Yersinia enterocolitica serotype O:3 is required for virulence and plays a role in outer membrane integrity. Mol Microbiol **31**:1443-62.
212. **Smith, C. M., Z. Arany, C. Orrego, and E. Eisenstadt**. 1991. DNA damage-inducible loci in Salmonella typhimurium. J Bacteriol **173**:3587-90.
213. **Soncini, F. C., and E. A. Groisman**. 1996. Two-component regulatory systems can interact to process multiple environmental signals. J Bacteriol **178**:6796-801.
214. **Stanley, T. L., C. D. Ellermeier, and J. M. Slauch**. 2000. Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects Salmonella enterica serovar typhimurium survival in Peyer's patches. J Bacteriol **182**:4406-13.
215. **Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt**. 2000. Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol Microbiol **36**:1206-21.
216. **Stewart, E. J., F. Aslund, and J. Beckwith**. 1998. Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins. Embo J **17**:5543-50.
217. **Svensson, M., C. Johansson, and M. J. Wick**. 2001. Salmonella typhimurium-induced cytokine production and surface molecule expression by murine macrophages. Microb Pathog **31**:91-102.
218. **Taira, S., P. Riikonen, H. Saarilahti, S. Sukupolvi, and M. Rhen**. 1991. The mkaC virulence gene of the Salmonella serovar typhimurium 96 kb plasmid encodes a transcriptional activator. Mol Gen Genet **228**:381-4.
219. **Takaoka, A., H. Yanai, S. Kondo, G. Duncan, H. Negishi, T. Mizutani, S. Kano, K. Honda, Y. Ohba, T. W. Mak, and T. Taniguchi**. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature **434**:243-9.
220. **Takemoto, T., Q. M. Zhang, and S. Yonei**. 1998. Different mechanisms of thioredoxin in its reduced and oxidized forms in defense against hydrogen peroxide in Escherichia coli. Free Radic Biol Med **24**:556-62.
221. **Tamayo, R., B. Choudhury, A. Septer, M. Merighi, R. Carlson, and J. S. Gunn**. 2005. Identification of cptA, a PmrA-regulated locus required for phosphoethanolamine modification of the Salmonella enterica serovar typhimurium lipopolysaccharide core. J Bacteriol **187**:3391-9.
222. **Testerman, T. L., A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S. J. Libby, and F. C. Fang**. 2002. The alternative sigma factor sigmaE controls antioxidant defences required for Salmonella virulence and stationary-phase survival. Mol Microbiol **43**:771-82.
223. **Tezcan-Merdol, D., T. Nyman, U. Lindberg, F. Haag, F. Koch-Nolte, and M. Rhen**. 2001. Actin is ADP-ribosylated by the Salmonella enterica virulence-associated protein SpvB. Mol Microbiol **39**:606-19.
224. **Thomsen, L. E., M. S. Chadfield, J. Bispham, T. S. Wallis, J. E. Olsen, and H. Ingmer**. 2003. Reduced amounts of LPS affect both stress tolerance and virulence of Salmonella enterica serovar Dublin. FEMS Microbiol Lett **228**:225-31.
225. **Trent, M. S., C. M. Stead, A. X. Tran, and J. V. Hankins**. 2006. Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res **12**:205-23.
226. **Turnbull, I. R., S. Gilfillan, M. Cella, T. Aoshi, M. Miller, L. Piccio, M. Hernandez, and M. Colonna**. 2006. Cutting edge: TREM-2 attenuates macrophage activation. J Immunol **177**:3520-4.
227. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli. Microbiol Rev **48**:60-93.

228. **Wang, J., V. C. Asensio, and I. L. Campbell**. 2002. Cytokines and chemokines as mediators of protection and injury in the central nervous system assessed in transgenic mice. Curr Top Microbiol Immunol **265**:23-48.
229. **Warren, J., P. Mastroeni, G. Dougan, M. Noursadeghi, J. Cohen, M. J. Walport, and M. Botto**. 2002. Increased susceptibility of C1q-deficient mice to Salmonella enterica serovar Typhimurium infection. Infect Immun **70**:551-7.
