SALMONELLA-HOST INTERACTIONS: THE INTERPLAY BETWEEN SALMONELLA SPI2, AND EICOSANOIDS

by

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Abstract

_Salmonella_ are Gram-negative facultative intracellular pathogens that cross the intestinal barrier, and are taken up by phagocytes where, they can replicate and spread to systemic sites. _Salmonella_ encode two type III secretion systems, _Salmonella_ pathogenicity island 1 and 2 (SPI1 and SPI2), which mediate the translocation of bacterial effectors into the host cell. SPI1 facilitates bacterial uptake into non-phagocytic cells and is involved in forming a special replicative niche, called the _Salmonella_ containing vacuole (SCV). SPI2 is required for maintenance of the SCV, macrophage replication and systemic disease.

A comprehensive study of the contribution of individual SPI2 effectors to virulence had not been previously done, and was therefore performed. Strains deficient in specific SPI2 genes were tested for alterations in virulence in a mouse model of typhoid fever, and in epithelial and macrophage cell infections. These experiments showed that many SPI2 effectors are required for replication in macrophages, and that ΔspvB, ΔssaR, and ΔspiC strains were attenuated in mice.

_Salmonella_ infection causes many perturbations to the host, including changes in metabolites, specifically arachidonic acid metabolism, which leads to the production of eicosanoids. The effects of _Salmonella_ infection of macrophages on eicosanoids were examined. _Salmonella_ infection increased the expression of prostaglandin synthases, but decreased thromboxane and leukotriene synthases. The SPI2 deletion strains were tested to determine involvement of SPI2 in arachidonic acid metabolism. The SPI2 effectors SseF and SseG, which are largely uncharacterized in macrophage infections, were mainly responsible for the induction of prostaglandins.

The effects of prostaglandins on _Salmonella_ infection were studied. It was found that 15-deoxy-Δ^{12,14}-prostaglandin-J_2_ (15d-PGJ_2_) significantly reduced _Salmonella_ colonization of
macrophages, but not epithelial cells. Furthermore, this occurs independently of SPI1, SPI2, and PPAR-γ. 15d-PGJ2 reduces cytokines and reactive nitrogen species produced by infected macrophages. A role for 15d-PGJ2 in Salmonella infection has not been previously demonstrated. This thesis examines the role of SPI2 in Salmonella virulence and arachidonic acid metabolism.
Preface

A version of Chapter 2 has been published. **Buckner MM**, Croxen MA, Arena ET, Finlay BB. A comprehensive study of the contribution of *Salmonella enterica* serovar Typhimurium SPI2 effectors to bacterial colonization, survival, and replication in typhoid fever, macrophage, and epithelial cell infection models. **Virulence**. 2011 May-Jun;2(3):208-16. Prof. Finlay provided insight and guidance for this project. With the supervision and assistance of Dr. Croxen I constructed the SPI2 deletion strains used in this chapter, and later in chapter 3. Dr. Arena assisted me with the mouse infections completed for this project. I completed all the cell culture infections. I wrote most of the manuscript with input from Prof. Finlay.

A version of chapter 4 is currently under review. **Buckner MM**, Antunes LC, Gill N, Russell SL, Shames SR, Finlay BB. 15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) mediates macrophage interactions with *Salmonella enterica* serovar Typhimurium. Submitted. For this manuscript I completed all the experiments. Prof. Finlay assisted with the conceptual aspects of the study. Dr. Antunes assisted with a variety of experiments and provided project guidance. Dr. Gill assisted with the ELISAs, Ms. Russell assisted with the CBA and FACS analysis. Dr. Shames performed the LDH release assay. I wrote most of the manuscript with the input of Prof. Finlay.

Versions of the figures in Appendix A were published. Antunes LC, Arena ET, Menendez A, Han J, Ferreira RB, **Buckner MM**, Lolic P, Madilao LL, Bohlmann J, Borchers CH, Finlay BB. Impact of *Salmonella* infection on host hormone metabolism revealed by metabolomics. **Infect Immun**. 2011 Apr;79(4):1759-69. Under the supervision of Dr. Antunes I
performed the qRT-PCR on liver samples to look at the expression of COX2, TBXAS1, and PTGES.

Publications arising from my PhD work:


The mouse work presented in this thesis was approved by the UBC Animal Care Committee, certificate number: A09-0168
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<th>Meaning</th>
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<tbody>
<tr>
<td>15d-PGJ₂</td>
<td>15-deoxy-Δ^{12,14}-prostaglandin-J₂</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ALOX5</td>
<td>Arachidonate 5-lipoxygenase</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>Arachidonate 5-lipoxygenase activating protein</td>
</tr>
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<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
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<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
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<td>BMMO</td>
<td>Bone marrow macrophage</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
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<td>CD-18</td>
<td>Integrin β2</td>
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<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disorder</td>
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<td>COX1</td>
<td>Cyclooxygenase 1 (also called PTGS1)</td>
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<td>COX2</td>
<td>Cyclooxygenase 2 (also called PTGS2)</td>
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<td>CRS peptide</td>
<td>Cryptdin-related sequence peptide</td>
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<td>CYP4F</td>
<td>Cytochrome P450, family 4, subfamily F (leukotriene-B4 20-monooxygenase)</td>
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<td>Full Form</td>
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<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<td>EP2</td>
<td>Prostaglandin E2, receptor subtype 2</td>
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<tr>
<td>EP4</td>
<td>Prostaglandin E2, receptor subtype 4</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HIV/AIDS</td>
<td>Human immunodeficiency virus/acquired immunodeficiency syndrome</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory transcription factor 3</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>LAMP (1,2,3)</td>
<td>Lysosomal-associated membrane protein (1, 2, 3)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA4H</td>
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</tr>
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<td>LTC4S</td>
<td>Leukotriene C4 synthase</td>
</tr>
<tr>
<td>M-cells</td>
<td>Microfold cells</td>
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<td>MCP1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph nodes</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
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<td>NEAA</td>
<td>Non-essential amino acids</td>
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<td>NF-κB</td>
<td>nuclear factor kappa beta</td>
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<td>NK cells</td>
<td>Natural killer cells</td>
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<td>NLR</td>
<td>Nod-like receptor</td>
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<td>NOD-like receptor family, CARD-containing 4</td>
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<td>Pathogen associated molecular pattern</td>
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<td>PCR</td>
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<td>PG</td>
<td>Prostaglandin</td>
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<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>Prostaglandin D synthase</td>
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<td>PTGES</td>
<td>Prostaglandin E synthase</td>
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<td>Cyclooxygenase 2 (also called COX2)</td>
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<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<tr>
<td>ROI</td>
<td>Reactive oxygen intermediates</td>
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<td>SCV</td>
<td><em>Salmonella</em> containing vacuole</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td><em>Salmonella</em> genomic island</td>
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<td><em>Salmonella</em> induced filaments</td>
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<td><em>Salmonella</em> pathogenicity island 2</td>
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<td>Signal transducer and activator of transcription 1</td>
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<td>T3SS</td>
<td>Type III secretion system</td>
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<td>TBXAS1</td>
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<td>TBXB2</td>
<td>Thromboxane B₂</td>
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<td>TGN</td>
<td>Trans-Golgi network</td>
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<tr>
<td>T-helper cell</td>
<td>T helper cell</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>Trans-membrane</td>
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<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<td>TIR-domain-containing adapter-inducing interferon-β</td>
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<td>Vacuolar ATPase</td>
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</table>
Acknowledgements

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Chapter 1: Introduction

This Introduction section will cover general aspects of Salmonella biology, including information about Salmonella disease, prevalence, mechanisms of pathogenesis, and host response. Specific aspects of Salmonella and host biology that pertain to individual chapters will be discussed in more detail in the chapter introductions and discussions.

1.1 Disease relevance

1.1.1 Disease characteristics of Salmonellosis

Within the family of Enterobacteriaceae is the genus Salmonella, which encompasses a group of bacteria that cause severe disease worldwide. Salmonella cause two general categories of disease in humans: gastroenteritis, caused by non-typhoid Salmonella replicating in the intestine; and enteric fever, caused by the systemic spread of Salmonella. The gastroenteritis associated with Salmonella infection includes severe diarrhea lasting between 3-13 days, abdominal pain and cramps, and in some cases a short fever (Onwuezobe, Oshun et al. 2012). Symptoms can start between 6-72 hours post-infection, and a high inoculum is usually required to cause disease (Onwuezobe, Oshun et al. 2012). Enteric fever, including typhoid and paratyphoid fever is a febrile illness commonly found in developing countries with inadequate sanitation and water services (Parry, Hien et al. 2002, Effa, Lassi et al. 2011). Symptoms can include fever, headache, diarrhea, abdominal pain, nausea, and vomiting (Effa, Lassi et al. 2011).

Non-typhoidal Salmonella infection can lead to clinical complications that affect a variety of body systems including meninges, bones, joints, adrenal glands, aorta, heart, kidneys and lungs (Onwuezobe, Oshun et al. 2012). Complications associated with enteric fever include

*Salmonella* infection is very severe and life threatening in patients who are immunocompromised. Patients with HIV/AIDS in Malawi have a very high mortality rate (47%) and a very high recurrence rate (43%) from non-typhoidal *Salmonella* (Gordon, Banda et al. 2002). In addition to HIV/AIDS, immunosuppression leading to severe *Salmonellosis* can also be caused by malaria infections, severe anemia, malnutrition, malignancy, and immunosuppressive therapy (Hohmann 2001, Morpeth, Ramadhani et al. 2009).

1.1.2 *Salmonella* prevalence

In 2000, there were an estimated 21.7 million cases of typhoid fever, and 5.4 million cases of paratyphoid fever (Crump and Mintz 2010). Enteric fever is predominantly found in areas with poor sanitation. The greatest burden of disease is thought to affect children and adolescents in south central and south eastern Asia (Crump and Mintz 2010). In particular, China, India, Indonesia, Pakistan, and Vietnam have high levels of typhoid fever (Ochiai, Acosta et al. 2008). In Sub-Saharan Africa the characterization of enteric fever has been difficult, but there is evidence of increasing systemic diseases caused by non-typhoidal *Salmonella* (Mweu and English 2008). The prevalence of enteric fever in Latin America has recently shown signs of decreasing, likely due to economic transitions and improved sanitation (Crump and Mintz 2010).

While enteric fever tends to be found in developing countries, there is still a large proportion of *Salmonellosis* caused by contaminated food and water in developed nations including the United States of America and Canada. The USA Center for Disease Control (CDC), reports that from 2009-2010 there were 1,527 foodborne disease outbreaks, and *Salmonella* was the second leading cause, causing 30% of outbreaks (CDC 2013). In addition,
Salmonella outbreaks were responsible for the most hospitalizations associated with foodborne diseases (CDC 2013). In Canada, in 2005 there were 30 Salmonella outbreaks reported, resulting in 6,096 reported cases, which is likely an underestimate (Gill, Reilly et al. 2005). On a global scale, there are an estimated 93.8 million cases annually of gastroenteritis caused by Salmonella, associated with 155,000 deaths (Majowicz, Musto et al. 2010).

1.1.3 Economic burden

In addition to the serious detrimental affects on human life, there is also a significant financial burden associated with Salmonella induced disease. In the US, a retrospective study was done looking at 1993-2001, and found that the total economic burden of Salmonella associated disease was $2.8 billion US dollars per year (Bishwa, Angulo et al. 2004). In Canada, the annual cost of all food-borne illnesses, which includes Salmonella as well as other bacteria and viruses, was estimated to be around $3.7 billion Canadian dollars (Thomas 2008). For Salmonella specifically, it is estimated that the cost in Canada is around $640 million Canadian dollars per year based on underreporting, while in 2005 the cost of confirmed cases was $17 million Canadian dollars (Majowicz, McNab et al. 2006, Thomas, Majowicz et al. 2006). While there is some variation in estimated costs between sources, the fact remains that there is great economic costs associated with Salmonella induced disease.

1.1.4 Importance of studying Salmonella

Salmonella is a globally relevant pathogen, which affects both the developed and developing countries. Salmonella species cause both intestinal and extra-intestinal infections that lead to morbidity and mortality. In places with inadequate sanitation, enteric fever is widespread, affecting mostly children in Asia, Africa, and Latin America. In North America, and Europe there continue to be outbreaks of foodborne Salmonella causing gastroenteritis. Taken together
Salmonella disease cause serious burdens on the population, both in sickness, death, and financial burden. The scientific community continues to explore the mechanisms that enable these bacteria to cause disease. Studies into host-pathogen interactions, antibiotic resistance, improved sanitation, co-infections, and food handling are all key to controlling this pathogen. This thesis presents new data on Salmonellas interactions with the host and host small molecule hormones. These data contribute to our understanding of how this bacterial pathogen manipulates the host and causes disease.

1.2 Types of Salmonella

1.2.1 Salmonella classification

Salmonella are Gram negative, flagellated, facultative anaerobes that can grow either within host cells or independently of host cells. The Salmonella genus fits into the family of Enterobacteriaceae, along with commensals and other pathogens including E. coli, Shigella, and Yersinia (Sanderson 1976). The Salmonella genus is generally divided into two species: S. bongori and S. enterica and recently a third species was proposed, S. subterranean, but does not seem to be widely accepted (Shelobolina, Sullivan et al. 2004, Su and Chiu 2007, Achtman, Wain et al. 2012). A number of evolutionary subspecies have emerged among S. enterica, including subspecies I, II, IIIa, IIIb, IV, VI, and VII (Boyd, Wang et al. 1996, Popoff 1997). These subspecies are further divided into serovars (Ferreira, Buckner et al. 2012). Over 1500 serovars have been classified as S. enterica subspecies I, including serovar Typhimurium, Typhi, Paratyphi A, and Paratyphi C (Popoff, Bockemuhl et al. 2004, Ferreira, Buckner et al. 2012). Comparative genomics revealed 96 to 99.5% identity between S. enterica serovars (Edwards, Olsen et al. 2002). Although closely related, these serovars differ in host range, the degree of

1.2.2 Host range of *Salmonella*

*S. enterica* subspecies I colonizes a broad range of hosts, from reptiles to warm-blooded animals (Popoff 1992). The idea of host-adapted refers to the bacterium’s ability to cause disease, and circulate in a host population, without the need for reintroduction (Kingsley 2002). The specificity of *Salmonella enterica* serovars Typhi and Paratyphi are restricted to humans, while serovar Typhimurium has a broad host range, causing gastrointestinal disease in humans, and systemic disease in mice (Wick 2011). Because *S. Typhimurium* causes a systemic bacterial disease in mice, it has long been used as a model to study human enteric fever.

1.3 Genetic elements in *Salmonella*

1.3.1 *Salmonella* genomic islands

*Salmonella* has a variety of genetic elements including genomic and pathogenicity islands, plasmids, and phages. Genomic Islands are distinct regions within the bacterial genome, which differ in G+C content, and arise from gene transfer events (Ferreira, Buckner et al. 2012). Genomic islands contribute to bacterial fitness, while a subset of genomic islands, termed pathogenicity islands, contribute to bacterial virulence (Dobrindt, Hochhut et al. 2004, Kelly 2009).

*Salmonella* Genomic Island 1, called SGI1, is an integrative mobilizable element associated with multiple drug resistance (MDR) (Doublet, Praud et al. 2009, Kelly 2009, Yang, Zheng et al. 2009). SGI1 is a complex class 1 integron that is 43 kilobases with 44 open reading frames (ORFs), and a cluster of MDR genes (Boyd, Peters et al. 2001, Doublet, Boyd et al. 2005,
Mulvey, Boyd et al. 2006, Doublet, Praud et al. 2009, Kelly 2009). SGI1 has been found in many
S. enterica serovars including Typhimurium, Agona, Albany, Paratyphi B, Meleagridis,
Newport, Kentucky, Kingston, Virchow, Derby, Ceero, Kiambu, Emek, Dusseldorf, Haifa, and
Infantis (Boyd, Peters et al. 2001, Mulvey, Boyd et al. 2006, Doublet, Praud et al. 2009, Ferreira,
Buckner et al. 2012).

1.3.2  *Salmonella* pathogenicity islands

*Salmonella* Pathogenicity Islands (SPIs) are large regions of DNA that are associated
with virulence (Bishop, Baker et al. 2005, Ferreira, Buckner et al. 2012). SPIs often have a
mosaic-like structure, indicating that multiple insertion events lead to their formation (Bishop,
Baker et al. 2005). SPI1 through 5 are fairly well characterized in *S. Typhimurium* (Ferreira,
Buckner et al. 2012) and are briefly discussed here. At least 21 different pathogenicity islands
have so far been identified (de Jong, Parry et al. 2012).

SPI1 is around 40 kb, with 29-30 genes and a G+C content of 47%, while the *Salmonella*
core genome has a G+C content that averages around 52% (Marcus, Brumell et al. 2000, Hensel
2005, Ellermeier and Slauch 2007). SPI1 is found in many strains of *S. bongori* and *S. enterica*
(Hensel 2005). Interestingly, there have been *Salmonella enterica* strains identified that lack
SPI1, but still cause human disease (Ginocchio, Rahn et al. 1997, Hu, Coburn et al. 2008). SPI1
encodes a type III secretion system (T3SS), its associated proteins, and an iron uptake system
(Zhou, Hardt et al. 1999, Hensel 2005). The proteins associated with the SPI1-T3SS include the
T3SS apparatus, some of the translocated proteins, termed effectors, and the regulators
(Ellermeier and Slauch 2007, Kelly 2009). Some SPI1 effectors are encoded outside of SPI1,
including *sopA, sopB, sopD,* and *sopE2,* interestingly, *sopE* is encoded by a phage found in some
*Salmonella* strains (Hensel 2005). The SPI1 T3SS plays an important role during infection, as it

*Salmonella* have another T3SS, encoded by SPI2, which also encodes a two component regulatory system, and effectors (Bishop, Baker et al. 2005, Hensel 2005, Ellermeier and Slauch 2007, Kelly 2009). The SPI1 and SPI2 encoded T3SS are different and likely arose from separate gene transfer events (Hensel 2005, Kelly 2009, Ferreira, Buckner et al. 2012). SPI2 is around 40 kb, with 42 ORFs and is divided into two components, a 14.5 kb region encodes 5 *ttr* genes, which encode for tetrathionate respiration proteins, and a 25 kb region with 4 operons encoding the secretion system regulators (*ssr*), secretion system chaperones (*ssc*), secretion system apparatus (*ssa*), and the secretion system effectors (*sse*) (Hensel, Shea et al. 1997, Hensel, Nikolaus et al. 1999, Hensel 2005, Kelly 2009, Ferreira, Buckner et al. 2012). Many of the effectors secreted by the SPI2 T3SS are encoded outside of SPI2 (Figueroa-Bossi, Uzzau et al. 2001). SPI2 is important for the proliferation of the bacteria post-invasion and is required for intracellular survival within macrophages in a special vacuole called the *Salmonella* containing vacuole (SCV) (Santos, Tsolis et al. 2003, Ellermeier and Slauch 2007, Kelly 2009, Buckner, Croxen et al. 2011, van der Heijden and Finlay 2012). In general, SPI2 protects bacteria within the SCV by preventing the co-localization of the phagocyte oxidase and the inducible nitric oxide synthase with the SCV (Vazquez-Torres, Jones-Carson et al. 2000, Chakravortty, Hansen-Wester et al. 2002, Hensel 2005).

SPI3 is a 17 kb region with a highly variable G+C content of on average 47.5% (Blanc-Potard, Solomon et al. 1999, Hensel 2005, Kelly 2009, Ferreira, Buckner et al. 2012). SPI3 is found in all serovars, but has extensive variation in structure and location (Blanc-Potard, Solomon et al. 1999, Fierer and Guiney 2001, Amavisit, Lightfoot et al. 2003, Kelly 2009).

SPI4 is 25 kbs in length, with a G+C content of 44.8%, and is conserved between S. enterica serovars, with some variation in organization (Wong, McClelland et al. 1998, Amavisit, Lightfoot et al. 2003, Hensel 2005, Kelly 2009, Ferreira, Buckner et al. 2012). Encoded in SPI4 are a single-stranded DNA binding protein and superoxide response regulatory genes (soxSR) (Marcus, Brumell et al. 2000). It also encodes a type 1 secretion system, which may be important for survival within macrophages (Schmidt and Hensel 2004, van Asten and van Dijk 2005).

SPI5 is a 7.6 kb region with a G+C content of 43.6%, and is involved in enteropathogenesis, as mutations in SPI5 reduce enteritis (Marcus, Brumell et al. 2000, Hensel 2005, Kelly 2009, Ferreira, Buckner et al. 2012). SPI5 also encodes SopB and PipB, two effectors secreted by the SPI1 and SPI2-encoded T3SS respectively (Schmidt and Hensel 2004, McGhie, Brawn et al. 2009). SopB triggers fluid secretion leading to diarrhea, and is found in both S. bongori and S. enterica (Knodler, Celli et al. 2002). In contrast, pipAB are found in S. enterica, but not in S. bongori (Knodler, Celli et al. 2002). pipB2, is a homologue of pipB, and is associated with the formation of Salmonella-induced filaments within macrophages (Knodler and Steele-Mortimer 2005).

1.3.3 Salmonella phages

Salmonella has many phages, which contribute to both fitness and virulence. Gifsy 1 and Gifsy 2 are lambdoid prophages discovered in S. enterica serovar Typhimurium (Figueroa-Bossi and Bossi 1999, Slominski, Calkiewicz et al. 2007). Gifsy 2 contributes directly to systemic
virulence, and encodes *sodC*, *gtgE* and *gtgB/sseI*, some of which are secreted by the SPI1 and SPI2-encoded T3SS (Figueroa-Bossi and Bossi 1999, Ho, Figueroa-Bossi et al. 2002, Coombes, Wickham et al. 2005, Slominski, Calkiewicz et al. 2007, Ferreira, Buckner et al. 2012). SodC is a periplasmic Copper/Zinc superoxide dismutase important for bacterial survival within macrophages and virulence in mice (De Groote, Ochsner et al. 1997, Figueroa-Bossi, Uzzau et al. 2001). Variations of SodC, SodCI and SodCII, have been identified in *S. Typhimurium* strain 14028 (Krishnakumar, Kim et al. 2007).

