From Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

CHEMICAL GENETICS AND IMMUNOMODULATION IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM INFECTION

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Karolinska Institutet

Stockholm 2015

Cover: Immunofluorescence microscopy of RAW264.7 macrophages infected with *Salmonella* Typhimurium 16 hours post infection, untreated (upper left) or treated with omeprazole (lower left), INP0010 (upper right) and both omeprazole and INP0010 (lower right). Green-*Salmonella*; red-actin; blue-nuclei. Collage assembled in a puzzle form by Viorel Banesaru.

Published by Karolinska Institutet.

ISBN 978-91-7549-871-3

Printed by AJ E-Print AB 2015

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Speranța Puiac Bănesaru

Doctoral dissertation for the degree of PhD in Medicine at Karolinska Institutet will be publicly defended in the lecture hall **Atrium**, Nobels väg 12B, on **May 6th**, 2015, at **09.00 am**

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ABSTRACT

Salmonella sp. are versatile bacteria that survive and replicate in a plethora of environmental niches and hosts, including humans. The illnesses caused by various serovars in humans range from mild gastroenteritis to severe systemic disease such as typhoid fever that still cause an important number of casualties especially in developing countries. *Salmonella enterica* serovar Typhimurium is the research model for typhoid fever and has provided many valuable insights into the molecular pathogenesis of salmonellosis. Its ability to cause disease relies on expression of specific virulence factors, often coded for by distinct genomic entities termed "*Salmonella* pathogenicity islands" (SPIs). The invasion of the intestinal lining is mediated by SPI1, while SPI2 enables the bacteria to survive, replicate and disseminate in the host inside phagocytes. Both SPI1 and SPI2 code for two distinct type three secretion systems (T3SSs), which translocate into the host cell bacterial effectors that hijack host cells functions.

Resistance to first line antibiotics has been reported in attempts to treat salmonellosis, even extended spectrum β–lactamase resistance. Hence, there is a need for new treatment alternatives. We report in **Paper I** a new antimicrobial property of the proton pump inhibitor omeprazole, which interfered with SPI2-mediated intracellular replication of *S.* Typhimurium in RAW264.7 murine macrophages. Its effect was bacteriostatic and manifested if the compound was applied at early stages of infection. Along with interference with bacterial virulence, omeprazole also posed a strong anti-inflammatory effect on macrophages. In **Paper II** we further characterized the immunomodulatory effect of omeprazole, alone or in combination with INP0010, another small molecular virulence inhibitor previously shown to inhibit the activity of SPI1 and SPI2 T3SSs. The two compounds had either antagonistic or synergistic effects on bacterial virulence and on host cell responses such as expression of inducible nitric oxide synthase (iNOS), nitric oxide (NO), pro-inflammatory cytokines and reactive oxygen species (ROS), depending on the dose and the model of infection used. Notably, in contrast to omeprazole, INP0010 enhanced the inflammatory responses of macrophages including NO production and hence part of its inhibitory effect on bacterial intracellular replication might be due to up-regulation of NO in infected cells.

Paper III reveals that bacterial thioredoxin 1, a reductase important for maintaining protein redox homeostasis, contributes to virulence of *S.* Typhimurium by participating in the activity of SPI2 T3SS. SPI2 and thioredoxin 1 had a convergent contribution to intracellular replication of *S.* Typhimurium both *in vitro* and *in vivo*. Catalytic as well as non-catalytic functions of thioredoxin 1 appeared important contributors to *S.* Typhimurium virulence, depending on the infection model used.

The outcome of the infection at individual cell level is dictated by the ever oscillating balance between host cell antimicrobial responses and the ability of bacteria to overcome them. In **Paper IV** we show that phagocytes infected with *S.* Typhimurium have a heterogeneous pattern of iNOS expression, which is not dependent on active virulence proficient bacteria and that the majority of infected cells are iNOS irresponsive, both *in vitro* and *in vivo*. Our study underlines the need of shifting from bulk to individual cell analysis when studying host-pathogen interactions.

LIST OF SCIENTIFIC PAPERS

I. **Speranta Puiac**, Aurel Negrea, Agneta Richter-Dahlfors, Laura Plant and Mikael Rhen. Omeprazole antagonizes virulence and inflammation in *Salmonella enterica*-infected RAW264.7 cells. *Antimicrob Agents Chemother*. 2009 Jun; 53(6):2402-9.

II. **Speranta Puiac**, Xiao Hui Sem, Aurel Negrea, Mikael Rhen. Small-molecular virulence inhibitors show divergent and immunomodulatory effects in infection models of *Salmonella enterica* serovar Typhimurium. *Int J Antimicrob Agents*. 2011 Nov; 38(5):409-16.

III. Aurel Negrea, Eva Bjur, **Speranta Puiac**, Sofia Eriksson-Ygberg, Fredrik Åslund, Mikael Rhen. Thioredoxin 1 participates in the activity of the *Salmonella enterica* serovar Typhimurium pathogenicity island 2 type III secretion system. *J Bacteriol*. 2009 Nov;191(22):6918-27.

IV. **Speranta Puiac Banesaru**, Naeem Anwar, Johan Nordfjell, Aurel Negrea and Mikael Rhen. Heterogeneity in phagocyte inducible nitric oxide synthase induction in response to infection with *Salmonella enterica* serovar Typhimurium. *Manuscript.*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Georgoudaki AM, Khodobandeh S, **Puiac S**, Persson CM, Larsson MK, Lind M, Hammarfjord O, Nabatti TH, Wallin RP, Yrlid U, Rhen M, Kumar V, Chambers BJ. CD244 is expressed on dendritic cells and regulate their functions. *Immunol Cell Biol*. 2015 Feb 3; [Epub ahead of print]

Guidi R, Levi L, Rouf SF, **Puiac S**, Rhen M, Frisan T. *Salmonella enterica* delivers its genotoxin through outer membrane vesicles secreted from infected cells. *Cell Microbiol*. 2013 Dec; 15(12):2034-50.

CONTENTS

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
ATR	Acid tolerance response
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CFUs	Colony forming units
CXCL	Chemokine (C-X-C motif) ligand
DCs	Dendritic cells
FcγR	Fragment crystallisable gamma receptor
IFN-γ	Interferon gamma
IFNR	Interferon receptor
Ig	Immunoglobulin
IkB- α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IRF-1	Interferon response factor-1
Jak	Janus kinase
LAMP	Lysosomal-associated membrane protein
LB	Luria Bertani
LBP	Lipopolysaccharide binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M6PR	Mannose 6-phosphate receptor
MAPK	Mitogen-activated protein kinase
MD-2	A protein that associates with Toll-like receptor 4 involved in LPS responsiveness
MDa	Mega Dalton
MDR	Multidrug-resistant
MEK	Mitogen extracellular signal-regulated kinase

MHC	Major histocompatibility complex
mLN	Mesenteric lymph nodes
MyD88	Myeloid differentiation primary response gene 88
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMMA	*N*-monomethyl-L-arginine
NO	Nitric oxide
Nramp	Natural resistance-associated macrophage protein
NTS	Non-typhoidal *Salmonella*
phox	Phagocyte NADPH oxidase
PMNs	Polymorphonuclear leukocytes
PP	Peyer's patches
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCV	*Salmonella* containing vacuole
Sif	*Salmonella*-induced filaments
SPI	*Salmonella* pathogenicity island
STAT	Signal transducer and activator of transcription
T3SS	Type three secretion system
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

1 INTRODUCTION

1.1 *SALMONELLA* AND SALMONELLOSIS

Salmonella is a Gram-negative, rod shaped, facultative intracellular bacteria very closely related to *Escherichia,* with whom it shares 90% of the genome. The divergence of the two bacteria from a common ancestor apparently has occurred around 100 million years ago. Since that event *Salmonella* have acquired by horizontal gene transfer multiple virulence determinants that contributed to the adaptation of bacteria to different environmental conditions, including colonization, invasion, persistence and spread in different hosts. The main evolutionary phases of host adaptation were acquisition of genes that confer the bacteria invasion capacity, followed by colonization of deeper tissues and ultimately adaptation to a wide range of hosts including warm-blooded vertebrates [1, 2].

The taxonomy of *Salmonella* is complicated and still evolving. Based on molecular methods it has been established that the genus *Salmonella* has three species, *S. bongori*, *S. enterica* and *S. subterranea* [3, 4]. While *S. bongori* is a pathogen of cold-blooded animals, *S. enterica* can infect and cause disease in a broad range of hosts from nematodes to humans and is further divided in 6 subspecies, *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*. *S. subterranea* was isolated from a low pH, nitrate and uranium contaminated sediment and was recently approved as the third *Salmonella* specie [5].

The different subspecies are further classified based on the O (somatic) antigen into more than 50 serogroups; moreover, the combination of O and H (flagellar) antigen repertoire gives rise to over 2400 serovars. Additionally, the presence of Vi (capsular) antigen provides another mean of classification of the different serotypes. Most of the human-associated serovars belong to *S. enterica* ssp. *enterica* [3].

Despite the increase in sanitation over the last century there are still high numbers of salmonellosis cases all over the world [6-8]. The disease is usually contracted via the faecal-oral route, by ingestion of contaminated water, various foods such as vegetables and animal-derived products or by contact with pets [9-17]. Based on disease manifestations *Salmonella* serovars are categorized as typhoidal and non-typhoidal (NTS) [8, 18].

Typhoid fever, also called enteric fever, is caused by the human restricted serovars Typhi and Paratyphi [6, 18] with the infection dose ranging between 10^3-10^9 bacteria. This is a serious invasive life-threatening disease, which is endemic in the developing countries that still lack

appropriate sanitation and clean water supplies. Its clinical manifestations include high fever, abdominal pain, nausea, diarrhoea, headache, rash, anorexia and dry cough [19, 20] and it is estimated to affect around 27 million people annually, causing more then 200 000 deaths [6, 7, 21]. 2-5 % of the typhoid fever patients remain chronically infected and become carriers and spreaders of the bacteria even thought they don't have the symptoms of the disease anymore [20]. In this stage the bacteria are primarily located in the gallbladder and the biliary tract [22].

In contrast to typhoid fever, NTS salmonellosis in humans are reported all over the world, with an estimation of 93.8 million cases and 155 000 deaths in the developing countries annually [8]. The infection dose varies between 10^6-10^9 bacteria and the usual manifestation of NTS infection is a self-limited gastroenteritis with watery diarrhoea, nausea, vomiting and fever. In some NTS cases, often in immunodeficient patients, the bacteria can cause bacteraemia and systemic infection with symptoms similar to typhoid fever [23]. NTSs can be caused by many serovars that are not restricted to a specific host, such as Typhimurium, Enteritidis, Dublin and Cholerasuis [21].

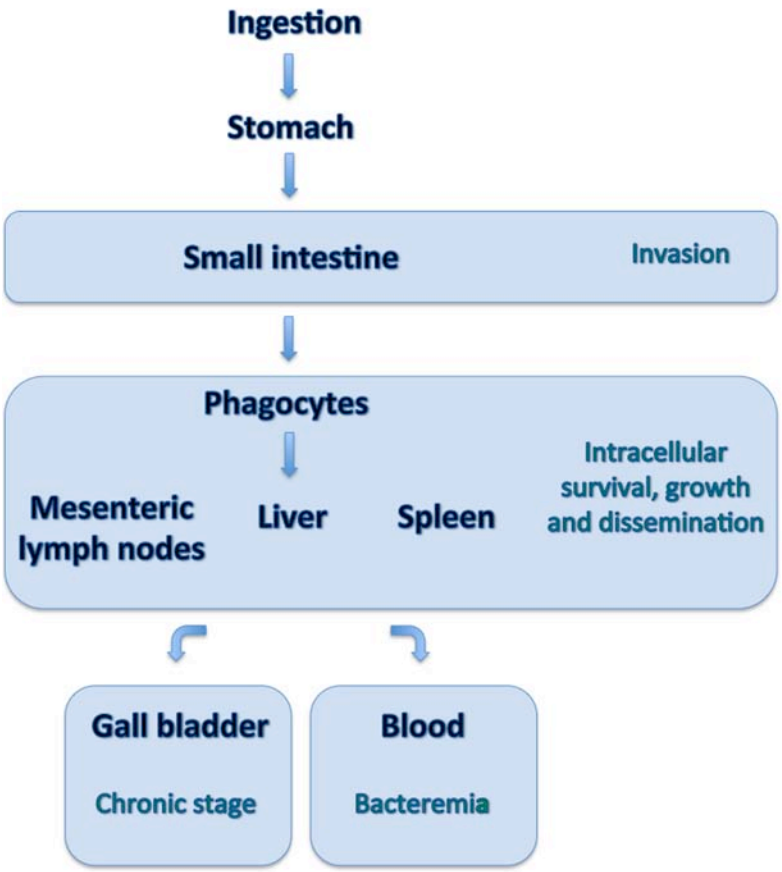

Figure 1. The stages of *Salmonella* pathogenesis

A simplified scheme of salmonellosis pathogenesis is depicted in **Figure 1**. The bacteria reach the host by the oral route, survive the gastric acid barrier then colonize the lumen of the small intestine and predominantly at the Peyer's patches (PP) attach to and invade the mucosa of the small intestine. The intestinal epithelium is breached by either one of the following means, i) engulfment by intraluminal dendritic cells (DCs); ii) bacteria-mediated invasion of specialized M cells or iii) bacteria-mediated invasion of ordinary intestinal epithelial cells [24-30]. After crossing the epithelium NTS strains usually cause a local inflammatory response that leads to infiltration of polymorphonuclear leukocytes (PMNs) and ultimately diarrhoea. In contrast, the initial invasion of the intestinal mucosa by typhoidal strains usually does not cause a high inflammatory response [21, 31-34]. After gaining access to the underlying lymphoid tissues bacteria are taken up by phagocytes (macrophages, DCs, neutrophils) and use this intracellular niche for survival, replication and dissemination throughout the reticuloendothelial system [34, 35]. In order to accomplish all the above the bacteria express several virulence factors that will be detailed in chapter 1.3.3.

1.2 PREVENTION AND THERAPIES

1.2.1 Prevention

The first line of salmonellosis prevention is increasing sanitation and hygiene, safe food, clean water supplies and better diagnostics for early detection [36]. Along with these, vaccination represents the additional tool for prevention of typhoid fever. There are three available vaccines against *S.* Typhi, a killed whole cell-typhoid-parathyphoid A and B parenteral vaccine [37], the Ty21a live attenuated oral vaccine [38] and a Vi polysaccharide capsule-based vaccine [39, 40]. The first whole cell-typhoid-parathyphoid A and B vaccine [37] was effective but had strong side effects. Therefore its use is now very limited. The Ty21a live attenuated and the Vi polysaccharide capsule vaccines are safer and have a similar moderate effectiveness of approximately 70% in older children and adults for about 3 years after vaccination. A booster dose is recommended every third year. While the Ty21a vaccine is licensed for children over 5 years old, the Vi-polysaccharide vaccine can be used for pre-school children older than 2 years [41-50]. To overcome the lack of protection for children under 2 years old, a new conjugated Vi vaccine (Vi-rEPA) is in current development [42, 51].

Since the killed whole cell-typhoid-parathyphoid A and B parenteral vaccine is not used anymore due to safety reasons, there are no other available vaccines against *S.* Paratyphi and the *S*. Typhi vaccines provide very little cross-protection [40].

Regarding NTSs there are vaccines against *S*. Enteritidis and *S.* Typhimurium [21, 52] that are effective in poultry, but not in humans or other hosts.

1.2.2 Treatment and antibiotic resistance

Due to severity and life-threatening aspects of typhoid fever the treatment with antibiotics is a must and it is started as soon as the diagnosis is made.

Antibiotics started to be used since the middle of the 20th century both in human and veterinary medicine and their use has dramatically decreased the death rates in many infectious diseases. Due to selective pressure, only a few years after antibiotics were being largely used, antimicrobial resistance emerged in many bacteria. The resistance is acquired either by endogenous spontaneous mutation or by horizontal transfer of resistance genes harboured by mobile genetic elements such as plasmids.

The golden standard for typhoid fever treatment until mid-1970s was chloramphenicol, which reduced the mortality from 10% to less than 2% in developed countries [53, 54].

In 1972 the first epidemic due to emergence of chloramphenicol resistant strains occurred in Mexico, followed in the next years by several outbreaks in locations from Central America, Indian subcontinent and South-East Asia [53, 55-57]. The resistance was thought to be due to acquisition of a plasmid that harbours the chloramphenicol acetyl transferase gene from *Escherichia coli*.