230. **Vassiloyanakopoulos, A. P., S. Okamoto, and J. Fierer**. 1998. The crucial role of polymorphonuclear leukocytes in resistance to Salmonella dublin infections in genetically susceptible and resistant mice. Proc Natl Acad Sci U S A **95**:7676-81.
231. **Waterman, S. R., and D. W. Holden**. 2003. Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. Cell Microbiol **5**:501-11.
232. **Vazquez-Torres, A., and F. C. Fang**. 2000. Cellular routes of invasion by enteropathogens. Curr Opin Microbiol **3**:54-9.
233. **Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang**. 1999. Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature **401**:804-8.
234. **Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang**. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. J Exp Med **192**:227-36.
235. **Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang**. 2000. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287**:1655-8.
236. **Webb, J. L., M. W. Harvey, D. W. Holden, and T. J. Evans**. 2001. Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. Infect Immun **69**:6391-400.
237. **Vernikos, G. S., and J. Parkhill**. 2006. Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. Bioinformatics **22**:2196-203.
238. **West, N. P., P. Sansonetti, J. Mounier, R. M. Exley, C. Parsot, S. Guadagnini, M. C. Prevost, A. Prochnicka-Chalufour, M. Delepierre, M. Tanguy, and C. M. Tang**. 2005. Optimization of virulence functions through glucosylation of Shigella LPS. Science **307**:1313-7.
239. **Wick, M. J.** 2002. The role of dendritic cells during Salmonella infection. Curr Opin Immunol **14**:437-43.
240. **Vieira, O. V., R. J. Botelho, and S. Grinstein**. 2002. Phagosome maturation: aging gracefully. Biochem J **366**:689-704.
241. **Wiese, A., K. Brandenburg, A. J. Ulmer, U. Seydel, and S. Muller-Loennies**. 1999. The dual role of lipopolysaccharide as effector and target molecule. Biol Chem **380**:767-84.
242. **Wilmes-Riesenberg, M. R., J. W. Foster, and R. Curtiss, 3rd**. 1997. An altered rpoS allele contributes to the avirulence of Salmonella typhimurium LT2. Infect Immun **65**:203-10.
243. **Vodovotz, Y., D. Russell, Q. W. Xie, C. Bogdan, and C. Nathan**. 1995. Vesicle membrane association of nitric oxide synthase in primary mouse macrophages. J Immunol **154**:2914-25.
244. **Wong, K. K., M. McClelland, L. C. Stillwell, E. C. Sisk, S. J. Thurston, and J. D. Saffer**. 1998. Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a Salmonella pathogenicity island located at 92 minutes on the chromosome map of Salmonella enterica serovar typhimurium LT2. Infect Immun **66**:3365-71.
245. **Worley, M. J., K. H. Ching, and F. Heffron**. 2000. Salmonella SsrB activates a global regulon of horizontally acquired genes. Mol Microbiol **36**:749-61.

246. **Wosten, M. M., L. F. Kox, S. Chamnongpol, F. C. Soncini, and E. A. Groisman**. 2000. A signal transduction system that responds to extracellular iron. Cell **103**:113-25.
247. **Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield**. 2000. Salmonella enterica serovar typhimurium waaP mutants show increased susceptibility to polymyxin and loss of virulence In vivo. Infect Immun **68**:4485-91.
248. **Yrlid, U., and M. J. Wick**. 2000. Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. J Exp Med **191**:613-24.
249. **Yu, Z., W. Zhang, and B. C. Kone**. 2002. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor kappaB. Biochem J **367**:97-105.
250. **Zhang, W., T. Kuncewicz, Z. Y. Yu, L. Zou, X. Xu, and B. C. Kone**. 2003. Protein-protein interactions involving inducible nitric oxide synthase. Acta Physiol Scand **179**:137-42.
251. **Zhou, D., L. M. Chen, L. Hernandez, S. B. Shears, and J. E. Galan**. 2001. A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol Microbiol **39**:248-59.
252. **Zhou, Z., S. Lin, R. J. Cotter, and C. R. Raetz**. 1999. Lipid A modifications characteristic of Salmonella typhimurium are induced by NH4VO3 in Escherichia coli K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. J Biol Chem **274**:18503-14.
253. **Zughaier, S. M., S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens**. 2005. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. Infect Immun **73**:2940-50.