Gifsy 1 encodes virulence factors including *gipA* and *gobG* (Stanley, Ellermeier et al. 2000, Coombes, Wickham et al. 2005, Slominski, Calkiewicz et al. 2007, Ferreira, Buckner et al. 2012). GogB is secreted by both the SPI1 and SPI2-encoded T3SS, and was found to localize to the cytoplasm (Coombes, Wickham et al. 2005). GipA is induced in the small intestine, and is involved in *Salmonella* growth and survival in Payer’s patches (Stanley, Ellermeier et al. 2000).

Other phages have been identified in the *Salmonella* genome, including Gifsy3, and SopEΦ (Figueroa-Bossi, Uzzau et al. 2001, Chan, Baker et al. 2003, Ferreira, Buckner et al. 2012). Gifsy3 was found in *S. Typhimurium* strain 14028 and contains the SPI1 and SPI2-secreted effector SspH1 (Figueroa-Bossi, Uzzau et al. 2001). SopEΦ is found in serovar Typhimurium and encodes SopE, a protein secreted by the SPI1-encoded T3SS, which contributes to bacterial invasiveness (Kropinski, Sulakvelidze et al. 2007).

### 1.3.4 *Salmonella* plasmids

*Salmonella* serovars also contain plasmids. In particular, a virulence plasmid is found in serovars Abortusovis, Abortusequi, Choleraesuis, Dublin, Enteritidis, Gallinarum, Sendai and Typhimurium (Porwollik and McClelland 2003, Chu and Chiu 2006, Ferreira, Buckner et al. 2012). Frequently encoded on the virulence plasmid of *Salmonella* is the *spv* operon, which is
found on the chromosome in *S. enterica* subspecies I, II, IIIa, IV, and VII (Porwollik and McClelland 2003, Chu and Chiu 2006). This operon contains 5 genes important for the intracellular replication of the bacteria including SpvB, an ADP-ribosylating protein that modifies actin within the host cell and is secreted by the SPI2 T3SS (Matsui, Bacot et al. 2001, Hochmann, Pust et al. 2006, Browne, Hasegawa et al. 2008, McGhie, Brawn et al. 2009). In serovar Typhimurium, the *spv* operon is a major contributor to virulence (Matsui, Bacot et al. 2001, Buckner, Croxen et al. 2011).

1.4 **Mechanisms of pathogenesis**

1.4.1 **Disease progression**

*Salmonella* are transmitted primarily by contaminated food or water or contact with animals that carry the bacteria (Mastroeni and Grant 2011, Chai, White et al. 2012, Lee and Greig 2012). After ingestion of *S. enterica*, bacteria cross the intestinal barrier, are taken up by phagocytes, replicate, and spread to systemic sites in cases of enteric fever (Kohbata, Yokoyama et al. 1986, Jones, Ghori et al. 1994, Vazquez-Torres, Jones-Carson et al. 1999, Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008). *Salmonella* replicate in the liver and spleens of infected individuals forming foci of infection which progress to bacterial septicemia (Sheppard, Webb et al. 2003). *Salmonella* are excreted in the infected host’s feces, thus allowing for the completion of the infective cycle (Buchwald and Blaser 1984). After *Salmonella* induced gastroenteritis, humans usually excrete *Salmonella* for about 5 weeks (Buchwald and Blaser 1984).
1.4.2 *Salmonella* replication in the intestine

When *Salmonella* first enter the gastrointestinal tract they must survive the acidic conditions in the stomach ensuring passage to the intestine. *Salmonella* have an acid tolerance response which, when activated allows for bacterial survival in low pH environments, such as that found in the stomach (Audia, Webb et al. 2001, Alvarez-Ordonez, Begley et al. 2011). The acid tolerance response in *Salmonella* involves a variety of mechanism including proton pumps, lysine and arginine decarboxylases, acid shock proteins, which are controlled by a variety of regulatory genes including RpoS, Fur, PhoP/Q, and OmpR (Alvarez-Ordonez, Begley et al. 2011). In addition, *Salmonella* is able to alter its membrane composition to maintain fluidity and prevent oxidative damage (Alvarez-Ordonez, Begley et al. 2011). Once *Salmonella* reach the intestine, the bacteria must interact with the host’s normal gut microflora. In the mouse, it has been shown that the composition of the microflora is a significant factor in determining *Salmonella* disease outcome (Sekirov, Tam et al. 2008, Ferreira, Gill et al. 2011). In fact, it has now become accepted practice to shift the mouse microflora by treatment with antibiotics to allow for a variety of mouse models of Salmonellosis using *Salmonella enterica* Typhimurium (Grassl, Valdez et al. 2008, Kaiser, Diard et al. 2012). *Salmonella* is able to use the inflammation it induces to outcompete the microbiota by using threathionate as a terminal electron acceptor and ethanolamine as a carbon source (Winter, Thiennimitr et al. 2010, Thiennimitr, Winter et al. 2011).

*Salmonella* crosses the epithelial barrier of the host’s intestine (Haraga, Ohlson et al. 2008). A few mechanisms of bacterial uptake have been proposed including crossing the intestinal barrier through M-cells (Mastroeni and Grant 2011). M-cells, or microfold cells, are specialized epithelial cells found overlying Peyer’s patches in the intestine, and in the case of
*Salmonella*, provide an ideal entry point (Jones, Ghori et al. 1994, Haraga, Ohlson et al. 2008). *Salmonella* is able to mediate its own uptake and replication in epithelial cells in a SPI1 dependent manner (van der Heijden and Finlay 2012). A population of *Salmonella* has been found to replicate in the cytosol of epithelial cells, and these bacteria, when released into the intestinal lumen, are hyperinvasive (Knodler, Vallance et al. 2010). Beneath M-cells are resident gut phagocytes, which provide an opportunity for *Salmonella* uptake and systemic spread (Haraga, Ohlson et al. 2008).

### 1.4.3 *Salmonella* systemic spread

Once inside the phagocyte, *Salmonella* replicates within a special SPI2 mediated phagocytic vacuole called the *Salmonella*-containing vacuole (SCV) (Kuhle and Hensel 2004, Browne, Hasegawa et al. 2008, Poh, Odendall et al. 2008). Replication within phagocytes allows the bacteria to disseminate through the reticuloendothelial system, moving primarily to the mesenteric lymph nodes, spleen, bone marrow and liver (Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008, Mastroeni and Grant 2011). *Salmonella* continue to replicate within these phagocytes in systemic organs, creating foci of infection (Sheppard, Webb et al. 2003, Grant, Restif et al. 2008, Mastroeni and Grant 2011). The bacteria can also replicate inside specialized cells in the liver, termed Kupffer cells (Mastroeni and Grant 2011). From the liver, bacteria are able to gain access to the gallbladder through the ducts and vasculature (Parry, Hien et al. 2002, Gonzalez-Escobedo, Marshall et al. 2011).

*Salmonella* can replicate within the bile, and can be found at high levels in the gallbladders of a portion of infected mice (Menendez, Arena et al. 2009, Antunes, Andersen et al. 2011, Buckner, Croxen et al. 2011). Menendez et. al. (2009) found that *Salmonella* are actually able to replicate within the gallbladder epithelium (Menendez, Arena et al. 2009). In
addition, *Salmonella* can form biofilms on gallstones in the presence of bile (Prouty, Schwesinger et al. 2002). It is thought that *Salmonella* in the gallbladder contribute to intestinal re-seeding, transmission, and asymptomatic carrier states (Menendez, Arena et al. 2009, Gonzalez-Escobedo, Marshall et al. 2011). Therefore, colonization of the gallbladder may be an important step in completing the infective cycle. However, there is still some debate among *Salmonella* experts as to the importance of the gallbladder as a bacterial reservoir.

1.5 Virulence mechanisms

*Salmonella* has a variety of virulence mechanisms that allow this bacterium to cause such widespread disease. Perhaps the most well studied virulence mechanisms in *Salmonella enterica* Typhimurium are the two type 3 secretion systems encoded on *Salmonella* pathogenicity islands 1 and 2 (Kuhle and Hensel 2004, Geddes, Worley et al. 2005, Coombes, Lowden et al. 2007, Browne, Hasegawa et al. 2008, Haraga, Ohlson et al. 2008, van der Heijden and Finlay 2012).

1.5.1 Type III secretion systems

Type III secretion systems (T3SS) are frequently referred to as molecular syringes that allow the translocation of proteins directly from the bacterial cytoplasm into the host cell cytosol. T3SSs play an essential role in *S. enterica* systemic spread and pathogenesis by transporting virulence proteins (effectors) from the bacteria into the host cell to promote bacterial survival and colonization (Freeman, Ohl et al. 2003, Kuhle and Hensel 2004, Geddes, Worley et al. 2005, Coombes, Lowden et al. 2007, Browne, Hasegawa et al. 2008, Haraga, Ohlson et al. 2008). The SPI1 T3SS is involved in bacterial uptake into non-phagocytic cells, such as epithelial cells, although evidence is mounting that the roles of SPI1 and SPI2 are not as segregated as was once thought (Lilic and Stebbins 2004, Patel and Galan 2005, Ly and Casanova 2007). SPI2 is

1.5.2 *Salmonella* pathogenicity island 1

The SPI1 secretion system is essential for bacterial uptake into non-phagocytic cells. SPI1 secretion is under the control of the regulators HilA and InvF (Lee, Jones et al. 1992, Eichelberg and Galan 1999). SPI1 is important during the early phases of infection, and it is associated with bacterial internalization and early SCV formation (van der Heijden and Finlay 2012). Three of the genes associated with SPI1, *sipB*, *sipC*, and *sipD* encode translocases that are required for intimate association with host cells and secretion (Lara-Tejero and Galan 2009). Once the SPI1 T3SS is active, a variety of effectors are involved in mediating bacterial internalization. See Figure 1.1 for a schematic representation of SPI1 mediated effects and effectors. SipA, SipC, SopB, SopE, and SopE2 are all involved in the induction of actin rearrangements and membrane ruffling which allow internalization of *Salmonella* (Patel and Galan 2005). Once the bacteria are internalized, SptP is involved in restoration of the actin cytoskeleton to its normal state (Patel and Galan 2005). SPI1 is also involved in the early formation of the SCV. SopB, SipC, SopA, SopD, and SptP are all involved in mediating the recruitment of early SCV markers, fusion with vesicles, removal of late lysosome markers, and the formation of macropinosomes (Bakowski, Cirulis et al. 2007, McGhie, Brawn et al. 2009, van der Heijden and Finlay 2012). Thus the activities of the SPI1 T3SS are vital to the establishment of *Salmonella* in non-phagocytic cells.
Figure 1.1 SPI1 mediated effects
SPI1 mediated bacterial entry into host cells and formation of early *Salmonella* containing vacuole. Reproduced from van der Heijden and Finlay, Future Microbiology, June 2012, Vol. 7, No. 6, Pages 685-703 with permission of Future Medicine Ltd.
1.5.3 *Salmonella* pathogenicity island 2

SPI2 effectors mediate many functions during *Salmonella* infection, including regulation of the vacuolar membrane, increasing the motility of infected cells, targeting the SCV to the trans-Golgi network (TGN), forming an actin meshwork surrounding the SCV, and inducing the formation of *Salmonella* Induced Filaments (SIF), which are long filamentous structures originating at the SCV (Brumell, Goosney et al. 2002, Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008). For a schematic representation of SPI2 mediated effects, see Figure 1.2. SIFs are membranous structures that extend from the SCV along microtubules (Ruiz-Albert, Yu et al. 2002), and may be involved in maintaining the size of the SCV and the acquisition of nutrients (Poh, Odendall et al. 2008). SIFs also have similar content and markers to the SCV (Gorvel and Meresse 2001). Multiple SPI2 effectors are thought to be involved in the formation of SIFs, including SseF, SseG, SseJ, and SifA (Brumell, Goosney et al. 2002, Kuhle and Hensel 2002, Ruiz-Albert, Yu et al. 2002, McGhie, Brawn et al. 2009). SpiC is a cytoplasmic effector that is thought to induce an IL-10 response (Uchiya, Barbieri et al. 1999, Uchiya, Groisman et al. 2004, Browne, Hasegawa et al. 2008). SpiC may also play a role in vesicle trafficking and is required for the secretion of SseB, SseC and SseD (Uchiya, Barbieri et al. 1999, Freeman, Rappl et al. 2002, Browne, Hasegawa et al. 2008). SpiC is a component of a pH sensing complex which controls effector secretion (Yu, McGourty et al. 2010). The *Salmonella* T3SSs are vital to pathogenesis, and are therefore a very important topic to study. SPI2 effectors, and their role in *Salmonella* pathogenesis will be discussed in more detail in the introduction of Chapter 2.
Figure 1.2 SPI2 mediate effects
SCV maturation and Salmonella replication. (VAP – Vacuole-associated actin polymerization). Reproduced from van der Heijden and Finlay, Future Microbiology, June 2012, Vol. 7, No. 6, Pages 685-703 with permission of Future Medicine Ltd.
1.6 Host response to *Salmonella*

1.6.1 Mucosal defense

For *Salmonella* to establish systemic infection, the bacteria first have to cross the intestinal barrier. To do this, the bacteria must pass through the mucus layer, produced by goblet cells, and survive a battery of anti-microbial peptides (AMP), such as α- and β-defensins (cryptdins in mice), CRS peptides, lysozymes, cathelicidins, angiogenin, and Reg IIβ/γ (Broz, Ohlson et al. 2012). Paneth cells secrete many of the AMPs, but upon activation other epithelial cells can also produce AMPs (Broz, Ohlson et al. 2012). AMPs have been found to contribute to the control of *Salmonella*, but are not considered to be a major factor in prevention of infection or clearing of the bacteria (Dougan, John et al. 2011). Once *Salmonella* has crossed the epithelial barrier it must face the GALT (gut-associated lymphoid tissue), which is composed of T and B cells, dendritic cells, macrophages, and neutrophils (Broz, Ohlson et al. 2012).

The Peyer’s patches, a component of the GALT, are one of the major routes of entry for *Salmonella*. Peyer’s patches are aggregated lymphoid follicles covered by a special form of epithelium, called the follicle associated epithelium, which includes M-cells (Broz, Ohlson et al. 2012). M-cells transport antigens and bacteria, such as *Salmonella*, via transcytosis to lymphocytes or antigen presenting cells that wait beneath the M-cells (Haraga, Ohlson et al. 2008, Broz, Ohlson et al. 2012). Antigen presenting cells can then activate B cells and T cells and induce memory cells leading to the induction of the adaptive immune response (Broz, Ohlson et al. 2012).

*Salmonella* are taken up by macrophages, where they stimulate the production of IL-18 and IL-23, which amplify the immune response leading to increased IFN-γ, IL-22, and IL-17 production (Broz, Ohlson et al. 2012). IL-12 and IL-18 secretion by activated macrophages
contributes to the recruitment of NK cells and T helper cells, which secrete additional IFN-γ, leading to further activation of macrophages in a positive inflammatory feedback loop (Eckmann and Kagnoff 2001, Srinivasan, Salazar-Gonzalez et al. 2007, Dougan, John et al. 2011). IFN-γ performs a variety of functions, including leading to the activation of macrophages and making them more capable of clearing intracellular pathogens such as *Salmonella* (Monack, Bouley et al. 2004, Dougan, John et al. 2011). The production of IFN-γ and the subsequent activation of macrophages is crucial to macrophage clearance of *Salmonella* (Mittrucker and Kaufmann 2000, Eckmann and Kagnoff 2001). IL-23 stimulates further release of IL-17 and IL-22 from antigen experienced T cells, IL-22 then stimulates the production of AMPs and mucus from paneth and goblet cells respectively (Broz, Ohlson et al. 2012). IL-17 production can stimulate epithelial cells to produce CXC chemokines, which recruit neutrophils to the site of *Salmonella* invasion (Broz, Ohlson et al. 2012). The increase in IL-22 and IL-23 also stimulates the production of AMPs and neutrophil recruitment (Broz, Ohlson et al. 2012).

Neutrophils are crucial for the successful clearance of *Salmonella*, and are recruited to the site of infection by multiple factors including IL-1β (Broz, Ohlson et al. 2012). Neutrophils are able to successfully kill extracellular *Salmonella* (Broz, Ohlson et al. 2012). One of the mechanisms used by neutrophils to kill *Salmonella* is by the production of reactive oxygen species (Miao, Leaf et al. 2010). While neutrophils are effective at killing *Salmonella*, they also cause tissue damage and may stimulate chloride secretion, leading to diarrhea (Tsolis, Adams et al. 1999, Broz, Ohlson et al. 2012).

Dendritic cells (DC) can also be found beneath the M-cells in Peyer’s patches. DCs provide a link between the innate and the adaptive immune responses by migrating to lymph nodes and activating T cells (Bueno, Riquelme et al. 2012). Mature DCs also secrete cytokines

NRAMP1 (natural resistance-associated macrophage protein 1) is an important host factor that determines the outcome of *Salmonella* infection (Mittrucker and Kaufmann 2000). NRAMP1 is a proton/divalent cation transporter, involved in iron metabolism and ROS detoxification found in the vacuolar membrane (Kehres, Zaharik et al. 2000, Dougan, John et al. 2011). Mice lacking NRAMP1 are extremely susceptible to *S. Typhimurium* (Monack, Bouley et al. 2004, Valdez, Diehl et al. 2008).

1.6.2 Pattern recognition receptors

Pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) play an important role in the recognition of *Salmonella* and help mediate the connection between the innate and adaptive immune responses of the host (Kawai and Akira 2010, de Jong, Parry et al. 2012). PRRs recognize signals called pathogen associated molecular patterns (PAMPs), and danger associated molecular patterns (DAMPs), PRRs then initiate signaling cascades that lead to the activation of the host immune response (Bueno, Riquelme et al. 2012, de Jong, Parry et al. 2012). In brief, in cases of Salmonellosis PRR signaling leads to the migration and activation of macrophages and neutrophils to the site of infection, and the
production of a variety of cytokines including IL-6, IL-1β, IL-12, IL-18, TNFα, and IFN-γ, and the activation of iNOS (Dougan, John et al. 2011, de Jong, Parry et al. 2012). In particular, the IFN-γ response has been found to be crucial for successful clearance of Salmonella (Muotiala and Makela 1990, Monack, Bouley et al. 2004). TNFα has also been found to be important throughout Salmonella infection, as mice lacking TNFα had worsened infections at early time points, and mice treated with anti-TNFα antibodies during later phases of infection lead to relapses (Mastroeni, Villarreal-Ramos et al. 1993, Everest, Roberts et al. 1998, Dougan, John et al. 2011).

The PAMPs produced by Salmonella include its T3SS, flagella, fimbriae, LPS, lipoproteins, curli, and bacterial DNA (Broz, Ohlson et al. 2012, de Jong, Parry et al. 2012). The S. Typhi Vi capsule prevents immune recognition by restricting PRR access to PAMPS (Wilson, Raffatellu et al. 2008). TLRs 1, 2, 4, 5, 6, and 10 are all found on the cell membrane, while TLRs 3, 7, 8, 9, 11, and 13 are found on early and late endosomes (Broz, Ohlson et al. 2012). Once TLRs bind their ligands, they associate with adaptor molecules, including MyD88 and TRIF, and then initiate signaling cascades leading to the activation of the NF-κB and IRF3 transcription factors, and the production of IL-8, IL-10, pro-IL-1β, pro-IL-18, and IFN-γ (Medzhitov 2001, Broz, Ohlson et al. 2012). TLR4, which recognizes LPS, and TLR5, which recognizes flagella, have been found to be important for Salmonella disease, as mice lacking these TLRs have increased susceptibility to Salmonella (Vazquez-Torres, Vallance et al. 2004, Coburn, Grassl et al. 2007, de Jong, Parry et al. 2012). Enterocytes express TLR5 on their basolateral sides, which allow them to recognize flagellin from invading Salmonella, while being unaffected by commensal bacteria in the intestinal lumen (Gewirtz, Navas et al. 2001).
Mice lacking TLR2, and/or TLR4 were more susceptible to *Salmonella* infection; however, when TLR2, 4 and 9 were all knocked out *Salmonella* was found to colonize poorly (Arpaia, Godec et al. 2011). This study showed that *Salmonella* required TLR signaling, which induces acidification of the vacuole, a signal *Salmonella* uses to initiate the expression of virulence genes (Arpaia, Godec et al. 2011). Taken together, it has become clear that PRRs are important for the successful clearance of the pathogen, primarily through initiating the immune response and the production of cytokines, such as IFN-γ and TNFα, which activate immune cells to kill *Salmonella*. It also seems that *Salmonella* has evolved mechanisms to exploit some aspects of these host responses to regulate its own virulence.

### 1.6.3 Inflammasome

The inflammasome is comprised of a complex of Nod-like Receptors (NLRs), caspase-1, and ASC (apoptosis-associated speck-like protein containing a CARD), and is crucial for the host response against *Salmonella* (Broz, Ohlson et al. 2012, de Jong, Parry et al. 2012). The inflammasome, when active, cleaves pro-IL-1β and pro-IL-18 to their active forms, and can also lead to pyroptosis (Miao, Leaf et al. 2010, Broz, Ohlson et al. 2012). Pyroptosis is a pro-inflammatory form of programmed cell death which, in the case of *Salmonella* infection, leads to the release of intracellular bacteria into the extracellular milieu, providing an opportunity for neutrophils to kill *Salmonella* (Lara-Tejero, Sutterwala et al. 2006, Bergsbaken, Fink et al. 2009, Miao, Leaf et al. 2010). *Salmonella* Typhimurium induce the NLRC4- and NLRP3-inflammasomes to release IL-1β and IL-18, and mice lacking these NLRs are more susceptible to *Salmonella* infection (Broz, Newton et al. 2010, Bueno, Riquelme et al. 2012). In addition, mice lacking caspase-1 are also more susceptible to *Salmonella* infection (Lara-Tejero, Sutterwala et al. 2006). The *Salmonella* SPI1 rod-like protein PrgJ was found to bind to NAIP2, which
promotes NLRC4 inflammasome activation (Kofoed and Vance 2012). In addition, flagellin secreted by the SPI1 T3SS can bind NAIP5 and 6, leading to NLRC4 inflammasome activation, resulting in pyroptosis, IL-1β, and IL-18 secretion (Lightfield, Persson et al. 2008, Zhao, Yang et al. 2011, Bueno, Riquelme et al. 2012, Kofoed and Vance 2012). Thus Salmonella infection leads to increased inflammation via the induction of the inflammasome, resulting in the release of IL-1β and IL-18, and the initiation of the pyroptotic pathway.