Chloramphenicol use was replaced by ampicillin and trimethoprim in patients infected with chloramphenicol resistant strains [58]. Yet, already in 1989 strains of *S*. Typhi resistant to chloramphenicol, ampicillin and trimethoprim emerged in several developing countries and termed multidrug-resistant (MDR) strains. The multi-resistance was encoded on a plasmid from H_1 incompatibility group [59-64].

In the early 1990s fluoroquinolones started to be used and the drug of choice was ciprofloxacin, especially for endemic regions of MDR *S.* Typhi and for patients that travelled to those areas [58, 63, 65]. Already in 1991 cases of MDR strains resistant to ciprofloxacin emerged [63, 66, 67] and since then ciprofloxacin and nalidixic acid

resistance has been reported [63, 68]. The resistance to these drugs is due to mutations in the DNA gyrase (*gyrA* or *gyrB*) or topoisomerase (*parC* and *parE*) genes and also due to decreased permeability and efflux of the compounds; quinolones resistance has been recently proved to be transferred also by plasmids carrying the *qnr* gene [69, 70].

The antibiotics largely used after the emergence of fluoroquinolone resistance are the cephalosporins, which belong to the β-lactams class of antibiotics. Acquisition of plasmid-encoded extended spectrum β-lactamase lead to resistance to β-lactams, initially in NTS strains, but recently resistance to the third-generation cephalosporin ceftriaxone has been noted even in *S*. Typhi [71].

In humans, an NTS infection does not require antibiotic treatment, only when complications such as bacteremia and systemic spread of the bacteria occur or for risk groups such as infants, elderly and immunocompromised patients. Moreover, it has been shown that the use of antibiotics prolongs the time when the bacteria are excreted, in recovery after NTSs [21, 72]. NTS gastroenteritis is usually self-limited and only in severe cases fluids and electrolyte replacement is needed.

However, since the symptoms of NTS infections are common to other bacterial infections and in some locations from developing countries the diagnostics methods are not always available, antibiotics are misused for NTSs. Antibiotics are also used for treatment of salmonellosis in cattle, pigs and poultry when other treatments fail. Therefore the NTS strains constitute a reservoir of continuously evolving antimicrobial resistance that can be transferred to typhoidal strains.

1.2.3 New therapeutic strategies and chemical genetics

A drawback of antibiotic usage is the induction of a strong selective pressure in bacteria that eventually leads to acquisition of resistance, not only among pathogens but also amongst the normal commensal flora. Therefore we are facing now an urgent need of new therapeutic strategies to combat infectious diseases.

One such approach is represented by "**chemical genetics**". Even though the concept is old and has been mainly used in the context of eukaryotic biology, in the last decade it is more and more utilized to describe the use of small molecular compounds that interfere with the expression or function of specific virulence factors possessed by pathogenic microorganisms [73, 74].

Application of such compounds that are not lethal to the pathogen but rather disarm it would substantially decrease the selective pressure so that acquisition of resistance would dramatically decrease. Moreover, since they target specific virulence-associated traits they would not affect the avirulent commensals.

For instance, one virulence factor that is conserved among several Gram-negative pathogens is the type 3 secretion system (T3SS), which will be detailed in chapter 1.3.3. Up to date there are several reports of small compounds that interfere with T3SS-mediated virulence in pathogens such as *Yersinia pseudotuberculosis* [75, 76], *Chlamydia trachomatis* [77] and *Shigella flexneri* [78]. In *S.* Typhimurium compounds such as selected salicylidene acylhydrazides [79-81], a thiazolidinone [82] and the proton pump inhibitor omeprazole [83] have been shown to inhibit T3SS-mediated virulence.

Another example is the small molecule virstatin that interferes with adhesion and toxin expression of *Vibrio cholerae* [84].

Although most of these compounds have physicochemical properties that need to be improved before being used in clinical studies and become established treatments, they are promising candidates in the attempt of switching from antibiotics to virulence blockers in infectious diseases treatment. Additionally, they represent valuable tools to be used in on going research investigations of pathogens.

1.3 *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM - THE RESEARCH MODEL FOR TYPHOID FEVER

The best-characterized *S. enterica* serovars are Typhi and Typhimurium. As mentioned previously, *S.* Typhi is human restricted and causes the systemic disease typhoid fever, while *S.* Typhimurium has a broad range of hosts ranging from amoebae to man. Most of the details of the molecular pathogenicity in *Salmonella* infection have been discovered from studies of *S.* Typhimurium infection in cell culture settings and mouse models.

1.3.1 Mouse models

The mouse models have been instrumental for detailing systemic bacterial infections because *S.* Typhimurium causes in mice an infection that apparently parallels *S.* Typhi infection in humans [85]. Also, the possibility to apply gene “knockouts” has brought in the opportunity

to study the infection in hosts lacking selected factors, such as inducible nitric oxide synthase (iNOS) or phagocyte NADPH oxidase (phox).

BALB/c is the most widely used mouse strain for investigating *S.* Typhimurium infections. These mice have an innate susceptibility to several pathogens as compared to other strains. This is thought to be due to low production of interferon gamma (IFN-γ) and a preferential Th2-type cytokine response, which is important for resistance to extracellular pathogens [86]. They also lack functional Nramp1 that makes them less capable of hindering intravacuolar bacterial replication [87, 88].

To analyse visceral dissemination, mice are typically infected per orally [89]. With a reasonable high infection dose (10^8 bacteria or so) bacteria can be detected in the PP one day post infection. The pathology of the small intestine resembles much the one caused by *S.* Typhi in humans; enlarged PP, thickening of the ileal mucosa and a diffuse enteritis that is mainly localized at the terminal ileum and PP, while the other areas on the intestinal epithelium are mainly intact. Further on, bacteria spread to mesenteric lymph nodes (mLN), liver and spleen where they multiply in professional phagocytes such as macrophages. Several days after infection bacteria can reach 10^9-10^{10} colony forming units (CFUs) per organ, causing hepato- and splenomegaly. Bacterial multiplication at these sites causes acute abscesses that gradually become enlarged and transform in granulomata that have mononuclear leukocytes peripherally and are centrally necrotized [85]. The lesions of the hepatic tissue due to inflammation ultimately lead to death of the animal.

As rodents do act as a natural host of *S.* Typhimurium, this model is considered to be less "artificial". Yet, it has it's own limitations. Naturally, *S.* Typhimurium causes in humans enteritis rather than typhoid fever and hence humans and mice mount different host responses to infection with this serovar. Also, there are virulence genes present in *S.* Typhi but absent in *S.* Typhimurium and *vice versa*.

Nevertheless the strengths of this model outweigh the drawbacks; it has been and still is a very useful model to discover and study major bacterial virulence factors [90] and to test typhoid fever vaccine candidates [91].

1.3.2 Cell culture models

In vitro cell culture models revealed many aspects of the molecular pathogenesis of *Salmonella* infection. They represent very simplified models that brought in light small but important pieces of the "puzzle" that is the complex interaction of the pathogen with the host cells within the progression of the infection.

Table 1. Cell lines commonly used in *Salmonella* research (adapted from Hurley *et al*., 2003 [92] and ATCC collection data).

Name of cell line	Provenience	Advantages	Disadvantages
CaCo2	Human epithelial colorectal adenocarcinoma	Can form polarized confluent monolayer that resembles the enterocytes of the small intestine	Do not always grow as readily Difficult to transfect
HeLa	Human epithelial cervix adenocarcinoma	Human provenience Robust in culture	Not of intestinal origin Aberrant karyotype Easily contaminates other cell cultures
HT29	Human epithelial colorectal adenocarcinoma	Can form polarized confluent monolayer	Heterogeneous, contain a small subpopulation of mucus-secreting cells and columnar absorptive cells
MDCK	Canine epithelial kidney	Can form polarized confluent monolayer Suitable transfection host	Not of human or mouse origin Not of intestinal origin
T84	Human epithelial colorectal carcinoma	Can form polarized confluent monolayer	Do not always grow as readily Difficult to transfect
J774-A.1	Murine reticulum cell sarcoma, macrophage	Phagocytosis capable Suitable transfection host Robust in culture Mouse origin	
RAW264.7	Murine Abelson leukaemia, macrophage	Phagocytosis capable Permissive for *Salmonella* intracellular growth Suitable transfection host Robust in culture Mouse origin	
THP-1	Human monocytic leukaemia, monocyte	Suitable transfection host Human origin	

Bacteria-mediated invasion of epithelial cells has been studied using immortalized epithelial cell lines such as HeLa, Caco2, HT29, MDCK and T-84 [89, 92, 93]. Macrophage cell lines

such as RAW264.7 and J774-A.1 have been instrumental to investigate the details of the intracellular stage of *Salmonella* pathogenesis and its interplay with the host [89, 94]. **Table 1** summarizes some features of commonly used cell lines important in the context of *Salmonella* research.

The experimental method widely used for *in vitro* cell culture infection experiments with *S.* Typhimurium is the **gentamicin protection assay**. Typically, for assays using epithelial cell lines the bacteria are grown prior to be applied to cell monolayers in conditions that promote expression of virulence factors that mediate the invasion. After application of bacteria to the cells bacterial invasion is let to occur for 15 min – 1 hour, after which gentamicin is applied. This antibiotic does not penetrate intact eukaryotic cells; therefore the bacteria that are located intracellular are protected by its killing action. When studying intracellular phase of *Salmonella* within macrophages, non-invasive bacteria are applied to cells, and similarly after they have been phagocytosed by macrophages gentamicin is applied to kill the remaining extracellular bacteria and protect the ones that have been internalized. At desired time points after the infection the numbers of intracellular bacteria are determined by counting the CFUs from the cell lysates plated on Luria Bertani (LB)-agar plates [89, 95].

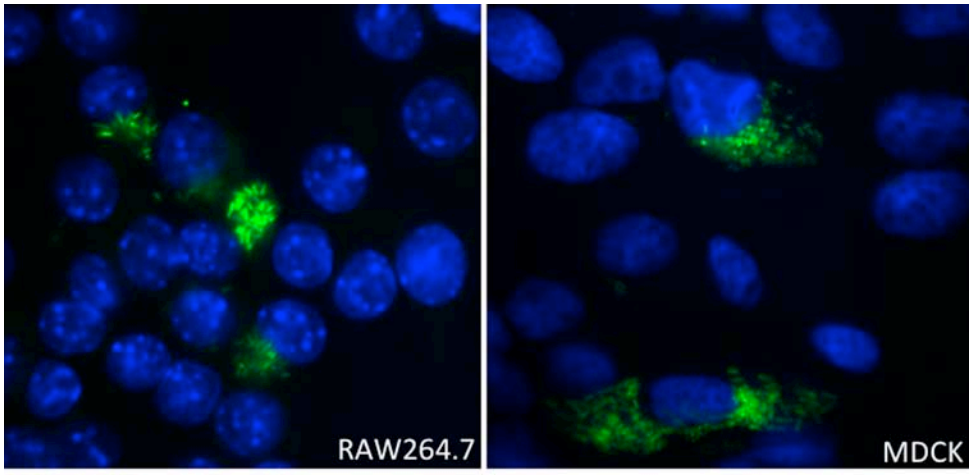

Figure 2. Immunofluorescence microscopy of two widely used cell lines 16 hours post infection with GFP-expressing *S.* Typhimurium by the gentamicin protection assay.

1.3.3 Virulence factors

S. Typhiurium and *S.* Typhi share about 89% of the genes while the differences include 479 genes that are unique to Typhimurium [96] and 601 genes unique to Typhi [97] (**Figure 3**). The differences include virulence genes belonging to the *Salmonella* pathogenicity islands (SPIs), the Vi polysaccharide capsule, plasmids, prophages and phage remnants.

In *Salmonella* many virulence-associated genes are clustered in distinct genomic regions termed "*Salmonella* pathogenicity islands", or SPIs. These regions are usually found next to a tRNA gene and differ from the rest of the genome in their lower G+C content, suggesting that they were acquired through horizontal gene transfer. Currently there are 21 known SPIs in *Salmonella* serovars; *S.* Typhimurium and *S.* Typhi share 11 SPIs (1-6, 9, 11, 12, 13 and 16) [98].

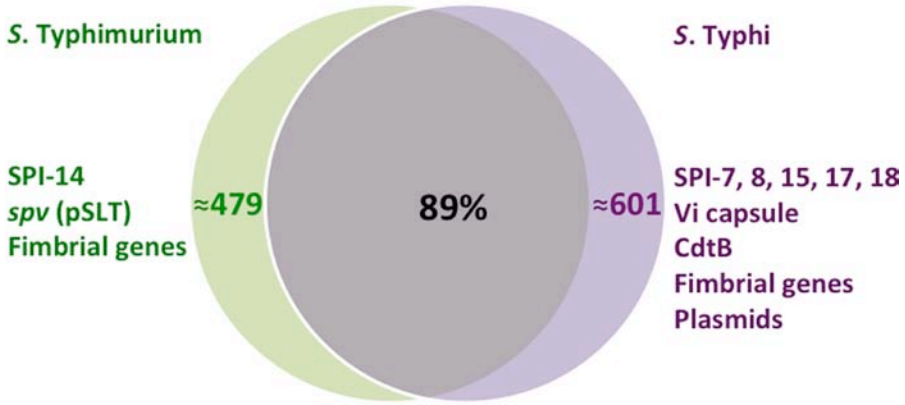

Figure 3. *S.* Typhimurium and *S.* Typhi share the majority of their genome, including several virulence factors. Yet, they do have unique genes that might explain the differences in host tropism and disease manifestations (adapted from Sabbagh *et. al*, 2010 [98]).

SPI1 and SPI2 are considered the hallmarks of virulence in both serovars. They both encode an extraordinary supra-molecular secretory apparatus that is the **T3SS**. Similar T3SSs are found in several Gram-negative pathogens [99] and they are evolutionary related to the flagella apparatus. Their function is to translocate bacterial effector proteins into host cells that will interfere with host functions and enable bacterial invasion, survival and dissemination in the host [100]. Visualization of T3SS structure reveals a syringe like appearance, with a wide base embedded in the bacterial membrane and a thin extracellular needle [101]. T3SS has a size of approximately 3.5 MDa and is composed of around 20 protein components that are forming the inner membrane export assembly, the basal body

that is a hollowed shell which spans the inner and outer bacterial membrane, the extracellular needle and the translocon complex that is creating the contact with host cell membrane and allows translocation of effectors [102] (**Figure 4**). The assembly and function of T3SSs are tightly regulated.

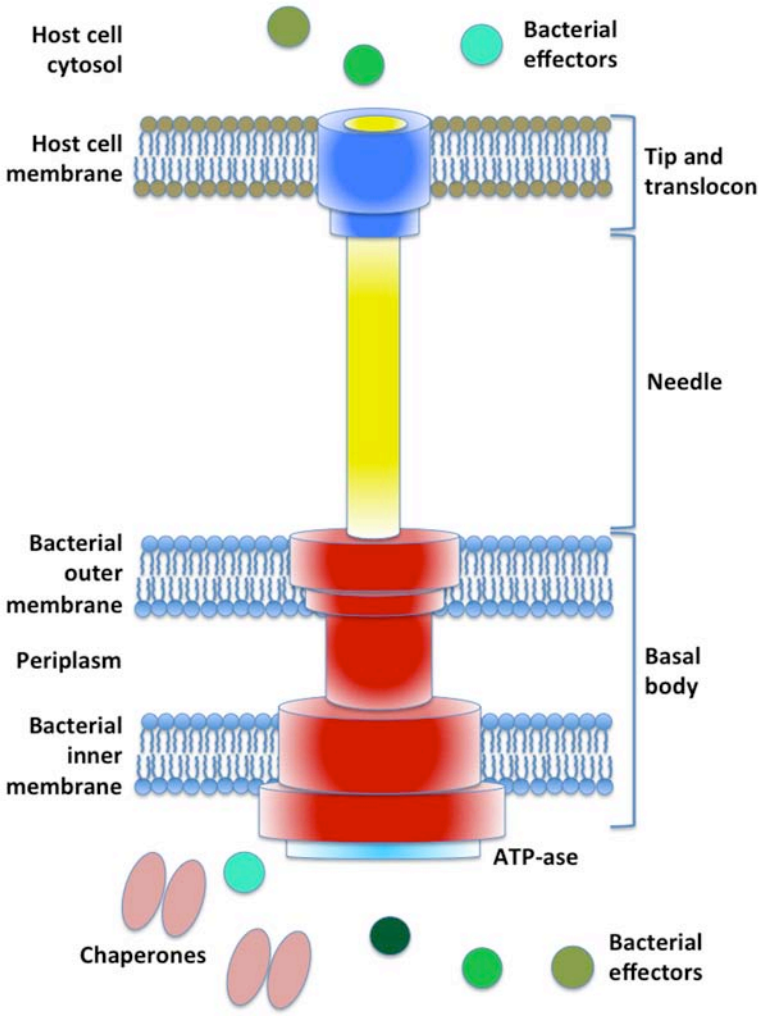

Figure 4. Schematic representation of T3SS (based on Galan *et al*., 2001 [102])

1.3.3.1 SPI1

SPI1 is a 40 kb cluster that codes for genes mainly involved in the invasion of the intestinal epithelial lining [103]. The genes of the structural components and primary effectors of SPI1 T3SS are encoded by three operons; the *prg/org* and *inv/spa* operons that code for the needle-like machinery and the *sic/sip* operon that codes for the translocon and several protein

effectors. Apart from the ones encoded by SPI1, *Salmonella* also secretes via SPI1 T3SS proteins coded by other loci than SPI1. Along with these main components, SPI1 encodes also regulators such as HilA, HilC, HilD and InvF and chaperones that stabilize and protect the effector proteins from degradation and undesired interactions [104, 105].