1.6.4 Reactive oxygen intermediates

Reactive oxygen intermediates (ROI) are able to rapidly clear Salmonella (Jantsch, Chikkaballi et al. 2011). The primary target of ROI is DNA (Fang 2011). Salmonella has evolved to avoid SCV association with NADPH oxidase, which forms ROI, in a SPI2 dependent mechanism (Vazquez-Torres, Xu et al. 2000). SPI2 prevents the NADPH oxidase subunit, Cytb558, from associating with the SCV (Gallois, Klein et al. 2001). In fact, one minute after phagocytosis, a difference in association with NADPH oxidase can be seen between wild-type Salmonella and Salmonella lacking SPI2 (Gallois, Klein et al. 2001). Additionally, SPI2 prevents the co-localization of Salmonella with hydrogen peroxide (Fang 2011). Salmonella has additional defenses, including three classes of proteins that are able to detoxify ROI, catalases, peroxiredoxins, and superoxide dismutases (Fang 2011).

1.6.5 Reactive nitrogen species

Reactive nitrogen species (RNS) are also important for containing Salmonella infection (Henard and Vazquez-Torres 2011, Jantsch, Chikkaballi et al. 2011). RNS are cytotoxic to Salmonella through a variety of mechanisms, including arresting DNA replication, causing DNA damage, inhibiting SPI2 transcription, and inhibiting the PhoPQ regulated acid tolerance response (Bourret, Song et al. 2009). Salmonella LPS triggers the JAK/STAT signaling pathway,
and IFN-γ production, which mediate the upregulation of the inducible nitric oxide synthase (iNOS) (Henard and Vazquez-Torres 2011). IFN-γ activated macrophages produce high levels of RNS, which prevents SPI2 activation, allowing SCV association with phagolysosomes, and thus leading to increased antimicrobial activities of the macrophage (Mitrucker and Kaufmann 2000, McCollister, Bourret et al. 2005, Bourret, Song et al. 2009, Henard and Vazquez-Torres 2011). Furthermore, mice treated with iNOS inhibitors were found to have higher *Salmonella* counts in the liver and spleen (MacFarlane, Schwacha et al. 1999). At low pH, RNS can also interfere with the acid tolerance response by inhibiting the PhoPQ two-component regulator system (Henard and Vazquez-Torres 2011). NO also leads to the repression of SPI2 via PhoPQ independent mechanisms (Henard and Vazquez-Torres 2011). *Salmonella* has evolved ways to deal with the RNS produced by the host, including a NO$_2^-$ transporter, and nitrate reductases (Henard and Vazquez-Torres 2011). Additionally, *Salmonella* avoids SCV association with iNOS containing vesicles in a SPI2 dependent matter (Henard and Vazquez-Torres 2011).

### 1.7 Conclusions

In conclusion, *Salmonella* species and serovars cause disease in people all around the world. This pathogen has become a model organism for the study of bacterial-host interactions, and as such, the scientific community has gained a great deal of insight into pathogenicity from the study of this bacterium. *Salmonella* are able to survive within the host intestine, outcompeting the normal flora, and *Salmonella* are also able to survive and replicate within the host’s phagocytic immune cells. This versatile pathogen has successfully evolved to control the host’s response to infection, and represents an excellent system to probe and study many questions about bacterial pathogenicity.
Chapter 2: The contribution of individual Salmonella pathogenicity island 2 effectors to virulence

2.1 Abstract

Salmonella enterica serovars are Gram-negative bacterial pathogens responsible for human diseases including gastroenteritis and typhoid fever. After ingestion, Salmonella cross the intestinal epithelial barrier, where they are phagocytosed by macrophages and dendritic cells, which then enables their spread to systemic sites during cases of typhoid fever. Salmonella use two type III secretion systems (T3SS) encoded by Salmonella pathogenicity islands (SPI) 1 and 2 to inject virulence proteins into host cells to modify cellular functions. SPI1 is involved in host cell invasion and inflammation, whereas SPI2 is required for intracellular survival and replication within phagocytes, and systemic spread. In this chapter the contribution of many known SPI2 effectors was examined in an in vivo model of murine typhoid fever and cell culture models of macrophage and epithelial cell infection. Unmarked, in-frame deletions of SPI2 effectors were engineered in S. enterica serovar Typhimurium and the ability of the 16 different mutants to colonize and replicate was examined. In the typhoid model, we found that the ΔspvB and ΔspiC mutants were attenuated for colonization of intestinal and systemic sites, while the ΔsseF mutant was attenuated in systemic organs. In epithelial cells, all mutants replicated to the same extent as the wild type. In macrophages, ΔspiC, ΔsteC, ΔspvB, ΔsseK1/K2/K3, ΔsifA, and ΔsifB strains replicated poorly in comparison to wild-type Salmonella. This study provides the first thorough comparative screen of the majority of the known SPI2 effectors evaluated under
the same conditions in various models of infection, providing a foundation for comparative examination of the roles and interactions of these effectors.

2.2 Introduction

*Salmonella enterica* are Gram-negative, facultative intracellular pathogens that can cause a wide range of human illness, from enteric/typhoid fever (caused by serovar Typhi or Paratyphi) to gastroenteritis (caused by serovar Typhimurium) (Freeman, Ohl et al. 2003, Kuhle and Hensel 2004). In cases of typhoid fever, after ingestion of *S. enterica*, the bacteria cross the intestinal barrier, predominantly through microfold cells (M-cells) in Peyer’s patches, where they are taken up by phagocytes. In these cells *Salmonella* replicates within a special phagocytic compartment termed the *Salmonella*-containing vacuole (SCV) (Kuhle and Hensel 2004, Browne, Hasegawa et al. 2008, Poh, Odendall et al. 2008). Within the phagocyte, the bacteria disseminate to systemic sites (Kohbata, Yokoyama et al. 1986, Jones, Ghori et al. 1994, Vazquez-Torres, Jones-Carson et al. 1999, Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008) through the reticuloendothelial system, moving primarily to the mesenteric lymph nodes (MLNs), spleen, and liver (Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008).

Type 3 secretion systems (T3SS) play an essential role in *S. enterica* systemic spread and pathogenesis by transporting virulence proteins, termed effectors, from the bacteria into host cells to promote bacterial invasion and survival (Freeman, Ohl et al. 2003, Kuhle and Hensel 2004, Geddes, Worley et al. 2005, Coombes, Lowden et al. 2007, Browne, Hasegawa et al. 2008, Haraga, Ohlson et al. 2008). *S. enterica* serovar Typhimurium utilizes two T3SS, one encoded by *Salmonella* Pathogenicity Island (SPI) 1 and one by SPI2 (Kuhle and Hensel 2004, Geddes, Worley et al. 2005, Coombes, Lowden et al. 2007, Browne, Hasegawa et al. 2008, Haraga,
Ohlson et al. 2008). SPI1 is involved in bacterial uptake into non-phagocytic cells, such as epithelial cells, whereas SPI2 is activated within phagosomes and is required for survival within phagocytes and consequently, systemic disease (Ochman, Soncini et al. 1996, Beuzon and Holden 2001, Freeman, Ohl et al. 2003, Geddes, Worley et al. 2005, Coombes, Lowden et al. 2007, Haraga, Ohlson et al. 2008). Growing evidence suggests that the roles of SPI1 and SPI2 are not as segregated as previously thought, (Lilic and Stebbins 2004, Patel and Galan 2005, Ly and Casanova 2007) including the finding that SPI2 is also activated inside the intestinal lumen (Brown, Vallance et al. 2005).

SPI2 effectors mediate many cellular functions during Salmonella infection, including regulation and maintenance of the vacuolar membrane, increasing the motility of infected cells, targeting the SCV to the trans-Golgi network, forming an actin meshwork surrounding the SCV, and inducing the formation of Salmonella Induced Filaments (SIFs), which are long filamentous structures originating at the SCV (Brumell, Goosney et al. 2002, Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008). Known roles of SPI2 effectors have been extensively reviewed (See references (Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008, McGhie, Brawn et al. 2009, van der Heijden and Finlay 2012)).

SPI2 is regulated by the kinase SsrA, which activates the transcription factor SsrB, which in turn activates SPI2 gene transcription (Walthers, Carroll et al. 2007). SPI2 is also regulated by the PhoPQ two component regulatory system (Miller, Kukral et al. 1989). Once the T3SS is in place, effectors are secreted which mediate functions including maintenance of vacuolar membrane, SCV positioning, actin meshwork formation, and the formation of SIFs (van der Heijden and Finlay 2012). SifA targets SKIP and Rab7, and is involved in down regulating the recruitment of kinesin to the SCV, it plays an integral role in SIF formation, and in maintaining
SpiC is involved in maintaining the SCV position, as it interacts with TassC and Hook 3 to control trafficking (Shotland, Kramer et al. 2003, Haraga, Ohlson et al. 2008). SpiC also plays a role in sensing the environment and permitting effector secretion (Yu, McGourty et al. 2010). SseJ is another effector which is implicated in SCV positioning, it has acyl transferase activity, is involved in lipid and cholesterol metabolism, and is important for bacterial replication and SIF formation (Ruiz-Albert, Yu et al. 2002, Ohlson, Fluhr et al. 2005, Nawabi, Catron et al. 2008, van der Heijden and Finlay 2012). SseF and SseG are involved in SCV positioning (van der Heijden and Finlay 2012), and are discussed in more detail in Chapter 3.

An actin meshwork is formed around the SCV, and is important for bacterial replication (van der Heijden and Finlay 2012). A few effectors that are involved in the formation of this meshwork are SseI, SspH2, SpvB, and SteC (Haraga, Ohlson et al. 2008, van der Heijden and Finlay 2012). SseI and SspH2 both bind to filamin and localize with the actin cytoskeleton (Srikanth, Mercado-Lubo et al. 2011). In addition to filamin, SspH2 also binds to profilin and interacts with G-actin to enhance actin polymerization (Miao, Scherer et al. 1999, Miao, Brittnacher et al. 2003, Haraga, Ohlson et al. 2008, van der Heijden and Finlay 2012). SpvB is an ADP-ribosylating protein which ribosylates monomeric actin, reversing mesh formation (Lesnick, Reiner et al. 2001). SteC has kinase activity that is important for the formation of the actin meshwork (Poh, Odendall et al. 2008).

The elucidation of the role of SPI2 effectors in pathogenesis is important for our understanding of how Salmonella manipulate the host to successfully replicate and cause disease.
Most of the information about SPI2 effectors comes from individual studies examining only one or a few effectors. In this chapter, the SPI2 effectors were systematically individually knocked-out, and tested in a variety of models. This enables a direct comparison of the contribution of individual SPI2 effectors to *Salmonella*’s ability to colonize and replicate in several different infection models. This work confirms the importance of SPI2 effectors in macrophage replication and systemic spread. The work presented in this chapter provides a basis for the systematic comparison of SPI2 effectors, and provides the foundation for this thesis.

### 2.3 Methods and materials

#### 2.3.1 Construction of bacterial strains, plasmids and growth conditions

*Salmonella enterica* serovar Typhimurium and *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) agar at 37°C, supplemented with the appropriate antibiotics (100 µg/mL streptomycin, 30 µg/mL chloramphenicol, 50 µg/mL kanamycin). Overnight cultures were grown in LB broth at 37°C with aeration with the appropriate antibiotics. *E. coli* MC1061λpir was used for standard cloning, *E. coli* SM10λpir was used for conjugation, and *S. Typhimurium* SL1344 strain was used to construct all of the in-frame deletions (Table 2.1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061λpir</td>
<td>hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 gal1 galK rpsL thi λpir</td>
<td>(Rubires, Saigi et al. 1997)</td>
</tr>
<tr>
<td>SM10λpir</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu pir⁺</td>
<td>(Miller and Mekalanos 1988)</td>
</tr>
<tr>
<td>Plasmids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRE112</td>
<td>cat sacB oriV&lt;sub&gt;R6K&lt;/sub&gt; oriT&lt;sub&gt;RP&lt;/sub&gt;</td>
<td>(Edwards, Keller et al. 1998)</td>
</tr>
<tr>
<td>pZA23MCS</td>
<td>neo p15a P&lt;sub&gt;A1LacO-1&lt;/sub&gt;</td>
<td>(Lutz and Bujard 1997)</td>
</tr>
</tbody>
</table>
PCR was done with Pfu Turbo DNA Polymerase (Stratagene, 600252) to amplify ~500 bp flanking regions of each gene (Table 2.2). PCR products were then joined using overlapping PCR (Croxen, Sisson et al. 2006). Using restriction digestion with SacI and KpnI and T4 DNA ligation, this PCR product was cloned into the suicide vector pRE112, (Edwards, Keller et al. 1998) which was transformed into E. coli MC1061λpir (Rubires, Saigi et al. 1997). Following confirmation of the correct insert by DNA sequencing (BigDye Terminator v3.1 Cycle Sequencing, ABI, 4337456; NAPS unit, DNA Sequencing Laboratory, UBC), the suicide plasmids were transformed into E. coli SM10λpir (Miller and Mekalanos 1988) so that they could be mobilized into SL1344 by conjugation. Initial plasmid integration was identified by recovering chloramphenicol resistant colonies. Plasmid loss was selected for following growth for 4 hours without chloramphenicol, and by selecting for sucrose resistant colonies. The desired genotype was confirmed by PCR and DNA sequencing. Deletion strains were constructed to be unmarked, and therefore did not contain any additional antibiotic resistance markers.

**Table 2.2 Oligonucleotides used to construct SPI2 gene deletions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>sifB 5’ flanking</td>
<td>CTGAGCTCGCCGTCCACATTTCT</td>
<td>TCTCCGATAGTAATTGGCAT</td>
</tr>
<tr>
<td>sifB 3’ flanking</td>
<td>GCCAATTACTATCGGGAGAGGC</td>
<td>GACTGGTACCGAGCCCTGAAG</td>
</tr>
<tr>
<td>spiC 5’ flanking</td>
<td>CTGAGCTCGGTAAGCAGATAGC</td>
<td>CATGAATCCCTCCTCAGACAT</td>
</tr>
<tr>
<td>spiC 3’ flanking</td>
<td>ATGTCTGAGGAGGATTCATGCAC</td>
<td>GACTGGTACCTTACACTCAGG</td>
</tr>
<tr>
<td>sseF 5’ flanking</td>
<td>GTACGGTACCGCGCATCTGACAG</td>
<td>ACTTGCCGTCGAGGAATATG</td>
</tr>
<tr>
<td>sseF 3’ flanking</td>
<td>CATATTCGTCAGCGGCAAGTAGT</td>
<td>TAGAGCTCATCCCATCCATACC</td>
</tr>
<tr>
<td>sseG 5’ flanking</td>
<td>GTACGGTACCGCAATACACTATTC</td>
<td>CTGAGCATTTGGGCTAACAGG</td>
</tr>
</tbody>
</table>
Gene | Forward oligonucleotide | Reverse oligonucleotide
--- | --- | ---
*sseG* 3’ flanking | CCTGTTAGCCCAAATGCTCAGCCA GAACACGTGCGCCG | TAGAGCTCAGCAGCGCCATA GCCTCT
*sseF/G* 5’ flanking | GTACGGTACCGCGCATCCTGACAG TAATGG | ACTTGCCGCTGACGGAATATG
*sseF/G* 3’ flanking | CATATTCGCCGTCAGCGCAAGTCCA GAACACGTGCGCCG | TAGAGCTCAGCAGCGCCATA GCCTCT
*sseI* 5’ flanking | CTAGGGTACCGCAGCGCAGCTTCG TCAGGCGG | TAGAGCTCAGCAGCGCCATA GCCTCT
*sseI* 3’ flanking | AGCGGATGTCTTCCCCGCCCATCTAT TCCTATAATAGTAAAAATGTAAG | CTGAGCTCCCGGCCTCAGAT GCTCATCA
*ssPH2* 5’ flanking | GTACGCTACCGCGACCCGGAACCAGA TGACACA | GCTTCAATATGAAAGGGCAT
*ssPH2* 3’ flanking | ATGCCCTTTCATATTGGAAGCGTT CAGTGGCGCAGGT | TAGAGCTCGTGGGCGCATGTT GTCATCT
*slrP* 5’ flanking | CTGAGCTCGTTGCCACGTTACGC GAG | TAGAGCTCGTGGGCGCATGTT GTCATCT
*slrP* 3’ flanking | ACTAATATACATACATCGCAAGC GCCTACTGGCGATAG | GACTGGTACCGGCAGGAGA TCAATGTA
*steC* 5’ flanking | CTGAGCTCCGGCTATCTCCGGCAGG TG | GCAACTATGATTTCCGATCTG
*steC* 3’ flanking | CAGATCGGAAATCATAGTGCTGGA GGACTCTTGGGGCT | GACTGGTACCGGCAGGAGA TCAATGTA
*spvB* 5’ flanking | CTGAGCTCGAGAGGCCAGAGCGTG CG | GCACTATGATTTCCGATCTG
*spvB* 3’ flanking | ACTCTGGCCTCAGACGAGGAGGT ACTCAACTCATAG | GCACTATGATTTCCGATCTG
*sseA* promoter | CTCGAGTGTAGTGAGTGAGTGATGTA | TACGAAATTCACGATGATAAT TAAACGTGC
*sseF* complement | GCACGTTAATTATCTCTCTCGTAAT TCCAGAAGGAATGAAAATG | TACGAGTTAATTATCTCTCGTAAT TCCAGAAGGAATGAAAATG

The δsseF strain was complemented by first PCR amplifying the *sseA* promoter region and the *sseF* gene (Table 2.2), then joining the products by overlapping PCR. BamH1 and Xho1 restriction digest followed by T4 DNA ligation was used to clone the fragment into pZA23MCS (Lutz and Bujard 1997). This plasmid (pZA23-sseF) was then electroporated into the δsseF *Salmonella* strain. The cloning was confirmed by restriction digest and DNA sequencing.
2.3.2 Epithelial cell infections

HeLa cells (American Type Culture Collection, ATCC; Manassas, VA) were seeded at 7x10^4 cells/well, and CaCo2 (ATCC) were seeded at 2.5x10^5 cells/well in 24-well plates and grown for 24 hours at 37°C, 5% CO₂. All cells were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (Thermo Scientific, SH3024301) and 10% fetal bovine serum (FBS) (Thermo Scientific, SH3039603), 1% non-essential amino acids (NEAA)(Gibco,11140), and 1% GlutaMax (Gibco, 35050).

For HeLa infections, bacteria were grown overnight, sub-cultured for 3 hours, then diluted and 10^5 bacteria were added to each well in DMEM for 10 min. Cells were then washed 3 times with phosphate buffered saline (PBS) +/- DMEM with no bacteria was then added for 20 min. Cells were incubated with medium containing 50 μg/mL gentamicin for 90 minutes, then 10 μg/mL gentamicin for the remainder of the 2 and 6 hour time points. Bacterial counts at 2 hours post infection were used as measures of bacterial invasion. Counts at 6 hours were chosen to calculate the replication index as our group has found that HeLa cells begin to die at later time points due to bacterial load (Unpublished data). Bacteria were released from HeLa cells using lysis buffer containing 1% Triton X-100 (BDH, R06433) and 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, 151213) in PBS, diluted in PBS and plated for colony counts on LB plates containing 100 μg/mL of streptomycin.

For CaCo2 infections, overnight cultures were sub-cultured for 3 hours, then 10^6 bacteria were added to each well in DMEM for 20 minutes. Cells were then incubated with 100 μg/mL gentamicin in DMEM for the remainder of the 2-hour time point. Six hour samples were treated with 10 μg/mL gentamicin for the remainder of the time. Samples were washed and lysed with
lysis buffer, then diluted in PBS and plated for colony counts. Calculations were done the same way as for the HeLa cell infections.

2.3.3 Macrophage infections

RAW264.7 cells (ATCC) were seeded at 1x10^5 cells/well in 24-well plates and grown for 24 hours at 37°C, 5% CO₂. All cells were grown in DMEM with high glucose (Thermo Scientific, SH3024301) and 10% FBS (Thermo Scientific, SH3039603), 1% NEAA (Gibco, 11140), and 1% GlutaMax (Gibco, 35050).

For RAW264.7 infections, 10^6 bacteria from an overnight culture were added to each well in DMEM for 20 minutes. Cells were then incubated in medium containing 100 µg/mL gentamicin for 60 min, then 10 µg/mL gentamicin for the remainder of the 3 and 24 hour time points. Bacterial counts at 3 hours were used as measures of invasion, and the 24-hour time point was used to calculate the replication index, since it provided enough time for Salmonella to replicate, as we have found that Salmonella begin to replicate in RAW264.7 cells at 10 hours post infection. Bacteria were released from RAW264.7 cells using lysis buffer, diluted in PBS and plated for colony counts.

2.3.4 Typhoid fever model

Overnight cultures of Salmonella were diluted in 100 mM HEPES with 0.9% sodium chloride (pH 8.0). Between 5 and 10 8-week-old female C57BL/6 mice (The Jackson Laboratory) were infected orally with 3-5x10^7 bacteria in 100 µl. All animal infections were performed in accordance with ethical requirements of the University of British Columbia’s Animal Care Committee. Mice were euthanized 3 and 5 days post infection and tissue samples, including the respective intestinal contents, were removed for bacterial quantification. The tissues that were collected for this study were: liver, spleen, mesenteric lymph nodes, cecum,
colon, and ileum. Samples were homogenized using a mixer mill (MM 301, Retsch, Haan Germany) for 10 minutes at a frequency of 30/s. Samples were diluted in PBS and plated for colony counts. Bile counts were obtained by extracting bile from the gallbladder, bile was diluted in PBS and plated for colony counts.

2.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0. (GraphPad Software). Data were analyzed by nonparametric Mann-Whitney t tests with 95% confidence intervals. For each figure, the term “measurements” refers to the combination of both technical and experimental replicates. Each experiment was repeated a minimum of two times, with multiple technical replicates. Thus, for example “8 measurements” would refer to two separate experiments with 4 technical replicates each.

2.4 Results - Salmonella SPI2 mutant replication in epithelial cells

2.4.1 Replication in HeLa epithelial cells

*S. enterica* serovar Typhimurium actively invade and replicate within epithelial cells, causing multiple changes in cellular organization (Garcia-del Portillo, Zwick et al. 1993). The panel of SPI2 mutants (Table 2.3) was generated and examined for their ability to replicate in HeLa epithelial cells. The replication index was determined by comparing colony forming units (CFU) at 2 and 6 hours post infection. All SPI2 mutants showed no significant differences in intracellular replication when compared to the wild-type strain (Figure 2.1), confirming that SPI2 effectors are not necessary for bacterial replication in epithelial cells. The ΔssaR strain, which contains a mutation in the SPI2 apparatus that prevents translocation of SPI2 effectors (Brumell, Rosenberger et al. 2001), was used as a control. This strain has been used previously as a SPI2
control strain (Brumell, Rosenberger et al. 2001, Coombes, Wickham et al. 2005, Grassl, Valdez et al. 2008). Invasion of Salmonella mutants was determined by enumerating CFU 2 hours post-infection. No significant differences were seen in CFU at this early time point (data not shown).

Table 2.3 Salmonella Typhimurium strains constructed and used for this study

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<tr>
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<td>(Brumell, Rosenberger et al. 2001)</td>
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Figure 2.1 Replication of SPI2 deletion strains in HeLa epithelial cells

HeLa cells were infected with exponentially growing *Salmonella enterica* Typhimurium, with a multiplicity of infection of 14. Error bars represent standard errors of the means. Results shown are the averages of a minimum of 8 measurements. Fold replication was determined by comparing bacterial counts at 2 and 6 hours post-infection.

2.4.2 Replication in CaCo2 epithelial cells

HeLa cells are widely used for *Salmonella* experiments, however, because they are cervical epithelial cells, CaCo2 intestinal epithelial cells were also used in this study. The ability of the SPI2 mutants to replicate in CaCo2 intestinal epithelial cells was examined. Similar to what was seen in HeLa cells, no significant differences were seen between the replication index of the SPI2 mutants and wild-type *Salmonella* (Figure 2.2). The ΔssaR strain was also used as a control in this model. Together, the HeLa and CaCo2 data confirm that SPI2 effectors are not required for *Salmonella* colonization of epithelial cells.
Figure 2.2 Replication of SPI2 deletion strains in CaCo2 epithelial cells
CaCo2 epithelial cells were infected with exponentially growing Salmonella enterica Typhimurium, with a multiplicity of infection of 10. Results shown are the averages of a minimum of 8 measurements. Error bars represent standard errors of the means. Fold replication was determined by comparing bacterial counts at 2 and 6 hours post-infection.

2.5 Salmonella SPI2 mutant replication in macrophages

2.5.1 Replication in RAW264.7 macrophages

S. Typhimurium exploit macrophages and dendritic cells for replication and transport within the host in a SPI2-dependent manner (Haraga, Ohlson et al. 2008). Therefore, the replication of the SPI2 effector mutants (Table 2.3) in the RAW264.7 macrophage cell line was examined. Macrophages were infected with S. Typhimurium strains and harvested for colony counts. The replication index was determined by comparing CFU at 3 and 24 hours post...
infection. As expected, the ΔssaR strain, which is used as a control that has no SPI2 effector secretion, replicated significantly less than wild-type Salmonella (p=0.0118; Figure 2.3). Some SPI2 effector deletion strains were found to have lower levels of bacterial replication in the macrophages, whereas other mutations had little or no effect. The ΔsseFG double mutant, ΔsseF, ΔsseG, ΔslrP, ΔsteA, ΔsspH2, Δssel, and ΔgogB mutant strains did not affect bacterial replication. However, the ΔsifA, ΔsseK1/K2/K3, ΔsteC, ΔspiC, ΔsifB, and ΔspvB strains replicated at levels significantly lower than the wild type (p=0.0052, p=0.0279, p=0.0460, p=0.0048, p=0.0459, p=0.0248, respectively; Figure 2.3). These data show that SifA, SseK1, SseK2, SseK3, SteC, SpiC, SifB and SpvB are important for enabling bacterial replication within cultured macrophages.

Figure 2.3 Replication of SPI2 deletion strains in RAW264.7 macrophages
Macrophages were infected with Salmonella enterica Typhimurium SPI2 deletion strains, with a multiplicity of infection of 10. Results shown are the averages of a minimum of 8 measurements. Fold replication was determined by comparing bacterial counts at 3 and 24 hours post-infection. Error bars represent standard errors of the means. (*p < 0.05).
2.6 *Salmonella* SPI2 mutant replication in murine typhoid fever model

2.6.1 Colonization of intestinal tract

Because of the important role that SPI2 plays in macrophage replication and systemic spread (Haraga, Ohlson et al. 2008, McGhie, Brawn et al. 2009, van der Heijden and Finlay 2012), the SPI2 mutants (Table 2.3) were tested in a mouse model of typhoid fever. The CFU were examined in systemic and intestinal sites of C57BL/6 mice 5 days post oral infection. As expected, the ΔssaR strain was significantly less abundant in the intestine (\(p<0.0001\)) than the wild-type strain. Interestingly, colonization of the majority of SPI2 mutants was not significantly different from that of the wild type at intestinal sites (Figure 2.4). In the colon, the colonization of the ΔspiC and ΔspvB strains was significantly lower than that of the wild type (\(p=0.0024\) and \(p=0.0009\) respectively; Figure 2.4). In the cecum, the ΔspiC and ΔspvB strains also colonized at lower levels than the wild-type strain (\(p=0.0006\), and \(p=0.0006\) respectively; Figure 2.4). In the ileum, the ΔspiC, ΔsseI, and ΔspvB strains colonized at significantly lower levels than the wild-type strain (\(p=0.0166\), \(p=0.0405\), and \(p=0.0011\) respectively; Figure 2.4). Taken together, these data suggest that the SpiC and SpvB proteins are important for *S. Typhimurium* colonization of intestinal sites in the typhoid fever model, while SseI appears to play a role in colonization of the ileum.
Figure 2.4 Colonization of the intestinal tract by SPI2 deletion strains

Bacterial counts of *S. Typhimurium* SPI2 mutants recovered from intestinal organs of C57BL/6 mice after oral infection. Mice were infected via oral gavage and sacrificed 5 days post-infection. Organs collected include ileum, cecum, colon. Counts are given as colony-forming units (CFU) per milligram of tissue. Error bars represent standard deviation from the means. A minimum of 5 mice was used for each SPI2 mutant. (**p < 0.001, *p < 0.05).
2.6.2 Colonization of gallbladder

*Salmonella* has been shown to replicate in epithelial cells in the gallbladder, and these bacteria can be found in the luminal bile of murine gallbladders (Menendez, Arena et al. 2009). The role of SPI2 effectors in bile replication has not been previously determined. Therefore, bacterial counts in the bile of infected mice were examined by extracting bile from the gallbladders and determining CFU. No statistically significant differences were found among the SPI2 effector deletion strains in bile (Figure 2.5). Despite the lack of significance, the results show a similar trend where the ΔssaR, ΔsseF, and ΔspvB strains replicated at levels lower than wild-type *Salmonella*. Because oral infection was used for all gallbladder infections, it is interesting that these strains, which are attenuated in systemic sites, were not significantly attenuated in the bile. However, this apparent effect could be a result of the low proportion of gallbladders that are infected, even during wild-type infections (Menendez, Arena et al. 2009). Because of the large variation in CFU within replicates in this model, statistical significance was not obtained, however the potential for biological significance is evident.
Figure 2.5 Colonization of gallbladder by SPI2 deletion strains
Bacterial counts of *S. Typhimurium* SPI2 mutants recovered from bile of C57BL/6 mice after oral infection. Mice were infected via oral gavage, sacrificed 5 days post-infection, gallbladders were removed, and bile was extracted. Counts are given as colony-forming units (CFU) per microliter of bile. Error bars represent standard errors of the means. A minimum of 5 mice was used for each SPI2 mutant.

2.6.3 Colonization of systemic organs

Since SPI2 is required for systemic spread during typhoid fever, we examined bacterial burden at systemic locations, including the liver, spleen, and mesenteric lymph nodes (MLN). The ΔssaR strain colonized at significantly lower levels than the wild-type strain in the liver, spleen, and MLN (p=0.0002, p=0.0002, and p=0.0004, respectively; Figure 2.6). In the liver, the ΔspiC and ΔspvB strains colonized at levels lower than the wild-type-infected mice (p=0.0028 and p=0.0023, respectively; Figure 2.6). In the spleen, the ΔspiC, ΔsseF, and ΔspvB strains did not colonize as well as the wild type (p=0.0028, p=0.0253, and p=0.0018, respectively; Figure 2.6). In the MLN, the ΔspiC, ΔsseF, and ΔspvB strains colonized significantly less than the wild
type ($p=0.0086$, $p=0.0107$, and $p=0.0102$, respectively; Figure 2.6). Therefore, as seen in the intestinal tract, the $\Delta \text{spiC}$ and $\Delta \text{spvB}$ strains were attenuated in systemic organs. Interestingly we found that the $\Delta \text{sseF}$ strain was attenuated in the MLN and spleen, but not in the intestinal organs. This data suggests an important role for the SpiC, SpvB, and SseF proteins in $S.$ Typhimurium virulence in mice.
Figure 2.6 Colonization of systemic organs by SPI2 deletion strains

Bacterial counts of *S. Typhimurium* SPI2 mutants recovered from systemic organs of C57BL/6 mice after oral infection. Mice were infected via oral gavage and sacrificed 5 days post-infection. Organs collected include liver, spleen and mesenteric lymph nodes (MLN). Counts are given as colony-forming units (CFU) per milligram of tissue. Error bars represent standard deviation from the means. A minimum of 5 mice was used for each SPI2 mutant. (*p < 0.05).
2.6.4 Early colonization in the typhoid model

To examine the early phases of *Salmonella* colonization in typhoid fever, we determined levels of colonization for three *Salmonella* strains, wild type, ΔsseF, and ΔspvB. The wild-type strain was used, which is invasive and replicates at high levels. At 3 days post-infection the wild-type strain colonized at moderate levels in all organs tested (Figure 2.7), the colonization at three days was lower than what we saw at five days post-infection (Figure 2.6). The ΔspvB strain was tested as this strain is known to be severely attenuated in systemic disease (McGhie, Brawn et al. 2009). We found that the ΔspvB strain colonized at significantly lower levels in the liver (p=0.0079), spleen (p=0.0159), colon (p=0.0317), and ileum (p=0.0317) (Figure 2.7). Finally, the ΔsseF strain was also examined, as the role of this effector in systemic disease is less well characterized and had a modest attenuation systemically at 5 days post-infection (Figure 2.6). We found that the ΔsseF strain only colonized at significantly lower levels in the colon (p=0.0317), while in the cecum, ileum and MLNs there is a trend for slightly lower levels of colonization (Figure 2.7). Therefore, these data generally support what was seen at 5 days post-infection, with ΔspvB strain being attenuated, and the ΔsseF strain colonizing at slightly lower levels than wild type.
Figure 2.7 Colonization of select strains early during infection
Bacterial counts of S. Typhimurium SPI2 mutants recovered from systemic and intestinal organs of C57BL/6 mice 3 days post-infection. Mice were infected via oral gavage. Counts given represent colony forming units per milligram of tissue. Error bars represent standard errors from the means, each group contained 5 mice. (*p < 0.05). Mesenteric lymph nodes (MLN).

2.7 Discussion

SPI2 effectors are known to be important for Salmonella replication within macrophages and systemic spread. This chapter provides a thorough and comparative analysis of SPI2 effectors in three different models of infection, namely an in vivo murine model for typhoid fever and three cell culture infection models, two using epithelial cells and the other macrophages. Many of the studies of Salmonella effectors published to date have focused on only one or a few SPI2 effectors, thus making comparisons between studies difficult. Therefore, the aim of this study was to test as many SPI2 deletion strains as possible under the same conditions, allowing for direct comparisons of the roles played by different SPI2 effectors during infection. The data

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confirms previously identified phenotypes and further characterizes other previously untested effectors.

In this study the ΔspvB strain was attenuated in both the typhoid model and the macrophage replication model, which supports the well-characterized role of this effector as an ADP-ribosylating protein that transfers ADP-ribose onto monomeric actin, which is needed for virulence (Lesnick, Reiner et al. 2001, Browne, Hasegawa et al. 2008). The ΔspvB strain colonized at statistically higher levels than the ΔssaR strain in the systemic sites, but not in the intestinal sites. This study shows that the ΔsseK1/K2/K3 triple knock-out strain was attenuated in macrophages, but not in any sites in the murine model or in the cultured epithelial cell model. This confirms previous reports that SseK1 and SseK2 are not required for systemic infection in mice (Kujat Choy, Boyle et al. 2004). Our macrophage results with the ΔsseK1/K2/K3 strain correlates with that found previously (Brown, Coombes et al. 2011). Brown et. al.(Brown, Coombes et al. 2011) did see a slight attenuation of the ΔsseK1/K2/K3 strain using a competitive index. The model presented here uses mouse infections with only one bacterial strain, therefore it is possible that by using a competitive index, more subtle colonization differences may be seen.

Because SpiC is thought to interact with proteins in the host cell, manipulating host cell function, (McGhie, Brawn et al. 2009) it was included in this screen. The ΔspiC strain was attenuated in both the typhoid and the macrophage replication model in this study. This information supports the model proposed by Yu et al. (Yu, McGourty et al. 2010), whereby SpiC is involved in sensing the pH of the environment and permitting effector translocation. The results of these screens support the current understanding that SpiC activity is needed for SPI2 effector secretion, and its importance during infection. It should also be noted that the ΔspiC strain replicated at levels significantly higher than the ΔssaR strain, which does not secrete any
SPI2 effectors (Brumell, Rosenberger et al. 2001). This work also highlights the importance of SpiC for bacterial replication in macrophages.

SseF and SseG are involved in the formation of SIFs and may play a role in microtubule bundling (Guy, Gonias et al. 2000, Kuhle and Hensel 2002, Kuhle, Jackel et al. 2004). This work also provides evidence for the slight attenuation of the ΔsseF strain in the mouse typhoid fever model. However, the ΔsseF, ΔsseG, and ΔsseFG strains do not replicate significantly less than the wild-type strain in the macrophage model. It is striking that the ΔsseF strain was attenuated, but there was not a statistically significant attenuation in the ΔsseG and ΔsseFG strains in the typhoid model, as the SseF and SseG proteins have been shown by biochemical assays to directly interact with each other (Deiwick, Salcedo et al. 2006). However, it has also been shown that while SseF and SseG frequently co-localize, they are also found separately within the cell (Kuhle, Jackel et al. 2004). Because the ΔsseF strain was attenuated in systemic organs, this mutation was complemented, and was found to replicate at levels comparable to wild type.

It is interesting that the ΔspiC and ΔspvB strains were attenuated in both intestinal and systemic sites. This led us to speculate that these effectors may have roles in both anatomical sites. It is also possible that attenuation of these strains in the intestine influences their replication at systemic sites, or perhaps systemic attenuation leads to lower levels in the intestine due to low levels of intestinal re-seeding. In addition, we found that at 3 days post infection, the ΔsseF, and ΔspvB strains did not appear to be as attenuated as at 5 days, indicating these effectors may play an important role in replication at systemic sites.

Salmonella counts in the bile were examined to determine if there were correlations with the systemic sites, which could support the concept that the gallbladder is involved in the re-seeding of the intestine by systemic bacteria (Everest, Wain et al. 2001, Lawley, Bouley et al.
After examining the SPI2 deletion strains in the gallbladder, no statistically significant differences in the number of bacteria in bile were seen when compared to wild type, likely due to the fact that only some mice had their gallbladders infected. Though not statistically significant, this data does suggest some interesting trends. Firstly, the ΔssaR strain does not effectively colonize systemic sites, and is also seen at low levels in the bile. The same pattern is seen for the ΔspvB and ΔsseF strains. This supports the notion that bacteria in the gallbladder correlate to the numbers of systemic bacteria, and a threshold of *Salmonella* must be reached before bacteria begin to colonize the gallbladder (Menendez, Arena et al. 2009).

This study provides an overview of SPI2 effector contributions to *Salmonella* pathogenesis. Furthermore, it provides a tool to allow *Salmonella* researchers to compare and contrast the roles of SPI2 effectors tested under the same conditions in a Typhoid murine model, a macrophage replication model, and two epithelial cell replication models.

### 2.8 Summary

In summary, these results confirm that SPI2 effectors are not required for epithelial cell replication and that multiple SPI2 effectors are required for macrophage replication. The *in vivo* studies show that some SPI2 effectors may in fact play a role in intestinal colonization, as well as systemic spread of the disease (Table 2.4). Interestingly, none of the mutants tested here were significantly attenuated for growth in bile, despite their attenuation in both systemic and intestinal sites. This study enables direct comparison between SPI2 effectors in various models of infection and provides groundwork for further studies on the roles of *Salmonella* SPI2 effectors, and the contribution of these SPI2 deletion strains to other aspects of infection.
Table 2.4 Summary of SPI2 deletion strains contribution to virulence

In this table, (+++) indicates strains that replicated/colonized as well as wild-type *Salmonella*. (++) indicates significantly lower levels of replication with p<0.05. (+) indicates significantly lower levels of replication than the wild-type *Salmonella* with p<0.001. (nd) indicates data not determined.

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<thead>
<tr>
<th>Strain</th>
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<th>Typhoid fever model</th>
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Chapter 3: Arachidonic acid metabolism is altered during *Salmonella* infection of macrophages

3.1 Abstract

*Salmonella enterica* are Gram-negative bacterial pathogens responsible for enteric fever and gastroenteritis. *Salmonella* cross the intestinal epithelial barrier and are taken up by phagocytes, enabling their spread to systemic sites. *Salmonella* use two type three secretion systems encoded by *Salmonella* pathogenicity islands (SPI) 1 and 2 to inject effectors into the host cell. SPI1 is required for the invasion of non-phagocytic cells, whereas SPI2 is required for intracellular survival and replication within phagocytes, in a specialized phagosome called the *Salmonella* containing vacuole (SCV). There are still many aspects of *Salmonella*’s interactions with the host that are not fully characterized. In the host, arachidonic acid metabolism leads to the production of eicosanoids, a collection of small molecule hormones including the prostaglandins (PG), leukotrienes, and thromboxanes. Our group has previously found that arachidonic acid metabolism is increased and that the transcription of enzymes involved in eicosanoid synthesis is altered during *Salmonella* infection of mice. In this chapter, the interaction between eicosanoids, with a focus on the PGs, and *Salmonella* is examined more closely. PGs are produced by multiple cell types and are involved in many functions, including inflammation. PG synthesis begins through the activity of the cyclooxygenase (PTGS/COX) enzymes, COX1 and COX2, which mediate the rate-limiting step in the production of all PGs and thromboxanes. COX1 is constitutive, while COX2 is induced by inflammatory stimuli. This chapter demonstrates that *Salmonella* infection induces the production of COX2 and PGE
synthase (PTGES) transcripts in macrophages, while the expression of thromboxane B\textsubscript{2} synthase (TBXAS1) is reduced. The enzymes involved in leukotriene synthesis in macrophages were decreased by *Salmonella* infection. In addition, the SPI2 deletion strains described in the previous chapter were tested for their ability to alter the expression of TBXAS1, PTGES, and COX2. Two of the effectors secreted by the SPI2 T3SS, SseF and SseG, were found to be partially responsible for the induction of COX2 seen during *Salmonella* infection. SseF and SseG are involved in the formation of tubular structures extending from the SCV, termed *Salmonella* induced filaments (SIF), and the localization of the SCV, but no role for these effectors in PG production has been identified to date. This chapter demonstrates not only the involvement of arachidonic acid metabolism in *Salmonella* infection, but also shows that *Salmonella* is able to modulate it in a SPI2 dependent manner.