SPI1 is induced by the conditions that prevail in the small intestine microenvironment, such as low oxygen, high osmolarity, neutral to alkaline pH and presence of bile [106]. In order to invade, the bacteria attach to the intestinal epithelial surface using fimbriae and adhesins. Subsequently, SPI1 T3SS is assembled and more than 19 bacterial effectors are delivered into the host cells. The result of the interaction between the effectors and the host is the bacterial uptake by the non-phagocytic cells of the small intestine epithelial lining. This is achieved by effector-mediated induction of actin cytoskeleton rearrangements and membrane ruffling followed by internalization of the bacteria. The main bacterial effectors involved are SipA [107] and SipC [108] that bind actin, induce its nucleation, polymerization and stabilize the F-actin filaments. SopE, SopE2, SopB interact with and activate the Rho family GTPases Cdc42 and Rac1, central regulators of the host cell actin cytoskeleton, and that subsequently leads to rearrangements of the actin filaments and formation of membrane ruffles [102, 109, 110]. After bacterial uptake by the host cell the activation of the Rho family GTPases and the actin cytoskeleton rearrangements are reversed by the action of the effector SptP that inactivates Rac-1 and Cdc42 [111].

Another feature following invasion is the induction of an inflammatory response followed by infiltration of PMNs. This is also due to activation of Rho GTPases that trigger several mitogen-activated protein kinase (MAPK) pathways and activate the transcription factors NF-kB and AP-1 that direct the production of pro-inflammatory cytokines such as IL-8 [112]. Other SPI1 effectors such as SopA, SopD and SipA as well as secreted flagellin further contribute to inflammation by stimulation of PMNs transmigration, induction of fluid accumulation and diarrhoea [102].

The orchestrated induction and function of SPI1 is tightly regulated. The key regulator of SPI1 gene expression is HilA [113] and in BALB/c mice deleting *hilA* results in the same phenotype as deleting the whole SPI1 locus [114]. HilA activates the transcription of *inv/spa* and *prg/org* promoters; subsequently InvF encoded by the *inv* operon activates the transcription of *sic/sip* operon as well as other genes located outside SPI1. The expression of HilA is positively controlled by the regulators HilC and HilD encoded by SPI1, and by RtsA encoded outside SPI1 on the chromosome [105]. HilA negatively regulates its own expression. Another negative regulator of SPI1 hence HilA, is HilE encoded outside SPI1,

which exerts its function through binding to HilD and inhibiting its up-regulating action on HilA [115].

1.3.3.2 SPI2

SPI2 is the second identified pathogenicity island in *S*. Typhimurium, with size roughly similar to SPI1. Its function enable the bacteria to adapt, survive and replicate inside host cells, ensuring a successful intracellular life-style which is one of the most remarkable features of *Salmonella*. SPI2 codes for a second T3SS, effector proteins, chaperones and the two-component regulatory system SsrA/SsrB [116].

After breaching the intestinal epithelial barrier *Salmonella* serotypes that cause a systemic illness are phagocytosed by professional phagocytes such as macrophages, neutrophils and dendritic cells that were attracted and concentrated at the site of bacterial invasion as a consequence of inflammatory cytokines secreted by the epithelial cells. Once inside the cells the bacteria reside initially in a spacious phagosome that tightens up around the bacteria and results in a vacuolar compartment called the *Salmonella* containing vacuole (SCV). The conditions encountered by the bacteria in the developing SCV, such as low pH and poor nutrient status, are the ones that induce SPI2 [117, 118]. Subsequently SPI2 T3SS is assembled at the interface bacteria-SCV membrane and through the needle complex the bacteria is able to deliver around 30 effector proteins into the host cell cytosol. In contrast to SPI1 effectors, the function of the SPI2 effectors is still poorly understood. A list of the effectors secreted by SPI2 T3SS and their function is given in **Table 2**. Notably, mutants lacking some of the individual effectors do not show significant attenuation in mice or in cell lines. Therefore, many of them seem to have redundant functions [119]. Several effectors secreted by SPI2 T3SS are encoded outside SPI2 [120]. The concerted action of SPI2 effectors controls the fate of SCV, and the manipulation of host cell factors leads to establishment of SCV as a unique intracellular niche that allows bacterial survival and growth, quite distinct from a normal phagosome.

Table 2. **The effectors secreted by SPI2 T3SS** (adapted from Haraga *et al*., 2008 [121]; Figueira and Holden, 2012 [122]).

Effector	Function
CigR	Unknown
GtgA	Unknown
GogB	Unknown
PipB	Unknown
PipB2	Contributes to Sif formation and extension in epithelial cells
SifA	Induces Sif formation in epithelial cells, maintains integrity of the SCV and down-regulates kinesin recruitment to the SCV
SifB	Unknown
SopD2	Contributes to Sif formation in epithelial cells and maintenance of SCV integrity
SpiC	Inhibits endosomal trafficking
SpvB	Inhibition of actin polymerization, macrophage cytotoxicity, P-body disassembly in infected cells
SpvC	Dephosphorylation of MAP kinase
SpvD	Unknown
SseF	Contributes to Sif formation and microtubule bundling in epithelial cells
SseG	Contributes to Sif formation and microtubule bundling in epithelial cells
SseI (SrfH)	Contributes to host-cell dissemination
SseJ	Maintenance of SCV integrity, deacylase activity
SseK1	Unknown
SseK2	Unknown
SseK3	Unknown
SseL	Macrophage delayed cytotoxicity, down-modulation of NF-kB-dependent cytokine production, altered lipid metabolism in infected cells
SspH2	Inhibits the rate of actin polymerization and contributes to virulence in calves
SteA	Unknown
SteB	Unknown
SteC	Unknown
SteD	Unknown
SteE	Unknown

SCV biogenesis and maturation has been thoroughly studied using different macrophage and epithelial cell lines and several studies shown that SCV transiently interacts with early endocytic pathway, quickly lose early endosomal markers and gain late endosomal markers such as LAMP-1, LAMP-2, LAMP-3, the GTPase Rab7 and the vacuolar ATPase. The SCV also excludes M6PR, the receptor that delivers lysosomal hydrolases [123-128]. SCV associates with a set of Rab GTP-ases distinct from a typical phagosome and this is correlated

with bacterial control of endosomal recycling pathways and continuous remodelling of SCV, not only to prevent fusion with undesired endocytic compartments but also to remove unwanted factors such as M6PR-positive membrane [128]. The consensus so far has been that SCV does not fuse with lysosomes [129, 130], even though other studies have found the opposite [131]. Moreover, more recent studies have shown that in HeLa cells SCV significantly associates with lysosomes as early as 30 minutes post infection and up to several hours after, and proposed that the fusion with the lysosomes is rather delayed than prevented [132] or that SCV fuses with enzyme-depleted lysosomes [133].

The action of SPI2 effectors directs SCV to a perinuclear location proximal to the Golgi apparatus, induces host cytoskeleton rearrangements by regulating actin and microtubule motors to create a protected "bunker" that allows bacterial replication inside the host cells [128]. In macrophages, SPI2 mediates also the bacterial protection against the toxic action of reactive oxygen and nitrogen species (ROS, RNS) [134, 135].

In epithelial cells another phenotype produced by intracellular *Salmonella* is the formation of *Salmonella*-induced filaments (Sif), which are formed along microtubule scaffolds. The secreted effectors involved in Sif formation are SifA, SseF and SseG [119, 136, 137].

SPI2 gene expression is mainly regulated by the two-component regulatory system SsrA/SsrB. Conditions like low pH, low osmolarity, low Ca^{2+} and Mg^{2+} are sensed by the membrane sensor kinase SsrA and the signal is transmitted to the transcriptional activator SsrB which induces SPI2 expression [138].

1.3.3.3 The interplay and regulation of SPI1 and SPI2

Genetics studies of the two T3SSs suggest they were acquired independently; even so, probably the host environments posed a selection pressure that led to their cooperation [102]. Although SPI1 and SPI2 are induced and play crucial roles in different phases of *Salmonella* molecular pathogenesis, evidence of crosstalk and overlapping functions for certain effectors have been reported. Several SPI1 effectors (SipA, SopA, SopB, SopD and SopE2) persist or are expressed in the intracellular phase and add to SPI2 effector functions [139, 140]. For example, the SPI1 effector SipA has been detected in association with SCV and seems to play a role together with SPI2 effector SifA in directing SCV to the perinuclear location [141]. SopB, another SPI1 effector protein is involved in early stages of SCV maturation [142] and stimulates NO production in macrophages [143].

Likewise, it has been shown in mice infected with *S.* Typhimurium that expression of SPI2 T3SS begins in the intestinal lumen even before intestinal penetration, probably to prepare the bacteria for the intracellular phase of the disease [144]. Moreover, impairment of the SPI2 T3SS apparatus decreases the expression of several SPI1 T3SS genes and affects the ability of the bacteria to invade epithelial cells [145, 146].

Regulation of gene expression in response to environmental stimuli in *Salmonella* and other Gram-negative pathogens is controlled by several **two-component systems**, which consist of a membrane-bound kinase that senses a specific environmental stimulus, and a response regulator that activates differential expression of target genes. SPI1 and SPI2 expression is also regulated by several two-component systems.

PhoP/PhoQ two-component system is activated in SCV by low pH and low divalent cations and regulates directly or indirectly over 200 genes, including genes involved in intracellular survival and growth, invasion, motility and resistance to antimicrobial peptides [147, 148]. PhoPQ plays an important role in controlling both SPI1 and SPI2 gene expression. It represses SPI1 by down-regulating HilA, but it also actives the SPI1 operon *orgBC* that is expressed intracellularly and might be involved in later stages of the infection [149]. Activation of PhoPQ system by the conditions prevailing in the SCV leads to activation of SPI2 expression through induction of SPI2 regulator system SsrA/SsrB; PhoP binds to SsrB promotor to induce SsrB expression and controls SsrA post-transcriptionally [150].

Another two-component system that controls expression of SPI1 and SPI2 genes is EnvZ/OmpR, which was initially characterized for sensing osmolarity variations in the environment and controlling the expression of the outer membrane proteins OmpC and OmpF. OmpR response regulator affects SPI1 expression by inducing *hilC* expression and post-transcriptional HilD activation [105], while regulation of SPI2 is due to OmpR direct binding to *ssrAB* promotors and subsequent induction of their expression [151, 152].

SirA/BarA is also an important two-component regulatory system mainly required for SPI1 induction and bacterial invasion, but also involved in carbohydrate metabolism, motility and biofilm formation. It is not known exactly which environmental signals are sensed by the sensor kinase BarA. Subsequently, SirA directly binds to *hilA* and *hilC* promoters to induce their expression [105, 153]. SirA also up-regulates HilD expression post-transcriptionally by inducing expression of the two small regulatory RNAs CsrB and CsrC, which bind to CsrA. CsrA is an RNA binding protein that binds to *hilD* mRNA and inhibits SPI1 gene expression [154].

1.3.3.4 Spv

Another important virulence factor of *S*. Typhimurium that is not present in *S.* Typhi is the *spv* gene cluster, located on a large plasmid called pSLT. The *spv* operon contains the *spvA*, *spvB, spvC* and *spvD* structural genes, and *spvR* that codes for a positive transcriptional regulator. The *spv* genes are induced and expressed inside SCV and are important for intracellular survival and bacterial systemic dissemination in mice [155]. *spvB*, *spvC* and *spvR* are the genes mainly important for the virulence of the *spv* locus [156]. SpvB is a toxin that targets actin and prevents its polymerisation by ADP-ribosylation of actin monomers [157], whereas SpvC has phosphothreonine lyase activity and interferes with MAPK signalling of infected host cells [158]. Both SpvB and SpvC are secreted by the SPI2 T3SS [158, 159].

1.3.3.5 LPS

Lipopolysaccharides (LPSs) are the main constituents of the Gram-negative bacteria cell wall. In *Salmonellae* LPS is composed of the glycolipid lipid A that anchors LPS in the outer membrane of bacteria, an oligosaccharide core and the polysaccharide O-antigen. LPS is conserved among different bacteria species, but the number of acyl chains on lipid A, the composition of sugars of the core and the O-antigen confer a high diversity between different bacteria. Bacterial strains that are mutated to lack the O-antigen produce a "rough" colony morphology on LB agar plates while the ones that have the O-antigen are "smooth" [160].

LPS protects the bacteria by conferring membrane stability and resistance to harmful agents from the environment including antibiotics and antimicrobial peptides. For pathogenic bacteria such as *Salmonella*, LPS constitutes a virulence factor important in the interplay with the host by inducing pro-inflammatory responses and protecting bacterial membrane from action of complement membrane attack complex [160-164].

Depending on the environmental conditions, *Salmonella* is able to modify its LPS to increase bacterial fitness. The major regulators of LPS composition are the PhoP/PhoQ and PmrA/PmrB two-component systems [165, 166]. The environmental conditions that are detected by the PmrB sensor are high Fe^{3+}, high Al^{3+} or a mild acidic pH. Subsequently, PmrB autophosphorylates and transfer the phosphoryl group to the response regulator PmrA that activates expression of genes involved in LPS modification such as *pbgP*, *ugd*, *pmrC*. PmrA activation can be also achieved indirectly via the PhoP/PhoQ system that activates

expression of PmrD, which is a protein that regulates and promotes PmrA activity post-transcriptionally [165, 167].

1.3.4 Host responses and *Salmonella*' s solutions

In order to cause disease *Salmonella* has to successfully pass several challenges encountered inside the host. The first host defence encountered by the bacteria is the acidity of the **stomach**. This condition leads to activation of an adaptive response called the acid tolerance response (ATR) [168] which causes expression of acid shock proteins that support bacterial survival and passage of the stomach barrier.

In the **small intestine** *Salmonella* encounter challenges like peristalsis, other competing bacteria, digestive enzymes, bile salts and especially antimicrobial peptides. It is not entirely clear how the bacteria face all these challenges to gain access to the epithelial cells lining of the small intestine. Some mechanisms to escape the microbicidal effect of antimicrobial peptides are modification of LPS in order to create a more positively charged membrane that would reject the positively charged antimicrobial peptides [166, 169] and bacteria-mediated down-regulation of antimicrobial peptides such as α-defensins and lysozyme [170].

Once the bacteria has gain access to the epithelial cells in the small intestine they adhere to the cells and induce bacterial internalization mediated by SPI1 T3SS. The host responses induced by SPI1 effectors, along with actin cytoskeleton rearrangements that induce bacterial internalization, are production of pro-inflammatory cytokines such as IL-8, CXCL1/2/3, CXCL5 and CCL2 that leads to recruitment of neutrophils and macrophages to the site of infection [171]. These cells actively phagocytose the bacteria that gain access to the lamina propria. In turn, the phagocytes further produce other inflammatory cytokines like IL-1, TNF-α, IL-6 and chemoattractants (CCL3/4, CXCL1/2) [112, 121, 171] that leads to recruitment of more inflammatory cells and other events associated with acute inflammation. For instance, ROS produced by phagocytes upon inflammation react with intestinal thiosulfate and form tetrathionate, a new respiratory electron acceptor utilized by *S.* Typhimurium and which it confers a growth advantage over the gut microbiota [172]. The SPI1 effector SipB contributes to intestinal inflammation by stimulating production of IL-1β and IL-18 through binding and activating caspase-1 [173]. After crossing the epithelial barrier *Salmonella* actively restores the actin cytoskeleton, turn-off MAPK signalling, inhibit NF-kB nuclear translocation and subsequently down-regulate cytokine production by the action of the SPI1 effectors SptP, SspH1 and AvrA [111, 174-176]. The bacteria-mediated down-regulation of intestinal

inflammation in *Salmonella* pathogenesis might suggest that on a long run the bacteria could potentially tend to evolve towards asymptomatic colonization of the small intestine, a feature associated with parasitism or commensalism [121].