### 3.2 Introduction

*Salmonella enterica* are Gram-negative, facultative intracellular pathogens that can cause enteric (typhoid) fever and gastroenteritis. (Freeman, Ohl et al. 2003, Kuhle and Hensel 2004). After ingestion of *S. enterica*, the bacteria cross the intestinal barrier, are taken up by phagocytes, replicate, and in systemic disease spread to sites including the liver, and spleen. (Kohbata, Yokoyama et al. 1986, Jones, Ghori et al. 1994, Vazquez-Torres, Jones-Carson et al. 1999, Kuhle and Hensel 2004, Haraga, Ohlson et al. 2008). *Salmonella* replicates in a special vacuole called the *Salmonella*-containing vacuole (SCV) (Kuhle and Hensel 2004, Browne, Hasegawa et al. 2008, Poh, Odendall et al. 2008, van der Heijden and Finlay 2012). *Salmonella* use two type 3 secretion systems (T3SS) encoded by SPI1 and SPI2 to mediate bacterial uptake and replication (Haraga, Ohlson et al. 2008, van der Heijden and Finlay 2012). SPI2 is primarily

SseF and SseG are two effectors translocated by the SPI2 T3SS and they contain 2 and 3 hydrophobic domains, respectively (Salcedo and Holden 2003, Abrahams, Muller et al. 2006, Muller, Chikkaballi et al. 2012). They are involved in the formation of SIFs and play a role in microtubule bundling (Guy, Gonias et al. 2000, Kuhle and Hensel 2002, Kuhle, Jackel et al. 2004, Muller, Chikkaballi et al. 2012). The first 127 amino acids of SseF, including the first transmembrane (TM) domain, are thought to contribute to the translocation of the protein, whereas the second TM domain has been found to be important for the recruitment of dynein, a microtubule motor protein, microtubule bundling, the intracellular positioning of the SCV, and SIF formation in epithelial cells (Abrahams, Muller et al. 2006). A 6 amino acid residue in the second transmembrane domain has recently been found to be required for the proper formation of SIFs, and for the localization of *Salmonella* microcolonies close to the microtubule organizing center in epithelial cells (Muller, Chikkaballi et al. 2012). Infection with *Salmonella* strains lacking SseF and SseG leads to the formation of pseudo-SIFs, which are non-continuous structures with punctuate association with LAMP1, LAMP2, LAMP3 and V-ATPase in epithelial cells (Kuhle and Hensel 2002). LAMPs (Lysosomal-associated membrane protein) are endosome markers, which associate with the SCV and SIFs during wild-type *Salmonella* infection (Steele-Mortimer, Meresse et al. 1999). SseF and SseG co-localize with LAMP, and the
distribution of SseF is dependent on a functional microtubule cytoskeleton (Kuhle, Jackel et al. 2004). SseF and SseG frequently colocalize in epithelial cells, but are occasionally found separately (Kuhle, Jackel et al. 2004). However, the functions of SseF and SseG can be completed by a fusion protein of both effectors, implying that the functions of these proteins are linked during normal Salmonella epithelial cell infection (Muller, Chikkaballi et al. 2012). When SseG was expressed via transfection in host cells, it was found to co-localize with the Golgi apparatus; however, when the protein was expressed via infection, it did not localize with the Golgi (Kuhle, Jackel et al. 2004). In another study, also completed in epithelial cells, SseG was involved in the localization of Salmonella with the Golgi apparatus in epithelial cells and was necessary, but not sufficient, for SCV localization with the trans-Golgi network (Salcedo and Holden 2003). Taken together, these data show that SseF and SseG play an important role in the formation of SIFs and SCV localization in epithelial cells; however little is known about their role in macrophages.

Our group has recently completed a metabolomics study of Salmonella infection in mice, which found that many host hormone pathways are affected by this pathogen (Antunes, Arena et al. 2011). Specifically, it was found that arachidonic acid (AA) metabolism is increased during Salmonella infection of mice (Appendix A Figure 1). AA metabolism leads to the production of eicosanoids, which are a class of lipid hormone mediators which can act via paracrine and autocrine mechanisms and can be produced by most cells (Funk 2001). They include the prostaglandins (PGs), thromboxanes, leukotrienes, and others (Matsuoka and Narumiya 2008). Among the diverse effects mediated by eicosanoids, PGs in particular, are the induction and resolution of acute inflammation induced by bacterial infection (Yoshikai 2001). Macrophages and dendritic cells are thought to secrete PGs in response to LPS (Bowman and Bost 2004).
PGs are derived from arachidonic acid (AA), which is released from cell membranes into the cytosol by phospholipase A2 (PLA2) (Figure 3.1) (Funk 2001). AA is then modified by the cyclooxygenase enzymes (COX), which is inserted into the membrane of the endoplasmic reticulum or nuclear envelope (Funk 2001, Wan and Coveney 2009). Two isoforms of COX have been identified, COX1 and COX2. COX1 is constitutively expressed in most tissues, whereas COX2 is induced by pro-inflammatory agents, including bacterial components (Ghosh, Misukonis et al. 2001, Yoshikai 2001). Two waves of COX2 expression have been identified, the first is involved with inducing inflammation and high levels of PGE₂, the second wave is much stronger and is associated with resolution of inflammation and high levels of PGD₂ and 15d-PGJ₂ levels (Gilroy, Colville-Nash et al. 1999). After AA is modified by COX, one of several PG synthase enzymes will act upon it to form different PGs. Prostaglandin D₂ (PGD₂) is formed by PGD synthase (PTGDS), PGE₂ by PTGES, and so forth. Once PGs are made, they are released from the cell via PG transporters (Funk 2001). See Figure 3.1 for a schematic of arachidonic acid metabolism and the production of the prostaglandins.
Figure 3.1 Arachidonic acid metabolism
Enzymes involved in the pathways that were examined in this study are printed in grey italics, and include: PLA2G4A, ALOX5, ALOX5AP, LTA4H, CYP4F, LTC4S, PTGS2/COX2, PTGES, and TBXAS1.

The role of PGs in inflammation is very complex, and not fully understood. PGs can play both anti- and pro-inflammatory roles depending on the context, and are important for multiple pathophysiological functions (Funk 2001, Matsuoka and Narumiya 2008). Both COX1 and COX2 were found to play a protective role in DSS induced colitis in mice (Morteau, Morham et al. 2000). A pro-inflammatory role is traditionally attributed to COX2; this can be seen in the pharmaceutical use of COX2-specific inhibitors. Among all PGs, the role of PGE2 in inflammation is perhaps the best studied. PGE2 is the most abundant PG and is traditionally thought to play an immunosuppressive role (Sakata, Yao et al. 2010). This is seen in its inhibition of IL-1 and TNF-α production and its induction of IL-6 and IL-10 production by macrophages (Strassmann, Patil-Koota et al. 1994, Kim and Hahn 2000, Treffkorn, Scheibe et al.)
2004, Sakata, Yao et al. 2010). However, there is also evidence that PGE$_2$ may play an immunoactivator role by facilitating $T_{H1}$ differentiation and $T_{H17}$ cell expansion in vitro, a process mediated by the PGE$_2$ receptors 2 and 4 (Yao, Sakata et al. 2009, Sakata, Yao et al. 2010). PGE$_2$ and PGI$_2$ work in conjunction with histamine and bradykinin to increase vascular permeability and blood flow at the site of inflammation (Ikeda, Tanaka et al. 1975, Yoshikai 2001). However, high levels of PGE$_2$ reduce vascular permeability, and therefore the effect of PGE$_2$ may be concentration-dependent (Yoshikai 2001). The role of PGE$_2$ in LPS-induced inflammatory responses remains uncertain as the addition of exogenous PGE$_2$ leads to the expression of CD14 receptors, which augments cellular response to LPS (Iwahashi, Takeshita et al. 2000), while inhibiting LPS-induced TNF-$\alpha$ and TLR2 expression (Yoshikai 2001). PGE2 also enhances the production of IL-10 by macrophages stimulated by LPS (Strassmann, Patil-Koota et al. 1994, Yoshikai 2001). In acute inflammation, PGE$_2$ is a central mediator of LPS-induced fever; it enhances IL-1$\beta$ production by macrophages, and in conjunction with TNF-$\alpha$ stimulates the production of IL-12 by dendritic cells, which leads to the activation of NK cells and $T_{H1}$ cell differentiation (Yoshikai 2001, Matsuoka and Narumiya 2008). PGE$_2$ also inhibits the production of IL-12p70, while leading to reduced expression of the IL-12 receptor (Bowman and Bost 2004). Overall, PGE$_2$ is thought to play a role in the early phases of inflammation (Yoshikai 2001).

The role of PGs during Salmonella infection is beginning to emerge. Salmonella, among other entero-invasive bacteria, triggers the release of PGE$_2$ (Bowman and Bost 2004). The activity of PLA2, which releases AA from membranes, is required for Salmonella invasion into epithelial cells (Pace, Hayman et al. 1993). The addition of a COX inhibitor had no effect on invasion of a wild-type Salmonella strain (Pace, Hayman et al. 1993). In bone marrow derived
macrophages, an increase in COX2 expression was seen in response to both live and killed *Salmonella*, but an increase in PGE2 was only seen with viable *Salmonella*, and this increase was blocked by COX2 inhibitors (Bowman and Bost 2004). Interestingly, *Salmonella*-derived LPS was not as potent of an inducer of PGE2 as live bacteria (Bowman and Bost 2004). Studies looking at PGs and *Salmonella in vivo* have shown that COX2 mRNA levels are increased in mouse lymph nodes three days post infection in comparison to mice treated with UV-inactivated bacteria (Bowman and Bost 2004). It has also been shown that macrophages and dendritic cells are responsible for the increased COX2 expression in mesenteric lymph nodes upon *Salmonella* infection (Bowman and Bost 2004). In addition proteomic analysis revealed an increase in COX2 production by macrophages in response to *Salmonella* (Shi, Chowdhury et al. 2009). *Salmonella*-infected mice survive for longer periods when treated with COX2 inhibitors, although *Salmonella* counts in the mesenteric lymph nodes are higher (Bowman and Bost 2004). Furthermore, our group has recently shown that the PG pathway is significantly altered by *Salmonella* infection of mice (Antunes, Arena et al. 2011). Specifically, we found that the production of PGE2, TXB2, and 15d-PGJ2 were increased in the feces of infected mice (Appendix A Figure 2).

Uchiya and Nikai (2004) found that the SPI2 effector SpiC was responsible for part of the increase in COX2 caused by *Salmonella* infection of J774E macrophage cells (Uchiya and Nikai 2004). They showed that the increase in COX2 was dependent on ERK1/2 signaling and found a SPI2-dependent increase in the expression of EP2 (the PGE2 receptor) in response to *Salmonella* infection. In addition, they found a reduction in the growth of *Salmonella* in cells treated with a COX2 inhibitor. One major caveat of this work is that the authors did not address the possibility that a SpiC deletion strain does not translocate many SPI2 effectors (Yu, Ruiz-Albert et al.
2002). Recently it was found that SpiC is a component of a pH sensing complex which controls effector secretion (Yu, McGourty et al. 2010).

In this study, the effect of *Salmonella*, and specifically the role of SPI2, in arachidonic acid metabolism was examined. The effect of *Salmonella* infection on the expression of a variety of enzymes involved in arachidonic acid metabolism was explored to define which pathways are involved in Salmonellosis. The panel of SPI2 deletion strains constructed in the previous chapter was used to explore the role of SPI2 in modulating PG synthesis. This was done in part to address the concerns with Uchiya and Nikai’s 2004 study (Uchiya and Nikai 2004), which indicated that SpiC was required for COX2 induction, and to further characterize the role of SPI2 during *Salmonella* infection. While studying the effect of multiple SPI2 mutants on PG gene expression in macrophages using quantitative real-time PCR, it was shown that SseF and SseG are responsible for a large portion of the induction of COX2 seen during *Salmonella* infection. Finally, the effect of the addition of specific eicosanoids to macrophage cultures on colonization by *Salmonella* was determined.

3.3 Methods and materials

3.3.1 Chemical reagents

Streptomycin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). 15-deoxy-Δ^{12,14}-prostaglandin J2 (15d-PGJ_{2}), thromboxane B2, prostaglandin E2, and aldosterone were purchased from Cayman Chemicals (Ann Arbor, USA).

3.3.2 Cell culture and *Salmonella* infections

RAW264.7 cells (American Type Cell Culture, Manassas, USA) were seeded at 1x10^5 cells per well in 24-well plates and grown for 24 hours at 37°C, 5% CO₂. All cells were grown in
Dulbecco’s modified Eagle medium (DMEM) with high glucose (HyClone, Waltham, USA) and 10% FBS (HyClone), 1% NEAA (Gibco, Carlsbad, USA), and 1% GlutaMax (Gibco).

For infections, overnight cultures of *Salmonella enterica* serovar Typhimurium SL1344, grown in LB with streptomycin (Sigma-Aldrich) at 37°C with aeration, were diluted 1:100 in LB and sub-cultured for 3 hours at 37°C with aeration. Bacteria were pelleted and resuspended, and 10⁶ bacteria were added to each well in DMEM for 30 minutes. Cells were washed 3 times with PBS, then incubated in DMEM containing 100 µg/mL gentamicin for 60 min, then 10 µg/mL gentamicin for the remainder of the 24 hour time point. The ΔsseFG deletion strain was complemented, as done previously in Chapter 2, section 2.3.1.

### 3.3.3 RNA extraction and cDNA synthesis

After 24 hours of infection, macrophage cells were washed 3x with PBS. RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), with the on-column DNA digestion (Qiagen). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). All procedures were carried out according to the manufacturers instructions. RNA samples were all stored at -80°C, and cDNA samples were stored at -20°C.

### 3.3.4 Quantitative real-time PCR (qRT-PCR)

For qRT-PCRs, the QuantiTect SYBR Green PCR Kit (Qiagen) and the Applied Biosystems (Foster City, USA) 7500 system was used. Reactions contained forward and reverse primers at 0.4 µM each. All results were normalized using the mRNA levels of the acidic ribosomal phosphoprotein PO as baseline (Antunes, Arena et al. 2011). Averages of the data obtained with untreated samples were normalized to 1 and the data from each sample was normalized accordingly. Primer sequences are listed in Table 3.1.
### Table 3.1 qRT-PCR primers for eicosanoid pathways

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2G4A</td>
<td>TTGGTCCCAGTTGCAGAAAT</td>
<td>GAAGGCACAGAGAGGCTGA</td>
</tr>
<tr>
<td>PTGS2</td>
<td>GGGGTGTCCCTCAACTTCTTTCA</td>
<td>TGGGGAGCAGTTCAGTTGA</td>
</tr>
<tr>
<td>(COX2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBXAS1</td>
<td>ATGTCCAGATACGGCAGACC</td>
<td>GAGGCTTCTGAAAGAGGTTG</td>
</tr>
<tr>
<td>PTGES</td>
<td>ATGAGTACACGAAGCCGAGG</td>
<td>CCAAGTTACAGGAGTGACCAGGGG</td>
</tr>
<tr>
<td>ALOX5</td>
<td>ATATCTCGGGGCGAGATCCTT</td>
<td>GTAAGAAGCTGGAGCAGC</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>CACAAGGAGAGAAGGAGG</td>
<td>GGGAGAAGCAGTTCAGAGG</td>
</tr>
<tr>
<td>LTC4S2</td>
<td>CCTTCGTGCAGAGATACCTC</td>
<td>GGCAACATGAGGACAGAAGT</td>
</tr>
<tr>
<td>LTC4S3</td>
<td>CCTTCGTGCAGAGATACCTC</td>
<td>CTAATATTCTGCCTGTGGGCTTG</td>
</tr>
<tr>
<td>LTC4S4</td>
<td>TTACCCTGGCGTCGAAGAC</td>
<td>GCTCTTCTGGCATTCCAGTCAC</td>
</tr>
<tr>
<td>LTA4H2</td>
<td>ACCTTGACTTTCTCAAAGGG</td>
<td>CACAGGGAGGAGAATGCTGCG</td>
</tr>
<tr>
<td>LTA4H3</td>
<td>CTTTGCACGTCTCCAGAGG</td>
<td>GGTCCAGTCACAGGAGGAGA</td>
</tr>
<tr>
<td>LTA4H4</td>
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<tr>
<td>CYP4F16</td>
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<tr>
<td>CYP4F18</td>
<td>ACAGGAGTCCTCCTCTGAGC</td>
<td>TGAGAAGCCTCCGCTCC</td>
</tr>
</tbody>
</table>

### 3.3.5 CFU determination

To determine bacterial colonization, infected cells were lysed in 250 µL of 1% Triton X-100 (BDH, Yorkshire, UK), 0.1% sodium dodecyl sulfate (Sigma-Aldrich). Serial dilutions were plated on LB plates containing 100 µg/mL of streptomycin (Sigma-Aldrich). Plates were incubated for approximately 15 hours at 37°C, then bacteria were enumerated.

### 3.3.6 Hormone addition to Salmonella infection

Macrophages were seeded as above, with 20 µM aldosterone, PGE<sub>2</sub>, TBXB<sub>2</sub>, and 2 µM 15d-PGJ<sub>2</sub> in medium, and incubated for 24 hours. Cells were checked for confluency, to ensure hormones were not causing significant cell lifting. Salmonella infections were carried out as above, with all DMEM media containing 20 µM or 2 µM of hormones, as above. 2 µM 15d-PGJ<sub>2</sub>...
was used because 20 µM was found to cause increased cell lifting, while 2 µM had no effect. At 24 hours post-infection, macrophages were lysed and bacteria enumerated as above.

3.3.7 Statistical analysis

Data were analyzed by nonparametric Mann-Whitney t tests with 95% confidence intervals using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, USA). For each figure, the term “measurements” refers to the combination of both technical and experimental replicates. Each experiment was repeated a minimum of two times, with multiple technical replicates. Thus, for example “8 measurements” would refer to two separate experiments with 4 technical replicates each, “4 measurements” would refer to two separate experiments with two technical replicates each.

3.4 Results - *Salmonella* infection alters eicosanoid production

3.4.1 Prostaglandin and thromboxane pathways are affected by *Salmonella* infection

To begin to understand how arachidonic acid metabolism is affected by *Salmonella* infection, the expression of enzymes in this pathway was examined (See Chapter 3 Introduction, Figure 3.1). In the prostaglandin and thromboxane pathways, the expression levels of four enzymes were determined by quantitative real-time PCR (Figure 3.2). The expression of phospholipase A2 (PLA2G4A), which allows for the release of arachidonic acid from the membrane, was largely unaffected by *Salmonella* infection of macrophages. From this point arachidonic acid can enter either the prostaglandin/thromboxane pathway or the leukotriene pathway. Interestingly, the levels of TBXAS1 mRNA, leading to thromboxane synthesis, were significantly reduced during *Salmonella* infection. In contrast, the expression levels of COX2
and PTGES were both significantly increased (Figure 3.2). This data supports the idea that the prostaglandin pathway is induced during *Salmonella* infection.

**Figure 3.2 Expression of enzymes involved in the prostaglandin and thromboxane pathways**
Expression levels from RAW264.7 macrophages infected with *Salmonella* at 24 hours post-infection were examined using qRT-PCR. Data was normalized to uninfected samples. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. (**p<0.001, ***p<0.0001). (UI – uninfected, SL – wild-type *Salmonella* SL1344).

### 3.4.2 Leukotriene pathway is repressed during *Salmonella* infection

While the prostaglandin pathway is activated by *Salmonella* infection, the leukotriene pathways are inhibited by *Salmonella* infection of macrophages (Figure 3.3). The expression of ALOX5, the enzyme mediating the initial step in the leukotriene pathway, was unaffected by
Salmonella infection. However, its regulator, the ALOX5 activating protein (ALOX5AP), was significantly down regulated, implying a reduction in the activity of ALOX5 at the post-transcriptional level. The expression of the three isotypes of LTC4S that were tested (LTC4S2, LTC4S3, and LTC4S4) was reduced during Salmonella infection. The expression of two of the isotypes of LTA4H (isotypes 2 and 3) were not significantly different between infected and uninfected samples; however, isotype 4 was significantly reduced. In addition, 3 of the 4 isotypes of CYP4F were all down regulated by Salmonella infection. Together, this data shows that the expression of genes involved in the leukotriene pathway is reduced during Salmonella infection.
Figure 3.3 Expression of enzymes involved in the leukotriene pathway
Expression levels from RAW264.7 macrophages infected with *Salmonella* at 24 hours post-infection were examined by qRT-PCR. Data was normalized to uninfected samples. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. (**p<0.001). (UI – uninfected, SL – wild-type *Salmonella* SL1344).
3.5 The role of SPI2 in prostaglandin and thromboxane production

3.5.1 Involvement of SPI2 effectors in TBXAS1 repression

Nine of the SPI2 deletion strains used in the previous study and chapter (Buckner, Croxen et al. 2011) were tested for their effect on TBXAS1 expression in RAW264.7 macrophages (Figure 3.4). Interestingly, most of the deletion strains tested induced higher levels of TBXAS1 expression than the wild-type strain. The ΔspiC and ΔspvB strains induced significantly higher levels of TBXAS1 (p<0.001). This is not surprising, as previously it has been shown that these are significantly impaired in colonizing macrophages (Figure 2.3) (Buckner, Croxen et al. 2011). In addition, the ΔsteC strain was also found to colonize macrophages at lower levels than wild-type Salmonella. Therefore, the lower bacterial burden may be why these strains have similar levels of TBXAS1 transcripts to uninfected samples. The ΔsseFG, ΔsseG, ΔsspH2, ΔsteA, ΔsteC, and ΔslrP strains also caused an increase in the expression of the TBXAS1 transcript compared to wild-type Salmonella. Together, these data indicate that SPI2 effectors may have a role in the reduction of TBXAS1 transcripts seen during macrophage infection with Salmonella.
Figure 3.4 SPI2 effectors affect TBXAS1 expression
The expression of TBXAS1 was determined by qRT-PCR after infection of RAW264.7 macrophages for 24 hours with Salmonella strains lacking individual SPI2 effectors. Data was normalized to uninfected samples. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. All deletion strains were statistically compared to wild-type SL1344 expression. (*p<0.05**p<0.001, ***p<0.0001).

3.5.2 Involvement of SPI2 effectors in PTGES expression
Fifteen SPI2 deletion strains were tested for their effect on the induction of PTGES transcripts in RAW264.7 macrophages infected for 24 hours (Figure 3.5). Two of the deletion strains, ΔpipB2 and ΔgogB both had increased induction of PTGES when compared to wild-type Salmonella. Conversely, the ΔsifB, ΔsseK1/2/3, ΔsteC, and ΔspvB strains, all of which have previously shown reduced bacterial colonization of macrophages (Figure 2.3), showed no changes in PTGES induction when compared to wild-type Salmonella. However, the ΔspiC strain, which is thought to not secrete any SPI2 effectors (Yu, McGourty et al. 2010), and had
significantly reduced bacterial colonization of macrophages (Figure 2.3), showed reduced levels of PTGES mRNA. Thus, it is possible that SPI2 effectors, and not the mere presence of bacteria in the cells, may be responsible for the induction of PTGES. Potential SPI2 effector candidates for this role in PTGES induction include ΔsspH2, ΔsseF, ΔsseG, (ΔsseFG) and ΔslrP, as all of these strains colonize macrophages well but show reduced induction of PTGES. Together, these data raise the possibility that SPI2 effectors may function to control a portion of the expression of PTGES.