1.3.4.1 Salmonella and the phagocytes

The next stage of *Salmonella* infection is the intracellular phase where the bacteria face the challenges of the **intracellular milieu of phagocytes**. *S.* Typhimurium is able to reside in phagocytes such as neutrophils, DCs and macrophages.

Neutrophils harbour bacteria in early stages of infection and constitute a reservoir from which bacteria can escape to more permissive cell-types [177]. The mechanisms by which the bacteria are able to escape killing by neutrophils are poorly understood. More likely that the bacteria are protected from neutrophils actions by residing in cells such as macrophages or DCs.

Salmonella is phagocytosed by **DCs** in the submucosal tissue and at PP. DCs are phagocytes important in antigen presentation and subsequent triggering of adaptive immune responses, bridging innate and adaptive immunity. Following uptake of bacteria they migrate to secondary lymphoid organs, undergo maturation that includes up-regulation of major histocompatibility complex (MHC) and costimulatory molecule expression and then interact with T cells. The bacteria prevent antigen presentation by surviving in SCV and avoiding lysosomal degradation or by inducing apoptosis by the action of the effector SipB. *Salmonella* is also able to regulate the polyubiquitination and the expression of MHC II on the DCs surface, which may reduce the ability of DCs to present antigens to CD4 T cells [178]. To circumvent this, uninfected DCs can act as bystander antigen presenting cells and engulf antigenic material from neighbour cells that have undergone bacteria-induced apoptosis [179]. *In vitro* studies have shown that another mechanisms by which *S.* Typhimurium killing and processing for antigen presentation can occur is by targeting the IgG-coated bacteria to FcγRs receptors on the DCs surface [180]. Bacterial internalization by this route affects the ability of SCV to avoid lysosomal degradation. While most of the bacteria are safe within SCV, a small proportion escape in the cytosol due to damaged SCV and failure of bacteria to repair the damage [181]. In the cytosol the bacteria are recognized by the autophagy machinery, which leads to degradation of the pathogen by lysosomal proteases [182].

Even though bacteria prevent T cell activation by DCs in a SPI2-dependent manner [183], T cell activation still occurs and has an important role in clearance of the infection [184-186].

Apart from the above-mentioned functions of DCs, these cells also produce in response to *Salmonella* infection cytokines that activate and recruit other immune cells such as natural killer cells (NK), neutrophils, macrophages and T-cells [186]. Finally, DCs are the major contributors to translocation of the bacteria to mLNs, which reduces bacterial dissemination to systemic compartments and enables mounting of adaptive immune responses [187].

The most studied phagocytic cells that provide the niche for survival and dissemination of *Salmonella* are the **macrophages**. The capacity to survive inside macrophages is a vital requirement of *Salmonella* virulence both *in vitro* and *in vivo* [188]. As described above, upon phagocytosis SPI2 effectors divert the natural vacuolar maturation of SCV to prevent lysosomal fusion and degradation of the bacteria. The main features of macrophage responses to *S.* Typhimurium infection and the bacterial counteracts are discussed bellow.

1.3.4.2 Oxidative and nitrosative stress

Apart from the destructive action of lysosomal proteases bacteria also face another challenge in the phagocyte intracellular milieu, which is the microbicidal action of **ROS** and **RNS**.

Production of ROS relies on the activity of phox, a multimeric enzyme complex that is assembled on the phagosome membrane following phagocytosis. Using NADPH as the electron donor, phox reduces molecular oxygen to the superoxide radical (O_2^-), which subsequently dismutates to hydrogen peroxide (H_2O_2). H_2O_2 can be further converted to hydroxyl radicals ($OH^\bullet$) in the Fenton reaction or to hypochlorous acid (HOCl) by myeloperoxidase action [189]. Chronic granulomatous disease patients that have a mutation in the gp91 phox subunit gene are impaired in ROS production and suffer from recurrent and atypical infections, which demonstrates the importance of ROS in controlling infections, including NTS infections [190].

iNOS is a cytosolic enzyme that catalyses the production of nitric oxide (NO) in a reaction where L-arginine is oxidised to L-citruline, in the presence of oxygen and NADPH. NO auto-oxidation results in production of other RNS such as nitrogen dioxide ($NO_2^\bullet$), dinitrogen trioxide (N_2O_3) and S-nitrosothiols that have enhanced antimicrobial potential [191].

Effective iNOS inducers are the bacterial LPS as well as pro-inflammatory cytokines IFN-γ, TNF-α, IL-1 and IL-2 [192, 193]. LPS stimulation of Toll-like receptor 4 (TLR4) induces production of NO as well as production and secretion of the main pro-inflammatory cytokines. Upon binding to LPS-binding protein (LBP), LPS is delivered to the high-affinity receptor CD14. TLR4 in collaboration with the extracellular protein MD-2 interacts with the CD14-LPS complex and via adaptors that include MyD88 and IRAK induce an intracellular signalling cascade that leads to activation of MAPK and NF-kB pathways that converge and induce iNOS transcription [193]. IFN-γ induces iNOS transcription by interaction with IFNR1-IFNR2 complex that activates Jak-STAT pathway that leads to synthesis of the transcription factor IRF-1, which is responsible for stimulation of iNOS induction [194, 195].

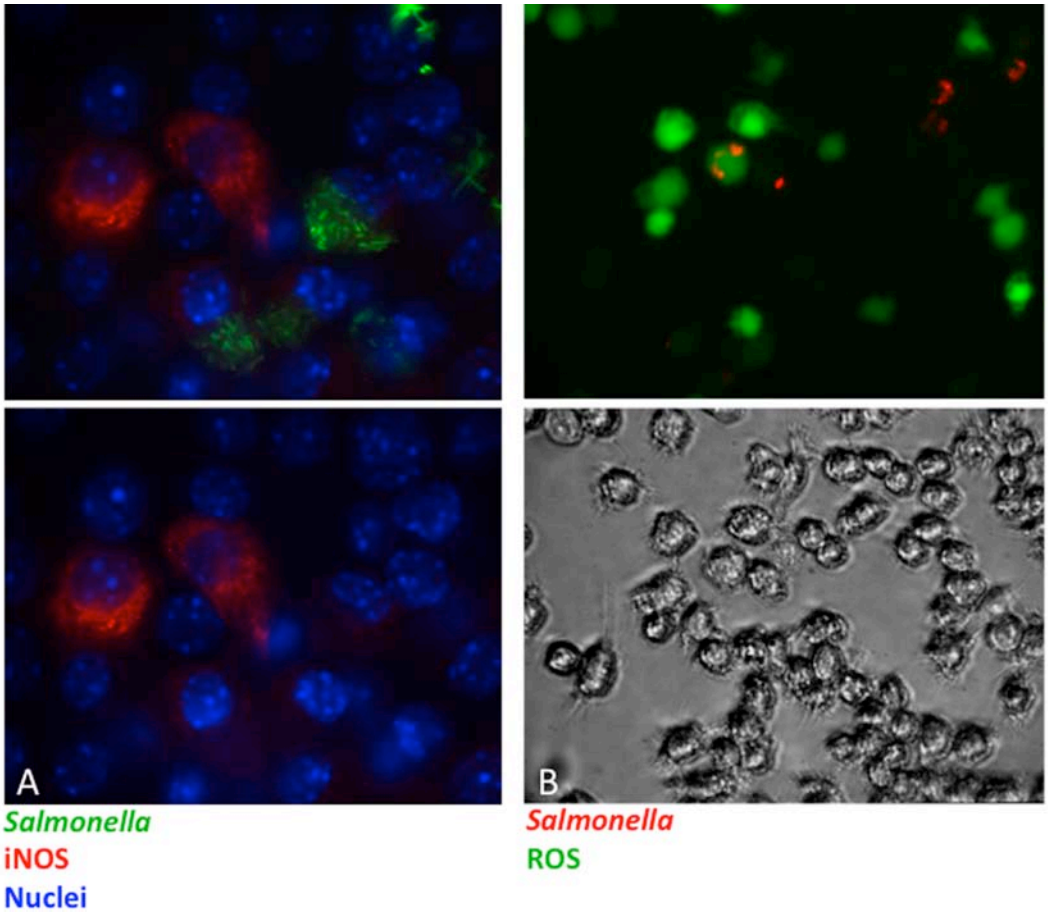

Figure 5. Immunofluorescence microscopy of RAW264.7 macrophages 16 hours post infection with *S.* Typhimurium, revealing iNOS expression (**A**) and ROS production (**B**).

Phox and iNOS activities synergise. NO can further diffuse across the phagosomal membrane and react with superoxide radicals produced by phox to form peroxynitrite ($ONOO^-$) that can be further converted to other potent RNS [189]. *In vivo* studies using gp21phox deficient,

iNOS deficient, double iNOS/gp21phox deficient and their congenic wild-type mice as well as *in vitro* studies using peritoneal macrophages from the above mentioned mice strains, revealed that phox activity plays an important role in reducing the initial numbers of intracellular bacteria at early time points of infection, while iNOS is important to control bacterial replication at later stages of infection [196, 197]. Therefore phox and iNOS have a delineated temporal action even though their products interact to form potent toxic radicals.

The toxic effect of ROS and RNS is due to their ability to damage a wide range of microbial targets like DNA, RNA, proteins, vitamins and lipids [191, 198-200]. The main antimicrobial action of ROS is the induction of DNA base oxidation and strand breaks, several oxidative modifications of proteins including oxidation of thiol groups and interaction with the iron-sulfur clusters of metabolic enzymes, which results in cytosolic iron release that can further sustain the Fenton reaction and potentiate ROS production [199, 201, 202]. RNS have also a broad spectrum of antimicrobial properties such as inhibition of bacterial respiration, DNA replication and specific metabolic pathways like the tricarboxylic acid cycle, as a result of their interaction with DNA, protein metal centres and thiols [203-205].

1.3.4.3 Salmonella ROS/RNS stress management

Salmonella has evolved various evasive, protective and repairing mechanisms in order to survive intracellular oxidative and nitrosative stress. Normally upon phagocytosis of pathogens, the membrane and cytosolic components of phox are mobilised and assemble to discrete cytosolic vesicles, which migrate and fuse with the phagosome. *S.* Typhimurium creates its own "special" phagosome that is SCV and it has been proposed that SCV evades the fusion with phox containing vesicles [135, 206] in a SPI2-dependent manner, even though more recent studies revealed that phox assembly on SCV occurs even in SPI2-proficient bacteria and suggested that *Salmonella* rather rely on its own arsenal of detoxifying enzymes to cope with ROS [207]. These enzymes differ by their location and substrate specificity. The periplasmic (SodCI, SodCII) [208] and cytoplasmic (SodA, SodB) [209, 210] superoxide dismutases convert O_2^- to H_2O_2 that is further degraded to water and oxygen by catalases (KatE, KatG, KatN) and peroxidases (AhpC, TsaA, Tpx) [211, 212] in the bacterial cytoplasm.

RNS production of murine macrophages in response to *S.* Typhimurium infection begins at around 6-8 hours post infection [213] (Paper IV-Banesaru *et al*., Manuscript). It has been shown that *S.* Typhimurium is able to subvert its intracellular co-localization with iNOS by

the action of SPI2 T3SS [134]. Moreover, bacteria are able to detoxify NO by the action of flavohaemoglobin Hmp, which catalyses the oxygen-dependent denitrosylation of NO to nitrate (NO_3^-) or the oxygen-independent reduction of NO to nitrous oxide (N_2O), which are less toxic. In the presence of NO Hmp synthesis is significantly up-regulated and *hmp* mutants are impaired in growth both in LB and inside macrophages [214, 215]. Apart from Hmp *Salmonella* possesses other NO detoxifying enzymes such as the flavorubredoxin NorV, the periplasmic cytochrome *c* nitrite reductase NrfA [213, 216] and the glutathione-dependent formaldehyde dehydrogenase that can decrease the levels of *S*-nitrosoglutathione formed during nitrosative stress [217]. Constitutive detoxification of NO by thiol-based scavenging systems such as the homocysteine, cysteine and tripeptide glutathione is another mean of directly removing RNS [218].

Apart from avoidance and detoxification of ROS and RNS *Salmonella* have the capacity to repair the damage produced to macromolecules such as DNA and proteins as a result of their interaction with the toxic reactive species. DNA repair is conducted by the action of RecA/RecBCD and base excision repair (BER) [219-221] systems. The oxidative modifications of thiol groups of proteins perturb the redox balance in the bacterial cell, the activation status of enzymes and redox-sensitive signal transduction. *S*. Typhimurium and other Gram-negative bacteria are equipped with several oxidoreductases that repair oxidative damage and regenerate antioxidants, such as the methionine sulfoxide reductase and the thioredoxin, glutathione/glutaredoxin and periplasmic Dsb systems [95, 222, 223]. All these reductases use NADPH as the electron source; hence an important role has been attributed also to the glucose 6-phosphate dehydrogenase (G6PD) encoded by the *zwf* gene, which catalyses the first enzymatic step in the pentose phosphate cycle that provides reducing equivalents in the form of NADPH along with ribose for nucleoside synthesis [224].

The coordinated expression of genes responsible for resistance to or repair of oxidative damage is performed by the regulatory proteins OxyR and SoxRS [225-228]. Cysteine oxidation or S-nitrosylation leads to OxyR activation, whereas SoxR is activated by the reversible oxidation of an iron-sulphur cluster. The oxidised proteins can bind to DNA and activate transcription of different oxido-protective enzymes. Moreover, the response regulator SsrB functions also as a redox sensor; oxidation of o specific cysteine in SsrB increases the bacterial fitness in a murine model of *Salmonella* infection [229].

2 AIMS OF THE THESIS

The focus of the thesis is to explore new potential chemical inhibitors of *S.* Typhimurium virulence and to detail aspects of the interplay between the bacteria and host cells. Specifically, the general aims are:

i. To probe for the ability of commercially available proton pump inhibitors to prevent *S.* Typhimurium intracellular replication

ii. To characterize and compare two small-molecular weight virulence inhibitors in their effect on *S.* Typhimurium intracellular replication and on infected host cells

iii. To establish the role of thioredoxin 1 in *S.* Typhimurium virulence

iv. To investigate at the level of individual cells the iNOS responses of phagocytes infected with *S.* Typhimurium

3 RESULTS AND DISCUSSIONS

3.1 PAPER I

Omeprazole antagonizes virulence and inflammation in *S.* Typhimurium-infected RAW264.7 cells

Chemical interference with expression or function of virulence factors using small-molecular compounds falls under the concept of chemical genetics. Such approaches are not only providing tools for fundamental research investigations but also have the potential to pinpoint new potential strategies and molecular targets for pharmaceutical interference with virulence and hence associated disease. Along with low Mg^{2+}, low phosphate, low nutrients level, acidification of the vacuolar compartment in which *Salmonella* resides in is a condition that induces SPI2 expression, subsequent T3SS assembly and secretion of the bacterial effectors responsible for creating the SCV, a protected niche for propitious survival and growth of the pathogen [117, 118]. As mutants defective in SPI2 cannot replicate inside host cells, another way of interference with intracellular replication would be to alter the conditions that lead to SPI2 induction.

In *Salmonella* identification of compounds that target T3SS functionality and resulting virulence has given new hopes in the challenge to design new antibiotics in an era endangered by the continuous emergence of bacterial strains resistant to available antimicrobials [81, 230]. Even though most of them still have major physicochemical constrains that makes them not suitable to be used *in vivo*, they still provide a valuable base for further improvements and design of new generations of molecules.

Another important pool to be explored for new pharmaceutical effects, including antimicrobial effects, are the compounds that are used as active ingredients in already commercially available drugs. The big advantages with this approach is that these compounds are safe to be used *in vivo* since they have already been tested for potential harmful side-effects and have known ADMET properties (absorption, distribution, metabolism, excretion and toxicity).

Using the above rationale, we set to test the potential use of the proton pump inhibitor omeprazole to alter the pH of SCV and in this way interfere with intracellular molecular pathogenicity traits of *S.* Typhimurium. Omeprazole is a benzimidazole compound commercially available and widely used in treatment of peptic ulcer that binds irreversibly to the H^+/K^+-ATPase of the stomach parietal cells and in this way suppresses acid secretion.

Inhibition of vacuolar H^+-ATPase by the macrolide bafilomycin A1 in *S.* Typhimurium infected macrophages have previously generated discrepant reports; while Rathman *et al.*, 1996 [231] shown that it drastically reduces the intracellular growth of the bacteria, in a later study the effect of bafilomycin application on intracellular replication of *S.* Typhimurium differed depending on the cell line used [232].

We set to test the effect of the two proton pump inhibitors on intracellular replication of *S.* Typhimurium in a cell culture infection model using RAW264.7 murine macrophages and the gentamicin protection assay. In our study application of a similar concentration of bafilomycin A1 as previously reported resulted in a drastic reduction of intracellular bacteria recovered 16 hours post infection. Moreover, bafilomycin reduced the intracellular pool of non-replicating bacteria, when tetracycline was added to block the bacterial protein synthesis. This implies that bafilomycin imposes a bactericidal effect on intracellular bacteria. Omeprazole on the other hand, apart from significantly reducing the intracellular growth of bacteria in a dose-dependent manner, had no further diminishing effect on the number of bacteria recovered from infected cells treated with tetracycline, indicating that its effect is rather bacteriostatic. To further analyse its effect we performed infections of RAW264.7 macrophages with a variant of *S.* Typhimurium 14028 bearing a plasmid that besides an ampicillin resistance marker has a temperature sensitive replicon. As the bacteria divide at 37°C the plasmid will segregate and bacterial replication can be measured by the determination of the proportion of ampicillin resistant bacteria; the less ampicillin resistant progenitors, the more replication rounds. In this experiment, the bacteriostatic nature of omeprazole's effect was supported by its ability to reduce both intracellular bacterial growth and the segregation of the plasmid.

To analyse the kinetics of intracellular bacterial growth we determined the growth yields at 2, 4, 6, 8, 10 and 16 hours post infection. A net growth was detectable starting with 8 hours post infection. Application of omeprazole at the above mentioned time points revealed that its growth yield reducing effect occurs if the compound is applied at early time points post infection and diminishes if added at later time points. Moreover, its effect was substantial even if the cells were only pre-treated with the compound for 3 hours before infection and then omitted (data not shown). These results lead us to conclude that the drug is effective only if applied before initiation of bacterial replication. Hence it might interfere with the virulence factors responsible for promoting and sustaining *S.* Typhimurium replication inside host cells, such as SPI2 [116]. Therefore we next tested the effect of omeprazole on selected SPI2 mutants that are strongly (Δ*sifA*, Δ*ssaV*) or partially (Δ*sseG*, Δ*sseF*) impaired in

intracellular replication. *ssaV* codes for a major component of SPI2 T3SS, whereas *sifA*, *sseG* and *sseF* code for secreted effector proteins. The rationale of using these mutants was that if omeprazole would have an additional inhibitory effect on the intracellular basal replication of *ssaV* or *sifA* mutants, it would indicate that the compound acts via a different pathway than SPI2; if omeprazole would not add to the replication inhibition of these mutants, it would be an indication that it might act through interference with SPI2. The results revealed that omeprazole application caused no further decrease in growth for *sifA* and *ssaV* mutants, which implies that the compound is indeed interfering with SPI2 or SPI2 T3SS. The intracellular growth of *sseF* and *sseG* mutants was only partially reduced, and application of omeprazole further reduced it; this is in accordance with previous reports which shown that gene deletion of some SPI2 effectors does not provide full attenuation of virulence and hence the function of several SPI2 effectors is redundant [119, 233].

Apart from interfering with SPI2, another way by which the intracellular bacterial loads could be reduced is due to the microbicidal effect of ROS and RNS produced by macrophages in response to infection. Moreover, it has been reported before that bafilomycin [234] and lansoprazole [235], a proton pump inhibitor closely related to omeprazole, affect the pro-inflammatory responses of macrophages. Therefore we tested also in our experimental setup the effect of bafilomycin and omeprazole on NO production and pro-inflammatory cytokines secretion. Bafilomycin up-regulated NO production of infected cells, whereas surprisingly omeprazole inhibited it. Omeprazole also inhibited iNOS expression, delayed IκB-α degradation and decreased the secreted levels of IL-6 and TNF-α. These are outstanding and ideal properties of a potential new antimicrobial; interference with virulence while down-regulating the inflammatory activation of the host.

Besides the different effect on NO production, the two compounds also differently affected the viability of macrophages. While bafilomycin increased the proportion of early and late apoptotic cells as well as the LDH release of cells, omeprazole had no deleterious effects on cell integrity and viability. Hence, the detrimental effect of bafilomycin on cells might explain the strong bactericidal effect on intracellular bacteria, by the gentamicin getting access to the intracellular bacteria due to damaged cell integrity.

Even though our initial hypothesis was that omeprazole would interfere with vacuolar acidification of macrophages, acridine orange staining of omeprazole treated macrophages revealed that the compound had no effect on vacuolar acidification.

Omeprazole has been previously reported to have antibacterial properties on *Helicobacter* [236] and oral streptococci [237] and also anti-parasitic effect on *Leishmania* [238]. We describe a new effect of omeprazole, interference with SPI2-mediated intracellular fitness of *S.* Typhimurium by a novel bacteriostatic mechanism, which does not results from increased antimicrobial function of macrophages, but on the contrary in the absence of inflammatory activation of infected macrophages.

3.2 PAPER II

Small-molecular virulence inhibitors show divergent and immunomodulatory effects in separate infection models of *Salmonella enterica* serovar Typhimurium

In the last decade several screens have been performed to identify chemicals that would interfere specifically with expression or function of pathogen-associated virulence factors. For *Salmonella* one such virulence factor that represents an attractive target for interference is the T3SS [230]; as already described above, the bacteria possess two individual T3SSs that are coded by SPI1 and SPI2, respectively. Studies by our lab [81, 83] revealed two potent compounds that interfered with SPI2-mediated intracellular replication of *S.* Typhimurium in RAW264.7 macrophages; the benzimidazole proton pump inhibitor omeprazole and the salicylidene acylhidrazide INP0010. As a follow up we set to test and compare the two compounds alone or in combination on both virulence-associated traits of *S.* Typhimurium and on inflammatory responses of host, in different infection models.

In *Salmonella* research apart from macrophages many infection assays are done using epithelial cell lines. These are usually performed to investigate SPI1-mediated bacterial invasion but nevertheless *Salmonella* also undergoes SPI2-dependent intracellular replication inside the epithelial cells; moreover, virulence-associated phenotypes such as Sif formation have been described and characterized in epithelial cells [119, 136, 137]. Therefore we also tested INP0010 and omeprazole in MDCK epithelial cell culture model of infection. Surprisingly, omeprazole had no effect on while INP0010 increased the intracellular *S.* Typhimurium growth yield; application of both decreased the up-regulated growth yield caused by INP0010 application alone. This implies that the two compounds act in an antagonistic manner in this infection setup.

When tested in RAW264.7 macrophages, omeprazole and INP0010 both decreased the intracellular bacterial pool in a dose dependent fashion as previously shown. Application of

the two surprisingly tended to abolish the growth reducing effect at a lower concentration while at a higher dose it reduced the growth to the same level as when individual compounds were applied.

One of the major differences between epithelial cells and macrophages is the arsenal of inflammatory and antimicrobial effectors. In contrast to epithelial cells, macrophages respond to bacterial infection by activating inflammatory signalling pathways that lead to production of ROS, RNS and pro-inflammatory cytokines. As reported in Paper I [83] omeprazole had an anti-inflammatory effect on infected macrophages. Therefore we set to test the same parameters, such as iNOS expression, NO and ROS production and release of TNF-α also for INP0010, alone and in combination with omeprazole. The two virulence inhibitors had markedly different effects; opposed to omeprazole, INP0010 up-regulated iNOS expression and the levels of NO and TNF-α detected 16 hours post infection from the infected cells supernatants. Combination of omeprazole and INP0010 reduced the up-regulating effect of INP0010. Notably, their effect was not depended on viable *S.* Typhimurium, since the compounds had similar effects also when the cells were only stimulated with LPS. As for ROS, omeprazole did not alter the levels, while INP0010 had an up-regulating effect; the combination of the two had this time an additive up-regulating effect.

The interplay between *Salmonella* and its host has been long studied and there are aspects that are still not elucidated. The bacteria are able to circumvent ROS and RNS production by various mechanisms, mainly mediated by the expression and function of SPI2. In order to investigate the connection between the effects of the two compounds on host cells and on the intracellular replication of *S.* Typhimurium, we included in our infection setup the competitive iNOS inhibitor NMMA and the antioxidant and NO scavenger ascorbic acid. As expected both chemicals decreased the NO production. Addition of ascorbic acid or NMMA did not have any further effect on the decreased NO production posed by omeprazole and it decreased the high levels of NO in INP0010 treated regiments. The bacterial growth yields were not affected by NMMA or ascorbic acid in the omeprazole treated infected cells, but in the case of INP0010 treatments the addition of NMMA increased the number of intracellular bacteria. This suggests that at least partially, the inhibitory effect of INP0010 on the intracellular replication of *S.* Typhimurium is due to its elevating effect on NO production of infected cells.

We also compared the effect of omeprazole and INP0010 on the ability of intracellular GFP-expressing *S.* Typhimurium to secrete the hemagglutinin-tagged SseJ SPI2 effector from a plasmid. The control infection regiments revealed immunostaining for SseJ around the

bacteria and also at dispersed locations in the infected cells. In contrast, in the omeprazole treated samples the SseJ was detectable only in the rare cases at clusters of replicating bacteria and confined to these clusters. Interestingly, application of INP0010 revealed in some cells an elongated bacterial phenotype that did not stain for SseJ. Such a phenomena was also previously reported by Rosenberger *et al*., 2002 [239] in IFN-γ primed RAW264.7 macrophages at late stages of the infection (24 hours post infection) and seemed to be mediated by MEK kinase signalling and phox [239]. It is hence possible that the elongation of the bacteria is due to INP0010-mediated exacerbation of inflammation and oxidative stress responses in macrophages. Application of both INP0010 and omeprazole revealed a phenotype resembling the application of omeprazole alone, while addition of NMMA on INP0010 treated samples restored the control phenotype, thus reinforcing the idea that INP0010 mediates its effect on intracellular bacteria through up-regulation of NO.

We also tested the compounds in a *Caenorhabditis elegans* infection model. In a liquid-based assay the infection with *S*. Typhimurium results in nematode paralysis and reduced lifespan. Application of the individual compounds substantially reduced the paralysis of the worms, with INP0010 being more potent than omeprazole. Combination of the two resulted in an almost complete abrogation of paralysis in *S*. Typhimurium infected worms.

The conclusion of our study is that even though omeprazole and INP0010 are efficient reducers of the intracellular numbers of bacteria in the macrophage model of infection, their effect is more likely to be the result of independent mechanisms; at least part of INP0010 effect on bacterial growth is due to up-regulation of NO production in the host cells. The effect of the compounds is very much dependent on the cell types used in the infection setup. Also, the effect of both compounds seems to be related to the ability of the infected host to mount inflammatory and antimicrobial responses. Our study brings in light the importance of choosing the right infection models when screening for new virulence inhibitors and the idea that the effects of potential candidates on the host should be taken in account and thoroughly investigated.

3.3 PAPER III

Thioredoxin 1 participates in the activity of the *Salmonella enterica* serovar Typhimurium pathogenicity island 2 type III secretion system

Adaptation to the intracellular life-style is a major attribute of *Salmonella* virulence. Once intracellular, the bacteria face great challenges such as oxidative and nitrosative stress, as well as the lower pH of the SCV. As a consequence of the reactive radicals stress the bacteria has to maintain a proper surveillance of the redox status of proteins and repair the eventual alterations. One of the important reductases that contribute to maintenance of cellular redox homeostasis is thioredoxin 1 (TrxA). TrxA catalytically active site consist of two cysteine residues that can reduce protein disulphide bonds [240]. Apart from the cysteine related catalytical activity, TrxA has also a chaperone function [241].

Previous studies by our lab revealed that TrxA contributes to *S.* Typhimurium virulence in macrophages and mice [95]. In the present study we further investigated the role of TrxA in bacterial virulence. As intracellular virulence is mediated by SPI2, we set to determine the interconnection between TrxA and SPI2.

When the bacteria were grown *in vitro* in a minimal medium characterized by low pH, low concentration of Mg^{2+} and phosphate that mimic the conditions that prevail in SCV, we could demonstrate that *trxA* is co-induced with SPI2 and was needed for induction of the SPI2 T3SS apparatus gene *ssaG* and for the secretion of the SPI2 effector protein SseJ.

Next we set to determine the contribution of TrxA to SPI2 activity in infected RAW264.7 macrophages and MDCK epithelial cells. In order to bypass effects that would relate to intracellular replication, but still be able to detect significant numbers of intracellular bacteria, we performed the gentamicin protection assay with a higher multiplicity of infection (MOI 100:1) and restricted the infection to 8 hours, a time that coincides with the beginning of intracellular replication [83]. In macrophages, the *trxA* deficient bacteria showed more scattered bacterial ensembles that did not stained for SseJ, as compared to *S.* Typhimurium wild-type infection that revealed perinuclear located intracellular bacteria staining for SseJ. Complementation of *trxA* mutant with a plasmid that codes for wild-type TrxA restored the perinuclear location and staining for SseJ. Complementation with a catalytically inactive variant of TrxA did not restore the SseJ staining. Yet, when in parallel immunoblotting for SseJ of infected cell lysates was performed, complementation with the catalytically inactive TrxA gave rise to a small recovery of the SseJ expression as compared to the non-complemented *trxA* mutant. Surprisingly, when the same set of bacterial strains was used to

infect epithelial cells, the restoration of the wild-type phenotype was noted for both catalytically active and inactive TrxA complementation. This implies that the catalytical activity of TrxA is particularly important for proper SseJ secretion in macrophages, cells that do differ from the epithelial cells in their capability to mount antimicrobial oxidative and nitrosative stress, while the non-catalytic function of TrxA significantly contributes to SPI2 T3SS secretion of effectors such as SseJ in epithelial cells. Indeed, when we let the infection to proceed for 16 hours and determined the growth yields of the above-mentioned bacterial strains, the catalytically inactive TrxA proficient bacteria were unable to replicate in macrophages, while in epithelial cells the replication was restored to a smaller but significant level as compared to *trxA* mutant.

To investigate whether TrxA and SPI2 contribute to intracellular replication via a shared pathway we performed infection of macrophages and epithelial cells with wild-type bacteria, an *ssaV* mutant that act as a SPI2 null mutant, the *trxA* mutant and a double *trxA/ssaV* mutant. All the mutants revealed a similar degree of impairment in intracellular growth. This made us to conclude that TrxA and SsaV contribute through a convergent pathway to intracellular replication of *S*. Typhimurium.

When *trxA* and *ssaV* mutants were used to infect mice by per oral route they display a similar level of drastic attenuation in regard to colonization of mLNs, liver and spleen. To be able to detect potential more subtle differences in net growth *in vivo* we also performed competition experiments in which the mice were intraperitoneally challenged with 1:1 ratio mixtures of the mutant bacterial strains. These experiments revealed that TrxA provided an additional fitness in the SPI2-deficient background, while SPI2 did not additionally contribute to virulence in a TrxA-deficient background. This implies that SPI2 is inactive in TrxA-deficient bacteria. As for the catalytically inactive TrxA we also demonstrated that, paralleling the results from the epithelial cell line infection, it could partially complement the replication defect of the *trxA* mutant in liver and spleen of BALB/c mice.

Regarding the potential contribution of TrxA to SPI1 activity, we could not detect a significant effect of *trxA* deletion on SPI1 T3SS-secreted effectors profile or on invasiveness of MDCK epithelial cells even in the presence of H_2O_2.

The conclusions of **Paper III** are that both catalytically and non-catalytically activities of TrxA are important contributors to *S*. Typhimurium virulence, depending on the infection model used, and that SPI2 and TrxA have a convergent contribution to intracellular replication of *S*. Typhimurium both *in vitro* and *in vivo*.