Figure 3.5 SPI2 effectors affect PTGES expression
The expression of PTGES was determined by qRT-PCR after infection of RAW264.7 macrophages for 24 hours with *Salmonella* strains lacking individual SPI2 effectors. Data was normalized to uninfected samples. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. All deletion strains were statistically compared to wild-type SL1344. (*p<0.05, **p<0.001, ***p<0.0001).
3.5.3 Involvement of SPI2 effectors in COX2 expression

Twelve of the SPI2 deletion strains were tested for their induction of COX2 expression during macrophage infection (Figure 3.6). Six of the twelve SPI2 deletion strains tested showed reduced levels of PTGS2/COX2 induction, including ΔsseFG, ΔsseF, ΔsseG, ΔspvB, ΔspiC, and ΔslrP. Of these strains, both the ΔspvB and the ΔspiC colonize at lower levels, which could account for the reduced induction of COX2. Following this logic, it is surprising that the ΔsseF, ΔsseG, ΔsseFG, and ΔslrP strains are all associated with reduced COX2 expression, despite colonizing macrophages at levels similar to wild-type Salmonella. Conversely, ΔsifB, ΔsseK1/2/3, and ΔpipB2 all had higher levels of COX2 mRNA expression than wild-type Salmonella. Both the ΔsifB and ΔsseK1/2/3 strains colonized macrophages poorly, but here show increased COX2 expression.
The expression of PTGS2 (COX2) was determined by qRT-PCR after infection of RAW264.7 macrophages for 24 hours with *Salmonella* strains lacking individual SPI2 effectors. Data was normalized to uninfected samples. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. All deletion strains were statistically compared to wild-type SL1344. (*p<0.05, **p<0.001, ***p<0.0001).

### 3.6 The role of SseF and SseG in prostaglandin production

#### 3.6.1 SseF and SseG induce COX2 expression during *Salmonella* infection

Because the *ΔsseF, ΔsseG*, and *ΔsseFG* strains showed interesting phenotypes in the eicosanoid SPI2 screen (Figures 3.5 and 3.6), these strains were examined more closely. To ensure there were no polar or un-accounted for effects of the deletions of the *sseF* and *sseG* genes on the induction of COX2, the complemented *ΔsseFG* double mutant strain, was tested in this macrophage infection model (Figure 3.7). Similar to what was seen previously, the *ΔsseF, ΔsseG*, and *ΔsseFG* strains all had significantly lower levels of COX2 induction, below 50% of what was seen with wild-type *Salmonella*. Furthermore, by complementing these mutations, the
level of COX2 mRNA was significantly increased. In fact, levels in the complemented strain were even higher than in wild-type Salmonella. Together, these data show that SseF and SseG are necessary for the full induction of COX2 that is seen during wild-type Salmonella infection of macrophages.

![Graph showing relative expression of COX2](image)

**Figure 3.7 SseF and SseG are required for full induction of COX2**

The ΔsseF, ΔsseG, and ΔsseFG deletion strains and the SseFG complemented strain were examined for their role in COX2 induction. RAW264.7 macrophages were infected with bacterial strains for 24 hours, after which RNA was extracted and qRT-PCR analysis was performed for COX2 expression. Results shown are displayed as a percentage of wild-type expression and are averages of a minimum of eight measurements, with standard errors of the means shown. Data was statistically compared to wild-type SL1344. (*p<0.05, **p<0.001).

### 3.6.2 Effect on COX2 is not due to colonization defects

To ensure that the effect of the ΔsseF, ΔsseG and ΔsseFG deletions on COX2 expression was not due to any reductions in bacterial loads, CFUs of the double mutant and the complemented strain were closely examined (Figure 3.8). There were no differences in the numbers of the CFUs of the ΔsseFG double mutant when compared to the wild-type Salmonella
strain SL1344. The complemented strain did colonize the macrophages better than the wild-type, which may account for the level of COX2 induction that was even higher than wild-type *Salmonella*. In conjunction with the qRT-PCR study, this data indicates that the SPI2 effectors SseF and SseG are intricately involved in the induction of COX2 that is seen during macrophage infections.

Figure 3.8 Colonization of macrophages by ΔsseFG and complemented sseFG strain
RAW264.7 macrophages were infected with a multiplicity of infection of 10 for 24 hours. Then, cells were lysed and bacteria were enumerated. Results shown are displayed as a percentage of wild-type colonization and are averages of a minimum of eight measurements, with standard errors of the means shown. (*p<0.05, N.S. not significant).

### 3.7 Arachidonic acid metabolites alter macrophage colonization by *Salmonella*

The above data indicates the prostaglandin pathway is altered during *Salmonella* infection. To further characterize these associations, some of the downstream products of COX2, TBXAS1, and PTGES were tested. Thromboxane B$_2$ (TBXB$_2$), prostaglandin E$_2$ (PGE$_2$), 15d-prostaglandin J$_2$ (15d-PGJ$_2$) and the steroid hormone aldosterone were all added to macrophages
prior to infection with *Salmonella*. After 24 hours of infection bacteria were enumerated. The addition of TBXB2, PGE2 and 15d-PGJ2 all caused reductions in bacterial counts (Figure 3.9), with 15d-PGJ2 being the most potent at reducing *Salmonella* colonization. This is particularly interesting because the concentration of 15d-PGJ2 used was 10x lower than the other hormones. Aldosterone, which was used as a control because it is from a different hormone pathway, had no significant effects on *Salmonella* colonization. Because 15d-PGJ2 had such a drastic effect on *Salmonella* colonization, its role was further explored in the upcoming chapter.

![Figure 3.9](image)

**Figure 3.9 Addition of hormones to *Salmonella* infection of macrophages**
The effects of the addition of thromboxane B2, prostaglandin E2, and 15d-prostaglandin J2, all of which are downstream products of COX2, and the steroid hormone aldosterone to RAW264.7 macrophages during wild-type *Salmonella* SL1344 infection was studied. 24 hours post infection cells were lysed and bacteria were enumerated. Results shown are averages of a minimum of four measurements, with standard errors of the means shown. (*p<0.05).
3.8 Discussion

This chapter provides evidence that arachidonic acid metabolism is altered during *Salmonella* infection of macrophages at the mRNA level. Specifically, the prostaglandin pathway is upregulated by *Salmonella* infection, whereas the thromboxane and leukotriene pathways are generally downregulated by infection. Many of the SPI2 deletion strains that were tested had different effects on TBXAS1, PTGES, and COX2 expression when compared to wild-type *Salmonella*. Specifically, the ΔsseF, ΔsseG, and the ΔsseFG deletion strains induce COX2 significantly less than wild-type *Salmonella*, despite colonizing at similar levels.

The increased expression levels of COX2 and PTGES seen in macrophage infections correlate well with what was observed in the livers of *Salmonella* infected mice (Appendix A Figure 3) (Antunes, Arena et al. 2011). Furthermore, it fits with the established role of prostaglandins in inflammation and infection, as discussed in the introduction. Surprisingly, the expression of TBXAS1 was reduced in macrophages infected with *Salmonella*, whereas in mice we saw an increase in TBXAS1 expression in the liver (Appendix A Figure 3) (Antunes, Arena et al. 2011). This difference may be due to the fact that liver samples have a variety of cells, whereas the experiments presented here were performed in monoculture. The fact that this data shows no significant changes in PLA2G4A transcripts, which is the initial step in all the pathways examined here, leads to a few tentative conclusions. Firstly, it is possible that phospholipase A2 is being regulated at the post-transcriptional level. Cytosolic PLA2 can be regulated by phosphorylation and changes in calcium, which induce the proteins translocation to the membrane, thus allowing access to substrate (Ghosh, Tucker et al. 2006, Tucker, Ghosh et al. 2009). Secondly, it is possible that the release of arachidonic acid from the membrane by PLA2 is not increased, but that during infection the activity of pathways is shifted such that some are
shut down, while others are activated. This idea fits well with the data presented here, as the leukotriene and thromboxane pathways are down regulated, which would allow for the up regulation in prostaglandin production. In this chapter, I have focused on the pathways that were up regulated by *Salmonella* infection. However this should not discount the possibility that the down regulated pathways may also play an important role during infection.

SPI2 has been implicated in the regulation of COX2 during *Salmonella* infection by other groups (Uchiya and Nikai 2004); however, no specific effector other than SpiC has been identified. To try to elucidate if and how SPI2 was involved, the panel of SPI2 deletion strains was tested for an effect on TBXAS1, PTGES, and COX2. As expected, the deletion strains that colonized macrophages at lower levels were generally found to have expression levels that were similar to uninfect ed samples. Of significant interest is the PTGES data, which shows that some of the strains that colonize at lower levels, such as ΔsifB, ΔseK1/2/3, ΔsteC, and ΔspvB, still induced levels of PTGES similar to wild-type. Additionally, the ΔspiC strain, which does not secrete effectors (Yu, McGourty et al. 2010), had low levels of PTGES expression. Together, this implies that some of the SPI2 effectors may be responsible for inducing PTGES transcription. Because the ΔsspH2, ΔseF, ΔseG, ΔseFG, and ΔslrP strains all had reduced levels of PGTEs induction with high levels of bacterial colonization they are potential candidates for this role.

The expression levels of COX2 induced by the SPI2 deletion strains revealed some interesting trends, namely the potential role of SseF and SseG in inducing COX2 expression during *Salmonella* infection. The data presented in Figures 3.7 and 3.8 show that SseF and SseG are responsible for a large portion of the induction of COX2 seen during *Salmonella* infection. Viewed in light of Uchiya and Nikai’s 2004 paper (Uchiya and Nikai 2004) showing that SPI2 is
responsible for COX2 induction, our data suggest that the effectors SseF and SseG contribute to this during infection. This novel role for these proteins in macrophage infection should be further explored. A great deal of work has been done to structurally analyze SseF and SseG (Salcedo and Holden 2003, Abrahams, Muller et al. 2006, Muller, Chikkaballi et al. 2012). Also, work has been done looking at their function in the formation of SIFs and manipulation of microtubules (Guy, Gonias et al. 2000, Kuhle and Hensel 2002, Kuhle, Jackel et al. 2004, Abrahams, Muller et al. 2006). However, much of what we know about SseF and SseG comes from experiments in epithelial cells, not in macrophages. The data presented in this chapter provides a novel role for these effectors during Salmonella infection of macrophages.

Finally, the role of exogenous hormones added to macrophages infected with Salmonella was studied. Both thromboxane B₂ and prostaglandin E₂ reduced bacterial counts to similar levels. It is interesting that PGE₂ leads to a reduction in Salmonella counts, given that it has previously been shown (Bowman and Bost 2004) that live Salmonella induce the production and release of PGE₂. Surprisingly, 15d-PGJ₂ significantly reduced bacterial colonization of macrophages, and this phenomenon was studied more closely in the following chapter.

It is interesting to note that Salmonella infection causes an induction of COX2, which is at least partially dependent on SPI2 effectors, and leads to an increase in the production of prostaglandins. Increasing the concentration of prostaglandins leads to a reduction in bacterial colonization, as shown in Figure 3.9. This leads to the question of why would Salmonella have evolved a SPI2 dependent way to increase prostaglandins if they prevent bacterial colonization? Recently there has been mounting evidence that Salmonella needs the inflammatory response to effectively colonize the host (Arpaia, Godec et al. 2011). It is possible that Salmonella induce COX2, and thus PG synthesis, to prevent uncontrolled bacterial replication, to avoid killing the
host too quickly. Alternatively, the host may be using a sseFG mediated process to induce COX2, to control bacterial replication and thus protect itself. The elucidation of how SseF and SseG are inducing COX2 expression may shed more light on these questions. The data presented in this chapter, and the following chapter may fit into this complex interplay of finding the ‘right’ balance of inflammation.

Elucidation of the roles of SPI2 effectors in pathogenesis is important for the development of novel treatments and vaccines, as well as furthering our basic understanding of *Salmonella* biology and host interactions. While some *Salmonella* effectors have been studied extensively, little is known about many others. Understanding the role of SseF and SseG in *Salmonella*-induced PG production will be important for deciphering the interplay between the production of PGs in inflammation and *Salmonella*-host interactions.

### 3.9 Summary

In this chapter, the role of arachidonic acid metabolism, specifically the production of prostaglandins, during *Salmonella* infection was examined. The data presented here show that *Salmonella* infection induces the production of the mRNA for enzymes involved in the production of the prostaglandins. Conversely, the expression of genes involved in the production of the thromboxanes and leukotrienes was downregulated by *Salmonella* infection. Pertaining to the prostaglandin pathway, the data shows that SPI2 effectors may be playing a role in its upregulation. Specifically, this chapter shows evidence that SseF and SseG are partially responsible for the induction of COX2 seen during *Salmonella* infection. Finally, the addition of products downstream of COX2 leads to a reduction in bacterial colonization, confirming the
notion that the arachidonic acid pathway is an important component of the interplay between *Salmonella* and its host.
Chapter 4: 15d-PGJ$_2$ inhibits colonization of macrophages by *Salmonella*

4.1 Abstract

15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) is an anti-inflammatory downstream product of the cyclooxygenase enzymes. It has been implicated to play a protective role in a variety of inflammatory mediated diseases, including rheumatoid arthritis, neural damage, and myocardial infarctions. Here it is shown that 15d-PGJ$_2$ also plays a role in *Salmonella* infection. *Salmonella enterica* Typhimurium is a Gram-negative facultative intracellular pathogen that is able to survive and replicate inside phagocytic immune cells, allowing for bacterial dissemination to systemic sites. *Salmonella* species cause a wide range of morbidity and mortality due to gastroenteritis and typhoid fever. The previous chapter and Appendix A show that in mouse models of typhoid fever and macrophage infections, *Salmonella* causes a major perturbation in the prostaglandin pathway. Specifically, that 15d-PGJ$_2$ production was significantly increased in both liver and feces. This chapter shows that 15d-PGJ$_2$ production is also significantly increased in macrophages infected with *Salmonella*. Furthermore, the addition of 15d-PGJ$_2$ to *Salmonella* infected RAW264.7, J774, and bone marrow derived macrophages is sufficient to significantly reduce bacterial colonization. 15d-PGJ$_2$ reduces the inflammatory response of these infected macrophages, as evidenced by a reduction in the production of cytokines and reactive nitrogen species. The inflammatory response of the macrophage is important for full *Salmonella* virulence, as it can provide the bacteria cues for virulence. The reduction in bacterial colonization is independent of the expression of *Salmonella* virulence genes SPI1 and SPI2, and is independent of the 15d-PGJ$_2$ ligand PPAR-$\gamma$. In conclusion, this chapter shows that 15d-PGJ$_2$
mediates the outcome of Salmonella infection of macrophages, a previously unidentified role for this prostaglandin.

### 4.2 Introduction

Prostaglandins (PG) are a class of lipid hormones responsible for a wide range of functions within the body. PGs are synthesized from arachidonic acid that is released from the cell membrane by phospholipase A2 and then modified by the cyclooxygenase enzymes (COX1 and COX2) to enter the PG pathway (Figure 4.1) (Funk 2001, Yoshikai 2001). COX1 is constitutively active, whereas COX2 is induced under inflammatory conditions (Funk 2001). COX2-derived PGs are involved in a variety of pro- and anti-inflammatory processes (Funk 2001, Matsuoka and Narumiya 2008). The involvement of COX1 and COX2 in regulating inflammation is evidenced by the increased cardiovascular risk associated with the inhibition of COX2 (Cannon and Cannon 2012), and the increased susceptibility to colitis in mice lacking these two enzymes (Morteau, Morham et al. 2000). Two waves of COX2 activity have been identified: the first (early) activity is associated with the pro-inflammatory response, whereas the second wave mediates the resolution of inflammation (Gilroy, Colville-Nash et al. 1999), and is associated with high levels of PGD$_2$ and 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$ (hereafter referred to as 15d-PGJ$_2$) (Gilroy, Colville-Nash et al. 1999, Yoshikai 2001).
Figure 4.1 Arachidonic acid metabolism
Arachidonic acid metabolism and formation of prostaglandins and leukotrienes. 15d-PGJ$_2$ is non-enzymatically produced from PGD$_2$.


15d-PGJ2 inhibits the synthesis of iNOS in activated and peritoneal macrophages, which is at least partially dependent on NF-κB (Ricote, Li et al. 1998, Petrova, Akama et al. 1999, Castrillo, Díaz-Guerra et al. 2000). In RAW 264.7 and J774A.1 macrophages, 15d-PGJ2 increases ROS formation, which may inhibit phagocytosis and induce apoptosis at later time points (Castrillo, Mojena et al. 2001, Liu, Yu et al. 2012). Furthering its role as an anti-inflammatory mediator, 15d-PGJ2 reduces the production of cytokines (Jiang, Ting et al. 1998), and reduces the recruitment of bone marrow monocytes during liver inflammation (Han, Zhu et al. 2012). It was also found that 15d-PGJ2 reduces the phagocytic activities of bone marrow macrophages (BMMO) in vitro (Han, Zhu et al. 2012). Recently, the use of nanocapsules loaded with 15d-PGJ2 has proved an effective strategy to reduce neutrophil migration, IL-1β, TNF-α, and IL-12p70 production during inflammation (Alves, de Melo et al. 2011). In fact, 15d-PGJ2 is so vital to the resolution phase of the inflammatory process, that when it is added back to animals
treated with COX2 inhibitors, it is sufficient to restore the normal resolution that occurs after inflammation, which is prevented by COX2 inhibitors (Gilroy, Colville-Nash et al. 1999).

Since 15d-PGJ2 has been found to reduce inflammation in such a variety of models, it has been explored as a potential therapeutic in a number of inflammatory diseases. Liu and colleagues (2012) concluded that since 15d-PGJ2 reduces the general activity of both RAW264.7 and J774A.1 macrophages, it has the potential to be an effective therapeutic for inflammatory diseases (Liu, Yu et al. 2012). More specifically, the role of 15d-PGJ2 and its potential applications in therapy have been explored in rheumatoid arthritis, atherosclerosis, myocardial infarctions, cerebral injury, and gastrointestinal inflammation (Jiang, Ting et al. 1998, Ricote, Li et al. 1998, Surh, Na et al. 2011). 15d-PGJ2 has also been found to protect enteric glial cells from oxidative stress, to reduce hepatic inflammation and fibrosis, and to reduce symptoms of COPD in rats (Surh, Na et al. 2011, Abdo, Mahe et al. 2012, Han, Zhu et al. 2012). 15d-PGJ2 may also be useful in the treatment of cancers, as it has been found to inhibit cell growth and tumorigenicity (Bui and Straus 1998). In a model of periodontitis, 15d-PGJ2 nanocapsules were found to reduce inflammation caused by infection with Actinobacillus actinomycetemcomitans, but no effect on bacterial colonization was seen (Napimoga, da Silva et al. 2012).

15d-PGJ2 has been studied in models of sepsis and septic shock. In models of polymicrobial sepsis, 15d-PGJ2 treatment leads to increases in blood pressure, reductions in vascular injury, neutrophil infiltration, cytokine production, renal and liver dysfunction and injury, resulting in increased survival (Zingarelli, Sheehan et al. 2003, Dugo, Collin et al. 2004). In rat macrophages treated with heat killed S. aureus and E. coli, 15d-PGJ2 treatment leads to reductions in NO production, TBXB2 production, and ERK1/2 and NF-kB activity (Guyton, Zingarelli et al. 2003). In bacterial sepsis, PMN migration is reduced, and this was found to be
mediated by PPAR\(\gamma\), and 15d-PGJ\(_2\) treatment reduced PMN adherence to fibrinogen, another aspect of PMN migration (Reddy, Narala et al. 2008). The role of 15d-PGJ\(_2\) in microglial inflammatory response to \(S.\) \textit{aureus} was examined, and 15d-PGJ\(_2\) was found to inhibit a variety of cytokines including IL-1\(\beta\), TNF\(\alpha\), IL-12p40, and MCP1, while in this model the levels of PPAR\(\gamma\) were unaffected by either 15d-PGJ\(_2\) or \(S.\) \textit{aureus} treatment (Kielian, McMahon et al. 2004). The role of 15d-PGJ\(_2\) in \(H.\) \textit{pylori} infected epithelial cells was also studied, and it was found that 15d-PGJ\(_2\) treatment reduced JAK/STAT signaling, RANTES production, and NADPH oxidase activity (Cha, Lim et al. 2011). In this study, the involvement of PPAR\(\gamma\) was not determined (Cha, Lim et al. 2011). Interestingly, 15d-PGJ\(_2\) treatment of mice one day after infection with the influenza virus was found to significantly reduce morbidity and mortality, in a PPAR\(\gamma\) dependent fashion (Cloutier, Marois et al. 2012). In this study, 15d-PGJ\(_2\) reduced the production of chemokines and cytokines, as well as reducing viral titers (Cloutier, Marois et al. 2012). They also found that 15d-PGJ\(_2\) decreased inflammatory infiltrate in the lungs and reduced the production of IL-6, TNF\(\alpha\), CCL2, CCL3, CCL4, and CXCL10, but had no effect on IFN\(\gamma\) production (Cloutier, Marois et al. 2012). GW9662, a PPAR\(\gamma\) specific inhibitor, was used, and this inhibitor abolished the protection afforded by 15d-PGJ\(_2\) treatment (Cloutier, Marois et al. 2012). These studies show the potential use of 15d-PGJ\(_2\) in a variety of microbial associated disease conditions, however, it seems that there have not been any studies looking at the role of 15d-PGJ\(_2\) in \textit{Salmonella} infection.

\textit{Salmonella} is a Gram-negative enteric pathogen that is transmitted by contaminated food or water (Haraga, Ohlson et al. 2008). Once ingested, the bacteria replicate in the small intestine, and in cases of systemic disease, such as typhoid fever, the bacteria cross the intestinal barrier and are taken up by phagocytes (Haraga, Ohlson et al. 2008, McGhie, Brawn et al. 2009). By
means of the *Salmonella* Pathogenicity Island 2 (SPI2) type III-secretion system, *Salmonella* is able to replicate inside macrophages in a special vacuole termed the *Salmonella* containing vacuole (McGhie, Brawn et al. 2009, Buckner, Croxen et al. 2011, van der Heijden and Finlay 2012). From inside these macrophages, *Salmonella* is able to disseminate to systemic sites such as the spleen and liver, causing severe disease and bacteremia (Haraga, Ohlson et al. 2008).