3.4 PAPER IV

Heterogeneity in phagocyte inducible nitric oxide synthase induction in response to infection with *Salmonella enterica* serovar Typhimurium

S. Typhimurium evokes in infected phagocytes inflammatory responses and production of toxic radicals such as ROS and RNS by the enzymes phox and iNOS [134, 135, 206]. On the other hand, the bacteria have evolved mechanisms to prevent, evade and repair the damage produced by the reactive species. For instance, SPI2 activity seems to protect the intracellular bacteria against NO produced by the host cells through interference with iNOS localization [134], but in the same time high levels of NO produced by IFN-γ- stimulated macrophages appear to down-regulate SPI2 expression [242]. Moreover, it has been found that RNS modify a specific cysteine in the SPI2 activator protein SsrB [229]. At an individual host cell level, the outcome of the infection depends on who finally tilts the balance in its favour.

The most common assays used to assess the levels of iNOS expression and NO production in cell culture infection models of *Salmonella* are immunoblotting to detect iNOS expression from infected cell lysates and colorimetric measurements of NO from infected cells supernatants using Griess reagent, as described in **Paper I** and **II**. Still, the data we can extract in these ways will give us an overall picture of the responses at the cell population level. Previous unpublished data from our research group revealed that surprisingly the infected cells do not stain for iNOS when immunofluorescence microscopy was performed. Therefore we pursued to elucidate the activation status of individual cells from a population of phagocytes infected with *S.* Typhimurium and to assess if the heterogeneity of the responses is a *Salmonella* infection specific trait.

S. Typhimurium infection of RAW264.7 macrophages produced an uneven pattern of iNOS expression as analysed by immunofluorescence 16 hours post infection. In this regard, four distinct phenotypes were noted; infected cells expressing iNOS, infected cells being iNOS negative, uninfected cells being iNOS positive and uninfected cells not expressing iNOS. Surprisingly, the vast majority of iNOS responsive cells were not the ones infected, but rather uninfected cells neighbouring infected iNOS negative cells. The phenomenon was not dependent on the mean of bacterial internalization as both invasive *Salmonella* and non-invasive complement-opsonized bacteria resulted in the same pattern of iNOS responsiveness, noted as early as 4 hours post infection. A paracrine signalling was excluded because physical separation of the cells did not alter the proportion of cells belonging to the

four phenotypes. This suggests that the RAW264.7 macrophages might have an intrinsic heterogeneity with regard to iNOS responses.

To investigate if the heterogeneity in iNOS response was related to active virulence-proficient *S.* Typhimurium, we performed the infection of cells with wild type bacteria treated or untreated with tetracycline to block protein synthesis, the *ssaV* mutant that act as a SPI2 mutant and we included also the apathogenic laboratory strain of *E. coli* TG1. All the above mentioned infection regiments resulted in a similar pattern of iNOS resposive cells distribution, proving that the capacity of a given cell to mount iNOS is not dependent on *Salmonella* infection as such. Indeed, LPS or IFN-γ stimulation of uninfected cells resulted in a similar heterogeneous pattern of iNOS expression; only when the cells were stimulated with a combination of LPS and IFN-γ the iNOS response was fully restored in almost all cells.

Pre-stimulation with LPS did not alter the iNOS expression pattern of infected cells, while pre-stimulation with IFN-γ alone or in combination with LPS markedly enhanced the proportion of iNOS responsive cells.

Similar results were obtained also in BMDMs, infected and pre-stimulated as above. When we instead used TLR4-/- BMDMs, the iNOS response was completely abrogated for the *Salmonella* infection regiments while *E. coli* surprisingly generated very few iNOS responsive cells. Again, LPS pre-stimulation did not alter the responsiveness of the cells, whereas IFN-γ stimulation did evoke iNOS expression. These results suggest that LPS recognition is playing a central role in steering the capability of cells to express iNOS.

The *in vitro* results were further paralleled *in vivo*. Infected mouse livers displayed also a heterogeneous pattern of iNOS staining; still, the majority of infected cells were iNOS negative, while iNOS expression was mostly detected either in uninfected cells located in the vicinity of infected cells or in foci where massive tissue disruption was observed.

The conclusions from **Paper IV** are that RAW264.7 macrophages and murine BMDM have an intrinsic heterogeneous pattern of iNOS expression that is not dependent on *Salmonella* infection but that relates to TLR4. IFN-γ stimulation in combination with LPS restores an even distribution of iNOS responsive cells in a given population in the absence or presence of bacterial challenge. These might have also *in vivo* implications since a similar pattern of iNOS expression was found in *S.* Typhimurium infected mouse livers. Overall, our results underline the need of shifting from gross inspection to individual cell analysis when studying host-pathogen interactions.

4 ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor **Mikael Rhen** for his outstanding scientific guidance and expertise (you were my true Wikipedia, always giving me fast answers to both general and specific questions on our favourite bacteria and not only) and for uplifting my motivation and curiosity especially when the results were in my opinion weird (by saying "That's interesting!"). You are a true model for passion and devotion to science! It has been an honour to meet you and complete this journey thanks to your always kind and helpful supervision. Kiitos kaikesta Mika!

I am also thankful to my co-supervisor **Agneta Richter-Dahlfors** for your contagious and inspiring enthusiasm and for sending me to Stanford to learn the microfluidic device.

My sincere thanks to all the current and past members of **Rhen's lab**: **Kim** for the nice time and chats in the office and lab, for the helpful feedback on the thesis and for making me better understand "Finnish psychology"; **Sem**, **Naeem**, **Syed** and **Cesar** for being the best colleagues, thank you guys for contributing to my PhD time with scientific discussions and helpful advices and mostly by creating a joyful, friendly and homely atmosphere in the lab and office; **Relu** for introducing me to the lab and methods and letting me "steal" your secrets on various lab techniques; the German crew, **Sarah**, **Sabrina**, **Chriselle**, **Luisa**, **Pavel** and **Han** for good and joyful times in the lab.

Farhana, **Marie-Stephanie, Kim, Stephen** and **Jens** for the nice atmosphere in the office sprinkled with good jokes when I needed to take a break from writing the thesis.

My mentor and good friend **Laura Plant Fuentes**, thank you for all the help and advices both professional and personal throughout these years, you were there for me in times of crisis and joy! I could never have a better mentor.

All the nice colleagues with whom we shared the corridor(s) all these years:

Birgitta Henriques Normark and **Staffan Normark** and their group members (you are so many, please forgive me if by mistake I omitted someone) **Sandra**, **Martin**, **Laura S**, **Karina**, **Sarah**, **Anuj**, **Murat**, **Vicky**, **Susan**, **Katrin Pütsep**, **Marie**, **Mario**, **Priyanka**, **Federico**, **Shanshan**, **Sulman**, **Edmund**, **Peter**, **Anna** and **Maria** thank you for sharing lab meetings, ideas, reagents, instruments, fika and retreats and for making our corridor such a lovely "niche" of MTC.

Martin Rottenberg and his current and former group members “big” and “small” **Berit**, **Carina**, **Frank** and **Suman** for all the reagents, instruments, fika, lunches and celebrations we shared, those were really nice times!

Benedict and all the board members of KI Infection program, thank you for the retreats and interesting discussions on various matters, I really enjoyed collaborating with you!

I want to thank all the colleagues, collaborators, co-authors and people with whom I interacted during my PhD, each of you have contributed to this big puzzle!

All these would not have been possible without the love of God and my family from Romania and Sweden, thank you for your endless and unconditional love and support. **Mulţumesc din suflet! Vă iubesc mult!**

5 REFERENCES

1. Baumler, A.J., et al., *Evolution of host adaptation in Salmonella enterica.* Infect Immun, 1998. **66**(10): p. 4579-87.
2. Lavigne, J.P. and A.B. Blanc-Potard, *Molecular evolution of Salmonella enterica serovar Typhimurium and pathogenic Escherichia coli: from pathogenesis to therapeutics.* Infect Genet Evol, 2008. **8**(2): p. 217-26.
3. Su, L.H. and C.H. Chiu, *Salmonella: clinical importance and evolution of nomenclature.* Chang Gung Med J, 2007. **30**(3): p. 210-9.
4. Reeves, M.W., et al., *Clonal nature of Salmonella typhi and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of Salmonella bongori comb. nov.* J Clin Microbiol, 1989. **27**(2): p. 313-20.
5. Shelobolina, E.S., et al., *Isolation, characterization, and U(VI)-reducing potential of a facultatively anaerobic, acid-resistant bacterium from Low-pH, nitrate- and U(VI)-contaminated subsurface sediment and description of Salmonella subterranea sp nov.* Applied and Environmental Microbiology, 2004. **70**(5): p. 2959-2965.
6. Crump, J.A., S.P. Luby, and E.D. Mintz, *The global burden of typhoid fever.* Bull World Health Organ, 2004. **82**(5): p. 346-53.
7. Buckle, G.C., C.L. Walker, and R.E. Black, *Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010.* J Glob Health, 2012. **2**(1): p. 010401.
8. Majowicz, S.E., et al., *The global burden of nontyphoidal Salmonella gastroenteritis.* Clin Infect Dis, 2010. **50**(6): p. 882-9.
9. Bayer, C., et al., *An outbreak of Salmonella Newport associated with mung bean sprouts in Germany and the Netherlands, October to November 2011.* Euro Surveill, 2014. **19**(1).
10. Berger, C.N., et al., *Interaction of Salmonella enterica with basil and other salad leaves.* ISME J, 2009. **3**(2): p. 261-5.
11. Braden, C.R., *Salmonella enterica serotype Enteritidis and eggs: a national epidemic in the United States.* Clin Infect Dis, 2006. **43**(4): p. 512-7.
12. Cavallaro, E., et al., *Salmonella typhimurium infections associated with peanut products.* N Engl J Med, 2011. **365**(7): p. 601-10.
13. Haeusler, G.M. and N. Curtis, *Non-typhoidal Salmonella in children: microbiology, epidemiology and treatment.* Adv Exp Med Biol, 2013. **764**: p. 13-26.
14. Hohmann, E.L., *Nontyphoidal salmonellosis.* Clin Infect Dis, 2001. **32**(2): p. 263-9.
15. Mermin, J., et al., *Reptiles, amphibians, and human Salmonella infection: a population-based, case-control study.* Clin Infect Dis, 2004. **38 Suppl 3**: p. S253-61.
16. Jackson, B.R., et al., *Outbreak-associated Salmonella enterica serotypes and food Commodities, United States, 1998-2008.* Emerg Infect Dis, 2013. **19**(8): p. 1239-44.
17. Centers for Disease, C. and Prevention, *Outbreak of Salmonella serotype Saintpaul infections associated with multiple raw produce items--United States, 2008.* MMWR Morbidity and mortality weekly report, 2008. **57**(34): p. 929-34.
18. Crump, J.A. and E.D. Mintz, *Global trends in typhoid and paratyphoid Fever.* Clin Infect Dis, 2010. **50**(2): p. 241-6.
19. Stuart, B.M. and R.L. Pullen, *Typhoid; clinical analysis of 360 cases.* Arch Intern Med (Chic), 1946. **78**(6): p. 629-61.
20. Parry, C.M., et al., *Typhoid fever.* N Engl J Med, 2002. **347**(22): p. 1770-82.
21. Gal-Mor, O., E.C. Boyle, and G.A. Grassl, *Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ.* Front Microbiol, 2014. **5**: p. 391.

22. Gonzalez-Escobedo, G., J.M. Marshall, and J.S. Gunn, *Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state.* Nat Rev Microbiol, 2011. **9**(1): p. 9-14.
23. Mandal, B.K. and J. Brennand, *Bacteraemia in salmonellosis: a 15 year retrospective study from a regional infectious diseases unit.* BMJ, 1988. **297**(6658): p. 1242-3.
24. Jepson, M.A. and M.A. Clark, *The role of M cells in Salmonella infection.* Microbes Infect, 2001. **3**(14-15): p. 1183-90.
25. Jones, B.D., N. Ghori, and S. Falkow, *Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches.* J Exp Med, 1994. **180**(1): p. 15-23.
26. Niess, J.H. and H.C. Reinecker, *Dendritic cells in the recognition of intestinal microbiota.* Cell Microbiol, 2006. **8**(4): p. 558-64.
27. Takeuchi, A. and H. Sprinz, *Electron-Microscope Studies of Experimental Salmonella Infection in the Preconditioned Guinea Pig: II. Response of the Intestinal Mucosa to the Invasion by Salmonella typhimurium.* Am J Pathol, 1967. **51**(1): p. 137-61.
28. Vazquez-Torres, A., et al., *Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes.* Nature, 1999. **401**(6755): p. 804-8.
29. Wick, M.J., *The role of dendritic cells in the immune response to Salmonella.* Immunol Lett, 2003. **85**(2): p. 99-102.
30. Tam, M.A., et al., *Early cellular responses to Salmonella infection: dendritic cells, monocytes, and more.* Immunol Rev, 2008. **225**: p. 140-62.
31. Kraus, M.D., B. Amatya, and Y. Kimula, *Histopathology of typhoid enteritis: morphologic and immunophenotypic findings.* Mod Pathol, 1999. **12**(10): p. 949-55.
32. Nguyen, Q.C., et al., *A clinical, microbiological, and pathological study of intestinal perforation associated with typhoid fever.* Clin Infect Dis, 2004. **39**(1): p. 61-7.
33. Sprinz, H., et al., *Histopathology of the upper small intestines in typhoid fever. Biopsy study of experimental disease in man.* Am J Dig Dis, 1966. **11**(8): p. 615-24.
34. House, D., et al., *Typhoid fever: pathogenesis and disease.* Curr Opin Infect Dis, 2001. **14**(5): p. 573-8.
35. Weinstein, D.L., et al., *Differential early interactions between Salmonella enterica serovar Typhi and two other pathogenic Salmonella serovars with intestinal epithelial cells.* Infect Immun, 1998. **66**(5): p. 2310-8.
36. Bhan, M.K., R. Bahl, and S. Bhatnagar, *Typhoid and paratyphoid fever.* Lancet, 2005. **366**(9487): p. 749-62.
37. Engels, E.A., et al., *Typhoid fever vaccines: a meta-analysis of studies on efficacy and toxicity.* BMJ, 1998. **316**(7125): p. 110-6.
38. Germanier, R. and E. Fuer, *Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine.* J Infect Dis, 1975. **131**(5): p. 553-8.
39. Tacket, C.O., et al., *Safety and immunogenicity of two Salmonella typhi Vi capsular polysaccharide vaccines.* J Infect Dis, 1986. **154**(2): p. 342-5.
40. Waddington, C.S., T.C. Darton, and A.J. Pollard, *The challenge of enteric fever.* J Infect, 2014. **68 Suppl 1**: p. S38-50.
41. Acharya, I.L., et al., *Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of Salmonella typhi. A preliminary report.* N Engl J Med, 1987. **317**(18): p. 1101-4.
42. Anwar, E., et al., *Vaccines for preventing typhoid fever.* Cochrane Database Syst Rev, 2014. **1**: p. CD001261.
43. Black, R.E., et al., *Efficacy of one or two doses of Ty21a Salmonella typhi vaccine in enteric-coated capsules in a controlled field trial. Chilean Typhoid Committee.* Vaccine, 1990. **8**(1): p. 81-4.

44. Klugman, K.P., et al., *Immunogenicity, efficacy and serological correlate of protection of Salmonella typhi Vi capsular polysaccharide vaccine three years after immunization.* Vaccine, 1996. **14**(5): p. 435-8.
45. Levine, M.M., et al., *Duration of efficacy of Ty21a, attenuated Salmonella typhi live oral vaccine.* Vaccine, 1999. **17 Suppl 2**: p. S22-7.
46. Levine, M.M., et al., *Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation.* Lancet, 1987. **1**(8541): p. 1049-52.
47. Levine, M.M., et al., *Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in randomised controlled field trial.* Lancet, 1990. **336**(8720): p. 891-4.
48. Simanjuntak, C.H., et al., *Oral immunisation against typhoid fever in Indonesia with Ty21a vaccine.* Lancet, 1991. **338**(8774): p. 1055-9.
49. Wahdan, M.H., et al., *A controlled field trial of live Salmonella typhi strain Ty 21a oral vaccine against typhoid: three-year results.* J Infect Dis, 1982. **145**(3): p. 292-5.
50. Yang, H.H., et al., *Efficacy trial of Vi polysaccharide vaccine against typhoid fever in south-western China.* Bull World Health Organ, 2001. **79**(7): p. 625-31.
51. Thiem, V.D., et al., *The Vi Conjugate Typhoid Vaccine Is Safe, Elicits Protective Levels of IgG Anti-Vi, and Is Compatible with Routine Infant Vaccines.* Clinical and Vaccine Immunology, 2011. **18**(5): p. 730-735.
52. Desin, T.S., W. Koster, and A.A. Potter, *Salmonella vaccines in poultry: past, present and future.* Expert Rev Vaccines, 2013. **12**(1): p. 87-96.
53. Rowe, B., L.R. Ward, and E.J. Threlfall, *Multidrug-resistant Salmonella typhi: a worldwide epidemic.* Clin Infect Dis, 1997. **24 Suppl 1**: p. S106-9.
54. Woodward, T.E., J.E. Smadel, and et al., *Preliminary report on the beneficial effect of chloromycetin in the treatment of typhoid fever.* Ann Intern Med, 1948. **29**(1): p. 131-4.
55. Anderson, E.S., *The problem and implications of chloramphenicol resistance in the typhoid bacillus.* J Hyg (Lond), 1975. **74**(2): p. 289-99.
56. Olarte, J. and E. Galindo, *Salmonella typhi resistant to chloramphenicol, ampicillin, and other antimicrobial agents: strains isolated during an extensive typhoid fever epidemic in Mexico.* Antimicrob Agents Chemother, 1973. **4**(6): p. 597-601.
57. Paniker, C.K. and K.N. Vimala, *Transferable chloramphenicol resistance in Salmonella typhi.* Nature, 1972. **239**(5367): p. 109-10.
58. Mandal, B.K., *Modern treatment of typhoid fever.* J Infect, 1991. **22**(1): p. 1-4.
59. Albert, M.J., et al., *Multiresistant Salmonella typhi in Bangladesh.* J Antimicrob Chemother, 1991. **27**(4): p. 554-5.
60. Coovadia, Y.M., et al., *An outbreak of multiresistant Salmonella typhi in South Africa.* Q J Med, 1992. **82**(298): p. 91-100.
61. Mandal, B.K., *Treatment of multiresistant typhoid fever.* Lancet, 1990. **336**(8727): p. 1383.
62. Rowe, B., L.R. Ward, and E.J. Threlfall, *Ciprofloxacin and typhoid fever.* Lancet, 1992. **339**(8795): p. 740.
63. Tatavarthy, A., V.A. Luna, and P.T. Amuso, *How multidrug resistance in typhoid fever affects treatment options.* Antimicrobial Therapeutics Reviews: Infectious Diseases of Current and Emerging Concern, 2014. **1323**: p. 76-90.
64. Threlfall, E.J., et al., *Widespread occurrence of multiple drug-resistant Salmonella typhi in India.* Eur J Clin Microbiol Infect Dis, 1992. **11**(11): p. 990-3.
65. Eykyn, S.J. and H. Williams, *Treatment of multiresistant Salmonella typhi with oral ciprofloxacin.* Lancet, 1987. **2**(8572): p. 1407-8.
66. Rowe, B., L.R. Ward, and E.J. Threlfall, *Ciprofloxacin-resistant Salmonella typhi in the UK.* Lancet, 1995. **346**(8985): p. 1302.

67. Umasankar, S., R.A. Wall, and J. Berger, *A case of ciprofloxacin-resistant typhoid fever.* Commun Dis Rep CDR Rev, 1992. **2**(12): p. R139-40.
68. Al-Sanouri, T.M., et al., *Emergence of plasmid-mediated multidrug resistance in epidemic and non-epidemic strains of Salmonella enterica serotype Typhi from Jordan.* J Infect Dev Ctries, 2008. **2**(4): p. 295-301.
69. Medalla, F., et al., *Ciprofloxacin-resistant Salmonella enterica Serotype Typhi, United States, 1999-2008.* Emerg Infect Dis, 2011. **17**(6): p. 1095-8.
70. Ruiz, J., *Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection.* J Antimicrob Chemother, 2003. **51**(5): p. 1109-17.
71. Afzal, A., et al., *Current status of fluoroquinolone and cephalosporin resistance in Salmonella enterica serovar Typhi isolates from Faisalabad, Pakistan.* Pakistan Journal of Medical Sciences, 2012. **28**(4): p. 602-607.
72. Murase, T., et al., *Fecal excretion of Salmonella enterica serovar typhimurium following a food-borne outbreak.* J Clin Microbiol, 2000. **38**(9): p. 3495-7.
73. Rasko, D.A. and V. Sperandio, *Anti-virulence strategies to combat bacteria-mediated disease.* Nat Rev Drug Discov, 2010. **9**(2): p. 117-28.
74. Hung, D.T. and E.J. Rubin, *Chemical biology and bacteria: not simply a matter of life or death.* Current Opinion in Chemical Biology, 2006. **10**(4): p. 321-326.
75. Kauppi, A.M., et al., *Targeting bacterial virulence: inhibitors of type III secretion in Yersinia.* Chem Biol, 2003. **10**(3): p. 241-9.
76. Nordfelth, R., et al., *Small-molecule inhibitors specifically targeting type III secretion.* Infect Immun, 2005. **73**(5): p. 3104-14.
77. Muschiol, S., et al., *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, 2006. **103**(39): p. 14566-71.
78. Veenendaal, A.K., C. Sundin, and A.J. Blocker, *Small-molecule type III secretion system inhibitors block assembly of the Shigella type III secreton.* J Bacteriol, 2009. **191**(2): p. 563-70.
79. Hudson, D.L., et al., *Inhibition of type III secretion in Salmonella enterica serovar Typhimurium by small-molecule inhibitors.* Antimicrob Agents Chemother, 2007. **51**(7): p. 2631-5.
80. Layton, A.N., et al., *Salicylidene acylhydrazide-mediated inhibition of type III secretion system-1 in Salmonella enterica serovar Typhimurium is associated with iron restriction and can be reversed by free iron.* Fems Microbiology Letters, 2010. **302**(2): p. 114-122.
81. Negrea, A., et al., *Salicylidene acylhydrazides that affect type III protein secretion in Salmonella enterica serovar Typhimurium.* Antimicrobial Agents and Chemotherapy, 2007. **51**(8): p. 2867-2876.
82. Felise, H.B., et al., *An inhibitor of gram-negative bacterial virulence protein secretion.* Cell Host Microbe, 2008. **4**(4): p. 325-36.
83. Puiac, S., et al., *Omeprazole antagonizes virulence and inflammation in Salmonella enterica-infected RAW264.7 cells.* Antimicrob Agents Chemother, 2009. **53**(6): p. 2402-9.
84. Hung, D.T., et al., *Small-molecule inhibitor of Vibrio cholerae virulence and intestinal colonization.* Science, 2005. **310**(5748): p. 670-4.
85. Santos, R.L., et al., *Animal models of Salmonella infections: enteritis versus typhoid fever.* Microbes Infect, 2001. **3**(14-15): p. 1335-44.
86. Ulett, G.C., N. Ketheesan, and R.G. Hirst, *Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent Burkholderia pseudomallei.* Infect Immun, 2000. **68**(4): p. 2034-42.

87. Govoni, G., et al., *Genomic structure, promoter sequence, and induction of expression of the mouse Nramp1 gene in macrophages.* Genomics, 1995. **27**(1): p. 9-19.
88. Mazzolla, R., et al., *Differential microbial clearance and immunoresponse of Balb/c (Nramp1 susceptible) and DBA2 (Nramp1 resistant) mice intracerebrally infected with Mycobacterium bovis BCG (BCG).* FEMS Immunol Med Microbiol, 2002. **32**(2): p. 149-58.
89. Negrea, A., et al., *Thioredoxin 1 participates in the activity of the Salmonella enterica serovar Typhimurium pathogenicity island 2 type III secretion system.* J Bacteriol, 2009. **191**(22): p. 6918-27.
90. Hensel, M., et al., *Simultaneous identification of bacterial virulence genes by negative selection.* Science, 1995. **269**(5222): p. 400-3.
91. Miller, S.I., J.J. Mekalanos, and W.S. Pulkkinen, *Salmonella vaccines with mutations in the phoP virulence regulon.* Res Microbiol, 1990. **141**(7-8): p. 817-21.
92. Hurley, B.P. and B.A. McCormick, *Translating tissue culture results into animal models: the case of Salmonella typhimurium.* Trends in Microbiology, 2003. **11**(12): p. 562-569.
93. Hautefort, I., et al., *During infection of epithelial cells Salmonella enterica serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems.* Cell Microbiol, 2008. **10**(4): p. 958-84.
94. Eriksson, S., et al., *Salmonella typhimurium mutants that downregulate phagocyte nitric oxide production.* Cellular Microbiology, 2000. **2**(3): p. 239-250.
95. Bjur, E., et al., *Thioredoxin 1 promotes intracellular replication and virulence of Salmonella enterica serovar Typhimurium.* Infect Immun, 2006. **74**(9): p. 5140-51.
96. McClelland, M., et al., *Complete genome sequence of Salmonella enterica serovar typhimurium LT2.* Nature, 2001. **413**(6858): p. 852-856.
97. Parkhill, J., et al., *Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18.* Nature, 2001. **413**(6858): p. 848-52.
98. Sabbagh, S.C., et al., *So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi.* Fems Microbiology Letters, 2010. **305**(1): p. 1-13.
99. Hueck, C.J., *Type III protein secretion systems in bacterial pathogens of animals and plants.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 379-433.
100. Burkinshaw, B.J. and N.C. Strynadka, *Assembly and structure of the T3SS.* Biochim Biophys Acta, 2014. **1843**(8): p. 1649-63.
101. Kubori, T., et al., *Supramolecular structure of the Salmonella typhimurium type III protein secretion system.* Science, 1998. **280**(5363): p. 602-5.
102. Galan, J.E., *Salmonella interactions with host cells: Type III secretion at work.* Annual Review of Cell and Developmental Biology, 2001. **17**: p. 53-86.
103. Galan, J.E. and R. Curtiss, 3rd, *Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells.* Proc Natl Acad Sci U S A, 1989. **86**(16): p. 6383-7.
104. Akeda, Y. and J.E. Galan, *Chaperone release and unfolding of substrates in type III secretion.* Nature, 2005. **437**(7060): p. 911-915.
105. Ellermeier, J.R. and J.M. Slauch, *Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium.* Current Opinion in Microbiology, 2007. **10**(1): p. 24-29.
106. Lostroh, C.P. and C.A. Lee, *The Salmonella pathogenicity island-1 type III secretion system.* Microbes Infect, 2001. **3**(14-15): p. 1281-91.
107. Zhou, D., M.S. Mooseker, and J.E. Galan, *Role of the S. typhimurium actin-binding protein SipA in bacterial internalization.* Science, 1999. **283**(5410): p. 2092-5.

108. Hayward, R.D. and V. Koronakis, *Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella.* EMBO J, 1999. **18**(18): p. 4926-34.
109. Hardt, W.D., et al., *S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells.* Cell, 1998. **93**(5): p. 815-26.
110. Stender, S., et al., *Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell.* Mol Microbiol, 2000. **36**(6): p. 1206-21.
111. Fu, Y. and J.E. Galan, *A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion.* Nature, 1999. **401**(6750): p. 293-7.
112. Hobbie, S., et al., *Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by Salmonella typhimurium in cultured intestinal epithelial cells.* J Immunol, 1997. **159**(11): p. 5550-9.
113. Bajaj, V., C. Hwang, and C.A. Lee, *hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes.* Molecular Microbiology, 1995. **18**(4): p. 715-727.
114. Ellermeier, C.D., J.R. Ellermeier, and J.M. Slauch, *HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium.* Molecular Microbiology, 2005. **57**(3): p. 691-705.
115. Baxter, M.A., et al., *HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype.* Infect Immun, 2003. **71**(3): p. 1295-305.
116. Hansen-Wester, I. and M. Hensel, *Salmonella pathogenicity islands encoding type III secretion systems.* Microbes Infect, 2001. **3**(7): p. 549-59.
117. Beuzon, C.R., et al., *pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella typhimurium.* Mol Microbiol, 1999. **33**(4): p. 806-16.
118. Lober, S., et al., *Regulation of Salmonella pathogenicity island 2 genes by independent environmental signals.* Int J Med Microbiol, 2006. **296**(7): p. 435-47.
119. Kuhle, V., D. Jackel, and M. Hensel, *Effector proteins encoded by Salmonella pathogenicity island 2 interfere with the microtubule cytoskeleton after translocation into host cells.* Traffic, 2004. **5**(5): p. 356-370.
120. Hansen-Wester, I., B. Stecher, and M. Hensel, *Analyses of the evolutionary distribution of Salmonella translocated effectors.* Infect Immun, 2002. **70**(3): p. 1619-22.
121. Haraga, A., M.B. Ohlson, and S.I. Miller, *Salmonellae interplay with host cells.* Nat Rev Microbiol, 2008. **6**(1): p. 53-66.
122. Figueira, R. and D.W. Holden, *Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors.* Microbiology, 2012. **158**(Pt 5): p. 1147-61.
123. Garcia-del Portillo, F. and B.B. Finlay, *Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors.* J Cell Biol, 1995. **129**(1): p. 81-97.
124. Garvis, S.G., C.R. Beuzon, and D.W. Holden, *A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and Salmonella-containing vacuoles in macrophages.* Cellular Microbiology, 2001. **3**(11): p. 731-744.
125. Meresse, S., et al., *The rab7 GTPase controls the maturation of Salmonella typhimurium-containing vacuoles in HeLa cells.* EMBO J, 1999. **18**(16): p. 4394-403.
126. Rathman, M., L.P. Barker, and S. Falkow, *The unique trafficking pattern of Salmonella typhimurium-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry.* Infection and Immunity, 1997. **65**(4): p. 1475-1485.

127. Steele-Mortimer, O., et al., *Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway.* Cell Microbiol, 1999. **1**(1): p. 33-49.
128. Bakowski, M.A., V. Braun, and J.H. Brumell, *Salmonella-containing vacuoles: directing traffic and nesting to grow.* Traffic, 2008. **9**(12): p. 2022-31.
129. Buchmeier, N.A. and F. Heffron, *Inhibition of Macrophage Phagosome-Lysosome Fusion by Salmonella-Typhimurium.* Infection and Immunity, 1991. **59**(7): p. 2232-2238.
130. Hashim, S., et al., *Live Salmonella modulate expression of Rab proteins to persist in a specialized compartment and escape transport to lysosomes.* Journal of Biological Chemistry, 2000. **275**(21): p. 16281-16288.
131. Oh, Y.K., et al., *Rapid and complete fusion of macrophage lysosomes with phagosomes containing Salmonella typhimurium.* Infection and Immunity, 1996. **64**(9): p. 3877-3883.
132. Drecktrah, D., et al., *Salmonella trafficking is defined by continuous dynamic interactions with the endolysosomal system.* Traffic, 2007. **8**(3): p. 212-225.
133. McGourty, K., et al., *Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate Receptors and Lysosome Function.* Science, 2012. **338**(6109): p. 963-967.
134. Chakravortty, D., I. Hansen-Wester, and M. Hensel, *Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates.* J Exp Med, 2002. **195**(9): p. 1155-66.
135. Vazquez-Torres, A., et al., *Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase.* Science, 2000. **287**(5458): p. 1655-8.
136. Brumell, J.H., D.L. Goosney, and B.B. Finlay, *SifA, a type III secreted effector of Salmonella typhimurium, directs Salmonella-induced filament (Sif) formation along microtubules.* Traffic, 2002. **3**(6): p. 407-415.
137. Deiwick, J., et al., *The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche.* Infection and Immunity, 2006. **74**(12): p. 6965-6972.
138. Garmendia, J., et al., *The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the Salmonella typhimurium SPI-2 type III secretion system.* Microbiology, 2003. **149**(Pt 9): p. 2385-96.
139. Steele-Mortimer, O., et al., *The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells.* Cellular microbiology, 2002. **4**(1): p. 43-54.
140. Giacomodonato, M.N., et al., *SipA, SopA, SopB, SopD and SopE2 effector proteins of Salmonella enterica serovar Typhimurium are synthesized at late stages of infection in mice.* Microbiology (Reading, England), 2007. **153**(Pt 4): p. 1221-8.
141. Brawn, L.C., R.D. Hayward, and V. Koronakis, *Salmonella SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication.* Cell Host Microbe, 2007. **1**(1): p. 63-75.
142. Mallo, G.V., et al., *SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34.* J Cell Biol, 2008. **182**(4): p. 741-52.
143. Drecktrah, D., et al., *The Salmonella SPI1 effector SopB stimulates nitric oxide production long after invasion.* Cellular Microbiology, 2005. **7**(1): p. 105-113.
144. Brown, N.F., et al., *Salmonella pathogenicity island 2 is expressed prior to penetrating the intestine.* PLoS pathogens, 2005. **1**(3): p. e32.
145. Deiwick, J., et al., *Mutations in Salmonella pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents.* J Bacteriol, 1998. **180**(18): p. 4775-80.