Our group has recently performed a high-throughput metabolomics study to determine the effect of *Salmonella enterica* serovar Typhimurium infection of mice on the chemical composition of the body (Antunes, Arena et al. 2011). We found that the PG pathway was greatly perturbed by *Salmonella* infection and that 15d-PGJ\(_2\) production was greatly increased in infected mice (Appendix A, Figure A.1 and Figure A.2) (Antunes, Arena et al. 2011). Additionally, 15d-PGJ\(_2\) was identified in the previous chapter as having a role in *Salmonella* infection of macrophages (Figure 3.9). Therefore, the impact of this hormone on the pathogenesis of *Salmonella* was examined. This chapter shows that 15d-PGJ\(_2\) production is increased during *Salmonella* infection of cultured macrophages. Additionally, the roles of individual PGs on bacterial colonization of macrophages was examined, and it was shown that 15d-PGJ\(_2\) causes a marked decrease in *Salmonella* colonization, despite its well-known role in reducing macrophage activity. Like many activities of 15d-PGJ\(_2\), this effect on colonization is PPAR-\(\gamma\) independent. Furthermore, this chapter presents evidence showing that this reduction in colonization is not due to inhibition of SPI2. Altogether, this data shows a novel role for 15d-PGJ\(_2\), and provides further evidence for the importance of inflammation to *Salmonella* pathogenesis.
4.3 Methods and materials

4.3.1 Chemical reagents

Streptomycin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). 15d-PGJ$_2$ was obtained from Cayman Chemical (Ann Arbor, USA).

4.3.2 Tissue culture

RAW264.7 and J774 macrophages, as well as HeLa epithelial cells, were obtained from the American Type Culture Collection (Manassas, USA). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; HyClone, Waltham, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 1% non-essential amino acids (Gibco, Carlsbad, USA) and 1% GlutaMAX (Gibco). Cells were seeded approximately 20 hours before experiments in 24-well plates at a density of $10^5$ cells per well. 15d-PGJ$_2$ was dissolved in DMSO and concentrations of 2 µM were used, unless otherwise indicated. Controls without 15d-PGJ$_2$ contained the same amounts of DMSO. Wild-type Salmonella strain SL1344 (hereafter refered to as Salmonella) were grown in LB with streptomycin at 37°C with aeration. For infection assays, bacterial cells grown in LB, in mid-logarithmic growth were spun down and resuspended in phosphate-buffered saline (PBS) and diluted in tissue culture medium. Cells were infected at a multiplicity of infection of 10 for 30 minutes at 37°C, 5% CO$_2$. Subsequently, cells were washed with PBS and incubated at 37°C, 5% CO$_2$ in growth medium containing 100 µg/mL gentamycin (Sigma-Aldrich) for 1 hour. Medium was replaced to decrease the gentamycin concentration to 10 µg/mL for later time points. All media contained (or did not contain for controls) the indicated concentration of 15d-PGJ$_2$. At the appropriate times, supernatants were collected and cells were lysed in 250 µL of 1% Triton X-100 (BDH, Yorkshire, UK), 0.1% sodium dodecyl sulfate
Serial dilutions were plated on LB plates containing 100 µg/mL of streptomycin (Sigma-Aldrich) for bacterial enumeration.

4.3.3 Bone marrow macrophage collection and infection

Age-matched C57BL/6 female mice were sacrificed and femurs were removed. Femurs were cleaned, and marrow was removed in Hank’s balanced salt solution (Gibco) with 2% FBS. Animal experiments were approved by the Animal Care Committee of the University of British Columbia and performed in accordance with institutional guidelines. Cells were spun down and resuspended in BMMO media [DMEM (HyClone), 20% FBS, 2 mM Glutamax, 1 mM Sodium Pyruvate (Gibco), (5%) penicillin/streptomycin (Gibco), 20% L-conditioned media]. Cells were grown for 7-10 days before use. For infection, BMMO’s were seeded in 24-well plates at 1x10^6 cells/well in BMMO media without penicillin/streptomycin and L-conditioned media. BMMOs were infected with Salmonella at a multiplicity of infection of 10, and the gentamycin protection assay was completed as above. CFU was determined at 2, 6, and 10 hours post-infection.

4.3.4 Cytokine analysis and ELISAs

Cytometric bead assay (CBA) for mouse inflammation (BD Biosciences) was performed following the recommended assay procedure. Supernatants from macrophages infected with or without 15d-PGJ_2 were used for CBAs.

Enzyme-linked immunosorbent assays (ELISA) were performed on culture supernatants from uninfected and infected cells using commercially available ELISA kits to determine concentrations of 15d-PGJ_2 (Assay Designs, Ann Arbor, USA). ELISAs (BD Biosciences) were also used to examine the concentrations of cytokines (TNF-α, MCP1, IL-10, IL-6) in the supernatants of infected, 15d-PGJ_2-treated and untreated macrophages. Manufacturer’s recommendations and procedures were followed for all ELISAs.
4.3.5 Quantitative real-time PCR

RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), with the on-column DNA digestion (Qiagen). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). For qRT-PCRs, we used the QuantiTect SYBR Green PCR Kit (Qiagen) was used and the Applied Biosystems (Foster City, USA) 7500 system. Reactions contained forward and reverse primers at 0.4 μM each. All results were normalized using the mRNA levels of the acidic ribosomal phosphoprotein PO as baseline. Averages of the data obtained with untreated samples were normalized to 1 and the data from each sample (untreated or treated) was normalized accordingly. Primers used are listed in Table 4.1.

Table 4.1 qRT-PCR primers used for cytokine genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>GAGGATACCACCTCCACAGACC</td>
<td>AAGTCATCATCAGTTGTTCATA</td>
</tr>
<tr>
<td>IL-10</td>
<td>GGGTGCAGCGCTACGCCTGGA</td>
<td>ACCTGCTCCCAGGCCTTGCT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ATGGGATCATCTTGCTGGT</td>
<td>CCTGCTCAGGCTAGTGCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGGATCGGGCGCATAGAATC</td>
<td>CCACCACGCTTTCTGTGCT</td>
</tr>
</tbody>
</table>

4.3.6 Immunofluorescence microscopy

Macrophages were seeded as mentioned previously, but on glass coverslips. Infections were carried out as above. Cells were fixed using 4% paraformaldehyde (Canemco Supplies, Quebec, Canada) overnight. Cells were then stained using a rabbit, polyclonal, anti-Salmonella LPS antibody (BD Biosciences). Prolong Gold containing DAPI (Invitrogen) was used to attach coverslips to the slides. The Zeiss Axioplan Fluorescence Microscope was then used to enumerate the bacteria in each infected macrophage for a total of 50 infected macrophages per sample.
4.3.7 Cell viability assays

For Trypan blue exclusion assays, at the appropriate time points after infection, macrophages were released from the bottom of plates using cell scrapers, and stained with Trypan Blue (Gibco). The number of cells were then counted using the Countess automated cell counter (Invitrogen).

CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega) was performed on supernatants from infected or uninfected, 15d-PGJ\textsubscript{2} treated or untreated macrophages to determine the amount of LDH released. The manufacturer’s protocol was followed.

4.3.8 Salmonella growth in 15d-PGJ\textsubscript{2}

Salmonella was grown in LB overnight with aeration at 37°C in the presence or absence of 15d-PGJ\textsubscript{2}. Salmonella was also grown in DMEM with or without 15d-PGJ\textsubscript{2}, without aeration, in 5% CO\textsubscript{2} at 37°C for the indicated time points. Bacterial growth was monitored through measurements of absorbance at 600 nm.

4.3.9 hilA, phoP, ssrA reporter assays

Salmonella strains containing fusions between the promoters of hilA, ssrA or phoP and gfp, as previously described (Antunes, Buckner et al. 2010) were sub-cultured in liquid LB culture for 4 hours in the absence or presence of 2 \mu M 15d-PGJ\textsubscript{2}, and GFP production was analyzed through flow cytometry of bacterial cultures using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ), as indicated. All cultures contained carbenicillin (100 \mu g/ml) and were incubated at 37°C with shaking (225 rpm). In each experiment, 50,000 events were collected per sample. Also, the ssrA reporter plasmid was introduced into Salmonella strain MCS004, which constitutively expresses the mKO red/orange protein. This strain was then used to infect RAW264.7 macrophages, as indicated above. Macrophages were lysed and bacteria were washed with PBS containing 2%
FBS. GFP and RFP production was analyzed through flow cytometry, performed using an LSR II (BD Biosciences), and data were analyzed with FlowJo 8.7 software (TreeStar, Ashland, OR). In each experiment, 100,000 events were collected per sample.

4.3.10 **Reactive nitrogen species production**

To determine reactive nitrogen species, the Griess reaction was performed on supernatants taken from macrophages infected as indicated above.

4.3.11 **PPAR-γ inhibitor**

RAW264.7 macrophages were seeded as above and GW9662 (Cayman Chemicals) was used at 4 µM, where indicated. Infections were carried out as above.

4.3.12 **Statistical analysis**

Data were analyzed by nonparametric Mann-Whitney t tests with 95% confidence intervals using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, USA). For each figure, the term “measurements” refers to the combination of both technical and experimental replicates. Each experiment was repeated a minimum of two times, with multiple technical replicates. Thus, for example “8 measurements” would refer to two separate experiments with 4 technical replicates each.

4.4 **Results - 15d-PGJ₂ inhibits *Salmonella* colonization**

4.4.1 **Salmonella infection induces 15d-PGJ₂ production**

Our group has previously shown that the prostaglandin pathway is perturbed in mice infected with *Salmonella* (Appendix A) (Antunes, Arena et al. 2011). Specifically, the levels of 15d-PGJ₂ were increased during infection in both liver and feces (Appendix A, Figure A.2) (Antunes, Arena et al. 2011). To further characterize the interactions between the anti-
inflammatory molecule 15d-PGJ₂ and *Salmonella*, a simplified cell culture system was established. Because *Salmonella* actively replicates in macrophages, RAW264.7 macrophage cells infected with *Salmonella* were examined to determine if 15d-PGJ₂ production was induced in these cells, as observed in mice. Similar to mice, a significant increase in the amount of 15d-PGJ₂ produced by cultured macrophages in response to *Salmonella* was seen (Figure 4.2).

![Figure 4.2 15d-PGJ₂ produced in response to *Salmonella*](image)

**Figure 4.2 15d-PGJ₂ produced in response to *Salmonella***

15d-PGJ₂ is increased during *Salmonella* infection of RAW264.7 macrophages at 20 hours post-infection. Supernatants were collected at 2 and 20 hours post-infection and levels of 15d-PGJ₂ were determined through ELISA. Results shown are averages of four measurements, with standard errors of means. (**p<0.001).

### 4.4.2 Addition of 15d-PGJ₂ reduces *Salmonella* colonization of macrophages

The previous chapter indicated a potential role of 15d-PGJ₂ in bacterial colonization. To further characterize this increasing concentrations of 15d-PGJ₂ was added to RAW264.7 macrophages prior to and during *Salmonella* infection and colonization was monitored through bacterial enumeration by selective plating. A dose-dependent decrease in *Salmonella* colonization of macrophages 24 hours post infection was clearly seen (Figure 4.3A). To
determine the effect of 15d-PGJ$_2$ on *Salmonella* colonization throughout the course of infection and to understand the kinetics of this phenomenon, bacterial loads in macrophages were examined at 2, 6, 10, and 24 hours post infection in the absence or presence of 2 µM 15d-PGJ$_2$, added prior to and during infection. This showed that 15d-PGJ$_2$ reduces *Salmonella* colonization as early as 2 hours post infection, and continues to exert its effect until 24 hours post infection (Figure 4.3B). Immunofluorescence microscopy was also used to enumerate the *Salmonella* inside individual macrophages. By counting the bacteria inside 50 macrophages untreated or treated with 2 µM 15d-PGJ$_2$ at 2, 4, and 8 hours post infection significantly fewer *Salmonella* were seen in the 15d-PGJ$_2$ treated RAW264.7 macrophages (Figure 4.3C), confirming our CFU observations.
**Figure 4.3 Salmonella colonization is reduced by 15d-PGJ$_2$**

(A) *Salmonella* colonization of RAW264.7 macrophages with the addition of increasing concentrations of 15d-PGJ$_2$ at 24 hours post infection. (B) The effect of 2 µM 15d-PGJ$_2$ on *Salmonella* colonization of RAW264.7 macrophages over time as determined by CFU analysis. (C) Immunofluorescence microscopy was used to enumerate bacterial colonization in individual macrophages at 2, 4 and 8 hours post-infection. Averages of at least 8 measurements are shown with standard errors of the means. (*p<0.05, **p<0.001).

To confirm that 15d-PGJ$_2$ was not killing the macrophages, Trypan blue and LDH release assays were used to measure cell viability. The Trypan blue exclusion assay was used to count the number of cells in infected, 15d-PGJ$_2$ treated and untreated, macrophage cultures. No differences were seen between 15d-PGJ$_2$ treated and untreated controls (Figure 4.4A). An LDH-release assay was also used to ensure that 15d-PGJ$_2$ was not causing cell death at 24 hours post-infection. No significant difference was seen in the amount of LDH released by 15d-PGJ$_2$ treated
cells as compared to untreated control cells (Figure 4.4B). Therefore, the reduction in *Salmonella* colonization is due to 15d-PGJ\(_2\) and not to increased macrophage cell death.

**Figure 4.4 Macrophage viability**
(A) Enumeration of live RAW264.7 macrophages using Trypan Blue exclusion after treatment with 2 \(\mu\)M 15d-PGJ\(_2\) and infection with *Salmonella*. (B) LDH released from macrophages infected with *Salmonella* in the absence or presence of 15d-PGJ\(_2\).

### 4.4.3 15d-PGJ\(_2\) does not inhibit *Salmonella* growth directly

The above results indicate that 15d-PGJ\(_2\) inhibits *Salmonella* colonization of macrophages. This could occur through a number of distinct mechanisms, the simplest of which would be direct inhibition of bacterial viability and growth. To determine if this was the case, the effect of 15d-PGJ\(_2\) on *Salmonella* growth in culture media in the absence of macrophages was tested. 15d-PGJ\(_2\) did not affect the growth of *Salmonella* alone in either LB or DMEM (Figure 4.5), suggesting that the effect of this hormone on *Salmonella* colonization of macrophages is not due to a direct inhibition of *Salmonella* viability and growth.
Figure 4.5 15d-PGJ₂ does not affect *Salmonella* growth rate
*Salmonella* growth in (A) LB and (B) DMEM, with and without 2 µM 15d-PGJ₂ treatment. Averages of 4 measurements, with standard deviations are shown.

4.4.4 The effect of 15d-PGJ₂ on *Salmonella* colonization is dependent on cell type

To determine whether the effect of 15d-PGJ₂ was dependent on cell type, both HeLa epithelial cells and J774 macrophages treated with and without 15d-PGJ₂ were infected with *Salmonella*. 15d-PGJ₂ had no effect on *Salmonella* colonization of HeLa epithelial cells (Figure 4.6A), but, like that observed with RAW macrophages, 15d-PGJ₂ reduced colonization in J774 macrophages (Figure 4.6B). The original metabolomics study (Antunes, Arena et al. 2011) was done in mice, therefore a model that more closely resembled the murine infection model was devised. To do this, bone marrow derived macrophages taken from C57BL/6 mice, and were infected with *Salmonella* with or without 15d-PGJ₂ treatment. At 2, 6, and 10 hours post infection there was a significant reduction in *Salmonella* in the 15d-PGJ₂ treated samples (Figure 4.6C). Together this data shows that 15d-PGJ₂ reduces *Salmonella* colonization of RAW264.7, J774, and bone marrow macrophages, while having no effect on HeLa cells.
**Figure 4.6** 15d-PGJ$_2$ reduces colonization of bone marrow and J774 macrophages but not HeLa cells

(A) *Salmonella* colonization of HeLa epithelial cells treated with 15d-PGJ$_2$. (B) The effect of 15d-PGJ$_2$ on *Salmonella* colonization of J774 macrophages cells, as determined by CFU analysis at 24 hours. (C) *Salmonella* colonization of bone marrow macrophages at 2, 6, and 10 hours post-infection, with 15d-PGJ$_2$ treatment. Averages of at least 8 measurements are shown with standard errors of means. (*p*<0.05, **p**<0.001).

The effect of 15d-PGJ$_2$ on activated, IFN-γ pre-treated, RAW264.7 macrophages was also tested. In these activated macrophages *Salmonella* colonization was also significantly reduced by 15d-PGJ$_2$ treatment (Figure 4.7). This indicates that 15d-PGJ$_2$ is potent even in macrophages activated to kill invading bacteria. Interestingly the addition of IFN-γ did not appear to have a significant affect on overall *Salmonella* replication in RAW264.7 macrophages.
Figure 4.7 IFN-γ priming of macrophages does not alter the effect of 15d-PGJ₂ on colonization

The effect of 15d-PGJ₂ on Salmonella colonization of 2 ng/mL IFN-γ activated RAW264.7 macrophages 24 hours post infection. Averages of 8 measurements are shown with standard errors of means. (*p<0.05, N.S. indicates not significant).

4.5 15d-PGJ₂ affects the immune response of macrophages infected with Salmonella

4.5.1 15d-PGJ₂ reduces cytokine response to Salmonella

As the effect of 15d-PGJ₂ seemed to be restricted to macrophages, the effects of 15d-PGJ₂ on the macrophage inflammatory response was examined. A cytometric bead assay (CBA) was performed on supernatants from Salmonella infected RAW264.7 macrophages. The CBA experiment showed that the 15d-PGJ₂ treated macrophages produced significantly lower levels of TNF-α, MCP-1, IL-10, and IL-6, whereas levels of IFN-γ and IL-12 were unaffected (Figure 4.8A). This was confirmed using qRT-PCR (Figure 4.8B), and ELISA (Figure 4.8C). This indicates that 15d-PGJ₂ is in fact reducing specific cytokines produced in response to Salmonella infection.
Figure 4.8 15d-PGJ$_2$ treatment reduces cytokine production

The effect of 2 µM 15d-PGJ$_2$ treatment during *Salmonella* infection on cytokine production. RAW264.7 macrophages were examined at 24 hours post infection, cytokine production was determined by; (A) CBA assay, (B) quantitative real-time PCR, and (C) ELISA performed on supernatants from infected cells. Averages of 8 measurements are shown with standard errors of means. (*p<0.05, **p<0.001).
4.5.2 15d-PGJ$_2$ reduces production of RNS

Since 15d-PGJ$_2$ reduces the cytokines produced during infection, the next question was whether 15d-PGJ$_2$ would reduce other macrophage mechanisms aimed at responding to pathogens. To this end, the amount of RNS produced in response to *Salmonella* infection was tested. The data shows that RNS production was significantly reduced by the addition of 15d-PGJ$_2$ (Figure 4.9).

![Figure 4.9 15d-PGJ$_2$ reduces the production of reactive nitrogen species](image)

**Figure 4.9 15d-PGJ$_2$ reduces the production of reactive nitrogen species**
The Griess reaction was used to determine the amount of reactive nitrogen species produced by RAW264.7 macrophages treated with 2 µM 15d-PGJ$_2$ and infected with *Salmonella*. Averages of 8 measurements are shown with standard errors of means. (*p<0.05, N.S. indicates data was not significant).

4.6 15d-PGJ$_2$ does not affect *Salmonella* virulence gene expression.

There is a possibility that in addition to dampening the immune response, 15d-PGJ$_2$ may also have an effect on *Salmonella* virulence gene expression thus affecting the ability of *Salmonella* to invade and replicate in macrophages. Therefore, *Salmonella* reporter strains were
used to determine if the regulation of virulence genes was directly affected by 15d-PGJ$_2$ treatment. For these experiments, the SPI1 regulatory gene *hilA*, the SPI2 regulatory gene *ssrA*, and the two-component regulatory gene *phoP* were chosen to examine the expression of virulence genes in the presence of 15d-PGJ$_2$, as these genes play major roles in the regulation of the SPI1 and SPI2 virulence regulons during the infection process. To study their expression, reporter fusions between the promoters of these genes and *gfp* were used as previously described (Antunes, Buckner et al. 2010). The expression of these genes was not affected by the addition of 15d-PGJ$_2$ to LB (Figure 4.10A). Additionally, because SPI2 is highly induced inside the *Salmonella* containing vacuole, where it plays a major role in systemic virulence and the formation of a hospitable intracellular niche in phagocytes (Haraga, Ohlson et al. 2008, van der Heijden and Finlay 2012), the activity of SPI2 in 15d-PGJ$_2$ treated macrophages was determined. First 15d-PGJ$_2$ treated or untreated macrophages were infected with either the wild-type *Salmonella* strain, or the ΔssaR strain, which does not secrete any SPI2 effectors into the macrophage. Since 15d-PGJ$_2$ might be affecting *Salmonella* colonization by inhibiting SPI2, it was anticipated that infecting host cells with a strain already missing a SPI2 component would abolish the colonization defect seen with 15d-PGJ$_2$ treatment. Interestingly, this was not the case; in fact, macrophage colonization by the ΔssaR strain was inhibited to the same extent as the wild-type infections when compared to the samples that did not receive 15d-PGJ$_2$ (Figure 4.10B). To test if the pathway by which *Salmonella* was taken up by the macrophages was being affected by 15d-PGJ$_2$ treatment, macrophages were infected with a ΔinvA strain, an invasion mutant which does not secrete SPI1 effectors, and therefore bacterial uptake occurs through phagocytosis alone. The data shows that the ΔinvA strain’s colonization was inhibited by 15d-PGJ$_2$ to the same extent as wild-type *Salmonella*. In Figure 4.10B the data are expressed as a
percentage of the respective control samples, to illustrate that the extent of the inhibition caused by 15d-PGJ$_2$ is equivalent, even though the $\Delta$ssaR and $\Delta$invA strain colonized at a lower level than the wild-type *Salmonella* strain.

**Figure 4.10 Expression of *Salmonella* virulence genes are unaffected by 15d-PGJ$_2$**

(A) Wild-type *Salmonella* carrying *hilA*-, *phoP*-, and *ssrA*-gfp reporter transcriptional fusions were used to analyze the effect of 15d-PGJ$_2$ on virulence gene expression. Cultures were grown in LB for 4 hours. No changes in expression were seen. (B) 15d-PGJ$_2$ reduced bacterial colonization of macrophages by the wild-type *Salmonella* strain, the $\Delta$ssaR strain, and the $\Delta$invA strain. Data are expressed as a percentage of the respective control samples, to illustrate that the extent of the inhibition caused by 15d-PGJ$_2$ is equivalent in all strains. Averages of 8 measurements are shown with standard errors of means. (*p<0.05, **p<0.001, N.S. indicates data was not significantly different).

To further ensure that SPI2 expression was not affected in 15d-PGJ$_2$ treated macrophages the *ssrA* reporter fusion was used in bacteria constitutively expressing the mKO red/orange protein, and *ssrA* expression was examined after macrophage infection. There were no differences in *ssrA* expression in untreated or 15d-PGJ$_2$ treated macrophages (Figure 4.11). Therefore our data indicates that despite 15d-PGJ$_2$ generally reducing the macrophage inflammatory response, the expression of *Salmonella* virulence genes is not affected.
**Figure 4.11 15d-PGJ₂ does not alter ssrA expression after macrophage infection**

Flow cytometry analysis of ssrA::gfp gene expression in *Salmonella* after infection of RAW264.7 macrophages with or without 15d-PGJ₂ treatment. Averages of 8 measurements are shown with standard errors of means. (N.S. indicates data was not significant).

**4.7 15d-PGJ₂ affects *Salmonella* colonization via a PPAR-γ independent mechanism.**

15d-PGJ₂ is known to bind to and alter PPAR-γ activity, however, it is also not considered to be important in RAW264.7 macrophages. To ensure that PPAR-γ was not involved in this system, the PPAR-γ inhibitor GW9662 was added to RAW264.7 macrophages infected with *Salmonella* and treated with 15d-PGJ₂. Bacterial colonization was determined, as above. The inhibitor was unable to restore the colonization defect seen with 15d-PGJ₂ (Figure 4.12), indicating that the effect of 15d-PGJ₂ on macrophage colonization by *Salmonella* is PPAR-γ independent.
Figure 4.12 PPAR-γ inhibitor has no effect on *Salmonella* colonization
The effect of the addition of a PPAR-γ inhibitor to 15d-PGJ2 treated macrophages infected with *Salmonella* on bacterial colonization. Averages of 8 measurements are shown with standard errors of means. (*p<0.05, N.S. indicates data was not significant).

4.8 Discussion

The potential role of 15d-PGJ2 during bacterial infection was initially considered because of the results of a metabolomics screen recently performed by our lab (Antunes, Arena et al. 2011). This study and the previous chapter indicate that the PG pathway is highly responsive to *Salmonella* infection. Therefore further experiments were designed to examine if this pathway played a role in the establishment of infection by *Salmonella*. This chapter shows that at 20 hours post-infection macrophages produce high levels of 15d-PGJ2 in response to *Salmonella* infection, which coincides with the previous data showing that 15d-PGJ2 is highly induced by *Salmonella* infection in mice. It was then hypothesized that the high level of 15d-PGJ2 production observed would likely have a significant effect during the course of infection. To test this, 15d-PGJ2 was added exogenously and its effects on *Salmonella* colonization of macrophages were monitored.
The data demonstrated a significant impact of 15d-PGJ2 on *Salmonella* burden and also showed a dose-dependent decrease in *Salmonella* colonization, clearly indicating that 15d-PGJ2 is sufficient to prevent bacterial colonization of macrophages. Some reports have claimed that 15d-PGJ2 treatment causes apoptosis in macrophages (Hortelano, Castrillo et al. 2000), and in fact at high concentrations of 15d-PGJ2 we did begin to see increased macrophage cell death (data not shown). Therefore, 2 µM 15d-PGJ2 was used in experiments because this was the lowest concentration at which a decrease in colonization was seen without an increase in cell death (Figure 4.4).

The effect of 15d-PGJ2 on *Salmonella* colonization was not limited to RAW264.7 macrophages. In fact, 15d-PGJ2 reduced bacterial colonization in both J774 macrophages and bone marrow macrophages (BMMO) from C57BL/6 mice. BMMOs are considered more ‘physiologically relevant’, and are able to clear *Salmonella* more rapidly and effectively than RAW264.7 macrophages (data not shown). Surprisingly, 15d-PGJ2 did not affect *Salmonella* replication in HeLa epithelial cells. This could indicate that the 15d-PGJ2–induced resistance to *Salmonella* is cell type specific. It is possible that 15d-PGJ2 alters a macrophage specific response to bacteria, thus inhibiting bacterial infection. It is interesting to note that Straus and colleagues (Straus, Pascual et al. 2000) found that 15d-PGJ2 had a dramatically different effect on NF-κB inhibition in RAW264.7 and HeLa cells. The *Salmonella* life cycle inside of these two cell types is also very different (Haraga, Ohlson et al. 2008) and this may be the reason for the significantly different responses.

Previously published results indicate that 15d-PGJ2 is able to reduce the production of cytokines in response to LPS (Zingarelli, Sheehan et al. 2003, Surh, Na et al. 2011, Liu, Yu et al. 2012). Here, we show the same effect with live, replicating *Salmonella*. Specifically, we saw a
reduction in IL-10 production in 15d-PGJ₂ treated cells. IL-10 is increased via a SPI2 dependent mechanism during *Salmonella* infection, and may inhibit ROS and RNS in macrophages (Eckmann and Kagnoff 2001, Uchiya, Groisman et al. 2004). A reduction in the amount of IL-6 and MCP-1 produced by macrophages treated with 15d-PGJ₂ and infected with *Salmonella* was also seen. Interestingly, there was no change in IL-12, which can stimulate IFN-γ production (Eckmann and Kagnoff 2001). IFN-γ is very important for the defense against *Salmonella*, and is produced predominantly by NK cells and T cells (Eckmann and Kagnoff 2001). Since IFN-γ plays such an important role in anti-*Salmonella* defenses it was surprising to see that 15d-PGJ₂ did not significantly alter either IL-12 or IFN-γ production. TNF-α, which is known to be important for anti-*Salmonella* defenses and is involved in triggering NO production (Eckmann and Kagnoff 2001, Coburn, Grassl et al. 2007), was decreased with 15d-PGJ₂ treatment. Collectively, these data indicate a reduction in pro-inflammatory molecules.

Similar to the results presented in this chapter, Cloutier *et al.* found that 15d-PGJ₂ treatment reduced the production of IL-6, and TNFα in mice infected with the influenza virus, but also showed no effect on IFN-γ production (Cloutier, Marois et al. 2012). In addition, Kielian *et al.* showed that 15d-PGJ₂ selectively inhibited the inflammatory response of microglia in response to *S. aureus* (Kielian, McMahon et al. 2004). This group showed 15d-PGJ₂ dependent reduction in the production of IL-12p40, MCP1, and TNFα (Kielian, McMahon et al. 2004).

Interestingly, there is increasing evidence that *Salmonella* induced inflammation can actually benefit the pathogen in both intestinal colonization and systemic disease. Stecher *et al.* showed that intestinal inflammation is both necessary and sufficient in allowing *Salmonella* to outcompete the microbiota (Stecher, Robbiani et al. 2007). More specifically, Winter and colleagues (2010) showed that *Salmonella* induced gut inflammation resulted in the production
of tetrathionate, which *Salmonella* is able to use as an electron acceptor, thus showing a mechanism by which inflammation benefits this pathogen (Winter, Thiennimitr et al. 2010). *Salmonella* also gains a growth advantage by the production of ethanolamine and nitrate, which *Salmonella* is able to utilize (Thiennimitr, Winter et al. 2011, Lopez, Winter et al. 2012). It was also recently shown that *Salmonella* induces the recruitment of neutrophils to the intestinal lumen (Gill, Ferreira et al. 2012). These neutrophils produce neutrophil elastase, which shifts the microbiota to favour *Salmonella* colonization (Gill, Ferreira et al. 2012). At the systemic level, Arpaia and colleagues (2011) showed that TLR induced innate immunity in response to *Salmonella* induces virulence in the pathogen, allowing bacterial growth leading to systemic disease (Arpaia, Godec et al. 2011). These studies, like the one presented here, show that inflammation can actually be beneficial to *Salmonella*.

Another immune mechanism generally considered to be critical to the host’s defense against *Salmonella* is the production of reactive nitrogen species (RNS). RNS are normally produced during *Salmonella* infection and are integral to bacterial killing as they modify components of the bacterial electron transport chain, metabolic enzymes, transcription factors, DNA, and DNA associated proteins (Bourret, Song et al. 2009, Shi, Chowdhury et al. 2009, Henard and Vazquez-Torres 2011). Furthermore, IFN-γ pretreated macrophages have a stronger RNS response to *Salmonella* than untreated macrophages (Henard and Vazquez-Torres 2011). Intriguingly, both a reduction in RNS as well as a reduction in *Salmonella* burden in 15d-PGJ$_2$ treated macrophages was seen. In addition, this effect was seen in both untreated and IFN-γ treated macrophages, which is interesting given IFN-γ treated macrophages stronger RNS response. The reduction in RNS is also in line with the reduction in TNF-α that is caused by the addition of 15d-PGJ$_2$.  

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The data presented here also indicates that the 15d-PGJ\textsubscript{2} mediated changes in bacterial colonization are SPI2 independent. Initially the possibility of SPI2 involvement due to the apparent reduction in the inflammatory response of the macrophages was explored, but surprisingly SPI2 does not appear to be involved. The potential role of PPAR-\(\gamma\) in the 15d-PGJ\textsubscript{2} mediated reduction in \textit{Salmonella} colonization was examined. As expected, the effects of 15d-PGJ\textsubscript{2} on bacterial colonization were PPAR-\(\gamma\) independent. Furthermore, RAW264.7 macrophages do not appear to produce physiologically relevant amounts of PPAR-\(\gamma\) (Ricote, Li et al. 1998).

Research into the use of 15d-PGJ\textsubscript{2} for the treatment of inflammatory diseases is already underway, and recently the use of nanocapsules as a mechanism of delivery has shown promise (Alves, de Melo et al. 2011, Napimoga, da Silva et al. 2012). In the future, the use of these or other delivery mechanisms may provide a way to effectively administer 15d-PGJ\textsubscript{2} during \textit{Salmonella} infection. Such research may provide insights into a novel mechanism of treating Salmonellosis and possibly other bacterial infections. This chapter suggests a new role of 15d-PGJ\textsubscript{2}, namely the control of \textit{Salmonella} pathogenesis and replication within phagocytic immune cells. The role of 15d-PGJ\textsubscript{2} in bacterial infections is poorly characterized, and this work lays the foundation for further research into this area.

### 4.9 Summary

This chapter shows evidence for the role of 15d-PGJ\textsubscript{2} in \textit{Salmonella} infection of macrophages. 15d-PGJ\textsubscript{2} is produced by macrophages in response to \textit{Salmonella} infection. The addition of 15d-PGJ\textsubscript{2} to infected macrophages results in reduced \textit{Salmonella} colonization in RAW264.7, J774, and BMMO macrophages. Furthermore, the addition of 15d-PGJ2 reduces the
amount of IL-10, IL-6, TNF-α, MCP1, and RNS produced in response to infection. The reduction in colonization is independent of SPI1, SPI2, and PPAR-γ.
Chapter 5: Conclusion

Since Salmonella cause morbidity and mortality in both developed and developing nations worldwide, as well as a major financial burden, the study of this bacterial pathogen is of great significance globally. Therefore, understanding the basic biology of Salmonella, and how it interacts with the host to cause disease is critical for developing new and more effective ways to combat this pathogen. Furthermore, Salmonella is a model organism, and much of what the scientific community has learned from studying Salmonella can be applied to other pathogens and diseases. Increased knowledge in this area will hopefully lead to improved treatments and vaccines for Salmonella, but also potentially for other pathogens and disease conditions.

In this thesis the interactions of Salmonella with the host were explored. This was done by constructing a panel of SPI2 mutants, and examining their effect on virulence in the murine typhoid model, epithelial cell culture model, and macrophage infection model. These SPI2 deletion strains were also studied for their effect on arachidonic acid metabolism. In addition, the involvement of 15d-PGJ₂ in Salmonella infection was examined. A detailed discussion of the results from each of these studies is found at the ends of Chapters 2, 3, and 4. Therefore, this Conclusion explores the broader implications of this work.

In the literature, many studies have looked at SPI2 effectors; however most of these studies examine one or a few effectors in one or a few infection models. This makes comparison of effectors or models quite difficult. Therefore, the work presented in this thesis provides a tool for the Salmonella community to compare and contrast the roles of SPI2 effectors in both murine and cell culture models of infection. This information will be of use to researchers studying SPI2 and Salmonella pathogenesis. Already this work has been used by a variety of groups studying

The infectious disease community, and indeed much of the biology community, has for some time studied genomics and proteomics. Metabolomics, which is a growing field, enables researchers to determine how small molecules (the products and ligands of the proteome), are altered by infection. Metabolomics studies of *Salmonella* have revealed that many host metabolites are altered during infection (Antunes, Arena et al. 2011). These alterations have led to the identification of specific pathways that are affected by *Salmonella* infection.

In this thesis, the involvement of the arachidonic acid pathway in *Salmonella* infection, which was identified by metabolomics, was examined. Data is presented which confirms the involvement of arachidonic acid metabolism in *Salmonella* infection of macrophages. Specifically, the involvement of the leukotriene pathway was found to be down regulated in response to *Salmonella* infection, while the prostaglandin pathway was found to be upregulated. This finding is not surprising, as prostaglandins play a major role in inflammation and *Salmonella* causes significant inflammatory changes in the host. *Salmonella* has even evolved to induce and modulate the inflammatory response of the host for its own benefit. Since *Salmonella* SPI2-dependently modulates the host immune response, the effect of SPI2 deletion strains on the prostaglandin pathway were examined. Quite surprisingly, this study revealed that two of the SPI2 effectors, SseF and SseG, whose role in macrophage infection remains largely unknown, were found to modulate COX2 and PTGES expression, two proteins intricately involved in the production of all prostaglandins and prostaglandin E, respectively. This data, while surprising, is not without precedent, as another group found that COX2 induction was dependent on SPI2 (Uchiya and Nikai 2004), and the data presented in this thesis provides evidence that effectors
contributing to this are SseF and SseG. Some groups have seen a moderate decrease in replication of strains lacking \textit{sseF} and \textit{sseG} (Figueira, Watson et al. 2013); however, in the model and strains used in this thesis, no replication defect was seen.

Another area of this thesis showed that the addition of some of the downstream products of arachidonic acid metabolism were able to reduce bacterial colonization of macrophages. Specifically, 15d-PGJ$_2$ was found to significantly reduce \textit{Salmonella} colonization of macrophages. 15d-PGJ$_2$, a relatively poorly studied member of the prostaglandin family, is associated with an anti-inflammatory response and the resolution of inflammation. It is currently even being studied as a treatment option for a variety of inflammatory diseases. However, the role of 15d-PGJ$_2$ in bacterial infection is poorly studied. A small number of groups have looked at 15d-PGJ$_2$ as a treatment option in reducing the extreme inflammatory response seen in sepsis (Guyton, Zingarelli et al. 2003, Zingarelli, Sheehan et al. 2003, Dugo, Collin et al. 2004, Reddy, Narala et al. 2008). Recently, the role of 15d-PGJ$_2$ in \textit{H. pylori} and influenza infection has been studied, and both groups found that this molecule reduced the inflammatory response to infection (Cha, Lim et al. 2011, Cloutier, Marois et al. 2012). In this thesis, the novel role of 15d-PGJ$_2$ in modulating \textit{Salmonella} infection was explored. This data indicates that 15d-PGJ$_2$ is able to reduce \textit{Salmonella} colonization in a concentration dependent manner, and that this occurs in macrophages, but not epithelial cells. Since 15d-PGJ$_2$ reduces the inflammatory response, it is not surprising to see that it has an effect in macrophages, which play a critical role in \textit{Salmonella} infection. The effect of 15d-PGJ$_2$ appears to be independent of both SPI1 and SPI2. Taken together, the data presented in this thesis demonstrates a novel role for 15d-PGJ$_2$ in \textit{Salmonella} infection. This concept aligns with the prevailing thought that \textit{Salmonella} controls the extent of inflammation it induces, as \textit{Salmonella} infection leads to an increase in 15d-PGJ$_2$ production,
which in turn reduces *Salmonella* colonization. Alternatively, it is possible that this is a mechanism that is successfully used by the host to control *Salmonella* replication.

Major findings from this thesis include providing a tool for direct comparison of SPI2 effectors, providing evidence for the role of SPI2 effectors in modulating the activity of the prostaglandin pathway, and defining a previously unidentified role of a specific prostaglandin, 15d-PGJ₂, in modulating *Salmonella* colonization.

### 5.1 Future directions

This thesis addresses some of the aspects of *Salmonella* and specifically SPI2’s interactions with arachidonic acid metabolism, however many questions still remain. The work presented in Chapter 3 shows a novel phenotype for SseF and SseG. In the future, it would be interesting to characterize the mechanistic interactions of SseF and SseG with the prostaglandin pathway. Other groups have shown that deleting specific domains of SseF and SseG can alter their activities (Abrahams, Muller et al. 2006, Muller, Chikkaballi et al. 2012), therefore, these strains could be used in the COX2 induction model, to see which domains are responsible for the observed phenotype. The work in this thesis used relative expression levels as an indicator of PG pathway protein activity. Future experiments could look at the activity of these proteins, specifically COX2, during infection with the ΔsseF and ΔsseG strains. COX2 specific inhibitors may also prove beneficial in understanding the role of SseF and SseG in COX2 induction. It would also be interesting to look at the downstream products of COX2, and see how they are altered during infection with the ΔsseF and ΔsseG strains. In addition, it would be interesting to see if the SseF and SseG proteins are interacting with host proteins, specifically proteins in the
PG pathway. These experiments may help to reveal how SseF and SseG are inducing COX2 expression.

In addition to following up the SseF and SseG phenotypes, it would be interesting to look more closely at some of the other metabolic pathways identified during the metabolomics screen performed in our lab. The work in Chapters 3 and 4 further characterizes what was seen in that initial screen. Our lab has also looked at another pathway identified in the metabolomics screen, and has published work characterizing the impact of bile and bile metabolism on Salmonella and Salmonella infection (Antunes, Andersen et al. 2011, Antunes, Wang et al. 2012). The multitude of other pathways identified should be carefully examined, first with literature and database searches, then experimentally.

Chapter 4 describes a novel role for 15d-PGJ2 in Salmonella infection. To further characterize this, both mechanism and treatment options could be examined. To uncover more mechanistic aspects of the 15d-PGJ2 phenotypes, signaling pathways could be carefully examined. The NF-κB and Erk1/2 pathways have been shown to play a role in 15d-PGJ2 mediated effects (Castrillo, Diaz-Guerra et al. 2000, Rossi, Kapahi et al. 2000, Straus, Pascual et al. 2000, Guyton, Zingarelli et al. 2003, Zingarelli, Sheehan et al. 2003, Ruiz, Kim et al. 2004, Reddy, Narala et al. 2008, Cloutier, Marois et al. 2012). Therefore exploring the role of both NF-κB and ERK1/2 activity in the 15d-PGJ2 phenotypes identified here may provide excellent clues towards mechanism of action.

Recent work has also shown the use of 15d-PGJ2 filled nanocapsules to be an efficacious delivery method (Alves, de Melo et al. 2011, Napimoga, da Silva et al. 2012). It would be interesting to perform mouse experiments using 15d-PGJ2 filled nanocapsules during Salmonella infection. A variety of mouse models of Salmonellosis are currently used in our lab, including
the typhoid model, the gastroenteritis model, and the chronic fibrosis model. It would be interesting to test 15d-PGJ\(_2\) nanocapsules in all of these models, and see what the outcome is. Bacterial counts, inflammation, and survival could all be used as read-outs. Because of 15d-PGJ2s anti-inflammatory role, it would be particularly interesting to see the effect in the fibrosis and gastroenteritis model, since these models are associated with extensive intestinal inflammation. In addition to work on *Salmonella*, it would be interesting to see if 15d-PGJ\(_2\) alters the outcome of infection using other bacteria. *E. coli* and *C. rodentium* are pathogens commonly used in our lab, and the effect of 15d-PGJ\(_2\) could be examined with these other gastrointestinal pathogens. Hopefully future work will help to uncover the answers to many of the questions brought up by this thesis.
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Appendix
Figure A.1 Metabolic pathways affected by *Salmonella* infection
Mice infected with *Salmonella* were sacrificed 5 days post infection, and livers and feces were collected. Masses of interest were searched against the KEGG database using the MassTrix software (http://masstrix.org). Bars indicate the percentage of metabolites from each KEGG
pathway that was affected by infection. Black bars indicate metabolites from feces, and gray bars indicate metabolites from livers. From (Antunes, Arena et al. 2011)

**Figure A.2 Mouse fecal levels of eicosanoids**
Fecal levels of eicosanoids in uninfected (black bars) and infected (4 days; gray bars) samples, determined by ELISAs. Averages with standard errors of the means are shown. The numbers of uninfected mice used were 4 (15d-PGJ$_2$) and 5 (TBX$_2$ and PGE$_2$). The number of infected mice used was 4 in all cases. All differences were statistically significant ($P < 0.05$). Outliers were detected using the Grubbs’ test and removed. From: (Antunes, Arena et al. 2011)

**Figure A.3 Expression of COX2, PTGES, and TBXAS1 in mouse livers**
Relative transcript levels of enzymes involved in eicosanoid synthesis in uninfected (black bars) and infected (5 days; gray bars) livers. Averages of the results obtained from uninfected tissues were normalized to 1, and the levels for individual mice, uninfected and infected, were adjusted accordingly. Averaged results are shown. Bars indicate the standard errors of the means. Ten mice were used in all cases except for COX-2 determinations from uninfected mice ($n = 7$). All $P$ values were <0.002. From: (Antunes, Arena et al. 2011)