146. Hensel, M., et al., *Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2.* Molecular Microbiology, 1997. **24**(1): p. 155-167.
147. Rhen, M. and C.J. Dorman, *Hierarchical gene regulators adapt Salmonella enterica to its host milieus.* Int J Med Microbiol, 2005. **294**(8): p. 487-502.
148. Prost, L.R. and S.I. Miller, *The Salmonellae PhoQ sensor: mechanisms of detection of phagosome signals.* Cellular Microbiology, 2008. **10**(3): p. 576-582.
149. Aguirre, A., et al., *PhoP-induced genes within Salmonella pathogenicity island 1.* Journal of Bacteriology, 2006. **188**(19): p. 6889-6898.
150. Bijlsma, J.J. and E.A. Groisman, *The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica.* Mol Microbiol, 2005. **57**(1): p. 85-96.
151. Lee, A.K., C.S. Detweiler, and S. Falkow, *OmpR regulates the two-component system SsrA-ssrB in Salmonella pathogenicity island 2.* J Bacteriol, 2000. **182**(3): p. 771-81.
152. Fass, E. and E.A. Groisman, *Control of Salmonella pathogenicity island-2 gene expression.* Curr Opin Microbiol, 2009. **12**(2): p. 199-204.
153. Teplitski, M., R.I. Goodier, and B.M. Ahmer, *Pathways leading from BarA/SirA to motility and virulence gene expression in Salmonella.* J Bacteriol, 2003. **185**(24): p. 7257-65.
154. Fortune, D.R., M. Suyemoto, and C. Altier, *Identification of CsrC and characterization of its role in epithelial cell invasion in Salmonella enterica serovar Typhimurium.* Infect Immun, 2006. **74**(1): p. 331-9.
155. Gauthier, A., et al., *Transcriptional inhibitor of virulence factors in enteropathogenic Escherichia coli.* Antimicrobial Agents and Chemotherapy, 2005. **49**(10): p. 4101-4109.
156. Roudier, C., J. Fierer, and D.G. Guiney, *Characterization of translation termination mutations in the spv operon of the Salmonella virulence plasmid pSDL2.* J Bacteriol, 1992. **174**(20): p. 6418-23.
157. Tezcan-Merdol, D., et al., *Actin is ADP-ribosylated by the Salmonella enterica virulence-associated protein SpvB.* Molecular Microbiology, 2001. **39**(3): p. 606-619.
158. Mazurkiewicz, P., et al., *SpvC is a Salmonella effector with phosphothreonine lyase activity on host mitogen-activated protein kinases.* Mol Microbiol, 2008. **67**(6): p. 1371-83.
159. Browne, S.H., et al., *Identification of Salmonella SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice.* FEMS Immunol Med Microbiol, 2008. **52**(2): p. 194-201.
160. Alexander, C. and E.T. Rietschel, *Bacterial lipopolysaccharides and innate immunity.* J Endotoxin Res, 2001. **7**(3): p. 167-202.
161. Khan, S.A., et al., *A lethal role for lipid A in Salmonella infections.* Mol Microbiol, 1998. **29**(2): p. 571-9.
162. Yethon, J.A., et al., *Salmonella enterica serovar typhimurium waaP mutants show increased susceptibility to polymyxin and loss of virulence In vivo.* Infect Immun, 2000. **68**(8): p. 4485-91.
163. Bjur, E., S. Eriksson-Ygberg, and M. 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**(7): p. 1826-1838.
164. Thomsen, L.E., et al., *Reduced amounts of LPS affect both stress tolerance and virulence of Salmonella enterica serovar Dublin.* Fems Microbiology Letters, 2003. **228**(2): p. 225-231.

165. Chen, H.D. and E.A. Groisman, *The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications.* Annu Rev Microbiol, 2013. **67**: p. 83-112.
166. Guo, L., et al., *Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ.* Science, 1997. **276**(5310): p. 250-3.
167. Wosten, M.M. and E.A. Groisman, *Molecular characterization of the PmrA regulon.* J Biol Chem, 1999. **274**(38): p. 27185-90.
168. Bearson, B.L., L. Wilson, and J.W. Foster, *A low PH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress.* Journal of Bacteriology, 1998. **180**(9): p. 2409-2417.
169. Guo, L., et al., *Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides.* Cell, 1998. **95**(2): p. 189-198.
170. Salzman, N.H., et al., *Enteric salmonella infection inhibits Paneth cell antimicrobial peptide expression.* Infect Immun, 2003. **71**(3): p. 1109-15.
171. Eckmann, L. and M.F. Kagnoff, *Cytokines in host defense against Salmonella.* Microbes Infect, 2001. **3**(14-15): p. 1191-200.
172. Winter, S.E., et al., *Gut inflammation provides a respiratory electron acceptor for Salmonella.* Nature, 2010. **467**(7314): p. 426-9.
173. Hersh, D., et al., *The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2396-401.
174. Collier-Hyams, L.S., et al., *Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway.* J Immunol, 2002. **169**(6): p. 2846-50.
175. Haraga, A. and S.I. Miller, *A Salmonella enterica serovar typhimurium translocated leucine-rich repeat effector protein inhibits NF-kappa B-dependent gene expression.* Infect Immun, 2003. **71**(7): p. 4052-8.
176. Murli, S., R.O. Watson, and J.E. Galan, *Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of Salmonella with host cells.* Cell Microbiol, 2001. **3**(12): p. 795-810.
177. Cheminay, C., D. Chakravortty, and M. Hensel, *Role of neutrophils in murine salmonellosis.* Infect Immun, 2004. **72**(1): p. 468-77.
178. Lapaque, N., et al., *Salmonella regulates polyubiquitination and surface expression of MHC class II antigens.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 14052-7.
179. Yrlid, U. and M.J. Wick, *Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells.* J Exp Med, 2000. **191**(4): p. 613-24.
180. Tobar, J.A., P.A. Gonzalez, and A.M. Kalergis, *Salmonella escape from antigen presentation can be overcome by targeting bacteria to Fc gamma receptors on dendritic cells.* Journal of Immunology, 2004. **173**(6): p. 4058-4065.
181. Roy, D., et al., *A process for controlling intracellular bacterial infections induced by membrane injury.* Science, 2004. **304**(5676): p. 1515-8.
182. Birmingham, C.L., et al., *Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole.* J Biol Chem, 2006. **281**(16): p. 11374-83.
183. Tobar, J.A., et al., *Virulent Salmonella enterica serovar typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells.* Infect Immun, 2006. **74**(11): p. 6438-48.
184. McSorley, S.J., B.T. Cookson, and M.K. Jenkins, *Characterization of CD4(+) T cell responses during natural infection with Salmonella typhimurium.* Journal of Immunology, 2000. **164**(2): p. 986-993.

185. Mittrucker, H.W., A. Kohler, and S.H.E. Kaufmann, *Characterization of the murine T-lymphocyte response to Salmonella enterica serovar Typhimurium infection.* Infection and Immunity, 2002. **70**(1): p. 199-203.
186. Yrlid, U. and M.J. Wick, *Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon Salmonella encounter.* Journal of Immunology, 2002. **169**(1): p. 108-116.
187. Voedisch, S., et al., *Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar Typhimurium and limit systemic disease in mice.* Infect Immun, 2009. **77**(8): p. 3170-80.
188. Fields, P.I., et al., *Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent.* Proc Natl Acad Sci U S A, 1986. **83**(14): p. 5189-93.
189. Nathan, C. and M.U. Shiloh, *Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(16): p. 8841-8848.
190. Mouy, R., et al., *Incidence, severity, and prevention of infections in chronic granulomatous disease.* J Pediatr, 1989. **114**(4 Pt 1): p. 555-60.
191. Fang, F.C., *Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity.* J Clin Invest, 1997. **99**(12): p. 2818-25.
192. Chan, E.D. and D.W. Riches, *IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line.* Am J Physiol Cell Physiol, 2001. **280**(3): p. C441-50.
193. Lowenstein, C.J. and E. Padalko, *Inos (Nos2) at a Glance.* Journal of Cell Science, 2004. **117**(14): p. 2865-2867.
194. Saura, M., et al., *Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription.* J Mol Biol, 1999. **289**(3): p. 459-71.
195. Kamijo, R., et al., *Requirement for Transcription Factor Irf-1 in No Synthase Induction in Macrophages.* Science, 1994. **263**(5153): p. 1612-1615.
196. Mastroeni, P., et al., *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, 2000. **192**(2): p. 237-48.
197. Vazquez-Torres, A., et al., *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, 2000. **192**(2): p. 227-36.
198. Fang, F.C., *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.* Nat Rev Microbiol, 2004. **2**(10): p. 820-32.
199. Fang, F.C., *Antimicrobial actions of reactive oxygen species.* MBio, 2011. **2**(5).
200. Janssen, R., et al., *Responses to reactive oxygen intermediates and virulence of Salmonella typhimurium.* Microbes Infect, 2003. **5**(6): p. 527-34.
201. Imlay, J.A. and S. Linn, *DNA damage and oxygen radical toxicity.* Science, 1988. **240**(4857): p. 1302-9.
202. Tamarit, J., E. Cabiscol, and J. Ros, *Identification of the major oxidatively damaged proteins in Escherichia coli cells exposed to oxidative stress.* J Biol Chem, 1998. **273**(5): p. 3027-32.
203. Chakravortty, D. and M. Hensel, *Inducible nitric oxide synthase and control of intracellular bacterial pathogens.* Microbes and Infection, 2003. **5**(7): p. 621-627.
204. Pacelli, R., et al., *Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli.* J Exp Med, 1995. **182**(5): p. 1469-79.
205. Wink, D.A., et al., *DNA deaminating ability and genotoxicity of nitric oxide and its progenitors.* Science, 1991. **254**(5034): p. 1001-3.

206. Gallois, A., et al., *Salmonella pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane.* J Immunol, 2001. **166**(9): p. 5741-8.
207. Aussel, L., et al., *Salmonella detoxifying enzymes are sufficient to cope with the host oxidative burst.* Mol Microbiol, 2011. **80**(3): p. 628-40.
208. Fang, F.C., et al., *Virulent Salmonella typhimurium has two periplasmic Cu, Zn-superoxide dismutases.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(13): p. 7502-7507.
209. Farr, S.B. and T. Kogoma, *Oxidative stress responses in Escherichia coli and Salmonella typhimurium.* Microbiol Rev, 1991. **55**(4): p. 561-85.
210. Tsolis, R.M., A.J. Baumler, and F. Heffron, *Role of Salmonella typhimurium Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages.* Infect Immun, 1995. **63**(5): p. 1739-44.
211. Hebrard, M., et al., *Redundant hydrogen peroxide scavengers contribute to Salmonella virulence and oxidative stress resistance.* J Bacteriol, 2009. **191**(14): p. 4605-14.
212. Horst, S.A., et al., *Thiol peroxidase protects Salmonella enterica from hydrogen peroxide stress in vitro and facilitates intracellular growth.* J Bacteriol, 2010. **192**(11): p. 2929-32.
213. Eriksson, S., et al., *Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica.* Mol Microbiol, 2003. **47**(1): p. 103-18.
214. Bang, I.S., et al., *Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp.* J Biol Chem, 2006. **281**(38): p. 28039-47.
215. Gilberthorpe, N.J., et al., *NsrR: a key regulator circumventing Salmonella enterica serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages.* Microbiology, 2007. **153**(Pt 6): p. 1756-71.
216. Mills, P.C., et al., *A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects Salmonella enterica serovar Typhimurium against killing by NO in anoxic environments.* Microbiology-Sgm, 2008. **154**: p. 1218-1228.
217. Liu, L., et al., *A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans.* Nature, 2001. **410**(6827): p. 490-4.
218. De Groote, M.A., et al., *Homocysteine antagonism of nitric oxide-related cytostasis in Salmonella typhimurium.* Science, 1996. **272**(5260): p. 414-7.
219. Buchmeier, N.A., et al., *Recombination-deficient mutants of Salmonella typhimurium are avirulent and sensitive to the oxidative burst of macrophages.* Mol Microbiol, 1993. **7**(6): p. 933-6.
220. Richardson, A.R., et al., *The Base Excision Repair system of Salmonella enterica serovar typhimurium counteracts DNA damage by host nitric oxide.* PLoS Pathog, 2009. **5**(5): p. e1000451.
221. Cano, D.A., et al., *Role of the RecBCD recombination pathway in Salmonella virulence.* J Bacteriol, 2002. **184**(2): p. 592-5.
222. Denkel, L.A., et al., *Methionine sulfoxide reductases are essential for virulence of Salmonella typhimurium.* PLoS One, 2011. **6**(11): p. e26974.
223. Holmgren, A., et al., *Thiol redox control via thioredoxin and glutaredoxin systems.* Biochem Soc Trans, 2005. **33**(Pt 6): p. 1375-7.
224. Lundberg, B.E., et al., *Glucose 6-phosphate dehydrogenase is required for Salmonella typhimurium virulence and resistance to reactive oxygen and nitrogen intermediates.* Infection and Immunity, 1999. **67**(1): p. 436-438.
225. Hausladen, A., et al., *Nitrosative stress: activation of the transcription factor OxyR.* Cell, 1996. **86**(5): p. 719-29.

226. Vazquez-Torres, A., *Redox active thiol sensors of oxidative and nitrosative stress.* Antioxid Redox Signal, 2012. **17**(9): p. 1201-14.
227. Nunoshiba, T., et al., *Activation by Nitric-Oxide of an Oxidative-Stress Response That Defends Escherichia-Coli against Activated Macrophages.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(21): p. 9993-9997.
228. Pomposiello, P.J. and B. Demple, *Redox-operated genetic switches: the SoxR and OxyR transcription factors.* Trends Biotechnol, 2001. **19**(3): p. 109-14.
229. Husain, M., et al., *Redox sensor SsrB Cys203 enhances Salmonella fitness against nitric oxide generated in the host immune response to oral infection.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 14396-401.
230. Keyser, P., et al., *Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria.* Journal of Internal Medicine, 2008. **264**(1): p. 17-29.
231. Rathman, M., M.D. Sjaastad, and S. Falkow, *Acidification of phagosomes containing Salmonella typhimurium in murine macrophages.* Infection and Immunity, 1996. **64**(7): p. 2765-2773.
232. Steele-Mortimer, O., et al., *Vacuole acidification is not required for survival of Salmonella enterica serovar typhimurium within cultured macrophages and epithelial cells.* Infect Immun, 2000. **68**(9): p. 5401-4.
233. Kuhle, V. and M. Hensel, *Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2.* Cell Mol Life Sci, 2004. **61**(22): p. 2812-26.
234. Hong, J., et al., *Nitric oxide production by the vacuolar-type (H+)-ATPase inhibitors bafilomycin A1 and concanamycin A and its possible role in apoptosis in RAW 264.7 cells.* J Pharmacol Exp Ther, 2006. **319**(2): p. 672-81.
235. Hinoki, A., et al., *Suppression of proinflammatory cytokine production in macrophages by lansoprazole.* Pediatr Surg Int, 2006. **22**(11): p. 915-23.
236. Sjostrom, J.E., et al., *In vitro antibacterial activity of omeprazole and its selectivity for Helicobacter spp. are dependent on incubation conditions.* Antimicrob Agents Chemother, 1996. **40**(3): p. 621-6.
237. Nguyen, P.T., et al., *Antimicrobial actions of benzimidazoles against oral streptococci.* Oral Microbiol Immunol, 2005. **20**(2): p. 93-100.
238. Jiang, S., et al., *Antileishmanial activity of the antiulcer agent omeprazole.* Antimicrob Agents Chemother, 2002. **46**(8): p. 2569-74.
239. Rosenberger, C.M. and B.B. Finlay, *Macrophages inhibit Salmonella typhimurium replication through MEK/ERK kinase and phagocyte NADPH oxidase activities.* Journal of Biological Chemistry, 2002. **277**(21): p. 18753-18762.
240. Holmgren, A., *Reduction of disulfides by thioredoxin. Exceptional reactivity of insulin and suggested functions of thioredoxin in mechanism of hormone action.* J Biol Chem, 1979. **254**(18): p. 9113-9.
241. Kern, R., et al., *Chaperone properties of Escherichia coli thioredoxin and thioredoxin reductase.* Biochem J, 2003. **371**(Pt 3): p. 965-72.
242. McCollister, B.D., et al., *Repression of SPI2 transcription by nitric oxide-producing, IFNgamma-activated macrophages promotes maturation of Salmonella phagosomes.* J Exp Med, 2005. **202**(5): p. 625-35.